Plant molecular response to combined drought and nematode stress

Nicola Jane Atkinson

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The candidate confirms that the work submitted is her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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Nicola Atkinson. Growth, treatment, measurement and harvest of tomato plants and fruits. Extraction of nutritional compounds and preparation of tomato samples for HPLC, HPAEC-PAD and LC-MS analysis. HPLC analysis of carotenoids and HPAEC-PAD analysis of sugars. Writing of manuscript for publication.

Tristan Dew. Advice with experimental design. Running of samples on LC-MS and provision of LC-MS consumables. Writing of LC-MS materials and methods paragraph and editing of manuscript.

Caroline Orfila. Advice with experimental design and HPLC. Editing of manuscript.

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Abstract

Plants are adapted to respond to precise environmental stress conditions, activating specific molecular and physiological changes in order to minimise damage. Response to multiple stresses is therefore different to that to individual stresses. Simultaneous biotic and abiotic stress conditions are of particular interest, as the molecular signalling pathways controlling each interact and antagonise one another. Understanding such processes is crucial for developing broad-spectrum stress-tolerant crops.

This study characterised the molecular response of plants to the concurrent stresses of drought (abiotic stress) and infection with plant-parasitic nematodes (biotic stress). Drought stress increased susceptibility to infection with *Heterodera schachtii* in *Arabidopsis thaliana*. The whole-genome transcriptome response to these stresses was analysed using microarrays. Each stress induced a particular subset of differentially expressed genes. A novel programme of gene expression was activated specifically in response to a combination of drought and nematode stress, involving 2394 differentially regulated genes.

A diverse range of processes was found to be important in the response to multiple stresses, including plant hormone signalling, activation of transcription factors, cell wall modification, production of secondary metabolites, amino acid metabolism and pathogen defence signalling. Ten multiple stress-induced candidate genes were selected and their functions investigated using over-expression lines and loss-of-function mutants. Altered susceptibility to drought stress (*TCP9, AZI1, RALFL8*) and nematode infection (*TCP9, RALFL8, ATMGL, AZI1*) was observed in several of these lines.

The effect of combined drought and nematode infection on nutritional parameters of tomato fruits was analysed. Drought stress lengthened flowering time and negatively affected carotenoid accumulation. Infection with *Meloidogyne incognita* reduced yield and ripening time and had a positive effect on the accumulation of phenolic compounds. The stresses in combination increased fruit sugar content.

This work comprises the first whole-genome transcriptome study into combined abiotic and biotic stress. The results highlight the importance of studying stress factors in combination.

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Chapter 1. Introduction

1.1 The study of plant stress

Plants are continually faced with a variety of environmental pressures. Being sessile, they have evolved to respond rapidly and efficiently to these adverse conditions in order to survive and reproduce. Most plants grow in environments that are sub-optimal, which prevents the maximisation of their full genetic potential for growth and reproduction (Bray *et al.*, 2000; Rockstrom and Falkenmark, 2000). This is highlighted by analysing the difference between maximum crop yields compared to the average yield for that crop. For example, US wheat yields in a record year can be up to eight times as great as the average yield (Boyer, 1982). The yield difference can largely be accounted for by unfavourable environmental conditions, which when creating potentially damaging physiological changes within plants, are known as stresses (Shao *et al.*, 2008). Abiotic stress factors such as heat, cold, drought and salinity have a huge bearing on world agriculture and are thought to reduce average yields by over 50% for most major crop plants (Wang *et al.*, 2003). Further to this, plants must defend themselves from attack by a vast range of pests and pathogens, including fungi, bacteria, viruses, nematodes and herbivorous insects (Hammond-Kosack and Jones, 2000). Each stress elicits a complex cellular and molecular stress response system, activated within plants in order to prevent damage. Frequently plants in field conditions are exposed to multiple types of stress simultaneously, a situation demanding a new, adaptive response for each stress combination (Rizhsky *et al.*, 2004; Mittler, 2006). Current climate prediction models indicate an increased frequency of drought, flood and high temperature conditions known as heat waves (IPCC, 2008; Mittler and Blumwald, 2010). The increasing pressure on global food productivity as well as changing climatic conditions means that the study of plant stress tolerance is of crucial importance. Understanding the mechanisms of plant responses to stress will provide key opportunities for the development of future stress-tolerant crop varieties.

The model plant *Arabidopsis thaliana* is an extremely useful system in which to study stress responses because of its susceptibility to a wide variety of stresses (Sijmons *et al.*, 1991; Bartels and Sunkar, 2005; Morison *et al.*, 2008). Many molecular tools are available for *A. thaliana*, including an annotated genome sequence, whole genome

microarrays, available stocks of T-DNA insertion mutant lines and a large body of literature (Chaves *et al.*, 2003). These have facilitated the dissection of stress response pathways and the identification of stress-inducible genes. Global expression analysis using microarrays has now established that thousands of genes are involved in defence and the response to abiotic stress (Seki *et al.*, 2002; Bartels and Sunkar, 2005; De Vos *et al.*, 2005; Shinozaki and Yamaguchi-Shinozaki, 2007). Although some plant stress responses are specific to a particular stress, other regulatory systems are central to a generalised response system, thus providing targets for improving multiple stress tolerance (Swindell, 2006; Kilian *et al.*, 2007). As tolerance is largely controlled by genes associated with quantitative trait loci (QTLs), conventional breeding for improved yield under stress can prove difficult (Bartels and Sunkar, 2005; Bhatnagar-Mathur *et al.*, 2008). Despite this, drought-tolerant varieties of maize and rice have successfully been developed by crossing existing cultivars (Banziger *et al.*, 2006; WARDA, 2008; Badu-Apraku and Yallou, 2009). Knowledge derived from molecular studies in *A. thaliana* and other species, combined with modern advances in transgenic technology, will pave the way for further improvements in plant stress tolerance (Edmeades, 2008; Mittler and Blumwald, 2010).

1.2 Abiotic stress

Abiotic stress is caused by physical or chemical components of the environment (Bray *et al.*, 2000). Many abiotic stresses such as drought, salinity, oxidative stress and heat stress have a similar effect on plants, thus eliciting a similar molecular stress response. For example, drought and salt stress both exert oxidative stress on plant cells, leading to the build up of reactive oxygen species (ROS) which can cause denaturation of enzymes (Smirnoff, 1993). Drought, salinity and flooding all result in cellular osmotic stress (Wang *et al.*, 2003). Plants have developed several resistance mechanisms for minimising the effects of abiotic stress and preventing damage. These can be categorised into avoidance or tolerance mechanisms. Avoidance depends on strategic adaptations which prevent exposure to stress. In the case of dehydration avoidance, these may include longer roots, a waxy cuticle, sunken stomatal or early flowering (Taiz and Zeiger, 1991; Bray *et al.*, 2000). In arid environments plants are adapted to complete their life cycle in a very short time when water is available (Chaves *et al.*, 2003). Stress tolerance mechanisms allow plants to withstand stress, and involve processes such as stress perception, signalling and cellular osmotic adjustment (Bartels and Sunkar, 2005). Considerable progress has been made in understanding abiotic stress

resistance through the study of extreme stress tolerant plants such as the desiccation-tolerant 'resurrection' plant *Cratesostigma plantagineum* or the salt-tolerant *Mesembryanthemum crystalinum* (Bartels and Sunkar, 2005).

1.2.1 Drought stress

Drought affects up to a third of all arable land and is one of the most serious constraints to global crop production (Wang *et al.*, 2003; Bartels and Sunkar, 2005). Around 70 % of all available fresh water resources are used to irrigate crops, a figure which is expected to increase over the next 20 years as the global population increases, changes in climatic conditions occur and competition for water resources intensifies (Shiklomanov, 2000; Thomson, 2008; FAO, 2011). Understanding plant responses to drought stress is therefore of crucial importance. Drought stress, or water-deficit stress, is defined as a situation whereby plant water potential and turgor are reduced to a level at which normal functions are impaired (Shao *et al.*, 2008). This is characterised by cell dehydration, decrease in cell enlargement and growth, stomatal closure and limitation of gas exchange. Desiccation results from severe water deficit, and describes the point at which all free water is lost from the protoplasm (Wood, 2005).

1.2.2 Plant physiological responses to drought

The onset of drought stress causes several physiological changes within plants. One of the first responses is the closing of stomata. Plants must constantly balance the necessity for high stomatal conductance in order to assimilate carbon in the form of CO_2, with the equally important need to conserve water. However, high rates of transpiration during times of water deficit could lead to severe water loss, causing cavitation within the xylem and eventually death (Taiz and Zeiger, 1991), therefore closing the stomatal aperture is essential. This is achieved by a change in turgor of the guard cells in response to signals from dehydrated roots, in particular the phytohormone abscisic acid (ABA) (Chaves *et al.*, 2003). The inhibition of photosynthesis takes place soon after stomatal closure, and is thought to be caused both by the limitation of CO_2 due to the closing of stomata and by alteration in photosynthetic metabolism through down-regulation of enzymes (Taiz and Zeiger, 1991; Chaves *et al.*, 2003). As photosynthetic rates decline, the amount of light absorbed by leaves exceeds the amount which can be used in photosynthesis or photorespiration processes. Plants must dissipate the excess energy in order to avoid the build up of reactive oxygen species (ROS) which cause oxidative damage to photosynthetic apparatus (Apel and Hirt, 2004). This is achieved

by reducing the leaf area exposed to light through the inhibition of leaf growth, angling of leaves away from the sun or the abscission of older leaves. Furthermore, plants can thermally dissipate the absorbed light by reducing the efficiency of photosystem II (Chaves *et al.*, 2003). Growth inhibition in shoots occurs rapidly following the onset of drought stress, as cell expansion can only occur when cell turgor pressure is maintained (Shao *et al.*, 2008). The root-shoot ratio increases under water deficit to allow enhanced water absorption. Under conditions of sustained drought, root architecture changes to increase root density at a lower soil depth, thus allowing utilisation of remaining soil moisture (Hsiao and Xu, 2000). It has been proposed that ABA accumulation in roots during stress may antagonise ethylene-induced growth-inhibition, thus allowing the continued growth of roots. In shoots ABA does not accumulate to such a high level, causing ethylene to inhibit growth (Sharp and LeNoble, 2002). As a result of persistent drought conditions, a reduction in stem length, leaf area, fresh and dry weight and yield can occur (Taiz and Zeiger, 1991; Shao *et al.*, 2008). Some plants have adapted their mechanism of photosynthesis to allow greater water use efficiency, using two systems known as C_4 photosynthesis and crassulacean acid metabolism (CAM) (Chaves *et al.*, 2003). C_4 plants concentrate CO_2 in specialised bundle sheath cells, maximising its use under low concentrations that may arise during water stress conditions. CAM plants accumulate CO_2 during the night and close their stomata during the day, allowing survival in extremely arid environments.

1.2.3 The molecular response to drought stress

Plants also respond to drought stress at the cellular and molecular levels, activating signalling pathways and inducing genes with a range of functions in order to establish drought tolerance (Shinozaki and Yamaguchi-Shinozaki, 2007). The exact mechanism by which plants sense changes in cellular osmotic stress is unknown (Bartels and Sunkar, 2005). However, there is evidence that transmembrane osmosensors such as the histidine kinase AtHK1 may sense changes in osmotic potential, and that membrane proteins such as aquaporins may respond to changes in the physical membrane structure (Urao *et al.*, 1999; Tyerman *et al.*, 2002). Initial stress perception triggers signal transduction processes, including mitogen-activated protein kinases (MAPK) and Ca_{2+}-dependent protein kinase (CPK) cascades (Chaves *et al.*, 2003; Bartels and Sunkar, 2005; Zhang *et al.*, 2006). These function through a reversible chain of protein phosphorylation events, and are frequently used by eukaryotes as a mechanism for relaying external signals to cellular control systems. Following drought stress

perception, transcriptional changes occur within plant cells and genes encoding two types of protein are induced: 1) regulatory proteins, which further control the stress response and orchestrate downstream processes. These include transcription factors, protein kinases, protein phosphatases, enzymes involved in ABA synthesis and other signalling molecules; 2) functional proteins, which act directly to provide cellular stress tolerance through osmotic adjustment and the protection of membranes and proteins. These include heat shock proteins, late embryogenesis abundant (LEA) proteins, enzymes for osmolyte biosynthesis, water channel proteins and sugar and proline transporters (Chaves *et al.*, 2003; Wang *et al.*, 2003; Shinozaki and Yamaguchi-Shinozaki, 2007).

1.2.3.1 The ABA-mediated drought response

Most drought-inducible genes are regulated by the hormone ABA, which is synthesised *de novo* in response to drought stress and plays a crucial role in stress signalling (Shinozaki and Yamaguchi-Shinozaki, 2007). The gene encoding the key enzyme in ABA biosynthesis, 9-cis-epoxycarotenoid dioxygenase (NCED), is induced rapidly following stress, and when over-expressed in *A. thaliana* confers drought tolerance (Iuchi *et al.*, 2001; Seki *et al.*, 2007). The protein phosphatases 2C (PP2C) ABI1 and ABI2 are known to be negative regulators of ABA signalling. Specific point mutations in these genes (e.g. *abi1-1* or *abi2-1*) can cause ABA-insensitivity through alterations in their post-transcriptional regulation (Leung *et al.*, 1997). A model for ABA perception has recently been proposed whereby the binding of ABA into a receptor protein PYR/PYL/RCAR releases the PP2C proteins from inhibition of SnRK2 protein kinases, which go on to activate downstream targets including the leucine zipper (bZIP) transcription factors AREB1 (abscisic acid-responsive element binding protein 1) and ABF (ABRE binding factor) (Pardo, 2010). These two ABA-responsive transcription factors bind to a *cis*-acting element ABRE (abscisic acid-responsive element) in downstream drought response genes such as *RD29B*, thus activating their transcription and causing a range of physiological changes (Figure 1.1) (Yamaguchi-Shinozaki and Shinozaki, 1994; Uno *et al.*, 2000). ABA promotes synthesis of the drought-inducible transcription factors MYC2 and MYB2. These bind to promoters of target genes in downstream ABA-responsive genes as well as those in the jasmonic acid-mediated wounding and pathogen response pathway, providing one of the many points of interaction between the signalling pathways of these two hormones (Abe *et al.*, 2003; Anderson *et al.*, 2004). Another ABA-inducible gene, encoding the NAC

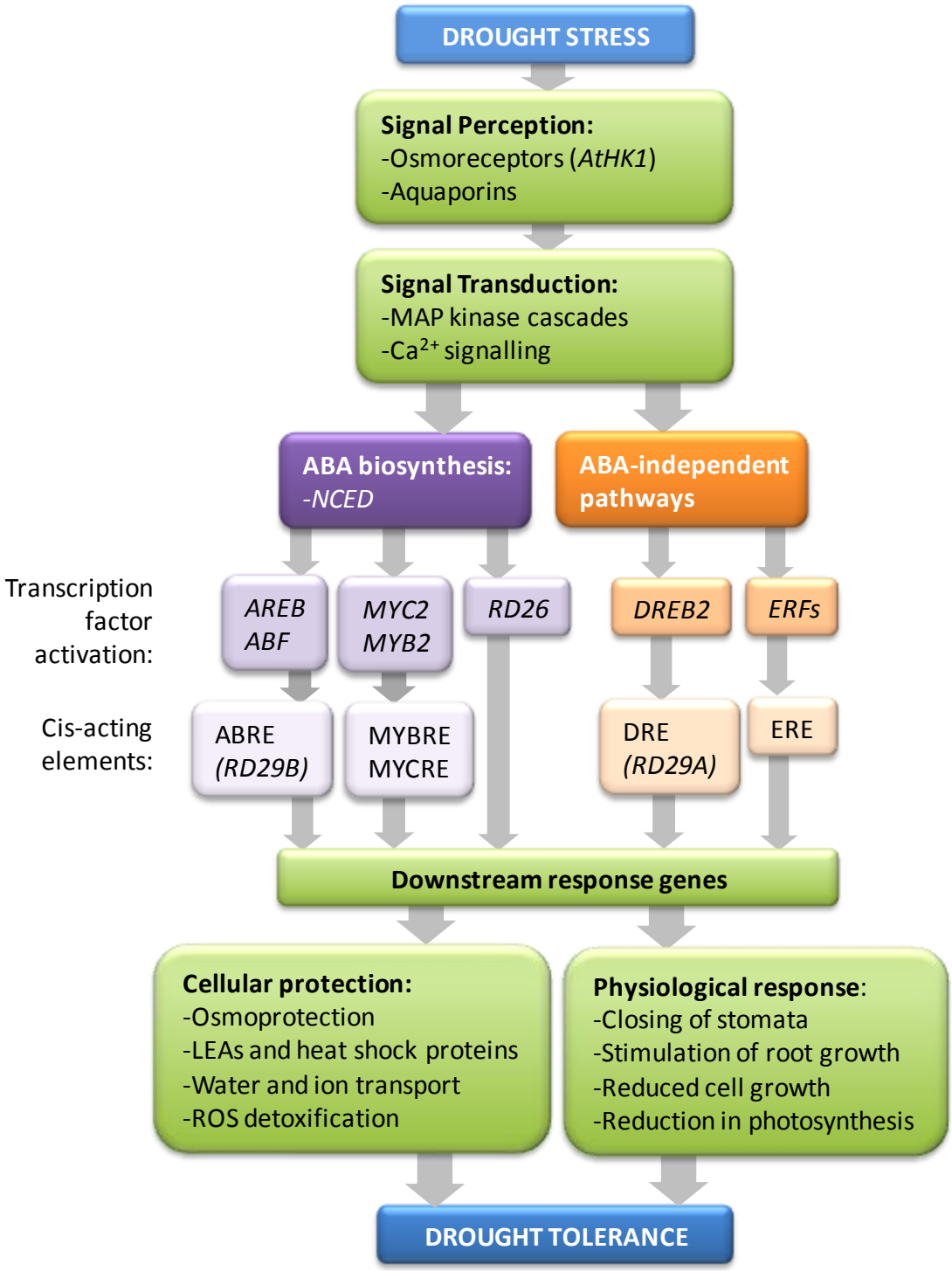


Figure 1.1. Drought stress response pathways. The perception of drought stress by a plant triggers signal transduction cascades and activates ABA-dependent and ABA-independent pathways. Various transcription factors are induced that bind to specific -*cis*-acting elements in down-stream response genes. These cause a range of cellular and physiological responses with the effect of protecting plant tissues against damage and allowing drought tolerance.

transcription factor RD26, is also activated by both ABA and jasmonic acid (Fujita *et al.*, 2004). This ABA-mediated induction of stress-responsive genes has been shown to be achieved partly through epigenetics (Chinnusamy *et al.*, 2008). Histones that are associated with the DNA become acetylated by ABA causing a conformational change in the chromatin, and allowing transcriptional machinery to access the promoters of genes such as *RD29A* and begin transcription. An additional stress signalling pathway that is dependent on ethylene has been discovered, which acts using a similar mechanism to the ABA-dependent pathway. Ethylene response factors (ERFs) when activated by stresses such as drought, salt and cold, trigger signal cascades by binding to ethylene response elements (EREs) in downstream genes. This pathway is thought to interact with the ABA-dependent one to control how plant organs respond to drought (Fujimoto *et al.*, 2000).

1.2.3.2 ABA-independent drought stress signalling

Genes have been identified that are induced by drought stress in the absence of a functioning ABA signalling pathway (Figure 1.1) (Shinozaki and Yamaguchi-Shinozaki, 2007). These ABA-independent drought-responsive genes have a conserved *cis*-acting element in their promoters known as a dehydration responsive element (DRE). Transcription factors of the AP2 family called dehydration responsive element binding proteins (DREBs), also known as C-repeat binding factors (CBFs), were identified that bind to the DRE sequences (Shinozaki and Yamaguchi-Shinozaki, 2007). DREB1 transcription factors are induced by cold stress, whilst DREB2s are induced by high salinity and dehydration (Liu *et al.*, 1998). The over-expression of *AtDREB1* in transgenic plants resulted in tolerance to freezing, drought and salt stresses. *AtDREB2*, however, only improved drought tolerance transgenically when over-expressed in its activated form, which occurs under abiotic stress as a result of post-translational modification (Sakuma *et al.*, 2006). A downstream effector gene that is induced by drought, cold and salinity, *RD29A*, contains both ABRE and DRE elements in its promoter, highlighting the inter-relatedness of the two pathways (Shinozaki and Yamaguchi-Shinozaki, 2007). *DREB* gene homologues have been identified in other species and play a similar role. In rice the four genes *OsDREB1A-D* are responsive to cold whilst *OsDREB2A* is induced by dehydration (Dubouzet *et al.*, 2003). In soybean *GmDREB2* is up-regulated by both cold and dehydration (Chen *et al.*, 2007). DREB-like factors have also been discovered in wheat, barley, tomato, pepper and millet

(Agarwal *et al.*, 2007), showing that the ABA-independent drought response pathway is highly conserved between both monocotyledonous and dicotyledonous plants.

1.2.3.3 The cellular and biochemical response to drought stress

Following activation of the multigene stress response system described above, various compounds are synthesised to maintain cell turgor and protect proteins from osmotic damage. The amino acid proline is a compatible solute that interacts with water molecules and acts to stabilise protein structures and membranes. It accumulates rapidly with drought and osmotic stress and is highly correlated with drought tolerance (Chaves *et al.*, 2003; Seki *et al.*, 2007). Hydrophilic globular proteins known as late embryogenesis abundant (LEA) make up the majority of stress-responsive proteins. Their transcription is ABA-responsive, and the proteins are thought to function in stabilising enzymes and membrane structures (Wang *et al.*, 2003; Bartels and Sunkar, 2005). Heat shock proteins (HSPs) act to bind and stabilise proteins that have become denatured during stress conditions. They also function as molecular chaperones which prevent protein aggregation (Bartels and Sunkar, 2005). Specific combinations of HSPs are induced following different types of abiotic stress, and are thought to play an important role in protecting plants from oxidative stress (Wang *et al.*, 2003; Rizhsky *et al.*, 2004). Other functional molecules produced to stabilise the structure and activity of proteins include glycine betaine, a methylated ammonium compound which creates a hydrating shell around macromolecular compounds; aquaporins, which facilitate osmosis by increasing water permeability of the cell membrane; and osmo-protecting sugars such as trehalose and mannitol (Wang *et al.*, 2003; Seki *et al.*, 2007). The abiotic stresses drought, heat and high salinity cause the build-up of reactive oxygen species (ROS) such as hydroxyl radicals, singlet oxygen, hydrogen peroxide and superoxide anion radicals, which are largely generated in the chloroplasts due to excess excitation energy and have damaging effects on membranes and macromolecules (Smirnoff, 1993; Bartels and Sunkar, 2005). Antioxidants are therefore produced by plants following stress to allow ROS detoxification. These include the enzymes superoxide dismutase, ascorbate peroxidise and catalases, and other small antioxidant molecules such as glutathione, carotenoids and anthocyanins (Noctor and Foyer, 1998; Wang *et al.*, 2003; Gadjev *et al.*, 2006).

1.3 Biotic stress

1.3.1 Pathogen recognition and signalling

Plants are constant targets for a broad range of herbivores and pathogens, and the study of biotic stress responses is fundamental for controlling plant disease in agriculture (Hammond-Kosack and Jones, 2000). Plants have developed sophisticated mechanisms for responding to pathogen attack and as a result, most plants are resistant to most pathogens (Dangl and Jones, 2001). As a result of pathogen infection plants activate both non-specific basal defence responses designed to limit pathogen spread, as well as specific responses tailored to individual types of pathogen (Figure 1.2). The basal defence response is triggered by the recognition of pathogen-associated molecular patterns (PAMPs) such as flagellins, and is known as PAMP-Triggered Immunity (PTI) (Pieterse *et al.*, 2009). In contrast, specific responses are governed by plant resistance genes called R-genes. These activate downstream defence mechanisms more efficiently than the basal response. When pathogens attack, the products of avirulence (*Avr*) genes in those organisms are recognised by specific R- gene products in the plant, leading to effective pathogen resistance termed an incompatible interaction (Jones *et al.*, 2006). If either the R-gene or the *Avr* gene is missing, then a compatible interaction ensues and disease occurs (Kaloshian, 2004). R-genes are highly polymorphic and are encoded by approximately 100 loci in *Arabidopsis* (Kaloshian, 2004; Jalali *et al.*, 2006). This allows a high level of specificity in recognising pathogens. Most R-genes contain a nucleotide-binding site (NBS) followed by several leucine-rich repeats (LRR) (Dangl and Jones, 2001). Following pathogen recognition various hormone-mediated signalling pathways are activated leading to a local and a systemic response and eventually the up-regulation of genes involved in defence, as shown in Figure 1.2 (Kaloshian, 2004). Transcription factors play an important role in modulating defence signalling within plants. In particular members of the WRKY, MYB, MYC, NAC and ERF families are all up-regulated in response to different biotic stresses (Jalali *et al.*, 2006). Induced defence responses include localised cell death (known as the hypersensitive response), strengthening of the cell wall by callose or lignin deposition and the production of antimicrobial secondary metabolites such as phenylpropanoid compounds, phytoalexins and glucosinolates (Jalali *et al.*, 2006; Bruce and Pickett, 2007; Pieterse *et al.*, 2009).

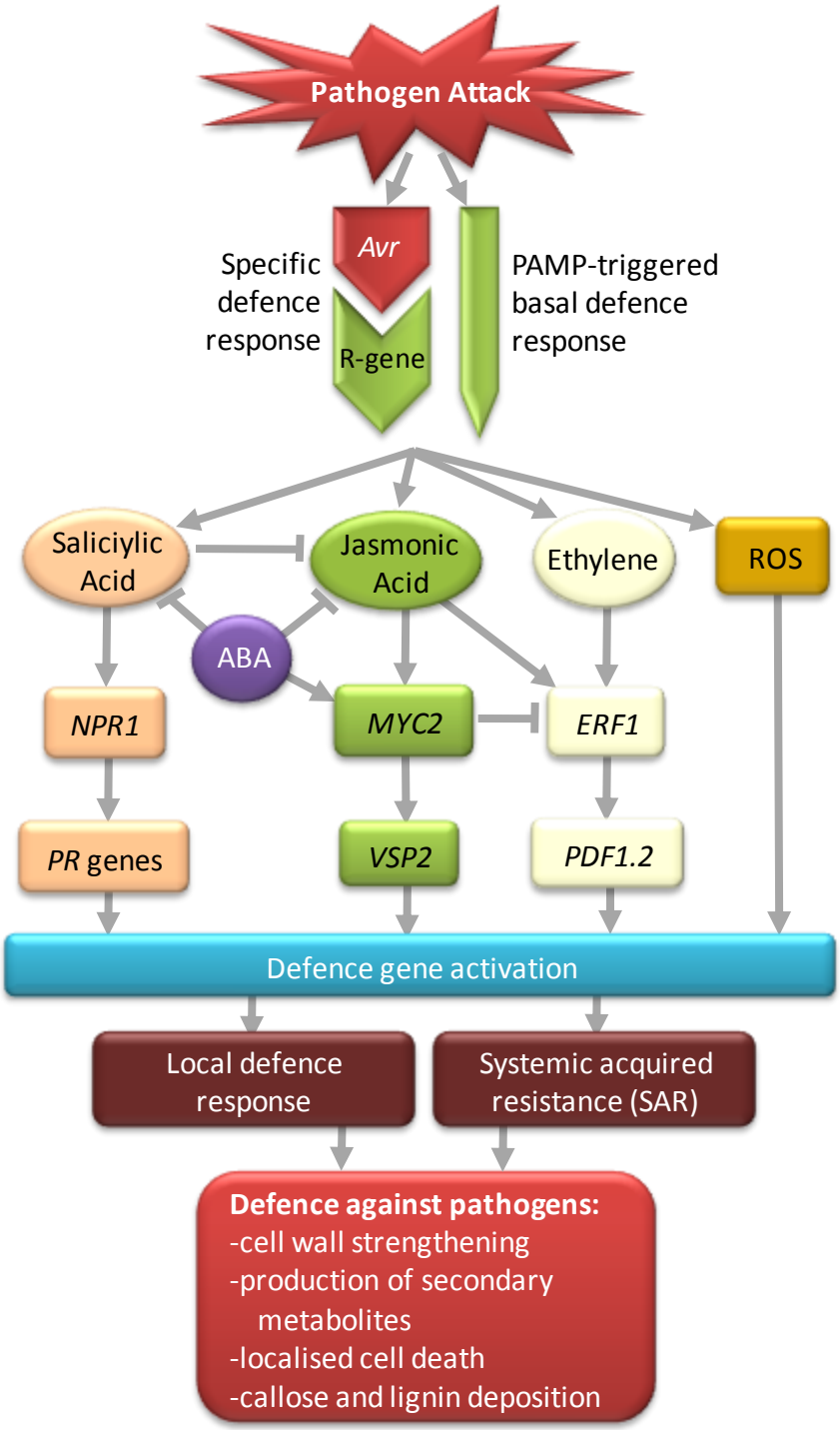


Figure 1.2. Biotic stress signalling and defence pathways. The model shows the interaction of hormones and down-stream signalling elements. Arrows indicate induction and bars indicate inhibition. Following infection basal PAMP- (pathogen-associated molecular patterns) triggered immunity is activated. When the product of a pathogen *avirulence* gene is recognised by an R-gene product, a specific pathogen response occurs which activates plant defences more efficiently. The hormones jasmonic acid, salicylic acid and ethylene are produced as well as ROS, and interact to create a pathogen specific response, inducing transcription factors and downstream defence genes and leading to a local and systemic defence response. ABA mainly inhibits biotic stress signalling despite positively regulating *MYC2.*

1.3.2 Hormones in plant defence

Defence responses are largely mediated through the accumulation of the phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene. The precise interplay of these compounds can dictate the nature of the defence response induced, allowing specificity to different types of pathogen (De Vos *et al.,* 2005; Pieterse *et al.,* 2009). The dissection of hormone-mediated defence pathways has been enabled through analysis of hormone signalling mutants (Figure 1.2) (Jalali *et al.,* 2006). Salicylic acid accumulates locally during pathogen attack as well as systemically. Immediately following pathogen recognition by R-gene products, the expression of signal molecules *EDS5, SID1* and *PAD4* is induced. This leads to SA production, which through expression of the transcriptional activator *NPR1* causes activation of downstream resistance genes such as pathogenesis-related (PR) genes. These can be used as markers of SA signalling (Cao *et al.*, 1997; Kaloshian, 2004; Jalali *et al.,* 2006; van Loon *et al.,* 2006). Mutants deficient in SA signalling show susceptibility to pathogens such as *Pseudomonas syringae*. In addition to the local pathogen-induced defence response, a mobile signal travels to distal parts of the plant where SA again accumulates, establishing a distal defence response to protect remote parts of the plant from secondary infection (Bostock, 2005). This protection system is known as systemic acquired resistance (SAR). The identity of the SAR signal has long been in question (Heil and Ton, 2008), but is now thought to be azelaic acid, a mobile metabolite that primes tissues to accumulate SA (Jung *et al.*, 2009; Parker, 2009).

Jasmonic acid (JA) is an oxylipin that is rapidly produced by plants in response to mechanical wounding or insect herbivory (Koo and Howe, 2009). It has a key role in defence, and when applied exogenously it can protect plants from herbivore attack (Baldwin, 1998). JA acts through activation of the transcription factors MYC2 and ERF1 to induce the transcription of downstream defence genes such as *PDF1.2* and *VSP2* (Koo and Howe, 2009; Pieterse *et al.,* 2009). There is evidence that JA is also essential for and in fact mediates the long-distance SAR signal (Truman *et al.,* 2007). JA and ethylene function synergistically in defence signalling, activating the same downstream defence genes and providing resistance to necrotrophic pathogens such as *Botrytis cinerea* and *Erwinia carotovora*. In contrast, SA mediates the response to biotrophic pathogens such as *P. syringae* (Anderson *et al.*, 2004; Pieterse *et al.,* 2009). Mutants deficient in either JA or ethylene signalling are susceptible to pathogens (Jalali

et al., 2006). The interaction between the SA defence pathway and the JA-ethylene pathway is mainly antagonistic, as SA-induced transcription factors can suppress JA-dependent gene expression, whilst JA-induced MYC2 is involved in the suppression of the SA response (Pieterse *et al.*, 2009). The complex crosstalk between defence-induced hormone signalling pathways becomes increasingly more apparent as further studies are carried out.

1.3.3 Plant-parasitic nematodes

An example of a biotic stress that has a major impact agriculturally is infestation by plant-parasitic nematodes. These pathogens can be studied extensively in the laboratory and thus provide an excellent model for biotic stress in plants. Nematodes are found throughout the world and infect almost every species of crop plant, causing a global loss of over $125 billion per year (Bird and Kaloshian, 2003). The most advanced plant-parasitic nematodes are biotrophic sedentary endoparasites, which invade and migrate through the root before initiating specialised feeding cells and becoming sedentary. Among these are the root-knot nematodes (e.g. *Meloidogyne* species) and the cyst nematodes (e.g. *Heterodera* and *Globodera* species). The host-range of cyst nematodes tends to be very specific, with the potato cyst nematode *Globodera pallida* only able to infect three major crops in the *Solanaceae* family, namely potato, tomato and aubergine (Turner and Rowe, 2006). *Meloidogyne* species are generally the most promiscuous with respect to host range, infecting a wide range of crops. This may account for *Meloidogyne* being the most damaging of plant-parasitic nematodes in terms of yield loss worldwide (Bird and Kaloshian, 2003). Symptoms of nematode infestation are generally characteristic of a plant with a damaged or malfunctioning root system, including reduced shoot growth and biomass accumulation, nutritional deficiencies that are evident in the foliage, chlorosis, temporary wilting, reduced photosynthesis and suppressed yields (Bird, 1974; Trudgill and Cotes, 1983; Trudgill *et al.*, 1990; Hammond-Kosack and Jones, 2000). Nematodes rarely cause the death of a plant, but they can severely affect plant water relations (Haverkort *et al.*, 1991; Smit and Vamerali, 1998). This becomes particularly apparent during times of water stress, whereby plants in nematode-infested soil may suffer greater yield loss (Audebert *et al.*, 2000). *A. thaliana* is a useful model for the study of plant-nematode interactions, allowing successful parasitism by several nematodes including the cyst nematode *Heterodera schachtii* and the root-knot nematode *Meloidogyne incognita* (Sijmons *et al.*, 1991).

1.3.3.1 The life cycle of plant-parasitic nematodes

Root-knot and cyst nematodes have similar life cycles but differ in their method of infection. Infective juveniles (J2s) of both migrate through the soil following the chemical gradient of root diffusates and enter plant roots near the tip. Cyst nematodes such as *Globodera pallida* then migrate intra-cellularly until they reach the differentiating vascular tissue. Migration is achieved using continued thrusts of a needle-like protrusible stylet at the nematode's anterior to rupture the plant cell wall and provide an opening through which the J2 can move, a process causing significant disruption to the plant tissue (Williamson and Hussey, 1996; Turner and Rowe, 2006). A suitable feeding cell is selected at the periphery of the vasculature into which the nematode injects secretions from the oesophageal glands. This causes re-differentiation of the cell into a large syncytium, a metabolically active cell with a dense granular cytoplasm (Jones, 1981). Up to 200 surrounding cells may be incorporated into the syncytium by partial dissolution of the cell walls and fusion of the protoplasts (Wyss and Grundler, 1992; Williamson and Hussey, 1996). The nematode ingests solutes from the feeding cell at frequent intervals using its stylet, undergoing three moults of 3–4 days before reaching the adult stage. The motile adult male then leaves the root while the female remains sedentary, her body swelling into a saccate shape and rupturing the root cortex (Turner and Rowe, 2006). The vermiform male is attracted to the female by the release of pheromones, and fertilises the eggs. Following this the female body tans to become a hardened cyst containing up to 500 eggs. This cyst eventually becomes detached when the root dies and can remain in the soil for up to 20 years until conditions become optimal for hatching (Williamson and Hussey, 1996; Turner and Rowe, 2006). The life cycle of a cyst nematode takes around 30 days for most species, although can vary from 15 days to up to 90 days depending on species, temperature and host plant. The life cycle is summarised in Figure 1.3.

Root-knot nematodes such as *Meloidogyne incognita* are less destructive in their invasion, as they migrate through the root inter-cellularly. When reaching the zone of cell division within the vascular cylinder each J2 establishes a permanent feeding site from a vascular parenchymal cell, known as a 'giant cell'. At this point the nematode loses musculature and becomes sedentary (Bird and Kaloshian, 2003). Giant cell re-differentiation is caused by nematode secretions injected into the cell through the stylet. The cells rapidly become multinucleate and enlarged through numerous rounds of

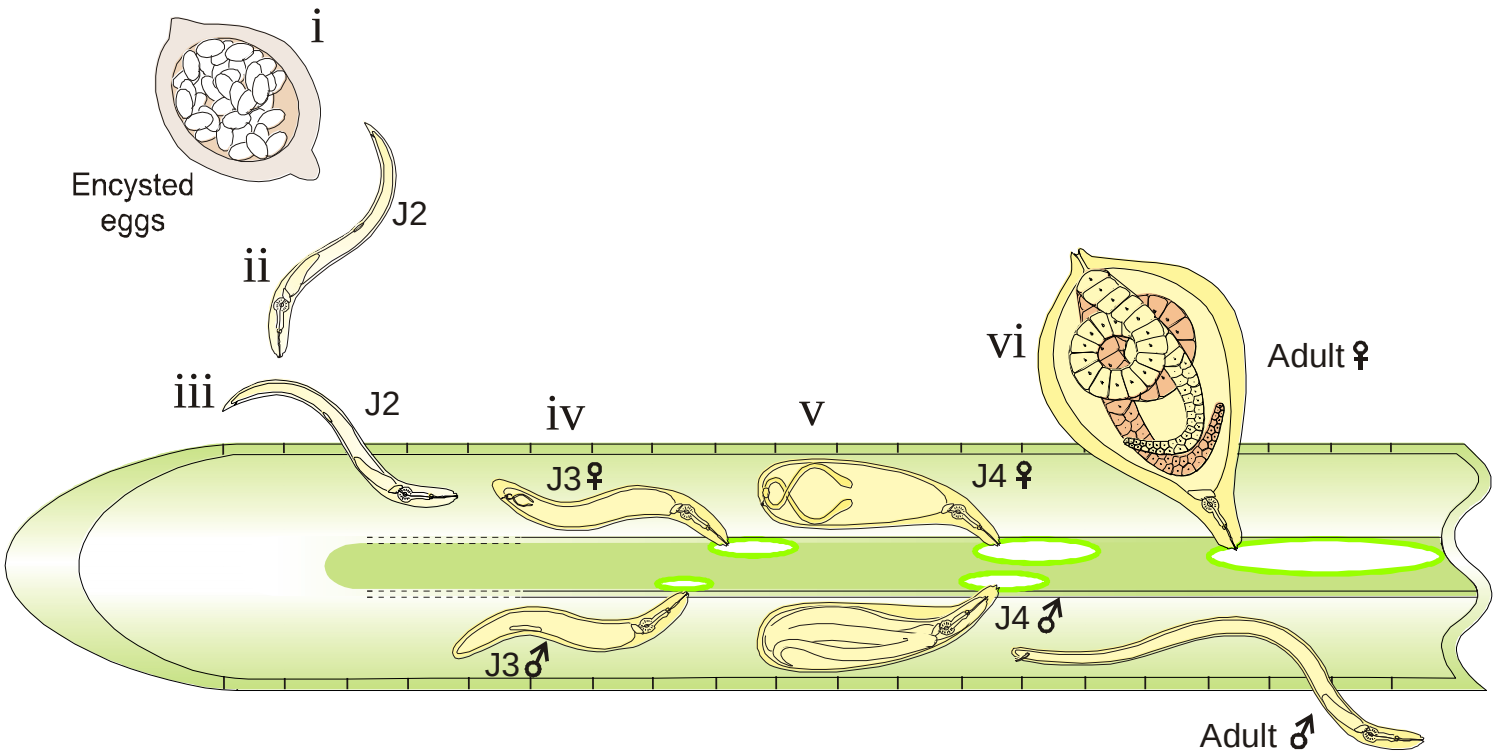


Figure 1.3. Life cycle of a cyst nematode. The life stages of a cyst nematode e.g. *Heterodera schachtii* are shown (from Lilley *et al.*(2005)).**(i)** Encysted eggs remain dormant in soil. **(ii)** Juveniles (J2) hatch under favourable conditions and are attracted towards a root. **(iii)** J2s enter the root tip and migrate intracellularly to the vascular cylinder. **(iv & v)** Nematodes establish feeding sites. The vermiform male develops and stops feeding. **(vi)** The male leaves the root and fertilizes the female, who grows and detaches from the root, forming a cyst containing the eggs.

mitosis without cytokinesis (Bird, 1996). The giant cells are used by the nematode as a nutrient sink, and their development is accompanied by the growth and division of root cortical and pericycle cells around the nematode, leading to the characteristic gall or 'root-knot' associated with these nematodes (Williamson and Hussey, 1996; Karssen and Moens, 2006). Feeding ceases when the nematode undergoes its first moult into the J3 stage and does not start again until the nematode has undergone two more moults in rapid succession and emerged as an adult female. When mature, females release several hundred eggs onto the surface of the root in a gelatinous matrix, from which the juveniles hatch. Juveniles at this stage can remain several weeks or months in the soil (Wyss and Grundler, 1992; Williamson and Hussey, 1996). The length of the *M. incognita* life cycle is host-dependent and typically completed in three to eight weeks.

1.3.3.2 Plant response and resistance to nematode infection

The establishment of nematode feeding sites within plant roots is associated with large-scale gene induction events within plant cells. In addition to this, the plant mounts both basal defence responses and where present, R-gene mediated resistance (Gheysen and Fenoll, 2002; Lilley *et al.*, 2005). Transcriptional changes in feeding cells are likely to be induced by secretions from the nematode dorsal gland. Secretions contain proteins that have an effect on plant cell cycle and cell division, as well as small signalling molecules that may induce the expression of plant genes to allow development of feeding sites (Goverse *et al.*, 1999; Gheysen and Fenoll, 2002; Wang *et al.*, 2005). Up-regulated plant genes in both cyst and root-knot nematode parasitism include cell cycle genes, cell wall modification, transcription factors, general metabolism, water transport and auxin response genes (Gheysen and Fenoll, 2002; Puthoff *et al.*, 2003; Jammes *et al.*, 2005). There is evidence that the development of nematode feeding sites requires intact auxin and ethylene response pathways, as auxin-insensitive and ethylene-insensitive mutants of *A. thaliana* are resistant to infection by *Heterodera schachtii* (Goverse *et al.*, 2000; Wubben *et al.*, 2001). Nematode secretions may actively manipulate auxin transport and production in feeding cells (Goverse *et al.*, 2000; Lilley *et al.*, 2005; Grunewald *et al.*, 2009). Auxin may also be important for nematode navigation through roots, and in the induction of giant cells by *Meloidogyne* sp., a process also associated with the production of flavonoids, which can act as regulators of auxin transport (Hutangura *et al.*, 1999; Curtis, 2007).

Infection with plant-parasitic nematodes activates the SA-mediated basal pathogen response system (Gheysen and Fenoll, 2002). In *A. thaliana,* the induction of pathogenesis-related (PR) genes is observed in plants infected with both *H. schachtii* and *M. incognita*, and in tomato a similar response occurs during parasitism with *M. incognita* and *M. javanica* (Bar-Or *et al.*, 2005; Sanz-Alferez *et al.*, 2008; Wubben *et al.*, 2008; Hamamouch *et al.*, 2011). Treatment with exogenous SA causes resistance to both cyst and root-knot nematodes in a variety of plant species whilst *A. thaliana* mutants deficient in SA signalling become hyper-susceptible to cyst nematodes (Wubben *et al.*, 2008; Gutjahr and Paszkowski, 2009; Molinari and Baser, 2010). These results confirm that plants activate SAR in response to nematode infection. However, it has also been proposed that nematodes may actually suppress SA-mediated plant defences, as in certain cases *PR* genes have been shown not to change or even to be down-regulated in local nematode-infected tissues (Jammes *et al.*, 2005; Ithal *et al.*, 2007a; Ithal *et al.*, 2007b; Wubben *et al.*, 2008; Hamamouch *et al.*, 2011). Several genes conferring natural resistance to nematodes have been cloned, namely $Hs1^{pro\text{-}1}$ from the wild relative of sugar beet *Beta procumbens*, *Gpa2* and *Gro1-4* from potato, *Hero A* from the wild relative of tomato *Solanum pimpinellifolium*, *rhg1* and *Rhg4* from soybean, and *Mi-1.2* from *S. peruvianum,* another wild relative of tomato (Cai *et al.*, 1997; Williamson, 1998; Concibido *et al.*, 2004; Sobczak *et al.*, 2005). These genes have been described in more detail by Fuller *et al.* (2008). When present R-genes cause an incompatible reaction often preventing the nematode from establishing a feeding site (van der Biezen and Jones, 1998). The *Mi-1.2* gene confers resistance to *Meloidogyne incognita* as well as potato aphid, and is dependent on the hormone salicylic acid for its response (Li *et al.*, 2006). The *Hero A* gene confers over 95% resistance to *Globodera rostochiensis* and over 80% resistance to *G. pallida* in tomato (Williamson and Hussey, 1996). When nematode infection occurs *Hero A* becomes up-regulated causing necrosis of the cells around the syncytium, which in turn stops the nematodes developing. The discovery and cloning of new nematode R-genes may create possibilities for transferring resistance to other crops, although as yet there are very few examples of the successful transfer of R-genes between species (Atkinson *et al.*, 2003). The transgenic expression of anti-feedant molecules such as cysteine proteinase inhibitors has proved effective in conferring resistance against several types of nematodes including *G. pallida, M. incognita, H. schachtii, Rotylenchulus reniformis, Radopholus similis* and *Pratylenchus penetrans* in crops such as potato (Urwin *et al.*, 2001), cavendish bananas

(Atkinson *et al.*, 2004) and rice (Vain *et al.*, 1998). Progress in developing transgenic resistance to nematode parasitism has been recently reviewed by Fuller *et al.* (2008).

1.4 Stresses in combination

1.4.1 The effect of multiple stress factors on plants

The effect of individual stress factors on plants and the molecular process controlling their responses have studied extensively. However, in field environments plants are often subjected to multiple concurrent stresses. A report by Mittler *et al.* (2006) described that in the US between 1980 and 2004 the total agricultural losses attributed to drought were worth $20 billion, but total losses due to drought combined with a heat wave totalled $120 billion, suggesting that the presence of a second stress factor can exacerbate the detrimental effects of the first. Plants have a high level of precision in sensing and responding to the specific environmental conditions encountered, allowing them to acclimate accordingly. Recent transcriptome analysis has shown that the molecular response of plants to multiple stresses is not additive, but results in a new pattern of gene expression that could not have been predicted by studying either stress individually (Rizhsky *et al.*, 2002; Rizhsky *et al.*, 2004). It has thus been proposed that each specific combination of stresses should be treated as a new type of stress, and studied accordingly (Mittler, 2006; Mittler and Blumwald, 2010). This is particularly important for the development of stress-tolerant crops, as plants that have been produced with enhanced tolerance to one particular stress may respond unpredictably when grown in field conditions.

Plants need to produce a tailored response to specific multiple stress conditions, as in many cases the individual stresses would normally elicit opposing reactions. For example, heat stress usually causes plants to open their stomata in order to cool the leaves, but under drought conditions this would be disadvantageous as more water would be lost (Rizhsky *et al.*, 2004). Similarly, increased transpiration caused by heat stress could enhance uptake of salt or heavy metals, heightening the damage from these factors (Mittler and Blumwald, 2010). The interaction between biotic and abiotic stresses presents an added degree of complexity, as the responses to these are largely controlled by different hormone signalling pathways which may interact and inhibit one another (Anderson *et al.*, 2004; Asselbergh *et al.*, 2008b). Most often, the exposure of plants to a pest or pathogen increases the effects of an abiotic stress such as water deficit

(Cockfield and Potter, 1986; Englishloeb, 1990; Khan and Khan, 1996; EnglishLoeb *et al.*, 1997; Smit and Vamerali, 1998; Audebert *et al.*, 2000), whilst in turn long-term abiotic stress can weaken plant defences and cause enhanced pathogen susceptibility (Amtmann *et al.*, 2008; Goel *et al.*, 2008; Mittler and Blumwald, 2010). For example, treatment of *A. thaliana* with drought stress allows greater infection levels of an avirulent isolate of *Pseudomonas syringae* (Mohr and Cahill, 2003). However, the presence of a biotic or abiotic stress may also have a positive effect on tolerance to the other (EnglishLoeb *et al.*, 1997; Anderson *et al.*, 2004; Wiese *et al.*, 2004; Asselbergh *et al.*, 2008a). For instance, infection with cucumber mosaic virus can improve drought and freezing tolerance in beets and tobacco species (Xu *et al.*, 2008), whilst drought stress can enhance resistance to the fungus *Botrytis cinerea* in tomato (Achuo *et al.*, 2006).

Considerable research has focussed on the development of plant varieties that are resistant to stress. The transgenic over-expression of stress-inducible genes such as *DREB1A* can be used to confer abiotic stress tolerance to a range of plants including *A. thaliana,* maize, wheat and rice (Kasuga *et al.*, 1999; Pellegrineschi *et al.*, 2004; Oh *et al.*, 2005; Al-Abed *et al.*, 2007; Bhatnagar-Mathur *et al.*, 2008), whilst resistance to pathogens has been achieved by manipulation of the SA-signalling pathway (Jung *et al.*, 2009; Pieterse *et al.*, 2009; Bechtold *et al.*, 2010; Hamamouch *et al.*, 2011). The effectiveness of these stress-tolerant plants when exposed to different or multiple stresses, however, is not well documented. The interaction between stress signalling pathways may interfere with induced tolerance mechanisms, perhaps providing an explanation for why some stress-tolerant plants fail to show the same level of tolerance when tested in field conditions (McKersie *et al.*, 1999; Mohamed *et al.*, 2001; Mittler, 2006). For example, the osmoprotectant proline accumulates in plant tissues in response to drought stress, and transgenic plants over-expressing a proline biosynthesis enzyme (P5C) have been developed that are resistant to osmotic stress (Kishor *et al.*, 1995; Bray *et al.*, 2000; Chaves *et al.*, 2003). However, Rizhsky *et al.* (2004) found that under a combination of drought and heat stress plant cells accumulated sucrose instead of proline, perhaps to protect hyper-active and therefore susceptible mitochondria from the build-up of potentially toxic P5C. Thus in the transgenic plants an added heat stress may counteract any osmoprotective benefit. Pathogen resistance may also be affected by differing abiotic conditions, as demonstrated by the inactivation of the nematode and aphid resistance gene *Mi-1.2* at temperatures above 28 ºC (Dropkin, 1969). An

increasing focus on the identification of multiple stress-tolerance genes is therefore needed for developing broad-spectrum stress tolerant traits for agriculture (Mittler, 2006; Fleury *et al.*, 2010; Mittler and Blumwald, 2010).

Many studies aimed at discovering multiple stress-responsive genes have been conducted by exposing plants to one stress or another and then comparing the lists of differentially regulated genes to find any commonalities (Seki *et al.*, 2002; De Vos *et al.*, 2005; Kilian *et al.*, 2007; Kant *et al.*, 2008). However, a landmark transcriptome study by Rizhsky *et al.* (2002) found that tobacco plants exposed to a combination of drought and heat respond very differently to drought stress or heat individually, activating an entirely new program of gene expression that was non-additive and could not have been predicted. A similar result was observed in *A. thaliana* (Rizhsky *et al.*, 2004). This confirms that in order to identify genes that are truly involved in multiple stress response, the stresses need to be applied simultaneously to the same plants (Mittler, 2006). Since then, several such studies have been carried out in various species, implicating certain genes, hormones and processes as important in controlling plant response to multiple stress and providing targets for the improvement of stress tolerance (Luo *et al.*, 2005; Hewezi *et al.*, 2008b; Priyanka *et al.*, 2010; Wang *et al.*, 2010; Grigorova *et al.*, 2011).

1.4.2 Interaction between biotic and abiotic stress signalling pathways

Plants must constantly balance the competing needs for growth and defence against environmental stresses. Growth and development is costly in resources but essential for reproduction and competition with other individuals. Defence against pathogens and environmental stresses requires a shifting of resources away from growth to produce secondary metabolites and other compounds that protect from stress (Herms and Mattson, 1992; Baldwin, 1998; Yasuda *et al.*, 2008). Response to a particular environmental insult requires a highly specific response, therefore plants activate stress response pathways that are most effective against the stress, whilst repressing defence responses that are unlikely to have a significant effect, in order to save valuable resources (Anderson *et al.*, 2004). Abiotic stresses such as drought often pose the greatest threat to plants' survival, therefore plants must be able to switch priority to respond to this stress at the expense of pathogen defence and growth (Asselbergh *et al.*, 2008b). This fine-tuning of stress response pathways may explain the non-additive effects observed when plants encounter multiple stresses, as described above, although

the exact mechanism for this is unknown. There is increasing evidence that stress signalling and response pathways interact and are controlled at the molecular level, a process governed by hormones, transcription factors, MAPK cascades and ROS (Anderson *et al.*, 2004; Fujita *et al.*, 2006; Asselbergh *et al.*, 2008b).

1.4.2.1 The role of hormones in controlling stress interaction

Abiotic stress responses are largely controlled by the hormone ABA whilst defence against biotic factors is specified by antagonism between the SA and JA/ethylene signalling pathways, as described in Sections 1.2.3.1 and 1.3.2. However, recent findings suggest that ABA acts both synergistically and antagonistically with biotic stress signalling, creating a complex network of interacting pathways with crosstalk at different levels (Fujita *et al.*, 2006; Asselbergh *et al.*, 2008b; Yasuda *et al.*, 2008). Treatment with ABA has been shown to increase susceptibility in *Arabidopis* to an avirulent *P. syringae* strain (Mohr and Cahill, 2003), in tomato to *B. cinerea* and *Erwinia chrysanthemi* (Audenaert *et al.*, 2002; Asselbergh *et al.*, 2008a), in rice to the blast fungus *Magnaportha grisea* (Koga *et al.*, 2004) and in potato to the pathogens *Phytophthora infestans* and *Cladosporium cucumerinum* (Henfling *et al.*, 1980). In contrast, a lack of ABA can cause a high level of pathogen resistance (Asselbergh *et al.*, 2008a). For example, the ABA-insensitive *A. thaliana* mutants *abi1-1* and *abi-2-1* showed resistance to the oomycete *Peronospora parasitica* and the fungal pathogen *Fusarium oxysporum*, respectively (Mohr and Cahill, 2003; Anderson *et al.*, 2004), whilst the tomato *sitiens* mutant which has reduced ABA levels showed enhanced tolerance to *B. cinerea* (Audenaert *et al.*, 2002). ABA treatment has been shown to repress the systemic acquired resistance (SAR) pathway both upstream and downstream of SA induction as well as inhibiting the accumulation of crucial defence compounds such as lignins and phenylpropanoids (Mohr and Cahill, 2007; Yasuda *et al.*, 2008). In return, SA can also interfere with abiotic stress signalling. The exogenous application of SA in maize leads to drought susceptibility (Nemeth *et al.*, 2002), whilst the artificial induction of SAR in *A. thaliana* leads to the suppression of abiotic stress responses (Yasuda *et al.*, 2008). Thus ABA is confirmed as a crucial regulator of pathogen response signalling. ABA also antagonises JA and ethylene defence signalling, as shown by the ABA-mediated repression of defence genes such as *PDF1.2*, an effect that cannot be reversed by the application of JA or ethylene (Anderson *et al.*, 2004). Ethylene treatment, in return, activates ABI1 and ABI1, two negative regulators of ABA signalling (Asselbergh *et al.*, 2008b). ABA is now considered a global regulator

of stress responses that can dominantly suppress pathogen defence pathways, thus controlling the switch in priority between the response to biotic or abiotic stress (Asselbergh *et al.*, 2008b).

1.4.2.2 Other molecular mechanisms controlling stress interaction

The hormone-regulated biotic and abiotic stress signalling pathways share several similar processes that may act as points of convergence and therefore control the specificity of stress responses (Mauch-Mani and Mauch, 2005). Transcription factors play a key role, of which MYC2 is thought to be most central. MYC2 is a positive regulator of JA-induced defence genes but represses genes induced by JA/ethylene signalling. It acts as a key repressor of the SA pathway. MYC2 has also been found to be activated by ABA, and *myc2* mutants lack ABA-responsive gene expression. Therefore MYC2 may act as a central regulator by which ABA controls biotic stress signalling pathways (Anderson *et al.*, 2004; Asselbergh *et al.*, 2008b; Pieterse *et al.*, 2009). This partial synergy between ABA and JA may explain the situations in which pathogen resistance is enhanced by abiotic stress, such as the finding that in barley drought stress increases resistance to *Blumeria graminis* (Wiese *et al.*, 2004), or that in *A. thaliana* ABA is necessary for defence responses against the oomycete *Pythium irregulare* (Adie *et al.*, 2007). The NAC transcription factor RD26 is induced by JA and pathogens as well as by ABA, drought and salinity (Fujita *et al.*, 2004; Fujita *et al.*, 2006). Findings suggest that RD26 may be involved in detoxification of ROS, providing a role for it in biotic and abiotic stress response. Other identified transcription factors that act in both pathways include tobacco TSI1, which when over-expressed confers pathogen and salt resistance (Park *et al.*, 2001); soybean ERF3, which confers resistance to salt, drought and pathogens in tobacco (Zhang *et al.*, 2009); *A. thaliana* BOS1, which is responsive to pathogen infection but is required for both biotic and abiotic stress responses (Mengiste *et al.*, 2003); and pepper RFP1, which functions as a defence regulator and confers osmotic stress tolerance (Hong *et al.*, 2007; Asselbergh *et al.*, 2008b). The manipulation of transcription factors provides one of the greatest opportunities for conferring multiple stress tolerance, as they control a wide range of downstream events (Pardo, 2010).

Mitogen-activated protein (MAP) kinase cascades are thought to be important in controlling crosstalk between different stress pathways, as each one can be activated in

response to more than one type of stress (Fujita *et al.*, 2006). In rice *MAPK5* is induced in response to ABA and causes the expression of genes leading to abiotic stress tolerance, at the same time negatively regulating pathogenesis-related (*PR*) genes involved in disease resistance (Zhang *et al.*, 2006). The rice MAPK gene *BWMK1* acts in both the pathogen response and wounding pathways (Hong *et al.*, 2007). MAPK cascades may be activated by reactive oxygen species (ROS) such as H_2O_2, which play a crucial role in signal crosstalk (Fujita *et al.*, 2006; Zhang *et al.*, 2006). ROS are important during pathogen response as well as accumulating as toxic side products of aerobic metabolism during abiotic stresses such as water deficit. Following pathogen infection, a large number of ROS are produced in a process known as the oxidative burst. This is thought to limit pathogen infection by contributing to cell death during the hypersensitive response, a process requiring the coordinated down-regulation of ROS scavenging mechanisms (Hammond-Kosack and Jones, 2000; De Gara *et al.*, 2003; Apel and Hirt, 2004; Fujita *et al.*, 2006). Plants have adapted to use ROS as stress signalling molecules (Fujita *et al.*, 2006). H_2O_2 is produced by membrane-bound NADPH-oxidases immediately on pathogen infection, then diffuses into cells and activates various plant defences. ROS also act as signals for ABA-induced stomatal closure during abiotic stress, and accumulate in tissues distal to pathogen infection in order to establish systemic immunity (Apel and Hirt, 2004). Recent evidence shows that Heat Shock Factors (HSFs) may act as molecular sensors to detect the presence of ROS and activate downstream stress-responsive genes (Miller and Mittler, 2006). These transcription factors and the Heat Shock Proteins (HSPs) they activate are highly specialised, with different HSF combinations activated by specific stresses or stress combinations (Rizhsky *et al.*, 2004; Miller and Mittler, 2006; Grigorova *et al.*, 2011). Research suggests they may be crucial in allowing plants to respond to different environmental conditions, and the over-expression of certain HSFs has been shown to confer resistance to multiple stresses (Nishizawa *et al.*, 2006).

1.4.3 The interaction of drought stress and nematode infection

Infection with plant-parasitic nematodes can exacerbate or ameliorate the effects of drought stress on plants, as their parasitism in roots severely disrupts plant water relations (Bird, 1974; Haverkort *et al.*, 1991; Smit and Vamerali, 1998). This has important implications for agriculture (Coyne *et al.*, 2001). Several studies have been carried out to examine the effect of combined nematode and drought stress on plant growth and development. In The Ivory Coast the nematode *H. schachtii* increased

drought-related losses in upland rice by contributing to reduced leaf water potential, stomatal conductance and leaf dry weight (Audebert *et al.*, 2000). A similar study investigated the effect of drought and the cyst nematode *G. pallida* on water use efficiency in potato. Both factors were found to negatively affect growth, although the combined effect was not additive, perhaps because the infected plants used less water, thus reducing drought stress (Haverkort *et al.*, 1991). *G. pallida* has also been shown to cause a retardation in potato root development, which in turn had the effect of reducing drought tolerance (Smit and Vamerali, 1998). However, field studies have often been unable to separate the effects of soil hydrology, irrigation and nematode community dynamics from the effects of nematode parasitism itself (Coyne *et al.*, 2001). To date no studies document the molecular interaction between drought stress and nematode infection in plants. As plants control the response to simultaneous biotic and abiotic stresses at the molecular level, it is clearly necessary to characterise the interacting molecular mechanisms in order to fully understand the relationship between these two stresses.

1.5 Project overview

This work aimed to characterise the molecular interaction between drought stress and nematode infection in plants. Initially, *A. thaliana* plants were exposed to either drought stress, nematode stress, or the two stresses in combination, under highly controlled laboratory conditions. A rapid dehydration treatment was used to elicit a drought stress response, whilst juvenile *H. schachtii* nematodes were used for infection. RNA was isolated from the roots and leaves of plants and examined in a microarray study using Affymetrix ATH1 GeneChips, allowing whole-genome transcriptome changes to be identified. Genes differentially regulated by each stress individually were compared against those documented in the literature. Genes were then identified that were specifically regulated by the two stresses in combination but not by either stress individually. Their gene ontology categories were explored, and over-represented groups identified. Further to this, ten genes of interest were selected for characterisation. These were genes most likely to play a key role in controlling the interaction between drought and nematode stress. Loss-of-function mutants were obtained for each of the ten genes and over-expression lines were created. The mutant lines were characterised under control conditions, and then tested for performance under one or both stress conditions in order to provide clues about gene function. The expression of candidate genes in hormone signalling mutants was also examined to establish their position in

known signalling pathways. In order to determine the effect of individual or combined stress on fruit nutritional compounds, tomato plants were exposed to drought stress, the nematode *M. incognita*, or a combination of the two stresses. The concentration of various nutritional compounds was measured in fruits from stressed plants, including carotenoids, flavonoids, chlorogenic acid and sugars. The effect on physiological and reproductive characteristics was also investigated.

Chapter 2. Characterising the response of *Arabidopsis thaliana* to combined nematode and drought stress

Aims

- Carry out physiological analysis of the effect of drought and nematode infection and their interaction on *A. thaliana* plants.
- Obtain RNA from root and leaf tissue of *A. thaliana* plants under individual or combined nematode and dehydration stress.
- Carry out microarray analysis on RNA using Affymetrix ATH1 GeneChip® arrays.
- Identify genes that are specifically regulated by a combination of dehydration and nematode stress.
- Verify microarray results and compare them to gene induction during drought stress using qRT-PCR.

2.1 Introduction

2.1.1 Analysing plant physiological and transcriptomic response to stress

There are several possible approaches to investigate the impact of environmental stresses on plants and the nature of the plants' response. Basic analysis of growth and physiological parameters of plants under stress can lead to conclusions about the mechanism of the stress response. In this way and by measuring populations under varying environmental conditions and pathogen load, important discoveries have elucidated the complexities of plant-nematode interactions (Bird, 1974; Wallace, 1974; Barker and Olthof, 1976; Vito *et al.*, 1986). More recently, detailed cellular, molecular and metabolomic analysis of roots and nematode feeding sites has provided information on the mechanism of parasitism and the production of hormones and metabolites by the plant in response (Jones, 1981; Glazer *et al.*, 1983; Wyss and Grundler, 1992; Williamson and Hussey, 1996; Bird and Kaloshian, 2003; Turner and Rowe, 2006). Physical measurements also play an important role in assessing the effect of drought or dehydration stress on plants. Water deficit has been assessed by measuring the water potential or relative water content (RWC) of the leaves, osmotic potential of the sap, turgor pressure of cells or through more modern techniques such as stomatal

conductance, which is a measure of gas exchange relating to photosynthesis (Barrs, 1968; Schulze, 1986; Rizhsky *et al.*, 2004). Growth characteristics such as fruit size, trunk diameter and leaf thickness are also measures of water deficit stress (Barrs, 1968). These techniques can determine comparative stress levels of plants that must be of the same species and in a similar environment. At the cellular level, our increasing understanding of guard cell function has provided insights into the regulation of plant water status (Schulze, 1986; Assmann and Wang, 2001), and the analysis of mutants deficient in normal plant water relations has since provided further key information on the hormones controlling the stress response (Tal and Imber, 1971; Quarrie, 1982; Leung *et al.*, 1997). These different investigatory techniques have also been used to characterise the effect of multiple stresses on plant physiology, for example in a study on the effect of *Heterodera schachtii* nematodes on drought-related losses in rice in the Ivory Coast (Audebert *et al.*, 2000), and when analysing water use efficiency and drought tolerance of potato plants under infestation by *Globodera pallida* (Haverkort *et al.*, 1991; Smit and Vamerali, 1998).

Molecular biology techniques and the widespread use of the polymerase chain reaction (PCR) allow researchers to characterise plants' response to encountered stress by analysing changes at the gene transcript level (Ingram and Bartels, 1996; Chaves *et al.*, 2003). Genes whose induction corresponds specifically to one type of biotic or abiotic stress have been identified and have become useful as marker genes for stress (Liu *et al.*, 1998; De Vos *et al.*, 2005; Sanz-Alferez *et al.*, 2008). For example, the dehydration-responsive element binding proteins (DREBs) are well-characterised transcription factors in the ABA-independent abiotic stress response pathway. In particular, the transcription of *DREB2* is rapidly induced in both root and shoots following dehydration stress, and can thus act as a marker gene for this type of stress, whilst *DREB1* is most highly induced by cold treatment (Liu *et al.*, 1998; Nakashima *et al.*, 2000; Agarwal *et al.*, 2006; Sakuma *et al.*, 2006). Marker genes are also useful in detecting a plant's response to nematode invasion. Pathogenesis-related (*PR*) genes have long been recognised as indicators of systemic acquired resistance (SAR) which is induced in response to infection by pathogens (Uknes *et al.*, 1992; Bowling *et al.*, 1994). These genes are induced in a salicylic acid-dependent manner following infection or treatment with chemical SAR inducers, although their exact role in pathogen response is unclear (Durrant and Dong, 2004; Yasuda *et al.*, 2008). Salicylic acid (SA) has been confirmed as important in plant-nematode interactions, a result of

observations that a reduction in SA allows greater nematode parasitism of *A. thaliana* by *H. schachtii*, whilst the pre-treatment of plants with exogenous SA gives a reduction in susceptibility (Wubben *et al.*, 2008). In response to infection by *H. schachtii*, *PR-1* is rapidly induced in leaves of *A. thaliana* plants, whilst *PR-2* and *PR-5* are induced in roots (Hamamouch *et al.*, 2011). It is proposed that SA accumulation and the induction of *PR-1* in roots may actually be suppressed as part of the down-regulation of defence genes induced by nematodes in feeding sites (Jammes *et al.*, 2005; Wubben *et al.*, 2008). Another recently identified marker of nematode infection is the dramatic up-regulation of the myo-inositol oxygenase genes *MIOX4* and *MIOX5* (40-fold and 400-fold, respectively), in syncytia (Siddique *et al.*, 2009; Szakasits *et al.*, 2009). In *A. thaliana* these genes are expressed almost exclusively in the pollen and nematode-infected root material, and are thus undetectable in uninfected roots.

As the quest to characterise plant stress responses in increasing detail continues, new techniques have become available whereby the transcript changes of thousands of genes can be measured in parallel. Transcriptomics have revolutionised the study of stress and also provide a basic platform from which to investigate other ‘omics such as metabolomics, proteomics, protein interactions and epigenetics (Denby and Gehring, 2005; Urano *et al.*, 2009; Deyholos, 2010; Urano *et al.*, 2010). Next-generation sequencing techniques such as high-throughput DNA sequencing, small RNA sequencing and DNA methylation sequencing have recently increased the capacity for the comprehensive analysis of genomes and transcriptomes, and allowed the technology to become more widespread and inexpensive (Shendure and Ji, 2008).

2.1.2 The use of microarray technology

Since the completion of the *A. thaliana* genome sequence in 2000 (Arabidopsis Genome Initiative, 2000), the range of molecular tools available to plant scientists has expanded at a dramatic rate, fuelling the determination to uncover the function of every gene and the interactions between them (Kennedy and Wilson, 2004). Microarrays represent a relatively simple platform by which to acquire a huge amount of information regarding regulation of genes and thus to draw inferences as to gene function. Their popularity has risen dramatically owing to their high-throughput method of creating a snapshot of the expression profile of a cell, monitoring tens of thousands of transcripts simultaneously (Kennedy and Wilson, 2004; Rockett and Hellmann, 2004; Busch and Lohmann, 2007). This information can be used in combination with other techniques to discover new

genes relating to a particular process, assess a plant's response to stress or environmental stimuli, discover natural variation between genotypes, or discover spatial and temporal patterns in normal gene expression (Lipshutz *et al.*, 1999; Busch and Lohmann, 2007). An excellent example of the use of microarrays in elucidating gene function in stress was shown by Luhua *et al.* (2008), who selected forty-one genes from a range of transcripts found by microarrays to be induced in response to oxidative stress in *A. thaliana*. These genes were experimentally over-expressed in plants, and a total of 70 % conferred tolerance to oxidative stress, thus confirming their function.

Microarrays contain many thousands of gene-specific oligonucleotide probes adhered to a solid chip. RNA or DNA samples to be tested are hybridised to the chip, whereby molecules complementary to the probes bind and are retained. Fluorescence is then emitted at a level relative to the transcript abundance (Schena *et al.*, 1995; Rensink and Buell, 2005; Clarke and Zhu, 2006). A variety of microarray platforms have been developed, broadly falling into two categories: Two-colour arrays are created by spotting DNA fragments onto a glass slide, and then hybridising two differently labelled RNA samples in order to measure relative gene expression. In contrast, one-colour synthetic oligonucleotide arrays are made by synthesising oligonucleotides directly onto silicon chips. Only one RNA sample is hybridised to each chip (Kennedy and Wilson, 2004). Synthetic oligonucleotide arrays allow the comparison of any number of mRNA samples, hybridised onto parallel arrays. This highly reproducible system has led to the development of commercially available GeneChips® for a variety of species (Lipshutz *et al.*, 1999; Rensink and Buell, 2005). Amongst these was the first *A. thaliana* Affymetrix GeneChip which consisted of approximately 8000 probes. This has now been replaced by the ATH1 GeneChip which comprises approximately 22,750 probe sets representing 23,750 genes, or more than 80 % of the entire genome based on the results of the *A. thaliana* sequencing project (Arabidopsis Genome Initiative, 2000; Redman *et al.*, 2004). Each probe set on the ATH1 chip consists of eleven 26-base probe pairs (www.affymetrix.com). During analysis, cRNA is synthesised and labelled with biotin. Hybridised biotinylated cRNA is stained with a fluorescent dye and a scanner is used to survey the resulting intensity data (Redman *et al.*, 2004).

Biological replicates are crucial in microarray experiments in order to firstly estimate biological variation between samples so that statistical analysis can be carried out, and

secondly to draw conclusions about the nature of the wider population from which the samples are taken (Clarke and Zhu, 2006). To minimise variation, each biological replicate should consist of pooled samples from different plants (Zhu and Wang, 2000). The accuracy of modern microarray technology means that most researchers forego the need for technical replicates (Clarke and Zhu, 2006). Before relative expression values can be obtained from the microarray probe intensities, data normalisation is necessary. Normalisation equalises the distribution of intensity values across all the arrays, thus controlling for differences in sample preparation, manufacture and processing of arrays, background noise and hybridisation conditions, and allowing comparison of expression data from different arrays (Quackenbush, 2002). Microarray analysis software such as GeneSpring now includes tools such as the multi-array averaging (RMA) algorithm, which combines normalisation with background correction and summarisation (Irizarry *et al.*, 2003). Background adjustment removes the effect of optical noise and non-specific binding, whilst summarisation combines the data from all the probes in the probe set to obtain a single expression value for each gene (Irizarry *et al.*, 2003)(GeneSpring GX Manual, Agilent Technologies, www.chem.agilent.com). Following the identification of differential expression values from microarray analysis, validation is usually carried out by means of an independent method such as qRT-PCR or RNA blot (Clarke and Zhu, 2006; Wise *et al.*, 2007).

Although drawing inferences as to gene function from one microarray experiment alone can be unreliable, combining data from multiple experiments vastly improves the power of any study (Kennedy and Wilson, 2004; Deyholos, 2010). Various public repositories now exist whereby gene expression data can be compared across a range of conditions, stimuli and developmental stages, such as NASCarrays, a service provided by the Nottingham Arabidopsis Stock Centre (NASC) (Craigon *et al.*, 2004). Data produced by most institutions offering large-scale expression services is made publicly available on these databases (Rensink and Buell, 2005). A set of guidelines has been established to ensure quality and reliability, known as the Minimal Information About Microarray Experiments (MIAME) (Brazma *et al.*, 2001). Databases have also been developed which permit easy comparison and visualisation of public microarray data, such as the web-based tool Genevestigator which shows expression profiles of genes over a range of developmental, spatial and environmental conditions (www.genevestigator.com) (Hruz *et al.*, 2008), and the Arabidopsis Co-expression Tool, a database which calculates co-expression coefficients for genes of interest

(www.arabidopsis.leeds.ac.uk) (Manfield *et al.*, 2006). The creative use of microarray data from model plants such as *A. thaliana* and the resulting identification of new genes will enhance our understanding of plant systems as a whole and may provide targets for the improvement of crop plants (Kennedy and Wilson, 2004; Denby and Gehring, 2005).

2.1.3 Transcriptomics in the study of dehydration and drought

Microarray technology has been employed many times in order to identify genes in *A. thaliana* that respond to water deficit stress. The first large scale study was carried out by Seki *et al*. (2002) and demonstrated the power of arrays in characterising plant stress responses. The authors constructed a full-length cDNA library of around 7000 genes hybridized onto an array. The transcriptomes of plants that had been exposed to either cold, salt or an intense dehydration treatment were then analysed. A total of 277 genes were found to be induced more than 5-fold by the dehydration treatment whilst 79 were repressed. Induced genes fell into two main categories; transcription factors such as DREB, WRKY and NAC; and also stress tolerance genes such as osmoprotectants, LEA proteins and heat shock proteins. Down-regulated genes were mainly involved in photosynthesis. In particular the large overlap in gene induction between the three different abiotic stresses was highlighted, leading researchers to speculate about the interaction between different stress signalling and response pathways. Other studies have characterised transcriptome response to water stress using slightly different experimental conditions. Kreps *et al.* (2002) induced 'drought' stress by subjecting seedlings to a hydroponic growth medium containing mannitol, known to produce osmotic stress. An Affymetrix 8k GeneChip was used to analyse the transcriptome, and 1008 genes were found to be differentially regulated. In a soil-based water deficit experiment using the same Affymetrix chip, 773 transcripts were found to be induced or repressed (Kawaguchi *et al.*, 2004). The difference in treatment methodology of these three studies is clearly reflected by the lack of overlap between the subsets of induced genes, as summarised by Bray, *et al.* (2004). Of all the differentially regulated genes identified, only 27 were commonly up-regulated in all three studies, whilst only 3 were commonly down-regulated. These facts highlight the extremely specific nature of plant stress responses and demonstrate that experimental approaches in the laboratory cannot be relied upon to represent the response of plants in field conditions to environmental stresses (Deyholos, 2010). Swindell *et al.* (2006) used the Affymetrix ATH1 full-genome array to characterise root and leaf tissue separately in a comprehensive study of

abiotic stress responses. In agreement with previous findings, there was a low correlation between the gene induction from osmotic stress due to mannitol and that of dehydration stress. Interestingly, of the 67 genes found to be commonly induced by all nine abiotic stresses, the majority of the root-specific changes occurred within 1 hour of stress induction, whilst most of the shoot-specific changes occurred 6 hours afterwards. Each of the 67 expression changes was specific to either roots or leaves. This and other findings have led to the suggestion that patterns of stress response are spatially and temporally specific, and that a generalised stress response occurs first in the roots of plants, and then later in the shoot tissue (Denby and Gehring, 2005; Swindell, 2006). Similarly in another whole-genome study, the majority of dehydration-induced gene changes in roots were found to occur 1 hour after exposure (470 genes in total) whilst the maximum change in leaves occurred after 3 hours (265 genes in total) (Kilian *et al.*, 2007). Microarrays have also been used to determine the role of hormones in stress-responsive transcriptome changes. Huang *et al.* (2008) found that of 1969 drought-responsive genes, approximately one third were also differentially regulated by ABA, underpinning the importance of this hormone in abiotic stress response. Furthermore the transcription of 197 genes was found to be affected by methyl jasmonate, which appeared to be the second most important hormone regulating this set of genes, a finding which further supports evidence of crosstalk between abiotic and biotic signalling pathways (Anderson *et al.*, 2004; Adie *et al.*, 2007).

2.1.4 Transcriptomics in the study of plant-nematode interactions

Microarray analysis has facilitated considerable advances in understanding of plant-pathogen interactions (Wise *et al.*, 2007). In particular, the dramatic re-programming of root cells into syncytia or giant cells during cyst nematode or root-knot nematode parasitism provides an excellent target for transcriptional profiling. Several studies have focussed on the modified transcriptomes of these cells compared to those of normal root cells (Hammes *et al.*, 2005; Szakasits *et al.*, 2009; Barcala *et al.*, 2010; Klink *et al.*, 2010). In *A. thaliana* the contents of syncytial cells created by *Heterodera schachtii* parasitism were extracted through micro-aspiration and analysed for transcript changes (Szakasits *et al.*, 2009). A remarkable total of 7231 genes were differentially regulated. Of these, cell wall modification genes such as expansins, pectate lyases and β-glucanases were found to be induced, as well as genes associated with high metabolic activity such as those encoding ribosomal proteins and translational proteins. These observations agree with other studies detailing the transcriptomes of root galls and giant

cells caused by *Meloidogyne incognita* parasitism of *A. thaliana* roots (Jammes *et al.*, 2005; Fuller *et al.*, 2007; Barcala *et al.*, 2010). Defence-related genes were found to be down-regulated in all the studies, apart from a group of plant defensin genes that were strongly expressed in syncytia (Szakasits *et al.*, 2009). The detection of gene down-regulation is a particular strength of microarrays, and these findings have contributed to the theory that nematodes may suppress the hosts' pathogen response system (Gheysen and Fenoll, 2002; Wise *et al.*, 2007). Jammes *et al.* (2005) found that 3373 genes were differentially regulated in the root gall by *M. incognita* parasitism, whilst fewer genes were differentially regulated in the giant cells alone (Barcala *et al.*, 2010). In addition, the importance of the up-regulation of transporter genes in root-knot nematode giant cells was confirmed using Affymetrix ATH1 arrays (Hammes *et al.*, 2005).

In comparison to the analysis of nematode feeding cells, the transcriptome analysis of whole roots of *A. thaliana* infected with cyst nematodes revealed far fewer changes, identifying only 128 genes with differential regulation (Puthoff *et al.*, 2003). The study used the original Affymetrix GeneChip, which only contains ~8200 gene probes, perhaps explaining the difference in magnitude of transcriptome change. However, of the 119 genes identified, only 57 were found to be in common with those identified in the syncytia alone. Therefore the remaining genes identified by Puthof *et al.* (2003) are likely to represent a systemic response, as the specific syncytia-related changes would be diluted too far to be perceptible in whole roots (Szakasits *et al.*, 2009). A microarray study on soybean infected with the cyst nematode *Heterodera glycines* demonstrates this phenomenon. Differentially regulated genes were characterised in the syncytia and also in the whole root 3 days post infection, and the overlap between subsets analysed. There were only 64 genes in common between the 351 transcript changes in the syncytia alone and the 3301 changes observed in the whole root system (Klink *et al.*, 2007). Even fewer genes were in common at a later time-point of 8 days post infection, at which point the systemic root response had risen to 6917 differentially expressed genes. Clearly the extraction of tissue from whole roots is not informative for characterising the nature of syncytia-specific changes, and vice versa. Various microarray studies on soybean and cowpea have established that similar genes are up- and down-regulated in nematode feeding sites in these crops as in *A. thaliana*, confirming the validity of using *A. thaliana* microarrays to gain insights into processes occurring in economically important crop plants (Alkharouf *et al.*, 2006; Wise *et al.*,

2007; Das *et al.*, 2010) In addition, the up-regulation of pathways such as methionine synthesis and scavenging, flavonoid biosynthesis, lignin biosynthesis and the phenylpropanoid pathway have been observed in infected soybean roots (Ithal *et al.*, 2007a; Ithal *et al.*, 2007b; Klink *et al.*, 2010).

To date, no reports describe systemic nematode-induced transcriptome changes in any distal plant tissues. However, in an illuminating study on the metabolomic response to *H. schachtii* by *A. thaliana*, clear systemic effects were observed in the leaves of infected plants (Hofmann *et al.*, 2010). Amino acids such as asparagine, glutamic acid and glycine were depleted in leaf tissue whilst accumulating greatly in syncytia, emphasising the strength of the nutrient sink in the developing feeding cell. The accumulation in leaves of certain metabolites such as raffinose and 1-kestose, a carbohydrate which does not normally accumulate in *A. thaliana* and which has been associated with pathogen defence (Van den Ende *et al.*, 2004), and the depletion of dehydroascorbic acid suggest the activation of plant-wide osmotic stress or defence responses (De Gara *et al.*, 2003).

2.1.5 Transcriptomics in the study of multiple stresses

Studies are frequently carried out exposing plants to individual abiotic or biotic stresses in parallel in an attempt to identify genes that may be central to a broad-spectrum stress response, or that may represent points of cross-talk between signalling pathways (Kreps *et al.*, 2002; Seki *et al.*, 2002; De Vos *et al.*, 2005; Swindell, 2006; Kilian *et al.*, 2007; Huang *et al.*, 2008). It has been speculated that these genes may be targets for improving stress tolerance in crop plants (Seki *et al.*, 2002; Denby and Gehring, 2005; Swindell, 2006). For example, the gene response of *A. thaliana* plants to 6 abiotic stresses, 4 hormone treatments and a fungal pathogen was compared using a 7000 full-length cDNA microarray, specifically with regard to cytochrome P450 proteins, thought to be involved in defence and stress responses (Narusaka *et al.*, 2004). The expression of these genes was found to be specific for each stimulus, and therefore overlap between induction patterns suggested points of cross-talk between signalling pathways. However, it is now known that the transcriptome response to combined stress factors is not merely additive. The imposition of two or more stress factors simultaneously can cause an entirely new program of transcript response that is not necessarily similar to that of either stress individually (Rizhsky *et al.*, 2002; Rizhsky *et al.*, 2004; Mittler and Blumwald, 2010). This may be particularly true in the incidence of combined abiotic

and biotic stresses, as the ABA-regulated stress signalling pathway and the defence-responsive jasmonic acid signalling pathway are known to interact and inhibit one another (Anderson *et al.*, 2004; Voelckel and Baldwin, 2004; Asselbergh *et al.*, 2008b; Ton *et al.*, 2009). Thus to truly identify genes that are central in the response to multiple stresses, it is crucial to study plants subjected to simultaneous stresses (Mittler, 2006). Analysis of the transcriptome in tobacco following infestation by two insect herbivores, a sap-feeding mirid (*Tupiocoris notatus*) and a chewing hornworm (*Manduca sexta)*, revealed a specific transcriptional effect when the two herbivores were applied together compared to when each was applied separately (Voelckel and Baldwin, 2004). This supports the existence of *trans*-activating factors which reorganise gene expression depending on the nature of the stress. Furthermore, gene expression patterns were different if the herbivores were applied sequentially compared to in parallel, suggesting a system of priming whereby the transcriptome exhibits a long-term change following biotic attack and serves as a kind of immunological memory. Another study analysed peanut plants infected with the fungus *Aspergillus parasiticus* and exposed to drought (Luo *et al.*, 2005). Using two expressed sequence tag (EST) cDNA libraries, 42 genes were up-regulated in response to both the fungus and drought simultaneously, whereas 52 genes were up-regulated by drought alone. As root damage due to drought is advantageous for this pathogen, it is proposed that the fungus may be able to repress ABA and drought signalling in order to achieve a higher infection rate. Rizhky *et al.* (2004) found using the Affymetrix ATH1 array that the response of *A. thaliana* plants to a combination of drought and heat stress produced a new pattern of gene activation, resulting in the differential expression of 772 genes that had not been activated by either drought or heat stress individually. A total of 765 genes that had been induced by drought alone ceased to be differentially regulated with the addition of heat stress, whilst 208 such genes were identified for heat stress alone. Amongst the genes that were specifically regulated by a combination of drought and joint stress were heat shock genes, LEA genes and genes involved in various defence pathways. The regulation of MYB transcription factors in particular was specific to each stress combination, as well as heat shock proteins. Heat shock proteins have since been found to play an important role in the specific response of crop plants such as maize and wheat to combined heat and drought stress (Hu *et al.*, 2010; Grigorova *et al.*, 2011). A comparable study in tobacco revealed a very similar pattern of physiological and molecular reaction to multiple stresses, suggesting that this mechanism of response is highly conserved amongst plants (Rizhsky *et al.*, 2002). These studies demonstrate the precision and

adaptability of plants in responding to specific environmental conditions, and highlight the need for this kind of analysis in order to fully understand the nature of stress responses. However, to date no whole-genome transcriptome study has detailed the response of plants to simultaneous biotic and abiotic stress.

In the current work, the response of *Arabidopsis thaliana* to concurrent dehydration and nematode stress was assessed through measurements of plant and nematode growth, the expression of stress marker genes, and the use of Affymetrix ATH1 GeneChips. The aim of the microarray study was to identify any genes that may be differentially expressed specifically in response to the combination of dehydration and nematode stress. Any such genes may play key regulatory roles and their discovery may provide important insights into the interaction of biotic and abiotic signalling pathways in plants.

2.2 Materials and methods

2.2.1 Species used

- *Arabidopsis thaliana* ecotype Columbia-0 (Lehle Seeds).
- *Heterodera schachtii*

2.2.2 Induction of drought and nematode stress in soil-grown *A. thaliana*

2.2.2.1 Growth of A. thaliana *in soil*

Seeds of *A. thaliana* were sown without sterilisation onto trays of compost with a depth of 5 cm. Growth took place in a greenhouse at 20-22 °C under 16 h/8 h light/dark cycles. Approximately 14 days after sowing the seedlings were removed from trays and individually re-potted into 9 cm pots containing compost (Sinclair Potting & Growing Medium, East Riding Horticulture) mixed with sand and loam soil at a ratio of 2:1:1. Each plant was watered with 30 ml tap water.

2.2.2.2 Maintenance of H. schachtii *stock cultures*

H. schachtii cysts were obtained by transplanting four-week-old cabbage seedlings into 50 % sand/loam containing *H. schachtii* cysts at a density of approximately 20-30 eggs/g. After approximately 3 months, aerial parts of the plants were removed and the soil was stored damp at 4 °C. Egg counts were performed on the infected soil to determine levels of infestation. This was carried out by extracting all the cysts from 100 g of soil, crushing them and re-suspending in water. The egg count per ml of water could then be determined using a Pieter's Counting Slide.

2.2.2.3 Infection of A. thaliana *with* H. schachtii *cysts in soil*

Approximately 14 days after sowing the seedlings were removed from trays and individually re-potted into 9 cm pots containing compost mixed with sand and loam soil containing cysts of *H. schachtii* at a concentration of 50 eggs/g. The growth characteristics of the plants were evaluated over the following 50 days by measuring rosette diameter and primary inflorescence height at regular intervals and counting the number of siliques per primary inflorescence and seed number per silique.

2.2.2.4 Imposition of drought stress

To evaluate the combined effect of nematode and drought, plants were subjected to five differing severities of drought stress. Following transplanting into individual pots, the soil moisture level of each pot was monitored using an SM200 Soil Moisture Sensor attached to an HH2 Moisture Meter (both from Delta-T Devices). Plants were then divided into five drought treatment groups as follows:

1 Well-watered

2 25 % Soil Moisture

3 15 % Soil Moisture

4 10 % Soil Moisture

5 5 % Soil Moisture

The well-watered treatment group was watered to field capacity every day (around 53 % soil moisture) throughout the experiment. Water was withheld from the other four treatment groups until the soil moisture level in each pot dropped to the appointed percentage. The stomatal conductance of plants was measured at this point using an SC-1 Leaf Porometer (Decagon Devices), as well as the rosette diameter and primary inflorescence height. Following this, the pots were maintained at that soil moisture level by adding an appropriate amount of water.

2.2.2.5 Extraction, sterilization and hatching of **H. schachtii** *cysts*

H. schachtii cysts were extracted from infected soil stocks using the Fenwick can method, whereby the cysts float and are collected using a 120 µm sieve, as described in Urwin *et al.* (1997). Collected cysts were sterilized for 30-60 minutes in 0.1 % malachite green solution at room temperature on a rotator, then rinsed extensively in running tap water. Cysts were then incubated for 24 hours at 4 °C in an antibiotic cocktail solution consisting of 8 mg ml^{-1} streptomycin sulphate, 6 mg ml^{-1} penicillin G, 6.13 mg ml^{-1} polymixin B, 5 mg ml^{-1} tetracycline and 1 mg ml^{-1} amphotericin. After sterilisation, cysts were washed in sterile distilled water and placed on a 30 µm mesh in a hatching jar in 3 mM zinc chloride at 20 °C in the dark. The zinc chloride solution was replaced every two days and the hatched juveniles were stored at 10 °C for up to a week prior to use.

2.2.2.6 Infection with juvenile H. schachtii

When each treatment group had reached the appointed soil moisture level, the plants were infected with juvenile *H. schachtii* nematodes. Hatched juveniles were watered directly onto the *A. thaliana* roots in the soil. Three large pipette tips were inserted to a depth of 2 cm next to the stem of each plant. A total of 500 *H. schachtii* J2s in 1 ml of sterile water were applied to each tip and washed down with a further 1ml of water. Control plants were mock-inoculated with 2 ml water. The pots were then maintained at the same level of soil moisture as appointed previously. Fourteen days after nematode infection the aerial parts of the plants were removed and the soil washed gently from the roots with tap water. The root systems were weighed.

2.2.2.7 Staining of nematodes with acid fuchsin

In order to clearly visualise and count nematodes on the plant roots, acid fuchsin was used as a stain. After washing to remove soil if necessary, roots were soaked in hypochlorite solution with 1% available chlorine for two to three minutes, depending on the thickness of root. The roots were then washed thoroughly in tap water and transferred to boiling acid fuchsin stain (0.035 %) for two minutes. After rinsing again in tap water, the roots were left to de-stain in acidified glycerol in Petri dishes. Parasitising nematodes could then be counted under a microscope.

2.2.3 Induction of dehydration and nematode stress in *A. thaliana* in tissue culture

2.2.3.1 Growth of A. thaliana *in tissue culture*

Seeds of *A. thaliana* were soaked for at least 30 minutes in sterile distilled water prior to sterilisation. The seeds were surface sterilised in 95 % ethanol for two minutes followed by 10 % bleach for five minutes, before washing five times in sterile distilled water. After sterilisation the seeds were kept in sterile distilled water at 4 °C in the dark for 48 hours. *A. thaliana* plants were grown in square Petri dishes (Sterilin) on half strength MS media (Duchefa) composed as follows:

4.4 g/l Murashige and Skoog basal medium (vitamins) (Duchefa)

10 g/l sucrose

10 g/l plant agar (Duchefa)

pH 5.7 adjusted with 1M KOH

Autoclaved at 120 °C for a minimum of 20 mins and cooled to 50 °C before use.

Growth took place in Sanyo Environmental Test Chambers at 20 °C under 16 h/8 h light/dark cycles. The average light intensity was 140 µmol/m^2/s and humidity was approximately 30 %. Four seeds were sown per 10 cm plate, and plates were held at an angle of approximately 70° to facilitate downward growth of the roots, as shown in Figure 2.1A.

2.2.3.2 Sterilisation of juvenile nematodes

Following hatching, juvenile *H. schachtii* nematodes were pelleted in siliconised 1.5 ml microfuge tubes (Axygen) and sterilised in 0.1% chlorhexidine digluconate (CD) and 0.5 mg ml^{-1} hexadecyltrimethyl-ammonium bromide (CTAB) for 25-32 minutes on a rotational mixer at room temperature. The nematodes were then washed three times in filter-sterilised tap water with 0.01 % Tween-20, and resuspended to a concentration of 1 nematode μl^{-1}.

2.2.3.3 Infection of **A. thaliana** *with* **H. schachtii** *in tissue culture*

At growth stage 1.08–1.12 (Boyes *et al.*, 2001) plants were challenged with sterile juvenile nematodes at five infection points on the root system. For the microarray experiment 35 nematodes were pipetted onto each infection point giving a total of 175 applied nematodes per plant. Control plants were mock-inoculated with sterile distilled water. A small square of GF/A paper (Whatman) was applied to each infection point to aid nematode penetration. This was removed after 48 hours. Figure 2.1B shows a tissue culture plate during nematode infection. Tissue sampling for RNA extraction took place 10 days after infection with the juveniles.

2.2.3.4 Imposition of dehydration stress on **A. thaliana** *in tissue culture*

At growth stage 3.2-3.5 plants were subjected to dehydration stress. Plants were removed from the agar, placed in an open Petri dish and subjected to a clean flow of air in a flow hood for 15 minutes as detailed in Seki *et al.* (2002), during which time they lost 10-15 % of their fresh weight. Figure 2.2 shows the plants during dehydration treatment. Subsequently the plants were placed back on the agar and returned to the growth cabinet for a further 30 minutes, to allow differential expression to take place. Control plants were removed from the agar and then immediately replaced and returned to the growth cabinet for 45 minutes before tissue sampling. Samples were harvested by

A

B

Figure 2.1. Tissue culture infections of *A. thaliana* with *H. schachtii*. (A) *A. thaliana* plants were grown upright on agar plates to facilitate downward growth of the roots. Growth took place at 20 °C in a Sanyo Environmental Test Chamber. **(B)** At growth stage 1.08-1.12 the plants were infected with 175 *H. schachtii* juveniles suspended in water. Squares of GF/A paper were applied at infection points to aid nematode penetration.

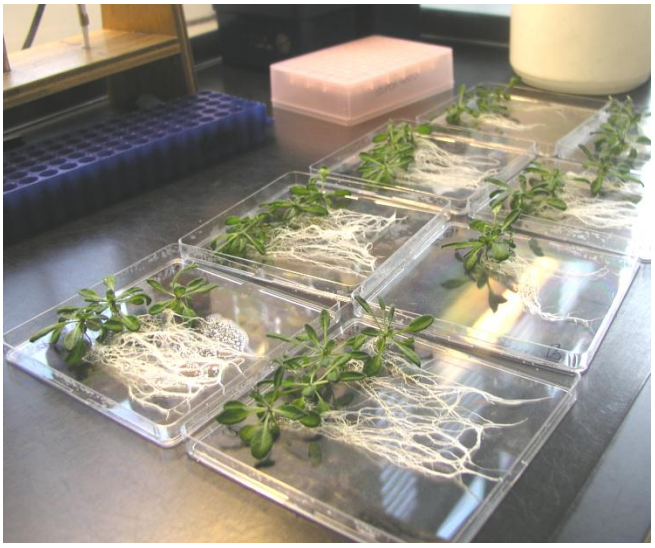


Figure 2.2. Dehydration of *A. thaliana* plants in tissue culture. Plants were subjected to dehydration stress by removing from agar plates and placing on a Petri dish lid in a clean flow of air for 15 minutes.

separating the roots and green parts of the *A. thaliana* seedlings and freezing each separately in liquid nitrogen before storing at -80 °C.

2.2.4 Extraction of total RNA

Total RNA was prepared from frozen leaf and root tissue of pooled plants using the Qiagen RNeasy Plant Mini Kit, according to the manufacturer's protocol. Larger samples were ground to a powder whilst frozen using a sterile, RNase-treated pestle and mortar. 100 mg of powder was then used in the extraction protocol. For smaller samples, 450 µl of RLT extraction buffer (supplied with the kit) with 10 µl/ml β-mercaptoethanol was added directly to the frozen plant material in a microcentrifuge tube with RNase-free sterile sand, and the tissue disrupted using a plastic pestle. Disrupted tissue was centrifuged through a QIAshredder spin column to remove cell debris and reduce lysate viscosity. Ethanol was added to the supernatant, which was then applied to an RNeasy Spin Column. The optional on-column DNase digestion was performed. The column was washed with the buffers RW1 and RPE to remove contaminants, and total RNA was eluted in 30 µl RNase-free water. The RNA was stored at -80 °C.

2.2.5 Reverse transcription of RNA

A NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) was used to estimate the concentration of RNA from a 1µl sample, at a wavelength of 260 nm. 260/280 nm and 260/230 nm ratios were calculated to assess the purity of the RNA. Following quantification, RNA was used in a reverse transcription reaction to create cDNA. 50ng-1µg RNA was first denatured at 70ºC in the presence of 10 pmol random primers. Then 200 units of BioScript MMLV reverse transcriptase (Bioline) was added along with 5x first strand buffer and 10mM dNTPs, and the reaction was incubated at 42ºC for 1 hour. The reaction was inactivated at 72ºC for 7 minutes, and the cDNA stored at -20ºC.

2.2.6 Semi-quantitative RT-PCR

The expression levels of stress marker genes were analysed using RT-PCR. Oligonucleotide primers were designed for the amplification of the *A. thaliana* genes *DREB1A*, *DREB2A*, *MIOX5* and *PR-1*. Nucleotide sequences were obtained from the TAIR SeqViewer website (http://www.arabidopsis.org/). Primer3 software (Rozen and

Skaletsky, 2000) was used to design primers which would amplify a segment from the coding region of the gene, of between 80 – 200 base pairs long. The maximum and minimum annealing temperatures of the primers were specified to be 58ºC and 60ºC respectively, with an optimum of 58ºC. The primers were 20 base pairs long and avoided runs of 3 or more of the same nucleotide in a row. Primers were obtained from Eurogentec, and their sequences are provided in Appendix 2.

PCR was carried out on cDNA created from root and leaf samples of plants undergoing dehydration or nematode stress. cDNA was amplified using BIOTAQ Red DNA Polymerase (Bioline). Cycling conditions are given in Appendix 1A and reagent volumes in Appendix 1B. Preliminary studies showed that after twenty-five cycles of PCR the product increase was still in its exponential phase for all the genes studied (data not shown). Thus the amount of end product yielded indicates semi-quantitatively the level of that gene transcript in the original sample as compared to the control sample. The housekeeping gene *ACTIN2* (At3g18780) was used for normalisation.

2.2.7 Agarose gel electrophoresis

Amplified DNA fragments were routinely electrophoresed in 1% agarose gels prepared with tris acetate EDTA (TAE) buffer (50 x stock comprised 242.2 g tris, 57.1 ml glacial acetic acid and 18.6 g EDTA disodium salt in 1 litre of water). Electrophoresis was carried out at 80 volts for 20-30 minutes in 1x TAE buffer. In order to visualise the DNA under UV light, 1 µl 10 mg/ml ethidium bromide was added to each 100 ml agarose gel. A 2-log DNA ladder (New England Biolabs) was used to estimate the size of DNA fragments.

2.2.8 Microarray experiment

2.2.8.1 Microarray experimental design

A microarray experiment was carried out to identify genes that were induced specifically in response to joint biotic and abiotic stress. Tissue was prepared for microarray analysis by imposing individual or combined nematode and dehydration stress on plants in tissue culture (Section 2.2.3). Forty plants were used in each treatment, which were divided into 5 pools of 8 plants (Figure 2.3). After the stress treatments had been carried out, tissue was sampled by separating roots and leaves and

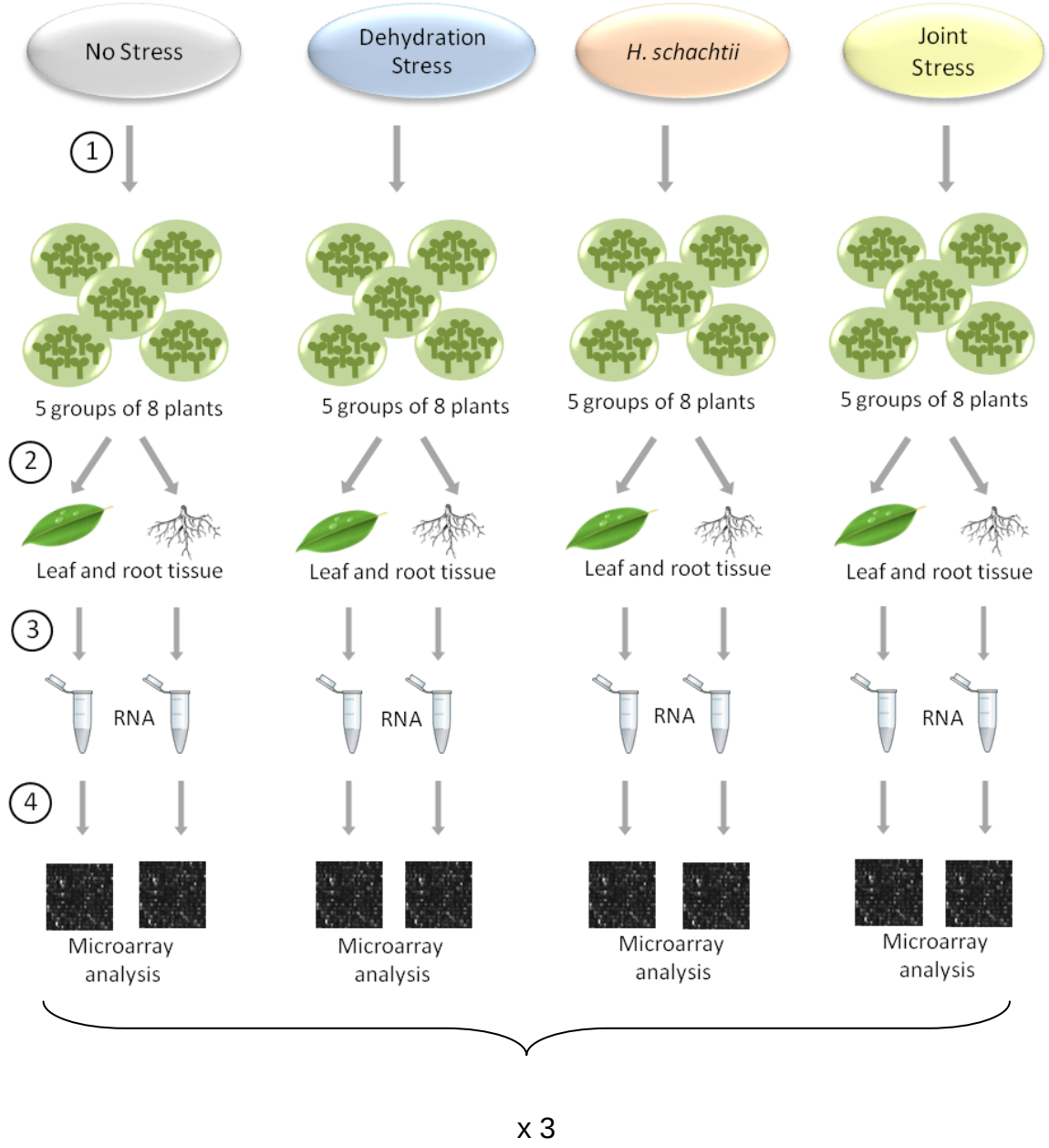


Figure 2.3. Schematic diagram of microarray experiment. 1. Plants were divided into four treatment groups comprising 40 plants each, in 5 groups. Treatments were dehydration stress, nematode stress, or both stresses in combination (joint stress). **2.** Following exposure to stress, roots were separated from leaf tissue and samples were quick-frozen in liquid nitrogen. **3.** RNA was extracted from each group of roots or leaves separately. **4.** A 4 µg aliquot from each pool was combined into one sample per treatment, which was used for microarray analysis. Analysis was carried out on Affymetrix ATH1 Chips, and the entire experiment was carried out in triplicate (24 arrays in total).

quick-freezing in liquid nitrogen. Each pool of 8 plants made up one sample. RNA was isolated from the pooled tissue samples and quantified using the NanoDrop. To ensure equal RNA contribution from all the plants, 4 µg RNA was then taken from each of the 5 pooled samples and combined to make a single 20 µg sample, which was used for a single microarray hybridisation. Microarray analysis was carried out on root and leaf tissue for each treatment, giving 8 arrays. The entire experiment was performed in triplicate, giving a total of 24 arrays.

2.2.8.2 Determination of RNA quality and preparation for shipment

A 2100 Expert Bioanalyser (Agilent) was used to analyse the quality of all RNA samples before use in microarray work. This instrument is able to evaluate RNA quantity and integrity using samples of only 1 µl. The protocol was carried out according to the manufacturer's instructions. Briefly, a gel matrix was combined with a fluorescent dye and loaded onto an RNA 6000 Nano Chip which consists of 12 wells connected by a series of microchannels. RNA samples were denatured at 70 ºC and loaded onto the chip along with a marker and a ladder in separate wells. A high voltage electric current was then applied, causing the gel matrix to behave like a denaturing gel. RNA molecules bound by the fluorescent dye were forced through the microchannels according to their size, and their fluorescence was measured as they passed the detector. The quality of RNA was assessed from an electropherogram, a plot of fluorescence levels against time. Pure, un-degraded RNA has a characteristic trace of two sharp ribosomal peaks against an otherwise flat baseline. Quantification can be achieved by comparing the peaks yielded in the samples to the known concentration of the ladder. RNA was then prepared for microarray analysis by ethanol precipitation. There were 24 samples, each containing 20 µg RNA. 2x volume of RNase-free ethanol and 0.1x volume of 3M potassium acetate were added to each sample, which was then incubated at -80ºC for 30 minutes. The sample was centrifuged and washed with 70% ethanol before being left to air dry. The precipitated samples were shipped on dry ice to the Nottingham Arabidopsis Stock Centre (NASC) for microarray analysis, where the integrity of the RNA was also checked.

2.2.8.3 Affymetrix ATH1 arrays and data analysis

Hybridisation of Biotin-labelled RNA to Affymetrix *Arabidopsis* ATH1 GeneChip arrays and array scanning were performed by the Nottingham *Arabidopsis* Stock Centre

transcriptomics service (Craigon *et al.*, 2004) following the standard Affymetrix protocol. Array data were provided by NASC in the form of CEL files. These contain the results of intensity calculations for each probe from the pixel values collected by the Affymetrix scanner. CEL files were imported in to GeneSpring GX10 and then baseline pre-processing, normalisation and summarisation was carried out using the RMA (Robust Multiarray Average) summarisation algorithm, as described in Irizarry *et al.* (2003). Each chip was normalised to the median of the control (unstressed) array.

2.2.8.4 Identifying differentially regulated genes

Having generated gene expression data using the RMA analysis, arrays were grouped according to treatment types, and data from replicate arrays combined. To identify genes differentially regulated between treatment arrays and the control unstressed arrays, un-paired T-tests were carried out using the GeneSpring GX10 software and *p*-values generated. Genes were considered up- or down-regulated if significantly different from the control where $p < 0.05$. The identification of subsets of differentially expressed genes overlapping between treatments was carried out in GeneSpring.

2.2.8.5 Ontological analysis of microarray data

To determine significant over- or under-expression of GOslim Biological Process categories, lists of gene locus IDs found to be up- or down-regulated were uploaded to the TAIR website and gene ontology annotation details were retrieved (http://www.arabidopsis.org/tools/bulk /go/index.jsp). Enriched or depleted ontology categories within the differentially regulated genes were identified by comparing the percentage of annotation counts in the list to the percentage of annotation counts across the whole genome. Chi-squared tests were then used to determine significant differences between categories.

2.2.9 Verification of microarray results

A subset of 12 differentially expressed genes were selected representing a wide range of positive and negative fold changes in response to joint stress, from both root and leaf tissue. The genes and their annotated functions are given in Table 2.1. AT1G61340, AT1G22190, AT4G27410, AT5G05410, AT4G25480 and AT5G51990 were up-regulated in leaves, whilst AT5G05410, AT4G25480 and AT5G51990 were up-

Gene ID	TAIR Function
AT3G18780	Constitutively expressed in vegetative structures (*ACTIN2*)
AT1G61340	F-box family protein
AT1G22190	Integrase-type DNA-binding superfamily protein
AT5G05410	Dehydration responsive transcription factor *(DREB2A)*
AT5G51990	DREB-family transcription factor (*CBF4)*
AT4G27410	Dessication-responsive NAC transcription factor (*RD26*)
AT4G25480	Dehydration responsive transcription factor *(DREB1A)*
AT1G52800	Fe(II)-dependent oxygenase superfamily protein
AT1G13080	Cytochrome P450 monooxygenase (*CYP71B2)*
AT3G48920	Member of the R2R3 factor gene family (*MYB45*)
AT2G38310	Member of PYR/PYL/RCAR family proteins which function as abscisic acid sensors (*PYL4*)
AT5G54040	Cysteine/Histidine-rich C1 domain family protein

Table 2.1. Genes used for microarray validation. Twelve genes were selected that showed differential expression in the microarray results. The expression of these genes was analysed in cDNA from plants under joint stress using quantitative RT-PCR, in order to confirm the validity of the microarray. *ACTIN2* was used as the normalisation gene. Primers were designed where possible to span exon boundaries to prevent the amplification of genomic DNA.

regulated in roots. AT1G13080 and AT2G38310 were down-regulated in leaves, while AT1G52800, AT5G54040 and AT3G48920 were down-regulated in roots. cDNA was synthesised from the same RNA used for the microarray experiment and the three biological replicates pooled. Expression levels of these genes in joint-stressed tissue in comparison to the control level were analysed using quantitative RT-PCR (qRT-PCR), in order to verify the results of the microarrays. The genes AT5G05410 (*DREB2A*) and AT4G25480 (*DREB1A)* were analysed in both root and leaf tissue, as they were highly up-regulated in both.

2.2.9.1 Preparation of cDNA

Template cDNA was prepared as detailed in Section 2.2.5, using the same RNA as was used for microarray analysis. However, an additional DNase digestion was first carried out to remove any traces of genomic DNA present in the sample, which could have been amplified during qRT-PCR. An 87.5 µl aliquot of RNA was combined with 2.5 µl DNase 1 (QIAGEN), 10 µl RDD buffer (supplied with kit) and made up to 100 µl with RNase-free water. The mixture was incubated at room temperature for 10 mins, and then purified using an RNeasy Plant Mini Kit (QIAGEN). The kit protocol was followed as described in Section 2.2.4, except that Buffer RLT without β-mercaptoethanol was used, and no additional DNase digestion carried out.

2.2.9.2 Quantitative RT-PCR

Primers for qRT-PCR were designed using Primer3 software. Short product lengths of 80-150 bp were preferable, and where possible primers were designed to span exon boundaries so that no genomic DNA would be amplified in the reaction. A full list of genes and primer sequences is detailed in Appendix 2. Primer stocks were prepared by combining forward and reverse primers for the same gene and diluting to a concentration of 7.5 pmol/µl each with sterile distilled water. qRT-PCR was carried out using a Stratagene Mx3005P instrument (Agilent Technologies) and using Brilliant II SYBR® Green 1-Step Master Mix (Agilent Technologies). The Master Mix contains all the components necessary for the reaction, including a buffer, $MgCl_2$, nucleotides, SureStart *Taq* DNA polymerase, SYBR Green (a fluorescent dye) and stabilisers. Reactions took place in 96-well polypropylene plates (Agilent Technologies) sealed with optical quality sealing film (Sarstedt). In each well the following components were combined: 12.5 µl SYBR Green Master Mix, 6.5 µl sterile distilled water, 1 µl primer

mix and 5 µl cDNA template. Plates were mixed for 2 mins at 400 rpm using a Mixmate (Eppendorf) and then centrifuged briefly. During the reaction plates underwent activation at 95 ºC for 3 minutes and then were cycled 40 times at 95 ºC for 30 seconds, 60 ºC for 30 seconds and 72 ºC for 30 seconds. Fluorescence data was collected at the 60 ºC annealing phase.

The efficiency of each primer pair was first confirmed by generating a standard curve using cDNA known to contain detectable amounts of all genes to be tested. cDNA was diluted to create a 3-fold dilution series of 5 standards. Each standard was tested in duplicate for each primer pair. Negative controls containing no cDNA were tested in duplicate with each gene as well as no RT controls to test for genomic DNA contamination. The specificity of each primer pair was analysed using a dissociation curve; one clean peak indicated a single product and thus specific binding. For analysis of samples, cDNA from roots or leaves under joint stress were diluted 1:10 and tested in triplicate. The expression levels of target genes were normalised to the housekeeping gene *ACTIN2*, and cDNA from unstressed control plants was used as a calibrator to calculate the change in expression. MxPro (Mx3005P v4.10) comparative quantitation software (Stratagene) was used to determine Ct values and fold changes. The Ct value represents the cycle number at which threshold fluorescence is reached. A 1 Ct difference between samples represents 2 x as much transcript when primer efficiency is 100 %. The default threshold fluorescence levels for determining Ct values were used. The fold change was then calculated by the MxPro software: The Ct value of *ACTIN2* was subtracted from that of the gene of interest to give the ΔCt value. The control ΔCt was then subtracted from the treatment (joint stress) ΔCt to give the ΔΔCt. When positivised this value represents the log2 fold change in expression level of the gene. The MxPro software also adjusts for the primer efficiency as calculated by the standard curve.

2.2.10 Validation of dehydration method as a model for drought

Wild-type plants were grown in compost and after two weeks transplanted into 9 cm pots (4 per pot). After 10 days water was withheld from half of the plants, until the soil moisture had dropped to 10-15 % (approximately 1 week). The stomatal conductance of plants was measured at this point, and was found to be 10-20 % of that of the control plants. Leaf samples were collected by pooling a medium-sized rosette leaf from each of

9 plants per treatment. Roots were washed to remove soil and 2 cm portions nearest the stem were sampled from a pool of 9 plants. RNA was extracted from the samples and cDNA synthesised. The samples were then analysed in triplicate for the expression of the 12 genes used in microarray validation, using qRT-PCR.

2.2.11 Statistical methods

The statistical methods employed throughout this thesis are described here, with the exception of the microarray analysis which is specifically described in Section 2.2.8.4. Data was analysed using SPSS statistical software (version 16.0). Results from several groups were analysed by ANOVA or Kruskal-Wallis H test. Data with a normal distribution and equal variance was tested by ANOVA and mean differences were compared between each stress treatment and the unstressed controls by the Student-Newman-Keuls (SNK) test. Data with a right-skewed distribution were normalized by taking the square root of the values before analyzing with ANOVA, whilst data with an extremely right-skewed distribution were normalised by transformation into $\log^{10}$ values. Non-parametric data and data with unequal variance was analysed using the Kruskal-Wallis H test, and differences between treatments determined by Mann-Whitney U test with a Bonferroni correction. When comparing data from two groups, t-tests were used for normally distributed data and Mann-Whitney U tests were used for non-parametric data. A p value of < 0.05 was considered statistically significant.

2.3 Results

2.3.1 The effect of *H. schachtii* infection on growth rate of *A. thaliana*

The relative growth rates of *A. thaliana* plants grown in *H. schachtii*-infested soil and un-infested soil were compared over a 60-day time course (Figure 2.4A). No difference was observed in the average diameter of the rosettes until 39 days after planting. Following this period the rosette diameter of the uninfected plants continued to increase, reaching the greatest size of 119 mm 53 days after planting. However after 39 days the nematode-infected plants failed to continue rosette growth, peaking 43 days after planting at 95 mm diameter, and then declining slightly in size due to leaf senescence. The last five measurements showed a significant difference between the infected and uninfected average rosette diameter. Similarly the height of the primary inflorescence was found to differ between nematode-parasitised plants and their un-infected counterparts (Figure 2.4B). The time taken for the primary inflorescence to emerge was no different between the two groups, but 56 days after planting a difference became apparent in the height of the inflorescence. The inflorescences of the plants infected with nematodes did not continue growth to the same extent as the controls, reaching a final height of 373 mm on average, whilst the control plants continued to an average of 442 mm. These differences between the two groups were significant at 56 and 60 days following planting. The number of siliques on the primary inflorescence was not found to differ between control and infected plants and neither was the seed yield in terms of seeds per silique (data not shown).

2.3.2 The interaction of drought and nematode infection rate in *A. thaliana*

Plants exposed to various levels of drought resulting from 5 different soil moisture levels showed significantly different photosynthesis and growth characteristics. Plants at 5 %, 10 % or 15 % soil moisture had a significantly lower stomatal conductance than those at 25 % soil moisture or well-watered plants (Figure 2.5A). The stomatal conductance of the well-watered plants was over 10 times that of the plants experiencing the most severe drought. The rosette diameter was also affected. Plants held at 5 % or 10 % soil moisture were significantly smaller than those at 15 %, which

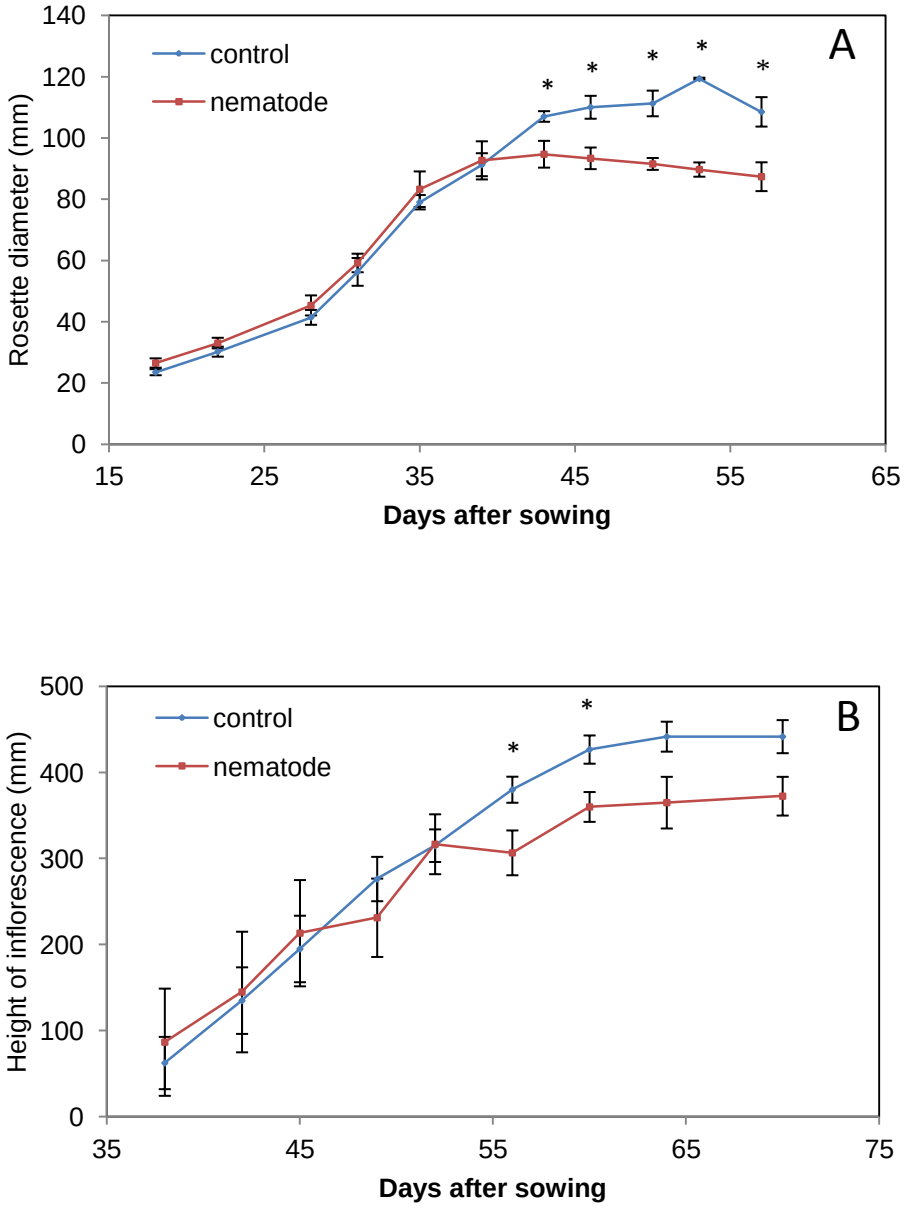


Figure 2.4. The effect of nematode infection on rosette diameter and inflorescence height of *A. thaliana*. Fourteen days after sowing, *A. thaliana* seedlings were transplanted into soil containing 50 eggs/g *H. schachtii* cysts. Measurements were taken of **(A)** the rosette diameter and **(B)** height of primary inflorescence until each had stopped increasing (57 and 70 days after sowing, respectively) (n = 5). Asterisks show significant differences between control and infected plants ($p < 0.05$).

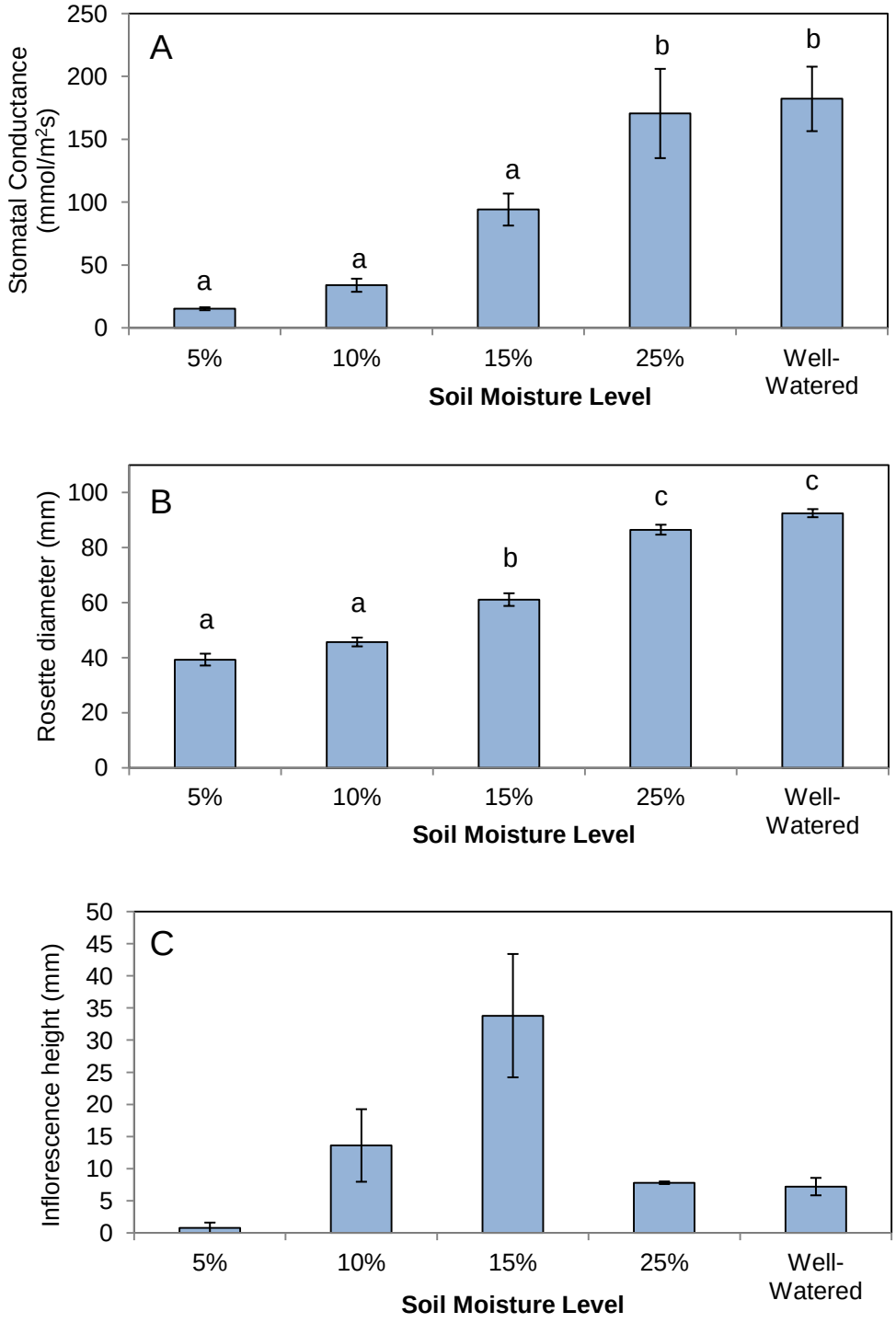


Figure 2.5. The effect of differing levels of soil moisture content on stomatal conductance, rosette diameter and inflorescence height in *A. thaliana*. Water was withheld from plants growing in compost to achieve varying levels of soil moisture. Well-watered plants were irrigated to field capacity, around 53 % soil moisture. When the soil moisture of each treatment group reached the appointed percentage, **(A)** stomatal conductance, **(B)** rosette diameter and **(C)** primary inflorescence height were measured. Soil moisture was measured using a SM200 Soil Moisture Meter and stomatal conductance was measured with an SC-1 Leaf Porometer. Means with different letters are significantly different at the 5 % level according to the SNK test. Error bars show the standard error of the mean (n = 5).

were in turn smaller than those at 25 % or well-watered plants (Figure 2.5B). Plants in the middle range of drought (15 % soil moisture) produced an inflorescence earlier than either the more severely drought-stressed or the well-watered plants, and were thus taller when measured on the 15th day after the initiation of drought stress, although not significantly (Figure 2.5C).

Plants at different severities of drought stress were infected with juvenile nematodes, which developed over 14 days through different stages of the parasitic life-cycle. Examples of these distinctive stages of *H. schachtii* infection on the *A. thaliana* roots are shown in Figure 2.6. The number of enlarged nematodes (fusiform or saccate) established within the root system was found to differ according to the severity of drought stress (Figure 2.7A). Plants at 5 % soil moisture had the least nematodes, averaging 11 per plant. Plants at 10 % soil moisture had significantly more nematodes, whilst plants at 25 % and well-watered plants had the most nematodes, averaging 40 and 44 per plant, respectively. Clearly the size of the root system would affect the extent of nematode infection. As root systems became smaller with increasing levels of drought stress, the number of nematodes was therefore calculated per mg of root tissue, thus correcting for differences in root system size (Figure 2.7B). An opposing trend was observed, whereby plants at 5 % soil moisture had the greatest number of nematodes per mg of root tissue, whilst those at 10 % and 15 % had significantly fewer, and plants at 25 % and well-watered plants had fewer again. This suggests that drought stress affects nematode parasitism levels in a manner unrelated to differences in root system size.

Figure 2.8 shows the proportion of nematodes at each life cycle stage on different groups of drought-treated plants. Nematodes on plants at 25 % soil moisture content had progressed the furthest through the life cycle. This was demonstrated by the greatest proportion of saccate and enlarged saccate nematodes compared to all the other treatment groups. Nematodes on the 5 % soil moisture-treated plants had progressed the least far, showing a significant reduction in the proportion of saccate females and a greater proportion of vermiform juveniles that had not yet established feeding sites. Nematodes on the well-watered plants showed an intermediate stage of progression whereby the proportion of each stage of the life cycle did not differ from that of any other treatment group.

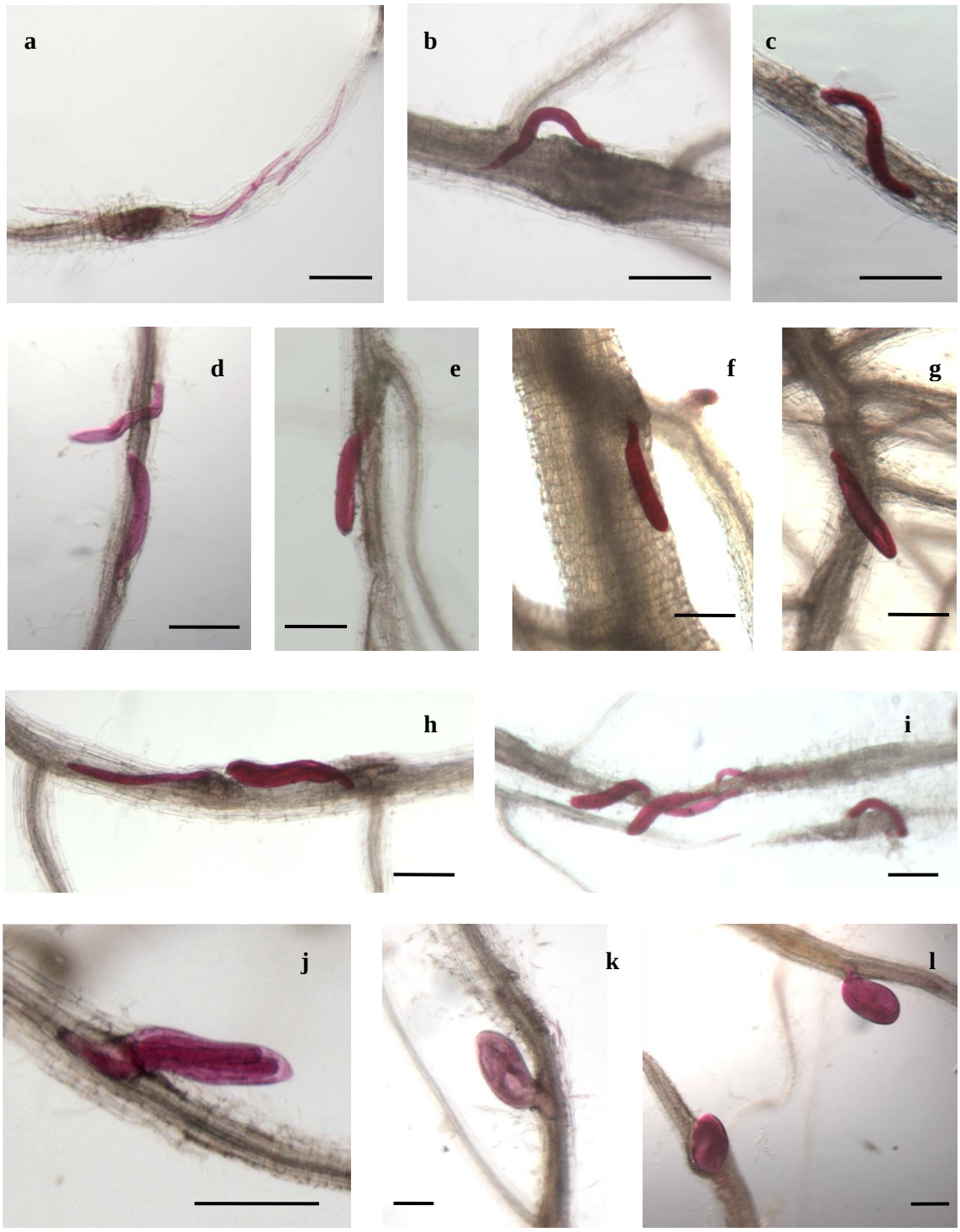


Figure 2.6. *Heterodera schachtii* nematodes parasitising *A. thaliana* roots. Nematodes are stained with acid fuchsin and appear pink. Scale bars represent 250 µm. **a)** Several J2 stage juveniles migrating through an *A. thaliana* root after penetrating the root tip. **b,c)** J3 stage nematodes after establishing feeding sites. **d,e,f,g)** J4 stage nematodes. **h,i)** *H. schachtii* of different life cycle stages infecting the same root. **j)** An adult male developing inside the J3 cuticle. The vermiform male leaves the root after this stage and fertilises the female. **k,l)** Adult egg-containing females. After fertilisation, females die and the cuticle tans to form the cyst, which detaches from the root into the soil.

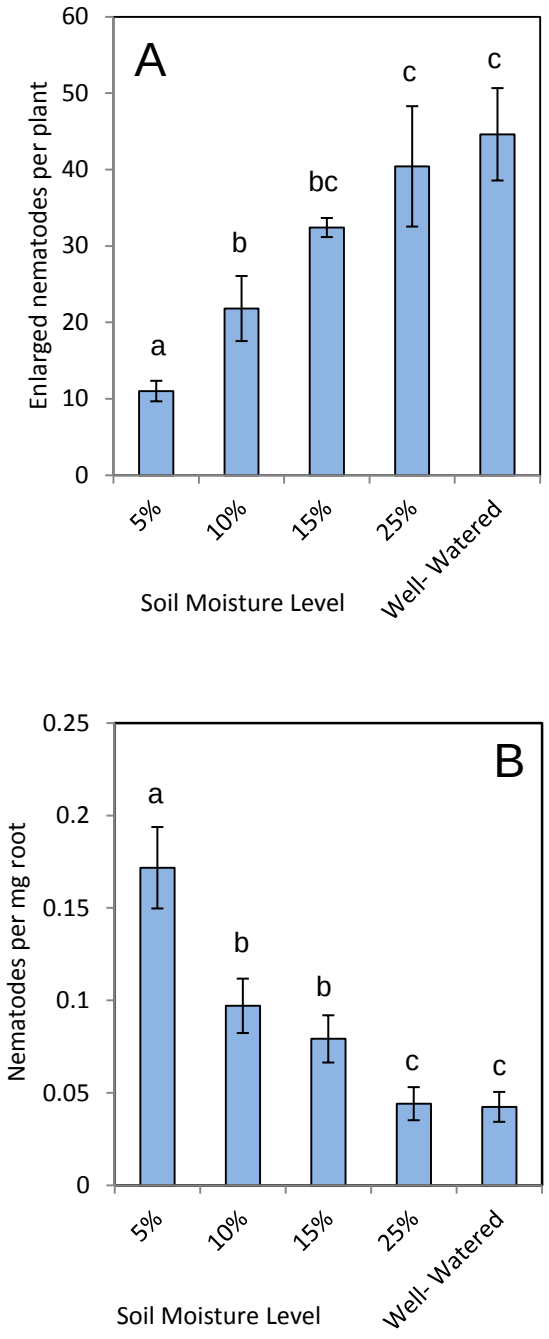


Figure 2.7. The effect of differing drought stress treatments on *H. schachtii* infection in *A. thaliana.* Well-watered plants and those at 4 different levels of drought stress were infected with 500 juvenile nematodes per plant. The nematodes were allowed to develop for 14 days and then were counted by staining the *A. thaliana* roots. **A)** The total number of enlarged (fusiform or saccate) nematodes was counted per plant. **B)** The entire root system was weighed and the nematodes per mg of root calculated. Means with different letters are significantly different at the 5 % level according to the SNK test. Bars displaying two letters show no difference to either group. Error bars show the standard error of the mean (n = 5).

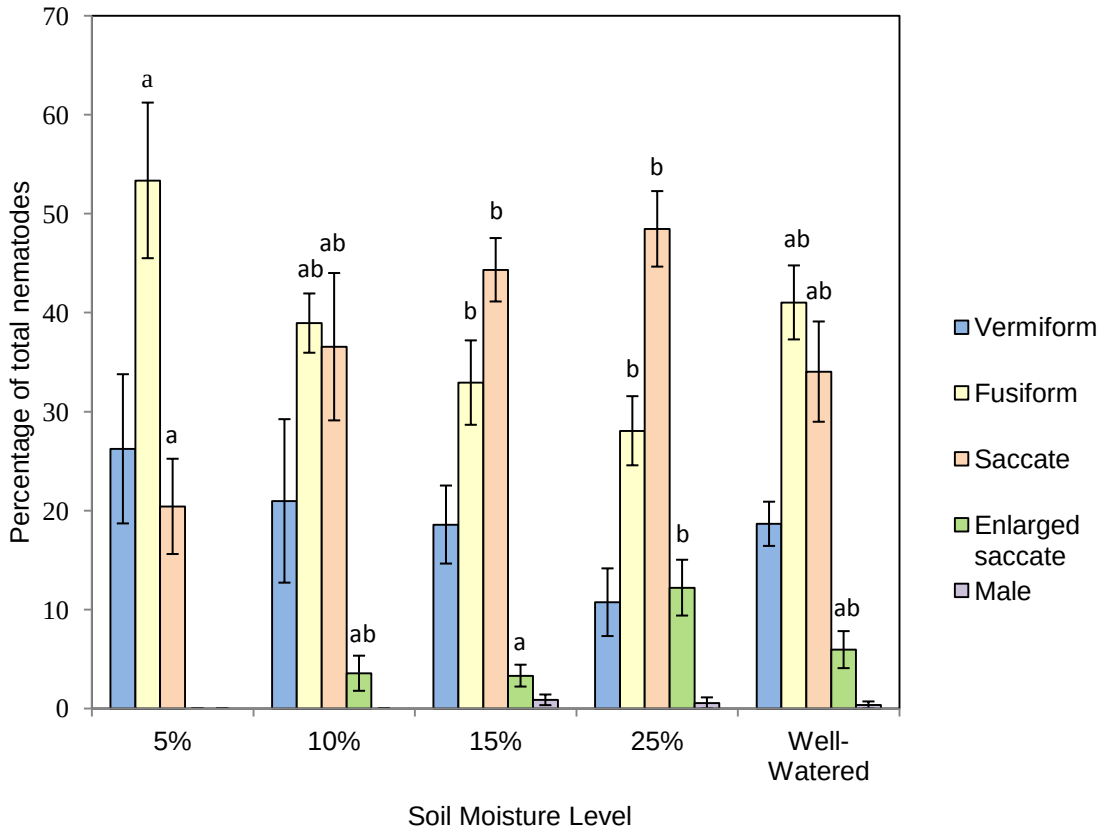


Figure 2.8. The effect of different drought treatments on the progression of nematode parasitism. The number of *H. schachtii* nematodes at each stage of parasitism was calculated as a proportion of the total. Different letters represent differences in the proportion of nematodes at a given parasitism stage compared to other drought treatment groups, according to the SNK test ($p < 0.05$) (n = 5). Bars displaying two letters show no difference to either group.

2.3.3 Induction of marker genes in response to stress treatments

The expression of certain marker genes was induced following stress treatments in tissue culture conditions. The results of semi-quantitative RT-PCRs on cDNA from leaf and root tissue are shown in Figure 2.9. The induction of *DREB1* can be seen in both dehydration-stressed leaf and root tissue (Figure 2.9A and B, respectively). *DREB2* was highly induced by dehydration in leaf tissue but only slightly induced in root tissue (this was confirmed by qRT-PCR (data not shown)). In root tissue, the relative transcript quantity of *MIOX5* was higher in cDNA from plants infected with the nematode *H. schachtii* than in uninfected tissue (Figure 2.10A). Similarly in leaf tissue, the expression of the SAR marker gene *PR1* was heightened in the nematode-infected plants compared to their non-parasitised counterparts (Figure 2.10B).

2.3.4 Confirmation of RNA quality

RNA extracted for use in microarray analysis was confirmed to be of high quality using the Bioanalyser. Figure 2.11 shows two typical electropherograms resulting from root and leaf RNA. Both electropherograms show a flat baseline indicating a lack of degradation. Two sharp peaks in the approximate ratio of 1:2 representing the 18s and 28s ribosomal RNAs are visible, indicating uncontaminated, intact RNA suitable for hybridisation onto a microarray chip. The leaf samples show three extra peaks just smaller than the 18s, which are produced by the chloroplast RNA. Quantification was achieved by comparison to the RNA ladder.

2.3.5 Microarray data quality and validity

Raw data files from the array scanner (CEL files) were normalised using the RMA algorithm. Figure 2.12 shows the spread of signal values across all the arrays following RMA normalisation. Median values were comparable across arrays, and variance was similar, ranging from 0.412 to 0.572. Consistency of hybridisation can be assessed by comparing the signal value of spiked-in biotin-labelled cRNA transcripts of bioB, bioC, bioD and creX. These controls are added in increasing concentrations into the array hybridisation cocktail. BioB is present at the lowest concentration and therefore its detection represents the level of assay sensitivity: It should be detected in at least 50 % of samples. When analysed, bioB was detected in all of the 24 arrays and the hybridisation controls were present in increasing signal values from bioB through bioC,

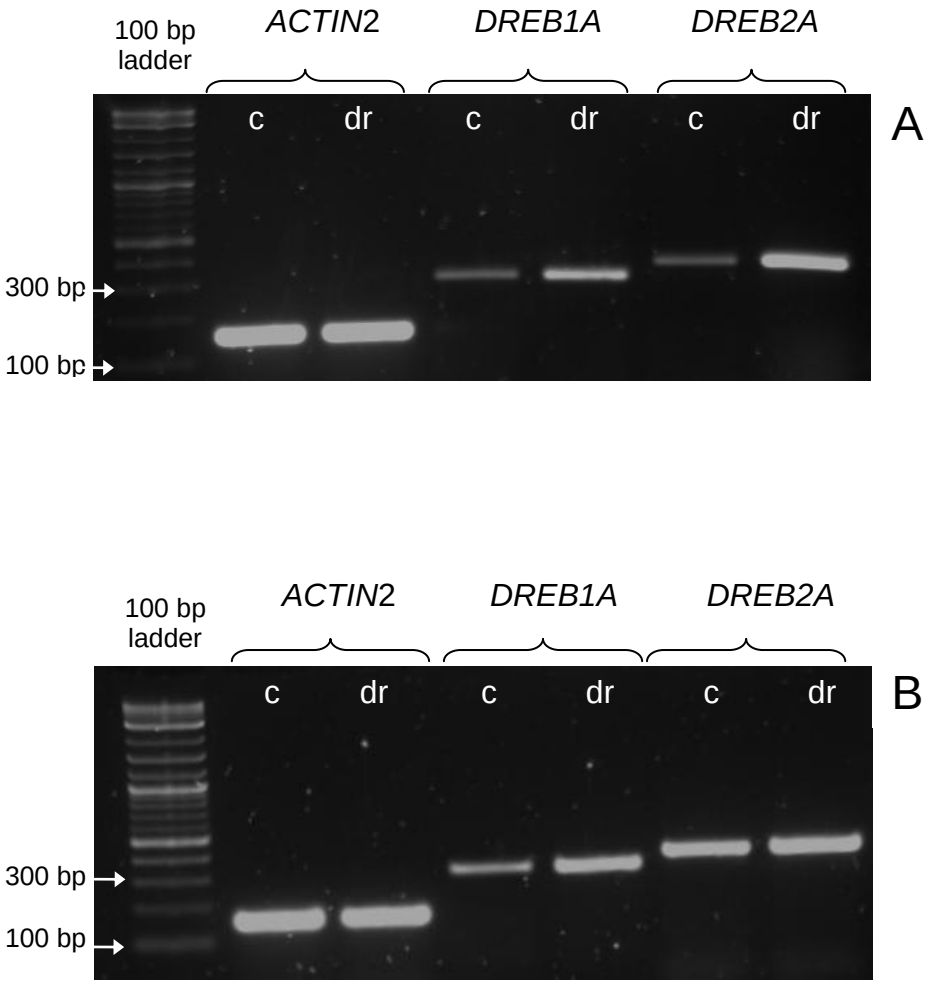


Figure 2.9. Induction of drought stress marker genes following dehydration treatment. Differences in *DREB1A* and *DREB2A* transcript level between control and dehydration-stressed plants were detected through semi-quantitative RT-PCR, using a program of 25 PCR cycles. **A)** PCR of leaf cDNA showing *ACTIN 2* (156 bp), *DREB1A* (312 bp) and *DREB2A* (368 bp) in control (c) and dehydrated (dr) plants. **B)** PCR of root cDNA showing *ACTIN 2*, *DREB1A* and *DREB2A* in control (c) and dehydrated (dr) plants.

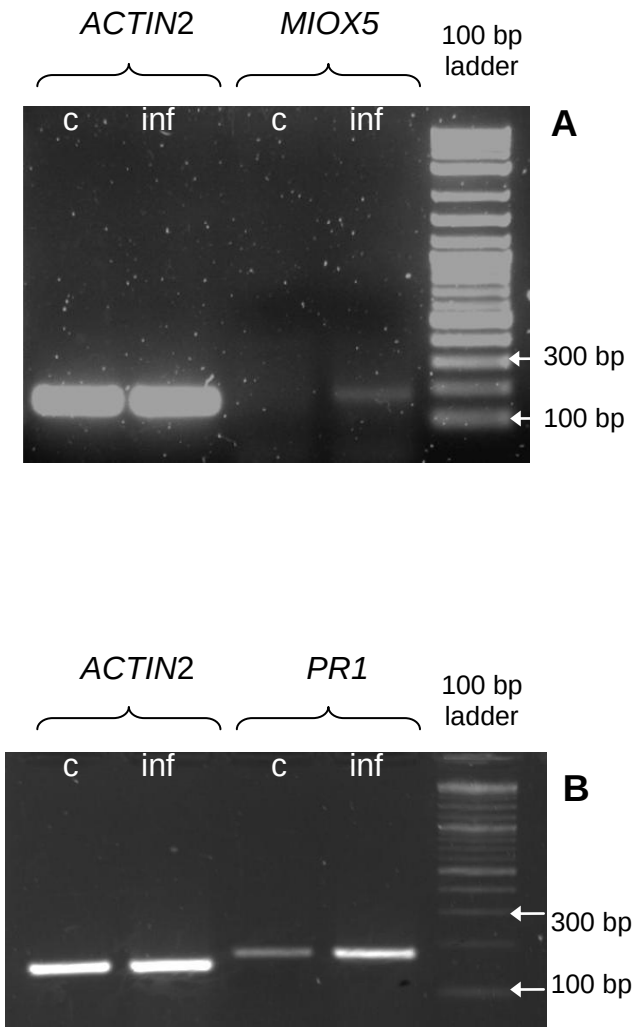


Figure 2.10. Induction of nematode and pathogen response marker genes following nematode infection. Differences in *MIOX5* and *PR1* transcript levels between control and nematode infected plants were detected through semi-quantitative RT-PCR, using a program of 25 PCR cycles. **A)** PCR of root cDNA showing *ACTIN 2* (156 bp) and *MIOX5* (191 bp) in control (c) and *H. schachtii*-infected (inf) plants. **B)** PCR of leaf cDNA showing *ACTIN 2* and *PR1* (177 bp) in control (c) and *H. schachtii*-infected (inf) plants.

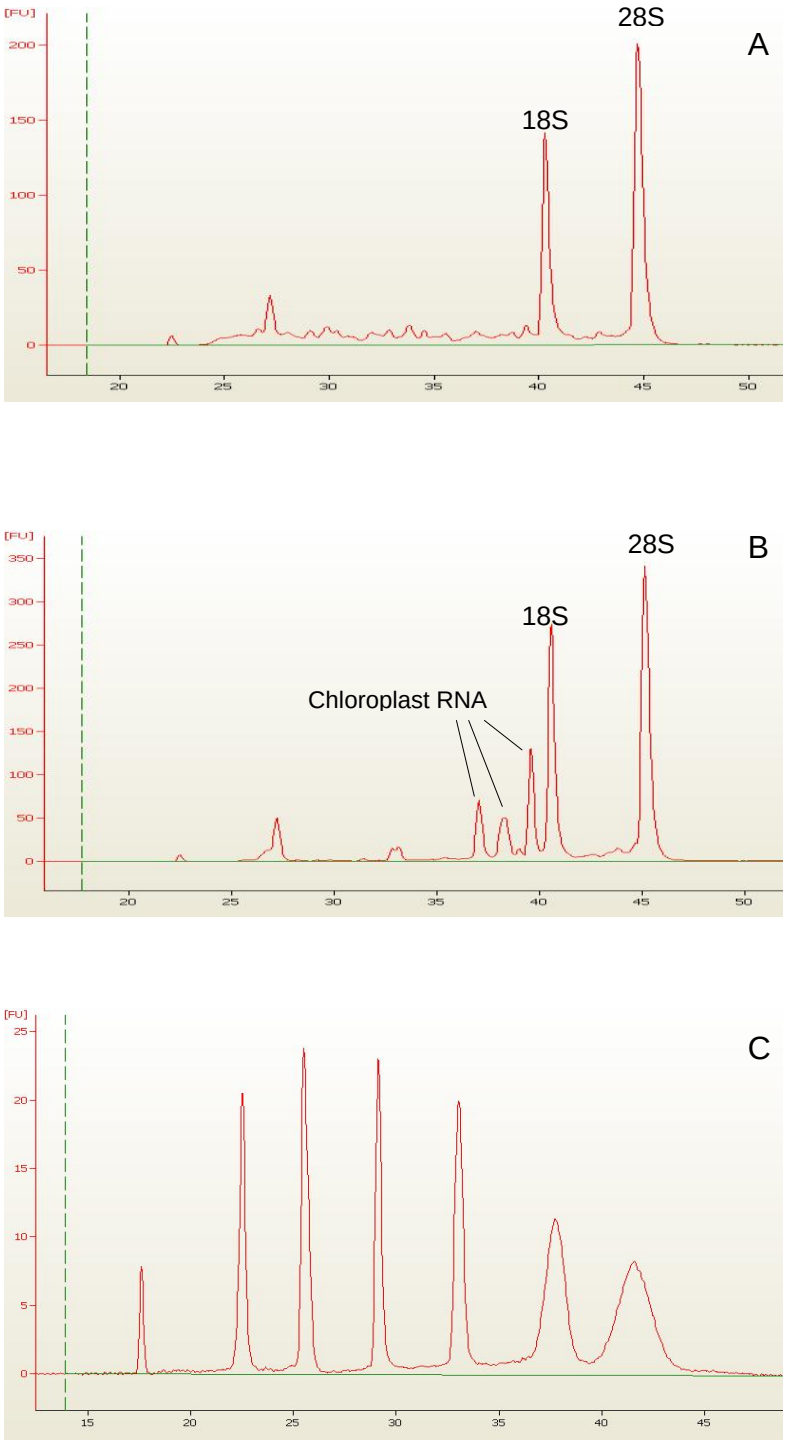


Figure 2.11. Representative Agilent Bioanalyser Electropherograms. The Agilent Bioanalyser was used to assess the quality of RNA samples before use in microarray analysis. The horizontal axis represents time and the vertical axis represents fluorescence. **A)** An electropherogram from a root RNA sample. The RNA is good quality as a flat baseline can be observed and a good 1:2 ratio between the two large rRNA peaks. **B)** RNA from a leaf sample, in which the 3 extra chloroplast RNA peaks can be observed. **C)** The RNA ladder.

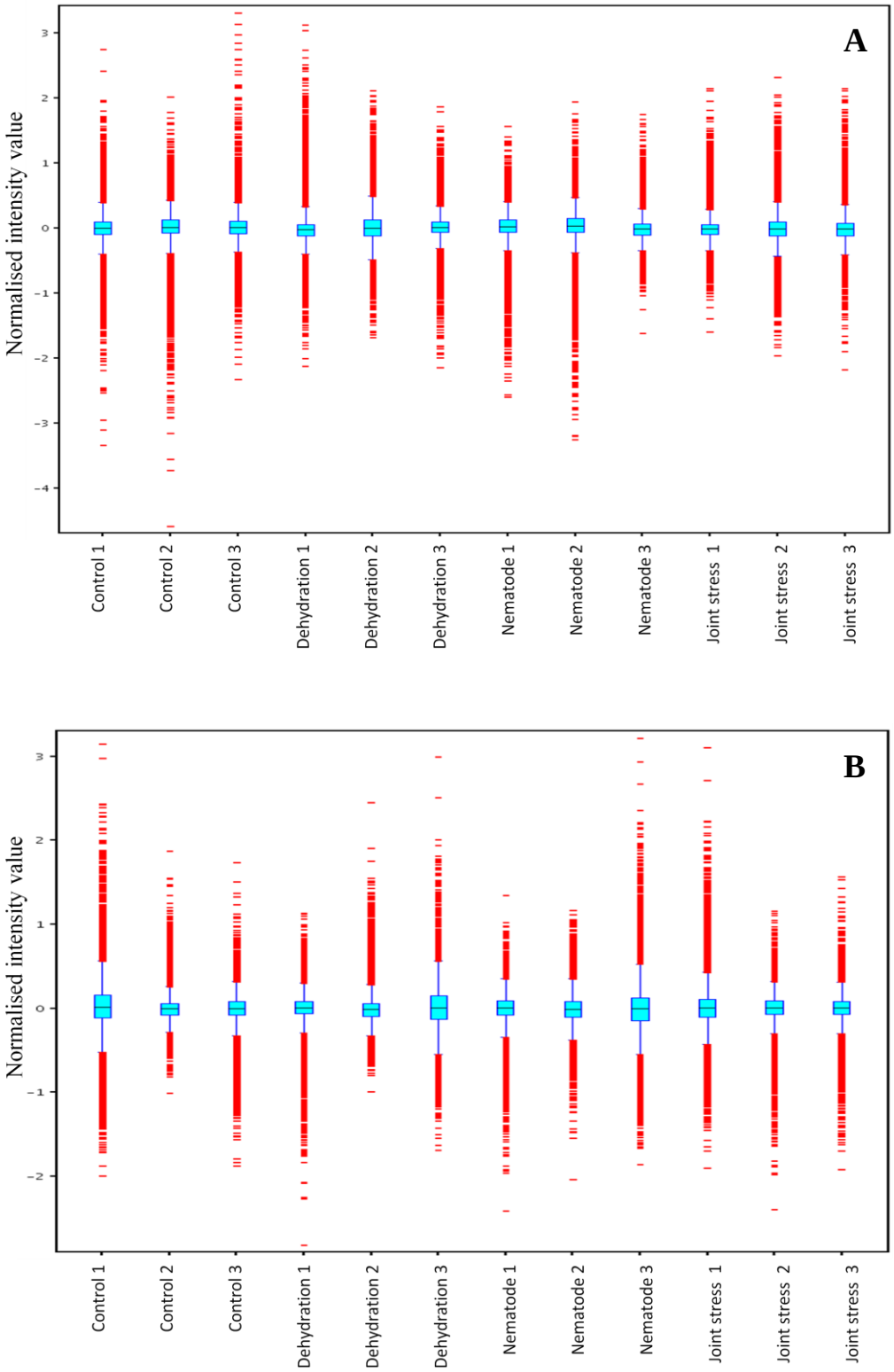


Figure 2.12. Normalised distribution of array data. Box plots showing the distribution of normalised intensity values for each array carried out on root (A) and leaf tissue (B). Median values are shown by black lines and blue boxes show the 25th and 75th percentile. Bars represent 1.5 standard deviations away from the median. Probes with intensity values beyond 1.5 times the standard deviation (outliers) are shown in red.

bioD and cre, indicating that no arrays had sub-optimal hybridisation (data not shown). Figure 2.13 shows the correlation between probe intensities from different arrays in the form of heat maps. Control arrays bear the closest correlation to nematode-treated arrays, whilst dehydration-treated and joint-stress treated arrays were closely correlated with each other. Correlation coefficients between biological replicates was always greater than 0.98. Between treatments the lowest correlation was between nematode and dehydration arrays in root (on average 0.968) and also between nematode and dehydration arrays in leaf (on average 0.974). These patterns are easily visualised in Figure 2.14, which shows examples of the data represented in scatter-plots. The normalised intensity value of each gene in a stress-treated array is shown against a control array, and two replicate control arrays against each other for reference. The plots comparing control arrays with either dehydration or joint stress arrays show greater deviation from the x = y line. The greatest disparity is shown when comparing a leaf array with a root array. In this case the gene expression values tend towards a negative correlation.

Data has been deposited in the public repository NASCArrays and is accessible at http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl with the reference number NASCARRAYS-489.

2.3.6 Identification of differentially expressed genes

Affymetrix *Arabidopsis* ATH1 GeneChip array hybridisations were carried out to examine changes in gene transcript level of *A. thaliana* plants subjected to dehydration, nematode stress, or their combination. Leaf and root tissue was examined separately. The numbers of genes differentially regulated by each treatment are shown in Table 2.2. A total of 3728 (1558 up, 2170 down) and 3174 (1519 up, 1655 down) genes displayed significant differential expression in leaf and root tissue ($p < 0.05$), respectively, in response to dehydration stress, representing 15 % and 13 % of the *A. thaliana* genome. Approximately 40 % of the differentially expressed genes in leaves were up-regulated (as opposed to down-regulated) whilst 50 % of the root genes were up-regulated.

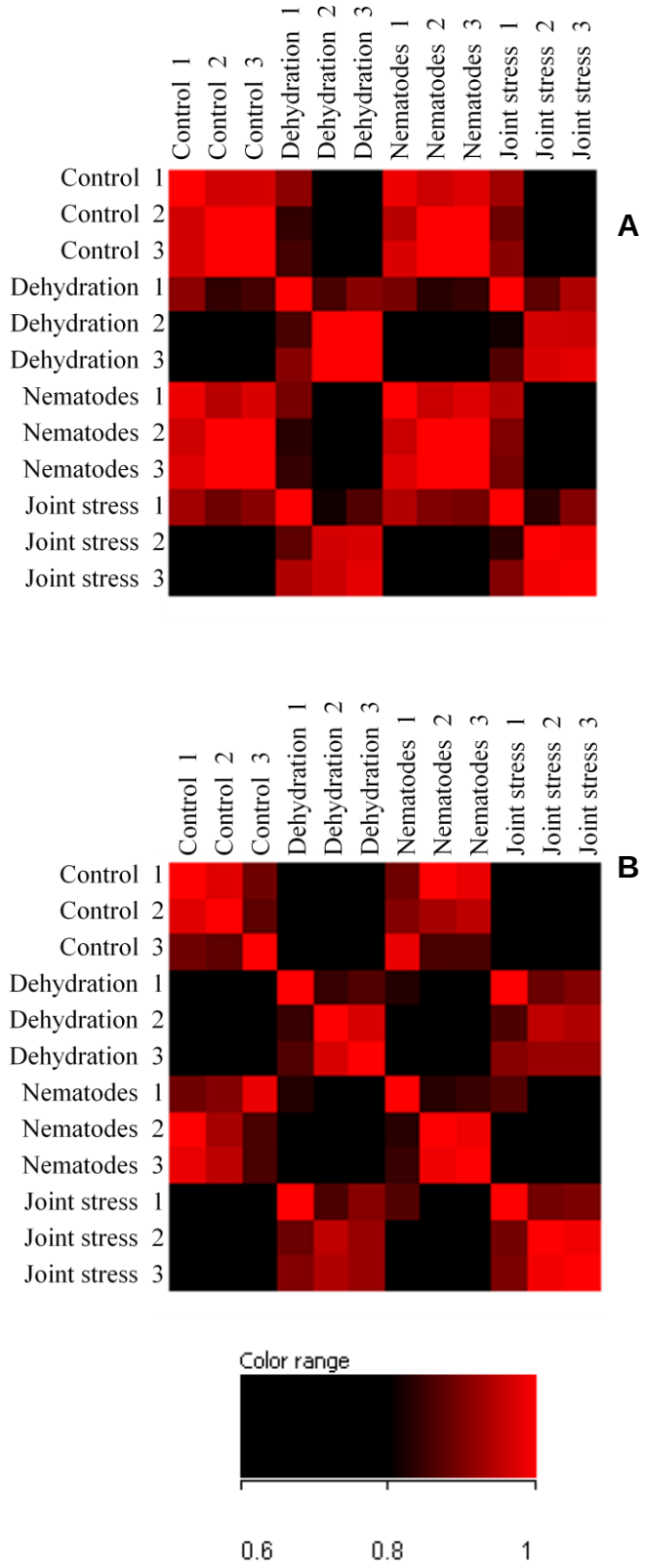


Figure 2.13. Heat map showing correlation between arrays for different treatments. Correlation between probe intensities from arrays carried out using (A) roots or (B) leaves of plants under different stress treatments is shown by colour. Black indicates a low correlation while red indicates a high correlation.

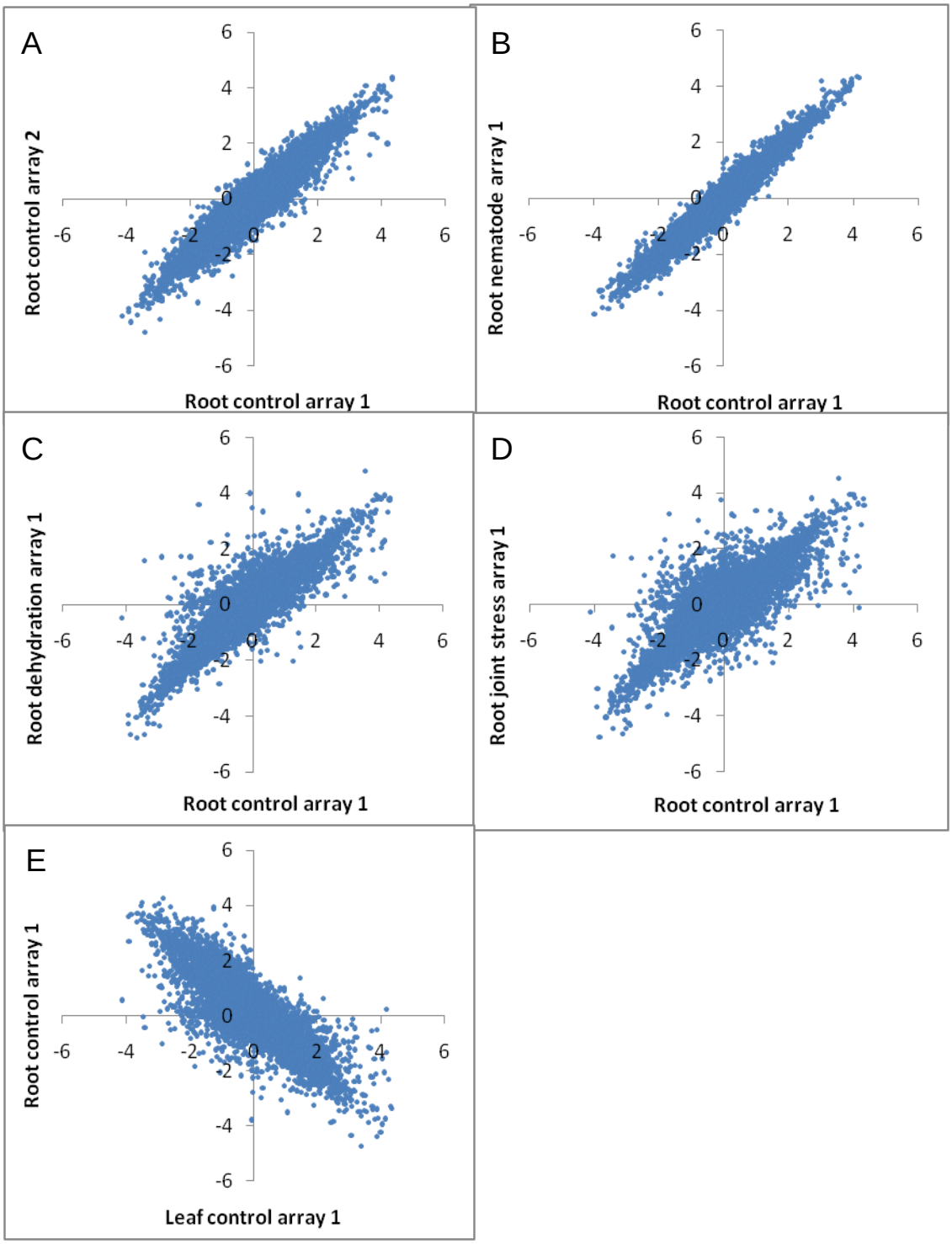


Figure 2.14. Scatterplots showing correlation of intensity data between arrays. X and Y axes show the normalised intensity data of all probe sets for (A) root control array 1 against root control array 2, (B) root control against root nematode stress, (C) root control against root dehydration stress, (D) root control against root joint stress and (E) leaf control against root control.

Treatment	Tissue	Number of genes significantly up-regulated	Number of genes significantly down-regulated
Nematode	Leaf	385	190
Dehydration	Leaf	1558	2170
Nematode + Dehydration	Leaf	1606	2014
Nematode	Root	260	278
Dehydration	Root	1519	1655
Nematode + Dehydration	Root	1278	1198

Table 2.2. Number of genes found to be differentially regulated by stress treatments. Whole-genome transcriptional analysis was carried out using Affymetrix ATH1 22 k arrays. Three replicate arrays were performed per treatment category. Genes were classed as differentially regulated if their averaged expression differed significantly from that in the unstressed control arrays ($p < 0.05$).

Around 24 % of the up-regulated genes were induced with a fold change of more than 2, whilst only 4 % of the down-regulated genes were repressed two-fold or more, indicating that the plants' response to dehydration is mediated more through induction of genes than repression. The most highly up-regulated genes in both roots and leaves were AP2 transcription factors, the relative transcript levels of which reached 19-fold in leaves and 31-fold in roots. Also very strongly induced was *DREB1B* (AT4G25490), ethylene-responsive element binding proteins, zinc finger family proteins and the MYB transcription factors *MYB2* (AT2G47190) in roots and *MYB74* (AT4G05100) in leaves. In leaves an F-box protein (AT1G61340) was also very strongly up-regulated. Amongst the down-regulated genes MYB and AP2 transcription factors as well as ethylene-responsive element binding proteins and zinc finger family proteins were also in the most highly repressed groups, but with fold changes of only 3 in roots and 5 in leaves, indicating that similar transcription factors may provide positive and negative regulation during stress responses. Up- and down-regulated genes were classified into gene ontology categories according to their Biological Process annotation. The enrichment or depletion of categories was assessed against the distribution of total gene counts across the whole genome, and several functional categories were found to differ significantly in their expected gene count. Over-represented GO categories of genes induced or repressed by each stress treatment are shown in Figures 2.15 and 2.16, respectively. The subsets up-regulated by dehydration stress in roots and leaves contained significantly higher numbers of genes involved in *response to stress, response to abiotic or biotic stimulus, transcription* and *signal transduction*, as may be expected for such a well characterised abiotic stress, whilst lower than the expected number of genes involved in *cell organisation and biogenesis, transport* (root only) and *DNA or RNA metabolism* (leaf only) were observed. Amongst the down-regulated genes, the categories *transport* and *signal transduction* were over-represented in roots, whilst *response to biotic and abiotic stimuli* and *electron transport or energy pathways* were enriched in leaves.

Nematode stress caused only 538 transcript changes in roots (260 up and 278 down) and 575 in leaves (385 up and 190 down). Approximately 50 % of these genes were up-regulated in roots (as opposed to down-regulated), whilst 65 % were up-regulated in

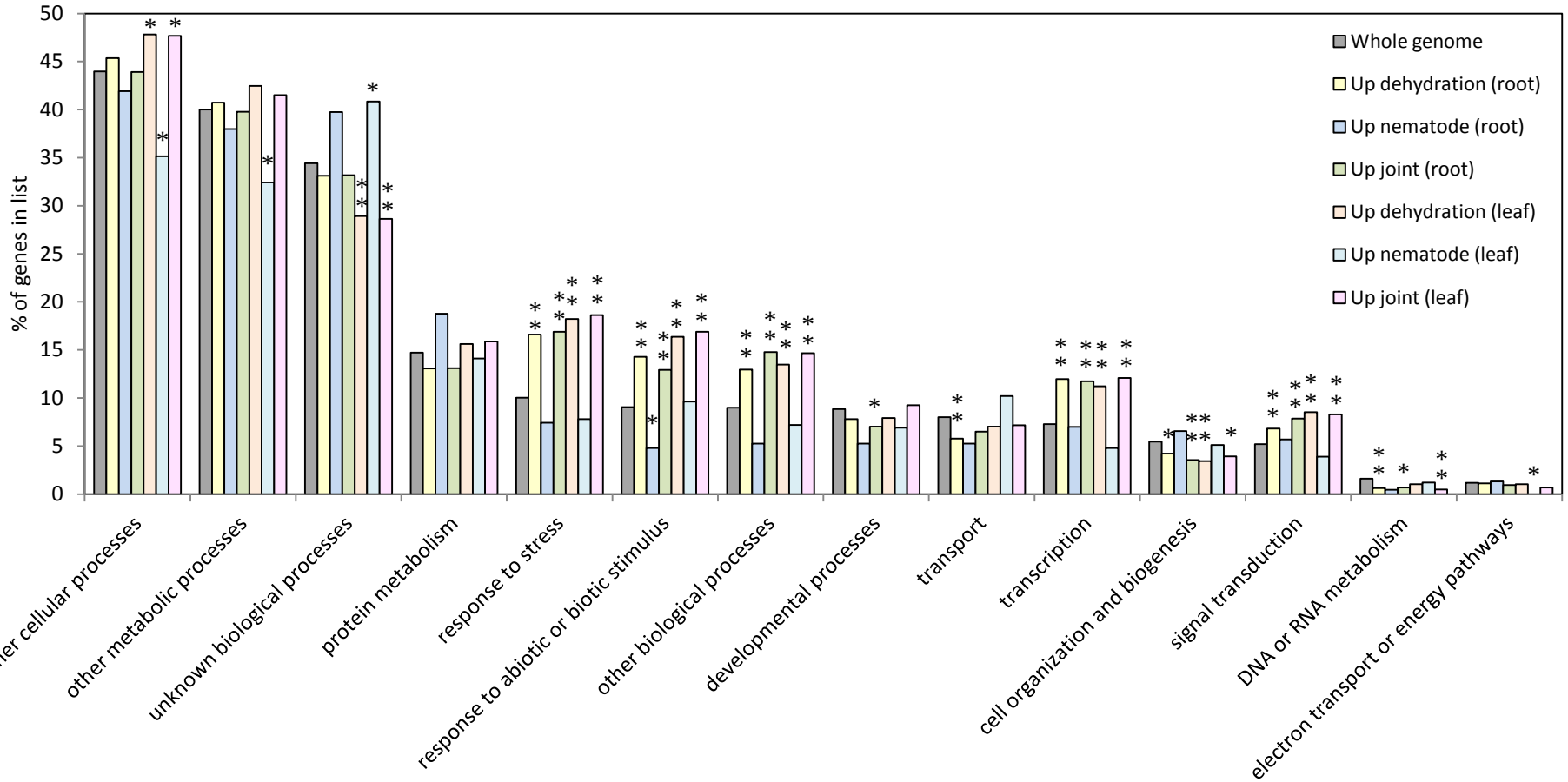


Figure 2.15. Classification of A. thaliana genes up-regulated by each stress treatment. Genes are classified by their Biological Process annotation as determined by the TAIR Go Ontology tool. The percentage of genes in each category is given, as well as the percentage of genes in the entire genome with that annotation. Significance was determined using Chi^2 tests of comparison. * = $p < 0.05$. ** = $p < 0.01$. Genes may be represented in more than one category due to multiple annotations.

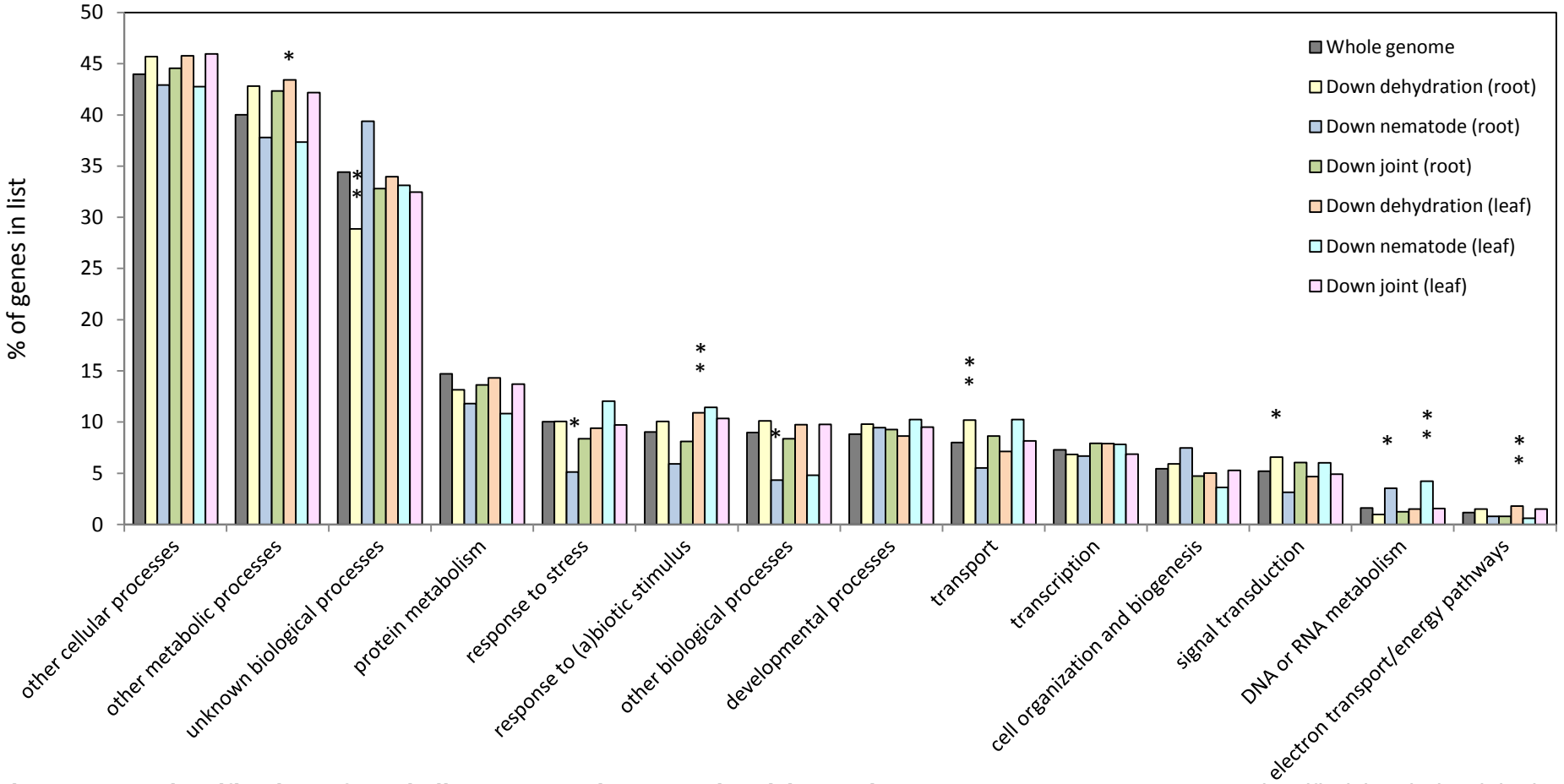


Figure 2.16. Classification of A. thaliana genes down-regulated by each stress treatment. Genes are classified by their Biological Process annotation as determined by the TAIR Go Ontology tool. The percentage of genes in each category is given, as well as the percentage of genes in the entire genome with that annotation. Significance was determined using Chi^2 tests of comparison. * = $p < 0.05$. ** = $p < 0.01$. Genes may be represented in more than one category due to multiple annotations.

leaves. None of the differentially regulated gene transcripts were changed by 2-fold or more, suggesting a much lesser magnitude of stress response than for dehydration. In roots, the most highly up-regulated genes were those encoding an extensin (AT1G26250), a cytochrome P450 (AT3G26220), a disease resistance protein containing a leucine-rich repeat (LRR) domain (AT5G47280), two UDP glucosyl transferase proteins and several unknown proteins. Abundant amongst the up-regulated genes in leaves were three pentatricopeptide (PPR) repeat containing proteins, another LRR family protein, a senescence-associated protein and several unknown proteins. The only GO category significantly enriched amongst genes up-regulated by nematode infection was *unknown biological processes*, whilst lower than expected numbers of genes were observed in the categories *electron transport or energy pathways* (in leaves) and *response to biotic or abiotic stimuli* (in roots). Amongst the down-regulated genes, the category *DNA or RNA metabolism* was significantly enriched in both leaves and roots, whilst there were fewer than expected genes in the *response to stress* category in roots alone. The difference in magnitude in terms of number and fold change of induced genes between the two stresses may reflect the fact that dehydration was the more severe stress, affecting the entire plant, whereas any changes elicited by the nematode infection were more subtle and therefore showed less of an effect over the whole plant.

Combined nematode and dehydration stress (joint stress) caused 2476 changes in roots (1278 up and 1198 down) and 3620 changes in leaves (1606 up and 2014 down). The most strongly up and down-regulated genes were very similar to those identified as a result of dehydration stress alone, as might be expected considering both sets of plants had experienced the same severe dehydration treatment. Furthermore, the enriched GO categories for genes induced by joint stress were similar to those identified by dehydration stress alone, as shown in Figure 2.15 and 2.16. The few differences were largely in the down-regulated gene subsets, for which the categories *transport, signal transduction, response to biotic or abiotic stimuli* and *electron transport or energy pathways* were no longer significantly enriched with the addition of the second stress, in comparison to dehydration stress alone.

2.3.7 Overlap between subsets of differentially expressed genes

Venn diagrams illustrate the overlap between sets of genes induced by each treatment (Figure 2.17). The diagram shows that there was little similarity between the response of *A. thaliana* to dehydration and to nematode stress. Of 1519 genes up-regulated in roots by dehydration stress and 260 elevated by nematode stress, an overlap of only 38 was found. In leaves, an overlap of only 54 transcripts was observed between 1558 and 385 transcripts induced by dehydration and nematode stress, respectively. Amongst these overlapping genes no significantly enriched GO categories were observed, although over half the genes had no assigned biological function. When the two stresses were applied together (joint stress), the subset of genes induced resembled far more closely that of dehydration stress compared to that of nematode stress, as was also indicated by the previous observations of number, fold change and GO categories of dehydration and joint stress induced genes. Of the transcripts elevated by joint stress in roots, a large proportion (837 genes) was also elevated during dehydration whereas only a few (25 genes) were also elevated during nematode stress. In leaves, 1100 of the transcripts up-regulated by joint stress were also elevated by dehydration, but only 60 by nematode stress. In leaves only 60 genes were induced or repressed by all three stresses. In roots the figure was 21. The lack of overlap between all the stress treatments demonstrates the specificity of plants when responding to different environmental stresses.

In addition to these overlapping transcript changes, each stress treatment induced its own set of specific changes that were not co-regulated by any other stress. Of particular interest were transcript changes that were induced specifically in response to a combination of dehydration and nematode stress, termed ‘joint stress specific’ genes. In roots 427 genes were specifically up-regulated by this stress combination, whilst in leaves 472 joint stress only genes were identified. Furthermore, 640 genes were down-regulated in roots specifically in response to combinatorial stress, and 855 in were down-regulated in leaves. The transcriptome of plants that underwent a combination of dehydration and nematode stress was thus different to that of plants subjected to dehydration or nematode stress alone. In order to test the hypothesis that certain regulatory genes may specifically control the response of plants to multiple stresses, it

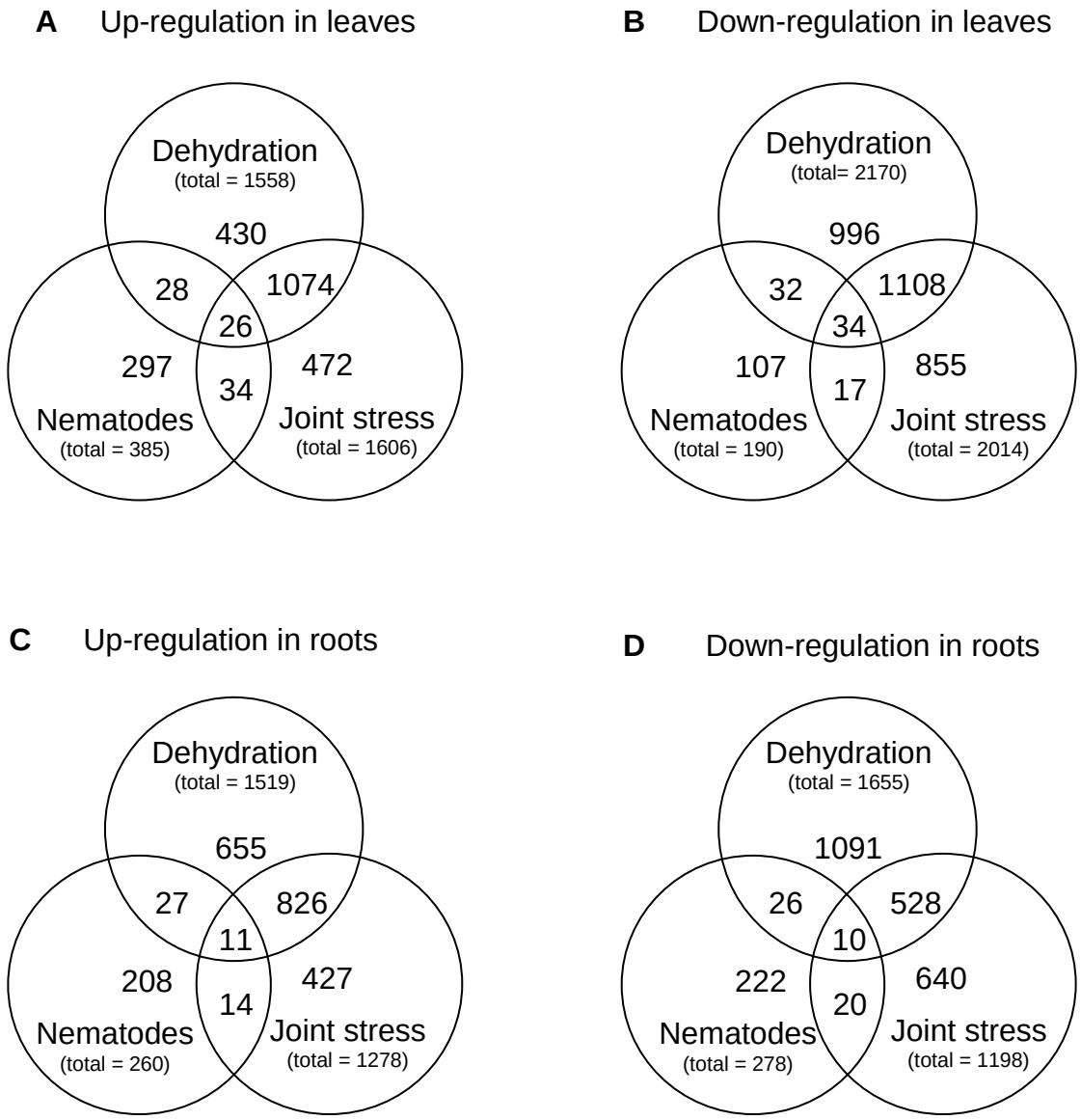


Figure 2.17. Venn diagrams showing overlap between categories of genes differentially regulated by stress treatments. Genes up- and down-regulated in leaves (A and B, respectively) and roots (C and D, respectively) are shown separately. Genes were included if their expression levels differed significantly from control arrays where $p < 0.05$. Overlapping circles represent genes that were up- or down-regulated by more than one stress treatment. Results shown are averaged from three replicate experiments.

was important to identify genes that had a separate pattern of expression under joint stress to that under individual stress. As the dehydration stress and joint stress induced a comparatively similar set of transcriptome changes, a new list of 'interaction' genes was created by comparing expression values *between* the dehydration and joint stress arrays, using the dehydration arrays as controls. This identified genes that were significantly induced or repressed in addition to their status under dehydration stress alone, and eliminated any of the original 'joint stress specific' genes that may have been just over the *p*-value of 0.05 due to dehydration stress but just under the *p*-value of 0.05 due to joint stress (i.e. not significantly differentially regulated between the two). Any genes whose expression was not significantly different between joint stress and nematode stress alone were removed from the list so that it contained only genes significantly differentially regulated by joint stress. The resulting 'interaction' gene lists comprised 385 genes that were up-regulated in roots, 522 genes that were down-regulated in roots, 566 genes up-regulated in leaves and 444 genes down-regulated in leaves. Of these, four smaller gene lists were created, comprising the 50 most highly up- or down-regulated genes in roots and leaves. These lists are given in Tables 2.3-6. Of the up-regulated 'interaction' genes, the highest fold change was 1.9 in leaves and 1.6 in roots. Of the down-regulated genes the highest fold changes were 2.4 and 2.3, respectively. Amongst the interaction genes were large groups of genes involved in functional processes such as cell wall re-modification, carbohydrate metabolism, heat shock response and disease resistance. Signal transduction and regulation genes were also prevalent, including transcription factors, pentatricopeptide repeat (PRR) containing genes and protein kinases. Figure 2.18 shows the enriched GO categories of the interaction genes. Amongst the interaction genes in roots, the process groups *developmental processes* ($p < 0.05$) and *transport* ($p < 0.05$) were significantly over-represented. Meanwhile the group of genes down-regulated in roots had significantly more *cell organisation and biogenesis* annotations than would be expected ($p < 0.05$). Down-regulated leaf interaction genes were enriched in *signal transduction* counts ($p < 0.01$), whereas amongst the up-regulated leaf interaction genes there was no significant enrichment of any process category. A comparison of the interaction genes found in leaves and roots was carried out, and 18 commonly up-regulated genes were identified, as well as 14 commonly down-regulated. Among the commonly up-regulated genes were those

AGI Code	Gene Function	Fold Change	*p*-value
AT2G20870	cell wall protein precursor, putative	1.911	0.034
ATCg01060	Encodes the PsaC subunit of photosystem I.	1.784	0.040
AT1G66850	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	1.735	0.004
AT3G15400	anther development protein, putative	1.490	0.007
AT3G01390	vacuolar ATP synthase subunit G 1 (VATG1) / V-ATPase G subunit 1 (VAG1) / vacuolar proton pump G subunit 1 (VMA10)	1.455	0.042
AT5G06630	proline-rich extensin-like family protein	1.448	0.034
AT3G07660	expressed protein	1.424	0.042
AT3G48040	Rac-like GTP-binding protein (ARAC8)	1.402	0.034
AT3G55420	expressed protein	1.392	0.034
AT4G34150	C2 domain-containing protein	1.383	0.029
AT3G26140	glycosyl hydrolase family 5 protein / cellulase family protein	1.381	0.008
AT4G21370	S-locus protein kinase, putative	1.380	0.047
AT5G48050	hypothetical protein	1.365	0.023
AT5G10430	arabinogalactan-protein (AGP4)	1.364	0.012
AT3G05480	cell cycle checkpoint control protein family	1.354	0.036
AT5G66140	20S proteasome alpha subunit D2 (PAD2) (PRS1) (PRC6)	1.350	0.036
AT3G16570	rapid alkalinization factor (RALF) family protein	1.346	0.045
AT3G48970	copper-binding family protein	1.345	0.040
AT5G62210	embryo-specific protein-related	1.343	0.044
AT4G30320	allergen V5/Tpx-1-related family protein	1.342	0.017
AT1G55410	pseudogene, CHP-rich zinc finger protein	1.340	0.004
AT4G25050	acyl carrier family protein / ACP family protein	1.340	0.017
AT1G49975	expressed protein	1.338	0.021
AT1G28630	expressed protein	1.335	0.015
AT2G40820	proline-rich family protein	1.330	0.048
AT3G12110	actin 11 (ACT11)	1.329	0.014
AT3G25620	ABC transporter family protein	1.327	0.023
AT1G78440	gibberellin 2-oxidase / GA2-oxidase (GA2OX1)	1.326	0.039
AT2G03890	phosphatidylinositol 3- and 4-kinase family protein	1.323	0.016
AT3G44020	thylakoid lumenal P17.1 protein	1.322	0.016
AT5G26350	transposable element	1.317	0.030
AT3G25820 /AT3G25830	myrcene/ocimene synthase, putative	1.316	0.012
AT4G32030	expressed protein	1.315	0.006
AT1G69690	TCP family transcription factor, putative	1.309	0.022

AT2G17610	transposable element	1.307	0.003
AT1G68875	expressed protein	1.304	0.003
AT2G35620	leucine-rich repeat transmembrane protein kinase, putative	1.296	0.008
AT5G60200	Dof-type zinc finger domain-containing protein	1.283	0.026
AT2G38905	hydrophobic protein, putative / low temperature and salt responsive protein, putative	1.271	0.046
AT1G36020	hypothetical protein	1.268	0.007
AT5G66090	expressed protein	1.266	0.007
AT3G61400	2-oxoglutarate-dependent dioxygenase, putative	1.255	0.008
AT2G45680	TCP family transcription factor, putative	1.254	0.048
AT3G16860	phytochelatin synthetase-related	1.252	0.007
AT1G74970	ribosomal protein S9 (RPS9)	1.248	0.006
AT1G29750	leucine-rich repeat transmembrane protein kinase, putative / serine/threonine kinase, putative (RKF1)	1.247	0.027
AT1G10200	transcription factor LIM, putative	1.245	0.013
AT5G60470	zinc finger (C2H2 type) family protein	1.244	0.012
AT5G56110	myb family transcription factor (MYB103)	1.240	0.035
AT4G19110	protein kinase, putative	1.236	0.003

Table 2.3. The 50 'interaction' genes that were most highly up-regulated in response to joint stress as compared to individual stress, in leaf tissue. A total of 566 genes were significantly up-regulated in leaves by the addition of a second stress (nematode infection) compared to plants under a single stress (dehydration) ($p < 0.05$). The genes are listed in order of fold change. The fold changes shown are an average of three replicates.

AGI Code	Gene Function	Fold Change	*p*-value
AT4G12470	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	-2.369	0.045
AT1G13470	expressed protein	-2.104	0.050
AT3G48640	expressed protein	-1.968	0.033
AT4G11890	protein kinase family protein	-1.855	0.019
AT1G57630	disease resistance protein (TIR class), putative	-1.731	0.031
AT4G19810	glycosyl hydrolase family 18 protein	-1.565	0.029
AT1G70140	formin homology 2 domain-containing protein / FH2 domain-containing protein	-1.544	0.016
AT1G62840	expressed protein	-1.509	0.033
AT3G47090 /AT3G47580	leucine-rich repeat transmembrane protein kinase, putative	-1.509	0.013
AT1G15670	kelch repeat-containing F-box family protein	-1.475	0.044
AT5G24530	oxidoreductase, 2OG-Fe(II) oxygenase family protein	-1.470	0.022
AT4G39830	L-ascorbate oxidase, putative	-1.465	0.033
AT2G44080	expressed protein	-1.456	0.030
AT5G39520	expressed protein	-1.456	0.042
AT2G16890	UDP-glucoronosyl/UDP-glucosyl transferase family protein	-1.443	0.000
AT1G66910 /AT1G66920	protein kinase, putative	-1.428	0.025
AT1G69550	disease resistance protein (TIR-NBS class), putative	-1.428	0.038
AT1G43910	AAA-type ATPase family protein	-1.416	0.018
AT2G32000	DNA topoisomerase family protein	-1.412	0.013
AT5G53890	leucine-rich repeat transmembrane protein kinase, putative	-1.405	0.031
AT5G46520	disease resistance protein (TIR-NBS-LRR class), putative	-1.404	0.018
AT2G43590	chitinase, putative	-1.404	0.040
AT3G16670	expressed protein	-1.399	0.013
AT4G35560	expressed protein	-1.388	0.032
AT3G47780	ABC transporter family protein	-1.386	0.031
AT2G28880	para-aminobenzoate (PABA) synthase family protein	-1.384	0.007
AT3G25010	disease resistance family protein	-1.379	0.043
AT3G26230	cytochrome P450 family protein	-1.375	0.024
AT5G10740	protein phosphatase 2C-related / PP2C-related	-1.373	0.023
AT3G47890 /AT3G47900 /AT3G47910	ubiquitin carboxyl-terminal hydrolase-related	-1.371	0.019
AT2G41370	ankyrin repeat family protein / BTB/POZ domain-containing protein	-1.366	0.005
AT3G26300	cytochrome P450 family protein	-1.363	0.017

AT2G03670	AAA-type ATPase family protein	-1.361	0.019
AT3G18930	zinc finger (C3HC4-type RING finger) family protein	-1.360	0.015
AT4G13900 /AT4G13920	pseudogene, similar to NL0D	-1.347	0.034
AT4G13810	disease resistance family protein / LRR family protein	-1.344	0.047
AT3G15352	cytochrome c oxidase copper chaperone-related	-1.337	0.022
AT1G18090	exonuclease, putative	-1.323	0.013
AT2G04630	DNA-directed RNA polymerase II, putative	-1.318	0.036
AT3G61880	cytochrome P450, putative	-1.315	0.013
AT4G38620	myb family transcription factor (MYB4)	-1.307	0.019
AT3G13420	expressed protein	-1.303	0.000
AT3G48720	transferase family protein	-1.302	0.003
AT5G13960	SET domain-containing protein (SUVH4)	-1.299	0.031
AT3G53350	myosin heavy chain-related	-1.293	0.009
AT1G31120	potassium transporter family protein	-1.277	0.003
AT2G02780	leucine-rich repeat transmembrane protein kinase, putative	-1.268	0.009
AT3G23110 /AT3G23120	disease resistance family protein	-1.267	0.022
AT4G01910	DC1 domain-containing protein	-1.264	0.020
AT5G65640	basic helix-loop-helix (bHLH) family protein	-1.263	0.031

Table 2.4.The 50 'interaction' genes that were most highly down-regulated in response to joint stress as compared to individual stress, in leaf tissue. A total of 444 genes were significantly down-regulated in leaves by the addition of a second stress (nematode infection) compared to plants under a single stress (dehydration) ($p < 0.05$). The genes are listed in order of fold change. The fold changes shown are an average of three replicates.

AGI Code	Gene Function	Fold Change	*p*-value
AT5G01690	cation/hydrogen exchanger, putative (CHX27)	1.646	0.046
AT3G54590	proline-rich extensin-like family protein	1.583	0.008
AT5G49440	expressed protein	1.523	0.004
AT4G08410	proline-rich extensin-like family protein	1.503	0.033
AT5G13330	AP2 domain-containing transcription factor family protein	1.501	0.018
AT3G54580	proline-rich extensin-like family protein	1.494	0.039
AT1G35580	CINV1: beta-fructofuranosidase, putative / invertase, putative / saccharase, putative / beta-fructosidase, putative	1.475	0.028
AT1G52800	oxidoreductase, 2OG-Fe(II) oxygenase family protein	1.47	0.007
AT1G80320	oxidoreductase, 2OG-Fe(II) oxygenase family protein	1.435	0.020
AT1G21310	proline-rich extensin-like family protein	1.43	0.018
AT1G52790	oxidoreductase, 2OG-Fe(II) oxygenase family protein	1.426	0.005
AT1G03550	secretory carrier membrane protein (SCAMP) family protein	1.424	0.023
AT4G13340	leucine-rich repeat family protein / extensin family protein	1.417	0.029
AT1G23720	proline-rich extensin-like family protein	1.411	0.004
AT3G52970	cytochrome P450 family protein (CYP76G1)	1.388	0.027
AT2G34600	expressed protein	1.379	0.048
AT5G55980	serine-rich protein-related	1.354	0.045
AT2G01690	expressed protein	1.354	0.035
AT1G33030	O-methyltransferase family 2 protein	1.352	0.050
AT5G01720	F-box family protein (FBL3)	1.352	0.040
AT4G30160	villin, putative	1.351	0.014
AT5G65040	senescence-associated protein-related	1.35	0.023
AT4G01890	glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein	1.342	0.015
AT5G56640	MIOX5	1.336	0.006
AT3G27300	glucose-6-phosphate 1-dehydrogenase / G6PD (ACG9)	1.331	0.045
AT4G09300	expressed protein	1.329	0.024
AT2G43580	chitinase, putative	1.317	0.023
AT3G02150	TCP family transcription factor, putative	1.314	0.040
AT1G65500	expressed protein	1.299	0.018
AT2G37780	DC1 domain-containing protein	1.299	0.042
AT3G15060	Ras-related GTP-binding family protein	1.298	0.021
AT5G34870	zinc knuckle (CCHC-type) family protein	1.298	0.011
AT2G22720	expressed protein	1.293	0.042
AT2G24430	no apical meristem (NAM) family protein	1.292	0.021
AT2G48130	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	1.289	0.026
AT1G62580	flavin-containing monooxygenase family protein / FMO	1.285	0.044

/AT1G63340	family protein		
AT1G36756	hypothetical protein	1.285	0.025
AT5G53840	F-box family protein (FBL13)	1.284	0.038
AT3G18460	hypothetical protein	1.284	0.039
AT1G12020	expressed protein	1.284	0.005
AT1G29400	RNA recognition motif (RRM)-containing protein	1.281	0.022
AT5G17910	expressed protein	1.281	0.014
AT2G05470 /AT2G12120 /AT5G28482	transposable element, pseudogene	1.277	0.004
AT3G63240	endonuclease/exonuclease/phosphatase family protein	1.277	0.002
AT5G65170	VQ motif-containing protein	1.277	0.044
AT5G15320	expressed protein	1.275	0.024
AT2G16005	MD-2-related lipid recognition domain-containing protein / ML domain-containing protein	1.274	0.045
AT2G35620	leucine-rich repeat transmembrane protein kinase, putative	1.273	0.035
AT1G63930	expressed protein	1.272	0.011

Table 2.5. The 50 'interaction' genes that were most highly up-regulated in response to joint stress as compared to individual stress, in root tissue. A total of 385 genes were significantly up-regulated in roots by the addition of a second stress (nematode infection) compared to plants under a single stress (dehydration) ($p < 0.05$). The genes are listed in order of fold change. The fold changes shown are an average of three replicates.

AGI Code	Gene Function	Fold Change	*p*-value
AtCg00350	chloroplast genome -	-2.265	0.016
AtCg00340	Encodes the D1 subunit of photosystem I and II reaction centers.	-1.633	0.041
AT5G12030	17.7 kDa class II heat shock protein 17.6A (HSP17.7-CII)	-1.621	0.009
AtCg00520	Encodes a protein required for photosystem I assembly and stability	-1.595	0.016
AT3G46530	disease resistance protein, RPP13-like (CC-NBS class), putative	-1.535	0.025
AT5G01370	expressed protein	-1.455	0.015
AT4G27370	myosin family protein	-1.441	0.036
AT1G53540	17.6 kDa class I small heat shock protein (HSP17.6C-CI) (AA 1-156)	-1.441	0.014
AtMg00640	encodes a plant b subunit of mitochondrial ATP synthase based on structural similarity and the presence in the F(0) complex.	-1.436	0.028
AT2G32860	glycosyl hydrolase family 1 protein	-1.428	0.033
AT3G19800	expressed protein	-1.423	0.049
AT3G63110	adenylate isopentenyltransferase 3 / cytokinin synthase (IPT3)	-1.419	0.040
AT2G41230	expressed protein	-1.418	0.050
AT3G53960	proton-dependent oligopeptide transport (POT) family protein	-1.416	0.031
AT2G02000 /AT2G02010	glutamate decarboxylase, putative	-1.400	0.029
AT5G07620	protein kinase family protein	-1.399	0.024
AT4G33810	glycosyl hydrolase family 10 protein	-1.396	0.023
AtCg00530	hypothetical protein	-1.389	0.048
AT2G32120	heat shock protein 70 family protein / HSP70 family protein	-1.389	0.036
AT1G66130	oxidoreductase N-terminal domain-containing protein	-1.388	0.045
AT2G38210 /AT2G38230 /AT2G38240	ethylene-responsive protein, putative	-1.388	0.043
AT3G42725	expressed protein	-1.387	0.033
AT2G37510	RNA-binding protein, putative	-1.377	0.008
AtCg00120	Encodes the ATPase alpha subunit, which is a subunit of ATP synthase and part of the CF1 portion which catalyzes the conversion of ADP to ATP using the proton motive force. This complex is located in the thylakoid membrane of the chloroplast.	-1.374	0.048
AT5G24710	WD-40 repeat family protein	-1.368	0.044
AT2G07711	pseudogene, similar to NADH dehydrogenase subunit 5	-1.362	0.010
AT2G31160	expressed protein	-1.354	0.031

AT2G19490 /AT3G32920	recA family protein	-1.350	0.032
AT4G36150	disease resistance protein (TIR-NBS-LRR class), putative	-1.347	0.039
AT1G48100	glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein	-1.344	0.003
AT4G39190	expressed protein	-1.333	0.002
AT4G25850 /AT4G25860	oxysterol-binding family protein	-1.332	0.006
AT5G37180	sucrose synthase, putative / sucrose-UDP glucosyltransferase, putative	-1.331	0.026
AT5G04890	small heat shock-like protein (RTM2)	-1.328	0.005
AT1G13810	expressed protein	-1.327	0.005
AtCg01120	encodes a chloroplast ribosomal protein S15, a constituent of the small subunit of the ribosomal complex	-1.325	0.006
AT1G04020	zinc finger (C3HC4-type RING finger) family protein / BRCT domain-containing protein	-1.299	0.017
AT1G20300	pentatricopeptide (PPR) repeat-containing protein	-1.297	0.003
AT1G70140	ATFH8: formin homology 2 domain-containing protein / FH2 domain-containing protein	-1.295	0.004
AT5G05250	expressed protein	-1.280	0.005
AT5G51760	protein phosphatase 2C, putative / PP2C, putative	-1.278	0.001
AT2G27290	transcriptional factor B3 family protein	-1.276	0.002
AT3G49890	expressed protein	-1.275	0.008
AT2G48110	expressed protein	-1.272	0.006
AT2G26040	Bet v I allergen family protein	-1.265	0.034
AT4G17430	expressed protein	-1.264	0.005
AT4G12240 /AT4G12250	zinc finger (C2H2 type) family protein	-1.259	0.012
AT4G25990	expressed protein	-1.259	0.005
AT2G40340 /AT2G40350	AP2 domain-containing transcription factor,putative(DREB family)	-1.258	0.045
AT4G16500	cysteine protease inhibitor family protein / cystatin family protein	-1.257	0.001

Table 2.6. The 50 'interaction' genes that were most highly down-regulated in response to joint stress as compared to individual stress, in root tissue. A total of 522 genes were significantly down-regulated in roots by the addition of a second stress (nematode infection) compared to plants under a single stress (dehydration) ($p < 0.05$). The genes are listed in order of fold change. The fold changes shown are an average of three replicates.

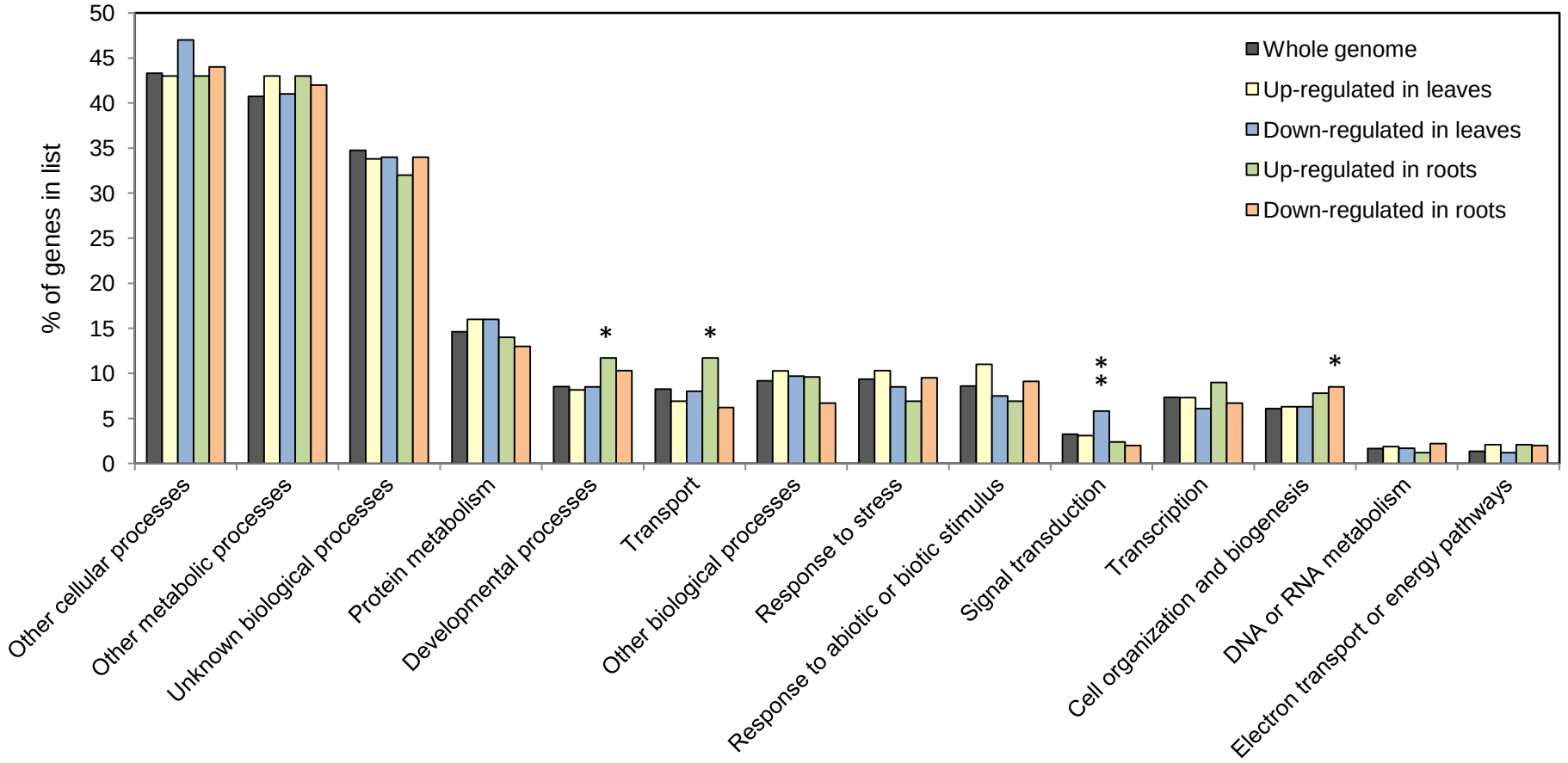


Figure 2.18. Classification of *A. thaliana* 'interaction' genes found to be differentially regulated by joint stress compared to individual nematode or dehydration stress alone. Genes are classified by their Biological Process annotation as determined by the TAIR Go Ontology tool. The percentage of genes in each category is given, as well as the percentage of genes in the entire genome with that annotation. Significance was determined using Chi^2 tests of comparison. *= $p < 0.05$. **= $p < 0.01$. Genes may be represented in more than one category due to multiple annotations.

encoding an LRR-domain protein (AT2G35620), an F-box family protein (AT5G53840) and a map-kinase (AT2G01450). The down-regulated genes included a MYB transcription factor (AT1G26780) a NAM transcription factor (AT2G27300) and a pentatricopeptide repeat-containing protein (AT3G29230). These interaction genes that were common to both roots and leaves were significantly enriched in the GO categories *transport* and *signal transduction.*

2.3.8 Verification of microarray result using qRT-PCR

Figure 2.19 shows the amplification plot, standard curve and dissociation curve for the internal control gene *ACTIN2* (AT3G18780). The results of the microarray show that this gene was not differentially expressed between any treatment groups. The slope of the standard curve was -3.133. This value was used by the MxPro software to calculate the primer efficiency of 108.5 %. The correlation (R^2) for the dilution series was 0.981 indicating a high level of accuracy. The melting temperature of the target amplicon was 78 ºC as shown by the dissociation curve. One clear peak was seen in this curve indicating a single amplified product. No product could be observed in the non-template control, confirming a lack of contamination or primer dimer. The qRT-PCR primer pairs used in this thesis gave an amplification efficiency value of between 90 % and 110 %, with the exception of *MYB45* which had an efficiency of 115 % and *DUF581* which had a value of 117 %. The R^2 correlation coefficients for all the standard curves ranged between 0.94 and 1.00. No product was observed in any of the non-template controls for any of the primer pairs.

The correlation between the fold changes obtained in the microarray analysis and qRT-PCR is shown in Figure 2.20. Genes were selected which showed a range of positive and negative fold-change values. The fold increase shown is between the control plants and plants subjected to joint stress. The R^2 value was 0.729. All of the genes showed the same direction of fold change using both systems, however almost all genes showed a somewhat greater magnitude of fold change when measured by qRT-PCR.

2.3.9 Correlation of dehydration-induced and drought-induced gene induction

Gene expression changes induced in soil-grown *A. thaliana* plants by drought treatment were compared to those induced by dehydration treatment in tissue culture. The

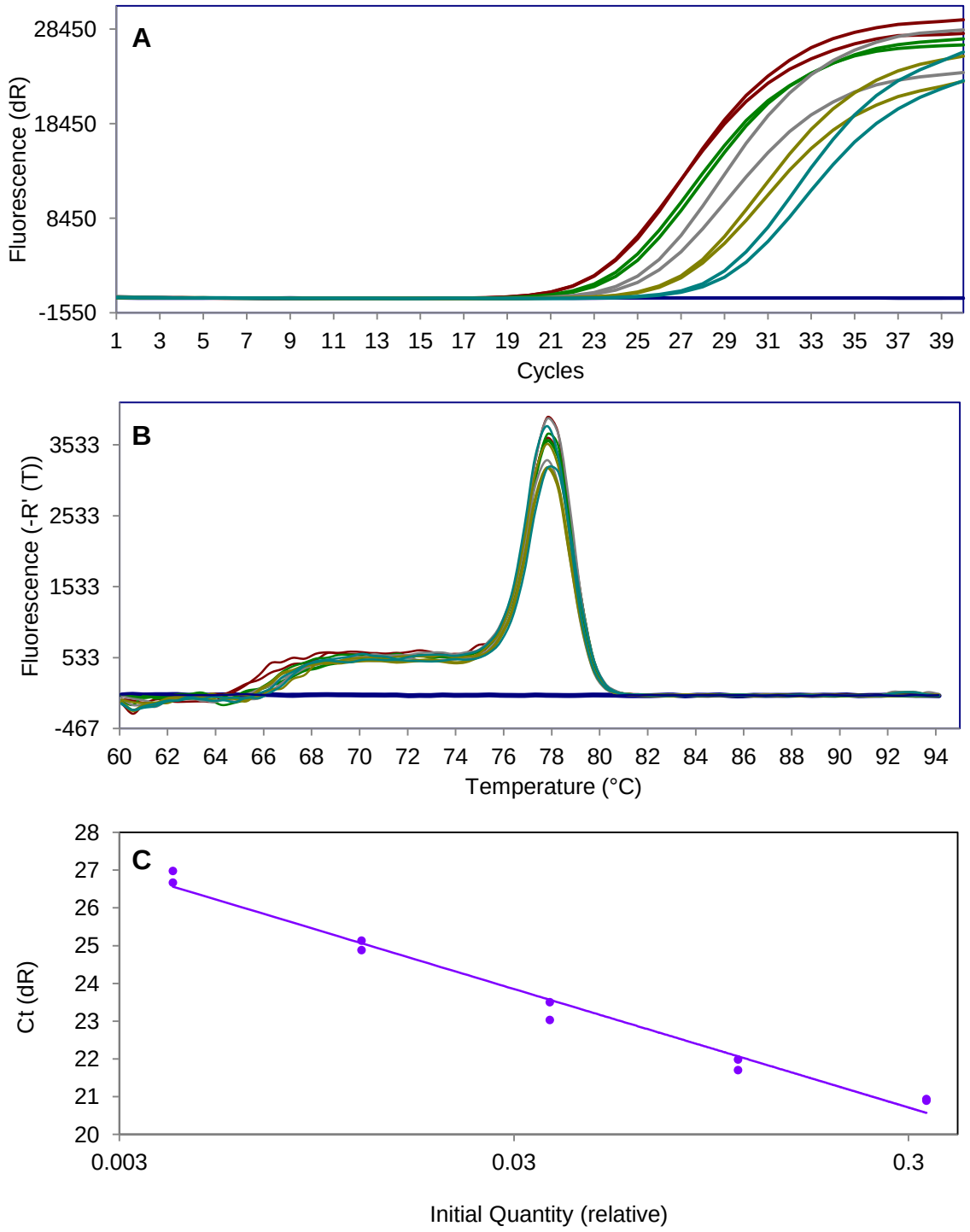


Figure 2.19. Representative plots created during qRT-PCR analysis for microarray validation. (A) Raw data curves generated by qRT-PCR. Relative starting quantity of RNA was calculated by measuring the cycle number (Ct value) at which samples reached threshold fluorescence, compared to the normalising gene *ACTIN2*. (B) A dissociation curve illustrating the specificity of qRT-PCR primers. The specific melting temperature of a product indicates its size, so a pure product gives one clear peak. The blue line represents the non template control. (C) A standard curve was created for each primer pair using a 3-fold dilution series. The *ACTIN2* standard curve pictured here had an R-squared value of 0.981 and the primers had an efficiency of 108.5 %.

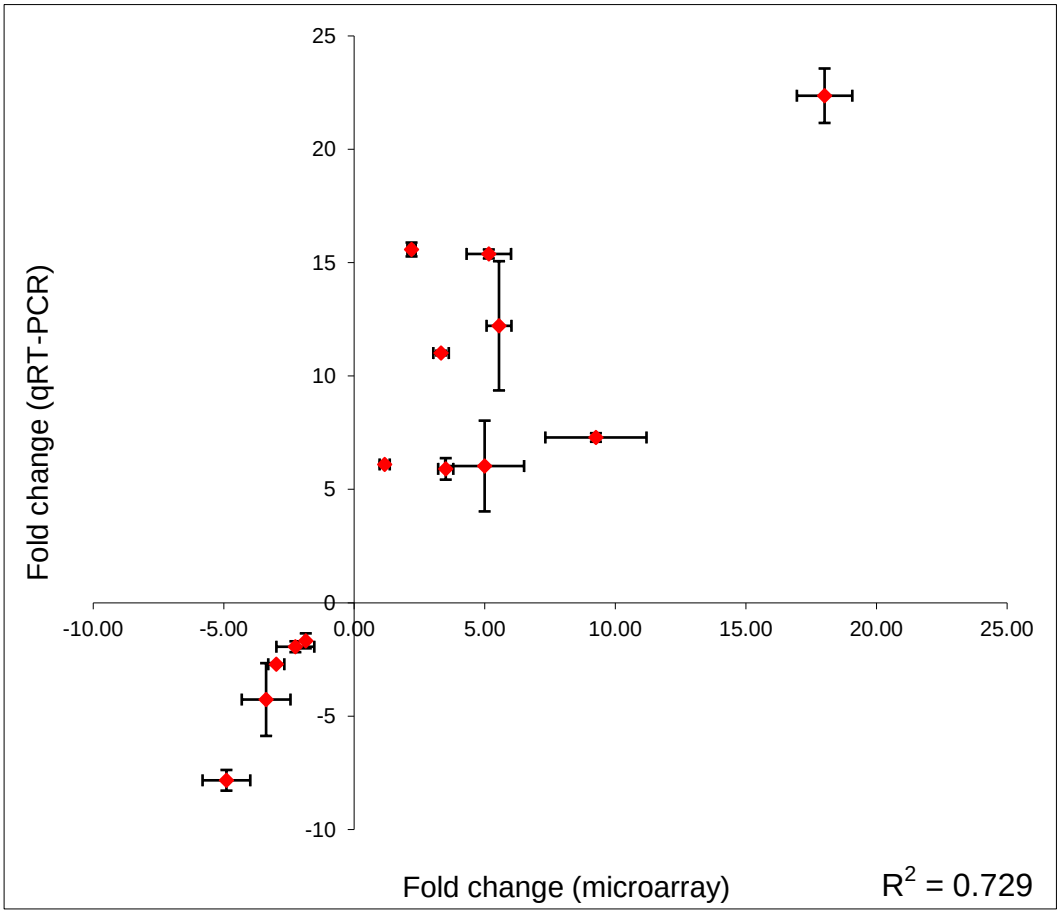


Figure 2.20. Correlation between microarray and qRT-PCR gene expression results. Fourteen genes were selected from the results of the microarray experiment. These represented a range of positive and negative fold changes that occured as a result of joint stress in comparison to control treatment. The expression levels of these genes were then analysed in the same RNA samples using qRT-PCR. A strong positive correlation was observed between results obtained from each method (R^2 = 0.727). Error bars represent technical replicates in qRT-PCR and biological replicates in array.

comparative fold changes of a range of positively and negatively induced genes are shown in Figure 2.21. Both axes represent data obtained by qRT-PCR. The R^2 value was found to be 0.408. All the genes showed the same direction of fold change in both systems with the exception of AT1G13080, a cytochrome P450 monooxygenase. This gene was negatively regulated by dehydration stress in tissue culture, but positively regulated by drought stress.

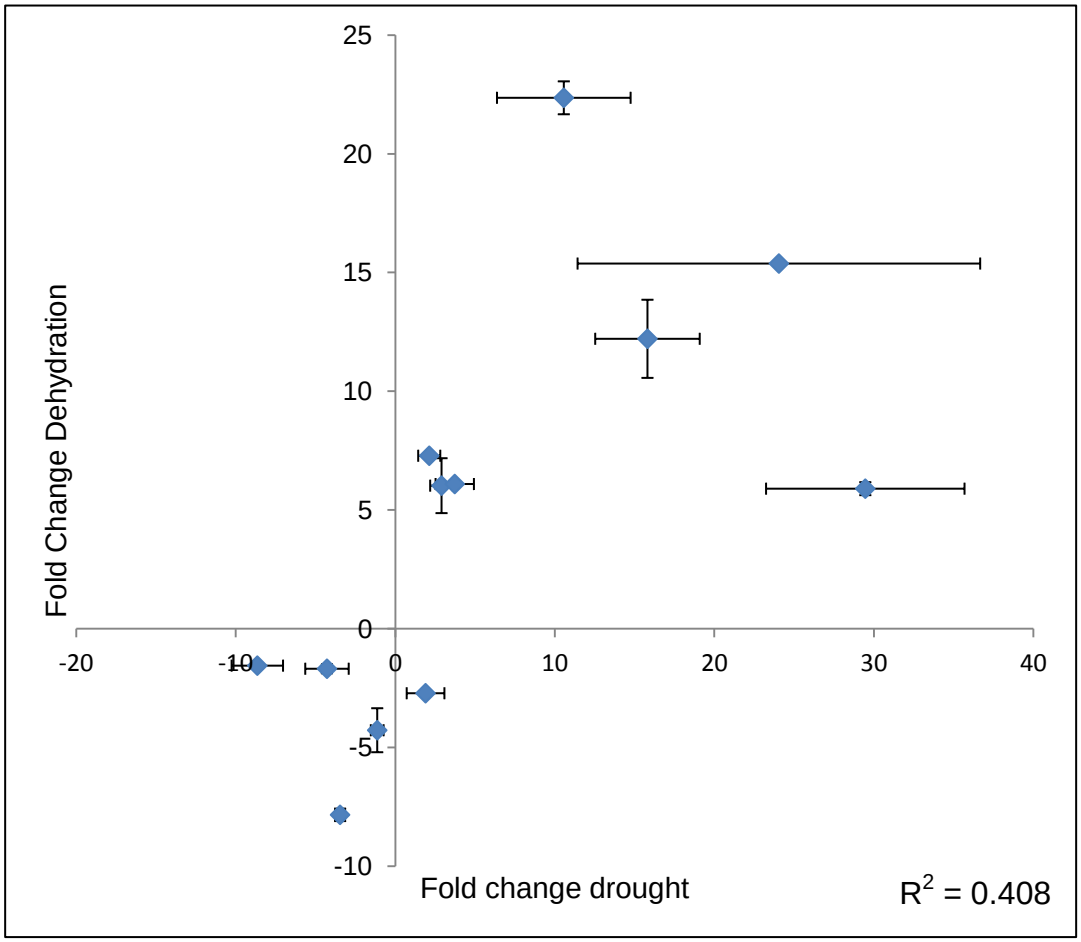


Figure 2.21. Correlation between gene expression changes due to dehydration treatment and drought. Twelve genes were selected from the results of the microarrray data representing a range of positive and negative fold changes. qRT-PCR was used to measure expression changes due to dehydration stress in leaves of plants grown in tissue culture and drought stress of plants grown in soil. Error bars represent technical replicates for dehydration and biological replicates for the drought study.

2.4 Discussion

2.4.1. Effects of stresses on *A. thaliana* growth and physiology

Plant-parasitic nematode infection is known to reduce growth and yield of crop plants, creating a significant problem in agriculture (Bird, 1974; Wallace, 1974; Barker and Olthof, 1976; Sasser, 1980; Vito *et al.*, 1986; Fasan and Haverkort, 1991; van der Putten *et al.*, 2006). As nematodes develop in the roots, nutrients in the phloem are diverted to the feeding cells and water transport is disrupted, thus affecting normal growth processes. In *A. thaliana*, most studies on the influence of nematode infection have focussed on the molecular and transcriptomic level (Puthoff *et al.*, 2003; Jammes *et al.*, 2005; Fuller *et al.*, 2007; Szakasits *et al.*, 2009; Klink *et al.*, 2010), rather than the physiological response. In the current study, exposing *A. thaliana* plants to nematode infection was found to cause significant differences in plant physiology compared to unstressed plants. Infection with the nematode *H. schachtii* led to a significantly smaller rosette size and a shorter inflorescence. It has been demonstrated by the use of fluorescent labelling that solutes are unloaded directly from the phloem of *A. thaliana* into syncytia of *H. schachtii* (Bockenhoff *et al.*, 1996). This, combined with the root growth retardation often seen due to nematode infection in various species (Haverkort *et al.*, 1994; Smit and Vamerali, 1998; Audebert *et al.*, 2000) may explain the observed effects on rosette size and inflorescence height, as fewer nutrients and less water would be available to the plant for directing to growth and reproductive processes.

Of all the environmental factors affecting plants, drought stress has the most severe effect on plant physiology and productivity (Shao *et al.*, 2008). When drought occurs, plants initiate several mechanisms to try and minimise water loss. Amongst the first responses are the closing of stomata and the inhibition of leaf growth (Chaves *et al.*, 2003; Rizhsky *et al.*, 2004; Shao *et al.*, 2008). These changes were observed in the current study as a result of the five different levels of drought stress, whereby the plants experiencing the most severe drought stress had the lowest stomatal conductance and the smallest rosette size. Maintained root growth is usually associated with drought stress, an adaption that allows plants to maximise water uptake (Chaves *et al.*, 2003). However, in this study the root systems of plants were observed to be smaller with increasing severity of drought stress. This may have been due to the relatively rapid

progression of the drought stress, which meant that long-term drought adaptations did not have time to develop.

The interacting effect of nematode infestation and drought has been shown to exacerbate crop stress compared to that observed for each individual stress, leading to reduced yields and biomass accumulation, although not always additively (Haverkort *et al.*, 1991; Audebert *et al.*, 2000; Coyne *et al.*, 2001). Nematodes can induce drought-like symptoms in plants due to root growth disruption, thus creating a complex interaction between the two stresses and making studies on their combined effect difficult (Fasan and Haverkort, 1991; Coyne *et al.*, 2001). Furthermore, nematode population density is affected by soil hydrology in field conditions (Coyne *et al.*, 2001). A study on potato tested the effect of drought and infection with the cyst nematode *Globodera pallida* on water use efficiency (Haverkort *et al.*, 1991). The results showed that both factors negatively affected growth, although the effect of two stresses together was not additive. This may have been partly due to the infected plants using less water and thus reducing drought stress. The authors noted that the reduction in dry matter accumulation due to nematode infection was greater than would be expected due to impaired water relations alone, and proposed that this may be due to increased carbon allocation to the nematode feeding cells. In the current study, when *H. schachtii* juveniles were applied to plants at differing levels of drought stress, the observed density of nematodes (established nematodes per mg of root tissue) was higher in plants experiencing severe drought. It might be expected that nematode motility would be impeded by the lower soil moisture content. However, this would have only increased the magnitude of the observed results. It is known that biotic and abiotic signalling pathways may interact and inhibit one another, a process controlled largely by hormones. The hormone ABA, although largely responsible for orchestrating plant response to abiotic stress, also has a prominent role in pathogen and disease resistance (Taiz and Zeiger, 1991; Finkelstein *et al.*, 2002; Asselbergh *et al.*, 2008b; Yasuda *et al.*, 2008; Ton *et al.*, 2009). The effect of ABA on pathogen response may be either positive or negative. For example, in early stages of infection with other pathogens, ABA initiates stomatal closure and induces callose deposition, both strategies that may limit the entry of pathogens (Ton *et al.*, 2009). However, ABA accumulation due to abiotic stress also results in the direct suppression of the salicylic acid (SA) controlled systemic acquired immunity (SAR) response and suppresses genes in the phenylpropanoid

pathway, including the production of lignin (Mohr and Cahill, 2007), whilst salicylic acid in return represses both the production of ABA and the activation of ABA-responsive genes (Yasuda *et al.*, 2008). ABA also suppresses the induction of jasmonic acid and ethylene-responsive defence genes (Anderson *et al.*, 2004). Reduced amounts of ABA have been shown to provoke increased resistance to *Pseudomonas syringae, Plectosphaerella cucumerina, Botrytis cinerea* and *Fusarium oxysporum* in *A. thaliana,* whilst increasing ABA levels reduce resistance to *Pseudomonas syringae* in *A. thaliana* and *Botrytis cinerea* in tomato, as summarised by Asselbergh *el al.* (2008a). Therefore the channelling of plant stress-response systems into ABA-regulated abiotic pathways in this experiment by imposing drought stress may have allow increased infection by nematodes and facilitated easier penetration of the roots. The stronger the drought stress, the more this was found to be the case.

In contrast to the positive effect of drought on nematode invasion, the progression of the established nematodes through the parasitic life cycle was affected negatively by drought treatments. Plants at the minimal level of drought stress (25 % soil moisture) had a significantly larger proportion of nematodes at later life cycle stages (saccate and enlarged saccate) compared to the most severely stressed plants (5 % soil moisture). This suggests that although nematodes found the severely drought-stressed plants easier to penetrate and establish feeding sites in, their development then became retarded by the lack of available water. While the combined effect of drought and nematode infection has not been documented in *A. thaliana,* similar studies have been carried out in crop plants. In a study on rice, a lower multiplication rate of the nematode *Heterodera sacchari* was observed on plants subjected to water stress (Audebert *et al.*, 2000). Another study demonstrated that there was no difference in the ability of the nematode *Heterodera avenae* to infect roots of oat plants following differing water regimes, but that again there was a difference in multiplication rates of nematodes. Nematodes infecting drought-stressed roots multiplied at a lower rate than under well watered conditions (Seinhorst, 1981). The proposed reason for this was the restriction of male nematode motility in dry soil, thus giving a reduced re-infection rate. However, the results shown here for *A. thaliana* suggest that the restriction of nematode multiplication occurs earlier, during the development of the female within the roots. The restriction in water transport due to drought may inhibit the supply of phloem nutrients to the feeding cell and thus play a role in the inhibition of nematode growth.

Interestingly, a contrasting study in potato showed increased nematode multiplication following drought stress (Fasan and Haverkort, 1991). Water status clearly plays an important role in the ability of the plant to resist nematode invasion and development, and vice versa, and more studies will need to be carried out to elucidate the exact nature of the interaction between stresses. In order to more fully understand these mechanisms of stress response, it is useful to examine molecular and transcriptomic events within plant cells (Wang *et al.*, 2003; Clarke and Zhu, 2006). The increased expression of marker genes for drought and nematode infection was tested and found to correspond with the imposition of dehydration and *H. schachtii* treatment in this experimental scenario, thus validating the method as a reliable model for environmental stress. Following this ascertainment, a whole-genome transcriptome study was embarked upon.

2.4.2 Microarray analysis and validation

Microarray analysis was carried out on RNA from *A. thaliana* leaf and root tissue following treatment with dehydration stress, nematode stress, or the two in combination. Affymetrix ATH1 GeneChip® technology provides the broadest opportunity for examining whole-genome transcript changes in *A. thaliana,* and was thus most suitable for detecting any molecular changes that may occur in plants in response to multiple simultaneous stresses (Redman *et al.*, 2004). The experiment was designed and carried out according to recommended guidelines for maximising microarray data quality (Clarke and Zhu, 2006; Wise *et al.*, 2007). RNA used in the microarray study was found to be of high quality, and hybridisation controls indicated a high level of consistency across arrays.

Validation of microarray data was carried out by qRT-PCR. Primer pairs used in qRT-PCR were all highly efficient, amplifying a single product each. The correlation between the two methodologies was found to be high ($R^2 = 0.729$), suggesting that the use of microarrays is a valid and effective way of characterising changes in gene expression in this experimental setting. The magnitude of fold change of selected genes was generally greater as measured by qRT-PCR. This effect has been noted previously in comparison studies between the two technologies, which indicate that qRT-PCR is usually more sensitive than microarray detection (Holland, 2002; Clarke and Zhu, 2006). For example, Czechowski *et al*. (2004) noted that the range of expression values of 1400 *A. thaliana* genes as measured by qRT-PCR was two orders of magnitude

higher than those obtained by Affymetrix chips. There is much debate in the literature as to the necessity of obtaining such corroborative data, particularly now that mass-produced oligonucleotide arrays are commercially available (Rockett and Hellmann, 2004; Clarke and Zhu, 2006; Wise *et al.*, 2007). It has been proposed that if microarray experiments are rigorously designed with an appropriate number of replicates and careful statistical analysis, there is no need for further validation (Wise *et al.*, 2007). Nevertheless, corroboration of microarray data is still the norm, and has even been stipulated as essential in order to publish in certain journals (Rockett and Hellmann, 2004).

2.4.3. Detection of differentially expressed genes

ATH1 GeneChip arrays generate expression values for each of the 23,750 genes represented on the chip. This information can be used to make assumptions about the relative abundance of different proteins within a cell, and therefore the molecular processes taking place due to any particular environmental condition. The criteria by which differentially expressed genes are identified depend on the experiment. Often a fold change is designated beyond which the gene is considered differentially regulated (Seki *et al.*, 2002; Rizhsky *et al.*, 2004; Kilian *et al.*, 2007). However, it has been observed that the genes induced with the largest fold changes are not necessarily the most important in a particular process, and that often stress responses are controlled by a large number of genes with small effects (Swindell, 2006). Therefore the act of stipulating a certain fold change cut-off may eliminate more subtly acting genes (Feder and Walser, 2005; Clarke and Zhu, 2006). Recently, the designation of a certain statistical significance level with which to identify transcript changes has been considered a more reliable method (Puthoff *et al.*, 2003; Huang *et al.*, 2008). This method was chosen for the current study, as many of the expression changes induced by nematode stress in particular were of small fold change, due to the method of sampling the whole root system. A *p*-value of 0.05 was initially stipulated as the cut-off mark for differential expression, but then for further analysis of gene lists fold change and expression level were also taken into account. A multiple testing correction such as the Bonferroni correction is often applied following statistical testing to reduce the false discovery rate (Swindell, 2006). However, for this dataset no such calculation was performed as the replication in triplicate combined with the *p*-value cut-off was deemed sufficient for successfully identifying biologically significant changes. Differentially

regulated genes were found to be induced by each stress individually and by the stresses in combination. These sets of genes will be commented on in the following three sections.

2.4.4 Dehydration as a model for drought stress

In field conditions the onset of drought is a slow process taking up to several weeks and going through various distinct phases, measurable by stomatal conductance and transpiration rates in comparison to the fraction of transpirable soil water (Sinclair and Ludlow, 1986). Care must therefore be taken when attempting to reproduce drought conditions over a short time period in the laboratory (Bhatnagar-Mathur *et al.*, 2008). Previous microarray studies investigating the molecular drought response in *A. thaliana* have imposed 'drought stress' using various different methods: Mannitol or polyethylene glycol (PEG) has been added to plants grown hydroponically in order to cause osmotic stress simulating drought stress (Kreps *et al.*, 2002; Hong *et al.*, 2008; Zhang *et al.*, 2008); plants grown on agar or in hydroponic systems have been removed from the growth media and subjected to a short period of severe desiccation in order to induce a rapid gene response (Seki *et al.*, 2002; Kilian *et al.*, 2007); and finally water has been withheld from soil grown plants until leaf relative water content is reduced to a certain level (Kawaguchi *et al.*, 2004; Huang *et al.*, 2008). Although all of these methods reduce the water potential of the plant tissue, none of the conditions are likely to occur in the field. Therefore the study of drought stress in the laboratory is a balance between achieving results that are relevant to plants in real environmental or agricultural situations, and establishing a methodology that can be highly controlled and produces consistent results (Feder and Walser, 2005; Deyholos, 2010). Findings from laboratory studies should be considered a model for drought stress rather than drought stress itself (Bhatnagar-Mathur *et al.*, 2008). In this study the dehydration method used by Kilian *et al.* (2007) and Seki *et al.* (2002) was adopted so that a consistent, highly controllable manner of inducing stress-related transcriptome changes could be achieved. Preliminary experiments had indicated that consistent dehydration of soil-grown *A. thaliana* by withholding of water was difficult to achieve in a controlled manner, leading to erratic and irreproducible changes in stress marker gene expression over an extended time course (data not shown). The results of the microarray study identified 3728 genes that were differentially regulated by dehydration stress in leaves whilst 3174 such genes were identified in roots, representing 15 % and 13 % of the genome, respectively. Early

microarray studies report similar proportions of differentially regulated genes in response to drought (Seki *et al.* (2002) 7%, Kreps *et al.* (2002) 13 % and Kawaguchi *et al.* (2004) 9%), despite very different methodologies. The overlap between these identified gene sets is low, resulting in only 30 genes that were commonly regulated by all three treatments (Bray, 2004). Huang *et al.* (2008) carried out the first whole-genome *A. thaliana* microarray on plants that had experienced water deficit stress in soil, a method likely to reflect environmental drought more closely than rapid dehydration methods. The 1651 differentially regulated genes identified in leaf tissue by Huang *et al.* (2008) were compared against those identified by dehydration stress in leaves in the current study. An overlap of 252 genes was discovered. This overlap may represent a core subset of genes which is induced in response to general water stress, whilst the other differentially expressed genes may be specific to the environmental conditions of each study. Of the 252 genes, many were of families known to have a regulatory role in stress signalling and transcriptional control, such as AP2, WRKY, MYB and NAM transcription factors, as well as factors responsive to the hormones giberellin, auxin, ABA and ethylene (Wang *et al.*, 2003; Seki *et al.*, 2007; Shinozaki and Yamaguchi-Shinozaki, 2007). Induced functional drought-tolerance proteins included late embryogenesis abundant (LEA) proteins, sugar transporters, protease inhibitors, as well as cytochrome P450s, pentatricopeptide (PPR) repeat-containing proteins and heat shock proteins (Chaves *et al.*, 2003; Wang *et al.*, 2003). Interestingly, of the 10 most highly up- and down-regulated dehydration-responsive genes in the current study, only 2 and 1 gene(s), respectively, were also identified by Huang *et al.* (2008). This suggests that the genes induced with the highest fold change may be specific to this method of dehydration stress, and that more moderately induced genes are more important to the slower drought stress. Neither *DREB1A* nor *DREB2A* were identified as differentially regulated by Huang *et al.* (2008). Of the genes differentially regulated by dehydration stress, 815 were common to both roots and leaves, almost a quarter of each of the gene lists. A reasonably large overlap would be expected, for example representing generalised cellular protection mechanisms such as solute production, enzyme stabilisation and sugar transport (Chaves *et al.*, 2003; Wang *et al.*, 2003). However, as root and shoot tissue behave differently under drought stress, with increased growth in roots but inhibition of growth, photosynthesis and stomatal closure occurring in leaves, a large proportion of tissue-specific genes would also be expected (Taiz and Zeiger, 1991; Chaves *et al.*, 2003; Shao *et al.*, 2008). Following the identification of gene

expression changes by microarray, the expression of dehydration responsive genes was analysed in plants grown in pots in soil that had been exposed to water deficit. A positive correlation was observed between the expression levels in the two systems (Figure 2.21). This result combined with the substantial overlap with the genes identified by Huang *et al.* (2008) indicates that the dehydration method employed by this study can be used as a model for understanding the processes occurring during drought stress in the field.

2.4.5 Transcriptomic response to nematode infection

Microarray studies have been used to characterise the nature of plant-nematode interaction, both at the level of the feeding cell (Hammes *et al.*, 2005; Szakasits *et al.*, 2009; Barcala *et al.*, 2010; Klink *et al.*, 2010) and of the whole root (Puthoff *et al.*, 2003; Klink *et al.*, 2007). New techniques such as laser capture and microaspiration of feeding cells have greatly expanded our knowledge of processes taking place in these specialised plant-pathogen interactions (Klink *et al.*, 2007; Szakasits *et al.*, 2009; Barcala *et al.*, 2010). Up to 7231 transcript changes have been identified in the syncytia of *Heterodera schachtii* infecting *A. thaliana* (Szakasits *et al.*, 2009), whilst up to 1284 genes are differentially regulated in root-knot nematode feeding sites (Jammes *et al.*, 2005; Fuller *et al.*, 2007). In soybean, up to 429 transcript changes have been noted in syncytia of *Heterodera glycines* (Ithal *et al.*, 2007a; Klink *et al.*, 2007). Direct comparison of the two methods of tissue sampling in soybean has revealed little overlap between the genes induced by cyst nematode infection in syncytia alone compared to whole root systems (Klink *et al.*, 2007). As root systems can only support a certain number of nematode feeding sites, the small quantity of tissue directly affected by the nematodes will comprise only a small proportion of the total root system. Thus when sampling whole roots, any feeding cell-specific effects are liable to be diluted and any changes observed are more likely to represent systemic responses to infection (Lilley *et al.*, 2005; Szakasits *et al.*, 2009). The aim of this study was to characterise the interaction between plant response to dehydration and to nematode stress. Any interaction was likely to occur at the systemic level rather than in the highly specialised and transcriptionally re-programmed nematode feeding cells. Therefore for the purposes of this study the whole root system was sampled. Puthof *et al.* (2003) used 8K Affymetrix GeneChips to identify expression changes in 128 genes in whole *A. thaliana* roots as a result of *H. schachtii* infection. In contrast, this study detected 538

differentially expressed genes. The differing genome coverage of the 8K GeneChip compared to the ATH1 GeneChip may account for the disparity in magnitude of results, as well as the fact that Puthof *et al.* (2003) sampled root tissue only 3 days post infection, whereas this study allowed 10 days of nematode development. Changes observed at the earlier time-point would have been before the syncytia were fully formed, and comprised the up-regulation of genes involved in cell wall modification and defence, whilst signal transduction genes proposed to play a regulatory role in plant defence response were down-regulated (Puthoff *et al.*, 2003). Cell wall modification genes such as extensins were also up-regulated in roots in the later time point studied here, suggesting that systemic changes to root cell walls continue even after the establishment of the syncytia (Gheysen and Fenoll, 2002). This may be a general defence mechanism aimed at preventing further nematode invasion, or may imply that cell wall modification to allow syncytia and nematode growth continues even 10 days post infection. Other notably up-regulated genes in roots were leucine rich repeat (LRR) family proteins, which are known for their role in defence responses, and of which R-genes are members (Dangl and Jones, 2001; Jalali *et al.*, 2006; Tameling and Joosten, 2007). LRR gene up-regulation has previously been observed as a result of nematode infection in *A. thaliana* and soybean, although their exact function is unclear (Fuller *et al.*, 2007; Klink *et al.*, 2007). The GO category of *DNA or RNA metabolism* was significantly enriched amongst down-regulated genes in both roots and leaves. These were genes involved in DNA replication, repair, recombination and methylation. This finding is in contrast to previous work which suggests that DNA replication is increased as a result of nematode-induced changes to the cell cycle during the establishment of the syncytia or giant cell, in the case of root-knot nematode infection (Gheysen and Fenoll, 2002; Puthoff *et al.*, 2003; Fuller *et al.*, 2007). Also abundant amongst the down-regulated genes in roots were pentatricopeptide repeat (PPR) proteins. Although generally classified as having *unknown biological function*, it is now known that this large family of proteins are involved in RNA editing and post-transcriptional control particularly of organellar RNA, and have been linked to the ABA response pathway in *A. thaliana* (Schmitz-Linneweber and Small, 2008; Liu *et al.*, 2010). In order to more fully characterise the systemic response to cyst nematode infection in roots, it may be prudent to actually excise the syncytia before extracting RNA, thus ruling out any changes in the feeding cell itself and allowing greater tissue specificity (Deyholos, 2010).

There are no reports in the literature describing transcriptome studies of aerial plant parts in response to nematode infection. Although fold increases were generally lower than those encountered in root tissue, a large number of genes were found to be significantly induced or repressed. Amongst the most highly up-regulated genes in leaves were a WRKY transcription factor, a MAP kinase and two more protein kinases. These may act as part of a systemic defence signalling or regulatory response to the distal nematode infection (Gheysen and Fenoll, 2002; Jalali *et al.*, 2006; Bruce and Pickett, 2007). Also induced were two ribosomal proteins, the induction of which characterises the increased metabolism associated with nematode infection in syncytia in *A. thaliana* and soybean (Klink *et al.*, 2007; Szakasits *et al.*, 2009). Amino acids and solutes in leaves become depleted as a result of the sink strength of the nematode feeding cell, thus the observed induction of ribosomal proteins in leaves may be in compensation for this loss (Hofmann *et al.*, 2010). Three of the 10 most highly induced genes in leaves were of unknown function. This fact combined with the significant enrichment of the GO category *unknown biological process* in leaves by nematode infection suggests that there may be additional processes involved in systemic signalling in response to nematodes, emphasising the need for further research in this area.

2.4.6 The response to multiple stresses

2.4.6.1 A new pattern of stress response

The effect of two or more concurrent environmental stresses can be far more detrimental to plants than an individual stress, and has led to severe agricultural losses (Craufurd and Peacock, 1993; Savin and Nicolas, 1996; Mittler, 2006). Increasing research is now being carried out into the response of plants to multiple stresses on a molecular level, a process which has been greatly facilitated by microarray technology (Rizhsky *et al.*, 2002; Rizhsky *et al.*, 2004; Voelckel and Baldwin, 2004; Luo *et al.*, 2005; Szucs *et al.*, 2010). The combination of drought and heat are particularly well studied, as these are potential breeding targets in several species (Craufurd and Peacock, 1993; Mittler, 2006; Barnabas *et al.*, 2008). However, the combination of abiotic and biotic stress factors on the plant whole-genome transcriptome has not previously been documented.

Here, the transcriptome of *A. thaliana* was analysed following a combination of dehydration and nematode treatments, and expression changes were compared to those influenced by each stress individually and to control plants. When the two stresses occurred in combination, a novel program of gene expression was observed. The levels of 1282 transcripts in leaves and 1112 transcripts in roots were found to be specifically induced or suppressed during a combination of drought and nematode stress (Figure 2.17). These 'joint stress specific' genes were not differentially regulated by either stress individually. Furthermore, a large proportion of the genes whose expression changed due to dehydration or nematode stress individually were no longer differentially regulated when the stresses occurred together (47 % of dehydration-induced and 85 % of nematode-induced). This finding supports the theory that plant responses to stress are highly specific and unique to the exact set of environmental conditions encountered (Rizhsky *et al.*, 2004; Mittler, 2006; Yasuda *et al.*, 2008; Ton *et al.*, 2009). Each type of stress elicits a different transcriptomic and metabolomic response. This has been demonstrated by a variety of transcriptome studies on plants under differing stress treatments. For example, little overlap was found between sets of genes differentially regulated by drought, cold, salinity, UV-B and osmotic stress (Kreps *et al.*, 2002; Seki *et al.*, 2002; Beck *et al.*, 2007; Kilian *et al.*, 2007) whilst plants treated with different biotic stresses also showed a highly specific response to each pathogen or herbivore (Voelckel and Baldwin, 2004; De Vos *et al.*, 2005). The ability of plants to recognise and respond to specific stress combinations may be extremely important when those stresses would elicit conflicting responses. For example, high temperature stress requires that plants open their stomata to release excess heat, whilst drought stress would necessitate the closing of stomata to conserve water. When occurring in combination, leaf temperature therefore becomes significantly higher than if the heat stress had occurred alone (Rizhsky *et al.*, 2004). Heat stress may enhance the effects of salinity or heavy metals through increased uptake due to increased transpiration, whilst the effects of drought and heavy metal stress can also exacerbate each other (Barcelo and Poschenrieder, 1990; Mittler, 2006). Many studies have aimed to identify genes important in multiple stress tolerance by comparing lists of genes induced by each stress individually (Cheong *et al.*, 2002; Seki *et al.*, 2002; De Vos *et al.*, 2005; Swindell, 2006; Kilian *et al.*, 2007; Kant *et al.*, 2008). With our current knowledge of how stress responses interact, this type of research is no longer considered sufficient for understanding multiple stress responses. It has thus been

proposed that each combination of two stresses be considered an entirely new kind of stress and studied accordingly (Mittler, 2006), a suggestion supported by the results of this study. The novel program of transcript response due to nematode and dehydration stress identified in the current research shows a very similar pattern to that discovered by Rizhsky *et al.* (2004), in which 772 *A. thaliana* genes were found to be differentially regulated by combined drought and heat stress. Another similarity to the current findings was the large number of genes whose expression changed due to drought or heat stress individually but were not induced when the stresses occurred together (48 % of drought-induced and 38 % of heat-induced). A similar result has been observed for biotic stresses. Tobacco plants exposed to simultaneous attack by two different pathogens, a sap-feeding mirid and a chewing hornworm, initiate a transcriptomic response that is different to that resulting from each pest individually (Voelckel and Baldwin, 2004). Plants clearly have a high degree of adaptivity in recognising simultaneous stresses and responding to them. As the first whole-genome study on combined biotic and abiotic stress response in plants, the work here emphasises the complex nature of interactions between stress signalling pathways, and underlines the need for further studies of this kind.

When dehydration and nematode stress were applied to plants in combination, the resulting gene expression profile resembled that of the plant under dehydration stress alone much more closely than under nematode stress alone. Only 15 % of nematode-induced genes were still differentially expressed during joint stress, compared to 53 % of dehydration-induced genes. There may be several reasons for this. Firstly, the effect of this method of dehydration stress was likely to have been stronger than that of the nematode stress, as water deficit causes rapid physiological changes throughout the plant, and cellular osmotic imbalance and turgor loss would have been widespread across plant tissues (Chaves *et al.*, 2003; Shao *et al.*, 2008). In contrast nematode stress, as we have seen in Section 2.4.5, had a less dramatic impact on the plants as highlighted by the lesser number of differentially expressed genes as well as smaller fold changes. Therefore the major stress on the plants during joint stress would have been the dehydration treatment. Inherent in this kind of experiment are discrepancies between the magnitudes of different kinds of stress treatment (Rizhsky *et al.*, 2004). Plants can only support a certain number of nematodes, so increasing the applied number of juveniles may not have produced a greater stress response over the whole root system (Barker and

Olthof, 1976; Szakasits *et al.*, 2009). However, the experiment still provides a valid model for multiple stress response, as in field conditions different environmental stresses would also occur in differing intensities. A further limitation of the experiment results from the necessity to initiate the two stresses sequentially rather than simultaneously. The lifestyle of plant-parasitic nematodes means that nematodes require several days to migrate through the root and establish feeding cells before eliciting the maximum stress response from the plant (Wyss and Grundler, 1992). To harvest tissue on the first day of nematode invasion would reveal mainly wound responses from the plant (Gheysen and Fenoll, 2002). Therefore in order to study these two stresses in combination it was essential to apply the nematodes before the drought stress. It is possible that nematode-infected plants may therefore have been 'primed' defensively and thus react differently to dehydration stress (Voelckel and Baldwin, 2004; Bruce and Pickett, 2007; Rouhier and Jacquot, 2008). A similar predicament was experienced by Rizhsky *et al.* (2004) when imposing 'simultaneous' drought and heat stress, whereby the drought had to be initiated in advance of the heat stress so that the water content of the leaves had time to reduce to the stipulated level. Sequential stress initiation may thus be a necessary compromise. It should be noted that the microarray experiment here was limited to a single time point, and that a more comprehensive picture may be revealed by more detailed analysis over an extended period of time throughout the development of both stresses (Swindell, 2006; Kilian *et al.*, 2007; Klink *et al.*, 2007). Another possible reason for the observed down-regulation of the nematode response that occurred when both stresses were applied together is the antagonistic crosstalk between biotic and abiotic signalling pathways, a process controlled largely by the hormones ABA, salicylic acid and jasmonic acid (Anderson *et al.*, 2004; Asselbergh *et al.*, 2008b; Yasuda *et al.*, 2008; Ton *et al.*, 2009). As described in Section 2.4.1, the induction of ABA during abiotic stress may down-regulate defence pathways including the SAR, known to be induced by nematode invasion (Wubben *et al.*, 2008; Yasuda *et al.*, 2008). These observations may explain why nematodes could invade drought-stressed roots more easily (Section 2.4.1 and Figure 2.7), whilst combined dehydration and nematode stress caused the transcriptomic repression of nematode-induced genes.

2.4.6.2 Functional categories of 'interaction' genes

In order to elucidate the mechanism of interaction between nematode and dehydration stress, the functional roles of specifically induced genes were analysed. As there was a

large overlap between genes induced by dehydration stress and those induced by joint stress, a list of 'interaction' genes was created comprising genes whose expression changed *between* dehydration and joint stress. The fold changes of the interaction genes between dehydration and joint stress were lower than those induced by the stresses in comparison to unstressed plants, with a maximum fold change of 2.4. This suggests that this novel mechanism of responding to multiple stresses is a system involving a large number of genes each with a marginal effect. As stress response systems are thought to be largely polygenic, the findings support the use of whole-genome transcriptome studies instead of focussing on selected gene subsets (Feder and Walser, 2005; Swindell, 2006). The overlap between interaction genes identified in leaves and roots was small. This phenomenon has been previously observed in the study of multiple stress response, and supports the hypothesis that different tissues have very different transcriptomic responses to stress (Kreps *et al.*, 2002; Deyholos, 2010).

Of the interaction genes identified in this study, several categories of gene function were highly prominent. These included both functional and regulatory elements. Of the functional processes, genes involved in cell wall modification, carbohydrate metabolism and a specific heat shock response were specifically induced by the combined stresses, whilst disease resistance mechanisms were mainly repressed. In roots, 24 up-regulated genes had cell wall-related functions, including extensins, pectinesterases, polygalacturonases and xyloglucan transferases. Extensins were also amongst the most highly up-regulated in leaves. Cell wall modifications such as the deposition of callose, lignin and pectin modification by methylesterases are known to play an important role in defence response, by effectively strengthening the barrier between cellular contents and potential attackers (Vorwerk *et al.*, 2004; Pelloux *et al.*, 2007). On penetration by pathogens, cell wall components are also released as signalling molecules to activate cellular defense (Vorwerk *et al.*, 2004), meaning that mutants with impaired cell wall modification enzymes are often susceptible to increased infection by pathogens (Pelloux *et al.*, 2007). Cell wall pectin methylesterases are known to be induced in response to infection by nematodes in *A. thaliana* and tobacco (Niebel *et al.*, 1993; Pelloux *et al.*, 2007). Cell wall modification is also important in abiotic stress responses. Different cell wall modification proteins are induced by abiotic stresses as well as pathogens (Pelloux *et al.*, 2007; An *et al.*, 2008). Over-expression of a pectin methylesterase inhibitor in pepper led to plants that were tolerant to drought. The exact mechanism of resistance is

unknown, but as the mutants also exhibited resistance to oxidative stress, this may have provided protection to plant cells undergoing other abiotic stresses (An *et al.*, 2008). Changes in cell wall composition and elasticity are furthermore important in maintaining cell turgor during drought stress (Piro *et al.*, 2003; Leucci *et al.*, 2008). The specific up-regulation of cell wall modification proteins in response to combined nematode and dehydration stress may therefore be a highly efficient means of adaptive tolerance, whereby under dual stress the plant response system moves towards a more general defensive mechanism that would provide tolerance to both types of stress. Carbohydrate metabolism genes were also abundant amongst the up- and down-regulated interaction genes in roots and leaves, particularly glycosyl and glycoside hydrolases. Rizhsky *et al.* (2004) found that under a combination of drought and heat stress, plants accumulated sucrose and other sugars as osmoprotectants instead of proline, which accumulates under drought stress alone (Chaves *et al.*, 2003; Wang *et al.*, 2003). As sugar accumulation becomes more important during severe dehydration, the observed move towards sugar metabolism may reflect the additive severity of the combined dehydration and nematode stresses (Hoekstra *et al.*, 2001). Heat shock factor (HSF) proteins are transcription factors which activate the expression of heat shock proteins, and their expression patterns under different stresses are thought to regulate plants response to specific stresses (Rizhsky *et al.*, 2004; Miller and Mittler, 2006; Nishizawa *et al.*, 2006; Hu *et al.*, 2010). Their activation may be triggered by the production of reactive oxygen species such as H_20_2 during stress (Miller and Mittler, 2006; Hu *et al.*, 2010). Rizhsky *et al.* (2004) discovered that certain heat shock proteins were specifically induced by a combination of drought and heat stress and that these changes were reflected by differences in expression levels of HSFs between stress treatments. In the current study 11 heat shock proteins were differentially regulated by the specific stress combination as well as one HSF (HSF7), which was down-regulated in roots. This difference in HSF and heat shock protein expression provides support for the importance of these proteins in controlling specific response to environmental stress, even in the absence of heat stress itself. Twelve interaction genes with ‘disease resistance’ annotations, including those with leucine rich repeat (LRR) domains, were highly down-regulated in leaf tissue, whilst five were down-regulated in roots. In contrast, only three ‘disease resistance’ genes were up-regulated in any tissue. LRR-proteins act as pathogen recognition receptors and signalling proteins, and are important in pathogen immunity and defence response (Dangl and Jones, 2001; Jalali *et al.*, 2006;

Tameling and Joosten, 2007; Padmanabhan *et al.*, 2009), so their down-regulation here suggests an active suppression of pathogen response pathways as a result of abiotic stress (Asselbergh *et al.*, 2008b; Yasuda *et al.*, 2008; Ton *et al.*, 2009).

Regulatory genes were also identified amongst the interaction genes. Transcription factors play an extremely important role in orchestrating stress responses (Zhu, 2002; Wang *et al.*, 2003; Shinozaki and Yamaguchi-Shinozaki, 2007). In particular certain factors, such as MYC2, act in both abiotic and biotic stress signalling pathways and are crucial in integrating signals from both ABA and JA (Fujita *et al.*, 2006; Ton *et al.*, 2009). Plasticity amongst transcription factor networks is thought to be key in specific stress responses (Rizhsky *et al.*, 2004). Transcription factors also appear crucial in governing the specific response of *A. thaliana* to combined dehydration and nematode stress. Amongst the interaction genes, 32 and 27 transcription factors were up-regulated in leaves and roots respectively, whilst 22 and 28 were repressed in those tissues. Ten of the transcription factors were from the MYB family, of which one was specifically down-regulated in both roots and leaves (*MYB117*). MYB transcription factors are associated with signalling in response to various stresses and are also involved in processes such as the production of the secondary metabolites anthocyanin, tannin and lignin, as well as controlling cell wall biosynthesis and protection against UV-B (Abe *et al.*, 1997; Jin *et al.*, 2000; Seki *et al.*, 2002; Patzlaff *et al.*, 2003; Kilian *et al.*, 2007; Dubos *et al.*, 2010). Many MYBs are induced by several stresses, including some that are specifically induced by a combination of drought and heat stress (Rizhsky *et al.*, 2004), and have thus been targeted as potential candidates for the improvement of broad-spectrum stress tolerance in plants (Jin *et al.*, 2000; Vannini *et al.*, 2004). The ectopic expression of the rice *MYB4* gene in *A. thaliana* resulted in plants that were resistant to several types of biotic and abiotic stress (Vannini *et al.*, 2006). The 10 MYB factors identified here may thus be central to the response to multiple stresses, potentially in the cell wall re-modification described earlier, amongst other functions. Also highly abundant were transcription factors from the no apical meristem (NAM) family, as well as the AP2, the zinc finger (C2H2 type), the basic helix-loop-helix (bHLH) and the Dof-type families. AP2 transcription factors include classic drought stress marker genes such as the DREB genes. Known for their role in abiotic stress signalling, these transcription factors have also been associated with methyl jasmonate signalling and defence against fungus (Kasuga *et al.*, 1999; Sakuma *et al.*, 2006; Lin *et*

al., 2007; Sun *et al.*, 2008). This suggests a role for AP2 in multiple stress response, a theory supported by the results of the current study. Protein kinases were abundant amongst the interaction genes, including several mitogen-activated protein kinases (MAPKs). Protein kinase cascades are an indication of active signalling and regulatory control and have been identified by previous studies into multiple stress response (Rizhsky *et al.*, 2004; Kilian *et al.*, 2007). MAPKs may also provide cross-talk and specificity between biotic and abiotic signalling pathways (Zhang *et al.*, 2006). The suppression of a MAPK in rice resulted in both enhanced resistance to fungal and bacterial pathogens at the same time as susceptibility to abiotic drought, salt and cold stress (Xiong and Yang, 2003). Thus the MAPKs and other protein kinase signalling genes identified here may be important in controlling the specific multiple stress response. Pentatricopeptide (PPR) repeat-containing proteins were abundant amongst down-regulated genes in roots (21 in total) and leaves (12), although were not amongst the most strongly repressed. Only 5 PPR repeat-containing proteins were up-regulated. PPR proteins carry out editing and post-transcriptional control particularly of organellar RNA (Schmitz-Linneweber and Small, 2008; Liu et al., 2010). Recently an important role for the PPR protein ABO5 was determined in ABA signalling to affect mitochondrial gene expression (Liu et al., 2010). Given that so many PPR genes were specifically induced here by a combination of dehydration and nematode stress, many more of these ubiquitous genes may have a role in stress-responsive regulation of translation as a result of changes in hormone concentration.

2.4.7 The limitations of microarrays

Despite the great popularity of microarray technology, many doubts have also been cast over its utility in providing a real picture of changes in cellular protein activity (Feder and Walser, 2005; Clarke and Zhu, 2006; Margolin and Califano, 2007; Fu *et al.*, 2009; Deyholos, 2010). Microarray studies make the basic assumption that mRNA levels are predictive of protein abundance (Feder and Walser, 2005; Margolin and Califano, 2007). However, correlation between proteomic data and transcript abundance measured by microarrays is surprisingly low, ($R = 0.24$) (Fu *et al.*, 2009). A study on transcription factor mRNA abundance found that only 20 % of mRNAs actually associate with their target, and suggested that the activity of most transcription factors is likely to be controlled post-trancriptionally or through phosphorylation (Herrgard *et al.*, 2003). It is now also known that translational control plays an extremely important role

in protein abundance, and only a small proportion of transcripts induced by stress have been found to be actively translated (Feder and Walser, 2005; Margolin and Califano, 2007; Deyholos, 2010). Furthermore, protein abundance does not necessarily correlate with protein activity (Feder and Walser, 2005). Glanemann *et al.* (2003) found that protein activity was difficult to predict from quantitative changes in mRNA abundance. Small non-coding RNAs called microRNAs are thought to play an important role in post-transcriptional regulation during stress, by degrading transcripts, re-modelling chromatin or preventing translation (Sunkar and Zhu, 2004). It may thus be more informative to examine the abundance of mRNA associated with polyribosomal complexes (i.e. in the process of being translated) than the total cellular abundance of mRNA (Kawaguchi *et al.*, 2004). Further criticism of using microarrays in the study of stress stem from the lack of specificity in tissue type sampled, and the vast differences in laboratory-induced stress treatments (Deyholos, 2010). In order to obtain a more accurate picture of cellular changes, many researchers now use metabolomics and proteomics to study stress responses (Seki *et al.*, 2002; Koussevitzky *et al.*, 2008; Shulaev *et al.*, 2008; Urano *et al.*, 2009; Hofmann *et al.*, 2010). The integration of this data with transcriptome results will provide the most powerful tool for characterising such complex plant processes (Deyholos, 2010; Urano *et al.*, 2010). Microarray analysis was used in the current study in order to provide a snapshot of transcriptome activity under three stress treatments and therefore an insight into how plants manage the interaction between stress response pathways. Rather than draw concrete conclusions from this experiment in isolation, the aim was to generate data on which to base further hypotheses and carry out future experiments. In the next chapter microarray data will be used alongside that from expression databases and other online resources in order to select candidate genes and further characterise the nature of plant multiple stress response.

2.4.8 Conclusions

Experimental conditions were developed to test the combined effect of drought and nematode stress on *A. thaliana* plants. The imposition of these stresses individually caused physiological and molecular changes concurrent with previous findings. Physiological studies showed that drought increased the ability of nematodes to parasitise *A. thaliana,* although the progression of the nematode through the parasitic life cycle was then slower than under conditions of less severe drought, highlighting the

complex interaction between these stresses. A whole-genome transcriptome study revealed large subsets of genes that were differentially regulated by each stress in roots and leaves, though the number of genes influenced by drought stress was substantially larger than that of nematode stress. When applied in combination, the two stresses induced a new pattern of gene response that included the differential regulation of 2362 'joint stress specific' genes that had not been regulated by either stress individually, a pattern previously observed in studies of combined heat and drought stress. The subset of genes induced by joint stress was more similar to that of individual drought stress than of nematode stress alone, suggesting possible repression of the biotic stress signalling response by the presence of the stronger abiotic stress. Of the 'interaction' genes (differentially regulated by joint stress compared to individual stress), many regulatory factors were identified that have previously been implicated in multiple stress response. This study supports the role of MYB and AP2 transcription factors as key regulators that govern crosstalk between biotic and abiotic stress responses, along with regulatory PPR proteins and the signalling molecules MAP kinases. These factors may thus be crucial in governing the recognition and response to this unique stress combination. Processes specifically induced by the combined stresses included cell wall modification, carbohydrate metabolism and a specific heat shock response, whilst disease resistance mechanisms were repressed. When encountering combined drought and nematode stress, plants may therefore initiate a new programme of response whereby biotic disease mechanisms are reduced whilst general measures providing resistance to a variety of stresses are activated, such as cell wall modification. The findings of this study emphasise the need to study stresses in combination in order to fully understand the nature of plant stress responses.

Chapter 3. Functional Analysis of Candidate Genes

Aims

- Select candidate genes that may be important in controlling the response of *A. thaliana* to multiple stresses.
- Generate over-expression lines and obtain loss of function mutants for each candidate gene.
- Characterise the phenotype of over-expression and knockout lines under control conditions.
- Carry out drought stress and nematode stress susceptibility assays on each line.
- Analyse the expression of candidate genes in hormone signalling mutants.

3.1 Introduction

3.1.1 The use of loss-of-function and constitutive expression mutants in determining gene function

One of the greatest targets for plant biologists is elucidating the function of all genes in *A. thaliana*, thus providing insight into fundamental plant processes (Parinov and Sundaresan, 2000; Kennedy and Wilson, 2004). Microarray experiments are extremely useful as the first step in this process. By applying a certain stimulus and then measuring whole genome transcriptome changes, previously uncharacterised genes can be identified that may be involved in the response to that environmental stimulus (Lipshutz *et al.*, 1999; Busch and Lohmann, 2007). However, with increasing evidence for disparity between mRNA abundance and protein activity, it is prudent to carry out further analysis in order to truly confirm the role of a single gene in a particular process (Feder and Walser, 2005; Clarke and Zhu, 2006; Deyholos, 2010). Artificially heightening or inhibiting the expression of a gene of interest by the manipulation of plants' DNA can provide insights about its function. *A. thaliana* is particularly tractable to this type of study due to its ease of transformation and its rapid generation time, combined with the availability of the genome sequence (Krysan *et al.*, 1999; Deyholos, 2010). In particular, gene inactivation is a very direct way of revealing function

(Carpenter and Sabatini, 2004). Lacking an efficient method for targeted gene replacement, the best technique for creating a high number of loss-of-function mutations in *A. thaliana* is large-scale insertional mutagenesis (Krysan *et al.*, 1999; Parinov and Sundaresan, 2000; Alonso *et al.*, 2003). This process involves the random insertion of several kilobases of *Agrobacterium* derived T-DNA or transposon constructs into the genome, which usually inactivate any gene that they insert into. T-DNA insertions are particularly useful as they create stable insertions on integration (Krysan *et al.*, 1999). As the full *A. thaliana* genome sequence is now available, the location of each T-DNA insert, and thus the identity of the disrupted gene, can be determined by sequencing the DNA flanking each insertion (Parinov and Sundaresan, 2000). Two such T-DNA mutagenesis projects created the Salk collection and the SAIL (Syngenta Arabidopsis Insertion Library) collection, which contain *A. thaliana* lines with a single T-DNA insertion in over 21,700 genes and 15-18,000 genes, respectively (Sessions *et al.*, 2002; Alonso *et al.*, 2003). This germplasm, and other similar libraries, is publicly available and provides a valuable resource for reverse genetics (Tissier *et al.*, 1999; Woody *et al.*, 2007). It is worth noting, however, that gene knock-out analysis does not always produce information regarding function. This can be due to redundancy that exists between genes of a similar type, the fact that a large number of genes may contribute in a small way to a particular phenotype, or that loss-of-function mutations may only produce phenotypic differences under specific experimental conditions (Feder and Walser, 2005).

Since its discovery in the early 1980s, the cauliflower mosaic virus (CaMV) 35S promoter has become widely used in plant molecular biology as a means of constitutively expressing genes of interest including foreign genes (Odell *et al.*, 1985; Benfey and Chua, 1990; Liu *et al.*, 1998; Kasuga *et al.*, 1999). Adapted to promote the constitutive transcription of viral genes on entering a plant cell, the 35S promoter drives the expression of an adjacent gene when inserted into plants on an expression cassette, and is expressed in all plant organs (Jefferson *et al.*, 1987). Promoter cassettes have been developed to improve the efficiency of the 35S promoter and link it to antibiotic resistance genes in a plasmid vector (Jefferson *et al.*, 1987; Mitsuhara *et al.*, 1996). One such vector is pBI121, which uses the 35S promoter to drive the expression of the β-glucuronidase gene (GUS). The vector acts as a reporter for successful plant transformation, and includes the neomycin phosphotransferase gene (*NPTII*) to confer

kanamycin resistance, as well as the 3' untranslated region of the nopaline synthase gene (*nos*) to provide a polyadenylation site and confer stability to the transcripts. The construct can be used as a general-purpose over-expression cassette by replacing the GUS gene with any gene of interest (Jefferson *et al.*, 1987). The 35S promoter has frequently been used in the study of stress responsive genes, for example in the discovery that when over-expressed in *A. thaliana*, the genes *DREB1* and *DREB2* confer freezing and dehydration tolerance, thus confirming their importance in abiotic stress responses (Liu *et al.*, 1998). Constitutive expression from the 35S promoter has also revealed the functions of genes such as heat shock factors, WRKY transcription factors, MYB transcription factors, MAP kinases and cysteine proteinase inhibitors in various stress response systems (Xiong and Yang, 2003; Vannini *et al.*, 2004; Nishizawa *et al.*, 2006; Zhang *et al.*, 2008; Qiu and Yu, 2009). Although indispensible for gene function analysis, the 35S promoter may have limited utility in creating stress-tolerant crops, as stunting is often observed in plants constitutively expressing stress tolerance genes (Kasuga *et al.*, 1999; Priyanka *et al.*, 2010). To avoid this negative impact during non-stress conditions, the stress-inducible *rd29A* promoter can be used instead of a constitutive promoter. This causes induction of the transgene only during conditions of stress (Kasuga *et al.*, 1999; Pellegrineschi *et al.*, 2004; Al-Abed *et al.*, 2007; Priyanka *et al.*, 2010).

3.1.2 The analysis of stress tolerance

Loss-of-function and constitutive expression mutants are thus frequently used to investigate gene function, for example after the identification of genes of interest by microarray. Often differences in plant growth, morphology and yield phenotypes can be identified under normal growing conditions (El-Lithy *et al.*, 2004). However, in the study of stress response, investigation of mutant genotypes often takes the form of stress susceptibility or tolerance assays. An excellent example of this process is described by Luhua *et al.* (2008), who identified 41 genes that were differentially regulated in H_2O_2-accumulating mutants. When over-expressed behind the 35S promoter, more than 70 % conferred oxidative stress tolerance on treatment with the chemicals paraquat or t-butyl hydroperoxide. Tolerance assays to characterise the stress-resistance of certain genotypes have been designed for virtually every type of biotic or abiotic stress, often to assess the effect of transferring a gene involved in stress tolerance from one species to another (Vannini *et al.*, 2004; Oh *et al.*, 2005; Vannini *et al.*, 2007; Qiu and Yu, 2009).

In order to impose drought and test plants' resistance, some authors report the application of mannitol or polyethylene glycol (Zhang *et al.*, 2008; Zhang *et al.*, 2009). However these systems have been criticised as actually imposing osmotic stress rather than drought (Bhatnagar-Mathur *et al.*, 2008). Most drought resistance assays carried out on *A. thaliana* use a method of withholding water for a specified time, re-watering and then scoring for survival. The exact length of time varies according to the growth conditions and soil type, but is generally between 14 – 21 days (Kasuga *et al.*, 1999; Iuchi *et al.*, 2001; Dubouzet *et al.*, 2003; Chini *et al.*, 2004; Fujita *et al.*, 2005; Chen *et al.*, 2007; An *et al.*, 2008; Hong *et al.*, 2008). This system has also been used for studying rice, the drought tolerance of which is assessed by withholding water for 4 – 6 days and then measuring characteristics such as leaf rolling and wilting, and tomato, which is scored for survival on re-watering (Lee *et al.*, 2003; Xiong and Yang, 2003; Oh *et al.*, 2005; Vannini *et al.*, 2007).

Resistance or susceptibility of certain plant genotypes to nematodes can also be assessed quantitatively, a process important in the development of nematode-resistant crops (Vain *et al.*, 1998; Urwin *et al.*, 2001; Atkinson *et al.*, 2003; Urwin *et al.*, 2003; Sobczak *et al.*, 2005; Goggin *et al.*, 2006; Li *et al.*, 2006). In order to provide a measure of the ability of nematodes to invade a plant, the number of nematodes established within the roots can be counted either directly under the microscope or by staining the roots with a compound such as acid fuchsin (Baum *et al.*, 2000; Liu *et al.*, 2005). Alternatively the size of nematodes within the root can be measured through a developmental time course (Urwin *et al.*, 1997). In order to assess the relative growth rate and fecundity of established cyst nematodes, the number of nematode cysts released into the soil following infection can be quantified by an egg count (Urwin *et al.*, 2001). To assess resistance to root-knot nematodes, the number of egg masses on the surface of the roots can be stained and counted, or the number of eggs themselves (Lilley *et al.*, 2004; Goggin *et al.*, 2006)

3.2 Materials and Methods

3.2.1 Species used

Arabidopsis thaliana Col-0

Escherichia coli DH5α

Agrobacterium tumefaciens GV3101

Heterodera schachtii

Myzus persicae (peach-potato aphid) Clone G

3.2.2 Selection of candidate genes

A small set of candidate genes was selected for further analysis using the results of the microarray experiments described in Chapter 2. Genes were selected according to several criteria. Firstly, in order to test the hypothesis that certain regulatory genes may specifically control the response of plants to multiple stresses, it was important to pick genes that were differentially regulated in response to joint stress compared to the individual stresses. Thus candidate genes were selected from the lists of 'interaction' genes given in Tables 2.3-6. The GO ontological categories of these genes were analysed using GeneSpring GX10, and their response to different biotic and abiotic stresses and hormones examined using Genevestigator V3 (https://www.genevestigator.com). This tool allows the visualisation of whole-genome expression data combined from thousands of publicly available microarray analyses (Hruz *et al.*, 2008). The expression profile of any gene can be examined under a wide range of stress, developmental or spatial conditions. Ten genes were selected in total. These were genes that had a particularly high fold change or a low *p*-value, indicating high reproducibility between replicates. In particular, genes were chosen if they were a member of a transcription factor family, or if they were strongly responsive to a hormone involved in stress signalling. This was because these groups were likely to be involved in regulatory pathways which may affect many downstream processes. Only one transcription factor was chosen per family of highly represented groups. The Arabidopsis Coexpression Data Mining Tool (www.arabidopsis.leeds.ac.uk) was used to identify genes co-expressing most highly with the selected candidate genes using data from 322 publicly

available ATH1 microarray experiments. Coexpression values are represented as Pearson Correlation Coefficients (Manfield *et al.*, 2006).

3.2.3 Obtaining loss-of-function mutants

In order to investigate the function of candidate genes, T-DNA insertion mutants were obtained for each gene where possible from the Nottingham Arabidopsis Stock Centre (NASC). The names of each mutant line and their corresponding genes are given in Table 3.1. Seven of the lines obtained were from the Salk collection of T-DNA insertion mutants, a genome-wide mutagenesis project carried out by the Salk Institute and aimed at determining the function of every *A. thaliana* gene (Alonso *et al.*, 2003). The At4g38620 (MYB4) T-DNA insertion mutant was provided by Prof. Cathie Martin (John Innes Centre, Norwich). This insertion line was derived from the SLAT lines (Sainsbury Laboratory *Arabidopsis Thaliana*), which were created by the mutagenesis of a Columbia population using a T-DNA construct containing a defective suppressor-mutator (dSpm) element (Tissier *et al.*, 1999). The mutant line obtained for At2g34600 came from the WiscDsLox collection of T-DNA mutagenised lines created using a Ds transposable element (Woody *et al.*, 2007), and is described by Sehr *et al.* (2010). No T-DNA insertion line was available for At1g61563.

3.2.3.1 Extraction of genomic DNA

DNA was extracted from plants of each T-DNA insertion line as follows. Leaf tissue was ground in 500 µl DNA extraction buffer (0.2 M Tris-cl (pH 9), 0.4 M lithium chloride, 25 mM EDTA and 1 % SDS) and the sample centrifuged for 5 minutes at high speed (13,100 rcf). A 350 µl portion was mixed with an equal amount of isopropanol and centrifuged for 10 mins at high speed. The liquid was removed and the DNA pellet left to air dry before re-suspending in 400 µl TE. 2 µl of DNA was used per PCR reaction.

3.2.3.2 Confirming homozygosity of T-DNA insertion lines

Primers were designed to amplify the T-DNA insertions in the mutant lines, thus confirming the presence of the transgenes and the homozygosity of the lines. Primers for the Salk lines were designed using the T-DNA Primer Design Tool from the Salk Institure Genomic Analysis Laboratory website (http://signal.salk.edu/tdnaprimers.2.html). Three primers are needed to detect the wild type and T-DNA insertion alleles:

Gene Name	Gene AGI Code	Mutant line	NASC ID	Location of insertion	Zygosity
DIR14	At4g11210	SALK_058728C	N653680	Exon	Homozygous
AZI1	At4g12470	SALK_085727C	N657248	Exon	Homozygous
F2H15	At1g17970	SALK_152907C	N667649	Promoter	Homozygous
ANACO38	At2g24430	SALK_103716C	N653811	Intron	Homozygous
JAZ7	At2g34600	WiscDsLox7H11	N849196	Exon	Homozygous
TCP9	At2g45680	SALK_026421C	N653815	Promoter	Homozygous
RALFL8	At1g61563	None available	-	-	-
ATMGL	At1g64660	SALK_074592C	N669846	Intron	Homozygous
DUF581	At5g65040	SALK_106042C	N656840	Exon	Homozygous
MYB4	At4g38620	SLAT *atmyb4*	-	Exon	Homozygous

Table 3.1. T-DNA insertion lines obtained for candidate genes. Mutant germplasm was obtained from the Nottingham Arabidopsis Stock Centre (NASC), or from the John Innes Centre (At4g38620).

The Left Border primer (LB) anneals to a region within the T-DNA insertion itself; the Right Primer (RP) anneals to a flanking sequence in the gene on the 3' side of the insertion; and the Left Primer (LP) anneals to a flanking region 5' of the insertion. Using the LB and RP in combination amplifies the allele with the T-DNA insertion, whilst LP and RP amplify the wild type allele. Appendix 2 shows the primer sequences used. PCRs were carried out (as detailed in Section 2.2.6) on DNA from plants of each T-DNA insertion line as well as wild type DNA. The At4g38620 insertion line was not tested as it had previously been confirmed (Jin *et al.*, 2000).

3.2.4 Creating CaMV 35S constitutive expression lines

Ten genes of interest were cloned into 35S over-expression vectors that were then used to transform wild type *A. thaliana* plants. The genes were first cloned into the entry vector pBlueScript SK- and then transferred to a pBI121 vector containing the CaMV 35S constitutive promoter. Schematic diagrams of the two vectors are shown in Figures 3.1 and 3.2. The pBlueScript SK- vector contains a multiple cloning site within a β-galactosidase gene, allowing blue/white colony selection on media containing X-gal. Restriction digest was used to cut the cassette out of pBlueScript and ligate into a pBI121 vector that had had the GUS gene removed by *Bam* HI and *Sac* I and replaced by linking DNA containing a *Kpn* I site. The neomycin phosphotransferase gene (*NPTII*) allows plant growth on kanamycin selection plates, whilst a kanamycin resistance gene allows bacterial selection.

3.2.4.1 Growth media and solutions

LB medium

10 g NaCl, 10 g tryptone, 5 g yeast extract. Addition of distilled water to 1 litre. For LB agar, 1 % bacteriological agar no. 1 was added. Autoclaved for sterilisation.

TE buffer

10 mM Tris.HCl, 1 mM EDTA ph 8.0. Autoclaved for sterilisation.

SOB solution

0.5 % yeast extract, 2 % tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$. Dissolve in distilled water and autoclave.

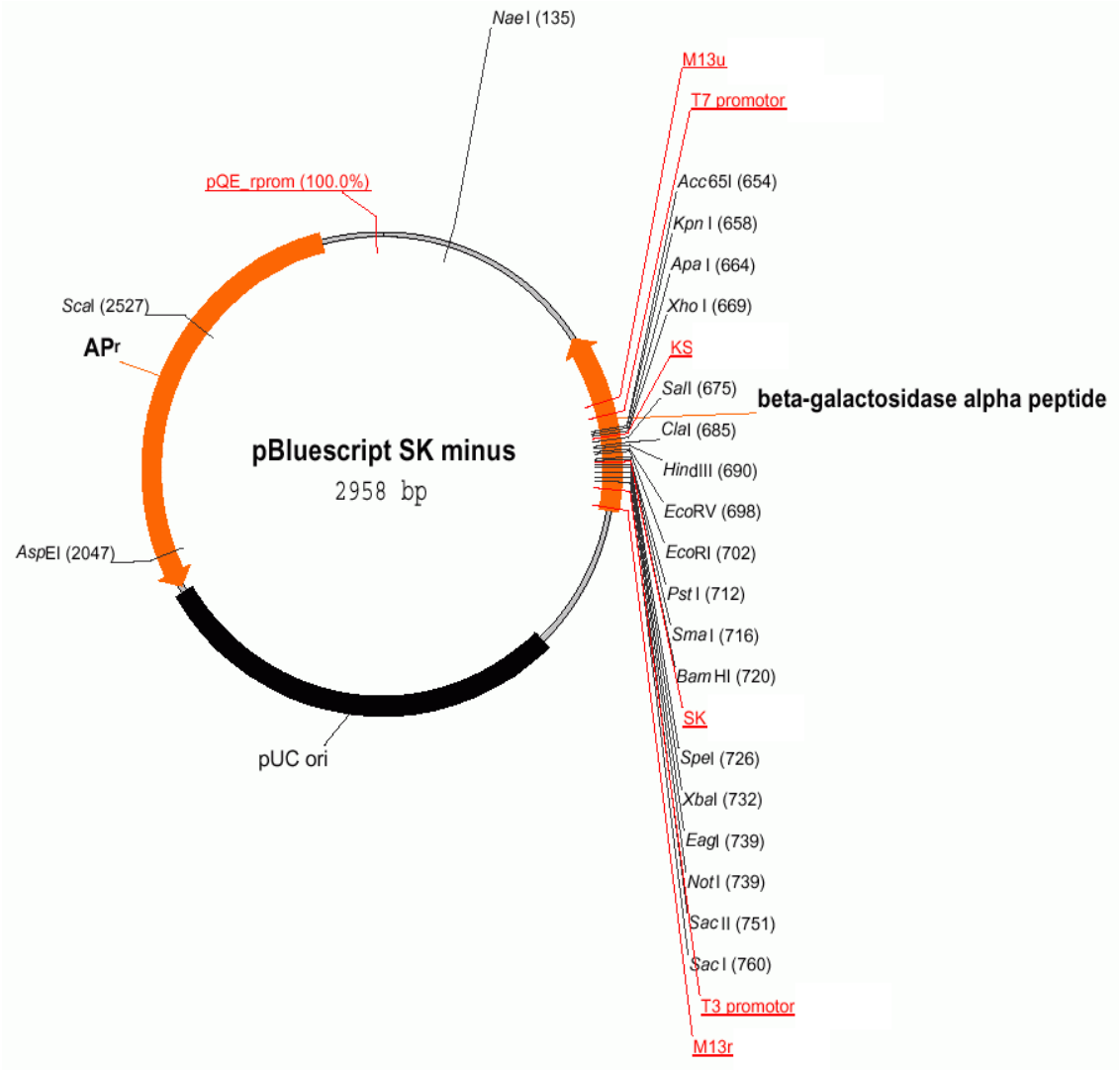


Figure 3.1. Schematic diagram of pBLUESCRIPT SK- vector. AP^r shows the location of the ampicillin resistance gene, which is used for the selection of positive *E. coli* colonies. M13u and M13r show the position of the M13 forward and reverse primers used for sequencing. pUC ori is the origin of replication. Inserting DNA into the Multiple Cloning Site within the β-galactosidase gene disrupts the production of functional β-galactosidase. Image taken from the BioSource ImaGenes website (http://www.imagenes-bio.de/info/vectors/pBluescript_SK _minus_pic.shtml).

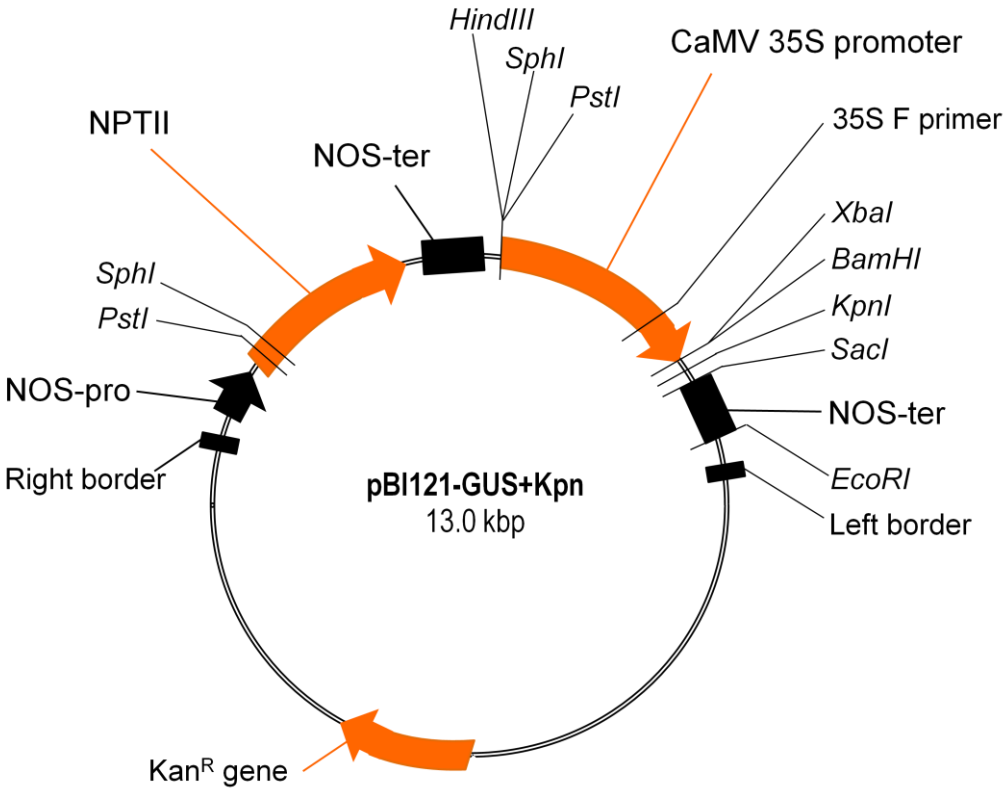


Figure 3.2. Schematic diagram of the pBI121-GUS+Kpn vector. The NPTII gene provides plant resistance to kanamycin, and is under the control of the bacterial nopaline synthase (NOS) promoter. Also present is a kanamycin resistance gene for bacterial selection. The CaMV 35S promoter allows the constitutive expression of a transgene inserted directly downstream.

TB solution

10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl dissolved in distilled water. Adjusted to pH 6.7 using KOH. Addition of $MnCl_2$ to a final concentration of 55 mM. Sterilised through a 0.45 µm filter and stored at 4 °C.

For alkaline lysis of bacterial cells:

Solution 1

1 % glucose, 10 mM EDTA pH 8.0, 25 mM Tris pH 8.0. Store at 4 °C.

Solution 2

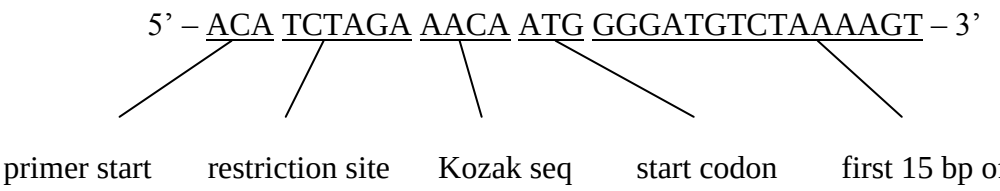
1 % SDS, 0.2 M NaOH.

Solution 3

11.5 ml glacial acetic acid added to 60 ml 5 M potassium acetate and 28.5 ml distilled water. Final concentration is 3 M potassium and 5 M acetate.

3.2.4.2 Design of primers for amplification of gene coding sequences

Primers were designed that would amplify the coding region of the ten genes of interest, as well as providing restriction endonuclease sites for digesting and ligating into the vector. Two restriction digest sequences were selected that were not present within the coding region of the gene, and that would allow insertion into the pBI121 vector in the correct orientation. The forward primer for each gene clone was designed as follows: An ‘ACA’ sequence at the start to allow the restriction enzyme to attach well to the DNA; the restriction enzyme sequence; the Kozak sequence ‘AACA’ which aids translation initiation; the ATG start codon of the gene; and around 15 bp into the gene of interest. For example the Forward primer for the At1g61563 gene was designed as follows:



The reverse primers consisted of the ‘ACA’ sequence, followed by the other chosen restriction site, followed by the stop codon at the end of the gene coding sequence, and around 15 bp back into the gene. The primers used for cloning are given in Appendix 2.

3.2.4.3 PCR for amplification of genes for cloning

Amplified fragments of the entire coding region of genes were created by PCR from leaf or root cDNA, depending on where the gene was found to be expressed most highly in the microarray experiment. Phusion High-Fidelity DNA Polymerase (NEB) was used to minimise errors during amplification. Due to an additional DNA-binding domain combined with the polymerase, the affinity of this enzyme is improved thus reducing errors and increasing processivity. The error rate of Phusion DNA polymerase is approximately 50-fold lower than the DNA polymerase from *Thermus aquaticus,* used in BioTaq Red, thus making it more suitable for cloning experiments (NEB, 2011). The reagents and PCR conditions are detailed in Appendix 1C and 1D. A portion of each PCR product was electrophoresed on an agarose gel (Section 2.2.7) to check that the band size was as expected. PCR products of the correct size were then purified using a QIAquick PCR purification kit (QIAGEN). Briefly, DNA fragments were adsorbed to a silica membrane whilst contaminants such as enzymes and salts were washed through using ethanol-based buffers. DNA was eluted in tris-buffer.

3.2.4.4 Restriction enzyme digests

Standard digests were carried out on all amplified DNA sequences in a volume of 60 µl, using 48 µl of purified PCR product, 6 µl of the manufacturer's recommended buffer for the combination of enzymes used, 1 µl (5-10 units) of each enzyme and 4 µl of sterile distilled water. Vectors were digested in volumes of 20 µl, consisting of 1 µl plasmid vector, 1 µl of each enzyme, 2 µl buffer and 15 µl sterile, distilled water. Incubation was carried out at 37 °C for 3 hours.

3.2.4.5 Phenol:chloroform extraction and ethanol precipitation

Phenol:chloroform was used to purify DNA by removing contaminants such as proteins. The product of each restriction digest was made up to 100 µl using sterile, distilled water and an equal volume of phenol:chloroform was added. The mix was vortexed and centrifuged at top speed for 3 minutes, and the aqueous top layer retained. Precipitation was carried out by mixing the DNA with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. The mix was incubated at -80 °C for 30 minutes and then centrifuged for 10 minutes. The liquid was removed and the pellet washed in 70 % ethanol before being air dried and re-suspended in sterile distilled water. PCR fragments were re-suspended in 8 µl and vectors in 14 µl.

3.2.4.6 DNA ligations

DNA fragments were ligated into digested vectors using T4 DNA Ligase (Invitrogen). The reaction consisted of 2 µl 5x buffer, 1 µl DNA ligase, 3 µl vector and 4 µl DNA insert. The reaction was incubated at 16 °C overnight.

3.2.4.7 Preparation of ultra-competent E. coli *cells*

Protocol based on Inoue *et al.* (1990) with some modifications. *E. coli* DH5α cells were cultured on an LB agar plate at 37 °C overnight. 10-12 large colonies were then transferred to a 1 L flask containing 250 ml SOB solution and grown at 19 °C with vigorous shaking until the OD_{600} reached 0.5. The flask was placed on ice for 10 mins and then the cells were pelleted by centrifuging at 1800 rcf for 10 mins at 4 °C. The cells were re-suspended by swirling in 80 ml ice-cold TB solution and placing on ice for a further 10 mins, before centrifuging again at 1800 rcf for 10 mins at 4 °C. The pellet was resuspended in 20 ml ice-cold TB and 1.4 ml DMSO was added. The cells were aliquoted into 100 µl portions, snap-frozen in liquid nitrogen and stored at -80 °C.

3.2.4.8 Transformation of ultra-competent E. coli *cells.*

After thawing the ultra-competent cells on ice, 5 µl of ligation product was added to a 100 µl aliquot of cells. The mixture was incubated on ice for 5 mins and then spread on top of pre-warmed LB agar plates containing the correct antibiotic. For selection of the pBlueScript vector, 100 µg/ml ampicillin and 40 µg/ml X-gal were added to the medium. For selection of the pBI121 vector, 25 µg/ml kanamycin was added. The plates were dried in a 37 °C incubator for 5 minutes and then incubated at 37 °C overnight.

3.2.4.9 Plasmid preparation

Single colonies were taken from the LB plates and grown overnight in liquid LB containing the appropriate antibiotic. In the case of transformation with the pBlueScript vector, only white colonies were used. DNA for restriction analysis was then prepared using the alkaline lysis method. First, 1.5 ml of LB broth containing the overnight bacterial culture was centrifuged for 1 min at maximum speed. The pellet was re-suspended in 100 µl of Solution 1. Then 200 µl of Solution 2 was added and mixed by inversion until clear and viscous. 150 µl of ice-cold Solution 3 was added and mixed by brief vortexing, before storing on ice for 2-5 minutes. The debris was pelleted by centrifuging for 5 minutes and the supernatant mixed with 2 volumes of ethanol. After

incubating for 5 minutes the mixture was centrifuged for 5 minutes, the liquid was removed and 500 µl of 70 % ethanol was added, mixed and re-centrifuged. The ethanol was then removed and the pellet allowed to air dry. The pellet was re-suspended in 50 µl of sterile, distilled water with 1 µl Ribonuclease A (Fermentas). A 5 µl portion of the plasmid preparation was digested with the corresponding enzymes and electrophoresed to confirm that the insert size was correct.

3.2.4.10 DNA Sequencing

Sequencing was carried out on plasmids that appeared to have the correctly inserted DNA fragment. Plasmid DNA was purified using a QIAquick PCR Purification Kit (QIAGEN). Samples were prepared at 50 ng/µl and primers provided at 1.6 pmol/µl. For sequencing of the pBlueScript vector the primers were M13F (5'-GTAAAACGACGGCCAGT-3') and M13R (5'-GGAAACAGCTATGACCATG-3'), and for pBI121 35S1 was used (5'-GATGTGATATCTCCACTGACG-3'). DNA sequencing was performed in the sequencing facility of the University of Leeds using the dye-labelled, dideoxy terminator method. An Applied Biosystems Cycle Sequencing Kit was used and the sequence analysed on an ABI377 Autosequencer (Applied Biosystems). The resulting chromatograms were analysed using Chromas Lite 2.01 software.

3.2.4.11 Transfer of insert from pBlueScript SK- to pBI121-GUS+KPN

Following sequencing, 5 µl of the pBlueScript vector with the gene correctly inserted was digested using the corresponding enzymes, and combined with the product of a pBI121 vector digest. The mixture was purified using phenol:chloroform, ligated, transformed into *E. coli.* and grown on kanamycin plates. Plasmid preparations were carried out on resulting colonies, followed by digestion to confirm fragment size and sequencing.

3.2.4.12 Preparation of **A. tumefaciens** *competent cells*

Agrobacterium tumefaciens GV3101 cells were inoculated into 5 ml LB containing 50 µg/ml rifampicin and grown overnight at 28 °C. A 2 ml portion of this culture was added to 50 ml LB (+ rifampicin) in a 250 ml flask, which was shaken at 28 °C at 200 rpm for around 5 hours until the culture reached OD_{600}=0.5-1. The culture was chilled on ice and transferred to a 50 ml tube, before centrifuging at 1800 rcf for 5 mins at 4 °C.

The supernatant was removed and the pellet was re-suspended in 1 ml ice-cold 20 mM $CaCl_2$ by gentle shaking. Cells were divided into 200 µl aliquots, quick-frozen in liquid nitrogen and stored at -80 °C.

3.2.4.13 Transformation of A. tumefaciens *competent cells*

After identification of pBI121 plasmids containing the correct insert, the plasmid DNA was transformed into *A. tumefaciens.* 1 µl DNA was added to *A. tumefaciens* cells which had been thawed on ice, and the mixture was incubated at 37 °C for 5 mins. Then 1 ml LB was added and the cells transferred to a 50 ml falcon tube which was shaken at 28 °C for at least 2 hours. The *A. tumefaciens* were spread onto LB plates containing rifampicin and kanamycin which were sealed with parafilm and incubated at 28 °C for 48 hours. Individual colonies were selected and streaked out onto fresh plates and grown again at 28 °C for 48 hours. PCR was carried out directly on the *A. tumefaciens* cells by re-suspending a small amount of bacterial growth in 10 µl sterile distilled water in a PCR tube. The tube was incubated at 99 °C for around 10 mins to lyse the bacteria, and then PCR reagents were added to the tube and a PCR reaction carried out (as detailed in Section 2.2.6).

3.2.4.14 Transformation of A. thaliana

A small amount of bacterial growth from transformed *A. tumefaciens* plates was re-suspended in 20 ml of LB containing rifampicin and kanamycin in a 50 ml flask, and shaken at 28 °C at 200 rpm for at least 16 hours. The 20 ml was used to inoculate 200 ml of fresh LB containing kanamycin in a 500 ml flask, and was shaken at 28 °C at 200 rpm for between 4-5 hours, until the A_{600}nm was between 0.5-0.8. The entire culture was centrifuged for 10 mins at 1800 rcf, and the supernatant decanted. The bacterial cells were re-suspended in 200 ml of 5 % sucrose solution and 100 µl of the surfactant Silwet L-77 (Lehle) was added. The cellular suspension was poured into a beaker and wild type *A. thaliana* plants at growth stage 6.0 – 6.5 (i.e. during flower production) (Boyes *et al.*, 2001) were inverted and dipped into the solution. The plants were agitated in the solution for 30 seconds so that all the flowers were covered. Approximately 8 plants per construct were transformed. The plants were covered with clear propagator lids for 2 days after transformation. The same plants were re-transformed 5-7 days later with freshly-cultured *A. tumefaciens* solution. Transformed plants were kept in the greenhouse for another 6-8 weeks to allow seed production. Seeds were collected from

each plant using plastic aracons to keep the seeds from each plant separate. These seeds formed the T_1 generation, as detailed in the table below.

Plant Generation	Definition
T_0	Wild type *A. thaliana* transformed with *A. tumefaciens*. These produce T_1 seed.
T_1	Plants grown from seeds of selfed T_0. These produce T_2 seed.
T_2	Plants grown from seeds of selfed T_1 These produce T_3 seed.

Definitions of transgenic *A. thaliana* generations (Rosso *et al.*, 2003).

3.2.4.15 Selection of A. thaliana *transformants and creation of homozygous lines.*

T_1 generation seeds were sterilised as detailed in Section 2.2.3.1 and stratified at 4 °C for up to 5 days. Approximately 0.5 ml volume of seeds for each construct were plated out onto a total of 10 petri dishes containing ½ MS media with 50 µg/ml kanamycin and 250 µg/ml cefotaxime. Plates were sealed with micropore tape and grown at 20 °C in growth cabinets. After approximately 2 weeks T_1 plants could be distinguished from plants unable to grow on kanamycin by their green colour, vigorous growth, and the presence of roots. Approximately 20 plants per construct were transferred to soil and grown in individual pots in greenhouse conditions. PCRs confirming the presence of the transgene were conducted at this point from leaf DNA samples, and leaf RNA samples were also collected in order to assess the over-expression level of the transgene. T_2 seeds were collected from each individual plant, and kept separately from each other as they represented different transgenic lines of the same construct. T_2 generation seeds were sterilised using the chlorine gas method, which is convenient for large numbers of seed lines. 50-100 seeds were placed in open PCR tubes in a glass chamber. In a glass beaker inside the chamber 100 ml of domestic bleach was combined with 3 ml HCl (approximately 37%) and mixed gently. The chamber was then sealed for 4 hours to allow sterilisation. Following this the open tubes were placed in a laminar flow hood for an hour to remove traces of chlorine gas. T_2 seeds from two or three lines per construct were sown on selective media. The resulting T_2 seedlings were either homozygous for

the transgene, heterozygous, or wild type at a ratio of 1:2:1, respectively. Seedlings possessing the transgene (homozygous or heterozygous) were planted into soil and their seeds collected again. When planted on selective media these T_3 seedlings would indicate the identity of their T_2 parent. If the T_2 parent had been homozygous then all the T_3 seeds would grow on selection. In this way a stable homozygous line was developed for each transgenic construct.

3.2.4.16 Confirmation of transgene identity and transcript level in planta

To confirm that T_1 plants had the transgene with the correctly inserted gene construct, DNA was extracted from leaf samples (as detailed in Section 3.2.3.1) and PCR carried out. The 35S1 primer was used in combination with the reverse cloning primer for each gene construct, so that the presence of a band would indicate the insertion of the correct construct. The PCR products were electrophoresed to confirm the presence of the band. qRT-PCR was carried out on RNA extracted from T_1 leaf samples in order to identify the transgenic line most highly expressing each transgene. RNA was isolated and reverse transcription carried out on around 10 lines per construct (Section 2.2.4 and 2.2.5), then qRT-PCR reactions were performed (Section 2.3.8) using the primers detailed in Appendix 2.

3.2.5 Phenotypic analysis of mutants

Seeds of each mutant and over-expression line as well as wild type seeds were sown in soil and then planted into 9 cm pots (4 plants per pot, 5 pots per genotype). A range of phenotypic measurements were recorded at various stages throughout the plant life cycle (adapted from El-Lithy *et al.* (2004) and Sakuma *et al.* (2006)). The measurements were as follows:

Rosette diameter at 16 days after sowing

Rosette leaf number 16 days after sowing

Length of time after sowing before emergence of primary inflorescence

Height of primary inflorescence 35 days after sowing

Final height of primary inflorescence

Dry weight of aerial plant material 35 days after sowing. Fully watered plants were cut from root system, weighed, dried in an oven for 24 hours, and weighed again.

Silique number on primary inflorescence 40 days after sowing

Seed number per silique (one fully-ripened silique selected from halfway up the primary inflorescence at maturity)

Root system characteristics were also measured. Plants were grown on upright ½ MS plates (as detailed in Section 2.2.3.1), and after two weeks the plates were scanned using a Hewlett Packard ScanJet 5370C and the images stored digitally. The following parameters were measured using Image-Pro Plus software version 7.0 (MediaCybernetics):

Length of the primary root

Number of lateral roots

Length of each of the lateral roots

Lateral root density

Total root system size (length)

3.2.6 *H. schachtii* trials with mutants

In order to determine resistance or susceptibility of mutant and over-expression genotypes to infection with the nematode *H. schachtii*, trials were carried out in tissue culture. A similar technique has been described by Baum *et al.* (2000). Twelve plants per genotype were grown on upright ½ MS plates (3 plants per plate), and at growth stage 1.05 plants were infected with 100 juvenile *H. schachtii* per plant (as detailed in 2.2.3.1-3). Nematodes were allowed to develop for 14 days. Following this, the root system was detached from the aerial plant parts, weighed, and stained using acid fuchsin (section 2.2.2.6). Nematodes on each plant were counted under a microscope and classified into the categories vermiform, fusiform, saccate, enlarged saccate, or male.

3.2.7 Drought trials with mutants

Drought susceptibility or resistance was analysed through the use of soil drought assays. Seeds of each genotype were sown in compost, and at wild type growth stage 1.05 plants were transferred to 25 x 40 cm trays containing compost:sand:loam at a ratio of 2:1:1 and to a depth of 5 cm. Mutant or over-expression lines were alternated with wild type plants to create a chequered pattern (Figure 3.3). The plants were watered to field capacity for 1 week, and then watering was ceased until the soil moisture level (as measured by the SM200 Soil Moisture Sensor) dropped to 3-4 %, which took an average of 2 weeks. The plants were then watered to field capacity again for 1 week and their percentage survival rate observed.

Figure 3.3. Plants arranged in tray for drought assay. Wild type plants were alternately placed between the mutant or overexpression line under analysis. White circles show wild type plants and red circles show mutant plant. Here the alternating pattern can be easily observed due to the characteristically slow growth of the 35S::At2g45680 line in contrast with the larger wild type plants. Water was withheld from the tray for approximately two weeks until the soil moisture was reduced to between 3 and 4 %. The plants were scored for survival following re-watering.

3.2.8 Joint stress trials with mutants

Response to joint stress was analysed in mutant phenotypes by imposing drought and nematode stress simultaneously. Seeds of mutant and over-expression lines as well as wild type were sown on compost. At wild type growth stage 1.05, seedlings of each genotype were divided into four treatment groups: 1) Plants without any stress treatment (control plants), 2) plants subjected to drought stress treatment, 3) plants subjected to nematode stress, and 4) plants subjected to both drought and nematode stress. Seedlings from the nematode-treated and joint-stress groups were potted up into compost containing 50 eggs/g *H. schachtii*, while control and drought-treated seedlings were transferred to normal compost and watered to field capacity. Following this, watering was suspended for the drought-stressed and joint-stressed groups until the soil moisture fell to 15-20 %, a process taking 8 days. The stomatal conductance of a sample of plants was measured when the soil moisture reached 15-20 %. Following this the pots were maintained at 15-20 % moisture for the remainder of the experiment by watering a small amount each day. Control and nematode-treated plants were watered to field capacity. Physiological measurements were taken over the course of 48 days following planting. These were:

Rosette diameter (measured every 4-5 days)

Date of inflorescence emergence

Height of primary inflorescence (measured every 4-5 days following emergence)

Final inflorescence height

Number of siliques on primary inflorescence at final height

Seed number per silique (one fully-ripened silique selected from halfway up the primary inflorescence at maturity)

3.2.9 Analysis of candidate genes in hormone signalling mutants

A. thaliana lines that were defective in hormone signalling were obtained in order to further characterise the role of the candidate genes. The following mutants were acquired from Dr. Hanma Zhang (Centre for Plant Sciences, University of Leeds): *abi2-1, abi4-1, CTR1* and *ein3-1*. The mutant *jar1-1* was obtained from NASC. Seeds were planted directly into compost (wild type, *CTR1, jar1-1*) or germinated on agar containing 1 µM ACC (*ein3-1*) or 3 µM ABA (*abi2-1* and *abi4-1*) and then transplanted into compost. At growth stage 1.05, plants were divided into four treatment groups:

control, drought, nematode and joint stress. Seedlings from the nematode stress and joint stress groups were potted up into compost containing 50 eggs/g *H. schachtii,* while control and drought-treated seedlings were transferred to normal compost and watered to field capacity. After waiting 2 weeks to allow establishment of nematodes, water was permanently withheld from the drought stress and joint stress treatment groups. When the soil moisture content reached 10-15 %, (at which point stomatal conductance readings were approximately 25 % of the control plants), tissue samples were taken from each of the plants and combined into pools containing tissue from 3 plants per genotype per treatment. RNA was isolated from each pool and reverse transcription carried out (Section 2.2.4-5). Semi-quantitative RT-PCR reactions (Section 2.2.6) were performed using primers for each candidate gene and cDNA from each hormone mutant. Significant results were repeated using qRT-PCR (Section 2.3.8, primers detailed in Appendix 2).

3.2.10 Aphid experiments

Nymphs of the peach-potato aphid *Myzus persicae* were obtained from the Scottish Crop Research Institute (now the James Hutton Institute, Invergowrie, Dundee). The aphids were clones of a wild population isolated in Scotland (Kasprowicz *et al.*, 2008). This population, known as Clones G, has a susceptibility to insecticide and a low tendency to form winged females except when feeding on peach species. Aphids were maintained as asexual clones on a potato plant sealed within a perspex box with a meshed window. In order to avoid the development of winged females associated with high population density, every few weeks a new colony was started on a fresh potato plant with 10 nymphs from the old colony.

3.2.10.1 Aphid fecundity assays

One-day old parthenogenically produced nymphs were used in aphid fecundity assays. Several large nymphs were removed from the stock plant and placed on a fresh potato leaf in a sealed, meshed beaker overnight. The resulting 1-day-old nymphs were used in fecundity experiments based on those described by Fenton *et al*. (2010) and De Vos *et al.* (2009). One nymph was placed on each *Arabidopsis* plant and the plant pot sealed with a meshed plastic lid (Figure 3.4). The plants were grown in greenhouse conditions for the next 15 days, after which the number of aphids on each plant was counted.

3.2.10.2 Systemic immunity assays

The effect of pre-treatment with a biotic stress on the fecundity of aphid feeders was assessed. Plants of differing genotypes were grown in compost for 2 weeks, before dividing into 3 treatment groups: Control, nematode pre-treatment, and aphid pre-treatment. The nematode pre-treatment group were transplanted into compost containing 50 eggs/g *H. schachtii* cysts, whilst other treatment groups were transplanted into normal compost. At this point four 1-day-old nymphs were applied to each plant in the aphid pre-treatment group as an immune system trigger, and removed 5 days later. Two weeks after transplanting, one 1-day-old nymph was applied to each plant in all three treatment groups. The number of aphids on each plant was counted after 15 days.

3.2.11 Analysis of 35S::RALFL8 over-expression line

3.2.11.1 Growth on auxin

To investigate the auxin response of 35S::RALFL8 plants, seeds were sown on medium containing various auxin-related chemicals. These included indole-3-acetic acid (IAA), one of the most important auxins in plants, at a concentration of 0.1 μM, 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin, at 0.1 μM, and α-(phenyl ethyl-2-one)-indole-3-acetic acid (PEO-IAA), a synthetic compound displaying anti-auxin activity, at 1 μM (Belin *et al.*, 2009). The plates were grown upright for 4 days and the roots photographed and measured for length.

3.2.11.2 Cross with axr3-1 mutant

Homozygous 35S::RALFL8 plants were crossed with homozygous *axr3-1* mutants. This was achieved by removing the stamens of flowers from one plant just before opening, then brushing the carpel with the pollen of a flower from the other genotype and collecting the seeds. Crosses were carried out using both 35S::RALFL8 and *axr3-1* plants as females/males. *Axr3-1* plants contain a dominant loss-of-function mutation in the AUX/IAA gene *AXR3*, which leads to auxin insensitivity. Seeds were obtained from Dr. Stefan Kepinski, Centre for Plant Sciences, University of Leeds. Seeds resulting from crosses were sown on media containing kanamycin, and their phenotype observed. PCRs were carried out using a 35S-specific primer to confirm the presence of the 35S::RALFL8 construct in seedlings.

Figure 3.4. Aphid fecundity assay. One day-old nymph of *Myzus persicae* was placed on each *A. thaliana* plant, and the pot sealed with a meshed lid. After 15 days the number of nymphs was counted to provide a fecundity value.

3.2.11.3 Analysis of pectin methylesterase expression

The expression level of the pectin methylesterases At2g47040, At1g69940 and At3g62170 was analysed in cDNA from 35S::RALFL8 over-expression line 4 using semi-quantitative RT-PCR (as detailed in Section 2.2.6). Primer sequences are detailed in Appendix 2.

3.3 Results

3.3.1 Selection of candidate genes

Ten genes were selected for further study from the list of interaction genes produced by the microarray experiment. Table 3.2 details the selected genes, their gene ontology categories and their differential regulation. Genes were selected that were amongst the most highly up- or down-regulated in response to joint stress compared to dehydration stress alone, or 'interaction' genes. The 10 selected genes were either transcription factors or strongly regulated by hormones, as these regulatory categories were highly represented and most likely to influence a range of signalling pathways. First, all the interaction genes were analysed for their response to hormone treatments in previous microarray experiments using Genevestigator. In these previous studies, seedlings had been treated with the hormones auxin, cytokinin, gibberellin, brassinosteroid, abscisic acid, jasmonate, ACC (an ethylene precursor) or ethylene and microarray analysis carried out on each (Millenaar *et al.*, 2006; Goda *et al.*, 2008). Germinating wild type and ABA-hypersensitive mutant seeds had also been treated with ABA and microarrays carried out (Nishimura *et al.*, 2007). Candidate genes of interest to the current study could then be selected according to a strong hormone response. Figure 3.5 shows the transcriptional response of the selected genes to hormone treatments in previous microarray studies collated in Genevestigator. *ATMGL* (At1g64660) was highly up-regulated by ABA treatment as well as ethylene treatment, although interestingly not in response to ACC. *AZI1* (At1g12470) was similarly up-regulated by ethylene but down-regulated by ABA. As expected, the jasmonate signalling repressor *JAZ7* (At2g34600) was highly up-regulated by MeJa. *MYB4* (At4g38620) and *DIR14* (At4g11210) were down-regulated by ABA treatment, as were *F2H15* (At1g17970) and *DUF581* (At5g65040), but only in seeds. *RALFL8* (At1g61563) was induced by ABA in seeds but repressed in leaves. The transcription of *ANAC038* (At2g24430) and *TCP9* (AT2G45680) was not influenced significantly by any of the hormone treatments.

3.3.2 Confirmation of homozygosity in T-DNA insertion lines

Each of the Salk T-DNA insertion lines obtained from NASC was found to be homozygous for the T-DNA insertion and no wild type locus of interest could be amplified by PCR. The WiscDsLox7H11 insertion allele in the *JAZ7* gene could not

AGI code	Affymetrix probe ID	Name	TAIR Description	Fold change: Dehydration-Joint stress			Fold change: from control		
				Differential regulation	Fold Change	*p*-value	Control-Dehydration	Control-Nematode	Control-Joint
AT1G64660	261957_at	ATMGL	A functional methionine gamma-lyase, a cytosolic enzyme catalyzes the degradation of methionine into methanethiol.	Up in roots	1.13	0.011	-1.01	1.08	1.11
AT4G11210	254909_at	DIR14	Disease resistance-responsive family protein involved in lignin biosynthesis. Molecular function unknown.	Up in roots	1.26	0.024	-1.11	1.13	1.14
AT1G61563	265007_s_at	RALFL8	Rapid alkalinization factor family protein.	Up in roots	1.23	0.018	1.01	-1.06	1.20
AT1G17970	255899_at	F2H15	Zinc finger (C3HC4-type RING finger) family protein	Up in leaves	1.23	0.007	1.12	1.04	1.24
AT4G12470	254818_at	AZI1	Involved in the priming of salicylic acid induction and systemic immunity triggered by pathogen or azelaic acid.	Down in leaves	-2.37	0.045	-1.43	-1.30	-1.53
AT5G65040	247212_at	DUF581	Senescence-associated protein-related. unknown function	Up in roots	1.35	0.024	1.06	1.05	1.43
AT2G24430	265685_at	ANACO38	No apical meristem (NAM) family transcription factor	Up in roots	1.29	0.021	1.10	1.18	1.41
AT2G34600	266901_at	JAZ7	Jasmonate-zim-domain protein. Transcriptional repressor in jasmonate signalling pathway	Up in roots	1.38	0.048	3.63	-1.11	4.98
AT2G45680	267515_at	TCP9	TCP family transcription factor	Up in leaves	1.25	0.048	1.44	1.10	1.56
AT4G38620	252958_at	MYB4	Myb family transcription factor involved in response to UV-B	Down in leaves	-1.31	0.019	1.93	-1.02	1.79

Table 3.2. Genes of interest selected for further analysis. Microarray analysis revealed sets of genes that were differentially regulated in response to joint nematode and dehydration stress compared to the stresses individually. From these lists, the above ten genes were chosen for further study. These genes were among the most highly differentially regulated, and were either hormone-regulated or transcription factors as these were over-represented categories. The fold change dehydration-joint stress is the difference in gene transcript level between the drought only array and the joint stress array. The *p*-value represents significance resulting from T-tests on array biological replicates (n=3). The fold change between control arrays and each stress is also shown. A negative value represents down-regulation.

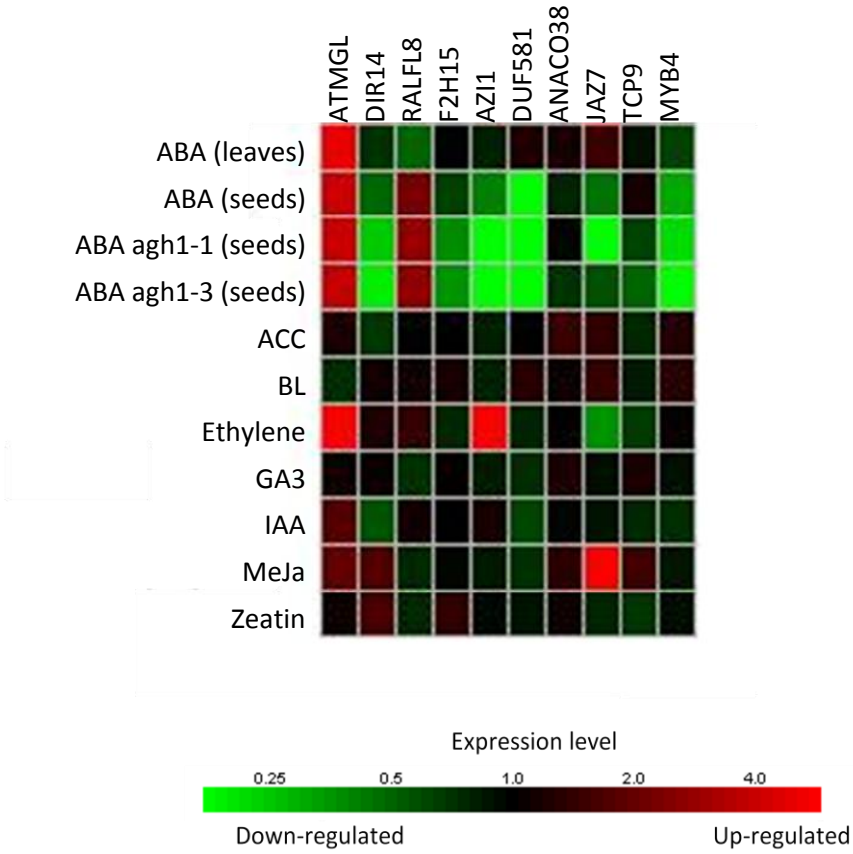


Figure 3.5. Response of 10 selected genes of interest to hormone stimulus over a range of microarray experiments compiled in Genevestigator. Gene induction is shown by red squares and gene repression is shown by green. In each experiment *A. thaliana* was exposed to a plant hormone indicated by the symbols: MeJA for methyl jasmonate; ABA for abscisic acid; IAA for indole acetic acid (auxin); ACC for 1-aminocyclopropane-1-carboxylic acid (ethylene synthesis pathway); GA3 for gibberellic acid; BL for brassinolide. All plants were wild type except *agh1-1* and *agh1-3* which are ABA hypersensitive mutants. The microarray experiments are described by Millenaar *et al.* (2006), Nishimura *et al.* (2007) and Goda *et al.* (2008).

be amplified by PCR, however the wild type allele could not be amplified in the mutant plants, confirming homozygosity for the T-DNA insertion. Gel images showing the amplification of each allele are shown in Figure 3.6. The mutant germplasm was thus used for subsequent experiments to determine the effect of inactivating the gene of interest.

3.3.3 Generation of *A. thaliana* CaMV 35S constitutive expression lines

The PCR amplification of ten genes of interest from *A. thaliana* cDNA produced fragments of the expected size for each gene, as shown in Figure 3.7. Successfully amplified PCR products were transferred to the entry vector pBlueScript SK- and produced white colonies when transformed into *E. coli* and grown on X-gal. Restriction digests verified the size of the insert, whilst the integrity and orientation was confirmed by sequencing the plasmid and comparing to published nucleotide sequences using the BLAST alignment tool. After the subsequent transfer of the coding regions into the 35S over-expression vector pBI121-GUS+KPN, a further round of restriction digests and sequencing reactions confirmed the correct transfer of the insert between vectors, as shown in Figure 3.8.

The pBI121 over-expression constructs were transformed into *A. tumefaciens*, where the presence of correctly sized inserts was confirmed by PCR. The *Agrobacteria* were then used to transform *A. thaliana*. Correctly transformed T1 seedlings were selected by their ability to grow on kanamycin, which indicated the presence of the T-DNA-derived *NPTII* gene. Seedlings without the T-DNA construct were pale in colour, did not form true roots, and did not develop past the cotyledon stage. The presence of the transgene in T1 plants was determined by carrying out PCR with primers specific to the 35S promoter on genomic DNA extracted from twenty plants per construct.

RNA was then isolated from each of twenty plants per construct and the expression levels of each gene of interest analysed by semi-quantitative RT-PCR. The 4-5 lines showing the highest expression levels on the gel image were subsequently analysed more accurately by qRT-PCR, whereby the relative transcript abundance of the genes of interest was determined in comparison to that of a wild type plant. The *A. thaliana* line with the highest transcript abundance for each construct was selected for future use.

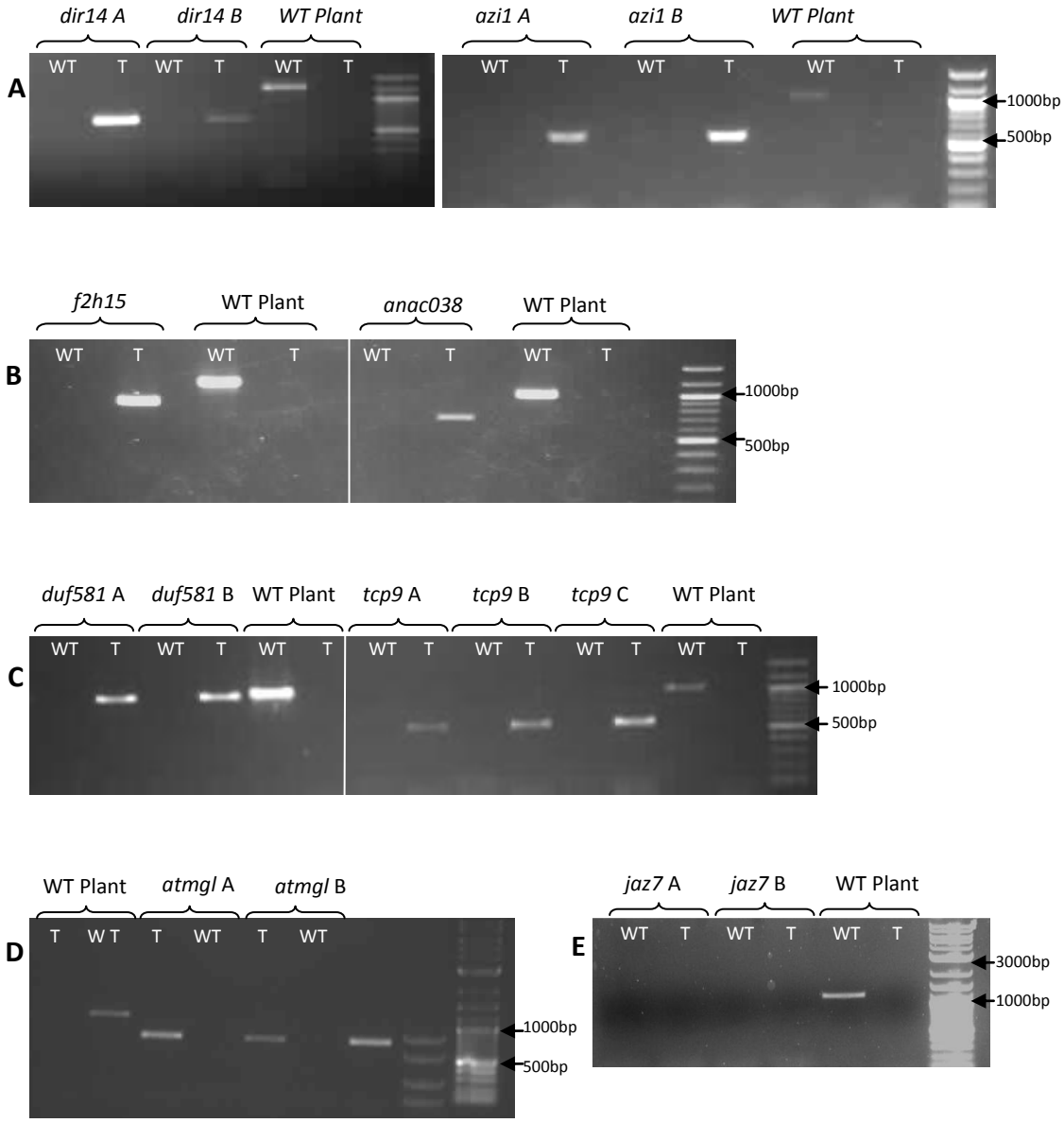


Figure 3.6. Confirmation of homozygosity in T-DNA insertion lines. PCR reactions were used to amplify the wild type allele and T-DNA allele from genomic DNA of each insertion mutant line. The wild type allele (WT) was amplified using primers annealing 5' and 3' of the T-DNA insertion location. The T-DNA allele (T) was amplified using a primer within the T-DNA insertion and 3' of the insertion. DNA from a wild type and at least one mutant plant was analysed for each gene. Plants homozygous for the transgene have two copies of the T-DNA insert and no copies of the wild type gene, whilst wild type plants have two copies of the wild type allele. **A)** *DIR14, AZI1* **B)** *FH215*, *ANACO38,* **C)** *DUF581, TCP9*, **D)** *ATMGL,* **E)** *JAZ7,* for this line, the T-DNA fragment could not be amplified in the mutant line, but these plants did not have any copies of the wild type gene.

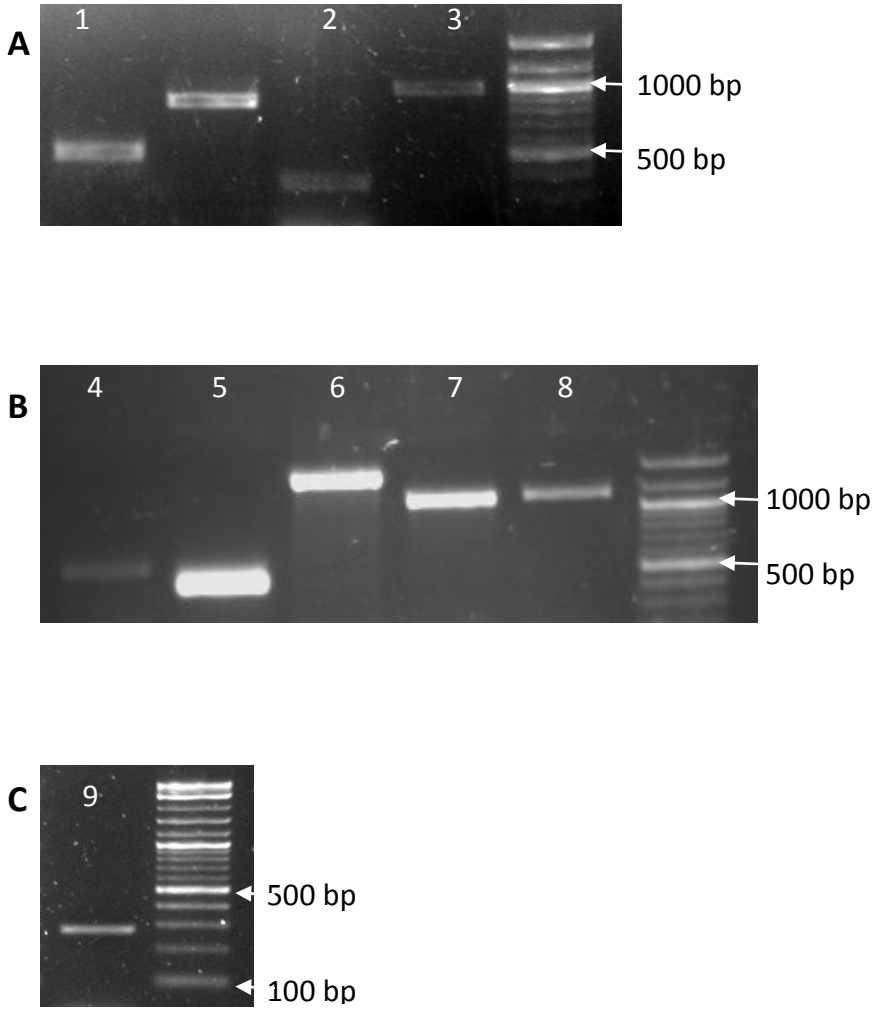


Figure 3.7. Cloning of ten candidate genes. The entire coding region of each gene of interest was amplified by PCR from cDNA using a proof-reading DNA polymerase. **A)** 1) *AZI1*, 2) *DUF581*, 3) *ANAC038*, **B)** 4) *DIR14*, 5) *JAZ7*, 6) *ATMGL*, 7) *TCP9*, 8) *F2H15*, **C)** 9) *RALFL8*. The cloned fragments were then ligated into the entry vector pBlueScript SK- and then transferred into a pBI121 vector containing the CaMV 35S constitutive promoter.

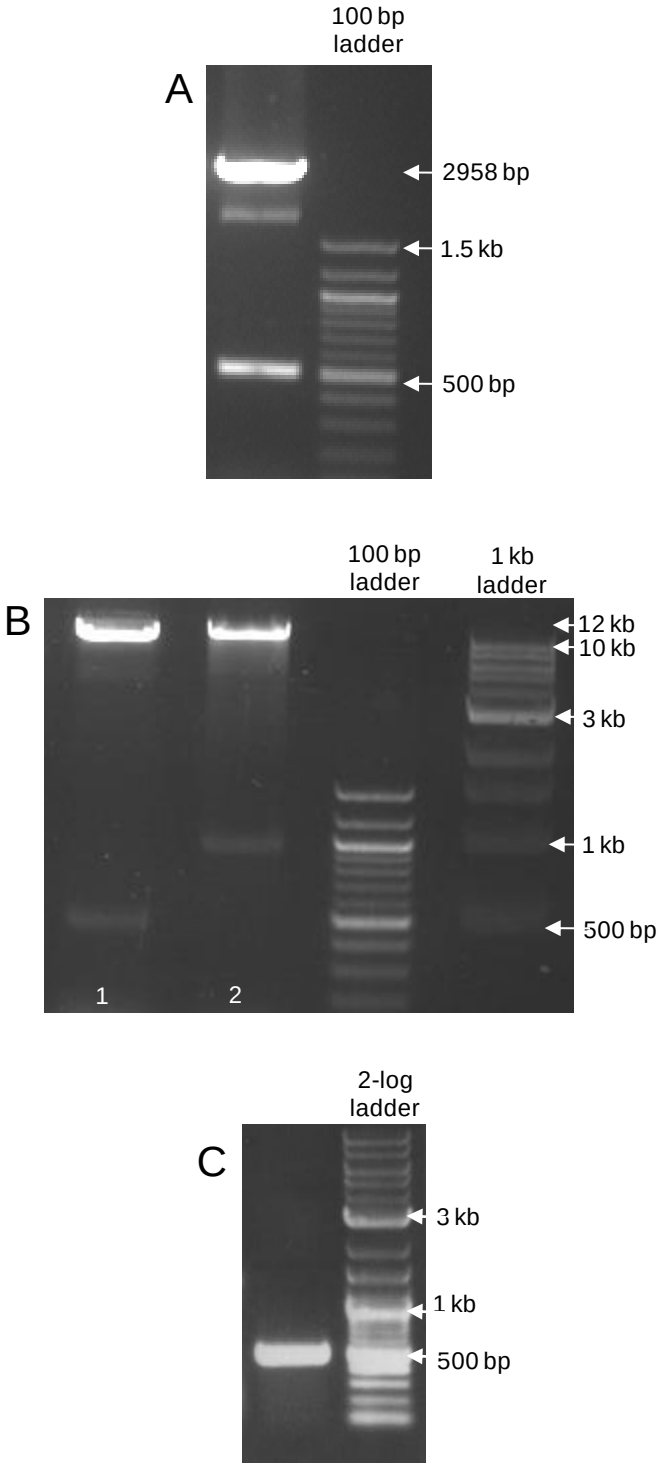


Figure 3.8. Digests of vectors during cloning of genes of interest. Example gels showing restriction digests of putatively recombinant vectors during cloning process. **A)** Digest of full length coding sequence of *AZI1* (486 bp) from pBlueScript vector (2958 bp). **B)** Digest of full length coding sequences of *AZI1* (486 bp) (lane 1) and *ANAC038* (951 bp) (lane 2) from pBI121 (12,000 bp). **C)** PCR of full-length *AZI1* from transformed *Agrobacterium* colony. This *Agrobacterium* was then used to transform *A. thaliana.*

Figure 3.9 shows these selected over-expression lines and the relative expression levels of each gene compared to wild type. Increase in transcript abundance ranged from a 4-fold increase in 35S::DUF581 compared to the wild type, to a 120,175-fold increase in 35S::RALFL8. Line 35S::MYB4 only produced one transformed T1 plant, and this actually showed a lower expression level of *MYB4* transcript than the wild type plant. A homozygous over-expression line for this gene was thus obtained from Prof. Cathie Martin (John Innes Centre, Norwich), which showed a fold-change increase of 4-fold compared to the wild type.

Homozygous lines were then created from each over-expression line, with the exception of 35S::JAZ7. In this line none of the T2 or T3 generation that grew on selection produced homozygous offspring, indicating that all the plants had been heterozygous. However, as the heterozygotes containing this construct were amongst the most highly over-expressing lines, experiments were carried out on the heterozygote instead. In plants expressing the 35S::RALFL8 construct, the phenotype of homozygotes was so extreme that plants did not grow well and often died. Heterozygotes were therefore also used in experiments on this line. The expression levels of all transgenes were analysed in the homozygous lines, and their relative abundance is also shown in Figure 3.9. Most lines showed higher transcript abundance of the transgene in the derived homozygous line than in the original heterozygote. However, the homozygous line 35S::DUF581 showed a transcript abundance which was actually lower than the wild type, perhaps due to gene silencing in the intermediate generation. This line was therefore excluded from future analysis.

3.3.4 Phenotypic characterisation of mutant and over-expression lines

3.3.4.1 DIR14 lines

T-DNA insertion mutants and 35S over-expression lines were grown under normal conditions and their phenotypic characteristics analysed. A summary of the phenotypes of the *dir14* mutant and 35S::DIR14 over-expression line is shown in Figure 3.10. The *dir14* mutant grew significantly slower than the wild type plant, as demonstrated by a reduction in leaf number, rosette diameter, inflorescence height and silique number, as well as a delay in inflorescence emergence. However, the final inflorescence height and the number of seeds per silique were not significantly different to wild type,

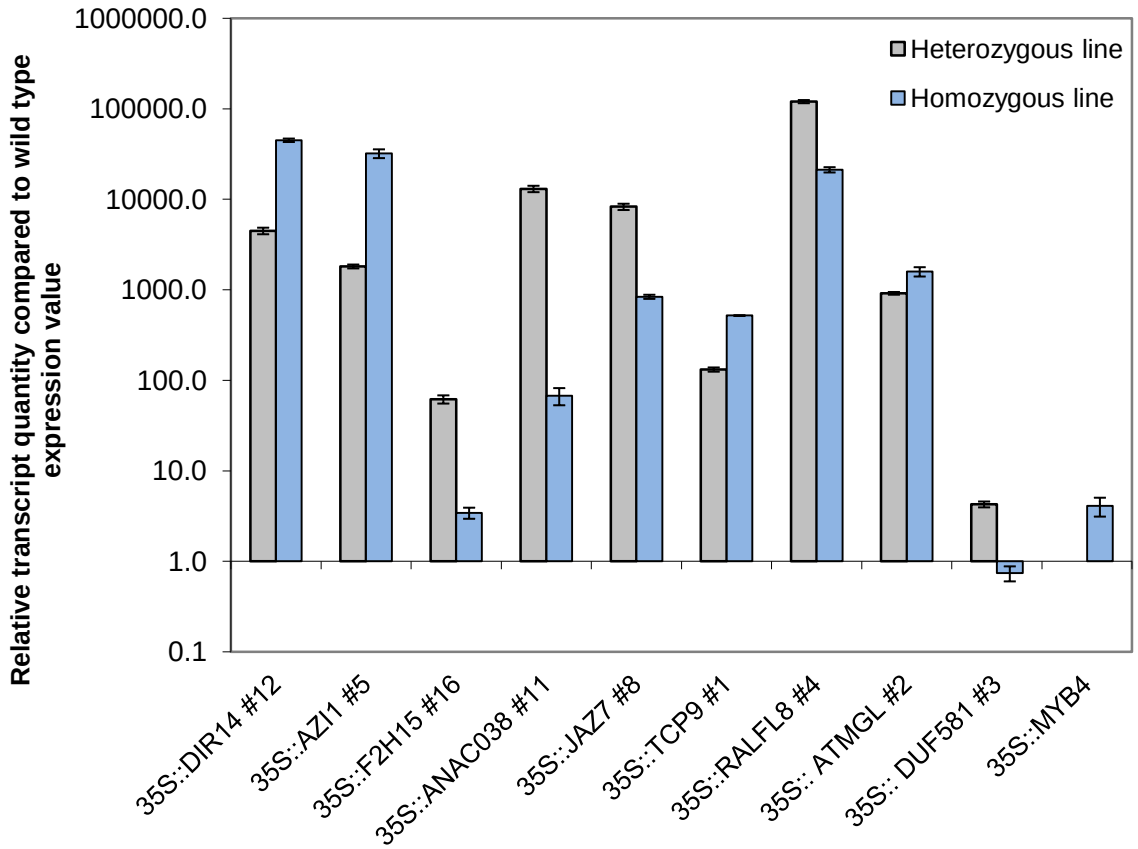
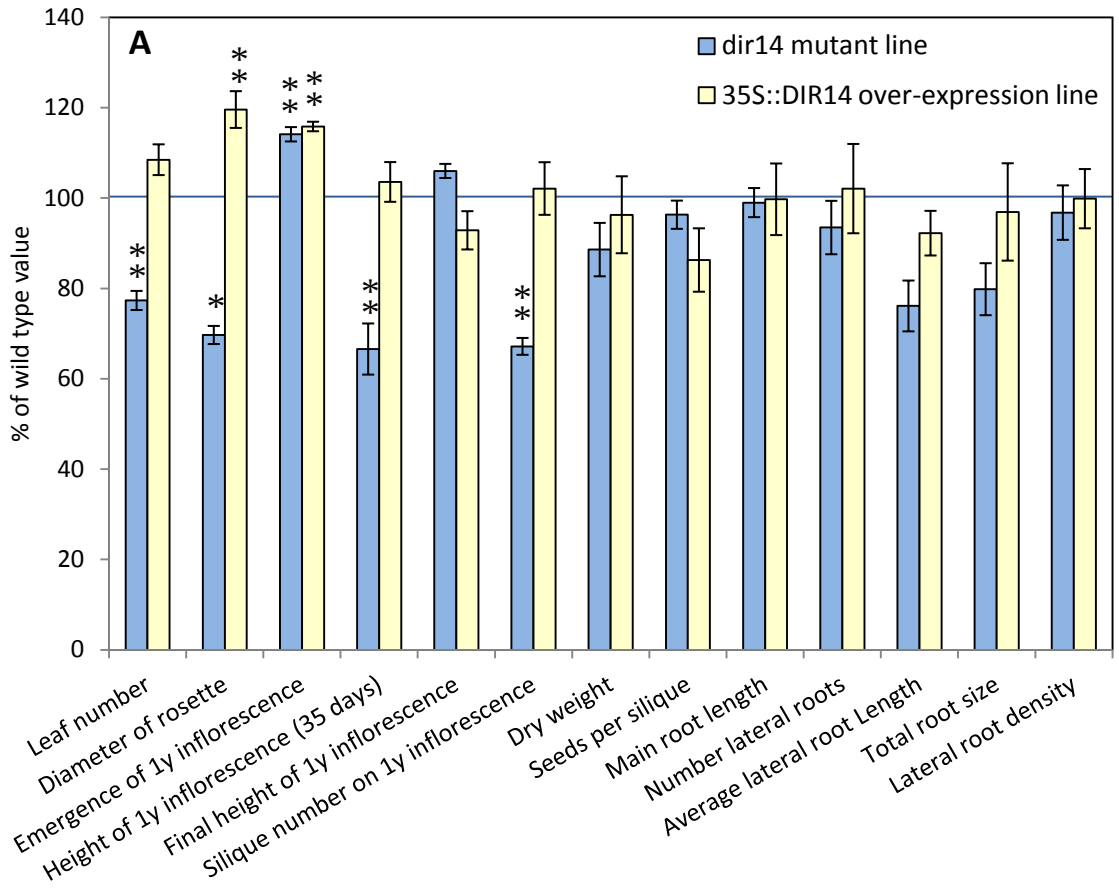


Figure 3.9. Relative transcript abundance of candidate genes in 35S over-expression lines. Each of 10 candidate genes was cloned in to a 35S promoter vector and used to transform *A. thaliana.* Five transformed lines per construct were analysed by qRT-PCR to select the one with highest expression. The transgene expression level in the most highly over-expressing line for each construct is shown here (grey bars). The number (e.g. #12) denotes which of the 20 lines was selected. Homozygous lines were created for each 35S line and tested again for expression level of transgene (blue bars). The expression level of each gene in wild type plants is always 1, therefore bars below the x-axis represent values lower than the wild type. No heterozygote was obtained for the 35S::MYB4 line. The Y-axis is a logarithmic scale, and error bars represent technical replicates in qRT-PCR. The homozygous 35S::DUF581 line had a lower expression level of *DUF581* than the wild type.



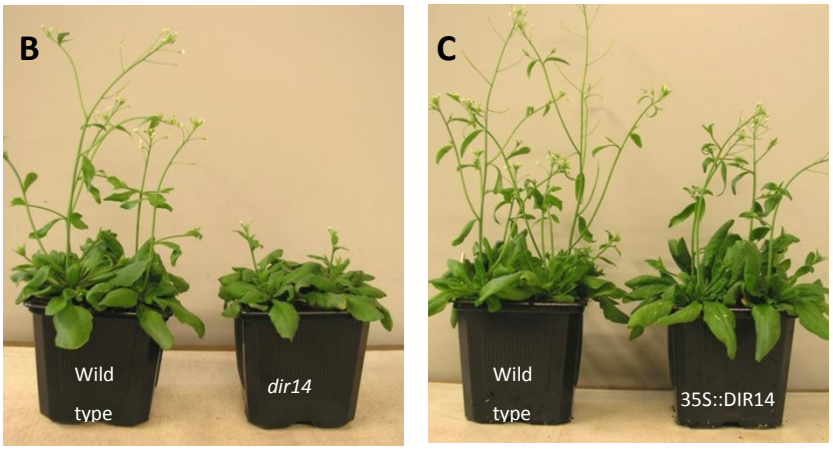


Figure 3.10. Phenotype of *DIR14* mutant and over-expression lines under control conditions. Mutant *dir14* and 35S::DIR14 plants were grown in soil to measure the phenotype of aerial parts of the plants, and on tissue culture plates to analyse the root systems. **A)** Phenotypic measurements are shown as a percentage of the value obtained for wild type plants grown in parallel. The line at 100 % represents the wild type value. Asterisks show a significant difference from the wild type (n=16)(** $p < 0.01$, * $p < 0.05$). **B, C)** Photographs show wild type and mutant plants 35 days after sowing.

suggesting that although slower growing, the ultimate size and productivity of the plant was not affected by the mutation. Interestingly, growth of the root system was not disrupted, as none of the root characteristics were significantly different from the wild type. The 35S::DIR14 line was notable due to its 20 % larger rosette size compared to the wild type plant as well as its slightly late inflorescence emergence. In other characteristics this over-expression line was no different to the wild type.

3.3.4.2 AZI1 lines

Both the *azi1* mutant and the 35S::AZI1 constitutive expression line exhibited slower growth in the aerial parts of the plant (Figure 3.11). The loss-of function mutant had a more severe phenotype, showing reduced leaf number, rosette diameter, height of 1y inflorescence, silique number and dry weight. It also had fewer lateral roots than wild type. The 35S line had fewer siliques and a lower dry weight than normal, although the root system was normal. Primary inflorescences in both the mutant and over-expression lines emerged later than the wild type, but seed yield was not affected.

3.3.4.3 F2H15 lines

Both the *f2h15* mutant and 35S::F2H15 over-expression line displayed somewhat slower rosette growth compared to the wild type, with significantly fewer leaves at 16 days as well as a smaller rosette diameter (Figure 3.12). The over-expression line produced a primary inflorescence later than the wild type, and the T-DNA insertion mutant had a significantly smaller inflorescence at 35 days. In contrast, the root characteristics of both lines were normal suggesting that the *F2H15* gene plays a minimal role in roots.

3.3.4.4 ANAC038 lines

The *anac038* knock-out mutant exhibited several characteristics of impaired growth, including significantly fewer rosette leaves, a smaller rosette diameter, a shorter primary inflorescence and fewer siliques per primary inflorescence (Figure 3.13). In addition the root growth was severely impaired, giving a shorter main root with a low density and length of lateral roots, contributing to a total root size that was only 44 % of the wild type. However, the total biomass accumulation, final inflorescence height and yield in terms of seeds per silique were not significantly affected by the mutation. The 35S::ANAC038 line also had a slightly disrupted growth pattern, with lower leaf

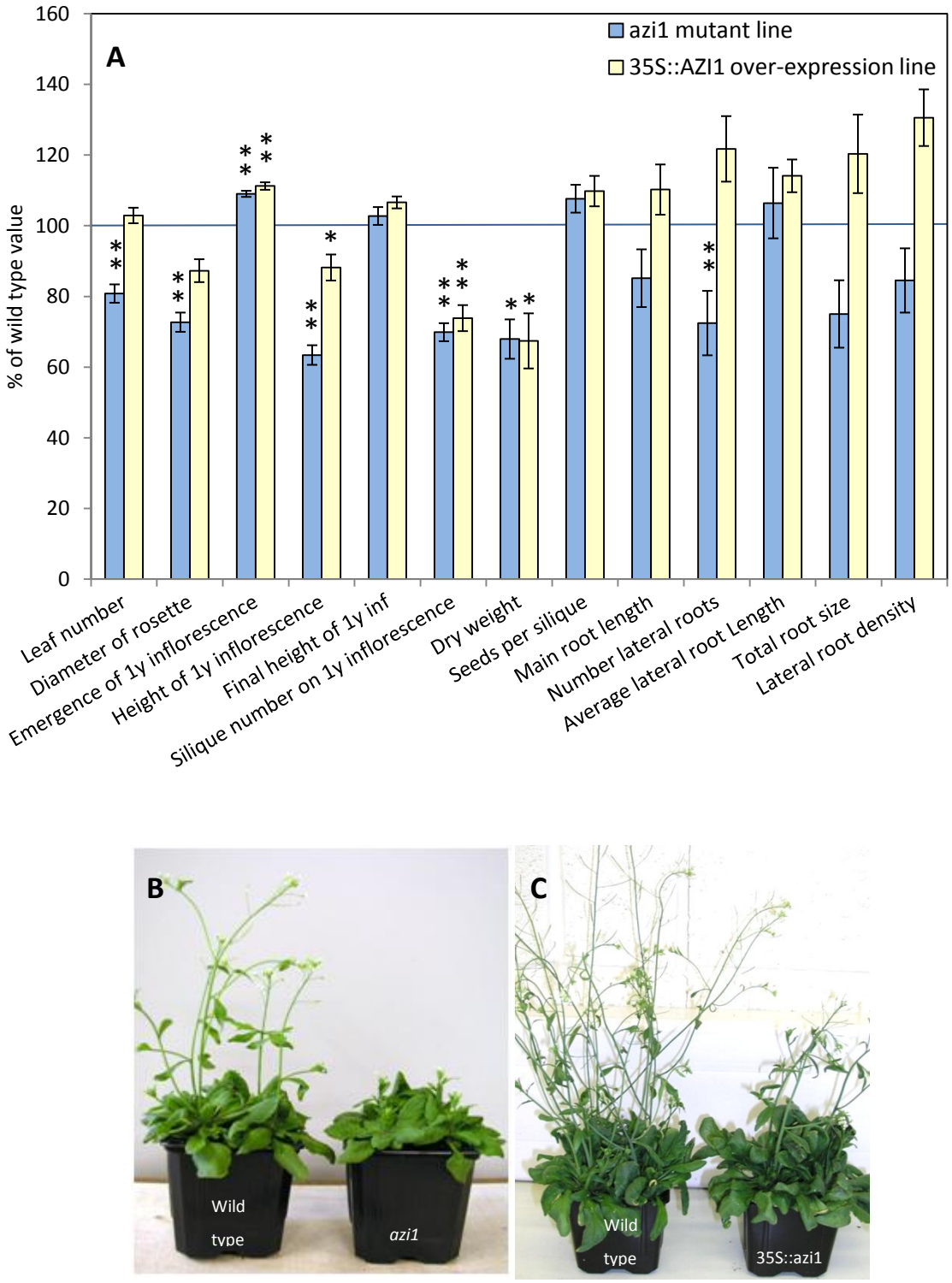


Figure 3.11. Phenotype of *AZI1* mutant and over-expression lines under control conditions. Mutant *azi1* and 35S::AZI1 plants were grown in soil to measure the phenotype of aerial parts of the plants, and on tissue culture plates to analyse the root systems. **A)** Phenotypic measurements are shown as a percentage of the value obtained for wild type plants grown in parallel. The line at 100 % represents the wild type value. Asterisks show a significant difference from the wild type (n=16)(** $p < 0.01$, * $p < 0.05$). **B, C)** Photographs show wild type and mutant plants 35 days after sowing.

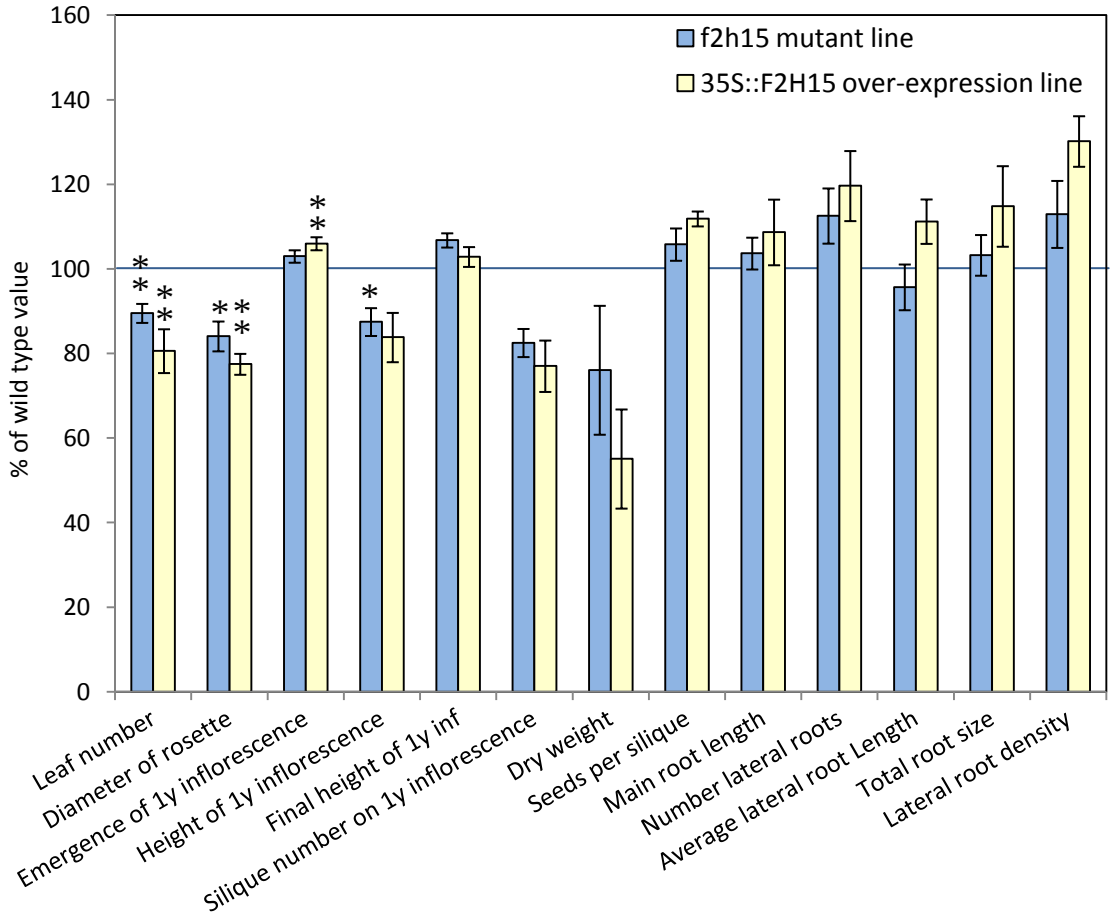


Figure 3.12. Phenotype of *F2H15* mutant and over-expression lines under control conditions. Mutant *f2h15* and 35S::F2H15 plants were grown in soil to measure the phenotype of aerial parts of the plants, and on tissue culture plates to analyse the root systems. **A)** Phenotypic measurements are shown as a percentage of the value obtained for wild type plants grown in parallel. The line at 100 % represents the wild type value. Asterisks show a significant difference from the wild type (n=16)(** $p < 0.01$, * $p < 0.05$). **B,C)** Photographs show wild type and mutant plants 35 days after sowing.

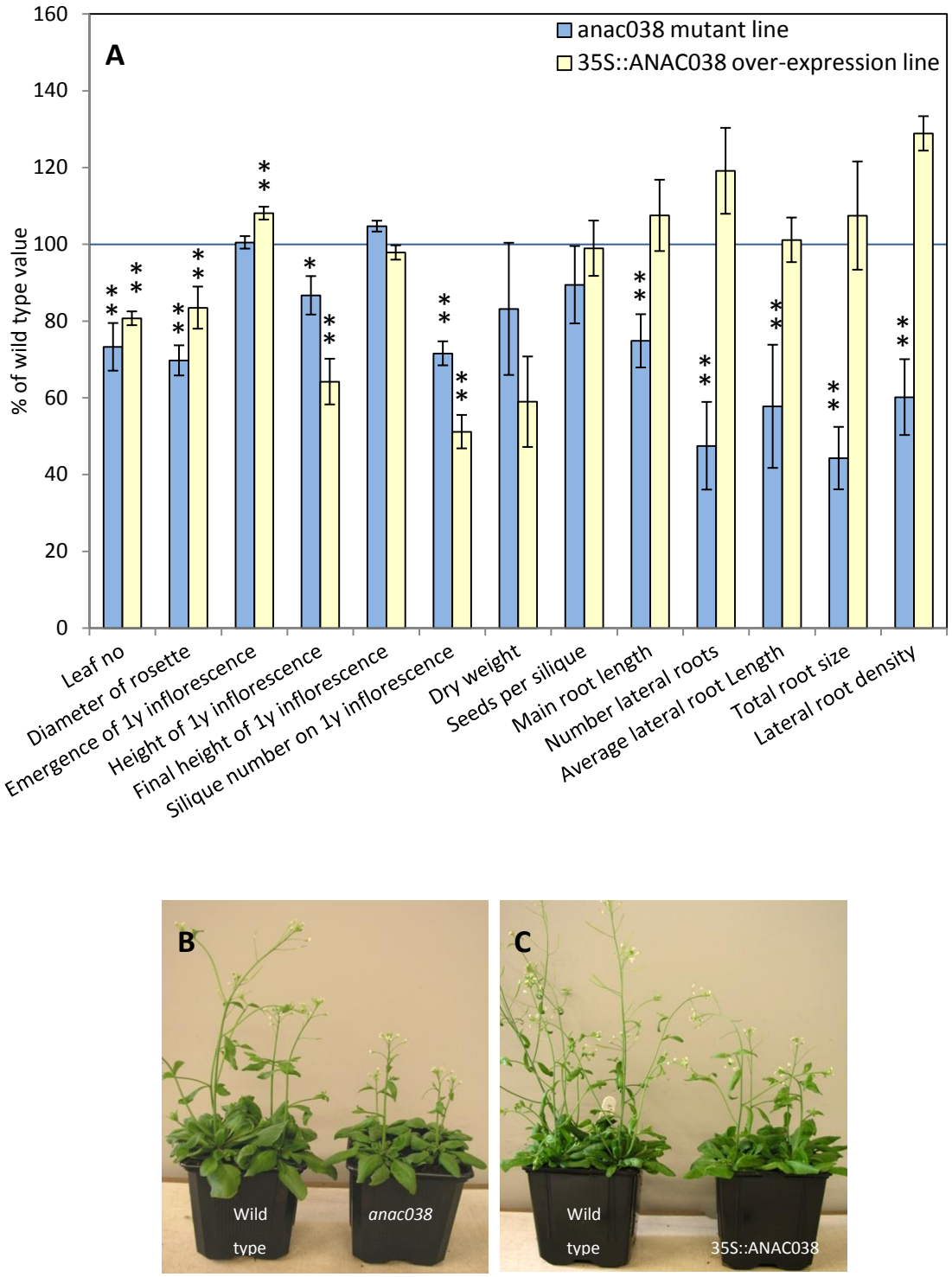


Figure 3.13. Phenotype of *ANAC038* mutant and over-expression lines under control conditions. Mutant *anac038* and 35S::ANAC038 plants were grown in soil to measure the phenotype of aerial parts of the plants, and on tissue culture plates to analyse the root systems. **A)** Phenotypic measurements are shown as a percentage of the value obtained for wild type plants grown in parallel. The line at 100 % represents the wild type value. Asterisks show a significant difference from the wild type (n=16)(** $p < 0.01$, * $p < 0.05$). **B,C)** Photographs show wild type and mutant plants 35 days after sowing.

number, rosette diameter, inflorescence height and silique number combined with later inflorescence emergence. The root system was no different from wild type plants.

3.3.4.5 JAZ7 lines

The *jaz7* T-DNA insertion line was significantly smaller during growth than the wild type, with a decreased rosette diameter, leaf number, inflorescence height at 35 days, and silique number (Figure 3.14). However, the final inflorescence height and the seed number per silique were comparable to the wild type values, so the final stature of the plant was unaffected by the mutation. The root dimensions were no smaller than the wild type, and in fact the mutant had significantly more lateral roots. In contrast the 35S::JAZ7 over-expression line displayed more vigorous growth than the wild type. It had more rosette leaves, a greater rosette diameter and a taller inflorescence during growth, and the final inflorescence height was greater than the wild type. Interestingly the seed yield was significantly reduced from an average of 57 seeds per silique in the wild type to 33 in the 35S plants. The over-expression line had fewer lateral roots than the wild type, but these were longer than normal.

3.3.4.6 TCP9 lines

Of all the genes studied, manipulation of the *TCP9* gene had the most severe phenotypic effect on *A. thaliana* plants. Both the mutant and over-expression line were greatly inhibited in growth (Figure 3.15). This inhibition manifested itself more mildly in the mutant *tcp9* line, which nonetheless had significantly reduced leaf number and rosette diameter, shorter inflorescence, fewer siliques and later inflorescence emergence. The final height of the inflorescence and the root system were normal, however. The 35S::TCP9 over-expression line had a more severe phenotype, being significantly inferior to the wild type in every characteristic measured (with the exception of lateral root length). The rosette was less than half the diameter of the wild type, and the inflorescences emerged on average 12 days later than normal (41 days after planting as opposed to 29 in the wild type). The dry weight of the plants was only 15 % of the wild type, and the siliques contained on average only 38 seeds compared to the wild type value of 63. The root system was markedly smaller and possessed significantly fewer lateral roots than the wild type.

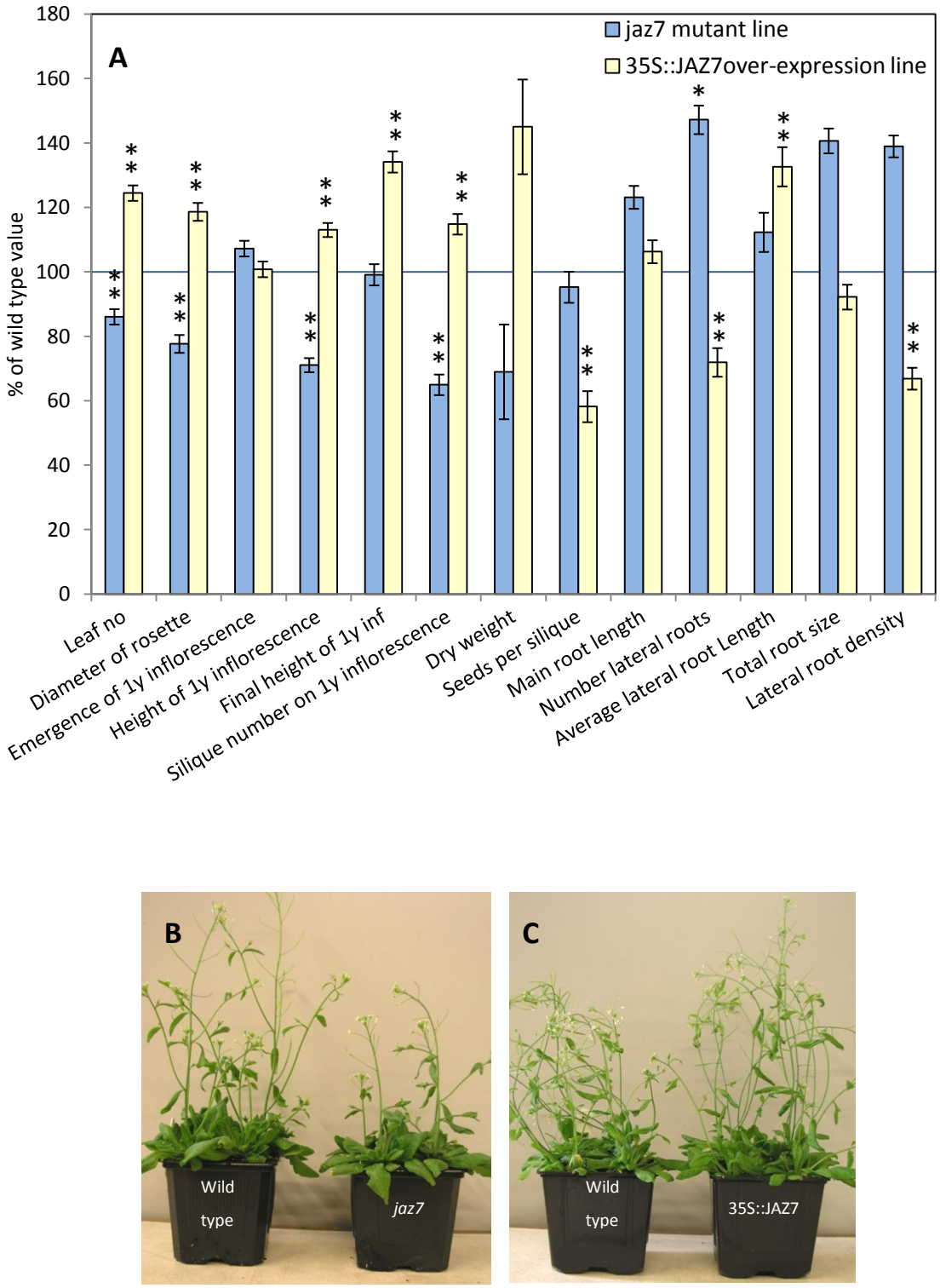


Figure 3.14. Phenotype of *JAZ7* mutant and over-expression lines under control conditions. Mutant *jaz7* and 35S::JAZ7 plants were grown in soil to measure the phenotype of aerial parts of the plants, and on tissue culture plates to analyse the root systems. **A)** Phenotypic measurements are shown as a percentage of the value obtained for wild type plants grown in parallel. The line at 100 % represents the wild type value. Asterisks show a significant difference from the wild type (n=16)(** $p < 0.01$, * $p < 0.05$). **B,C)** Photographs show wild type and mutant plants 35 days after sowing.

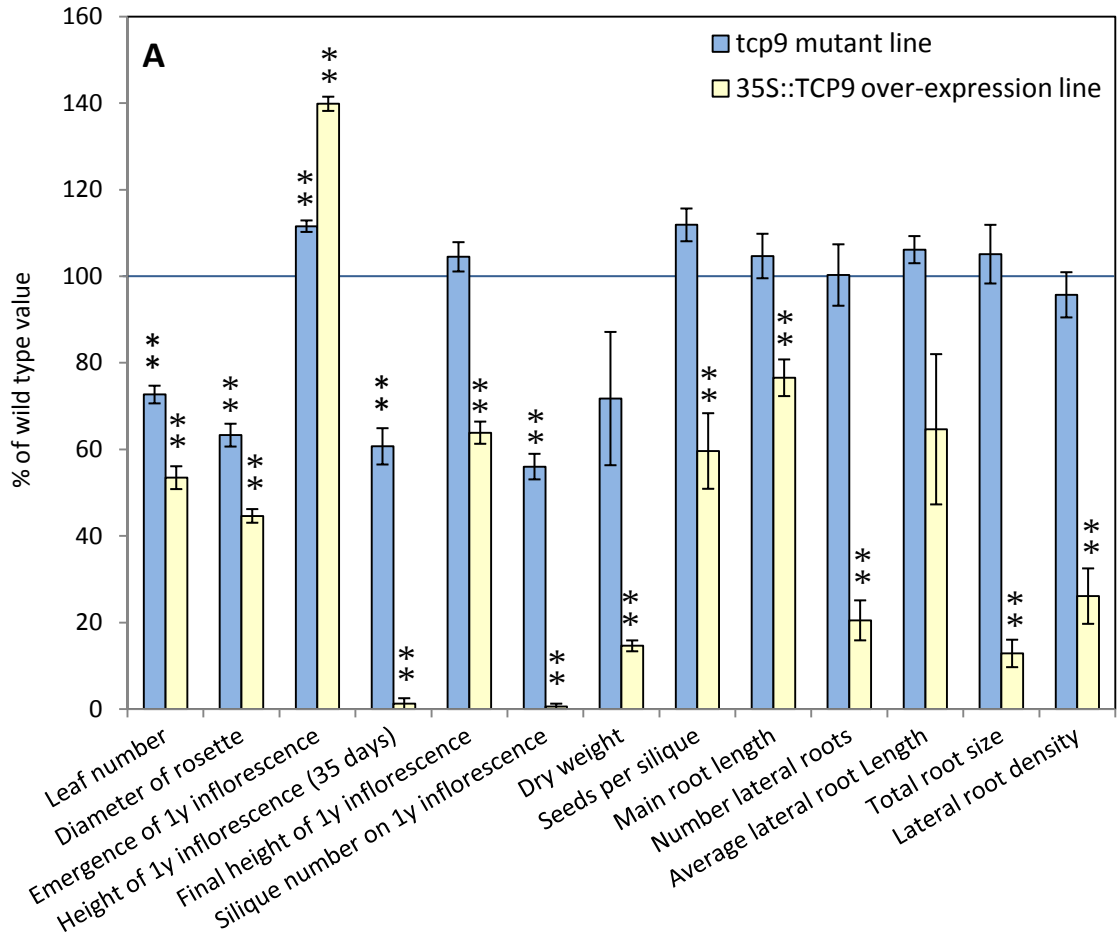


Figure 3.15. Phenotype of *TCP9* mutant and over-expression lines under control conditions. Mutant *tcp9* and 35S::TCP9 plants were grown in soil to measure the phenotype of aerial parts of the plants, and on tissue culture plates to analyse the root systems. **A)** Phenotypic measurements are shown as a percentage of the value obtained for wild type plants grown in parallel. The line at 100 % represents the wild type value. Asterisks show a significant difference from the wild type (n=16)(** $p < 0.01$, * $p < 0.05$). **B,C)** Photographs show wild type and mutant plants 35 days after sowing.

3.3.4.7 RALFL8 line

As no mutant could be obtained for this small gene, only the 35S::RALFL8 over-expression line was analysed. These plants had a severely stunted root system that was only 20 % of the size of the wild type (Figure 3.16). Main root length, number of lateral roots, total root size and lateral root density were significantly reduced. As may be expected, this impacted on the growth of the aerial parts of the plants. The rosette diameter was smaller than the wild type, whilst biomass accumulation was also greatly reduced, giving plants whose aerial parts weighed only 50 mg (dry weight) after 35 days in comparison to the wild type weight of 500 mg. The inflorescence was severely reduced in stature compared to the wild type, even when fully mature, and developed fewer siliques which in turn contained fewer seeds. The inflorescence stems were visibly thinner than normal.

3.3.4.8 ATMGL lines

Apart from a 15 % smaller rosette diameter at 16 days, the inactivation of the ATMGL gene due to the T-DNA insertion had no visible effect on the phenotype of *A. thaliana* plants (Figure 3.17). However, the over-expression of this gene caused several observable differences in development. The 35S::ATMGL plants were slightly reduced in diameter and had a very low rate of biomass accumulation resulting in a dry weight that was only 44 % of the wild type value. The seed yield was also significantly reduced, although the final inflorescence height was slightly higher than the wild type. The root system was unaffected by the excess *ATMGL* transcript.

3.3.4.9 DUF581 line

The *duf581* mutant line displayed a slower rate of growth and reduced stature (Figure 3.18). During growth the leaf number, rosette diameter, early inflorescence height, silique number and root system were significantly smaller than wild type. However, when the plants were mature they showed no discernable negative attributes, as the biomass accumulation, final inflorescence height and seed number were ultimately normal.

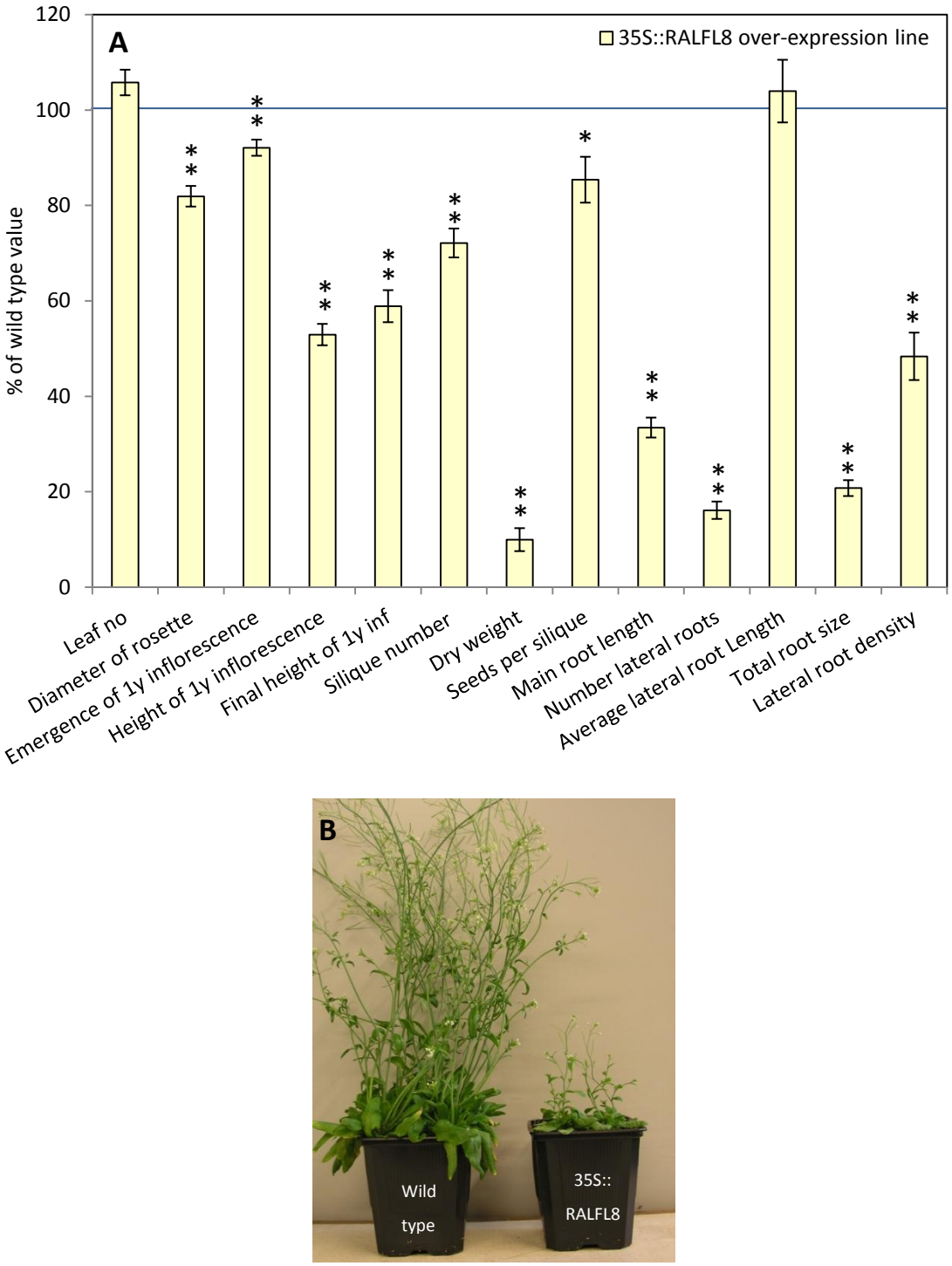
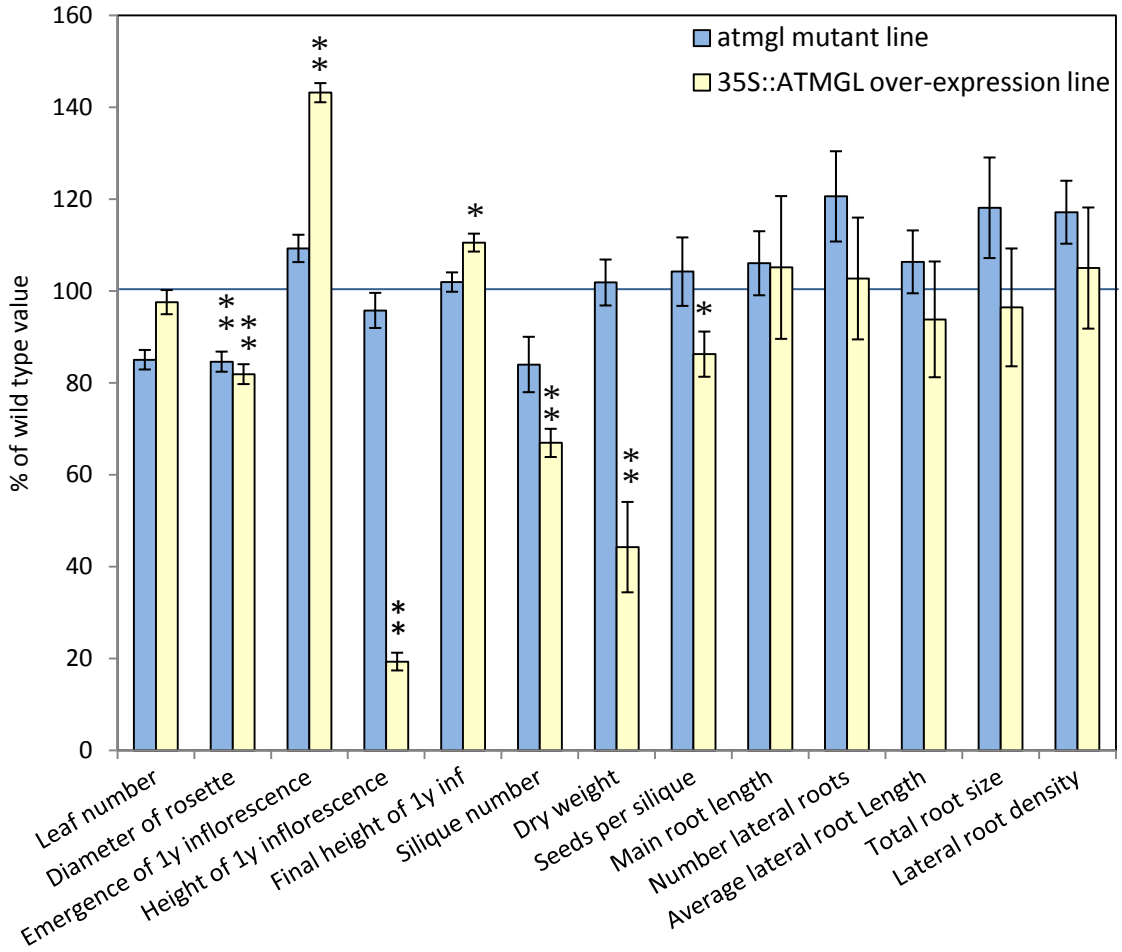


Figure 3.16. Phenotype of *RALFL8* over-expression line under control conditions. 35S::RALFL8 plants were grown in soil to measure the phenotype of aerial parts of the plants, and on tissue culture plates to analyse the root systems. **A)** Phenotypic measurements are shown as a percentage of the value obtained for wild type plants grown in parallel. The line at 100 % represents the wild type value. Asterisks show a significant difference from the wild type (n=16)(** $p < 0.01$, * $p < 0.05$). **B)** Photograph shows wild type and over-expression line 35 days after sowing.



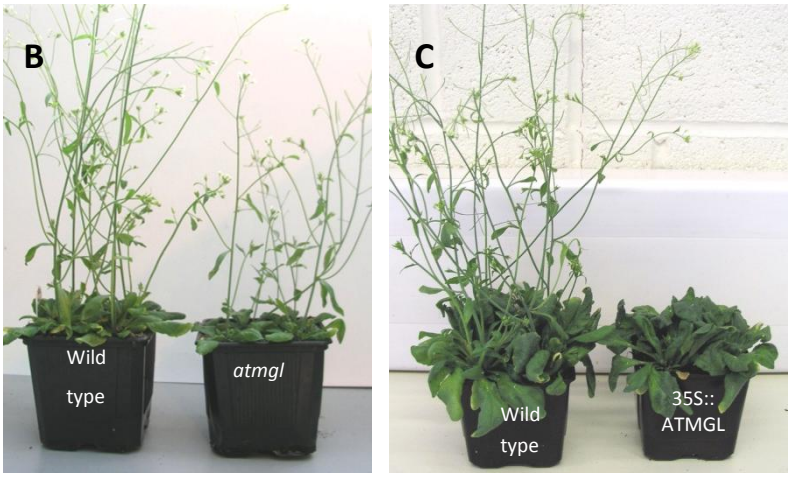


Figure 3.17. Phenotype of *ATMGL* mutant and over-expression lines under control conditions. Mutant *atmgl* and 35S::ATMGL plants were grown in soil to measure the phenotype of aerial parts of the plants, and on tissue culture plates to analyse the root systems. **A)** Phenotypic measurements are shown as a percentage of the value obtained for wild type plants grown in parallel. The line at 100 % represents the wild type value. Asterisks show a significant difference from the wild type (n=16)(** $p < 0.01$, * $p < 0.05$). **B,C)** Photographs show wild type and mutant plants 35 days after sowing.

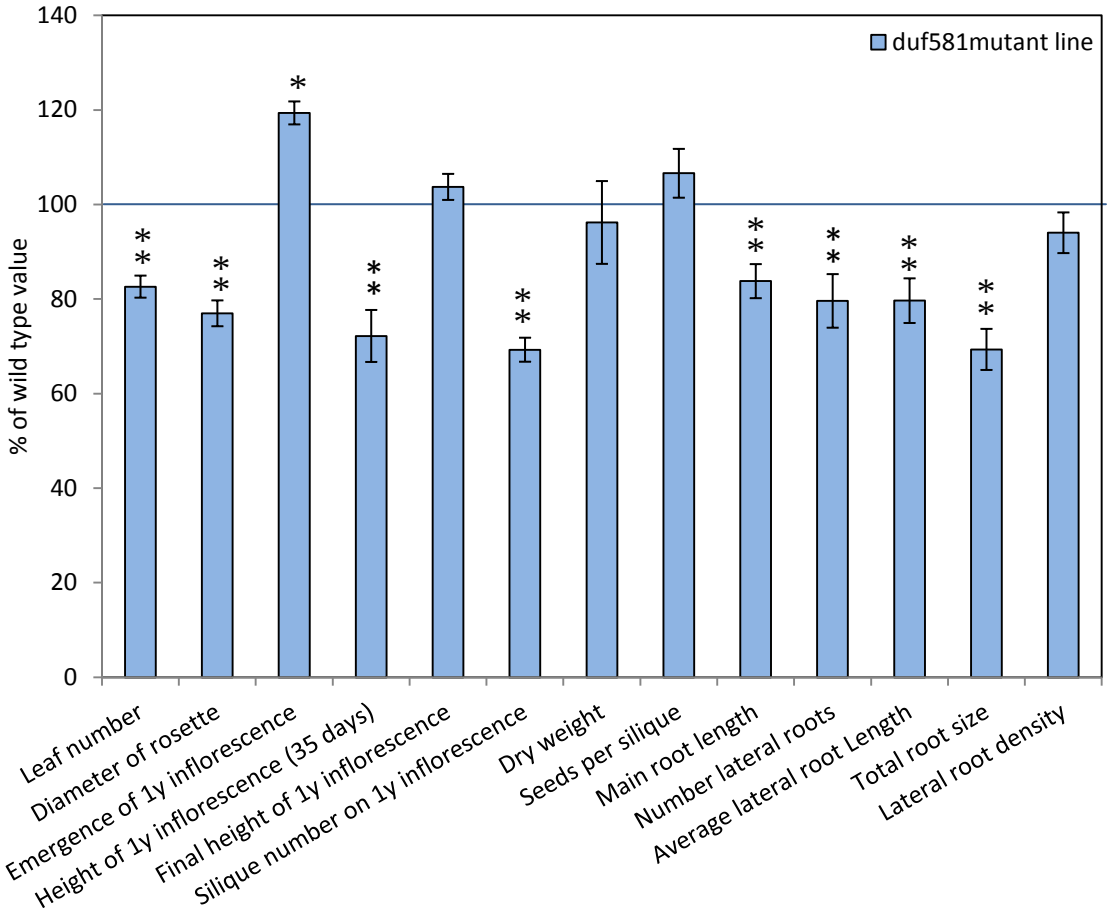


Figure 3.18. Phenotype of *DUF581* mutant line under control conditions. Mutant *duf581* plants were grown in soil to measure the phenotype of aerial parts of the plants, and on tissue culture plates to analyse the root systems. **A)** Phenotypic measurements are shown as a percentage of the value obtained for wild type plants grown in parallel. The line at 100 % represents the wild type value. Asterisks show a significant difference from the wild type (n=16)(** $p < 0.01$, * $p < 0.05$). **B)** Photograph shows wild type and mutant line 35 days after sowing.

3.3.4.10 MYB4 lines

Both the *myb4* mutant line and the 35S::over-expression line showed impaired growth characteristics in the aerial parts of the plants (Figure 3.19). The two lines had a significant reduction in leaf number, rosette diameter, inflorescence height, silique number and seed number. They also both produced inflorescences later than the wild type plants. In addition, the *myb4* mutant plants had a significantly reduced biomass accumulation, resulting in a dry weight that was only 48 % of the wild type plants.

3.3.5 Nematode resistance/susceptibility assays

Experiments to determine the susceptibility or resistance of mutant and over-expression lines to infection by *H. schachtii* were carried out in tissue culture. The number of nematodes to successfully parasitise the root system was counted. In Figure 3.20 the number of enlarged nematodes (fusiform or saccate) counted from each genotype is shown. Figure 3.20A shows the total number of nematodes as a percentage of the number observed on wild type plants. Figure 3.20B shows this proportion again but expressed per milligram of root, thus correcting for the size of the root system. Several genotypes appeared to differ in their susceptibility to nematode infection compared to the wild type. The 35S::TCP9 plants had fewer nematodes infecting them compared to wild type (5.0 enlarged nematodes per plant compared to 9.25). However, when expressed per milligram of root the 35S::TCP9 plants actually had over 10 times the infection rate of wild type plants (1.5 enlarged nematode per mg compared to 0.13), suggesting that the reason for the smaller number of nematodes was the greatly reduced size of the root system. Plants with the 35S::RALFL8 genotype had a greater infection rate than wild type plants. Furthermore, when expressed per mg of root the infection rate on 35S::RALFL8 plants was still significantly higher than the wild type (0.35 nematodes per mg compared to 0.09), suggesting a genuine nematode susceptibility caused by the over-expression of *RALFL8.* In contrast, plants over-expressing the *ATMGL* gene were found to be infected with significantly fewer nematodes than wild type plants (0.9 enlarged nematodes per plant as opposed to 3.8). This reduction in infection rate was maintained when expressed per mg of root tissue (0.01 compared with 0.04 in wild type), indicating that the smaller number of nematodes was not due to a smaller root system. Lastly, the 35S::DIR14 over-expression line showed some susceptibility to nematode attack. Although the total number of nematodes infecting the

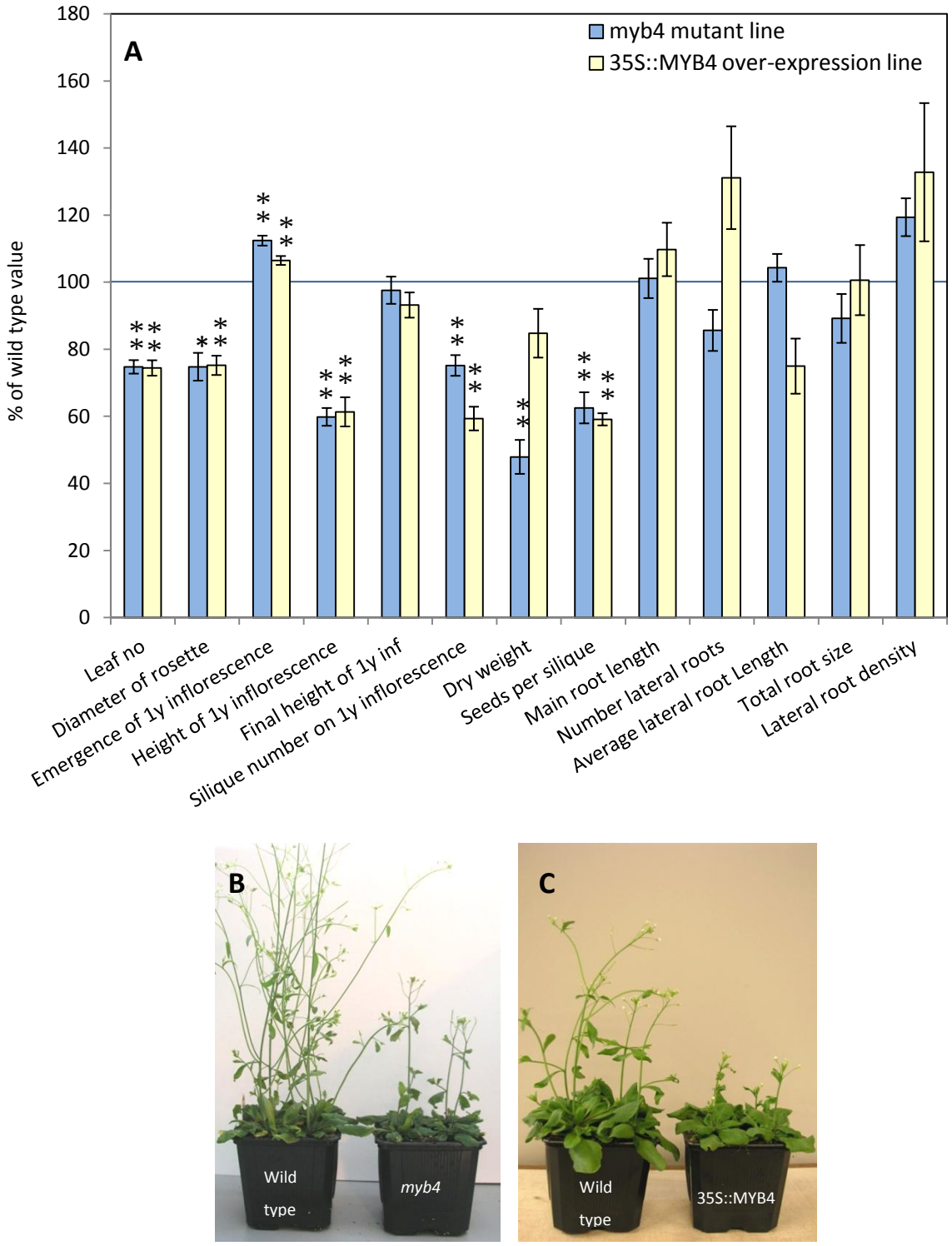


Figure 3.19. Phenotype of *MYB4* mutant and over-expression lines under control conditions. Mutant *myb4* and 35S::MYB4 plants were grown in soil to measure the phenotype of aerial parts of the plants, and on tissue culture plates to analyse the root systems. **A)** Phenotypic measurements are shown as a percentage of the value obtained for wild type plants grown in parallel. The line at 100 % represents the wild type value. Asterisks show a significant difference from the wild type (n=16)(** $p < 0.01$, * $p < 0.05$). **B,C)** Photographs show wild type and mutant plants 35 days after sowing.

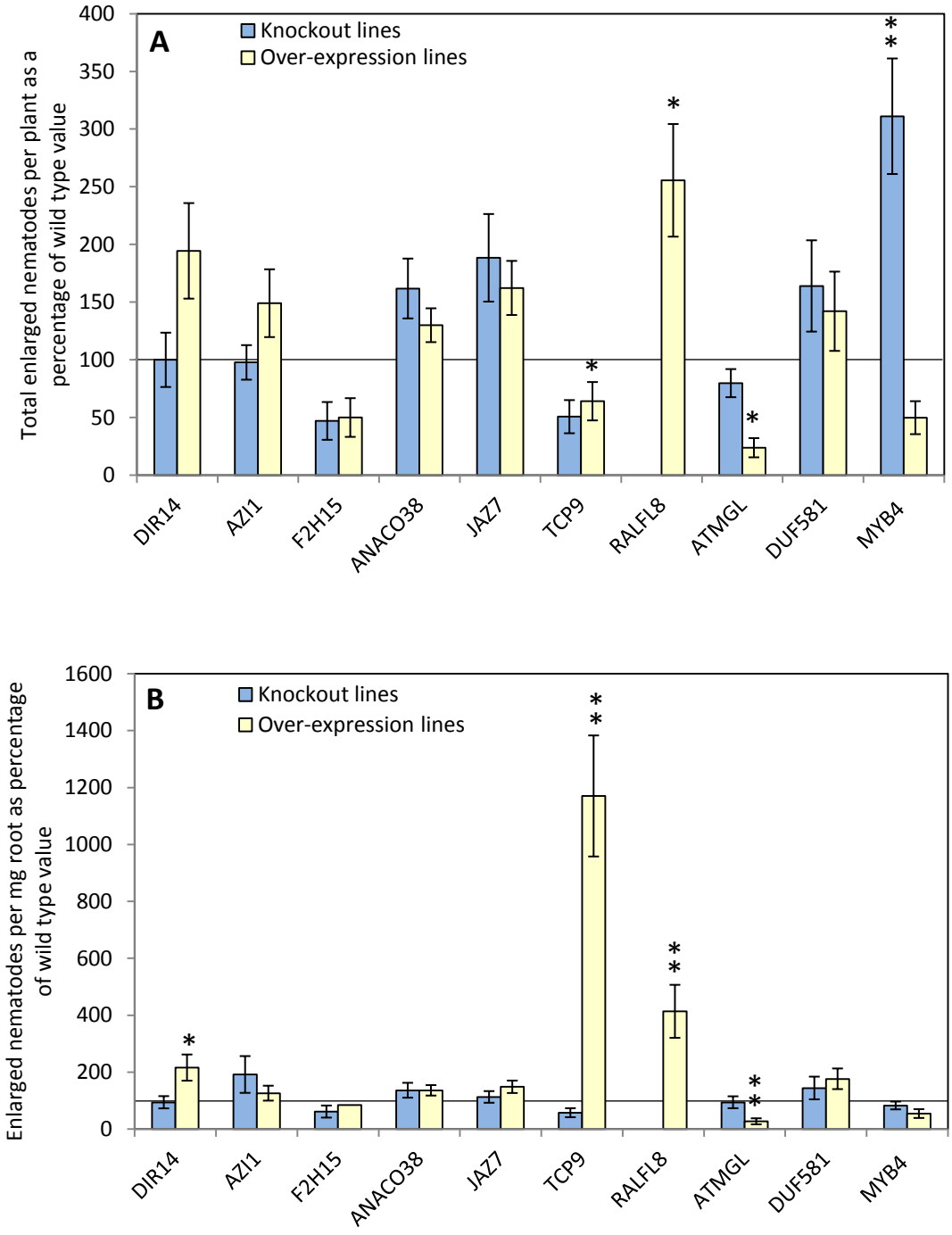


Figure 3.20. Nematode resistance assays. Mutant and over-expression lines for each candidate gene were exposed to 100 juvenile *H. schachtii* nematodes per plant. Nematodes were allowed to develop for 10 days and then roots were stained and the nematodes counted. Enlarged nematodes were those in the J3 or J4 stages. The line at 100 % represents the wild type value. **A)** The average number of enlarged nematodes counted on each genotype, expressed as a proportion of the wild type value (100 %). **B)** The number of enlarged nematodes per mg of root tissue, expressed as a proportion of the wild type value. Asterisks show a significant difference from the wild type (n=10-12) (** $p < 0.01$, * $p < 0.05$).

plants was not significantly greater than wild type, the fact that these plants had a slightly smaller root system meant that the infection rate per mg root was significantly higher.

3.3.6 Drought resistance/susceptibility assays

Experiments were carried out to test the susceptibility or resistance of each mutant or over-expression line to drought compared with wild type plants. After approximately two weeks without irrigation, plants were scored for their survival rate on re-watering. Four experiments demonstrated a significant difference in survival rate between the mutant or over-expression line and the wild type, as determined by Chi^2 tests. 35S::DIR14 showed an increased survival rate after drought compared to the wild type, whereas 35S::AZI1, 35S::TCP9 and 35S::RALFL8 all showed a diminished survival rate. These four experiments were repeated and the average survival score analysed. The combined survival score for the two repeats upheld the findings for 35S::AZI1, 35S::TCP9 and 35S::RALFL8, whereas the increased survival rate of 35S::DIR14 was no longer significant. Two examples of plant genotypes showing a difference in recovery rate after re-watering compared to the wild type are shown in Figure 3.21. The survival rates of all the genotypes in comparison to wild type are shown in Figure 3.22. Where experiments were repeated, the results shown are an average of the two survival scores.

3.3.7 Joint stress resistance/susceptibility assays

Several mutant and over-expression lines were selected for analysis under joint nematode and drought stress. These were lines that had shown significant results in individual nematode or drought stress assays, namely *azi1*, 35S::AZI1, *tcp9*, 35S::TCP9 and *jaz7*. Physiological measurements were taken from plants under imposed individual or joint stress. Due to a small sample size the data in this section should be considered preliminary.

To impose drought stress, plants were maintained at a soil moisture content of 15-20 %. The stomatal conductance of plants at this soil moisture level was measured and is shown in Figure 3.23. Almost all genotype groups showed a significant reduction in stomatal conductance in response to drought (both drought stress and joint stress

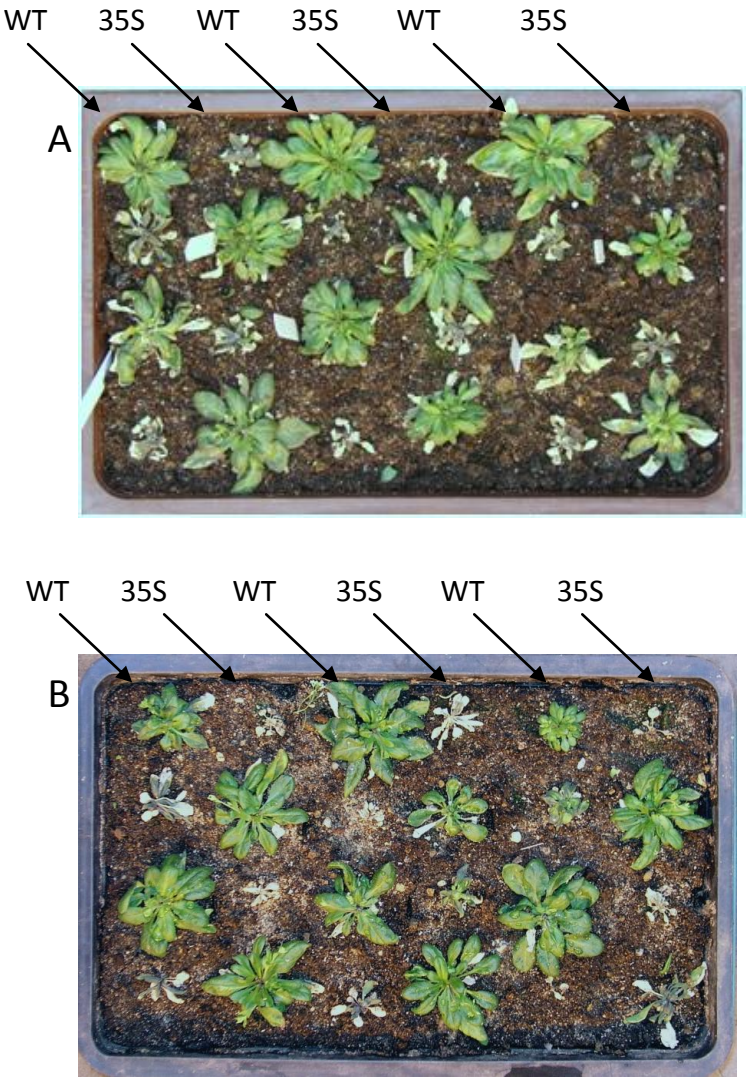


Figure 3.21. Examples of significant differences in drought survival rate. Irrigation was withheld from twelve 21-day old seedlings of each genotype until soil moisture content dropped to < 3% (approximately 2 weeks). Plants were re-watered and scored for survival after a further week. Arrows indicate the genotype of each diagonal line of plants. **A)** 35S::TCP9 over-expression and wild type plants one week after re-watering. In this assay all twelve wild type plants survived whilst only one 35S::TCP9 plant survived (top right), as determined by the presence of green leaves. **B)** 35S::RALFL8 over-expression and wild type plants one week after re-watering. In this assay all twelve wild type plants survived whilst only three 35S::RALFL8 plants survived. Inflorescences were removed from the plants for clarity.

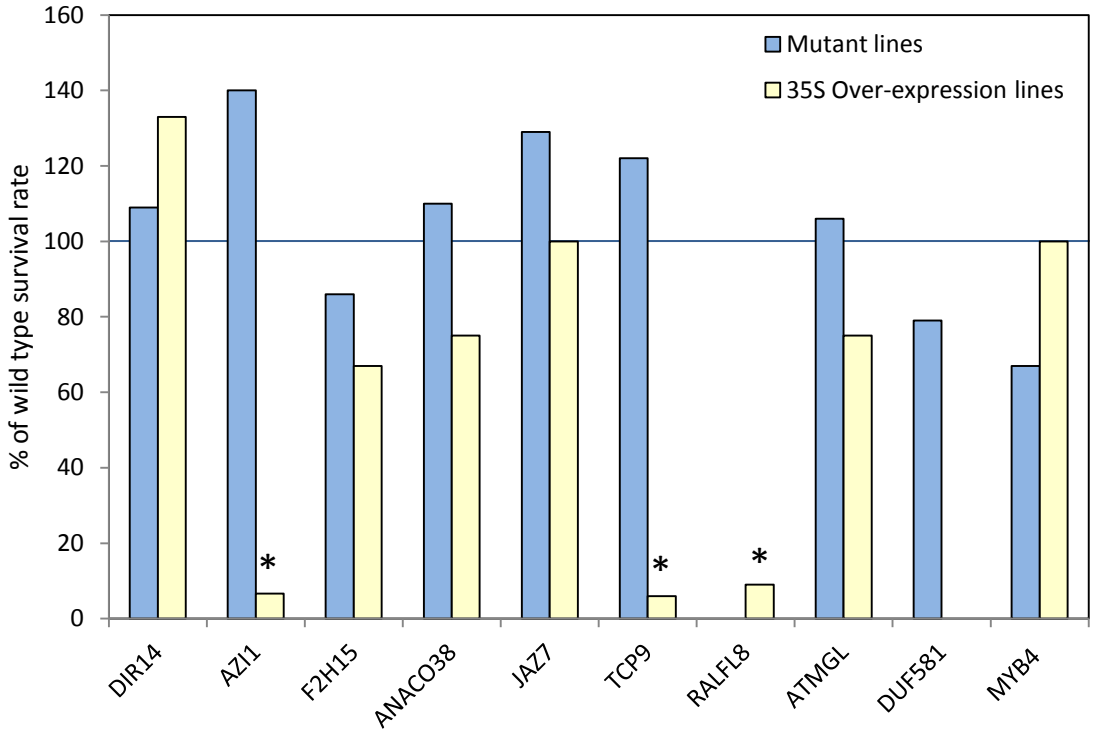


Figure 3.22. Survival rate of mutant and over-expression lines after drought. Plants of each genotype were scored for survival after approximately two weeks of drought and the value compared to that of wild type plants using Chi^2 tests (* = different to wild type value where $p < 0.05$). The line at 100 % represents the wild type value. Genotypes that differed significantly from wild type were tested a second time and the average results of the two assays are displayed in the graph.

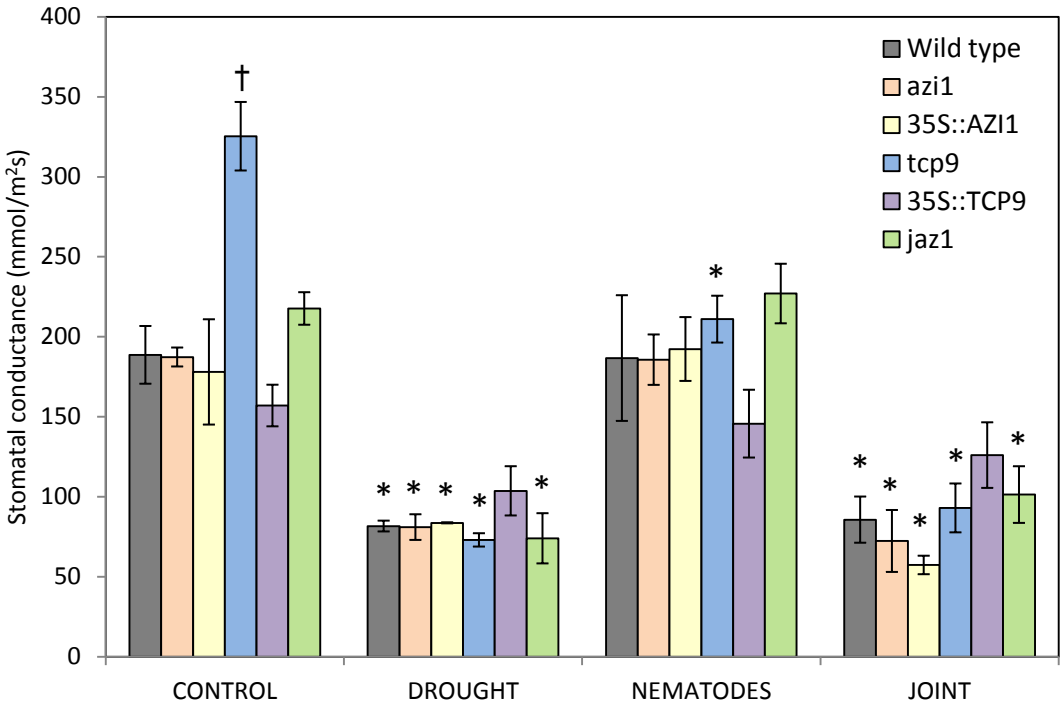


Figure 3.23. Stomatal conductance of mutant and over-expression lines in response to stress. Plants were exposed to drought stress by maintaining soil moisture level at 15-20% (compared to well-watered level 55-60 %). Nematode stress was imposed by planting seedlings into soil containing 50 eggs/g *H. schachtii* cysts. Joint stress comprised the two stresses in combination. * shows a difference from the unstressed stomatal conductance for that genotype, whilst † shows a difference from the wild type value for that treatment (n = 5) ($p < 0.05$).

treatment groups), implying that this level of stress was enough to have a distinct effect on normal stomatal aperture and thus photosynthesis. The one exception was 35S::TCP9, which showed no significant difference in stomatal conductance in response to any stress. Indeed there was no visible difference in these plants during drought stress, whereas the other genotypes looked distinctly less turgid. The genotype *tcp9* also showed a reduction in stomatal conductance due to nematode infection alone. The stomatal conductance of these plants when unstressed was significantly higher than the wild type.

The rosette diameter of each genotype under each type of stress was measured over 40-48 days following drought initiation, and is shown in Figure 3.24. In the wild type plants there was no difference between the sizes of plants under each stress treatment for the first 12 days. However, by 20 days the drought-treated plants (drought and joint stress treatment groups) were growing at a noticeably slower rate than the unstressed and nematode-treated plants. By 25 days the nematode-infected plants were also increasing in diameter more slowly than the unstressed plants. Older leaves then started to senesce, reducing the diameter. At the end of the experiment there was a clear separation in rosette diameter between each of the stress treatments. The nematode-stressed plants were on average 80 % of the unstressed size, the drought-stressed plants were 60 %, and the joint-stressed plants were only 53 % of the unstressed size. The mutant and over-expression lines in the experiment showed a similar pattern of growth inhibition due to stress treatments as the wild type (Figure 3.24). The drought and joint-treated plants produced rosettes that were ultimately 50-60 % of the size of the unstressed plants, whilst nematode-infected plants 70-80% of the final size. The inhibition of growth due to each type of stress in each plant genotype was calculated and compared to that in wild type plants by ANCOVA (analysis of covariance) using time as a co-variate. The growth reduction due to drought stress or joint stress was found to be no different from the wild type in any of the genotypes analysed. However, the growth inhibition due to nematode stress was significantly more severe in the *azi1*, *tcp9* and 35S::TCP9 plants than in the wild type. Furthermore, nematode-induced growth inhibition was significantly more severe in the *azi1* mutant than in the 35S::AZI1 over-expression line ($p < 0.05$) (data not shown).

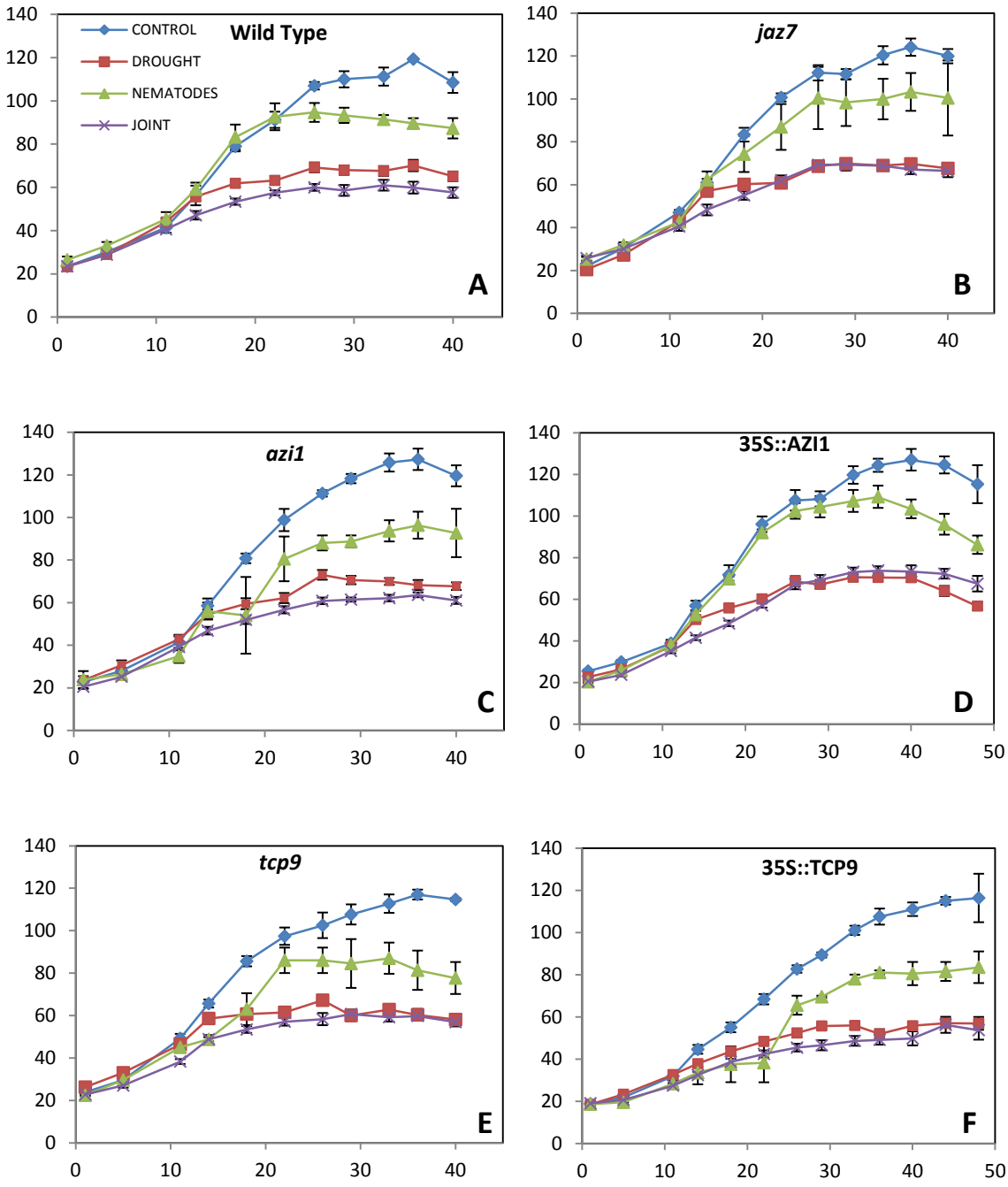


Figure 3.24. Rosette growth in over-expression and knock-out lines under different stress conditions. Soil-grown plants of different genotypes were exposed to *H. schachtii* nematodes (50 eggs/g), drought stress (soil moisture 15-20 %), or joint stress, and the rosette size measured over the following 50 days. **A)** Wild type, **B)** *jaz7*, **C)** *azi1,* **D)** 35S::AZI1, **E)** *tcp9,* **F)** 35S::TCP9. The X-axis represents the number of days after drought imposition. The Y-axis represents the rosette diameter in millimetres (n = 5).

The number of days taken for the primary inflorescence to emerge in all genotypes was noted, and is shown in Figure 3.25A. The 35S::TCP9 plants produced inflorescences significantly later than the wild type plants. However, there was no difference in inflorescence emergence between stress treatments for any of the genotypes studied. The final inflorescence height of all the plants was also measured (Figure 3.25B). The height of the wild type plants was significantly affected by drought and joint stress but not by nematode stress. The inflorescence height of the mutant and over-expression genotypes followed a similar pattern. However, the reduction in height of the *tcp9* and *jaz7* plants due to drought was significantly more severe than the wild type. Interestingly, in both these genotypes the detrimental effect of joint stress was less severe than in wild type plants. Similarly the *azi1* and 35S::AZI1 genotypes fared better under joint stress than the wild type plants.

The final number of siliques on the primary inflorescence was found to differ according to treatment in the wild type plants. Although not affected by either stress individually, when a combination of joint drought and nematode stress were imposed, the silique number was significantly reduced (Figure 3.26A). The effect of stress on silique number differed according to genotype. In *azi1* plants there was no reduction in silique number in joint stressed plants compared to unstressed plants. On the other hand, 35S::TCP9 plants were significantly more affected by both drought and joint stress than the wild type plants. Similarly, *tcp9* plants showed a greater reduction in silique number under drought stress than wild type plants.

When seed number per silique was analysed, there was found to be no effect of treatment in wild type plants (Figure 3.26B). However, plants over-expressing the *TCP9* gene were greatly affected by both drought and joint stress, producing almost no seeds at all under these conditions. In contrast *jaz7* plants produced a significantly greater than wild type yield under conditions of joint stress.

3.3.8 Analysis of candidate gene expression in hormone signalling mutants

Expression levels of the ten candidate genes were analysed in a variety of *A. thaliana* hormone signalling mutants under different stress conditions. Two ABA-insensitive lines were obtained. The first of these, *abi2-1,* contains a mutation in a protein

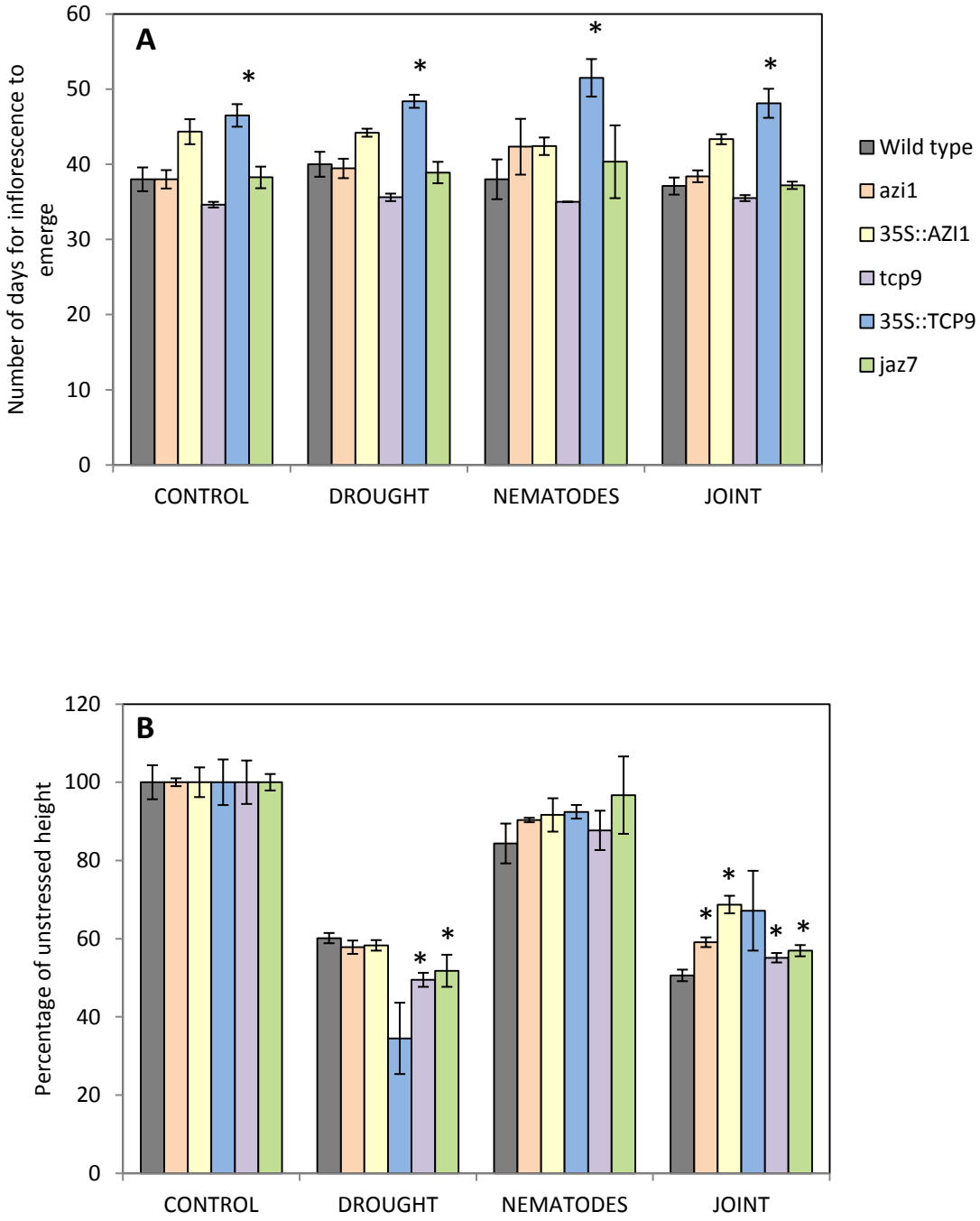


Figure 3.25. Effects of stress treatments on inflorescence development in different genotypes. (**A**) The number of days until the emergence of the primary inflorescence and (**B**) the final primary inflorescence height was measured in mutant and over-expression lines under different stress treatments. The final inflorescence height was calculated as a proportion of the unstressed height. Asterisks show significant differences to the wild type value for that stress treatment (n = 5) ($p < 0.05$).

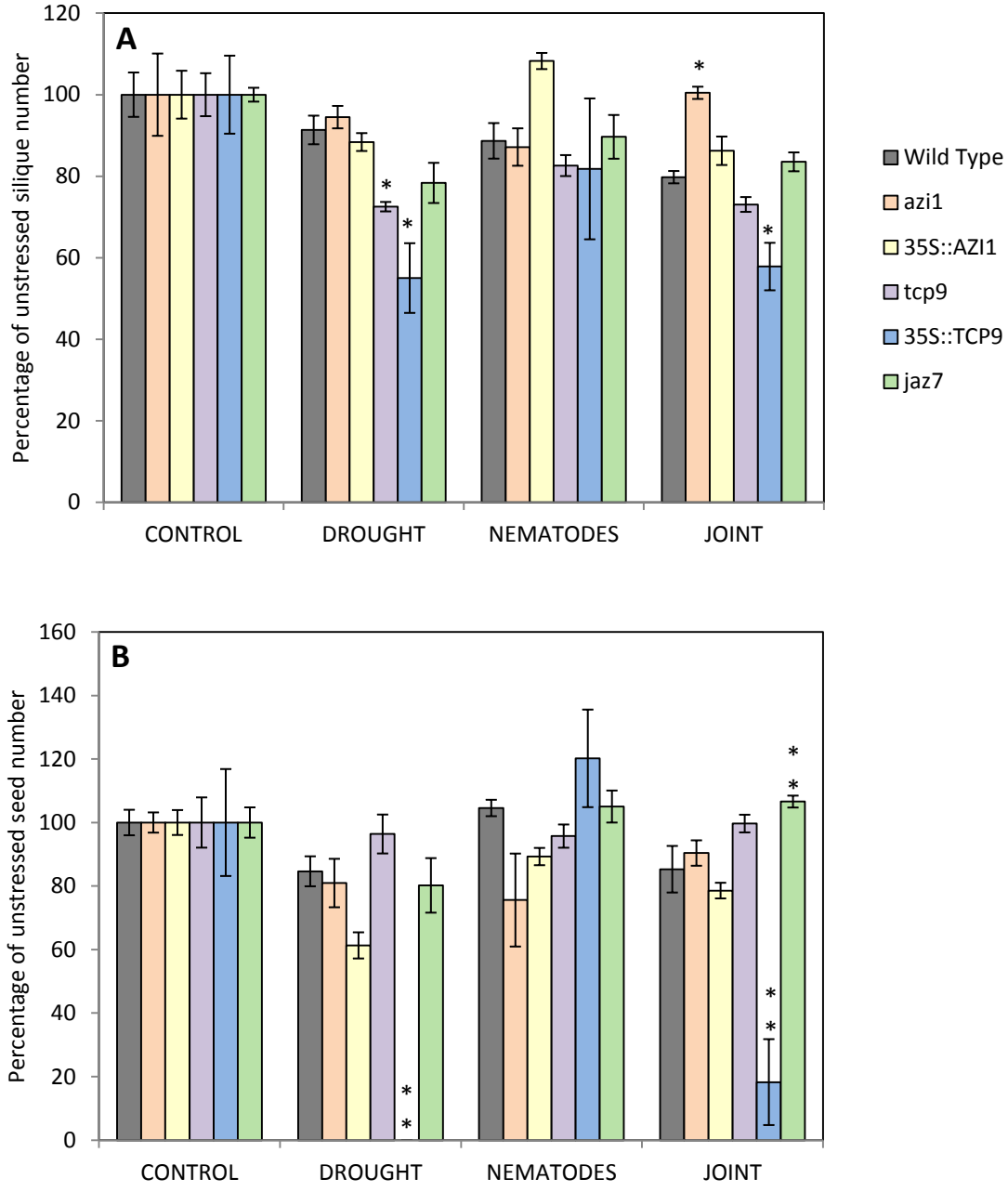


Figure 3.26. Effects of stress treatments on silique and seed development in different genotypes. (**A**) The number of siliques per primary inflorescence and (**B**) number of seeds per silique in different genotypes as a percentage of the unstressed value. Asterisks show significant differences to the wild type value for that stress treatment (n = 5) (* $p < 0.05$, ** $p < 0.01$).

phosphatase 2C involved in ABA signal transduction (Rodriguez *et al.*, 1998). The plants show resistance to ABA. This allows germination on high levels of exogenous ABA, and causes a stomatal phenotype leading to an enhanced rate of water loss from aerial tissues (Koornneef *et al.*, 1982). The *abi4-1* germplasm line has a mutation in a downstream AP2 transcription factor associated with ABA signalling. The plants show some resistance to ABA but have no stomatal phenotype (Finkelstein, 1994; Finkelstein *et al.*, 1998). Two ethylene signalling mutants were obtained, *ein3-1* and *ctr1*. The ethylene-insensitive mutant *ein3-1* has a mutation in the *EIN3* transcription factor, a key component of the ethylene signalling pathway due to its activation of downstream ethylene-responsive genes (Stepanova and Alonso, 2009). *Ein3-1* mutants show a loss of normal ethylene-mediated effects such as the 'triple response' of dark-grown seedlings as well as the inhibition of growth and increased senescence (Chao *et al.*, 1997). The *CTR1* gene encodes a MAPK kinase kinase that acts upstream of EIN3 as a negative regulator of ethylene signalling (Yoo *et al.*, 2009). The *ctr1* mutant displays a phenotype of constitutive ethylene response, notable by the plants' small, unexpanded leaves (Kieber *et al.*, 1993). In addition to the ABA and ethylene signalling mutants, the methyl jasmonate-resistant *jar1-1* mutant was studied. *JAR1* carries out the adenylation of jasmonic acid to form the active conjugate JA-Ile (jasmonoyl-isoleucine) during defence signalling. The mutant has reduced sensitivity to root growth inhibition caused by exogenous methyl jasmonate (Staswick *et al.*, 1992; Staswick *et al.*, 2002).

Each hormone signalling mutant was subjected either to no stress, nematode infection, drought stress, or joint stress. RNA was then extracted from aerial parts of the plants. The relative expression of candidate genes between hormone mutants in different conditions was analysed semi-quantitatively by RT-PCR (results not shown), and those seven that showed a differential regulation were analysed more accurately using qRT-PCR. The results are shown in Figures 3.27 and 3.28, and are summarised in a schematic diagram in Figure 3.29. The results from genes with no differential expression are not presented.

In this experiment plants were grown in soil and exposed to a drought stress far more natural than the rapid dehydration stress used in tissue culture. The expression of

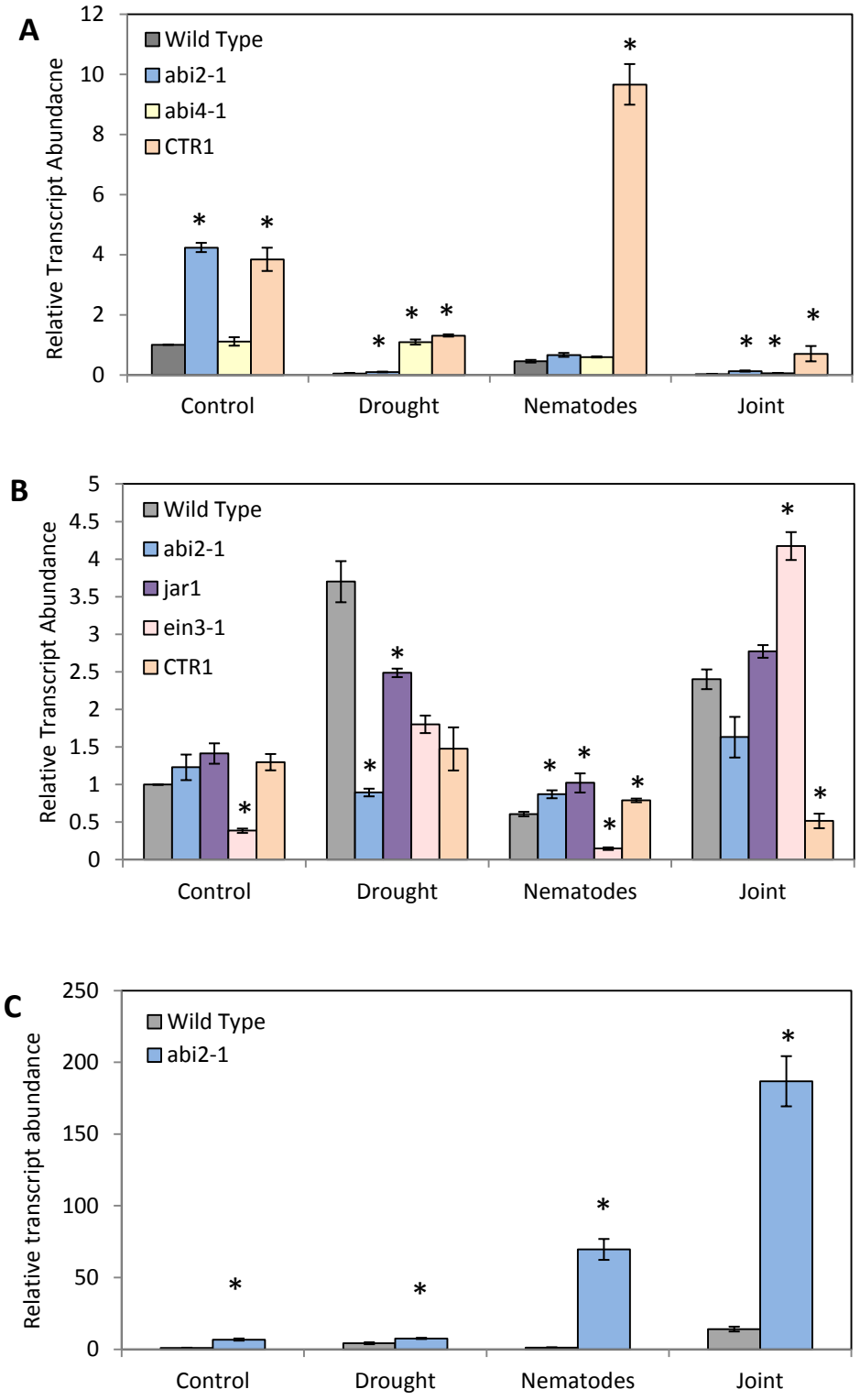


Figure 3.27. Relative transcript abundance of candidate genes in hormone signalling mutants (I). Wild type plants and hormone signalling mutants were grown in soil and exposed to different stress treatments. Analysis of candidate genes was then analysed by qRT-PCR. **A**) *AZI1*, **B**) *TCP9*, **C**) *DUF581*. All values are relative to the wild type value under control conditions. Asterisks show significant differences in candidate gene expression level between wild type and the hormone mutant for that treatment (n = 3) ($p < 0.05$).

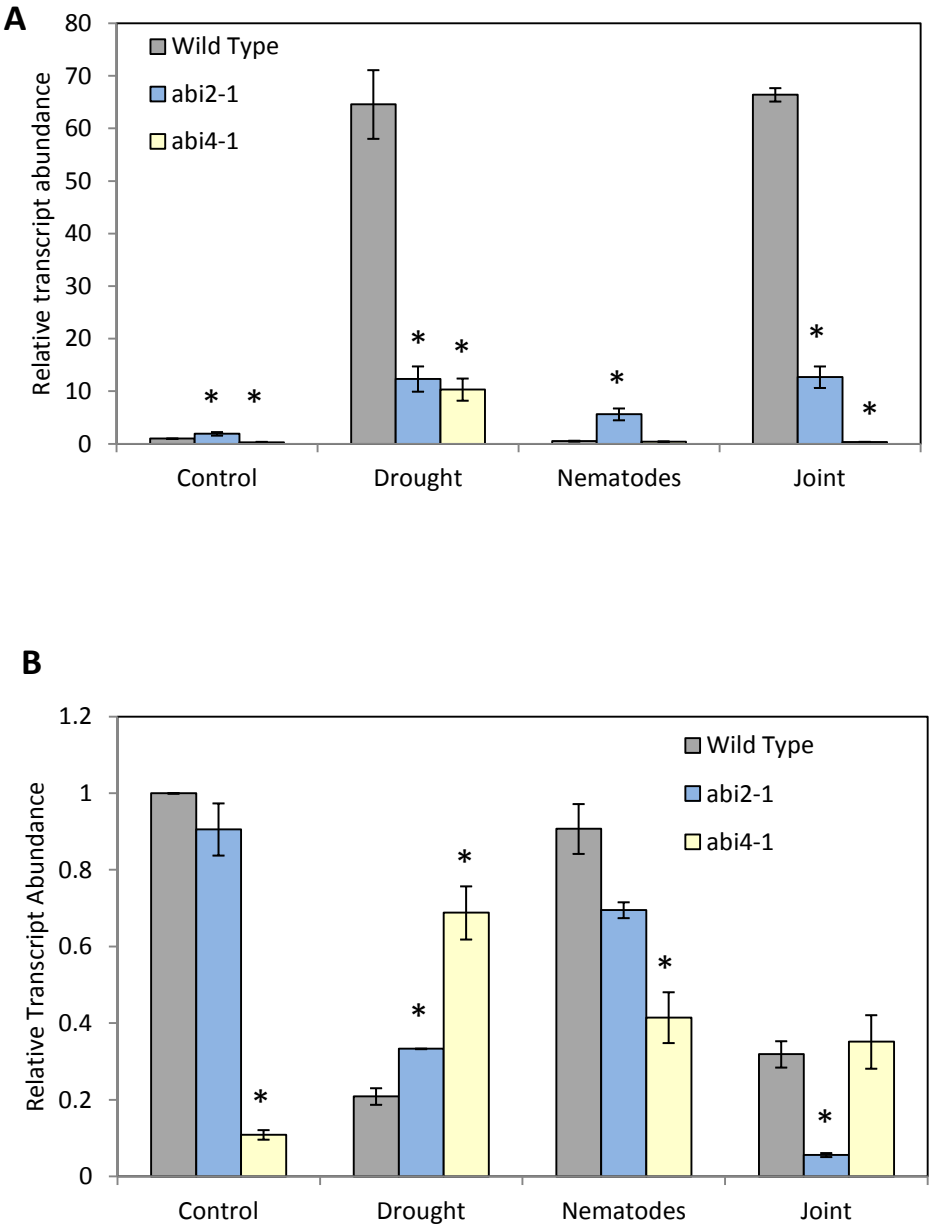


Figure 3.28. Relative transcript abundance of candidate genes in hormone signalling mutants (II). Wild type plants and hormone signalling mutants were grown in soil and exposed to different stress treatments. Analysis of candidate genes was then analysed by qRT-PCR. **A**) *ATMGL*, **B**) *MYB4*. All values are relative to the wild type value under control conditions. Asterisks show significant differences in candidate gene expression level between wild type and the hormone mutant for that treatment n = 3)($p < 0.05$).

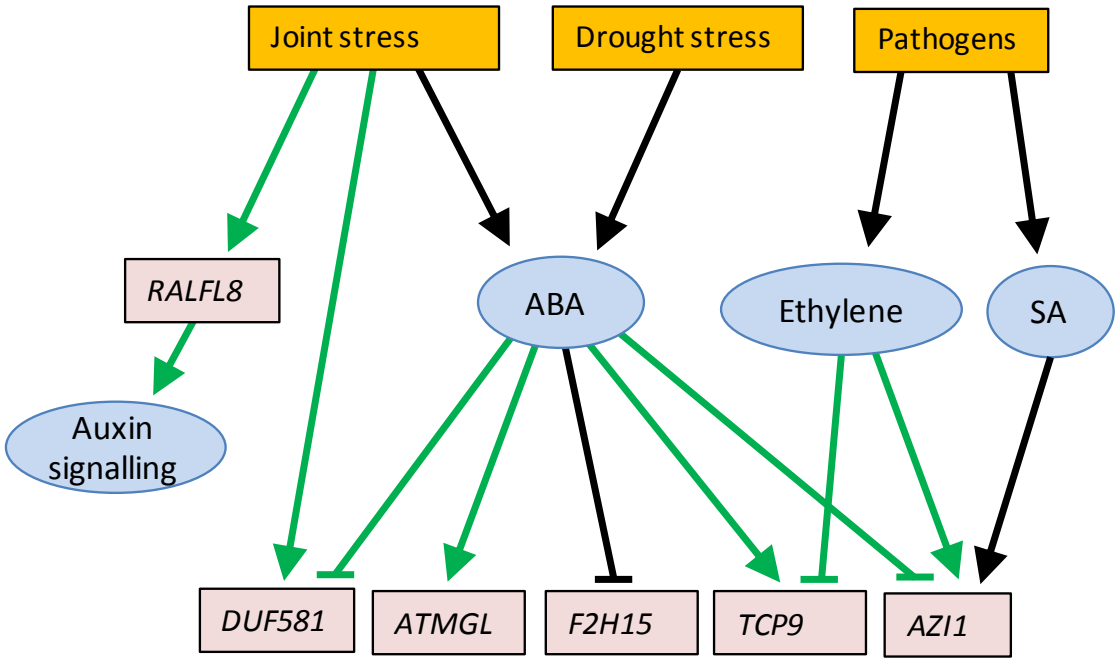


Figure 3.29. Schematic diagram showing effects of hormones on candidate genes. Hormones are shown in blue, along with their potential positive (arrow) or negative (bar) effect on genes of interest (pink). Black lines represent previously known effects and green lines represent the results of the current work. Stress factors are shown in yellow.

candidate genes under drought stress could thus be analysed and compared to that seen under dehydration stress induced for the microarray experiment.

AZI1

The expression of *AZI1* was differentially regulated in *abi2-1, abi4-1* and *CTR1* mutants (Figure 3.27A). In agreement with the results of the microarray, the expression of *AZI1* in wild type plants decreased under each type of stress, the biggest reduction being in plants under joint stress. In *abi2-1* mutants, the expression level of *AZI1* was four times higher than in the wild type in control conditions, but again decreased under each type of stress. In the *abi4-1* mutant the unstressed *AZI1* expression level was no different from wild type, but the relative levels increased under drought and joint stress. In the *CTR1* constitutive ethylene signalling mutant a higher level of *AZI1* was expressed under all treatments. Of particular note was the dramatic transcript increase during nematode stress, to ten times the wild type level.

JAZ7

No difference in *JAZ7* expression level was observed between wild type plants and *jar1-1* mutant plants under control conditions when analysed by qRT-PCR (data not shown).

TCP9

Expression of *TCP9* was significantly lower in wild type plants under joint stress than under drought stress alone (Figure 3.27B). This is in contrast to the microarray result, where expression in leaves under joint stress was significantly higher. The difference in *TCP9* regulation may arise from the differential effect of dehydration compared to drought treatment. *TCP9* expression was disrupted in several of the hormone signalling mutants. In the *abi2-1* mutant *TCP9* expression did not increase in response to stress. In the *jar1* mutant *TCP9* was not induced by drought stress as much as in the wild type, although under nematode stress the expression was slightly higher. In the ethylene signalling mutant *ein3-1,* the unstressed and nematode-stressed expression levels were lower than in the wild type, but the expression under joint stress was much higher. In the *CTR1* plants no stress-induced *TCP9* induction was observed, and in fact expression is reduced under joint stress. The opposite effect observed in the *ein3-1* and *CTR1* mutants under joint stress suggests a repression of *TCP9* by ethylene.

RALFL8

No significant difference was observed between *RALFL8* expression in wild type and the hormone signalling mutants *ein3-1* or *CTR1* when analysed by qRT-PCR (data not shown).

DUF581

Originally identified as up-regulated in roots in response to the combination of dehydration and nematode stress, *DUF581* expression showed a very similar pattern in leaves when exposed to drought and nematode stress in this experiment (Figure 3.27C). *DUF581* expression in wild type plants was unaffected by nematode stress, showed a small increase in response to drought (4-fold) and a large increase due to joint stress (14-fold). This gene may therefore play a similar role in roots and leaves. *DUF581* was expressed more highly in *abi2-1* ABA signalling mutants than in wild type plants. In the unstressed and drought-stressed *abi2-1* plants the expression was ~6-fold higher than in the wild type. With the application of nematodes the expression increased to 70-fold, but the highest expression was observed in the joint stressed plants, resulting in a huge fold increase of 180.

ATMGL

In roots, the results of the microarray showed a significant induction of *ATMGL* expression under joint stress compared to dehydration stress alone. In this experiment however, a high fold increase (~60-fold) in aerial tissues was observed in both drought- and joint-stressed wild type plants (Figure 3.28A). In both the ABA signalling mutants *abi2-1* and *abi4-1* this increase in expression was much less marked, if present at all. In *abi2-1* plants only a 12-fold increase was observed under both drought and joint stress, whereas in *abi4-1* the only increase in expression was due to drought. The results indicate that *ATMGL* induction due to drought stress may be dependent on functional ABA signalling.

MYB4

MYB4 was found to be down-regulated in the leaves of wild type plants as a result of drought or joint stress, in agreement with the microarray result. However, in contrast to the effect of dehydration, here there was no difference between the effect of drought and joint treatment (Figure 3.28B). In the *abi2-1* mutant the expression levels were similar to the wild type, although were higher under drought stress and lower under joint stress.

In *abi4-1* plants the expression level in unstressed or nematode-infected plants was much lower than the wild type, whilst in drought-stressed plants it was higher.

3.3.9 Aphid fecundity assays

Aphid fecundity assays were first carried out on wild type plants to determine if the presence of nematodes infecting the plant roots would affect the ability of the aphids to feed and reproduce. After 15 days the number of aphid nymphs recovered from infected plants was found to be no different from the number recovered from the uninfected controls (Figure 3.30A). The *DIR14* gene studied in this work is negatively regulated in a defence response elicited by the injection of aphid saliva into *A. thaliana* leaves (De Vos and Jander (2009), gene expression data analysed in Genevestigator). Aphid fecundity on the *DIR14* mutant and over-expression lines was therefore analysed to determine if the disruption of *DIR14* gene expression would affect aphid reproduction. Furthermore, analysis of the *azi1*, 35S::AZI1, *myb4* and 35S::MYB4 lines was also carried out (Figure 3.30B). No genotype caused a significant difference in aphid fecundity over the 15 days.

The *AZI1* gene is known to play a role in systemic immune signalling. Its expression is induced by the signalling molecule azelaic acid, which primes plants to accumulate salicylic acid upon pathogen infection (Jung *et al.*, 2009). Thus an experiment was carried out to test if this immune system priming would be compromised or accelerated in *azi1* or 35S::AZI1 plants. Plants were pre-infected with nematodes or aphid nymphs before testing the fecundity of a second set of aphids. There was no difference in aphid fecundity between the pre-treated and control plants in any of the genotypes, for either aphid or nematode pre-treatment (Figure 3.30C and D).

3.3.10 Analysis of root hair phenotype in 35S::RALFL8 line

As well as a severely stunted root system (Figure 3.16), 35S::RALFL8 plants were observed to have many more root hairs than the wild type. The hairs were also much longer (Figure 3.31). On close observation, the epidermal cells were seen to maintain a normal pattern of hair cells and non-hair cells. However, whereas in wild type plants not every hair cell produces a hair, the mutant produced an extra-long hair from every hair cell. The effect was more apparent due to the shorter epidermal cells. To determine

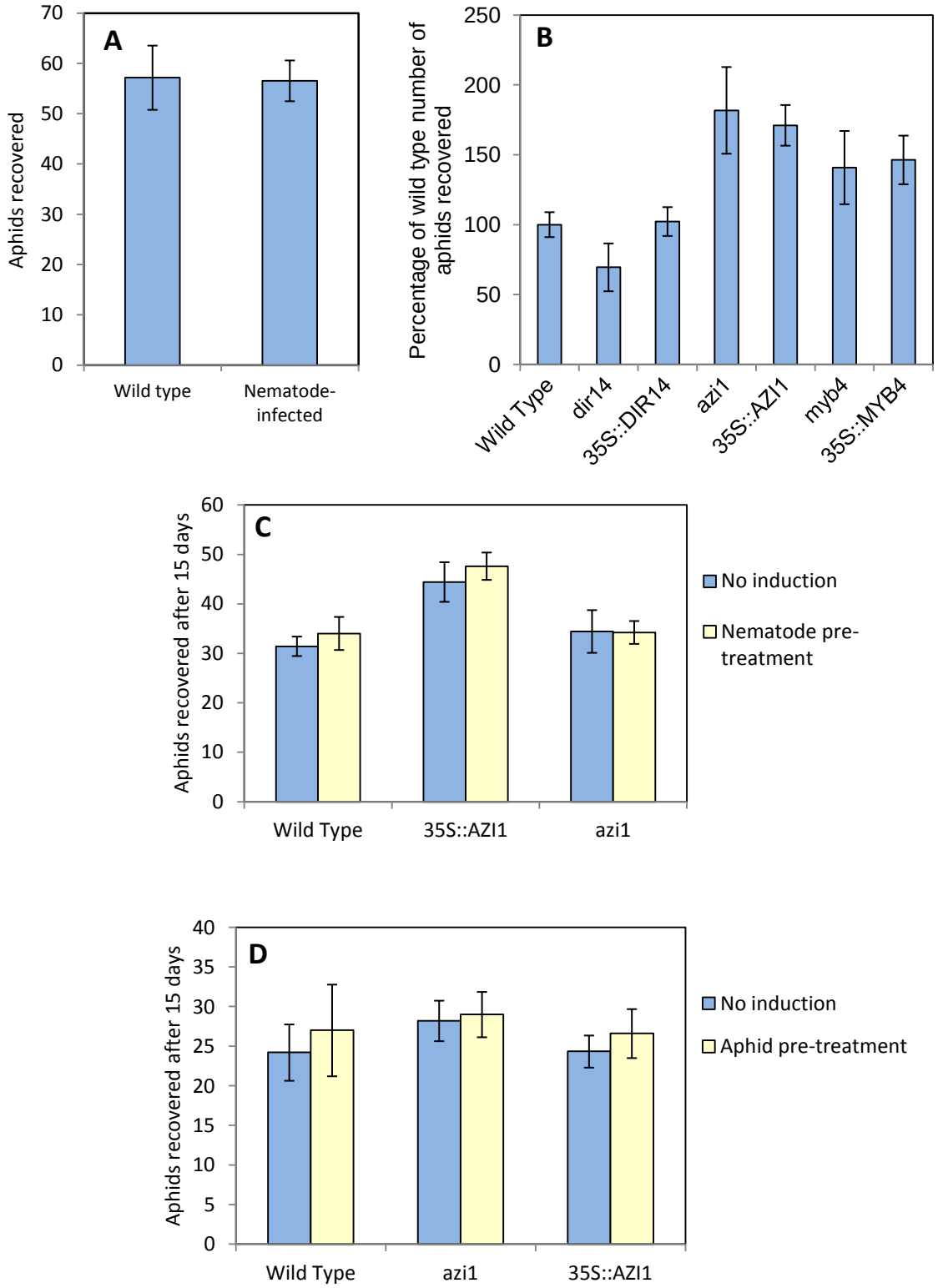


Figure 3.30. Aphid fecundity assays. A one-day-old nymph was placed on each plant and the number of progeny counted after 15 days. **A**) Number of aphids recovered from plants infected with *H. schachtii* compared to control plants (n=20). **B**) Fecundity of aphids on plants of different genotypes (n = 9). Fecundity of aphids on wild type and *AZI1* mutants that had been pre-treated with either (**C**) nematodes or (**D**) aphids (n = 5).

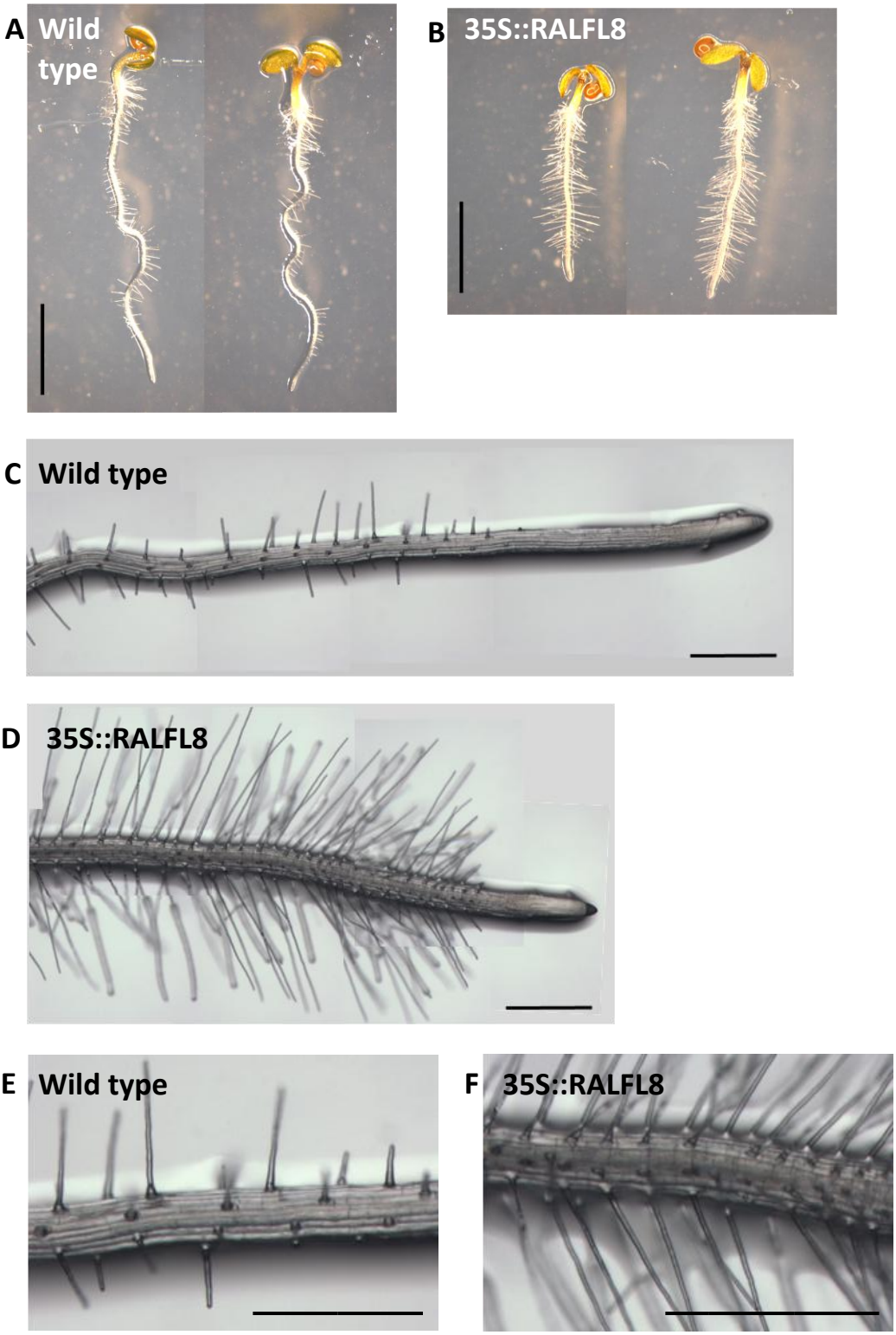


Figure 3.31. Root hair phenotype of 35S::RALFL8 plants. Wild type (**A, C, E**) and 35S::RALFL8 seedlings (**B, D, F**) were grown on half strength MS media and photographed after 4 days of growth. Long, dense root hairs are clearly visible on the mutant. High magnification of epidermal cells reveals a regular pattern of hair and non-hair cells in the mutant. Bars represent 2.5 mm (top pictures) and 500 μm (middle and lower pictures).

whether this was truly a result of *RALFL8* over-expression and not a secondary effect of the transgene insertion, several of the original 35S::RALFL8 lines, each representing a different insertion event, were analysed for the phenotype. All the lines showed a similar phenotype (data not shown). To determine whether this phenotype was related to a disruption in auxin signalling, plants were grown on auxin- and anti-auxin-containing media. Figure 3.32 shows the seedlings after 4 days of growth. The length of root was measured after 7 days of growth. In response to the natural auxin indole-3-acetic acid (IAA), wild type seedlings were stunted and developed more root hairs. The *RALFL8* over-expression line showed a similar reaction, producing denser root hairs than usual. The root length of both genotypes was reduced to 60 % of that on normal growth medium (Figure 3.33). When grown on the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) a more severe reaction was observed in both lines, whereby plants became extremely stunted with long, dense root hairs. The roots of each line were reduced to ~10 % of the normal length. On the anti-auxin α-(phenyl ethyl-2-one)-indole-3-acetic acid (PEO-IAA) the root length was not reduced in either genotype. However, the root hairs of the over-expression mutant were observed to be slightly shorter and less dense. Plants obtained by crossing homozygous 35S::RALFL8 and *axr3-1* mutants were analysed for their phenotype. *Axr3-1* plants are auxin-insensitive, as shown by stunted roots, a loss of gravitropism and a complete absence of root hairs. *35S::RALFL8/axr3-1* plants showed exactly the same phenotype as the *axr3-1* parents, indicating that the dominant *axr3-1* mutation over-rides the effect of over-expressing *RALFL8.* The phenotypes of plants were the same no matter which parental genotype had provided the male/female gamete. Growth on kanamycin and PCR confirmed that seedlings resulting from the cross still contained the 35S::RALFL8 construct. Co-expression analysis of *RALFL8* revealed an extremely high correlation with pectin methylesterase genes ($r < 0.99$) (Arabidopsis Coexpression Data Mining Tool). Expression analysis of three pectin methylesterase genes that are highly co-expressed with *RALFL8* (At2g47040, At1g69940, At3g62170) was carried out in the 35S::RALFL8 over-expression line. The results showed no discernable difference in expression in the 35S line compared to the wild type plants, suggesting that an increase in *RALFL8* expression does not regulate pectin methylesterases at the transcriptional level.

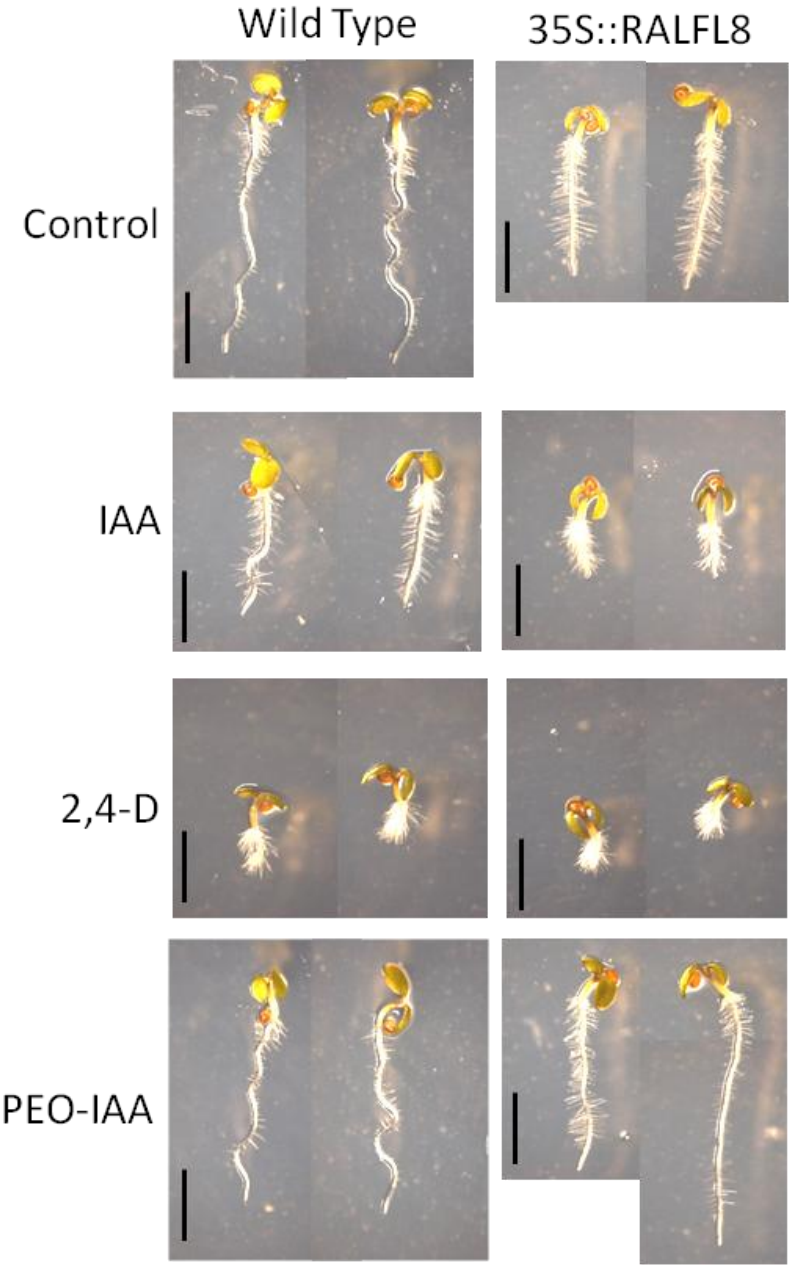


Figure 3.32. Effect of auxin on 35S::RALFL8 plants. Wild type and *RALFL8* over-expression line were grown on ATS media containing a variety of compounds. IAA (indole acetic acid) is a natural auxin. 2,4-D (2,4-dichlorophenoxyacetic acid) is a synthetic auxin which exerts a stronger effect on plants. PEO-IAA (α-(phenyl ethyl-2-one)-indole-3-acetic acid) is an anti-auxin agent. Plants were photographed 4 days after sowing. Black bars represent 2.5 mm.

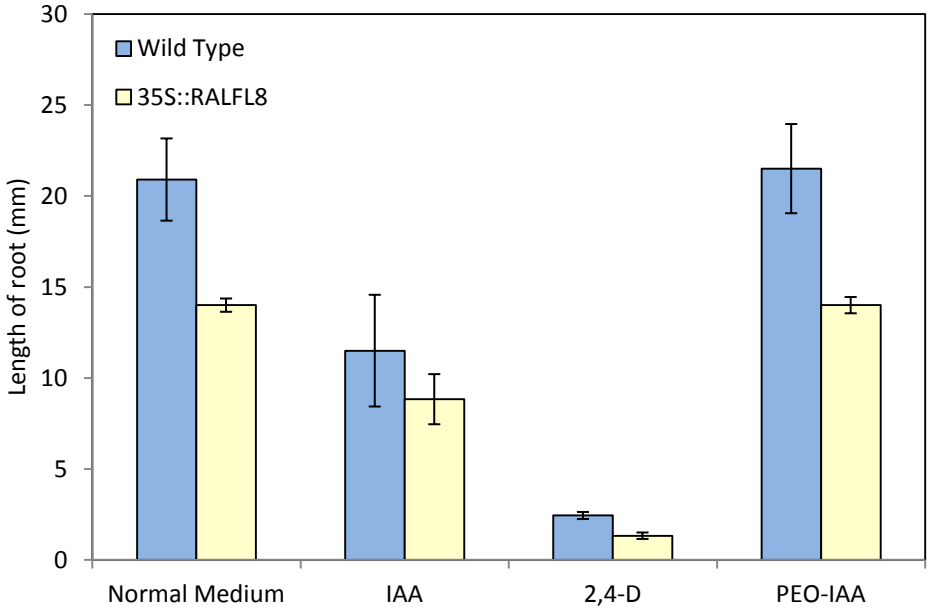


Figure 3.33. Effect of auxin on root length in 35S::RALFL8 over-expression line. Wild type and 35S::RALFL8 seedlings were grown on different media and the root length measured after 7 days (n = 8). IAA is a natural auxin, 2,4-D is a synthetic auxin and PEO-IAA is an anti-auxin agent.

3.4 Discussion

3.4.1 Functional characterisation of candidate genes

Ten candidate genes that may have roles in the response of *A. thaliana* to multiple stresses were selected from the results of the microarray experiment. Experiments were then carried out to elucidate the function of these genes using knock-out and over-expression lines. Many of the mutant lines incurred a growth or yield penalty under normal conditions, whilst stress resistance or susceptibility phenotypes were also observed. Candidate genes were found to be differentially expressed in hormone signalling mutants under different conditions, providing an insight into the regulatory pathways involved in their control. Further specific experiments were then carried out, for example to characterise the role of *AZI1* in immune system priming, or to investigate the root hair phenotype caused by over-expression of *RALFL8.* The results for each candidate gene are analysed individually in this section. Figure 3.29 is a schematic diagram showing the likely effects of hormones on each candidate gene.

3.4.2 The defence gene *DIR14* (At4g11210)

DIR14 is part of a disease-responsive family of genes known as a dirigent proteins. Dirigent proteins control the coupling of monolignols into lignins and lignans as part of the phenylpropanoid pathway (Davin and Lewis, 2000). This was first demonstrated in the plant *Forsythia suspensa,* in which DIR proteins were shown to direct the coupling of *E*-coniferyl alcohol to make the lignin pinoresinol (Davin and Lewis, 2000). Whereas lignins are mainly structural components of cell walls, lignans are a class of several thousand related metabolites which are produced as defence compounds against insects or pathogens, possessing antioxidant, antiviral, antibacterial and anti fungal properties (Burlat *et al.*, 2001). They also have beneficial properties for humans, for example in cancer prevention (Davin and Lewis, 2000). DIR proteins are particularly well-characterised in gymnosperms such as spruce and red cedar, accumulating in the outer stem tissues of Sitka spruce saplings. *DIRs* are induced up to 90-fold in bark and xylem tissues following insect attack, mechanical wounding or treatment with MeJA (Ralph *et al.*, 2007). Dirigent proteins have also been associated with cyst nematode infection. In soybean, several dirigent proteins became down-regulated in the syncytia of *Heterodera glycines,* although the synthesis of secondary metabolites lignin and suberin was

induced (Ithal *et al.*, 2007b; Klink *et al.*, 2007; Klink *et al.*, 2010). The largest families of DIR genes have been identified in *A. thaliana* (25 genes) and rice (54 genes) (Ralph *et al.*, 2006). Nothing is known about the specific function of *DIR14*, although sequence analysis shows that it bears closest resemblance to members of the spruce DIR-a subfamily. These genes have the greatest induction or repression in both bark and green tissues following herbivory by weevils or budworms, respectively (Ralph *et al.*, 2007). *DIR14* has been identified in experiments aiming to profile hormone-responsive genes in *A. thaliana.* It was significantly down-regulated in response to auxin in two different microarray experiments, whereas in response to cytokinin treatment its expression was induced (Rashotte *et al.*, 2003; Redman *et al.*, 2004; Goda *et al.*, 2008). Furthermore *DIR14* was down-regulated by ABA, a finding in accordance with the ABA-mediated down-regulation of lignin production in tomato (Mohr and Cahill, 2007; Goda *et al.*, 2008). The plant hormones auxin and ethylene are known to be important in the establishment of syncytia, and there is now evidence that cytokinins, gibberellic acid and jasmonic acid may all play a role (Goverse *et al.*, 2000; Lilley *et al.*, 2005; Ithal *et al.*, 2007b). Auxin and cytokinins also control crucial cell wall deposition processes, another possible explanation for the differential regulation of *DIR14* after treatment with phytohormones (Pesquet *et al.*, 2005).

In the current study, *DIR14* was up-regulated in response to joint stress compared to individual stresses in roots, and also slightly by nematode stress alone, although not to a significant level. *DIR14* may therefore be a defence gene that is induced by nematode feeding in order to increase the production of lignins and lignans. Lignin deposition is characteristic of a general defence mechanism in response to nematode infection, but has also been proposed to help protect cell walls from excess turgor pressure during syncytial development (Wuyts *et al.*, 2006a; Ithal *et al.*, 2007a; Klink *et al.*, 2007). Highly controlled by different hormones, the expression of *DIR14* may have been induced further by the specific pattern of hormone levels created by the combination of dehydration and nematode stress. In this specific stress combination, activation of the phenylpropanoid pathway may be beneficial in protecting against damage. The manipulation of *DIR14* had no effect on drought tolerance, substantiating its lack of a function in drought response. Furthermore, no difference in nematode tolerance or susceptibility was observed in these lines. Although the nematode density on the root

system was higher in the 35S::DIR14 line than in wild type plants, there was no difference in numbers per plant. *DIR14* has previously been shown to be down-regulated in response to aphid herbivory (De Vos and Jander, 2009). However, inactivating the gene had no effect on aphid fecundity. These results together suggest that although part of a hormone-regulated nematode and aphid-induced defence response, a level of redundancy may exist amongst dirigent proteins, meaning that manipulation of one does not noticeably affect the efficacy of the plant defence response. The *dir14* mutant exhibited a slower growth pattern in aerial parts of the plant, a symptom that may be due to disrupted lignin deposition in cell walls (Li *et al.*, 2010).

3.4.3 AZI1 (At4g12470), a signal in plant immune system priming

After attack from certain pathogens such as bacteria, fungus or viruses, plants activate a type of immunological 'memory' known as priming (Conrath *et al.*, 2006; Parker, 2009). Following a local resistance response to infection, a systemic defence system is activated throughout the whole plant leading to stronger protection and a more efficient response to subsequent attacks (Conrath *et al.*, 2006; Parker, 2009). This activation of systemic acquired resistance (SAR) in distal tissues requires salicylic acid, although this was ruled out as the signal molecule itself through grafting experiments which showed that mutant tobacco plants unable to accumulate salicylic acid were still capable of delivering priming signals to non-mutant scions (Vernooij *et al.*, 1994). The identification of the long-distance signal involved in priming has thus become an important target in understanding this process (Truman *et al.*, 2007; Parker, 2009). The mobile metabolite azelaic acid has been recently confirmed as important to systemic priming (Jung *et al.*, 2009). Azelaic acid was found to confer disease resistance when sprayed onto leaves, induced salicylic acid response genes, and could be transported to distal parts of the plant following foliar application. On investigation of azelaic acid effectors, *AZELAIC ACID INDUCED 1 (AZI1)* was found to be induced by azelaic acid. Mutant *azi1* plants failed to induce systemic immunity following azelaic acid application, although plants could still recognise a defence priming signal produced in the exudate of wild type plants (Jung *et al.*, 2009; Parker, 2009). Locally induced following pathogen infection, *AZI1* appears to be important for the regulation, modification or translocation of a mobile SAR signal. A lipid transfer protein, AZI1

may mobilise lipids that have been modified by azelaic acid, as signalling molecules in defence priming (Parker, 2009).

In the current study *AZI1* was found to be down-regulated 2.4-fold in leaves of plants exposed to joint dehydration and nematode stress compared to dehydration stress alone. In soil-grown plants, *AZI1* was down-regulated 2-fold in response to nematode stress, 17-fold in response to drought stress, and 30-fold in response to joint stress, suggesting the down-regulation of immune system priming in response to both biotic and abiotic stresses (Figure 3.27A). The expression of *AZI1* was 4-fold higher in the ABA-signalling mutant *abi2-1* but no different from wild type in *abi4-1*. This suggests that *AZI1* is negatively regulated by ABA, and that the repression is downstream of the ABA signal transduction gene *ABI2* but not dependent on the ABA-responsive AP2 transcription factor *ABI4* (see Section 3.3.8) (Finkelstein, 1994; Finkelstein *et al.*, 1998). The down-regulation of *AZI1* during drought stress may therefore be due to an increase in stress-responsive ABA accumulation (Shinozaki and Yamaguchi-Shinozaki, 1997; Bartels and Souer, 2004). However, in the absence of ABA (in the *abi2-1* mutant), *AZI1* was still repressed following each type of stress, resulting in a transcript level no different from the wild type. Therefore the stress-related repression of *AZI1* must occur independently of ABA, perhaps through DRE-response elements that act in the ABA-independent abiotic stress response pathways (Section 1.2.3.2, Shinozaki and Yamaguchi-Shinozaki (2007)).

Antagonistic cross-talk between the hormone signalling pathways of ABA, salicylic acid, ethylene and jasmonic acid allow plants to activate downstream genes that are highly specific to the type of stress encountered (Anderson *et al.*, 2004; De Vos *et al.*, 2005; Jalali *et al.*, 2006; Asselbergh *et al.*, 2008b; Yasuda *et al.*, 2008). Research shows that exogenously applied or salt stress-induced ABA can suppress salicylic acid production and prevent SAR induction, leading to pathogen susceptibility (Mohr and Cahill, 2007; Yasuda *et al.*, 2008). The down-regulation of *AZI1* due to drought stress in this study, even in *abi2-1* mutants, suggests that this abiotic stress-induced inhibition of SAR may partially be achieved in an ABA-independent manner. Conversely, the chemical induction of SAR can negatively influence the production of ABA and the activation of ABA-responsive genes (Yasuda *et al.*, 2008). The drought susceptibility observed in the current study in the 35S::AZI1 plants may therefore have been due to an

over-activation of the SAR priming system, which led to an inhibition of drought-responsive genes.

A local salicylic acid response is known to be important for resistance to cyst nematodes (Wubben *et al.*, 2008). *AZI1* was found to be down-regulated slightly by *H. schachtii* infection, whilst over-expression of *AZI1* conferred no resistance to nematodes. This suggests that nematode infection does not activate azelaic acid-associated priming, emphasising the specificity with which plants respond to different pathogens (De Vos *et al.*, 2005; Jalali *et al.*, 2006). However, a difference was observed in growth rate under nematode infection, whereby *azi1* plants were significantly more impaired than 35S::AZI1 plants (Figure 3.24). *AZI1* is positively regulated by ethylene, as shown in previous microarray work (Genevestigator, Figure 3.5) and as demonstrated here by the increase in *AZI1* expression under all four conditions in the constitutive ethylene mutant CTR1. The expression of *AZI1* was actually induced by nematode stress in this mutant, in contrast to its usual stress-induced down-regulation (Figure 3.27A). Ethylene plays an important role in both pathogen defence and nematode infection, acting as an antagonist of ABA, a positive regulator of pathogen response systems, and a necessary component of successful nematode parasitism events (Wubben *et al.*, 2001; Anderson *et al.*, 2004; Broekaert *et al.*, 2006). It is produced in the roots of nematode-infested tomato, soybean and *A. thaliana*, perhaps as an aid to the dissolution of cells walls during syncytia formation due to its role in cell wall degradation (Barker, 1999; Wubben *et al.*, 2001; Curtis, 2007; Klink *et al.*, 2010; Tucker *et al.*, 2010). Thus if ethylene is a positive regulator of the azelaic acid priming system, any increase in ethylene production due to nematode infection in the hypersensitive ethylene mutant CTR1 may have led to an increased induction of *AZI1*. However, the addition of the more severe drought stress may over-ride the ethylene response in the joint stress treatment group.

Experiments were carried out to determine whether *AZI1*-regulated defence priming was involved in the response of *A. thaliana* to the aphid *Myzus persicae.* Aphid feeding elicits two types of defence response system in plants. The first is a general response to tissue damage, similar to that induced by wounding and pathogen infection, which activates pathogenesis-related and general stress-related genes as well as salicylic acid and jasmonic acid (Moran and Thompson, 2001; Moran *et al.*, 2002; Smith and Boyko,

2007; Kusnierczyk *et al.*, 2008; De Vos and Jander, 2009). The second response is triggered by a specific elicitor in the aphid saliva, which induces a local resistance response in a system independent to salicylic acid, ethylene and jasmonic acid (De Vos and Jander, 2009). This resistance means that aphid fecundity is reduced on leaves which have previously been exposed to aphid feeders, although not on distal tissues. Plant-nematode and plant-aphid interactions have some commonalities, such as the secretion of similar enzymes to break down cell walls (Bird and Kaloshian, 2003). Therefore plant defence responses to both pathogens may be similar. Certain R-genes, such as *Mi-1.2,* can provide resistance to both types of pathogen (Kaloshian, 2004; Li *et al.*, 2006; Smith and Boyko, 2007). In this study *azi1* mutants and 35S::AZI1 over-expression lines were tested for effects on aphid fecundity. No difference was observed between wild type, knock-out and over-expression lines (Figure 3.30C and D). In a further experiment, plants pre-treated with either aphids or nematodes showed no resistance to a second foliar application of aphids, either in wild type plants or those with altered *AZI1* expression (Figure 3.30C and D). These results indicate that *AZI1*-mediated priming does not play a role in aphid defence response in *A. thaliana.* However, interpretation of the result is limited by the fact that no priming effect was observed in wild type plants. Jung *et al.* (2009) suggested that immune system priming acts on systemic tissues throughout the plant. In the light of findings reported by De Vos *et al.* (2009), in which aphid saliva only reduced subsequent aphid fecundity on local tissues, it may have been more pertinent to apply aphids to the same leaf on which the pre-induction had taken place. Combined attack by more than one pest can change the defence status of plants, and below-ground herbivores can activate defence responses that protect plants against foliar feeders (Bezemer *et al.*, 2005; Bruce and Pickett, 2007). For example, after feeding by the root nematode *Pratylenchus penetrans,* the survival and growth of shoot-feeding insect *Pieris rapae* was severely reduced on *Brassica nigra* mustard plants (van Dam *et al.*, 2005). In the current study, no effect of infection with *H. schachtii* was observed on the reproduction rate of foliar aphids, suggesting that the response to these two herbivores is sufficiently specific not to provide broad resistance (De Vos *et al.*, 2005). Alternatively, suppression of host defences by *H. schachtii* could explain the observed result. If *AZI1* was a target for such repression, this would explain why *AZI1* expression decreased in wild type plants infected with the nematode.

In conclusion, the *AZI1*-associated systemic immunity priming is a specific pathogen response that provides resistance to secondary infection (Jung *et al.*, 2009). When plants are exposed to abiotic stress or a different kind of biotic stress such as nematode infection, the *AZI1* pathway becomes down-regulated. Under these circumstances the priming system may be un-necessary, and thus resources become focussed on the potentially more damaging stress. Suppression of host defences by cyst nematodes may also play a role in *AZI1* repression. Down-regulation is controlled partially by ABA, but also through an ABA-independent pathway. Under multiple stress, *AZI1* becomes down-regulated in an additive fashion. In contrast, over-expression of *AZI1* leads to drought susceptibility. To truly understand its role in multiple stress, it would be interesting to apply simultaneous abiotic stress and biotic stress with the pathogen *Pseudomonas syringae*, a known elicitor of *AZI1*-regulated immunity (Jung *et al.*, 2009).

3.4.4 A zinc-finger family protein F2H15 (At1g17970)

Several zinc-finger family proteins were up-regulated in leaves specifically by a combination of dehydration and nematode stress. At1g17970 was selected as a candidate gene due to its relatively high fold change. The F2H15 designation refers to its chromosomal location. Genevestigator searches showed that *F2H15* was negatively regulated by ABA, possibly implicating it as a negative regulator of abiotic stress signalling (Shinozaki and Yamaguchi-Shinozaki, 2007). Investigation of the *f2h15* mutant revealed a marginally slow growth rate in aerial tissues compared to the wild type, but ultimately a normal level of biomass accumulation and seed yield. The 35S::F2H15 line also grew slightly slower than normal and flowered slightly later. Both root systems were normal, and neither plant line showed any stress tolerance or susceptibility phenotype.

F2H15 has a C3HC4-type RING finger domain. RING-finger domains are ubiquitous in the *A. thaliana* genome, featuring in 1.42 % of all proteins (Kosarev *et al.*, 2002). The RING domain is essentially a protein interaction domain, and is thought to be involved in a variety of functions including transcriptional or translational regulation and proteolysis (Kosarev *et al.*, 2002). Many RING proteins exhibit ubiquitin ligase activity, and may therefore be important for regulating gene function through protein degradation (Jackson *et al.*, 2000). An example of this is shown in the regulation of the

photomorphogenesis response (Deng *et al.*, 1991; Qin *et al.*, 2008). A RING-domain protein called COP1 represses photo-morphogenesis by degrading transcriptional activators of light-responsive genes. Similar processes occur in stress responses. To negatively regulate cold stress, the RING-finger protein HOS1 causes the ubiquitination and subsequent degradation of a transcription factor ICE1 that induces cold-responsive genes (Dong *et al.*, 2006). In drought stress, a recent study has shown that C3HC4-type RING domain genes *DRIP1* and *DRIP2* (DREB2A-interacting proteins 1 and 2) negatively regulate *DREB2A* (Qin *et al.*, 2008). In unstressed conditions *DRIP1* and *DRIP2* ubiquitinate DREB2A causing its proteolysis. During water stress, the ubiquitination process may be blocked either by stress signals themselves or by the conversion of DREB2A into its active form, preventing ubiquitination. Other RING-domain proteins are positive regulators of stress signalling. For example, the ubiquitin ligase AIRP1 positively regulates the ABA-mediated drought response, enhancing processes such as stomatal closure and root elongation (Ryu *et al.*, 2010). RHA2a is another RING-domain protein that positively regulates ABA signalling during seed germination as well as salt and osmotic stress (Bu *et al.*, 2009). *F2H15* may therefore encode a similar stress regulatory protein. Down-regulated by ABA, it could be induced by a specific combination of biotic and abiotic stress in order to repress normal drought signalling pathways and focus resources on the novel stress condition. However, no increase in *F2H15* expression was noted in ABA signalling mutants, suggesting that other factors are involved in its repression. It is difficult to deduce any direct function of *F2H15* due to the lack of a distinct phenotype in the knock-out and over-expression lines. Further experiments will be needed in order determine the role of this gene.

3.4.5 A stress-responsive NAC transcription factor, *ANAC038* (At2g24430)

A. thaliana possesses a large family of 105 largely uncharacterised transcriptional regulators known as NAC family proteins (Ooka *et al.*, 2003). Four of these were found here to be up-regulated in response to joint dehydration and nematode stress in roots, compared to each individual stress. Of these, *ANAC038* was induced with the highest fold change and was therefore selected as a candidate gene in this study. NAC family proteins share a common NAC (NAM, ATAF and CUC) domain. The first identified NAC genes were *NO APICAL MERISTEM* (*NAM*) in petunia and *CUP-SHAPED COTYLEDON* (*CUC2*) in *A. thaliana.* Both were found to be important in shoot apical meristem formation and floral development, as mutations in these genes caused floral

defects (Souer *et al.*, 1996; Aida *et al.*, 1997). *A. thaliana* NAC family proteins have since been classified into subgroups according to the amino acid sequence of their 5 NAC sub-domains. Different subgroups are proposed to play two main roles within plants (Ooka *et al.*, 2003). For example, the NAM and NAC1 subgroups are important in development and morphogenesis, whilst the ATAF subgroup responds to stress and wounding stimuli. Three NAC transcription factors were identified that bind to a promoter sequence in the *EARLY RESPONSE TO DEHYDRATION STRESS 1 (ERD1)* gene, a Clp protease regulatory subunit that is induced by dehydration as well as natural senescence (Tran *et al.*, 2004). The NAC proteins were induced by drought, high salinity and ABA, confirming the importance of NAC family proteins in stress response pathways. When over-expressed, each of the three genes conferred drought tolerance. In rice, the over-expression of the *SNAC1* NAC family gene allowed greater tolerance to drought and salinity (Hu *et al.*, 2006). Certain NAC family proteins are involved in the response to both biotic and abiotic stresses. For example, *RD26* is induced by drought, salinity, ABA and jasmonic acid (Fujita *et al.*, 2004). When over-expressed, this gene activates ABA- and abiotic stress-induced genes, as well as jasmonic acid responsive genes. RD26 is therefore an example of a NAC transcription factor which may mediate cross-talk between wounding and abiotic stress signalling pathways (Fujita *et al.*, 2004; Shinozaki and Yamaguchi-Shinozaki, 2007). Another NAC transcription factor called *ATAF1* mediates penetration resistance to the pathogen *Blumeria graminis* through the down-regulation of ABA synthesis (Jensen *et al.*, 2008). *ANAC038* is part of the NAM subgroup of NAC genes, of which the members are proposed to function in floral development (Ooka *et al.*, 2003). However, the analysis of a large number of microarray experiments in Genevestigator reveals a low to medium expression level of *ANAC038* throughout plant tissues, whilst in the current experiment *ANAC038* expression was found to be induced in root tissue. When *ANAC038* was inactivated, plants had a stunted root system, as well as slow-growing aerial parts. However, the seed yield of these and 35S over-expression plants was no different to wild type plants, implying no malfunction of floral development. These findings immediately suggest alternative functions for this transcription factor aside from those in floral development, as predicted by Ooka *et al.* (2003). It may have a role in both biotic and abiotic stress signalling in roots, leading to its increased expression in response to multiple stresses. Of all the candidate genes in this study, *ANAC038* was the least responsive to any hormone treatment (Figure 3.5). Several abiotic stress-related genes can act

independently of ABA signalling, including the *ERD1* gene mentioned previously (Kiyosue *et al.*, 1994; Shinozaki and Yamaguchi-Shinozaki, 2007). *ANAC038* may therefore be one of the NAC transcription factors that bind to *ERD1* to stimulate its expression. It would be interesting to examine down-stream stress response factors in this pathway in order to confirm the hypothesis (Shinozaki and Yamaguchi-Shinozaki, 2007). It is worth noting that when over-expressed or inactivated, *ANAC038* did not confer stress tolerance or resistance. It has been demonstrated that in order to fully induce *ERD1* expression, a novel zinc finger homeodomain transcription factor, *ZFHD1*, needs to be over-expressed alongside a NAC transcription factor (Tran *et al.*, 2007). These two act co-ordinately to induce *ERD1*, and when both are induced transgenically, a high level of drought tolerance is observed. To test this proposed role of *ANAC038* in ABA-independent stress signalling, *ZFHD1* could be over-expressed in 35S::ANAC038 plants to determine any drought tolerance phenotype. Alternatively, redundancy amongst the numerous NAC transcription factors may account for the lack of effect of an individual gene knock-out.

3.4.6 *JAZ7* (At2g34600) and jasmonate signalling

Jasmonates such as jasmonic acid (JA) are small signalling molecules which regulate the response to wounding, ozone, biotic stress and pathogen attack. They have also been associated with the response to water stress, as ABA is known to activate various JA-responsive genes (Chini *et al.*, 2007; Shinozaki and Yamaguchi-Shinozaki, 2007). In addition, jasmonates are involved in several developmental processes in plants, such as root growth, senescence, and secondary stem growth (Chini *et al.*, 2007; Chung *et al.*, 2009; Sehr *et al.*, 2010). Jasmonate signalling in plants is fine-tuned by a highly controlled negative feedback loop involving the transcriptional activator *MYC2* and transcriptional repressors known as JAZ (jasmonate ZIM-domain) proteins (Chini *et al.*, 2007; Chung *et al.*, 2008; Chung *et al.*, 2009). During low concentrations of JA-Ile (the active conjugate of jasmonic acid), JAZ proteins bind to MYC2 preventing it from activating transcription of down-stream JA-responsive genes. Following environmental cues such as wounding or pathogen attack, high levels of JA-Ile cause the binding of JAZ proteins to a complex known as SCF^{COI1}, signalling their degradation. As a result of JAZ protein inactivation, MYC2 is free to promote transcription of JA-responsive genes. Amongst these are the *JAZ* genes themselves, which go on to repress MYC2 in a self-regulatory system (Chung *et al.*, 2009). Evidence suggests that JAZ proteins play

an active role in plant-herbivore interactions, as JAZ proteins lacking the functional Jas motif confer insect susceptibility to *A. thaliana* plants (Chung *et al.*, 2008).

JAZ7 was identified in the current study as up-regulated by joint stress compared with dehydration stress alone in roots. *JAZ7* is specifically JA-inducible (Chini *et al.*, 2007). It is up-regulated strongly immediately following wounding, and weakly as a response to insect feeding, as part of a generalised JA response system (Chung *et al.*, 2008). It can also be induced by drought (Genevestigator), although the mechanism for this has not been described. JAZ7 does not interact directly with MYC2, prompting the hypothesis that other transcription factors may be targets of JAZ repression (Chung *et al.*, 2009). JAZ7 also functions in plant growth regulatory processes as a transcriptional repressor of secondary stem growth, which is triggered by stem mechanostimulation (Sehr *et al.*, 2010). JA-signalling pathways thus connect wounding and mechano-sensory growth pathways. A differential growth effect was observed here between the *jaz7* mutant and over-expression lines. The *jaz7* mutant grew slowly, had a reduced stature, more lateral roots and fewer siliques than the wild type (Figure 3.14). In contrast, the 35S line was larger, had fewer lateral roots and more siliques. The 35S line yielded fewer seeds than the wild type. JA inhibits growth by limiting meiosis, leading to the 'bonsai' phenomenon, whereby continued wounding and constantly high JA levels cause a severe stunting phenotype (Zhang and Turner, 2008). Thus the stunting in the *jaz7* mutant may be due to de-repression, and therefore activation, of jasmonate signalling, whilst in the over-expression line the excess repression by JAZ7 would prevent the transcription of JA-responsive genes allowing a higher growth rate than usual. Plants with a high level of JA signalling are more resistant to stress and yield more highly under stress conditions than normal plants (Baldwin, 1998), perhaps explaining why the *jaz7* mutant suffered no yield loss when exposed to combined drought and nematode stress, in contrast with the wild type (Figure 3.26B). No other resistance or susceptibility phenotypes were observed in the *jaz7* mutants. The *JAR1* gene is important for the synthesis of the active JA-Ile conjugate. However, in *jar1-1* plants the expression level of *JAZ7* was no different from wild type, a finding that has previously been reported (Chung *et al.*, 2008). *JAZ*-mediated transcriptional repression is therefore not dependent on *JAR1*.

In conclusion, *JAZ7* up-regulation during joint dehydration and nematode stress may be a result of two different signalling pathways. First, high JA-Ile levels resulting from nematode-induced wounding may cause the transcription of JA-responsive genes including *JAZs.* Second, JAZ proteins may be directly induced by the effects of water stress. Although well characterised in JA-signalling, the exact role of JAZ proteins in drought response remains to be elucidated. It will be interesting to further dissect the interactions between these two inter-connected signalling pathways.

3.4.7 A novel role for a TCP transcription factor in stress signalling (At2g45680)

Four TCP transcription factors were amongst the 'interaction' genes up-regulated in leaves. *TCP9* was chosen as a candidate gene in this study. Originally identified in 1999, TCP transcription factors are involved in controlling growth, cell proliferation and organ identity in developing tissues, and are distinguishable by their 59-amino acid basic helix-loop-helix domain which allows DNA binding and protein interactions (Giraud *et al.*, 2010; Martin-Trillo and Cubas, 2010). There are over 20 TCP family proteins in *A. thaliana,* falling into two classes according to the type of TCP domain. TCP9 is a Class I protein. Although the function of TCP9 has not been characterised, other Class I proteins have roles in seed germination, the transcription of chloroplast genes, mitochondrial phosphorylation, shoot morphogenesis, embryogenesis and photomorphogenesis. TCP proteins can be positive or negative regulators (Koyama *et al.*, 2007; Martin-Trillo and Cubas, 2010). A connection has also been made between TCP factors and the circadian clock, whereby TCP factors including TCP9 become down-regulated at night in order to coordinate organellar functions with the time of day (Giraud *et al.*, 2010). No connection between TCP transcription factors and stress responses has previously been made.

Despite its specific induction in the microarray experiment in response to joint stress, in experiments conducted in soil *TCP9* was induced by both drought and joint stress (Figure 3.27B). More was revealed about the function of TCP9 through the analysis of knock-outs, over-expression lines and hormone signalling mutants. 35S::TCP9 plants showed a dramatically altered phenotype compared to the wild type, with a severely reduced stature, much later flowering, a stunted root system, short siliques and far fewer seeds. The leaves were narrow, waxy and dark in colour, and plants were significantly more susceptible to both drought and nematode stress. The effect of drought or joint

stress on silique number and yield was also far more severe than in wild type plants (Figure 3.26). Small siliques have also been observed as a result of changes in *TCP3* expression (Koyama *et al.*, 2007). These results suggest a role for TCP9 as a negative regulator of growth and development, particularly of leaf structure, flowering time regulation, or silique morphogenesis. When a plant perceives drought stress, normal growth and proliferative processes are inhibited in order to focus resources on stress tolerance mechanisms (Taiz and Zeiger, 1991; Herms and Mattson, 1992; Chaves *et al.*, 2003). This includes limiting cell expansion, limiting of leaf expansion to reduce leaf area, stomatal closure, inhibition of photosynthesis and waxy deposition on leaf surfaces. TCP9 may therefore be up-regulated in aerial tissues by drought stress signals in order to negatively regulate shoot growth and proliferation. Its stress-induced expression was dependent on ABA, but inhibited by excess ethylene in agreement with a role in the abiotic stress response (Figure 3.27B). Stomatal function was impaired in 35S::TCP9 plants, as shown by a failure to reduce conductance following drought stress (Figure 3.23). In this line, *TCP9* expression was around 500 times the normal level, perhaps leading to the severe inhibition of growth observed. This under-developed and reduced stature, combined with a lack of stomatal closure, may be responsible for the stress susceptibility phenotype.

The *tcp9* T-DNA insertion line grew significantly more slowly and flowered later than the wild type, but the ultimate biomass accumulation, seed yield and root system were normal. The plants showed no susceptibility or tolerance to stress, although stomatal conductance was greater under control conditions. Most single Class I TCP mutants analysed so far in the literature have only yielded mild phenotypic effects if any, and this is thought to be due to genetic redundancy (Koyama *et al.*, 2007; Martin-Trillo and Cubas, 2010). In conclusion, the induction of TCP transcription factors such as TCP9 may play a role in the stress-induced inhibition of growth and developmental processes in aerial plant parts.

3.4.8 RALFL8 (At1g61563), a signal peptide in cell wall re-modelling

A gene with a very short coding sequence was found to be up-regulated in roots specifically by the combination of dehydration and nematode stress. Encoding only 82 amino acids, RAPID ALKALINIZATION FACTOR-LIKE 8 (RALFL8) is so-called due to its similarity to a tobacco gene named Rapid Alkalinization Factor (RALF).

RALF was originally identified in tobacco due to its ability to cause alkalinisation of cell culture medium (Pearce *et al.*, 2001). Known as a peptide hormone, it interacts with receptors on the cell surface leading to a signal transduction cascade and the blocking of proton pumps causing alkalinisation (Pearce *et al.*, 2010). When applied to seedlings of tomato or *A. thaliana*, RALF peptide induces immediate arrest of root growth (Pearce *et al.*, 2001). RALFs are thought to have basic physiological roles in plants aside from root stunting, but their exact function is unknown (Matsubayashi and Sakagami, 2006).

In *A. thaliana* at least 40 RALF-like genes have been identified. The results of this study shed light on the function of *RALFL8*, a previously uncharacterised gene in *A. thaliana*. Analysis of the 35S heterozygous over-expression line in which the transcript level of *RALFL8* was increased over 120,000-fold revealed a severe stunting phenotype. The root system was only 20 % of the size of the wild type, comprising a short main root and fewer lateral roots, indicating a similar function for RALFL8 as for tobacco RALF (Pearce *et al.*, 2001). Perhaps due to the root stunting, aerial parts of the plant were also of small stature, leading to a lower yield and minimal biomass accumulation compared to the wild type. In control conditions *RALFL8* is expressed almost exclusively in flowers during pollen development and is up-regulated up to 13-fold in pistils following pollination (Genevestigator, Boavida *et al.*(2011)). Co-expression analysis reveals an exceptionally high level of co-expression with pectin methylesterase family proteins. Pectin methylesterases (PMEs) are crucial for cell wall re-modelling in a variety of growth, reproductive and defence processes, as they catalyse the de-methylesterification of homogalacturonan domains within cell wall pectin. This allows the pectin either to form Ca^{2+} bonds and create a rigid gel, or to be targeted by pectin-degrading enzymes, either way affecting cell wall rigidity (Pelloux *et al.*, 2007). PMEs, including those that co-express highly with *RALFL8* (in particular At2g47040 and At1g69940), are responsible for pectin re-modelling during pollen tube growth and root hair growth, two very similar processes (Bosch and Hepler, 2005; Cole and Fowler, 2006). Cell wall re-modelling is also important for strengthening physical barriers during defence responses, and 75 % of PME transcripts have been found to vary in response to biotic and abiotic stresses (Pelloux *et al.*, 2007). The de-methylesterification of cell wall pectin is also thought to be important for lignin synthesis, a process involved in protecting tissues from drought stress (Taiz and Zeiger, 1991). Different PMEs are specifically active at varying pH levels, and their function can be modulated

by alkalinisation, a process which occurs during pollen tube development (Feijo *et al.*, 1999; Willats *et al.*, 2001; Pelloux *et al.*, 2007). A novel regulatory system can thus be proposed, whereby *RALFL8* acts as a signalling molecule that regulates the action of PMEs during pollen tube growth and in response to environmental stimuli by binding to the cell membrane and causing alkalinisation of the cell wall. This cell signalling role is supported by the finding that RALFL8 is a myristoylated protein (Boisson *et al.*, 2003). Myristoylation is an irreversible N-terminal modification involving the addition of a C14 fatty acid chain to the end of a polypeptide. It affects the membrane-binding properties of signal molecules and is common in disease resistance proteins such as LRR-repeat containing domains. The expression level of three PMEs was analysed in 35S::RALFL8 plants but found to be no different to the wild type levels, providing further evidence for RALFL8 regulation of PMEs through pH-induced activity changes rather than at a transcriptional level. As PME activity is involved in root tip elongation, the disruption of normal cell wall re-modelling may explain why excessive RALF-like protein causes root stunting (Pelloux *et al.*, 2007). De-esterification of pectins is associated with growth inhibition in a variety of species (Cosgrove, 1997). Furthermore, Staal *et al.* (2011) found that root surface pH varied along the root tip with distance from the meristem, whereby pH was lowest at the zone of cell elongation. Therefore the constitutive expression of an akalinisation factor may directly inhibit the expansion of cells in this zone.

The 35S::RALFL8 over-expression line was hyper-susceptible to parasitism by *H. schachtii* (Figure 3.20). PME activity has previously been associated with plant-parasitic nematodes. PMEs are up-regulated in giant cells of tomato roots infected with root-knot nematodes (Fosu-Nyarko *et al.*, 2009), as well as in the roots of *A. thaliana* plants infected with *H. schachtii* (Hewezi *et al.*, 2008a), whilst over-expression of *PME3* results in susceptibility to this nematode. *H. schachtii* nematodes secrete a cellulose binding protein which binds to and activates *PME3*, causing cell wall re-modification and the facilitation of nematode parasitism (Hewezi *et al.*, 2008a). Therefore increased *RALFL8* expression may allow a similar process to occur, leading to nematode susceptibility. The induction in nematode feeding sites of pollen-specific genes is not un-precedented. The myo-inositol oxidase genes *MIOX4* and *MIOX5* synthesise nucleotide sugars for incorporation into the cell wall of developing pollen, and yet are greatly up-regulated in syncytia of parasitising *H. schachtii* (Kanter *et al.*,

2005; Siddique *et al.*, 2009). Significant similarities may therefore exist in the re-modelling of cell walls in these two systems.

Previous studies have revealed an induction of *RALFL8* in response to drought or ABA treatment, as well as infection with the bacteria *P. syringae* (Genevestigator), supporting a role for it in multiple stress responses. 35S::RALFL8 plants were found to be highly susceptible to drought stress (Figures 3.21B and 3.22). The effects of RALFL8 in multiple stress response may again be mediated through PMEs. It is known that over-expression of a PME inhibitor in *A. thaliana* confers resistance to the fungus *Botrytis cinerea* (Lionetti *et al.*, 2007), whilst in pepper plants, over-expression of a PME inhibitor was found to confer resistance to both biotic and abiotic stresses (An *et al.*, 2008). In contrast, high PME activity has been associated with susceptibility to biotic and abiotic stress (Pelloux *et al.*, 2007). Therefore although *RALFL8* is induced by various stresses, excessive cell wall re-modelling in the over-expression line may cause a lack of esterified pectin, making the plant susceptible to a variety of stresses. *RALFL8* may be induced by drought stress in order to cause alkalinisation of the root surface. Water deficit in maize has been shown to increase the pH in the zone of elongation, which has the effect of limiting growth, perhaps as a stress response mechanism (Shabala and Newman, 1998; Staal *et al.*, 2011).

All the 35S::RALFL8 over-expression lines created had an extremely high number of root hairs, which were much longer than wild type hairs (Figure 3.31). As pollen tube growth and root hair growth are similar cell expansion processes that involve cell wall re-modelling, the observed phenotype could be due to the loosening of cell walls by PMEs, allowing excess root hair growth (Bosch and Hepler, 2005; Cole and Fowler, 2006). Another reason for excessive root hair growth may be that RALFL8 over-expression leads to increased auxin or ethylene sensitivity, as these hormones promote the growth of root hairs and the root tip (Tanimoto *et al.*, 1995; Knox *et al.*, 2003; Jones *et al.*, 2009). Exogenous auxin application results in longer root hairs whilst disruption of auxin transport or signalling leads to shorter root hairs (Jones *et al.*, 2009). The *RALFL8* over-expression line has the characteristics of a plant treated with exogenous auxin. An increase in auxin signalling may also explain the nematode susceptibility of 35S::RALFL8 plants, as auxin accumulates at the site of nematode feeding cells and is known to be necessary for successful parasitism (Goverse *et al.*, 2000; Curtis, 2007).

When 35S::RALFL8 plants were grown on medium containing the auxins IAA and 2,4-D, the reduction in root length was comparable to that of wild type plants, indicating no increased sensitivity. The anti-auxin agent PEO-IAA disrupted the auxin signalling of 35S plants giving fewer root hairs than on normal medium, implying that the auxin response is not constitutively active. RALFL8 may therefore act as a positive regulator of the auxin signalling pathway, with a role in signalling, metabolism or transport of auxin. Auxin responses are mediated through the interaction of Aux/IAAs and ARFs (auxin response factors) (Leyser *et al.*, 1996). When auxin is present Aux/IAAs are degraded, releasing ARFs to promote transcription of downstream genes. AXR3 is an Aux/IAA that is involved in the production of root hairs through its antagonism with SHY2 (Knox *et al.*, 2003; Jones *et al.*, 2009). In the *axr3-1* mutant a stable gain-of-function mutation prevents the auxin-mediated degradation of AXR3, thus causing auxin insensitivity and giving a phenotype with no root hairs, reduced root elongation and agravitropism (Leyser *et al.*, 1996; Rouse *et al.*, 1998). Crosses between *axr3-1* and 35S::RALFL8 produced offspring indistinguishable to *axr3-1* plants. The effect of *RALFL8* was blocked by the *axr3-1* mutation, despite its ectopic expression under a constitutive promoter. This strongly suggests that the RALFL8 signal peptide is involved in modulating events in the auxin response pathway. Root hair and pollen tube growth are positively regulated by auxin (Aloni *et al.*, 2006), therefore cell wall remodelling by RALFL8 may be a crucial component of this mechanism.

Ethylene positively regulates root hair formation as well as being necessary for successful nematode parasitism (Tanimoto *et al.*, 1995; Tucker *et al.*, 2010). A mutation in a gene negatively regulated by ethylene, *RHD1*, results in a phenotype very similar to that observed in the *RALFL8* over-expression line (Wubben *et al.*, 2001; Wubben *et al.*, 2004). *rhd1-4* plants have short roots, many root hairs and were hyper-susceptible to *H. schachtii* infection. They also have an increased sensitivity to ethylene and auxin. Experiments have shown that increased root hair length alone does not confer susceptibility to nematodes, but that the disruption of ethylene signalling causes both the extended root hair phenotype and the susceptibility (Wubben *et al.*, 2001). RALFL8 therefore appears to have the opposite effect, positively regulating the ethylene response. The alkalinisation of the root surface during growth inhibition in the root elongation zone is mediated by ethylene (Staal *et al.*, 2011). Therefore ethylene may induce alkalinisation in the root using RALF-like signalling molecules. However, there

was no change in *RALFL8* expression in the ethylene signalling mutant *ein3-1* or in the constitutive ethylene mutant *CTR1*. In addition, ethylene treatment tends to produce root hairs in non-root hair cells, and is therefore involved in regulating root hair patterning (Tanimoto *et al.*, 1995). In contrast, the *RALFL8* over-expression line only has hairs in the hair cells, displaying a normal pattern of differentiation (Figure 3.31). It is thus more likely that any disruption to ethylene signalling is secondary to, and may even result from, a change in auxin signalling.

In conclusion, the signal peptide *RALFL8* may act as a positive regulator of the auxin signalling pathway upstream of AXR3, causing cell wall remodelling during pollen tube and root hair growth events. When over-expressed *RALFL8* led to a hairy root phenotype and allowed susceptibility to nematode infection. It is induced by multiple stresses and may promote the activity of cell wall re-modelling enzymes in order to protect cells from stress-related damage, as well as strengthening the cell wall as a barrier to invading pathogens. *RALFL8* may also be induced by water stress in order to alkalinise the root surface and cause inhibition of root elongation. Perhaps due to the short length of *RALFL8,* no knock-out mutant was commercially available. RNAi technology may therefore provide a useful alternative for studying the effect of a loss of RALFL8 function, allowing further insight into role of short signal peptides in stress responses and auxin signalling.

3.4.9 ATMGL (At1g64660), a methionine gamma-lyase

ATMGL, encoding a methionine gamma-lyase, was up-regulated in roots in response to joint stress compared to either stress individually. In previous microarray studies, *ATMGL* had been induced by ABA, ethylene and methyl jasmonate (Genevestigator), thus making it an interesting candidate for involvement in the hormone mediated stress response. First described in 2006, ATMGL was implicated in methionine regulation due to its induction in response to high methionine levels, and the accumulation of 9 times the normal concentration of methionine in *atmgl* knock-out mutants (Rebeille *et al.*, 2006; Goyer *et al.*, 2007). Expressed throughout plant tissues, ATMGL is a cytosolic enzyme with two main functions: The catabolism of excess methionine to maintain cellular homeostasis; and the conversion of methionine to isoleucine (Rebeille *et al.*, 2006; Goyer *et al.*, 2007; Joshi and Jander, 2009) (Figure 3.34). Methionine and isoleucine, as well as being protein constituents, are fundamental for a range of cellular

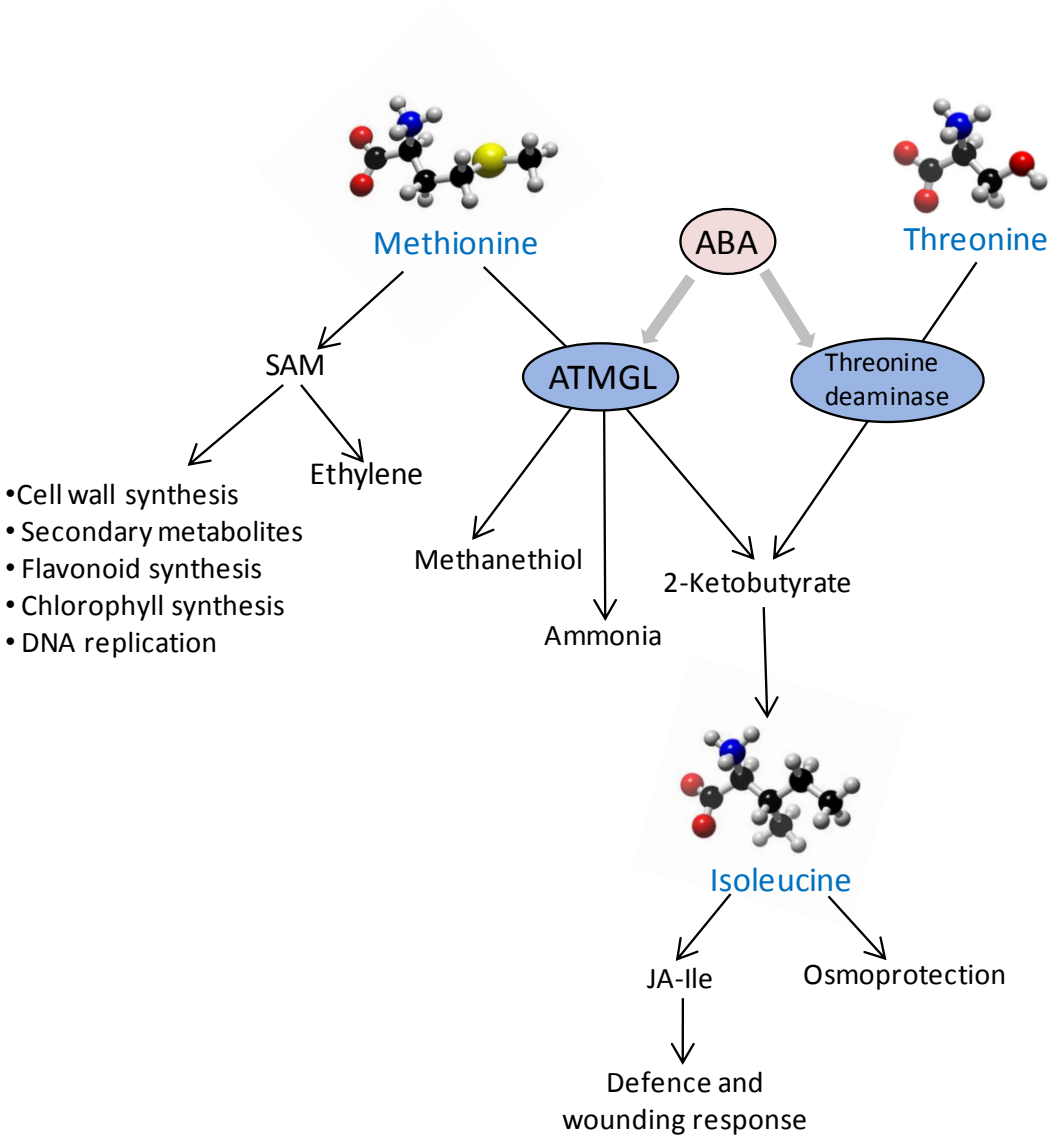


Figure 3.34. Methionine and isoleucine regulatory pathways. Methionine in plants is largely converted to SAM (S-adenosylmethionine) which acts as a methyl group donor in a variety of essential plant processes. SAM is also the precursor for ethylene. ATMGL (*Arabidopsis thaliana* methionine gamma-lyase) catabolises methionine to produce methanethiol, ammonia and 2-ketobutyrate. 2-Ketobutyrate is also produced from threonine via the enzyme threonine deaminase and is a precursor of isoleucine biosynthesis. Isoleucine is important for osmoprotection, and also combines with jasmonic acid to produce the active defence hormone JA-Ile. Both ATMGL and threonine deaminase are positively regulated by the hormone ABA.

processes within plants. Most methionine within plants is converted to S-adenosylmethionine (SAM), the primary biological methyl donor, which is the precursor for ethylene and has roles in DNA replication and methylation, cell wall synthesis, chlorophyll synthesis and secondary metabolites such as flavonoids, lignins, suberins and volatile compounds. (Joshi and Jander, 2009; Amir, 2010). However, ATMGL catabolises methionine alternatively to this pathway, producing methanethiol, ammonia and α-ketobutyrate (Rebeille *et al.*, 2006; Amir, 2010). The accumulation of α-ketobutyrate leads directly to the synthesis of isoleucine, whose production is thus regulated by ATMGL (via methionine catabolism) together with threonine deaminase (via threonine catabolism). Isoleucine combines with jasmonic acid to make the active form of the hormone, JA-Ile, which is crucial for plant defence (Koo and Howe, 2009). This involvement in the isoleucine synthesis pathway may also explain why *ATMGL* is induced in response to CaMV, phytopthora and flagellin (Genevestigator), as the JA-Ile conjugate would be important in response to these pathogens (Gfeller *et al.*, 2010).

The function of ATMGL during stress may be to regulate ABA-induced isoleucine biosynthesis. Amino acids are known to accumulate in order to protect plant cells from damage, acting as osmolytes, scavengers of reactive oxygen species, regulators of pH or as substrates for the synthesis of stress-related proteins (Nambara *et al.*, 1998; Joshi *et al.*, 2010). Isoleucine synthesis in particular occurs as a result of abiotic stress (Nambara *et al.*, 1998; Joshi and Jander, 2009). *ATMGL* is induced by drought stress, osmotic stress and salt stress in both roots and leaves (Rizhsky *et al.*, 2004; Less and Galili, 2008). Nambara *et al.* (1998) showed that in ABA deficient mutants, branched-chain amino acids such as isoleucine, valine and proline failed to accumulate in response to dehydration stress. The dramatic increase in drought-induced *ATMGL* expression in the current work was not observed in the ABA signalling mutants *abi2-1* and *abi4-1*, confirming the regulatory role of ABA. Drought-stressed *atmgl* plants accumulate less isoleucine than the wild type, although some accumulation still occurs, indicating redundancy in the pathway as a result of overlapping function with threonine deaminase (Joshi and Jander, 2009).

The analysis of *atmgl* knock-out plants revealed a phenotype that was no different to wild type plants except for a slightly smaller rosette size 16 days after sowing. Joshi *et*

al. (2009) also reported no phenotypic effects in *atmgl* mutants, although an increased level of methionine was observed in seeds and flowers. Methionine homeostasis operates on a negative feedback loop, meaning that in *atmgl* mutants methionine biosynthesis genes are accordingly down-regulated, providing an explanation for the lack of differential phenotype (Joshi and Jander, 2009; Amir, 2010). Previous findings indicate that *atmgl* mutants show no reduction in drought or salt tolerance and maintain normal isoleucine levels (Joshi and Jander, 2009). Here the *atmgl* mutant also exhibited no drought susceptibility, a phenotype again likely to result from redundancy with threonine deaminase (Joshi *et al.*, 2010). The over-expression of *ATMGL*, in contrast, severely affected the growth of aerial parts of the plant under normal conditions. The 35S line grew more slowly than normal, flowered later, accumulated less biomass and produced a smaller number of seeds. As 80 % of methionine is normally directed into SAM, the over-activity of ATMGL would convert excess methionine into the alternative pathway, depleting the pool used as methyl donors for essential plant processes such as DNA replication and methylation, cell wall synthesis and chlorophyll synthesis (Figure 3.34) and providing an explanation for the reduced growth phenotype. Although the 35S::*ATMGL* line showed no drought tolerance, these plants appeared resistant to nematode infection with *H. schachtii*, allowing only a quarter of successful infections compared to the wild type. There are several possible reasons for this. An enriched methionine concentration has been observed in the syncytia of *H. schachtii*, as well as an increase in transcription of methionine scavenging genes (Szakasits *et al.*, 2009; Hofmann *et al.*, 2010). As plant-parasitic nematodes are net consumers which depend on amino acids for protein synthesis from their hosts, the sink strength of syncytia is increased compared to normal root cells, leading to a high level of amino acid accumulation (Hofmann *et al.*, 2010). Amino acids may also accumulate in syncytia in order to protect against osmotic stress caused by water loss to the feeding nematode (Hofmann *et al.*, 2010). The depletion of available methionine by the over-expression of *ATMGL* may therefore inhibit nematode protein synthesis, preventing establishment and growth of nematodes. Ethylene is known to be important for the establishment of nematode feeding sites, as ethylene insensitive mutants show resistance to nematode parasitism whilst plants that over-produce ethylene have heightened susceptibility (Wubben *et al.*, 2001; Lilley *et al.*, 2005). Channelling of methionine into the alternative non-SAM catabolism pathway may limit the amount of

ethylene available and hinder parasitism. Finally, the increased production of isoleucine due to *ATMGL* over-expression may boost levels of JA-Ile, creating a heightened defence mechanism which could respond more effectively to the nematode invasion.

In conclusion, the homeostasis of methionine and isoleucine and the activity of ATMGL may have an important role in the response of *A. thaliana* to multiple stresses. Indeed Rizhsky *et al.* (2004) found that *ATMGL* was up-regulated in response to a combination of heat and drought stress. Under osmotic stress such as drought, ABA induces the expression of *ATMGL*, which converts cellular methionine into the osmolyte isoleucine. *ATMGL* is also induced in response to various pathogens. This may have the effect of increasing the amount of isoleucine available for converting into the active defence hormone JA-Ile, whilst limiting the flow of methionine into pathways beneficial to pathogens, such as conversion to ethylene. Too much *ATMGL* can have the effect of limiting growth and seed production. This may explain why in *A. thaliana* roots, an increase in *ATMGL* transcript was only observed in response to combined nematode and dehydration stress, when channelling methionine into the isoleucine pathway may provide protection from both biotic and abiotic stresses.

3.4.10 The role of a senescence-associated *DUF581* gene (At5g65040)

A previously un-characterised gene, At5g65040, was found to be one of the most highly up-regulated 'interaction' genes in roots. The gene contains a Domain of Unknown Function (DUF) category 581. Eighteen genes in total carry this domain, of which the function of very few has been elucidated (The Arabidopsis Information Resource (TAIR), www.arabidopsis.org). One of the DUF581-containing genes is Senescence-Associated Gene *SAG102* (At2g44670), which has led to the other DUF581 genes becoming labelled as 'senescence-associated protein-related'. This gene becomes up-regulated in senescing leaves and also in response to viral infection (He *et al.*, 2001; Espinoza *et al.*, 2007), and is considered to be a marker of oxidative stress (Aghdasi *et al.*, 2008). *SAG102* is up-regulated by ABA (Genevestigator), a hormone which positively regulates senescence. Another DUF581 gene (At1g22160) is positively regulated by drought stress and ABA (Huang *et al.*, 2008).

The DUF581 gene identified in this study (At5g65040) was induced to an extent in roots in response to drought stress, and further in response to joint stress. In the *abi2-1*

mutant the expression level was higher than in the wild type under all conditions (Figure 3.27C). The expression was particularly high in the ABA mutant in response to nematodes and joint stress, although there was no increase following drought stress. These findings suggest a multiple regulatory system for the DUF581 gene. ABA may repress its expression except under drought stress, whilst under nematode or joint stress another mechanism induces its expression. DUF581 transcript level was not affected in any other hormone mutant studied, so this additional regulation may be due to another signalling pathway such as that of salicylic acid. No stress tolerance or susceptibility was observed in the *duf581* knock-out mutant, although this mutant did suffer from a slight slow growth and stunted root phenotype. No over-expression line was studied, as the expression levels of DUF581 in the homozygous 35S line were found to be below that of the wild type. This may have been due to transgene silencing of the 35S promoter, a phenomenon that has previously been observed in tobacco and other plants (Elmayan and Vaucheret, 1996). From the evidence gathered here it is difficult to propose a function for DUF581 in plant response to multiple stress. However, a possible role for this gene may be in wounding and pathogen-related senescence. This would explain its induction due to nematode and joint stress in the absence of ABA, a hormone which would usually inhibit pathogen responses. The combination of biotic and abiotic stresses may create an enhanced drive towards senescence in certain tissues in order to protect remaining plant tissues from stress-related damage.

3.4.11 The role of *MYB4* (At4g38620) as a regulator of multiple stress response

MYB transcription factors control key regulatory mechanisms in the response of plants to various stresses, and are thought to be important in controlling cross-talk between different stress signalling pathways (Mattana *et al.*, 2005; Vannini *et al.*, 2007; AbuQamar *et al.*, 2009; Dubos *et al.*, 2010). MYBs are also involved in the stress-related production of secondary metabolites in the phenylpropanoid pathway such as anthocyanins and lignin, and in the regulation of cell wall biosynthesis (Jin *et al.*, 2000; Patzlaff *et al.*, 2003; Wuyts *et al.*, 2006a; Dubos *et al.*, 2010). Due to their role in different stresses, MYBs have been targeted as candidates for the improvement of broad-spectrum stress tolerance (Jin *et al.*, 2000; Vannini *et al.*, 2004). Ten MYB transcription factors were amongst the interaction genes found to be differentially regulated by joint stress compared to individual stress, of which *MYB4* had the highest fold change. *MYB4* actually showed a slight transcript increase as a result of nematode

or dehydration stress individually, but when the two stresses were applied together the gene was repressed compared to the control. However, when analysed in soil-grown plants, *MYB4* was repressed by both drought and joint stress. MYB4 is a transcriptional repressor that is known to regulate UV-B response. Plants protect themselves from UV damage by producing phenolic compounds such as flavonoids and hydroxycinnamate esters (Hemm *et al.*, 2001). When high UV-B levels occur, *MYB4* becomes down-regulated thus allowing the transcription of cinnamate-4-hydroxylase, the rate limiting step in the production of sinapate ester sun-protection compounds (Jin *et al.*, 2000; Hemm *et al.*, 2001). Essential for this process is SAD2, which traffics MYB4 to the nucleus, allowing it to bind to its own promoter in an auto-regulatory loop (Zhao *et al.*, 2007). SAD2 also plays a specific role in ABA signalling, meaning that *sad2* mutants are sensitive to ABA and to low water potential (Verslues *et al.*, 2006).

Homologues of *A. thaliana* MYB4 (*AtMYB4*) have been identified in other plants, where they also play a role in stress responses and the production of secondary metabolites. In pine, MYB4 induces lignification during wood formation. When ectopically expressed in tobacco this gene causes an increase in lignification, even in cell types not normally lignified (Patzlaff *et al.*, 2003). The *MYB4* gene from rice (*OsMYB4*) is of particular interest in stress signalling. Originally identified due to its up-regulation in cold-treated rice, when over-expressed in *A. thaliana* the gene conferred resistance to chilling and freezing stresses in accordance with its level of over-expression (Vannini *et al.*, 2004). The accumulation of sugars and compatible solutes also conferred tolerance to water deprivation (Mattana *et al.*, 2005). In *A. thaliana* plants over-expressing *OsMYB4*, 254 genes were found to be up-regulated, including those responsive to drought, salt and oxidative stress, but also to pathogen attack (Vannini *et al.*, 2006). Around 22% of these were gene expression regulators themselves. Induced genes included amino acid metabolism genes such as S-adenosylmethionine synthetase 1 (*SAM1*), defence genes such as *PR1*, cell wall re-modification genes such as pectin methyl esterase inhibitors, and many genes involved in the general phenylpropanoid pathways leading to production of flavonoids, lignins and anthocyanins. In addition to the stresses mentioned above, *OsMYB4* plants also displayed resistance to drought, salt, UV, ozone, viruses, bacteria and fungi (Vannini *et al.*, 2006). Plant response to UV-B radiation and herbivore defence are known to be linked. UV-B triggers the production of compounds such as flavonoids, phenolic

compounds, chlorogenic acid and other phenylpropanoid derivatives, which in turn inhibit insect foliar feeding, a process controlled by the defence hormone jasmonic acid (Caputo *et al.*, 2006; Izaguirre *et al.*, 2007). Flavonoid synthesis is also induced by infection with cyst and root-knot nematodes, and changes to the flavonoid biosynthetic pathway can affect nematode reproduction rate (Wuyts *et al.*, 2006a; Wuyts *et al.*, 2006b, Ithal *et al.*, 2007a; Jones *et al.*, 2007; Klink *et al.*, 2010). This may explain the close connection between different signalling pathways as influenced by *MYB4*. When over-expressed in tomato, *OsMYB4* conferred drought tolerance due to the accumulation of sugars and compatible solutes, as well as virus tolerance (Vannini *et al.*, 2007). The results suggest that *OsMYB4* is a central player in the coordination of multiple stress tolerance systems, and that this orchestration of cross-talk is conserved across species. Cinnamate-4-hydroxylase was also activated by *OsMYB4*, indicating a similar function as *AtMYB4* in the UV-B protection pathway but as a positive regulator instead of a negative one.

There is clearly a high level of similarity between the well-characterised *OsMYB4* and the little-studied *AtMYB4*. It has been suggested that rice *MYB* genes may act as transcriptional repressors in some plant tissues and activators in others (Suzuki *et al.*, 1997), therefore *AtMYB4* may act as a transcriptional repressor whilst *OsMYB4* is an activator. If this were true, one might have expected to see drought resistance or susceptibility in the *myb4* knockout and 35S::MYB4 over-expression plants, respectively, in the current study. This was not the case. However, Vannini *et al.* (2004) reported that cold and freezing tolerance occurred in a dose-dependent manner depending on the level of *OsMYB4* expression. As the 35S::MYB4 line used here had only a 4-fold induction, the drought assays used may not have been sensitive enough to detect any changes in stress tolerance. The down-regulation of *AtMYB4* in response to combined dehydration and nematode stress may represent a strategic shift in the plant stress response system. Dehydration and nematode stress individually elicit specific responses. However, certain defence mechanisms could clearly provide benefits under both types of stress, including the accumulation of secondary metabolites of the phenylpropanoid pathway such as lignins, cell wall re-modification, amino acid metabolism and solute accrual. Therefore when the two stresses occur together, the down-regulation of the negative repressor *MYB4* would allow a range of broad-

spectrum abiotic and biotic stress response systems to become activated, providing greater protection from any additional stresses.

Phenylpropanoid pathways are important for the development of normal pollen in *A. thaliana*, for example in the production of sporopollenin which is the major constituent of the pollen cell wall, as well as flavonoids which provide structure and UV protection (Preston *et al.*, 2004). Disruption of the expression levels of *AtMYB4* and the closely-related *AtMYB32* produces abnormal pollen grains (Preston *et al.*, 2004), an observation that could explain the fact that both *myb4* and 35S:MYB4 lines showed a reduced seed yield compared to wild type plants in this study (Figure 3.19). The negative impact on growth in both lines may have been due to a mis-allocation of resources into the phenylpropanoid pathway, thus interfering with normal plant processes.

3.4.12 Concluding remarks

Here several genes that may play a role in plants' response to multiple stresses have been identified. As the stress-induced fold change of each of these is relatively small, this suggests that the multiple stress response is controlled by a large number of genes, each with a small effect (Feder and Walser, 2005; Swindell, 2006). The orchestration of this effect is highly complex, involving the interaction of different hormones, transcription factors and signalling molecules, and influencing such processes as amino acid homeostasis, immune system priming, cell wall re-modelling, senescence and growth inhibition. In particular we have observed the importance of small signalling molecules and the role of the phenylpropanoid pathway in creating secondary metabolites such as lignins for defence and cell protection. As plants experience a greater number of concurrent stresses their defence systems may become more generalised, involving a wider variety of processes and physiological adjustments to create a broad-spectrum stress tolerance.

Chapter 4. The interaction of drought and nematode stress in tomato

Aims

- Investigate the effect of nematode infection on the response to drought stress and the effect of early drought stress on nematode susceptibility in tomato.
- Examine the effect of individual or combined drought and nematode stress on flowering and fruiting characteristics.
- Analyse the nutritional quality parameters of tomato fruits from plants subjected to joint drought and nematode stress by measuring concentrations of fruit carotenoids, flavonoids, chlorogenic acid and sugars.

4.1 Introduction

4.1.1 Tomato as a model crop to study stress interaction

A. thaliana is an excellent model system in which to study the molecular pathways involved in plant stress responses under laboratory conditions (see Section 1.1). The results obtained from such work provide a basis for the understanding of stress responses in agriculturally or economically important crop plants, ultimately presenting possibilities for improvement of stress tolerance in crops. Such transfer of knowledge from basic to applied plant science has been recognised as a priority for the establishment of future food security (Umezawa *et al.*, 2006; BBSRC, 2009; Mittler and Blumwald, 2010; Zurbriggen *et al.*, 2010). Tomato (*Solanum lycopersicum*) is an agricultural crop whose response to both drought and nematode infection has been well characterised. In particular it is an excellent host for nematodes of *Meloidogyne* spp, which disrupt water relations within the plant, inhibiting growth and causing a deleterious effect on fruit yield (Wallace, 1974; Barker *et al.*, 1976; Dorhout *et al.*, 1991). Tomato can also act as a host for the potato cyst nematode *Globodera pallida.* The pathogen-induced systemic acquired immunity (SAR) response in tomato is well studied, and its activation can be identified through the induction of pathogenesis-related (PR) genes (Kavroulakis *et al.*, 2005; Sanz-Alferez *et al.*, 2008). Agricultural

losses due to nematodes can be high, with up to 20 % yield loss reported in areas of the USA with high tomato cultivation such as California (Koenning *et al.*, 1999).

Tomato is commonly grown in parts of the world with a Mediterranean climate, characterised by warm temperatures and aridity. Competition for water resources is high, so increasing plant water use efficiency has become a valuable target (Costa *et al.*, 2007; Semel *et al.*, 2007; Favati *et al.*, 2009; Patane and Cosentino, 2010). Several successful attempts have been made to increase drought tolerance in tomato plants by inserting transgenes such as a rice MYB transcription factor or an *A. thaliana* CBF transcription factor (Lee *et al.*, 2003; Vannini *et al.*, 2007). The study of drought stress in tomatoes is facilitated by measurements such as stomatal conductance and leaf relative water content (RWC) (De Pascale *et al.*, 2007; Vannini *et al.*, 2007). Tomato is therefore an excellent system in which to investigate the interaction of biotic and abiotic stresses. A study of the interaction of *M. incognita* infection and mineral pollutants on tomato crops in India revealed a synergistic effect whereby nematode infection worsened the effects of pollution on foliage, whilst increasing pollution caused greater root galling (Khan and Khan, 1996). Other studies have reported an interaction effect between abiotic stresses and infection with fungal or bacterial pathogens. Evidence suggests that this is mediated by ABA, which accumulates in response to abiotic stress and can disrupt normal pathogen defence systems, purportedly by negatively regulating the salicylic acid defence pathway (Audenaert *et al.*, 2002; Achuo *et al.*, 2006; Asselbergh *et al.*, 2008a). For example, resistance to the bacteria *Erwinia chrysanthemi* and the fungus *Botrytis cinerea* is increased in the tomato ABA-deficient *sitiens* mutant, although drought stress can also enhance resistance to *B. cinerea* and *Oideum neolycopersici* (Audenaert *et al.*, 2002; Achuo *et al.*, 2006; Asselbergh *et al.*, 2008a). In the *sitiens* plants, the phenylpropanoid biosynthetic pathway is activated more strongly following pathogen attack, with greater accumulation of SA-induced defence gene transcripts such as *PR1*. (Audenaert *et al.*, 2002; Asselbergh *et al.*, 2008a). The effect of drought and the associated ABA accumulation may therefore influence nematode infection in tomato, a possibility that has not previously been investigated. Furthermore the effect of nematode infection on drought response systems remains to be elucidated.

4.1.2 The link between fruit nutritional compounds and plant stress

Tomatoes contain various compounds that are potentially beneficial to human health. As the world's third most important vegetable after potato and cassava (http://faostat.fao.org), tomato plays a significant role in diet and nutrition globally. With increasing interest in so-called functional foods, tomato has become the focus of many studies investigating the factors that influence nutritional quality. The levels of beneficial compounds in tomatoes are known to vary depending on the cultivar (Leonardi *et al.*, 2000; Giuntini *et al.*, 2008; Guil-Guerrero and Rebolloso-Fuentes, 2009; Slimestad and Verheul, 2009), ripening stage (Slimestad and Verheul, 2005; Riggi *et al.*, 2008) and growth conditions (Dumas *et al.*, 2003; Semel *et al.*, 2007; Dorais *et al.*, 2008; Favati *et al.*, 2009; Pernice *et al.*, 2010), as well as their exposure to environmental stress (Mitchell *et al.*, 1991; EnglishLoeb *et al.*, 1997; Ruelas *et al.*, 2006; Subramanian *et al.*, 2006; De Pascale *et al.*, 2007; Saito *et al.*, 2008; Lovdal *et al.*, 2010).

When plants are subjected to drought or osmotic stress, the resulting reduction in photosynthesis means that chloroplasts are exposed to excess excitation energy, triggering the production of active oxygen species such as singlet oxygen and hydrogen peroxide (Smirnoff, 1993; Noctor and Foyer, 1998). These products can be extremely harmful to plant cells, causing oxidative damage and inactivation of enzymes. In order to minimise damage, cells produce antioxidants that scavenge active oxygen species (Smirnoff, 1993; Noctor and Foyer, 1998). Several of these compounds confer health benefits related to their antioxidant activity when present in the diet, including carotenoids, flavonoids and other phenolic compounds (Hertog *et al.*, 1993; Mayne, 1996; Sawa *et al.*, 1999; Rao and Agarwal, 2000; Nijveldt *et al.*, 2001; Bassoli *et al.*, 2008). Breeding programmes and genetic manipulation studies have aimed to enhance antioxidant levels in tomato to increase consumer benefits (Romer *et al.*, 2000; Muir *et al.*, 2001; Niggeweg *et al.*, 2004; Frusciante *et al.*, 2007; Cle *et al.*, 2008).

Carotenoids are potent antioxidants abundant throughout plants such as tomatoes, and are important at times of water deficit in dissipating excess heat in chloroplasts (Smirnoff, 1993). Lycopene accounts for 80-90 % of total carotenoids in tomato and exhibits the highest ability to quench singlet oxygen species (Di Mascio *et al.*, 1989; Dumas *et al.*, 2003). In humans, lycopene consumption has been associated with a

reduction in the risk of prostate and other cancers, as well as protection against cardiovascular disease (Clinton *et al.*, 1996; Rao and Agarwal, 2000; Giovannucci, 2002). Another important carotenoid antioxidant in tomatoes, β-carotene, is the precursor for vitamin A, and its consumption has been correlated with a reduced risk of chronic disease such as of the cardiovascular system (Olson, 1989; Mayne, 1996). Flavonoids are a diverse group of phenolic secondary metabolites known to have several functions in plants. They act in the protection of plant tissues during oxidative stress and from UV-B damage; as anti-feedants induced during defence responses to insects, fungi or nematodes; during defence-induced lignification; as signalling molecules in establishing symbiotic relationships with rhizobia; and as regulators of auxin transport (Nicholson and Hammerschmidt, 1992; EnglishLoeb *et al.*, 1997; Williams *et al.*, 2004; Ruelas *et al.*, 2006; Treutter, 2006; Giuntini *et al.*, 2008). Flavonoid consumption is associated with protection against cardiovascular disease, cancer and age-related diseases in humans, where there is evidence that the antioxidant activity slows the ageing of cells and prevents lipid peroxidation (Hertog *et al.*, 1993; Manach *et al.*, 1995; Vinson *et al.*, 1995; Knekt *et al.*, 1996; Nijveldt *et al.*, 2001; Le Gall *et al.*, 2003). The most abundant flavonoids in tomato are chalconaringenin, which possesses anti-allergic properties, rutin, and naringenin (Yamamoto *et al.*, 2004; Slimestad *et al.*, 2008). Chlorogenic acid is one of the principle non-flavonoid phenolic compounds in tomatoes (Hung and Rohde, 1973; Niggeweg *et al.*, 2004). It is involved in the protection of plants from UV, accumulates following drought stress, and has been shown to be important in the response of resistant *Solanaceous* plants to infection with root-knot nematodes of *Meloidogyne* spp (EnglishLoeb *et al.*, 1997; Pegard *et al.*, 2005; Cle *et al.*, 2008). As well as being a potent and widespread antioxidant, chlorogenic acid has anticarcinogenic, antiviral and antidiabetic properties in humans (Laranjinha *et al.*, 1994; Sawa *et al.*, 1999; Farah and Donangelo, 2006; Bassoli *et al.*, 2008). Despite the association between consumption of these compounds and health benefits, dietary intervention studies have not always shown a causative effect on prevention of cardiovascular disease (Mayne, 1996; Giovannucci, 2002).

Due to the connection between plant antioxidants and human health benefits, and as a mechanism of reducing irrigation in arid areas, it has been proposed that a cultivation system exposing tomato plants to controlled levels of stress could be of use in improving the nutritional quality of fruits (Mitchell *et al.*, 1991; Costa *et al.*, 2007; De

Pascale *et al.*, 2007; Patane and Cosentino, 2010). Varied levels of success in increasing carotenoid and sugar concentrations have been reported as a result of water deficit or salinity stress (Zushi and Matsuzoe, 1998; Veit-Kohler *et al.*, 1999; Saito *et al.*, 2008; Pernice *et al.*, 2010). However, the results are often conflicting (Dumas *et al.*, 2003). Water deficit has been shown to cause a reduction in the levels of carotenoids such as lycopene in tomato fruits in some cases (De Pascale *et al.*, 2007; Riggi *et al.*, 2008), while in other studies water stress gave rise to a higher level of lycopene and total carotenoids (Matsuzoe *et al.*, 1998; Zushi and Matsuzoe, 1998; Pernice *et al.*, 2010). β-carotene levels increase (Riggi *et al.*, 2008) or remain unchanged in response to water stress (Zushi and Matsuzoe, 1998), or in one study decrease with moderate water stress but increase with severe water stress (Pernice *et al.*, 2010). Levels of flavonoids in tomato plants are also affected by water stress. Pernice *et al.* (2010) reported that although the accumulation of total flavonoids was heightened in fruits from plants under moderate water stress, the concentration of naringenin was actually lower under extreme water deficit. A study of phenolic compounds in the leaves of tomato plants revealed an increase in both rutin and chlorogenic acid due to water stress (EnglishLoeb *et al.*, 1997). However, one of the main functions of flavonoids in plants is as UV-protectants, and therefore the primary factors affecting variation in their accumulation tend to be UV levels and general light conditions (Stewart *et al.*, 2000; Giuntini *et al.*, 2008). The concentration of sugars in tomato fruits is often used as an assessment of nutritional quality, through contribution to flavour parameters and also because vitamin C is synthesised from sugars supplied through photosynthesis (Lee and Kader, 2000; Dorais *et al.*, 2008). Glucose and fructose concentrations in tomato fruits have been shown to increase in plants under water or salt stress, thus contributing to a higher fruit quality flavour (Gao *et al.*, 1998; Zushi and Matsuzoe, 1998; Auerswald *et al.*, 1999; Veit-Kohler *et al.*, 1999; Yin *et al.*, 2010). Despite these changes in nutritional compounds, even low levels of stress can have a negative impact on the yield and fruit ripening time, often counteracting the benefit of such measures (Mitchell *et al.*, 1991; Subramanian *et al.*, 2006; Dorais *et al.*, 2008).

There are no reports in the literature describing the effect of a combination of stresses on the nutritional qualities of tomato. The addition of a biotic stress factor to a system already imposing abiotic stress may confound any positive effects. Previous transcriptome studies on multiple stress response, as well as the findings observed in *A.*

thaliana in Chapters 2 and 3 of this thesis, have revealed that plants respond very differently to combined stress than to each individual stress, to the extent of activating an entirely new program of gene expression (Rizhsky *et al.*, 2004). It is also known that the signalling pathways for abiotic and biotic stress responses may interact and inhibit each other, allowing the plant to adapt most efficiently to the environmental situation (Anderson *et al.*, 2004; Asselbergh *et al.*, 2008b; Yasuda *et al.*, 2008). Therefore, it cannot be assumed that the concentrations of nutritional compounds that accumulate due to water stress or pathogen attack would be additive if the two stresses occurred together.

The purpose of this study was to examine the effect of concurrent drought stress and nematode infection on tomato plants. Plant growth, flowering, fruit ripening and fruit yield was measured in order to determine the possible synergistic or antagonistic effect of the two stresses. Furthermore, the effect of joint abiotic and biotic stress on nutritional quality parameters was determined through investigation of the levels of fruit carotenoids, flavonoids, chlorogenic acid and sugars.

4.2 Methods

4.2.1 Species used

- *Solanum lycopersicum* cv. Ailsa Craig (Tozer Seeds)
- *Solanum lycopersicum* cv. Shirley F1 (Tozer Seeds)
- *Globodera pallida* (Pa 2/3)
- *Meloidogyne incognita*

4.2.2 Growth of tomato

Seeds of tomato (*Solanum lycopersicum*) cv Shirley F1 or Ailsa Craig were sown in trays of compost in a greenhouse with a constant temperature of 25 °C. After two weeks plants were transferred to 9 cm pots containing standard 3-4 month slow-release fertiliser (25 g/L soil) and after another 2 weeks plants were transferred to 18 cm pots.

4.2.3 Maintenance and hatching of nematodes

4.2.3.1 Maintenance of nematode stock cultures

G. pallida cysts were obtained by planting a potato tuber (*Solanum tuberosum* cv Désireé) into soil containing *G. pallida* eggs at a concentration of 25-40 eggs/g. After 10-12 weeks, aerial parts of the plants were separated from the roots and the soil was left to dry before being stored at 4 °C. Egg counts were carried out as described in Section 2.2.2.2.

Colonies of *M. incognita* were maintained on soil-grown tomato plants in greenhouse conditions at 25 °C. Every 8 weeks the colony was propagated by planting new tomato seedlings into soil containing chopped roots of the previous plants. These roots contained mature *M. incognita* females carrying egg masses.

4.2.3.2 Extraction, sterilization and hatching of **Globodera pallida** *cysts*

G. pallida was hatched using a similar method to that used for *H. schachtii.* However, potato root diffusate was used as a hatching medium instead of zinc chloride. Root diffusate was obtained by applying tap water to 26-day old potato plants growing in perlite, and collecting the flow-through in a beaker. The diffusate was then diluted 1:4 with tap water and filter sterilised before use.

4.2.2.3 Collection of Meloidogyne incognita *pre-parasitic juveniles*

To obtain juveniles for use in plant infections, roots of tomato plants that had been infected approximately 8 weeks previously with *M. incognita* were washed to remove the soil and chopped into small pieces. Roots were laid on sections of nylon mesh held over funnels and placed in a misting chamber. A fine, warm mist of tap water encouraged hatching and washed the juvenile nematodes through the mesh, funnel and into 50 ml collecting tubes. As *M. incognita* juveniles began to hatch the tubes were replaced every 24 hours and the nematodes which had accumulated in the bottom were stored in tap water at 10 ºC.

4.2.4 Infection of tomato with *G. pallida* and *M. incognita*

Infection with juveniles: Nematode juveniles were watered directly onto the tomato roots in the soil. Three large pipette tips were inserted to a depth of 2 cm next to the stem of each tomato plant. A total of 500 *M. incognita* or 1000 *G. pallida* J2s in 1 ml of sterile water were applied to each tip and washed down with a further 1 ml of water. Control plants were mock-inoculated with 2 ml water.

Infection with *M. incognita* eggs: Root balls from tomato stock plants infected with *M. incognita* were removed from the soil when nematodes reached maturity and egg masses were visible on the surface of the roots. The root systems were washed to remove compost, finely chopped and an egg count carried out on a 1 g sample. This sample was shaken in 0.5% sodium hypochlorite for 5 minutes to remove the egg masses from the roots, and then the eggs were counted using a Pieter's Counting Slide. Nematode infection of tomato plants was carried out by mixing an exact weight of these infected roots with compost to achieve a final infection rate of 10 eggs per gram of soil.

Infection with *G. pallida* eggs: Young tomato plants were transplanted into soil containing *G. pallida* cysts at a concentration of 50 eggs/g.

4.2.5 Investigating the systemic response to nematode infection

One week following infection with juveniles of *G. pallida* or *M. incognita*, tissue samples were taken from leaves of Ailsa Craig tomato plants. Samples were taken from the youngest fully-unfurled leaf at the top of the plant and from the oldest leaf at the bottom of the plant. Samples were ground in liquid nitrogen with sterile, RNase-treated pestles and mortars, and RNA extracted from 100 mg of ground tissue as described in Section 2.2.4. Samples from 3 plants for each treatment were pooled, and cDNA was

synthesised (Section 2.2.5). Several pathogenesis-related (*PR*) genes were selected for expression analysis. Induction of these genes had previously been reported to change in response to nematode infection or fungal pathogens and can thus be used as marker genes for nematode-induced defence systems (Bar-Or *et al.*, 2005; Kavroulakis *et al.*, 2005; Sanz-Alferez *et al.*, 2008; Wubben *et al.*, 2008). The sequence of tomato pathogenesis-related genes was obtained from the NCBI website (http://www.ncbi.nlm.nih.gov), and qRT-PCR primers were designed to amplify four genes, *PR1*, *PR1a2*, *PR2b* and *PR3*. The housekeeping gene Eukaryotic Initiation Factor 3 (*EIF3*) was chosen as a normalisation gene. This gene had previously been used as a normaliser (Fuller *et al*. 2007, unpublished) and was originally identified from the TIGR Tomato Gene Index website (http://compbio.dfci.harvard.edu/tgi/gi/lgi/GenInfo. html). The expression levels of *EIF3* showed no significant difference between samples. The primer sequences and accession numbers are detailed in Appendix 2. qRT-PCR was carried out as detailed in Section 2.2.6 (and Appendix 1 A and B) to compare the expression of PR genes in infected leaves compared to un-infected controls.

4.2.6 Tomato drought physiology

Physiological measurements were taken of tomato plants under drought and nematode stress. Ailsa Craig plants were transplanted 28 days after sowing into either clean compost or compost containing 50 eggs/g *G. pallida* (12 plants each). Drought was imposed by the withholding of water starting 21 days after nematode infection. Drought stress was assessed by measuring the relative water content (RWC) of the leaves, as described by Vannini *et al.* (2007), at intervals of 3-4 days. Six typical leaves were selected ranging from the top to the bottom of the plant. The fresh weight (FW) of the leaves was recorded and then the leaves were placed on filter paper saturated with distilled water in a Petri dish for 24 hours to determine the turgid weight (TW). The leaves were then dried at 70 ºC for 24 hours to measure dry weight (DW). Relative water content was calculated as a percentage using the following formula:

$$RWC = (FW - DW) / (TW - DW)$$

The length of the five longest leaves of each plant was also measured 8 days after drought imposition.

4.2.7 Drought pre-treatment of tomato plants

Fourteen-day old seedlings of Ailsa Craig were transplanted into 9 cm pots containing moist compost:sand:loam at a ratio of 2:1:1. Half the plants were then maintained at field capacity whilst half the plants received no water for the following 14 days, comprising the drought pre-treatment. Sixteen plants were used per treatment. At this point stomatal conductance readings were taken, and the plants were then all watered to field capacity. After another 14 days of normal growth, plants were re-potted into 15 cm pots containing *M. incognita* eggs at a concentration of 10 eggs/g, as described in Section 4.2.4. The number of eggs per gram of infected stock plant root was determined to be 11899. Nematode infection of tomato plants was thus carried out by mixing 1.15 g of infected roots with 1368 g of compost to achieve a final infection rate of 10 eggs per gram of soil. Twenty-one days after infection, samples were harvested from 10 of the pre-treated and 10 of the control plants in order to determine nematode infection rate. The root systems of all the plants were washed and stained using acid fuchsin (Section 2.2.2.7). As the root systems were extremely large and heavily infected with nematodes, 20 x 5 cm sections were selected at random from each sample and the nematodes counted. The other 6 plants of each treatment type were left until the brown egg masses were visible on the surface of the roots (another 38 days). Whole root systems were then harvested to determine the reproductive success of the nematodes. The roots were shaken in sodium hypochlorite for 5 mins, then the resulting liquid sieved through a mesh to remove debris and the eggs counted.

4.2.8 Joint drought stress and infection with *M. incognita*

4.2.8.1 Plant stress treatments

Seedlings of tomato (*Solanum lycopersicum)* cv Shirley F1 were grown for five weeks and then divided into four treatment groups of 8 plants each: unstressed, water stress, nematode infection and joint stress (combined water stress and nematode treatment). Plants were transferred into 18 cm pots of either normal compost or compost containing 10 eggs/g *Meloidogyne incognita* (Section 4.2.4). All the tomato plants were irrigated to field capacity during the following 12 days, to allow time for the juvenile nematodes to hatch and invade the tomato root system. Water stress was then initiated in the water stress and joint stress treatment groups. The plants were submitted to a daily water regime whereby the well-watered plants received an equal amount of water to that

evapotranspired the previous day, as measured by weighing the entire pot after watering and again 24 hours later. Plants undergoing water stress treatment received only 80 % of the water evapotranspired the previous day. This treatment was continued for 3 weeks, after which all plants were watered to field capacity for the remainder of the experiment. Stomatal conductance was measured before and after the period of water stress. A schematic diagram of the experimental time span is shown in Figure 4.1.

4.2.8.2 Physiology measurements

The height of all plants was measured after the period of drought stress. Following this, the apex of the plant was removed after the 5th truss had emerged, and the plants supported using wires. Flowers were tagged on the day of anthesis, in order to determine the time taken for flowers to develop and also for fruits to ripen. Fruits were harvested on the first day of the red ripe stage, the ripening stage at which the fruit is usually consumed. Fruits are considered red ripe when red colour covers at least 90 % of the epidermis (Jones, 2008). Figure 4.2A shows the ripening stages of fruits from unstressed plants. Fruits were weighed on ripening. After the last fruits had ripened, the stomatal conductance of the plants was measured. Physiological measurements were combined from 8-9 plants per treatment group, and 4-5 fruits per truss.

4.2.8.3 Preparing samples for nutritional analysis

For the analysis of nutritional compounds, 18 fruits were sampled per treatment group. Of these, three tomatoes were harvested from each of 3 plants at truss position 2, and 3 fruits from each of 3 plants at truss position 5. Truss 2 was a lower region of the plant that produced fruits at an early time point (ripening approximately 108 days after planting). Truss 5 was at the top of the plant, and the fruits developed later (ripening approximately 126 days after planting). On harvesting, tomatoes were cut in half. Hexose sugars are soluble solids are found throughout the fruit, thus the entire tomato half including the locular jelly and seeds was used in the analysis of sugars (Jones, 2008). In contrast, 72-92 % of the lycopene content and 98 % of flavonols occur in the insoluble fraction or the tomato skin (Sharma and LeMaguer, 1996). Thus, for the analysis of flavonoids and phenolics, a section of epidermis and outer pericarp approximately 30 mm wide was sampled (Figure 4.2B).

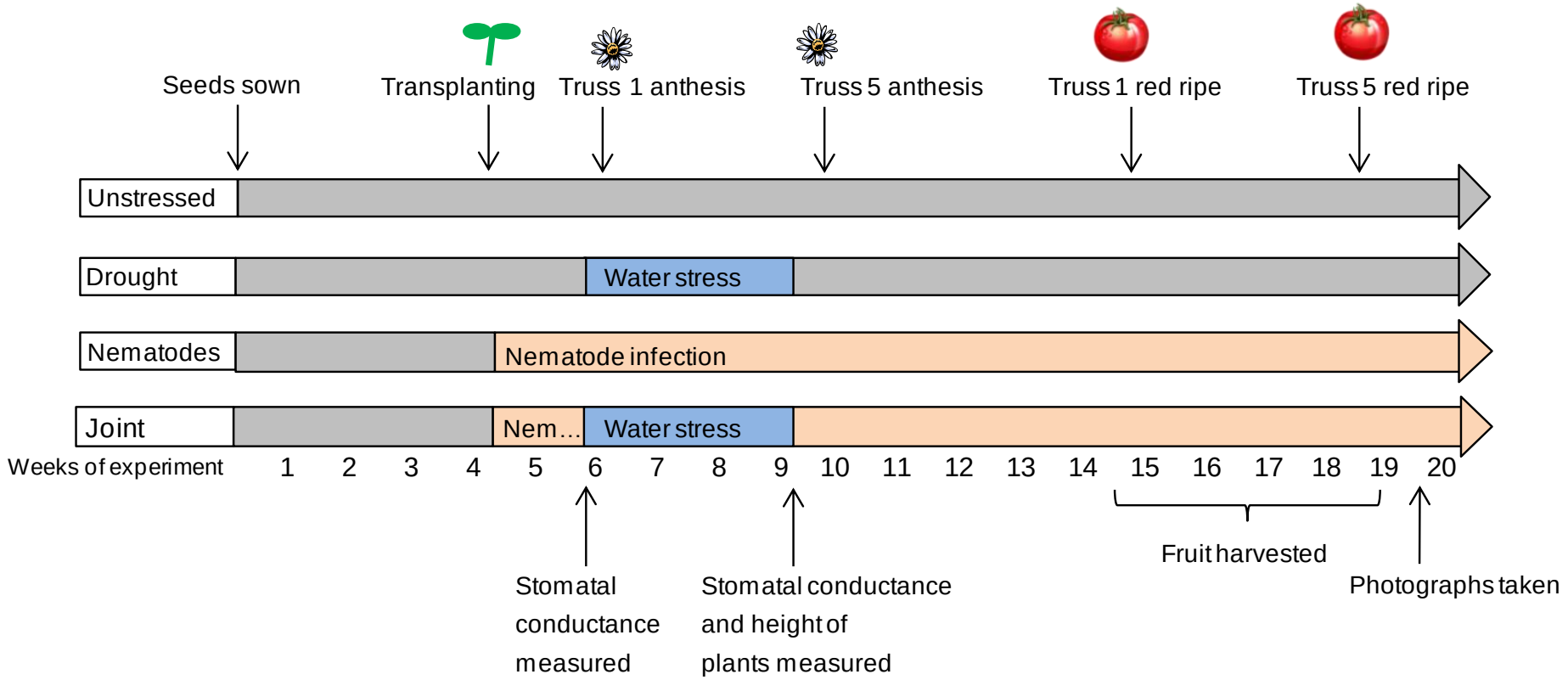


Figure 4.1. Schematic diagram showing experimental timescale of tomato nutritional analysis experiment. Seeds of *Solanum lycopersicum* cv Shirley F1 were grown in compost and then divided into four treatment groups. Plants for nematode infection were transplanted into soil containing 10 eggs/g *M. incognita* after 4 weeks of growth. Water stress was imposed on the drought and joint stress treatment groups by irrigating with only 80 % of the water evapotranspirated the previous day, whilst well-watered plants received 100 %. Truss 1 was the first truss to develop fruit, at the base of the plant, and Truss 5 was the last (the apex was removed after this point). Tomato flowers were tagged at anthesis to record fruit ripening time. Tomato fruits were collected for analysis at the red ripe stage.

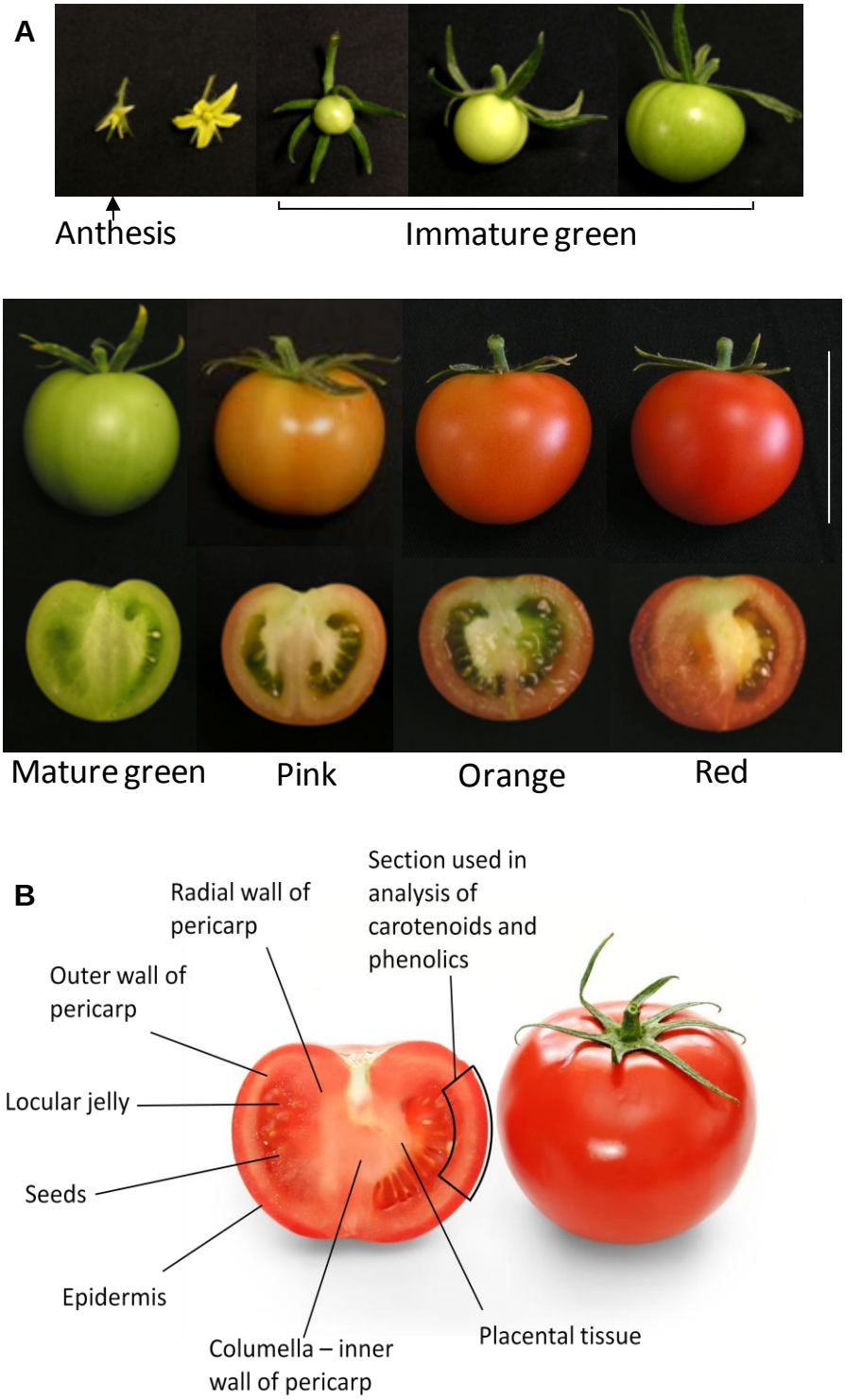


Figure 4.2. Development and anatomy of tomato fruit. A) Stages of tomato fruit development from anthesis (flowering) to red ripe stage. Fruits are considered red ripe when > 90 % of the epidermis shows a red colour (Jones 2008). Photographs are to scale and the white bar represents 5 cm. Under control conditions fruits took on average 60 days to ripen. **B**) Transverse section of a tomato fruit at red ripe stage. The main anatomical features are labelled. For the analysis of sugars the entire tomato half was sampled. For the analysis of carotenoids and phenolic compounds, a section was sampled consisting of the epidermis and outer pericarp. Photograph B is from www.digitalcut.pl

4.2.8.4 Extraction of phenolic compounds

Extraction of phenolic compounds (including chlorogenic acid and the flavonoids rutin, chalconaringenin and naringenin) was performed according to Giuntini *et al.* (2008) with some modifications. Peel-pericarp sections were freeze-dried using a LyoPro6000 lyophiliser (Heto) and ground to a powder in liquid nitrogen using a sterile pestle and mortar. A 25 mg portion of tomato powder was added to 2 ml 40 % aqueous ethanol containing 12.5 µg/ml of the internal standard morin (Apin Chemicals). This is a flavonoid which does not naturally occur in tomatoes. The sample was homogenised using an Ultra Turrax T-10 (IKA) for 5 minutes at approximately 20,000 rpm, and then centrifuged at 13,000 rcf for 10 mins. After centrifugation the supernatant was filtered using a 0.2 µm PTFE filter and used directly in LC-MS analysis. In order to validate the efficiency of this method, test analyses were also carried out on samples using 30 % or 20 % ethanol in the extraction process. In order to compare the possible loss of compounds through oxidation during sample storage, the effect of adding antioxidant was also evaluated. The antioxidants sodium metabisulphate or ascorbic acid were added to the extraction mixture at a concentration of 0.1 %. Samples were then incubated at 4 °C for 3 days, or used directly in LC-MS. In a subset of tomato segments the peel and pericarp were separated, weighed and analysed individually for phenolics.

4.2.8.5 LC-MS analysis of phenolic compounds

Quantification of tomato phenolic compounds was conducted using an LC-MS-MS system (liquid chromatography coupled with two phases of mass spectrometry). This system first separates compounds using standard reverse-phase HPLC, according to their polarity. The compounds are then vaporised into droplets and converted into ions (precursor ions) using a high voltage electrode (electrospray ionisation). Ions pass into the first Mass Spectrometry (MS) quadrupole and are filtered according to their mass/charge ratio. Ions then enter the collision cell where they are fragmented by collision with nitrogen to create product ions. The product ions are then separated by mass/charge ratio in the third quadrupole and passed through a detector. The system allows a high level of sensitivity and the ability to separate chemically similar compounds (Agilent Technologies, 2006). The HPLC system comprised a 1200 series micro-degasser, Binary SL pump, SL autosampler with a chiller module (set to 4^{o}C), column oven (set to 35^{o}C) and SL diode array detector (Agilent). A total of 5 µl of

tomato extract was injected onto a 150 x 2 mm 3 µm Luna PFP column (Phenomenex). Separation was achieved using an HPLC gradient of 0.2% aqueous formic acid (solvent A) versus 0.2% formic acid in LC-MS grade acetonitrile (solvent B). The flow rate was 0.3 ml/min. The gradient started at 15% solvent B, rising to 40% over 13 minutes and holding at 40% for another 2.2 min. To wash the column, the gradient then moved to 95% solvent B over 3.6 min, held for a further 3.6 min, then returned to 15% over 3.6 min. The column was re-equilibrated for a further 8.5 min before the next injection.

The eluent was directed into an Agilent 6410 triple quadrupole mass spectrometer (Agilent). The electrospray source was operated in negative mode, with a capillary voltage of 4000v, a drying gas temperature of 350^{o}C flowing at 11 litres/min, and a nebuliser pressure set to 30 psi. Tomato phenolic compounds of interest were quantified via multiple reaction monitoring (MRM), whereby the first and second MS analysers monitor for several specific user-defined precursor and product ions, respectively. Commercial standards were obtained for chalconaringenin, morin (both from Apin Chemicals), naringenin, chlorogenic acid and rutin (all from Extrasynthese) and used to determine optimal fragmentor and collision energy values for each compound, as well as the most favourable product ions to observe. The concentration of target phenolics was determined by creating standard curves spanning the full range of sample concentrations. The internal standard morin was used to normalise the response from other analytes.

4.2.8.6 Extraction of carotenes

Freeze-dried peel-pericarp sections were ground in liquid nitrogen and carotenes extracted in a two-phase separation as described by Lacker *et al.* (1999). Forty milligrams of tomato powder was mixed with 2.5 ml water and 2.5 ml MTBE (methyl tert-butyl ether), and extraction was carried out by shaking horizontally in a 15 ml tube at 37 °C for 10 mins. The organic phase containing the solubilised carotenes was centrifuged for 5 minutes at 13,000 rcf, diluted by half with MTBE, filtered using a 0.2 µm PTFE filter, and injected directly in HLPC.

4.2.8.7 HPLC analysis of carotenes

Quantitative determination of compounds was achieved using reverse phase HPLC. During reverse phase HLPC the analyte to be separated is introduced into a moderately

polar mobile phase which is then passed over a non-polar stationary phase adsorbed to a silica substrate within a column. The affinity of the analyte for the stationary phase depends upon its hydrophobicity. Polar compounds will elute quickly whereas more hydrophobic compounds are retained in the column longer. Being non-polar, carotenes can be separated using this system. The HPLC system consisted of a Shimadzu LC-20AD liquid chromatograph, autosampler, and SPD20A UV/VIS spectrophotometric detector (Shimadzu). Separation was accomplished using a YMC C_{30} carotenoid column (4.6 X 250 mm, 5µm particle diameter) (Waters). Due to the longer chain length this column improves retention times compared to its C_{18} predecessor, allowing a greater partitioning ability of carotene isomers, which are often structurally similar (Sander *et al.*, 1994). Chromatography was carried out according to Ishida *et al*. (2009) using an isocratic method and a mobile phase of MTBE/methanol/ethyl acetate (45:40:15) and a flow rate of 1 ml/minute for 27 minutes. Absorbance was measured at 450 nm, and chromatograms were analysed using LCsolution software (Shimadzu).

A standard curve was constructed using lycopene and β-carotene standards (Sigma-Aldrich) spanning the concentration range of the tomato samples. The exact concentration of the standards was calculated by using two identical aliquots of the compound and dissolving one in acetone and one in MTBE. The Beer-Lambert law was applied to the absorbance at 450 nm and the known absorption coefficient for each compound in acetone (140663 for β-carotene and 120600 for lycopene):

$$A = \varepsilon \times c \times d$$

where A is the absorbance, ε is the molar absorption coefficient ($Lmol^{-1}cm^{-1}$) of the compound at the specified wavelength, *c* is the molar concentration (mol/litre) of the compound, and *d* is the path length of the cuvette (Biehler *et al.*, 2010). The absorption coefficient of each compound in MTBE was then calculated, and the original exact concentration of the carotene standards deduced. Quantification of lycopene and β-carotene in the tomato samples was then achieved by comparing peak areas against the standard curves.

In order to identify smaller peaks in the chromatogram, a PDA-100 Photodiode Array Detector (Dionex) was employed to provide full absorption spectra at given time-points during chromatography. HPLC conditions were as specified above, and absorbance was

measured between 300 and 550 nm at a rate of 125 times per minute. Chromatograms were analysed using Chromeleon 6.5 software (Dionex).

4.2.8.8 Extraction of hexose sugars

Sugars were extracted from fresh tomato halves by homogenising the fresh fruit using an Ultra Turrax T-10 (IKA). Following this, 1 ml of homogenate was added to 4 ml 100 % ethanol and vortexed for 10 secs. Fucose (Sigma) was added as an internal standard at a concentration of 125 µg/ml. A hexose sugar occurring in yeast and some fungi, this compound is not naturally found in tomatoes. The samples were centrifuged at 600 rcf for 5 minutes and then 120 µl of the supernatant was evaporated and re-suspended in 600 µl water. The solution was filtered using a PTFE filter primed with methanol, and used in anion-exchange chromatography.

4.2.8.9 Ion-exhange chromatography for analysis of hexose sugars

Under conditions of high pH, small carbohydrates such as hexose sugars become ionised. They can thus be separated using anion-exchange chromatography, during which the ionised analyte is passed through a column containing a surface-charged ion-exchange resin. The carbohydrates are retained in the column and separated according to their pKa, the tendency of an acid to dissociate into charged ions. Once separated, carbohydrates are oxidised at an electrode and the resulting oxidation current measured. Samples were analysed using a Dionex system with a pulsed amperometric electrochemical detector (ED50) (Dionex). The anion exchange column used was a CarboPac PA20 (3 x 150 mm, Dionex), suitable for the analysis of mono- and disaccharides. Separation was carried out at a flow rate of 0.4 ml/minute, using a gradient of 60 mM NaOH for 10 minutes, during which time the sugars eluted, followed by 100 mM NaOH for 5 minutes to purge the column, and re-equilibration with 60 mM NaOH for the remaining 12 minutes. Chromatography was conducted at 30 °C.

Detection was achieved using a gold working electrode. Pulsed Amperometric Detection (PAD) comprises applying a 3-step potential waveform to the electrode, which cycles every second. The first voltage potential (E_{DET}) causes the analyte to become oxidised and results in the oxidation current. A large positive potential is then applied (E_{OX}), followed by a negative potential (E_{RED}), during which the electrode becomes oxidised and then reduced back to its reactive state. This prevents the build-up

of oxidation products on the electrode surface that can lead to loss in sensitivity (Johnson *et al.*, 1993). The waveform settings used are listed in Table 4.1, below. Chromatogram acquisition was performed using Chromeleon 6.5 software (Dionex). Standard curves constructed using glucose (Sigma) and fructose (BDH Chemicals) were used to calculate the concentration of these sugars in the samples, and were normalised using the internal standard fucose.

Step	Potential (mV)	Time (s)
E_{DET}	0.05	0.0 – 0.2
Sampling	0.05	0.2 – 0.4
E_{OX}	0.75	0.41 – 0.6
E_{RED}	-0.15	0.61 – 1.0

Table 4.1. The 3-step waveform used in HPAEC-PAD (High performance anion exchange chromatography with pulsed amperometric detection). The potential across the electrochemical detector cycles through the 3 voltages every second during chromatography. The oxidation current produced by the oxidation of sugars in the sample is measured during the first step (EDET), and the electrode is regenerated during the following two steps (EOX and ERED).

4.3 Results

4.3.1 The systemic response of tomato plants to *G. pallida* and *M. incognita* infection.

The molecular response to pathogens can be detected in tomato by analysing the expression of pathogenesis-related (*PR*) genes involved in systemic acquired immunity (SAR) (Sanz-Alferez *et al.*, 2008). Semi-quantitative RT-PCR revealed changes in the expression levels of *PR* genes in the leaves of tomato plants infected with the plant-parasitic nematodes *G. pallida* and *M. incognita.* In response to the cyst nematode *G. pallida,* the expression of *PR1a, PR2, PR1b* and *PR3* was induced (Figure 4.3A). *PR1b*, which was undetectable in the un-infected plants, was induced the most strongly. The root-knot nematode *M. incognita* caused a less dramatic change in gene expression, whereby the levels of *PR1a* and *PR2* were noticeably increased, but no difference was observed in *PR1b* (Figure 4.3B). *PR3* appeared to be slightly down-regulated as a result of the parasitism. The results indicate that the imposed level of nematode parasitism in the roots was enough to induce transcriptome changes and a systemic pathogen response.

4.3.2 The effect of nematode infection on drought physiology

Plants were exposed to infection with the nematode *G. pallida* in order to test the effect of parasitism on tomato drought physiology. After 28 days of infection, the infected plants were observed to have less foliage than uninfected plants. The average length of the longest 5 leaves was found to be significantly smaller in nematode-infected plants (Figure 4.4A). Plants were then subjected to drought stress by withholding irrigation. The relative water content (RWC) of leaves was measured over the course of 17 days following drought imposition in order to quantify the level of drought stress suffered by plants. The RWC of leaves from uninfected plants averaged 78 % at the start of the drought period, and dropped to 48 % by the end (Figure 4.4B). After this point the leaves were visibly damaged by water deficit and did not achieve turgidity during measurement of RWC. The RWC of leaves from infected plants was significantly lower than that of control plants at the start of the drought period, averaging only 68 %. However, the increasing drought stress appeared to affect the RWC of infected plants less than the control plants. Ten days after the drought period began, the infected plants

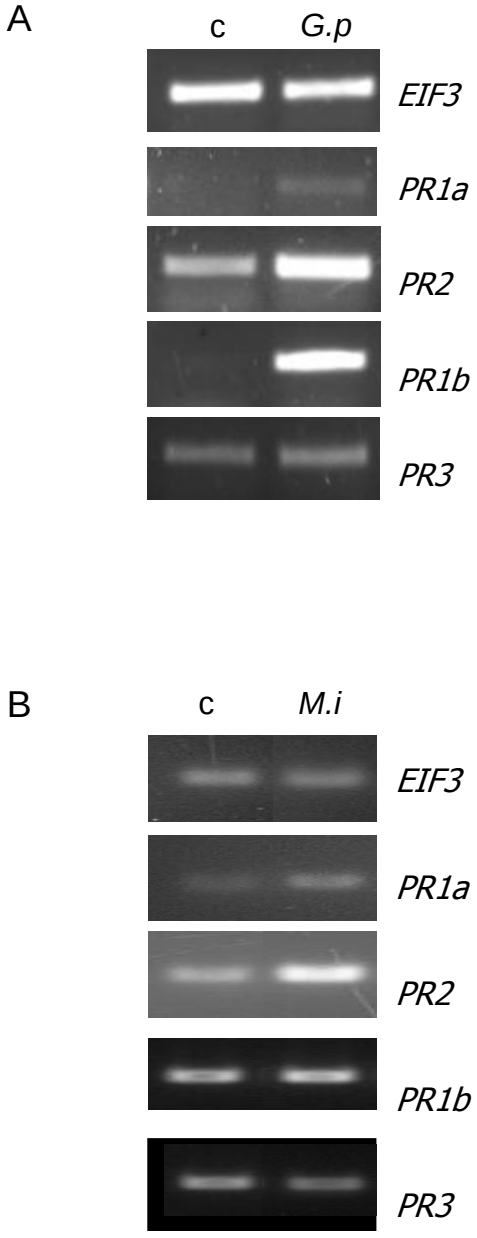


Figure 4.3. Induction of *PR* genes in leaves of nematode-infected tomato plants. Gel images show *PR* gene expression in control plants (c) and in plants that had been infected with **A)** *Globodera pallida* (*G.p*) or **B)** *Meloidogyne incognita* (*M.i*). The eukaryotic initiation factor 3 (*EIF3*) gene is included as a normaliser gene.

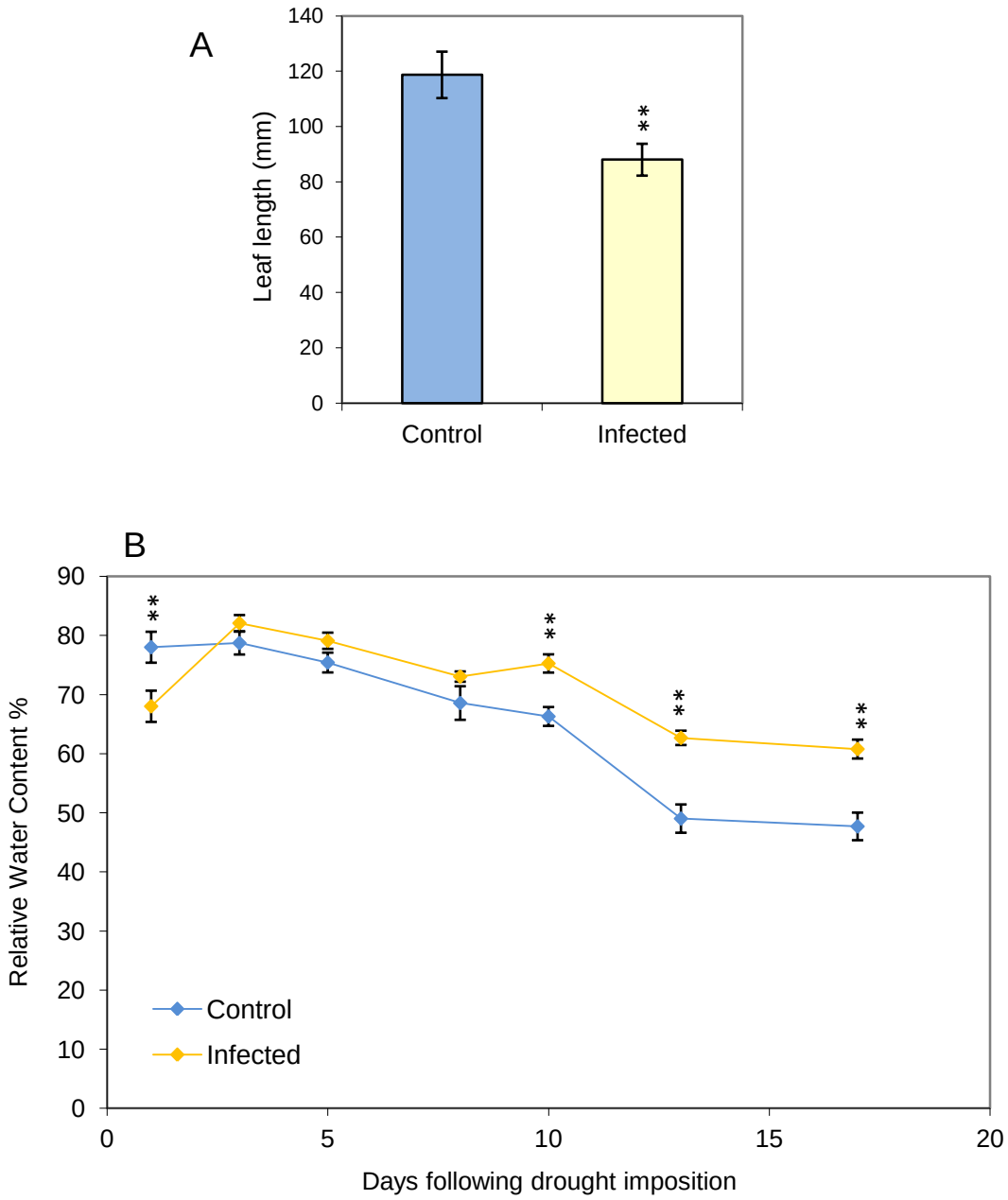


Figure 4.4. Effect of *G. pallida* infection on tomato leaf length and relative water content. A) Length of tomato leaves was measured 28 days after planting into soil containing 50 eggs/g *G. pallida* cysts. The length of the five longest leaves was averaged from each of 8 plants. **B)** Relative water content (RWC) of leaves from control and *G. pallida*-infected plants was measured over 17 days following imposition of drought by withholding irrigation. Six leaves were collected ranging from the top to bottom of the plant. Each data point represents a total of 12 leaves (from 2 plants). Asterisks show a difference between the control and infected plants according to unpaired T-tests ($p < 0.01$).

had a significantly higher RWC than the control plants, a difference that remained for the rest of the experiment. On day 17 the RWC of infected plants had only dropped to 60 %. In accordance with the difference in RWC, leaves from the non-infected plants wilted noticeably earlier than leaves from nematode-infected plants.

4.3.3 The effect of drought pre-treatment on nematode infection rate

Tomato plants were exposed to an early drought stress in order to determine whether or not drought pre-treatment affects the ability of nematodes to infect roots and reproduce. The drought treatment distinctly altered the physiology of treated plants, resulting in a stomatal conductance that was only 16 % of control plants on the last day of the drought period following 14 days without irrigation (Figure 4.5A). One day after re-watering, there was no difference between control and drought-treated plants, indicating recovery from the drought stress. Following the drought treatment and recovery, all plants were exposed to *M. incognita*. The number of nematodes successfully infecting tomato roots was counted 21 days post infection and found not to differ between the control and drought pre-treated plants (Figure 4.5B). The ability of nematodes to survive and reproduce within the roots was then analysed by counting the number of eggs in egg masses on the surface of the roots 59 days post infection. Again, no difference was observed between control plants and those that had experienced an early drought stress (Figure 4.5C), suggesting that drought pre-treatment does not affect the defence of tomatoes against nematode infection.

4.3.4 The effect of joint drought and *M. incognita* infection on tomato growth and reproductive physiology

Tomato plants were exposed to either individual drought, infection with *M. incognita*, or a combination of both stresses. *M. incognita* infection was initiated five weeks after sowing (Figure 4.1). This stress continued for the duration of the 20-week experiment, as the nematodes would have completed their life cycle of approximately six weeks and their juvenile offspring re-infected the plant roots (Bird and Kaloshian, 2003). Figure 4.6 shows *M. incognita* infecting tomato roots. The stress was more severe towards the end of the experiment as the nematodes increased in numbers. Drought stress consisted of a moderate water stress lasting three weeks during the time of flowering. The effects of the water stress were assessed by analysis of gas exchange and growth. At the end of the period of drought stress the stomatal conductance of the treated plants was only 30

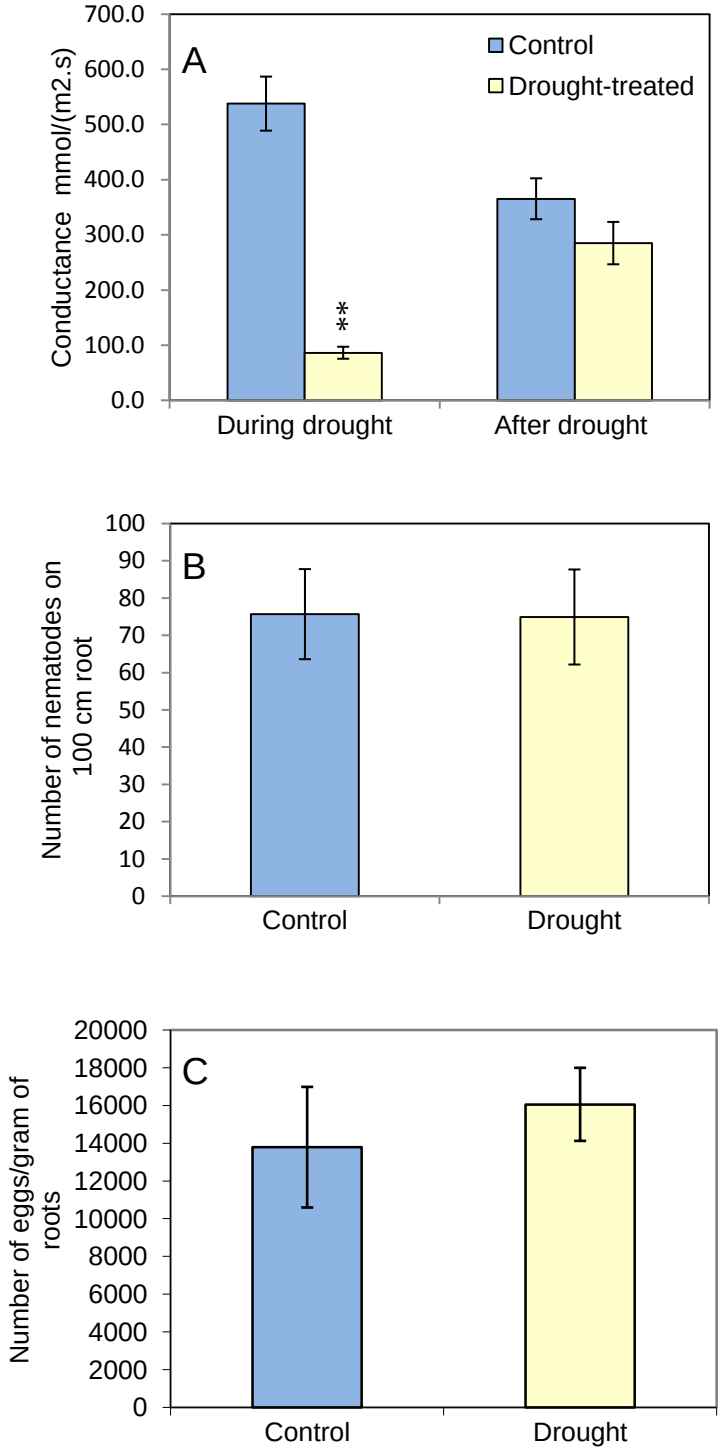


Figure 4.5. The effect of drought pre-treatment on nematode infection. Tomato plants were exposed to drought by withholding irrigation for 14 days and then potted into soil containing 10 eggs/g *M. incognita*. **A)** The stomatal conductance of control and drought-treated plants during the drought period and after re-watering. **B)** The number of enlarged nematodes counted on 100 cm sections of control and drought pre-treated roots 21 days post infection. **C)** The number of *M. incognita* eggs recovered from egg masses on the surface of control and drought pre-treated roots 59 days post infection. Asterisks show difference to control according to T-tests ($p < 0.01$).

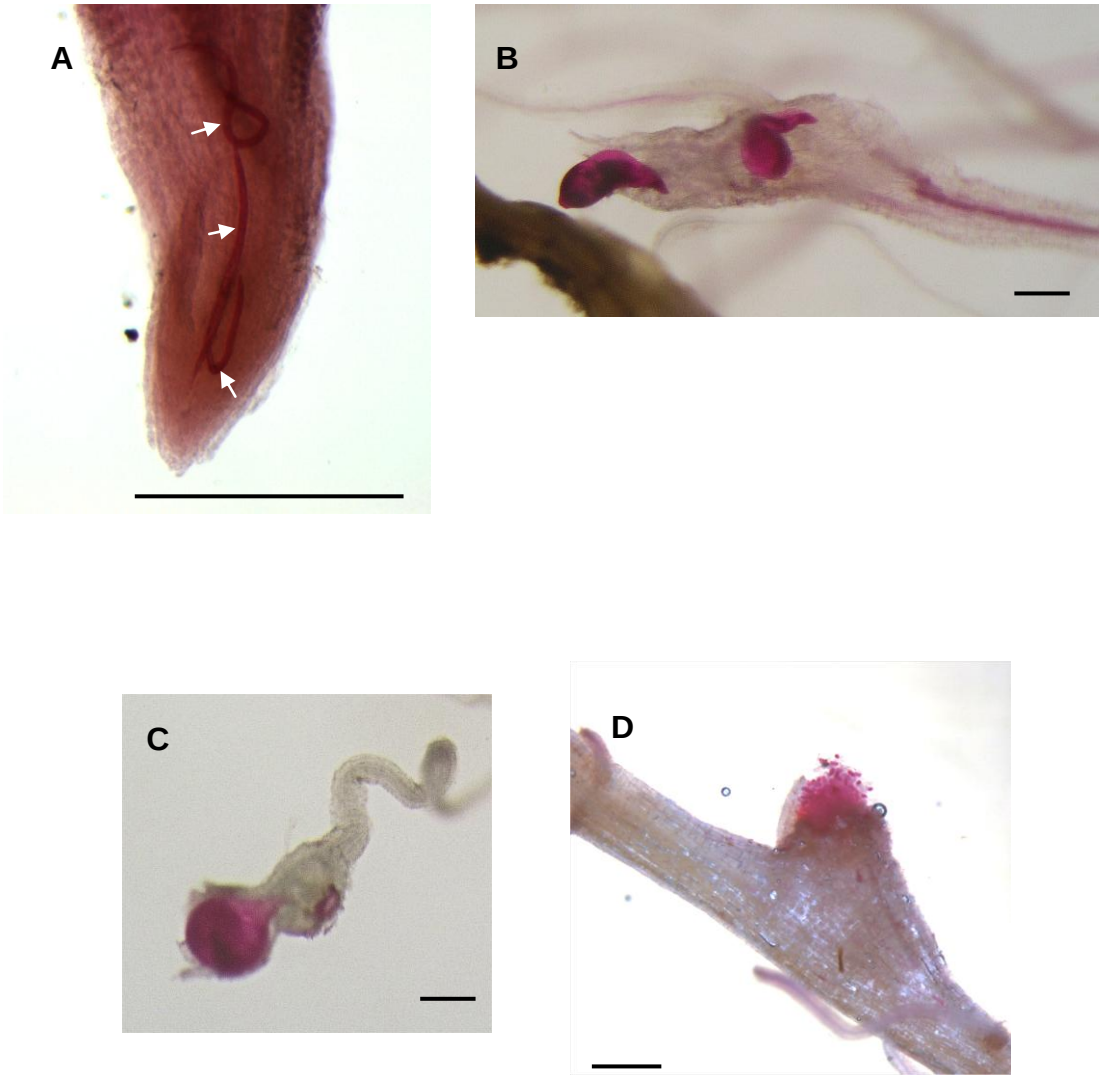


Figure 4.6. *Meloidogyne incognita* parasitising tomato roots. Nematodes are stained with acid fuchsin and appear pink. Scale bars represent 250 µm. **A)** Juvenile nematodes migrating through the root after penetrating the root tip (nematodes are indicated by arrows). **B, C)** Adult female *M. incognita* and the characteristic root gall associated with this species. **D)** A *M. incognita* egg mass on the surface of the root.

% of that of the well-watered plants (Figure 4.7A). This indicates a lower level of gas exchange due to the reduced aperture of the stomata, and thus a reduction of photosynthesis in a manner typical of plants undergoing drought (Chaves *et al.*, 2003; De Pascale *et al.*, 2007). The stomatal conductance of nematode-infected plants was no different from the control. Plants from all three stress treatments also showed a significant height reduction when measured after the period of water stress treatment, as compared to the unstressed plants (Figure 4.7B). Nematode stressed plants were on average 8 % shorter than unstressed plants, whilst drought stressed and joint stressed plants were 22 % and 21 % shorter, respectively.

The time taken for the plants to flower and fruit after planting was observed. Plants that had undergone drought stress or joint stress flowered significantly later than those that were well-watered, resulting in a delay of approximately two days (Figure 4.8A). The fruit ripening period, as defined by the number of days from anthesis to red ripe stage (as shown in Figure 4.2), was also severely affected by the stress treatments (Figure 4.8B). Water stress alone significantly increased the ripening time from 59.6 days to 62.6 days, whereas fruit from nematode-infected and joint-stressed plants ripened significantly faster (54.5 days, and 53.4 days, respectively). In addition, stress treatments affected the yield of tomatoes on an individual fruit weight basis. Drought stress alone did not influence the weight, but fruits from plants infected with nematodes and those undergoing joint stress were significantly lighter than those from their non-parasitised counterparts, with average weight decreasing from 50.2 g per fruit in the control group to 39.0 g in the nematode treatment and 38.3 g in the joint treatment (Figure 4.8C).

Fruits were collected from 5 trusses from each plant. After fruits from the 5th truss were harvested (approximately 20 weeks after planting, as shown in Figure 4.1), physiology of the plants was observed. Plants infected with nematodes lacked turgor and had diminished foliage compared with plants that had not been infected, as shown in photographs in Figure 4.9A. At this point the stomatal conductance of the water-stressed, nematode treated and joint stressed plants was 76 %, 41 % and 21 %, respectively, of the unstressed plants (Figure 4.9B). This implies a maintained level of stress throughout the experimental period, even after re-watering of drought-treated plants.

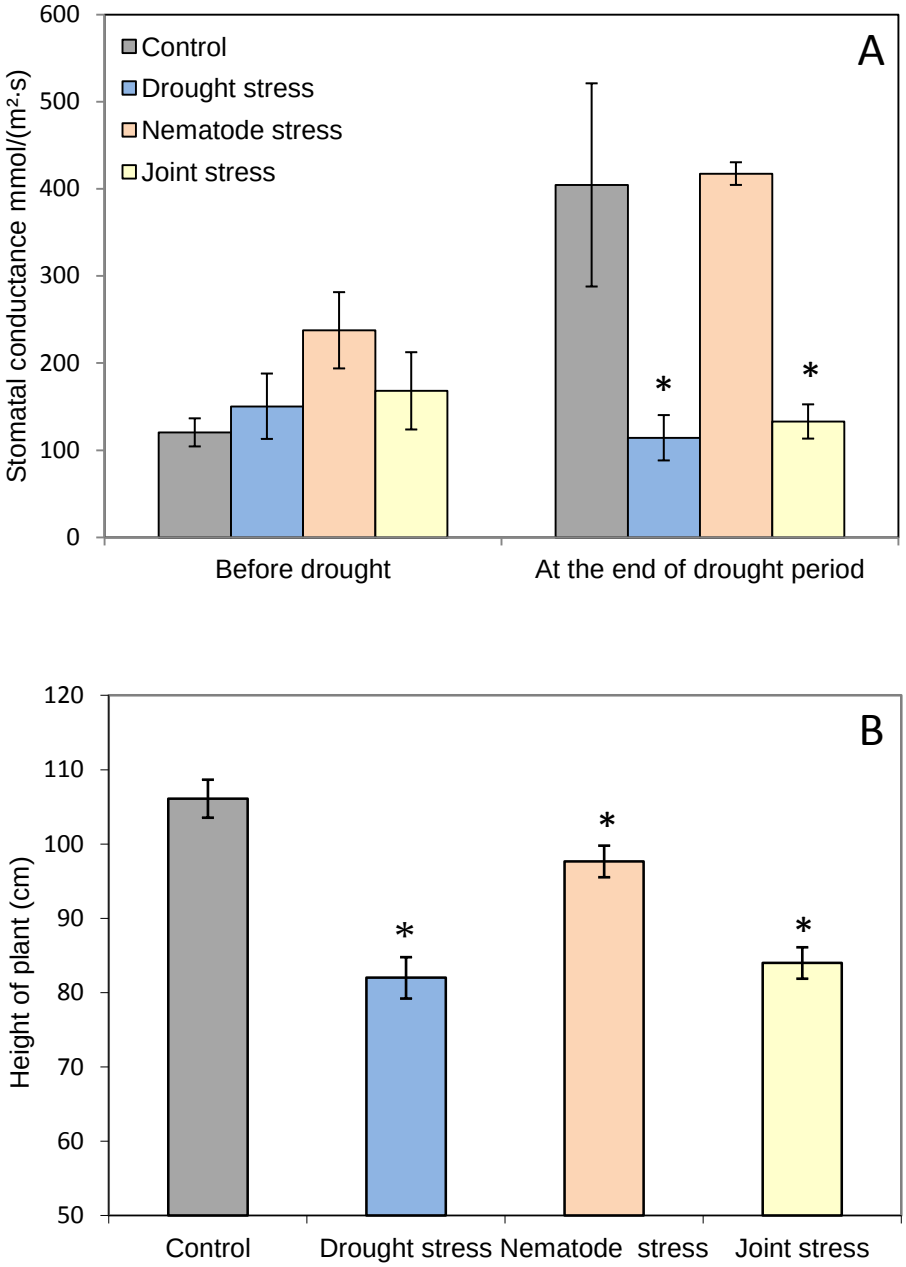


Figure 4.7. Effect of drought and *M. incognita* on tomato plant physiology. Tomato plants were exposed to infection with *M. incognita,* drought stress, or the two in combination. **A)** Stomatal conductance of plants before and at the end of a 3-week drought stress period during which treated plants received 80 % of water evapotranspired the previous day, whilst control plants received 100 % (n=3). **B)** Height of plants after period of drought treatment (n=8-9). Asterisks show a difference from the control according to the SNK test ($p < 0.05$).

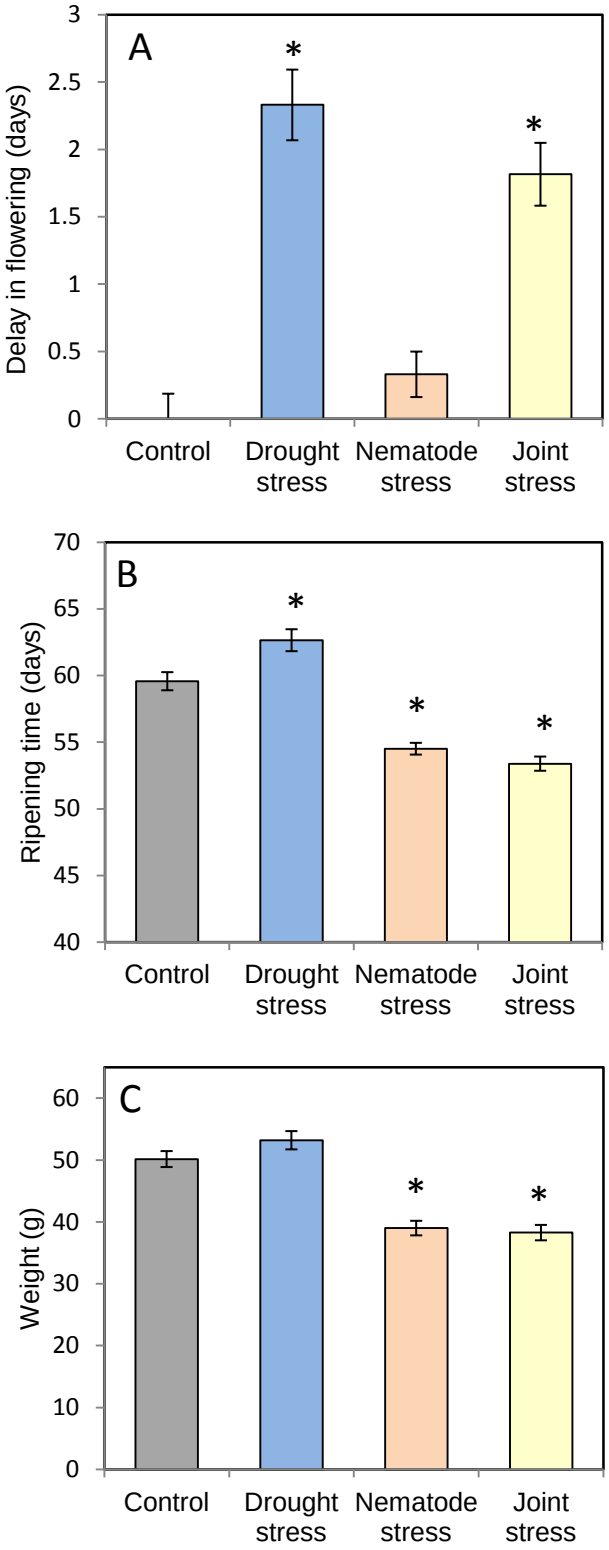
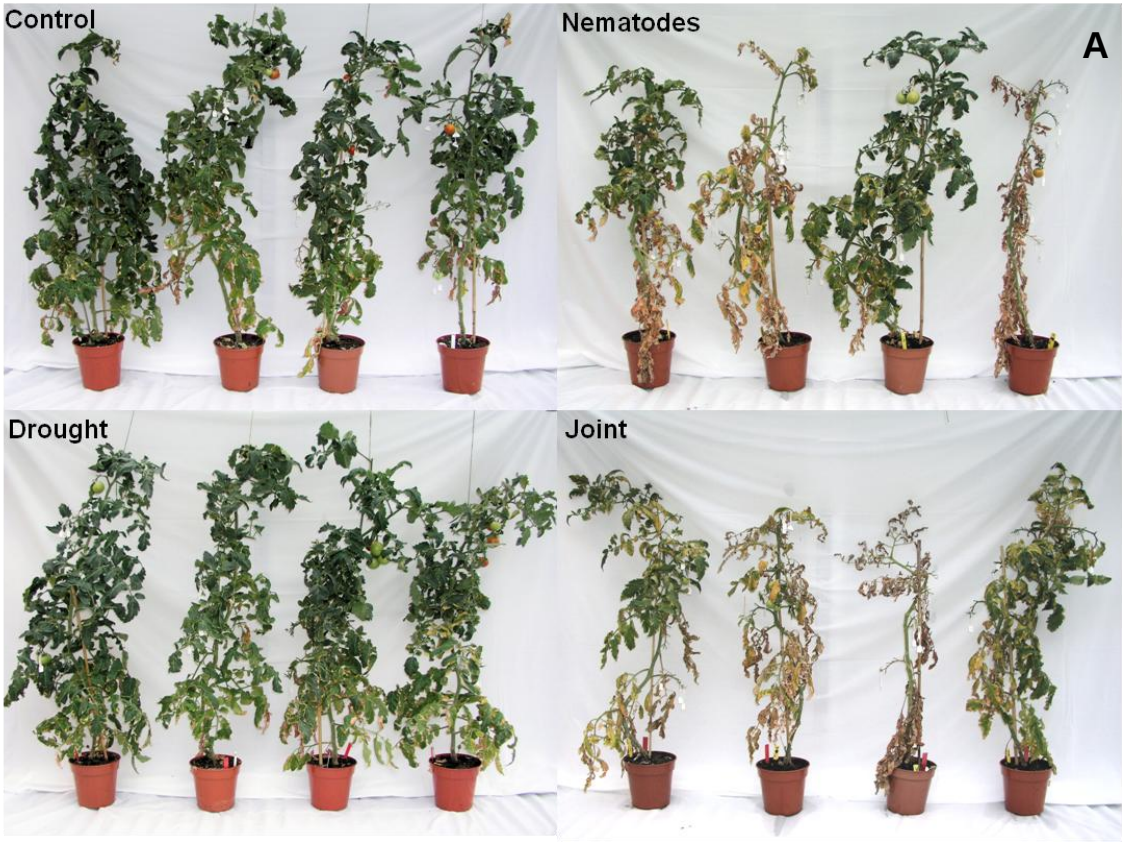


Figure 4.8. Effect of stress on flowering and fruit characteristics. Plants were treated with drought, nematode or joint stress, and flower and fruit characteristics measured. **A**) Delay in days until flowering time compared to control plants. **B)** Number of days taken for fruits to reach red ripe stage. **C)** Weight of individual fruits when harvested. Data shown are the mean values of fruits from all five trusses of 8-10 plants per treatment (n = 150-200). Asterisks show a difference from the control according to the Mann-Whitney U test ($p < 0.05$).



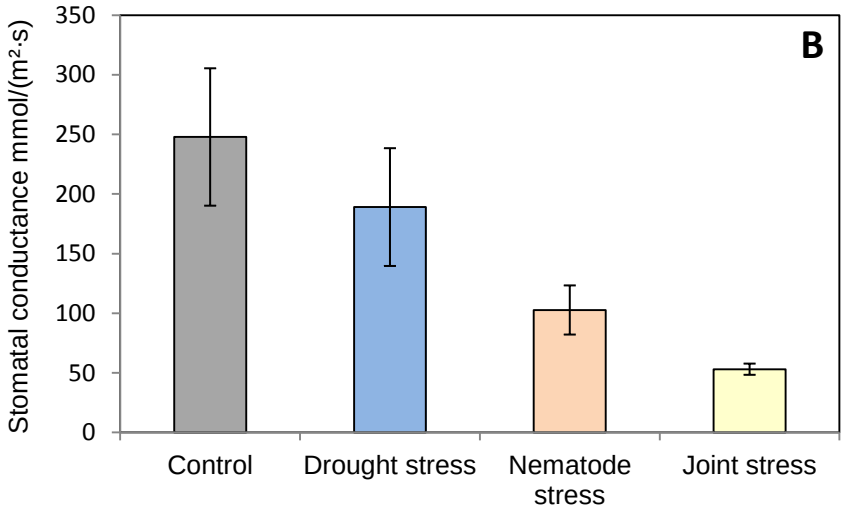


Figure 4.9. Physiology of tomato plants after prolonged stress. Following harvesting of fruits from the 5th (final) truss, around 20 weeks after planting, **(A)** plants were photographed and **(B)** the stomatal conductance measured (n=4). Plants infected with nematodes lacked turgor and showed a reduced foliage and enhanced senescence compared with control plants. Drought-treated plants were not visibly different to control plants.

The percentage dry matter in collected fruit segments was calculated by comparing fresh and freeze-dried weights. Early- (truss 2) and late-harvested (truss 5) fruit from drought-stressed only plants had a significantly lower percentage dry matter than control fruits (Figure 4.10). This is in contrast with previous studies that have found a higher proportion of dry matter in water-stressed fruits (De Pascale *et al.*, 2007; Riggi *et al.*, 2008). Both nematode and combined stress caused a differential effect on the dry matter accumulation in the tomato fruits. In fruits harvested early, there was a lower percentage dry matter as a result of these stress treatments, whilst in later harvested fruits there was a much higher proportion of dry matter than in control fruits, indicating that the more severe nematode stress at the later time point caused the plants to produce drier fruit.

4.3.5 Nutritional analysis of tomato fruits from plants exposed to joint stress

4.3.5.1 Analysis of phenolic compounds

The effect of plant stress treatments on the concentration of phenolic compounds in tomato fruits was investigated by analysing the levels of flavonoids (rutin, chalconaringenin and naringenin) and chlorogenic acid in peel/pericarp sections using LC-MS-MS. Compound identification was achieved by comparing the retention times with those of commercially available standards and by analysis of their unique fragmentation patterns into known daughter ions. Table 4.2 shows the molecular weight of each parent ion, the collision energies required, and the resulting transitions. The major transition was used to quantify the compound. Figure 4.11 shows an example of the total ion count resulting from liquid chromatography of a mixture of standards. The most abundant compound detected was chalconaringenin, followed by rutin, chlorogenic acid and then naringenin in trace amounts. This supports the results of previous studies which have found chalconaringenin and rutin to be the most abundant flavonoids in fresh tomatoes and chlorogenic acid to be the next most abundant phenolic antioxidant (Slimestad *et al.*, 2008).

A preliminary experiment was carried out to determine the necessity of adding antioxidant to samples to stabilise them during extraction and analysis, as is often described (Giuntini *et al.*, 2008; Dall'Asta *et al.*, 2009). A final concentration of 0.1 % of the antioxidants ascorbic acid or sodium metabisulphate was added to test samples

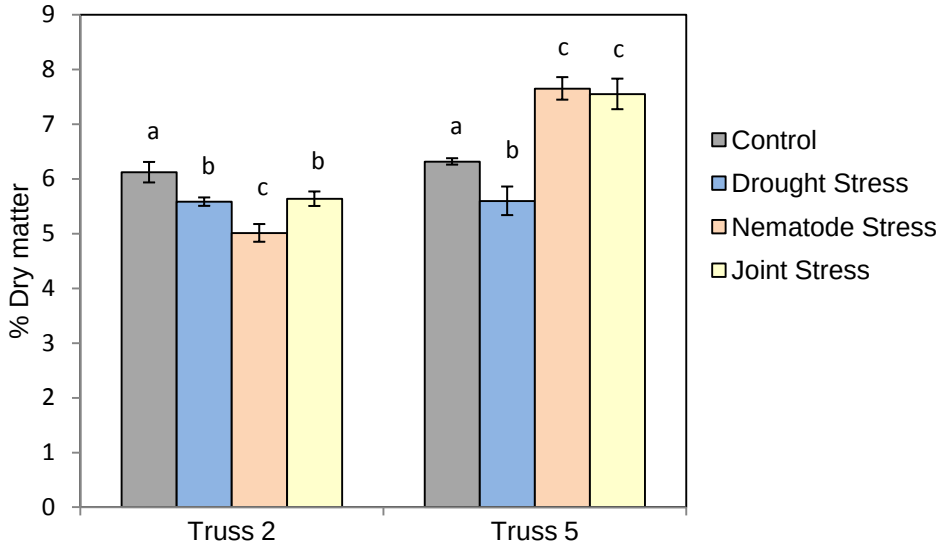


Figure 4.10. Percentage dry matter in tomato fruits following different stress treatments. After harvesting at red ripe stage, peel/pericarp segments were weighed before and after freeze-drying and the percentage dry weight calculated. Data shown are mean values from several plants (n = 10-20). Means with different letters are significantly different according to the SNK test for truss 2, and the Mann-Whitney U test for truss 5 ($p < 0.05$).

Phenolic Compound	Retention time	Precursor ion	Quantifier	Qualifier	Collision energy (V)
Chlorogenic acid	3.6	353.1	190.9		30
Rutin	7.1	609.1	299.9	300.9	25
Morin	11.5	301	150.9	124.9	25
Naringenin	14.8	271	150.9	118.9	12
Chalconaringenin	15.1	271	150.9	118.9	15

Table 4.2. Precursor ions and transitions observed in LC-MS analysis of phenolic compounds. Retention times for each compound refer to separation by liquid chromatography. Quantifier and qualifier ion result from fragmentation of the precursor ion using the collision energy specified. The major transition was designated as for quantification. Morin was included as an internal standard.

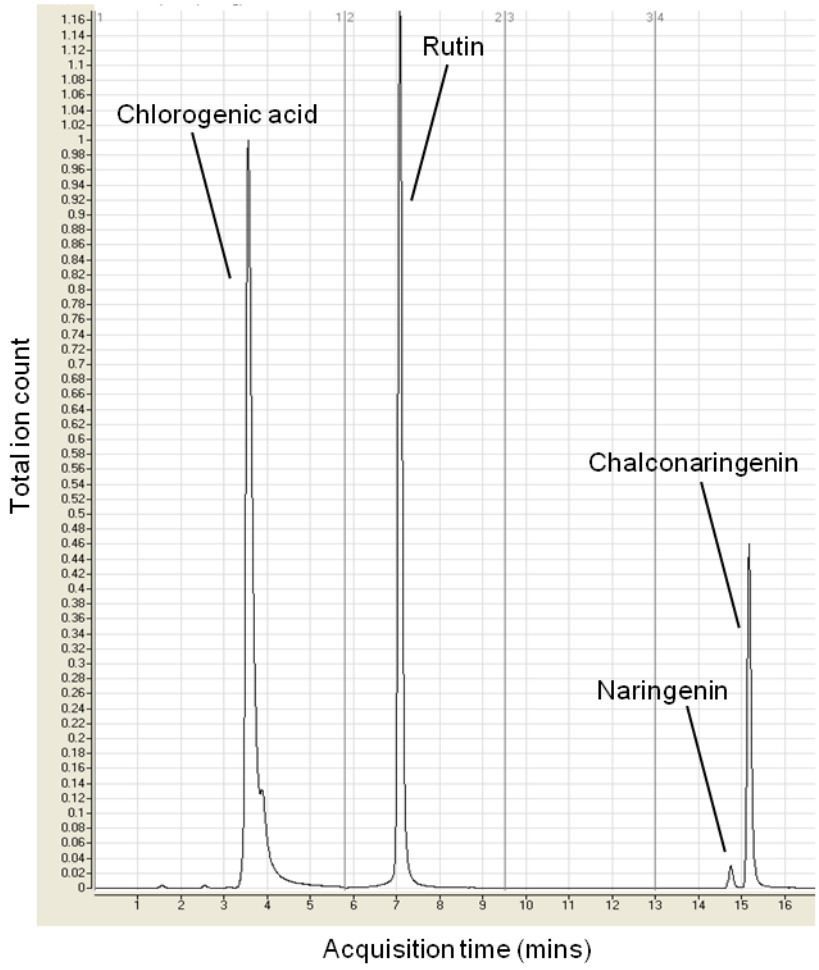


Figure 4.11. The total ion count resulting from liquid chromatography of phenolic compounds. Phenolic compounds were separated by reverse-phase HPLC using a gradient of increasingly non-polar mobile phase. The separated compounds were then filtered by mass spectrometry and fragmented into product ions of recognised mass/charge ratio to allow quantification. The graph shows separation of standards.

and the levels of phenolic compounds analysed initially and again after a 3-day interval. The levels of chlorogenic acid and rutin were negatively affected by the addition of either antioxidant (Figure 4.12). After storage for 3 days the levels did not change significantly. Naringenin levels were positively affected by antioxidant presence, and increased over the 3 days. Chalconaringenin concentration was not affected by antioxidants, although levels decreased over the 3 days. This suggests that over time chalconaringenin converts to its isomer naringenin, a process independent of antioxidants. The experiment was therefore continued without antioxidants. Flavonoids are reported to be most highly concentrated in the peel of tomatoes (Giuntini *et al.*, 2008), a finding corroborated in the current study. Peel and pericarp were measured separately in a subset of samples. Despite only accounting for 9 % of the sample weight, the peel contained 61 % of the rutin, 55 % of the naringenin, and 99 % of the chalconaringenin (Figure 4.13). This concentration in the epidermis of the fruit may allow the flavonoids to protect the tissues below from the damaging effects of UV-B (Treutter, 2006). In contrast, 9 % of the chlorogenic acid was present in the peel, indicating an equal concentration in the peel and pericarp.

Commercially available standards were used to construct standard curves for each compound, against which the concentration in tomato samples was calculated using morin as an internal control. The concentration of phenolic compounds was analysed as a proportion of both dry weight and fresh weight. It is important to examine both measurements, as previous work has shown that results can vary depending on whether fresh or dry weight is calculated (Mitchell *et al.*, 1991; Zushi and Matsuzoe, 1998; Riggi *et al.*, 2008). However, fresh weight (FW) was considered to be the most biologically relevant, and is the most often referred to (Slimestad and Verheul, 2009). Stress treatments were found to affect the levels of phenolic compounds in Truss 5 tomatoes, which were harvested at a late point in the experiment (Figure 4.14 and 4.15). Naringenin concentrations per fresh weight were heightened by nematode stress in Truss 5, showing an increase of 62 % (1.0 µg $100g^{-1}$ FW compared to a control value of 0.6 µg $100g^{-1}$ FW) (Figure 4.14A). Chalconaringenin concentration in fruits from nematode-stressed plants was not significantly different from the controls, however a significant difference was observed between the water stressed and nematode stressed fruits, resulting in an increase of 78 % (44.6 µg $100g^{-1}$ FW compared to 25.1 µg $100g^{-1}$ FW) (Figure 4.14C). Drought stress alone did not affect the levels of chalconaringenin

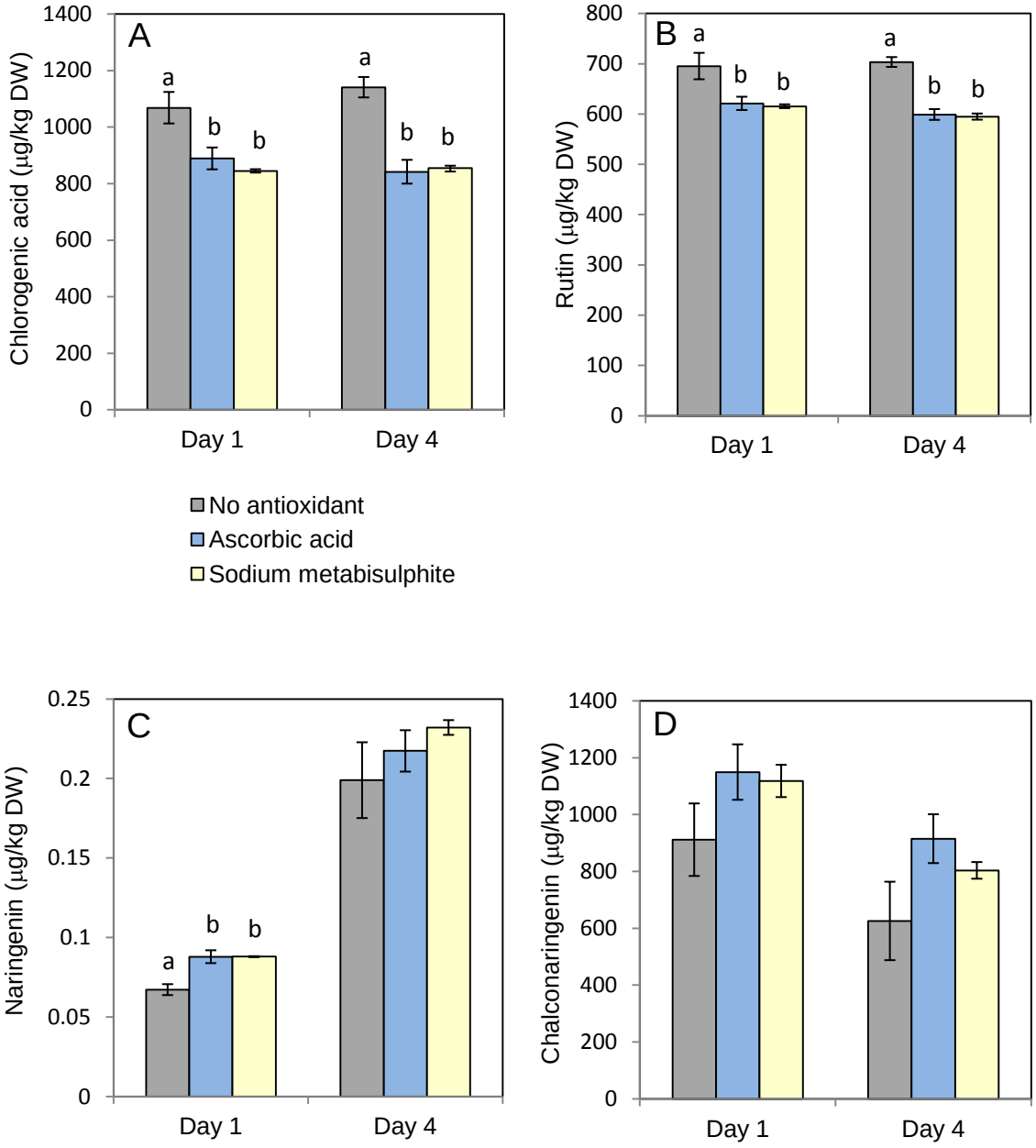


Figure 4.12. The effect of added antioxidant on stability of phenolic compounds. The antioxidants ascorbic acid or sodium metabisulphite were added to samples of tomato extract to determine the effect on compound stability. Samples were analysed by LC-MS before and after a 3-day incubation period with the antioxidant to determine the levels of the phenolic compounds **A)** chlorogenic acid, **B)** rutin, **C)** naringenin and **D)** chalconaringenin. Means with different letters indicate a difference to the control sample according to the SNK test ($p < 0.05$).

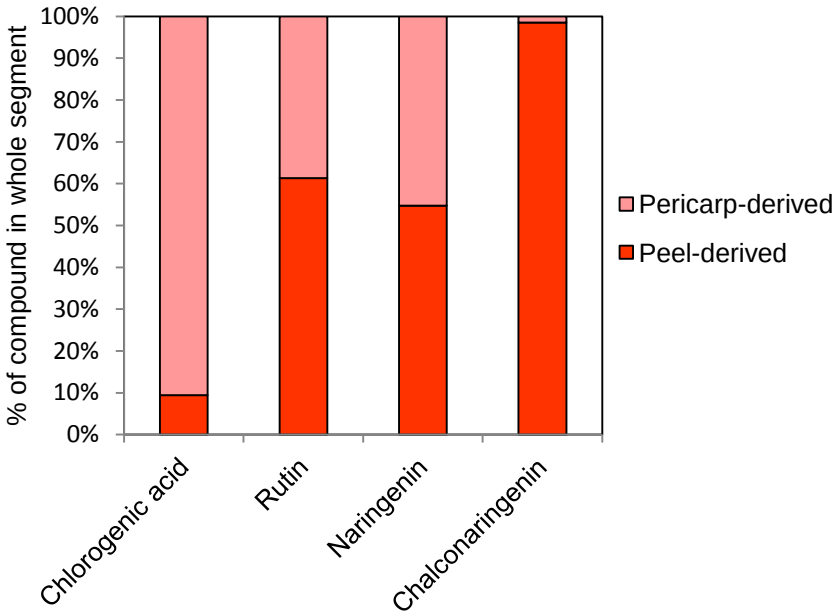


Figure 4.13. Distribution of phenolic compounds in peel and pericarp. Peel and pericarp samples were analysed separately by LC-MS to determine the relative concentration of phenolic compounds in each tissue. The proportion of the compound in the whole segment that was derived from peel or pericarp is depicted. The peel accounted for 9 % of the weight of the peel/pericarp segment.

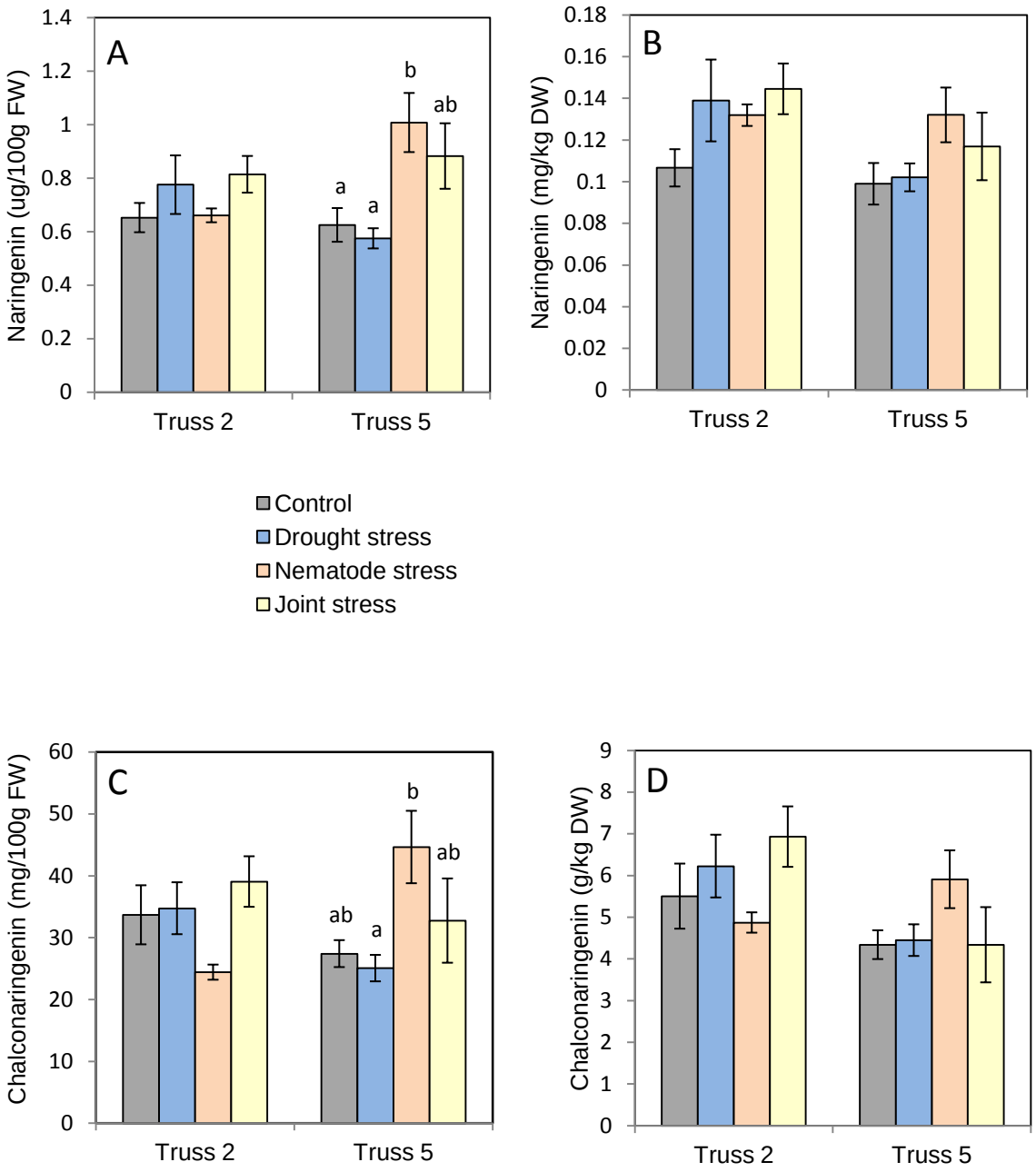


Figure 4.14. Concentration of naringenin and chalconaringenin in tomatoes after differing stress treatments. Concentrations of the phenolic compounds naringenin, expressed **A**) per fresh weight and **B)** per dry weight, and chalconaringenin, expressed **C**) per fresh weight and **D)** per dry weight in fruits from tomato plants subjected to drought, nematode or joint stress. Fruits were harvested either early (Truss 2) or late (Truss 5) in the experiment. Bars represent the standard error of the mean (n=9). Means with different letters are significantly different within the truss position according to the SNK test ($p < 0.05$) and bars displaying two letters show no difference from either group.

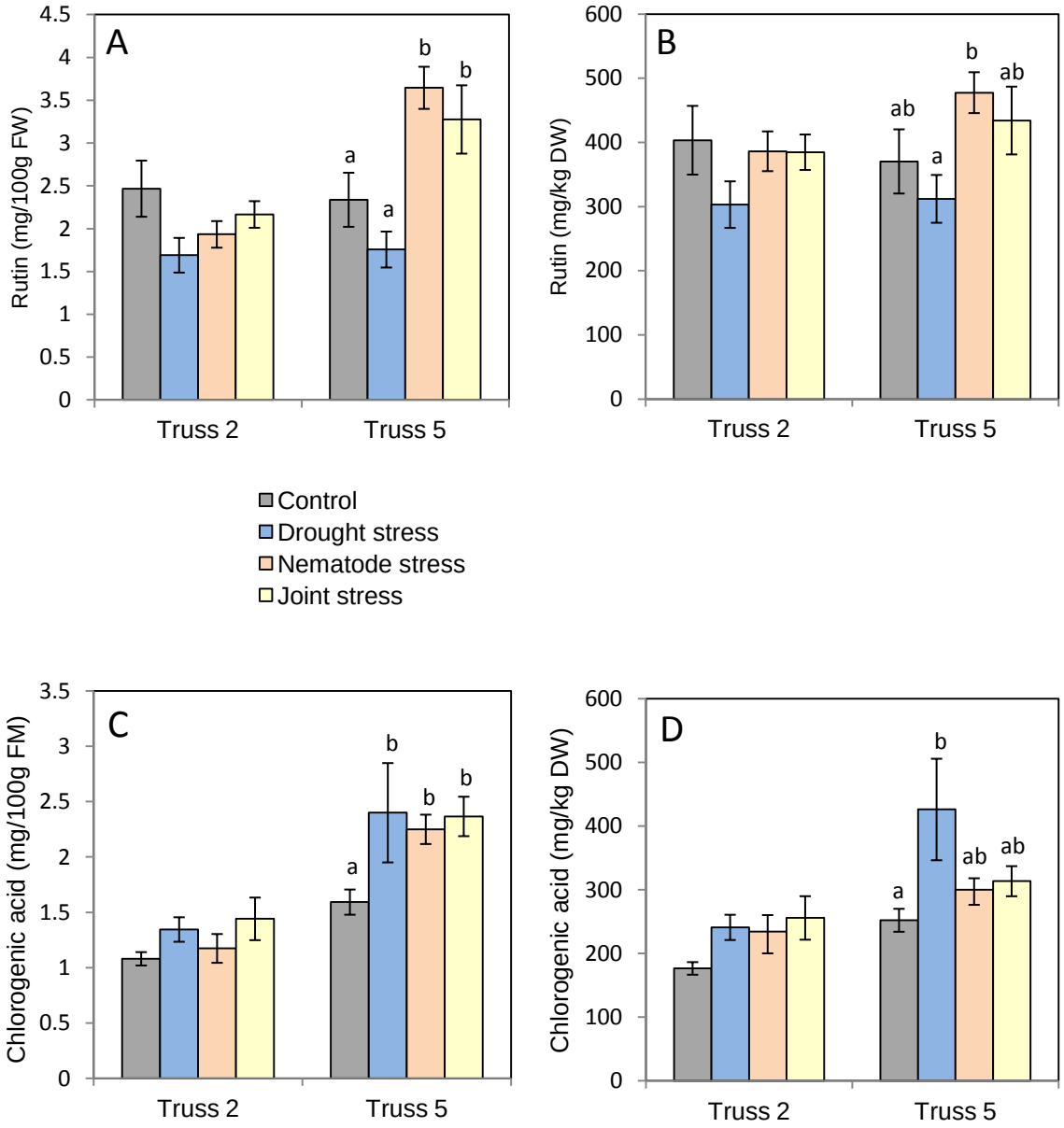


Figure 4.15. Concentration of rutin and chlorogenic acid in tomatoes after differing stress treatments. Concentrations of the phenolic compounds rutin, expressed **A**) per fresh weight and **B)** per dry weight, and chlorogenic acid, expressed **C**) per fresh weight and **D)** per dry weight in fruits from tomato plants subjected to drought, nematode or joint stress. Fruits were harvested either early (Truss 2) or late (Truss 5) in the experiment. Bars represent the standard error of the mean (n=9). Means with different letters are significantly different within the truss position according to the SNK test ($p < 0.05$) and bars displaying two letters show no difference from either group.

and naringenin. Furthermore when the two stresses were applied together, the heightened concentrations seen under nematode stress were reduced and thus not significantly different from the control or water stressed plants. Significantly higher levels of rutin expressed per fresh weight were observed in Truss 5 tomatoes from plants exposed to either nematode stress (3.6 mg $100g^{-1}$ FW) or joint stress (3.3 mg $100g^{-1}$ FW), compared to the controls (2.3 mg $100g^{-1}$ FW), resulting in an increase of 56 % and 40 % respectively (Figure 4.15A). No difference in rutin, naringenin or chalconaringenin concentrations was observed in fruits harvested at an early stage (Truss 2). The concentration of chlorogenic acid was significantly affected by all three stress treatments in Truss 5 fruits (Figure 4.15C). Water stress and nematode stress increased chlorogenic acid levels by 49 % and 46 %, respectively, compared to the control, whilst the two stresses in combination gave an increase of 51 % (control 1.6 mg $100g^{-1}$ FW, drought 2.4 mg $100g^{-1}$ FW, nematode stress 2.3 mg $100g^{-1}$ FW, joint stress 2.4 mg $100g^{-1}$ FW). Chlorogenic acid levels in fruits harvested early (Truss 2) were not affected significantly by any stress. When examined on a dry weight basis, no difference in naringenin or chalconaringenin were observed in the fruit of either truss due to any stress treatment (Figure 4.14 B and D). However, rutin concentration significantly differed between drought and nematode-infected Truss 5 plants, and chlorogenic acid concentration was significantly increased between control and drought-treated plants (Figure 4.15 B and D). A 2-way ANOVA was carried out to determine any interaction between the effect of treatment and truss position. The individual and combined truss results for all the nutritional compounds are shown in Table 4.3. An interaction was observed between stress treatment and truss position for the flavonoids rutin ($p < 0.01$), naringenin ($p < 0.05$) and chalconaringenin ($p < 0.01$), although not for chlorogenic acid. Truss position significantly affected rutin ($p < 0.001$) and chlorogenic acid ($p < 0.001$) concentration but not naringenin or chalconaringenin.

4.3.5.2 Analysis of carotenoids

Carotenoids were analysed in peel/pericarp sections of tomatoes from plants that had been exposed to single or combined stress. Compounds were separated using reverse-phase HPLC. As expected, the most abundant carotenoids in the tomato extract were lycopene and β-carotene (Figure 4.16). Their identities were confirmed by comparison to commercially available standards, as well as analysis of the absorption spectrum of each peak as measured by photodiode array detector, which obtains a full absorption

Truss Position	Treatment	Chlorogenic Acid	Rutin	Chalconaringenin	Naringenin	Lycopene	β-carotene	Glucose	Fructose
		mg/100g fresh weight				mg/100g fresh weight		mg/g fresh weight	
Truss 2	Control	1.08 ± 0.2	2.47 ± 1.0	33.68 ± 14.3	0.65 ± 0.2	11.29 ± 2.6^{a}	0.87 ± 0.2^{a}	13.70 ± 1.3	15.07 ± 1.2
Truss 2	Water Stress	1.35 ± 0.3	1.69 ± 0.6	34.74 ± 12.6	0.78 ± 0.3	7.50 ± 1.7^{b}	0.62 ± 0.1^{b}	13.47 ± 1.1	14.58 ± 1.3
Truss 2	Nematode	1.17 ± 0.4	1.93 ± 0.5	24.40 ± 3.7	0.66 ± 0.1	9.27 ± 3.0^{ab}	0.84 ± 0.2^{a}	13.12 ± 1.0	14.68 ± 1.1
Truss 2	Joint stress	1.44 ±0.6	2.17 ± 0.5	39.05 ± 12.3	0.81 ± 0.2	7.90 ± 2.0^{b}	0.72 ± 0.2^{ab}	14.22 ± 1.0	15.83 ± 1.3
ANOVA		ns	ns	ns	ns	**	*	ns	ns
Truss 5	Control	1.59 ± 0.3^{a}	2.34 ± 0.9^{a}	27.40 ± 6.5^{ab}	0.63 ± 0.2^{a}	7.58 ± 2.2^{a}	0.57 ± 0.1^{ab}	15.92 ± 2.4^{a}	17.87 ± 2.7^{a}
Truss 5	Water Stress	2.40 ± 1.3^{b}	1.76 ± 0.6^{a}	25.05 ± 6.1^{a}	0.57 ± 0.1^{a}	5.16 ± 0.9^{b}	0.53 ± 0.1^{a}	15.48 ± 1.7^{a}	17.61 ± 1.9^{a}
Truss 5	Nematode	2.25 ± 0.4^{b}	3.65 ± 0.7^{b}	44.64 ± 17.5^{b}	1.01 ± 0.3^{b}	9.04 ± 2.4^{a}	0.75 ± 0.1^{b}	17.31 ± 2.5^{a}	18.77 ± 2.5^{a}
Truss 5	Joint stress	2.37 ± 0.5^{b}	3.28 ± 1.2^{b}	32.74 ± 20.4^{ab}	0.88 ± 0.4^{ab}	7.31 ± 2.0^{a}	0.61 ± 0.1^{ab}	19.56 ± 2.1^{b}	21.81 ± 2.3^{b}
ANOVA		*	***	*	**	**	*	**	**
Truss 2 + 5	Control	1.34 ± 0.4^{a}	2.40 ± 0.9^{a}	30.54 ± 11.3	0.64 ± 0.2^{a}	9.44 ± 3.0^{a}	0.72 ± 0.2^{a}	14.81 ± 2.2^{a}	16.47 ± 2.5
Truss 2 + 5	Water Stress	1.84 ± 1.0^{b}	1.72 ± 0.6^{b}	30.18 ± 11.0	0.68 ± 0.3^{ab}	6.33 ± 1.8^{c}	0.58 ± 0.1^{b}	14.48 ± 1.7^{a}	16.10 ± 2.2
Truss 2 + 5	Nematode	1.71 ± 0.7^{b}	2.79 ± 1.1^{a}	34.52 ± 16.1	0.84 ± 0.3^{b}	9.16 ± 2.7^{ab}	0.79 ± 0.2^{a}	15.21 ± 2.8^{a}	16.72 ± 2.8
Truss 2 + 5	Joint stress	1.90 ± 0.7^{b}	2.72 ± 1.1^{a}	35.90 ± 16.7	0.85 ± 0.3^{b}	7.59 ± 2.0^{bc}	0.65 ± 0.2^{ab}	16.89 ± 3.2^{b}	18.82 ± 3.6
ANOVA	Treatment	**	***	ns	**	***	**	***	ns
	Truss position	***	***	ns	ns	***	***	***	***
	Treatment x Truss position	ns	**	**	*	ns	ns	*	ns

Table 4.3. Concentration of nutritional compounds in fruits from tomato plants subjected to individual or combined water and nematode stress. Concentrations of phenolic compounds and carotenoids are given as mg/100g FW ± SD. Sugar concentrations are given as mg/g FW ± SD. Means for each compound were compared between treatment groups for each truss separately and for the two trusses together. 'Truss 2 + 5' indicates the results of the two trusses together as analysed by two-way ANOVA. The significance of differences between factors is given as follows: ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Means with different letters are significantly different at the 5 % level according to the SNK test.

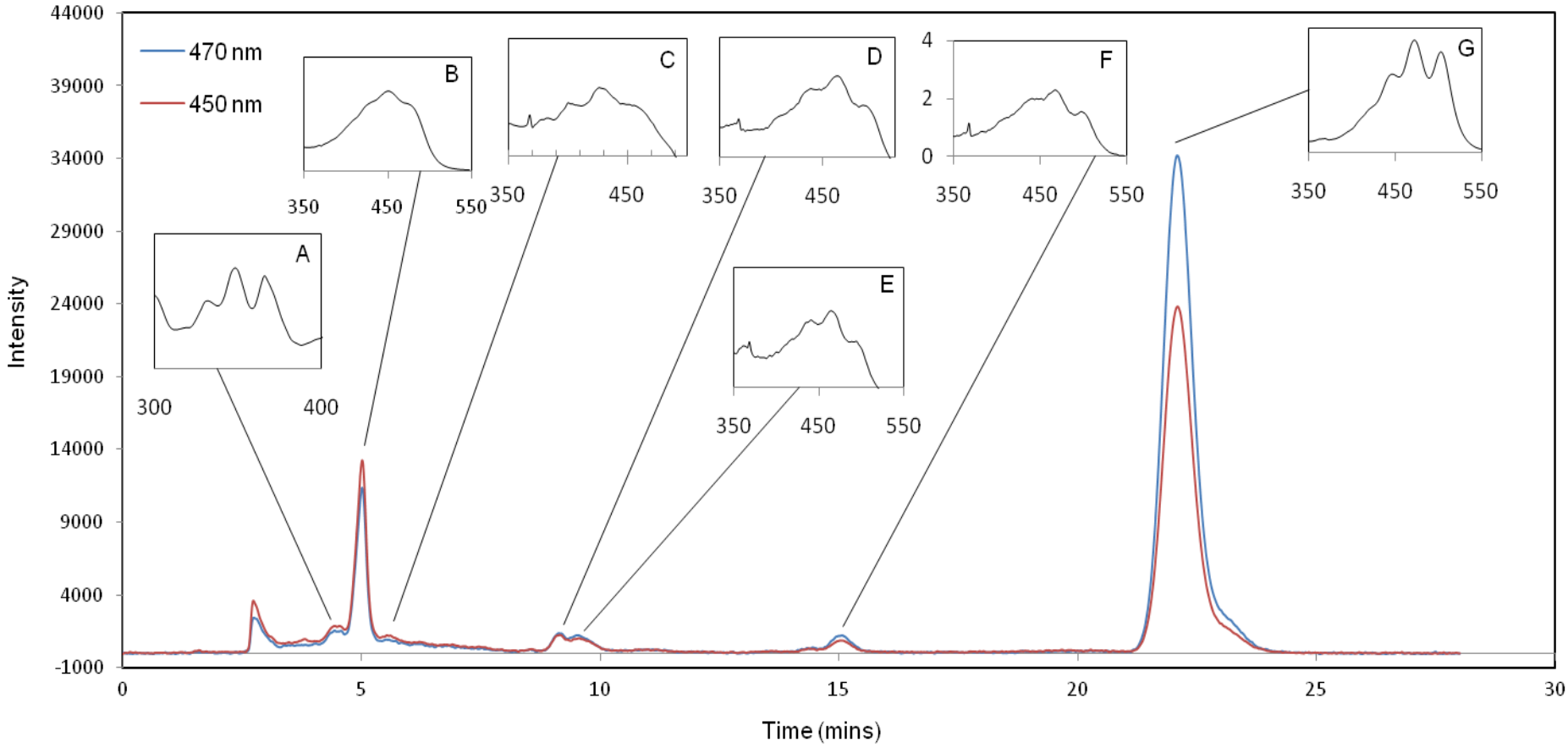


Figure 4.16. Chromatogram of carotenoid separation in tomato extract using reverse-phase HPLC. An isocratic method was employed, using a mobile phase of MTBE/methanol/ethyl acetate. Absorbance was measured at 450 nm and 470 nm. Inlayed chromatograms represent the absorbance spectrum of each peak as obtained by photodiode-array detector, allowing compound identification by comparison to known absorbance spectra (Johjima and Ogura, 1983; Ishida *et al.*, 2001). A = phytofluene, B = β-carotene, C = tetra-cis-lycopene, D - F = cis-lycopene, G = lycopene.

spectrum every half a second throughout the course of HPLC (Figure 4.16 B and G). Other carotenoid peaks in the chromatogram were tentatively identified by comparison to absorption spectra described by Ishida *et al.* (2001) and Johjima *et al*. (1983). Peaks are likely to represent stereo-isomers of lycopene (Figure 4.16 C-F) as well as the pigment phytofluene, for which no standard was available (Figure 4.16A). Standard curves were constructed for both lycopene and β-carotene, against which the concentration in tomato extract was calculated, again as a proportion of fresh weight (FW) and dry weight. The concentration of lycopene in the tomato samples ranged between 3.6 – 14.7 mg $100g^{-1}$ FW. β-carotene was present at approximately one tenth of the abundance of lycopene, varying from 0.3 – 1.2 mg $100g^{-1}$ FW. Similar concentrations for each compound have been reported by other authors in various studies of fresh tomatoes, as summarised by Dumas *et al.* (2003).

The relative levels of carotenoids were influenced significantly by different stress treatments. Lycopene concentration was significantly lower in Truss 2 fruits from plants that were exposed to drought or joint stress, resulting in a decrease in concentration of 34 % and 30 % respectively (from 11.3 mg $100g^{-1}$ FW in the unstressed controls to 7.5 mg $100g^{-1}$ FW in the drought stressed and 7.9 mg $100g^{-1}$ FW in the joint treatment) (Figure 4.17). The concentration was not affected by nematode stress in these fruits. In Truss 5, when the nematode stress became more severe, drought stress alone resulted in a 32 % decrease in lycopene concentration (7.6 mg $100g^{-1}$ FW in the control compared to 5.2 mg $100g^{-1}$ FW in the drought stress group) whilst joint drought and nematode stress had no effect. β-carotene levels followed a similar pattern in Truss 2, where a 28 % lower concentration was observed in the drought stressed plants compared to the control (0.9 mg $100g^{-1}$ FW in control compared to 0.6 mg $100g^{-1}$ in drought-stressed). The β-carotene concentration in joint stressed plants was also lower than unstressed controls but not to a significant level (P = 0.085). (Figure 4.17C). In Truss 5 a different pattern of results was observed for β-carotene. Although none of the stress treatments were significantly different from the control, in each case the β-carotene concentration with respect to the control was higher than in Truss 2. Truss position significantly affected both lycopene ($p < 0.001$) and β-carotene ($p < 0.001$) concentrations, giving lower concentrations in the later harvested fruits (Table 4.3), a finding previously documented by Dumas *et al.* (2003) (after Cabibel and Ferry, 1980). When results from the two trusses were analysed together the effect of the stress treatments became more

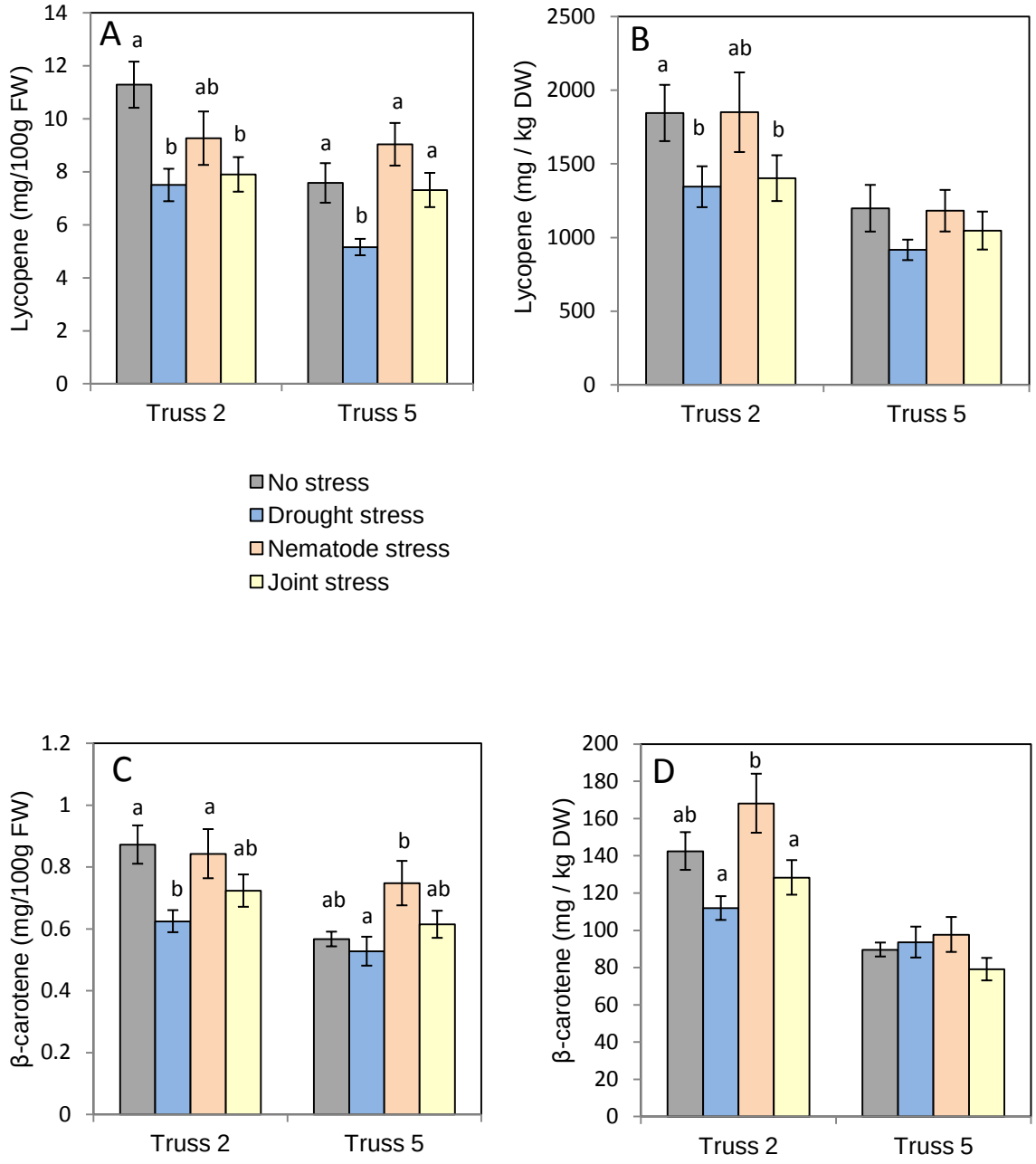


Figure 4.17. Concentration of lycopene and β-carotene in tomatoes after differing stress treatments. Concentrations of the carotenoids lycopene, expressed **A**) per fresh weight and **B)** per dry weight, and β-carotene expressed **C**) per fresh weight and **D)** per dry weight in fruits from tomato plants subjected to drought, nematode or joint stress. Fruits were harvested either early (Truss 2) or late (Truss 5) in the experiment. Bars represent the standard error of the mean (n=9). Means with different letters are significantly different within the truss position according to the SNK test ($p < 0.05$) and bars displaying two letters show no difference from either group.

significant for both lycopene ($p < 0.001$) and β-carotene ($p < 0.01$), although no interaction effect was observed between the stress treatments and the truss position (Table 4.3). When expressed as a proportion of dry weight, significant differences in both lycopene and β-carotene were still observed as a result of stress treatments in Truss 2 tomatoes. The lycopene concentration was significantly lower in drought stress and joint stress-treated fruits (Figure 4.17B). The β-carotene level in all stress-treated fruits was no different from the control, but the concentration in fruit from nematode-stressed plants was significantly higher than in drought-treated plants (Figure 4.17D).

4.3.5.3 Analysis of sugars

Sugars were extracted from fresh tomato homogenate and separated using ion-exchange chromatography (Figure 4.18). The hexoses glucose and fructose were identified as the main sugars in the tomato extract, and were quantified using standard curves with fucose as an internal control. Glucose and fructose were detected in the Truss 2 control tomato fruits at concentrations of 13.6 mg g^{-1} and 15.0 mg g^{-1} fresh weight, respectively. Truss 5 concentrations were approximately 20 % higher, at 17.1 mg g^{-1} and 19.0 mg g^{-1} respectively. These concentrations are similar to those previously described (Gao *et al.*, 1998; Zushi and Matsuzoe, 1998; Veit-Kohler *et al.*, 1999). Drought stress and nematode stress on their own had no effect on glucose or fructose concentration in tomato fruits. However, when the two stresses were applied in combination, a significantly higher concentration of both sugars was observed in Truss 5 fruits, resulting in a 23 % increase in glucose (from 15.9 mg $100g^{-1}$ FW to 19.6 mg $100g^{-1}$) and a 22 % increase in fructose (from 17.9 mg $100g^{-1}$ FW to 21.8 mg $100g^{-1}$) compared to the controls (Figure 4.19 A and C). An interaction was observed between the effects of stress treatment and truss position for glucose ($p < 0.05$) but not for fructose (Table 4.3). Hexose sugars are soluble and thus present in fluids throughout fruit tissues. Despite this, the concentration of sugars was also calculated as a proportion of dry weight (Figure 4.19 B and D). The pattern of differences in sugar concentration was altered due to the differing water contents of fruits. Although neither was different from the control, the fructose and glucose concentration of Truss 5 fruits under joint stress was significantly higher than under nematode stress.

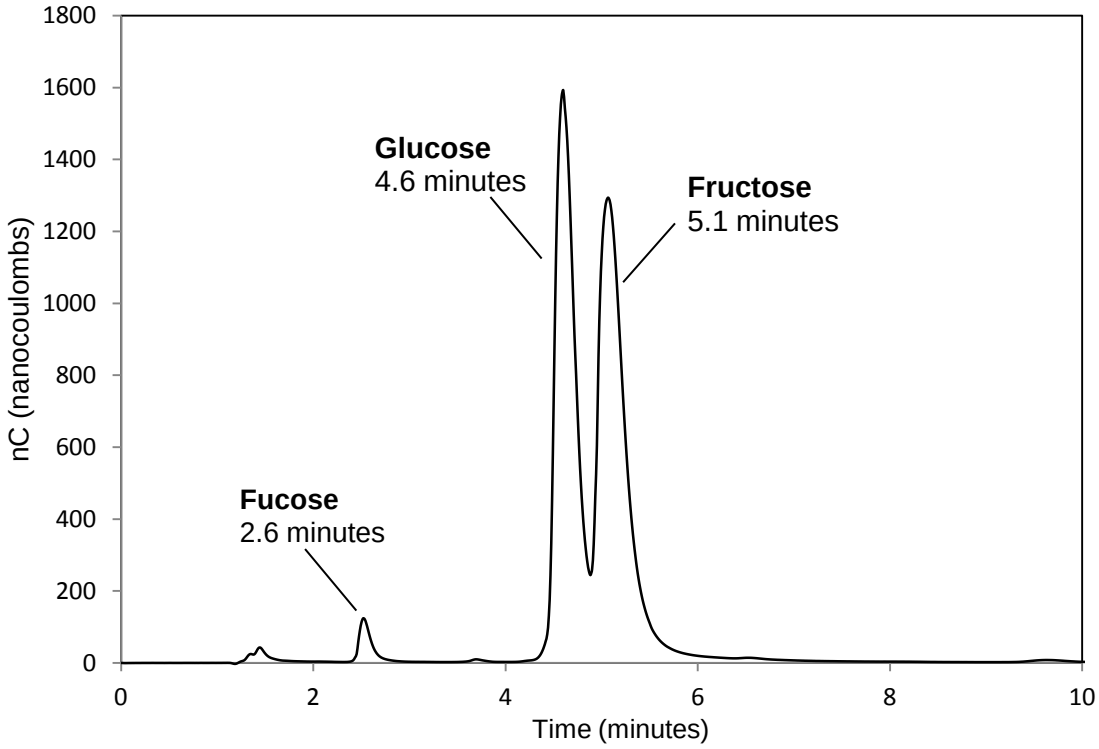


Figure 4.18. Separation of sugars by ion-exchange chromatography. Ion exchange chromatography was carried out at high pH to allow ionisation of hexose sugars. A 3-step potential waveform oxidised the sugars and the resulting oxidation current (nC) was detected using a gold electrode. Fucose was included in the tomato extract as an internal standard.

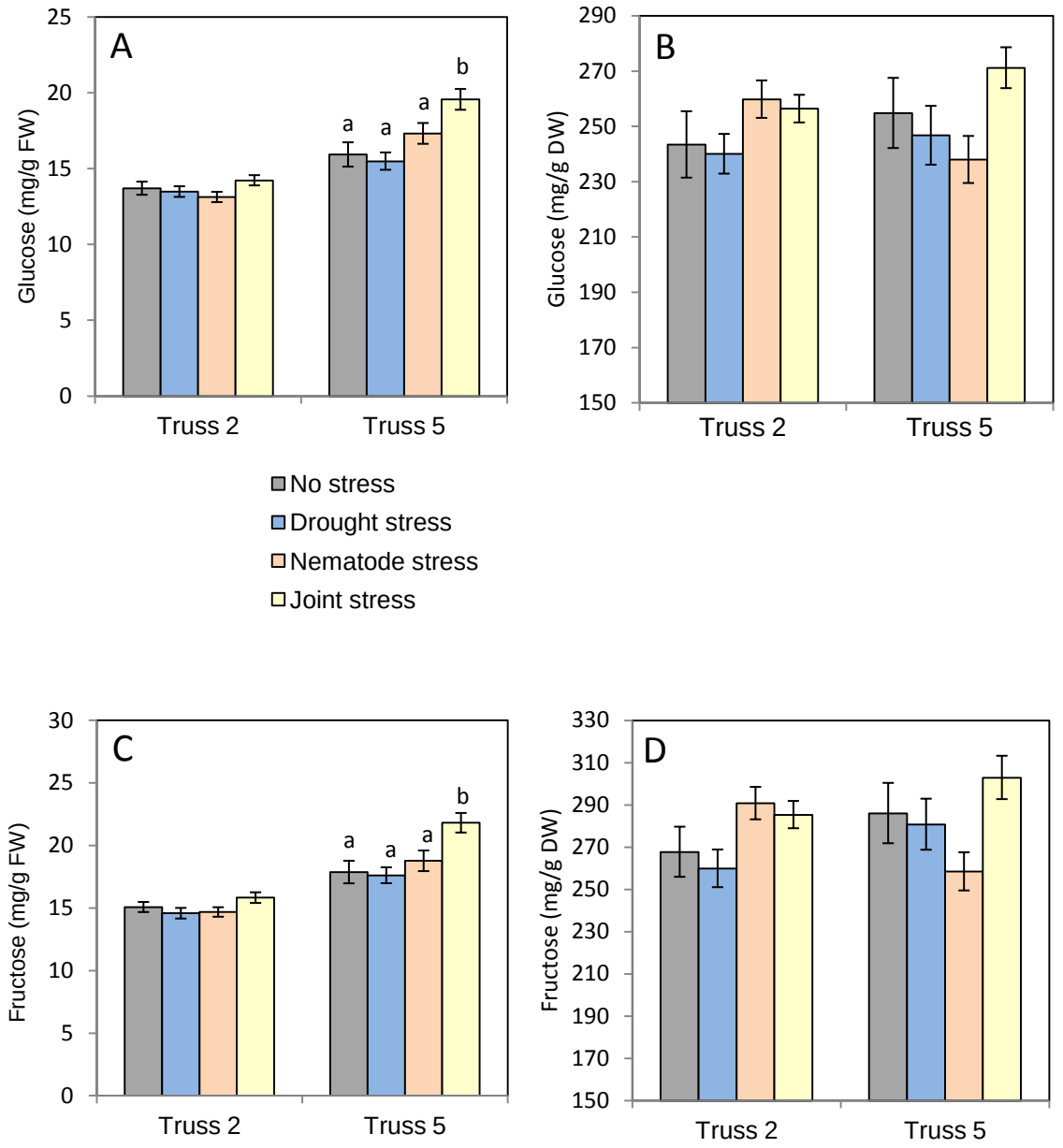


Figure 4.19. Concentration of glucose and fructose in tomatoes after differing stress treatments. Concentrations of the sugars glucose, expressed **A**) per fresh weight and **B)** per dry weight, and fructose expressed **C**) per fresh weight and **D)** per dry weight in fruits from tomato plants subjected to drought, nematode or joint stress. Fruits were harvested either early (Truss 2) or late (Truss 5) in the experiment. Concentrations are expressed per gram of fresh weight. Bars represent the standard error of the mean (n=9). Means with different letters are significantly different within the truss position according to the SNK test ($p < 0.05$) and bars displaying two letters show no difference from either group.

4.4 Discussion

4.4.1 Systemic response of tomato to *G. pallida* and *M. incognita*

Infection of tomato roots with the plant-parasitic nematodes *G. pallida* and *M. incognita* was of sufficient magnitude to induce a change in pathogenesis-related (*PR*) gene expression in distal leaf tissue (Figure 4.3). *G. pallida* induced the expression of the genes *PR1a, PR1b* and *PR2,* whilst *M. incognita* induced *PR1a* and *PR2*. The induction of *PR* genes following nematode infection is indicative of a salicylic acid (SA) defence response. SA is an inhibitor of cyst nematode parasitism in *A. thaliana*, and SA treatment has been shown to reduce susceptibility to *H. schachtii* in *A. thaliana* as well as inhibiting root galling by the nematode *M. incognita* in tomato (Wubben *et al.*, 2008; Molinari and Baser, 2010). In a previous study on leaves of tomato plants infected with *Meloidogyne javanica PR1* expression was also induced whilst no change was observed in *PR3*, although contrary to the findings here *PR2* was not induced (Sanz-Alferez *et al.*, 2008). Increases in *PR1* and *PR1b* have been previously induced in tomato in response to a potato cyst nematode *Globodera rostochiensis,* although **t**hese transcript increases were identified in root tissue (Sobczak *et al.*, 2005; Swiecicka *et al.*, 2009). In contrast to their up-regulation in distal tissues, PR genes are often suppressed in nematode feeding sites. There is evidence that this down-regulation may be a deliberate strategy by nematodes to repress the SA-mediated pathogen defence system (Bar-Or *et al.*, 2005; Jammes *et al.*, 2005; Sanz-Alferez *et al.*, 2008; Wubben *et al.*, 2008). The cyst nematode *G. pallida* elicited a stronger PR gene induction than the root-knot nematode *M. incognita.* The difference in magnitude may relate to the differing modes of invasion. Root-knot nematodes migrate through the root intercellularly, leaving no visible effect on plant cells in the invasion path (Karssen and Moens, 2006). In contrast cyst nematodes migrate intracellularly to gain access to the vascular cylinder, cutting open cell walls in a destructive manner using thrusts of the stylet (Wyss and Grundler, 1992). The higher level of wounding may thus cause a stronger activation of the systemic SA-mediated pathogen response system.

4.4.2 Interaction between drought and nematode stress

Experiments were performed to characterise the interaction between drought stress and nematode stress in tomato plants. Nematodes can induce drought stress symptoms in

their hosts due to the disruption of water transport, reduction in transpiration and stomatal closure (Wallace, 1987; Dorhout *et al.*, 1991; Fasan and Haverkort, 1991; Haverkort *et al.*, 1991; Ehwaeti *et al.*, 1998). Therefore, it may be expected that the combined effect of nematode and drought stress would be additive, as has been described before in tomatoes infected with *M. incognita* (Ehwaeti *et al.*, 1998). In contrast, plants whose roots were infected with *G. pallida* were observed to suffer less from the effects of drought than control plants. Wilting occurred later in the infected plants and the Relative Water Content (RWC) of leaves was significantly higher than controls after 10 days of drought stress (Figure 4.4). Infected plants had sparser foliage than the control plants, reflected by the significantly shorter leaf length. The disruption of water and nutrient uptake by roots infected with plant-parasitic nematodes can lead to stunted growth in solanaceous plants, including a reduction in plant dry weight, plant height, leaf number and leaf area (Wallace, 1974, 1987; Fasan and Haverkort, 1991; Williamson and Hussey, 1996; Ehwaeti *et al.*, 1998). This has been specifically demonstrated in tomato, where water uptake and flow through roots infected with *M. incognita* is significantly lower than uninfected roots (Dorhout *et al.*, 1991). It is therefore likely that the reduced foliage density and leaf length observed here was a direct result of nematode infestation. This smaller leaf area led to the infected plants using up available pot moisture more slowly than the controls, and thus experiencing drought later. This phenomenon has been previously observed in potatoes, where plants infected with *G. pallida* had lower transpiration rates and used up less water than their uninfected counterparts, thus suffering less from drought (Haverkort *et al.*, 1991). In order to truly determine the effect of nematode infection on tomato response to drought stress it would be prudent to control the water content of the soil so that infected and uninfected plants experienced a similar level of stress.

Early drought stress was found not to affect the ability of *M. incognita* nematodes to infect tomato roots and develop. The drought stress imposed significantly reduced tomato stomatal conductance, which then recovered following re-watering and before the nematode infection. ABA is present at high endogenous levels in tomato and increases two-fold in response to drought (Achuo *et al.*, 2006; Asselbergh *et al.*, 2008b). Changes in ABA levels can affect the resistance of plants to pathogens, as described in Section 4.1.1 (Asselbergh *et al.*, 2008b). The evidence suggests that ABA influences pathogen response pathways by suppressing SA-induced defences (Asselbergh *et al.*,

2008b). In potato, drought stress allowed a greater rate of *G. pallida* multiplication (Fasan and Haverkort, 1991). In tomatoes already infected with *M. incognita*, drought stress caused a slightly lower multiplication rate of nematodes, although the results were not significant (Ehwaeti *et al.*, 1998). In the current study it has been demonstrated that no long-lasting changes are made to nematode defence systems in tomato following an early drought stress. After re-watering, the elevated ABA concentrations may have returned to basal levels, thus not disrupting the pathogen response at the time of infection. Mohr and Cahill (2003) proposed that endogenous ABA levels at the time of the pathogen challenge are important for susceptibility. It would be interesting to investigate the ability of *M. incognita* to infect plants currently undergoing drought, although the lack of water itself may present problems for nematode motility within the soil as well as root penetration.

4.4.3 Effect of drought and nematode stress on flowering and fruiting characteristics

The exposure of tomato plants to individual or combined drought stress and infection with *M. incognita* affected flowering time, ripening time, fruit yield and fruit dry matter accumulation. The results demonstrated a different pattern of response depending on the stress encountered and the time of harvesting, as well as a complex interaction between the effects of the stresses in combination. The level of drought treatment imposed was sufficient to cause a physiological change in plants, as demonstrated by a difference in stomatal conductance. All stress treatments caused a significant stunting in height compared to the control plants, suggesting a shift from growth towards stress survival mechanisms (Herms and Mattson, 1992; Chaves *et al.*, 2003). Flowering time is known to be a period in which plants are particularly susceptible to changes in water availability (Barnabas *et al.*, 2008; Mittler and Blumwald, 2010). In the current work, water deficit caused the tomato plants to flower later than the unstressed controls (Figure 4.8A). The inhibition of growth caused during the drought period may have led to a delay in the establishment of normal developmental and reproductive patterns (Chaves *et al.*, 2003). In a continuation of this trend, fruit from drought-stressed plants also ripened more slowly (Figure 4.8B). However, in contrast to previous studies, there was no effect of water deficit alone on yield (Figure 4.8C). Severe water stress is known to negatively influence yield in terms of kilograms per plant or per hectare (Mitchell *et al.*, 1991; De Pascale *et al.*, 2007), although the weight of individual fruits often

remains the same (Veit-Kohler *et al.*, 1999) or can actually increase (De Pascale *et al.*, 2007), perhaps explaining why no reduction in fruit size was seen in this study. In an experiment on drought in potato, researchers discovered that an early drought stress had no effect on late harvest yield, although infection with nematodes did have a lasting detrimental effect (Fasan and Haverkort, 1991). Infection with nematodes in the current study caused a severe yield impediment, producing fruits that were 20 % lighter and that ripened much faster than the controls (Figure 4.8C). Plants can shorten their life cycle in order to escape dehydration, so perhaps the faster ripening time of nematode-infected fruit is an adaptive response to reduced water transport and water use efficiency by plants (Chaves *et al.*, 2003; Mittler and Blumwald, 2010). The reduction of yield in tomato plants infected with both *Meloidogyne* and *Globodera* spp is well characterised, occurring due to the disruption of water and nutrient transport from the roots (Barker *et al.*, 1976; Dowe *et al.*, 2004). These factors may also have affected the timing of senescence, which occurred earlier in nematode-infected plants (Figure 4.9).

The late-harvested fruits from infected plants had a significantly lower water content than the unstressed fruits, suggesting that water relations in the plant were disrupted to a greater extent by the severe nematode stress than by water stress itself (Figure 4.10). Water deficit actually increased the water content of tomato fruits, a surprising finding in light of previous studies that found the contrary to be true (Mitchell *et al.*, 1991; Riggi *et al.*, 2008). The earlier timing of the drought stress in the current study may account for differences between results. Interestingly, when water deficit and nematode infection occurred in combination, the plant's physiological response was most similar to that of water stress alone in the early tomatoes, but to nematode stress alone in the late-harvested tomatoes, a finding reflected by the results of yield analysis in potatoes infected with *G. pallida* and subjected to drought (Fasan and Haverkort, 1991). The results support the hypothesis that plant stress responses are specifically tailored to the exact combination of environmental stresses encountered, to the extent that the plant responds to whichever stress is most severe, over-riding the pathway for the lesser stress (Anderson *et al.*, 2004; Asselbergh *et al.*, 2008b).

4.4.4. Change in fruit nutritional quality parameters in response to stress

4.4.4.1 Phenolic compounds

Phenolic compounds such as flavonoids are important antioxidants produced in plants in response to both biotic and abiotic stress, and are also beneficial to humans when consumed (Hertog *et al.*, 1993; Knekt *et al.*, 1996; EnglishLoeb *et al.*, 1997; Williams *et al.*, 2004; Treutter, 2006; Giuntini *et al.*, 2008). Attempts have been made to raise flavonoid and chlorogenic acid levels transgenically, in order to increase nutritional quality (Muir *et al.*, 2001; Niggeweg *et al.*, 2004). This study found that as a result of severe nematode stress the levels of the flavonoids rutin, chalconaringenin and its isomer naringenin all increased significantly in tomato fruits (Figures 4.14 and 4.15). An interaction was observed whereby the effect of nematode stress was greater in truss 5 fruits. The activation of the flavonoid synthesis pathway has previously been described in response to infection with both cyst nematodes and root-knot nematodes, but localised in the roots during the establishment of the nematode feeding site (Wuyts *et al.*, 2006a; Ithal *et al.*, 2007a; Jones *et al.*, 2007; Klink *et al.*, 2010). Flavonoids themselves can act as nematode repellents and motility inhibitors for *M. incognita*, and changes to the flavonoid biosynthetic pathway can affect nematode reproduction rate (Wuyts *et al.*, 2006a; Wuyts *et al.*, 2006b). It has also been proposed that flavonoids may be necessary to influence local auxin transport pathways and thus allow the establishment of feeding cells (Hutangura *et al.*, 1999). However, nothing has previously been described about the influence of nematode infection on fruit flavonoids, as reported here. Plants may therefore respond to root-knot nematodes by activating a systemic defence system whereby flavonoid anti-feedants accumulate throughout plant tissues. Under severe biotic stress there may be a shift in carbon allocation towards the production of chemical defence compounds rather than growth.

Water stress has previously been reported to influence flavonoid levels in plants. Pernice *et al.* (2010) reported that although the accumulation of total flavonoids was heightened in fruits from plants under moderate drought, the concentration of naringenin was actually lower under extreme water deficit. Rutin and chlorogenic acid have been shown to accumulate in the foliage of tomato plants as a result of drought stress (EnglishLoeb *et al.*, 1997). In contrast, the current study found no such effect in the tomato fruits, with little or no change in flavonoid concentration observed as a result

of water stress. This suggests that the water status of the plant does not affect the process of stress-responsive flavonoid accumulation in the fruit. Interestingly when both stresses were applied to the plant in combination, the increase in flavonoid content was lower than under nematode stress alone. Therefore the water stress, although not significant in itself, may act to temper the biotic stress response induced by the nematodes, and thus maintain the flavonoid content at more normal levels. ABA accumulates in response to abiotic stress, and in turn inhibits the transcription of defence and pathogen-response genes (Zhu, 2002; Anderson *et al.*, 2004; Yasuda *et al.*, 2008). This phenomenon may thus explain the observed interaction of the two stresses, leading to the inhibition of the nematode-induced flavonoid accumulation.

Chalconaringenin was detected at a higher concentration than has been found in whole red tomatoes in previous studies, as summarised by Slimestad and Verheul (2009) where, depending on cultivar, the values ranged between 0.9 and 18.6 mg $100g^{-1}$. This difference can be attributed to the localisation of chalconaringenin in the peel, giving a higher concentration in peel/pericarp sections than in the whole fruit. Accordingly, studies examining peel in isolation have reported much higher concentrations (Iijima *et al.*, 2008). There is some debate as to whether naringenin is naturally present in ripe tomatoes, or if its detection is an artefact resulting from the spontaneous isomerisation of chalconaringenin during extraction, a process that can occur at low pH conditions (Mol *et al.*, 1985; Slimestad *et al.*, 2008). Many studies have previously treated chalconaringenin and naringenin as the same compound, reporting a single combined figure for both. This is now considered erroneous due to their very different spectral absorbencies (Slimestad and Verheul, 2009), and the fact that they can be separated via HPLC. In this study naringenin itself was detected at extremely low concentrations. This may be an indication of a more stable extraction procedure than previously documented, causing less isomerisation of chalconaringenin during sample preparation. Rutin and chlorogenic acid were detected at levels similar to those reported previously (Slimestad *et al.*, 2008; Slimestad and Verheul, 2009).

Chlorogenic acid has been shown to accumulate in tomato leaves in response to drought stress (EnglishLoeb *et al.*, 1997) and in tomato and pepper roots in response to root-knot nematodes (Hung and Rohde, 1973; Pegard *et al.*, 2005). Chlorogenic acid, though not itself a toxic compound, may be produced as part of a pool of available

phenylpropanoids which are broken down into activated defence components such as caffeic acid (Nicholson and Hammerschmidt, 1992; Pegard *et al.*, 2005). In the current study, the compound accumulated to a higher level under all three of the stress treatments in late-harvested fruits, indicating that chlorogenic acid is part of a generalised systemic stress system and not just a local response to pathogens. Its role as a potent antioxidant during other abiotic stresses such as UV-B exposure may explain its induction in fruit from drought-treated plants (Cle *et al.*, 2008).

Phenolic compounds are non-polar so are likely to associate with the insoluble fraction of tomato fruit. Analysis of flavonoid concentrations expressed per dry weight of fruit tissue revealed a similar pattern to that in fresh tissue for all the phenolic compounds (Figures 4.14B and D, 4.15B and D), although the differences between treatments were less pronounced, in some cases eliminating any significance. This indicates that differences in dry/fresh weight composition of tomatoes between treatment groups may partially account for differences in flavonoid levels, whereby drier fruit have a higher flavonoid level by default because they contain less water. In tomato products comprising dry tomato extract, therefore, the effect of environmental stress on fruit phenolic compounds may be less important.

4.4.4.2 Carotenoids

Studies on the effect of water stress on carotenoid levels in tomato fruits have previously revealed significant but inconsistent results (Dumas *et al.*, 2003), as described in Section 4.1.2. The analysis of carotenoids in the current study revealed a negative effect of water deficit stress on both lycopene and β-carotene accumulation, particularly in fruits harvested early, sooner after the period of drought stress (Figure 4.17). Truss 5 flowers appeared after the drought stress treatment had finished, perhaps accounting for the lesser effect on these late-harvested fruits. Carotenoids are important in the plant stress response as they act as scavengers for damaging oxygen radicals and also protect plant tissues by absorbing excess light (Smirnoff, 1993; Dorais *et al.*, 2008). Therefore, it could be expected that carotenoid levels would increase under osmotic stress conditions, as opposed to the observed decrease. It has been proposed that this inhibition in carotenoid accumulation may be related to the antagonism between abscisic acid and ethylene (Dorais *et al.*, 2008). Ethylene is crucial in regulating

carotenoid accumulation in response to UV-B stress, and lycopene and β-carotene in particular correlate positively with ethylene concentration in tomato fruits (Becatti *et al.*, 2009). Abscisic acid is produced rapidly in response to drought and osmotic stress in plants and is central in orchestrating stress response pathways (Zhu, 2002; Asselbergh *et al.*, 2008b). The signalling pathways of ethylene and abscisic acid are known to inhibit one another (Beaudoin *et al.*, 2000; Anderson *et al.*, 2004), and so the large-scale induction of abscisic acid in response to drought stress in the tomato plants may be the cause of the reduced carotenoid levels observed. A lack of ethylene may also explain the prolonged ripening time in the fruits that had undergone drought stress.

There was no significant effect of nematode infection on carotenoid levels in the tomato fruits. However, when examining the difference between carotenoid concentration patterns in the early and late fruits, some distinctions can be noted. Whereas lycopene level in joint stressed Truss 2 plants is significantly lower than the controls, in Truss 5 the relative level is increased and there is no difference from the controls. Furthermore, β-carotene levels in nematode-stressed Truss 2 tomatoes are no different to the controls, while in Truss 5 the level is significantly higher than the controls. These results indicate that as the nematode stress becomes more severe there is a positive influence on carotenoid accumulation. Root-knot nematodes are known to induce ethylene in infected tomato plants, and so the increase in carotenoid levels may be associated with an increase in ethylene production (Glazer *et al.*, 1983). As observed for phenolic compounds, the differences in carotenoid accumulation when expressed per dry weight were similar but less pronounced than those for fresh weight. The negative effect of drought stress on lycopene remained significant, underlying the importance of water availability in determining fruit nutritional quality.

4.4.4.3 Sugars

The concentration of hexose sugars in the tomato fruits was significantly increased as a result of combined water deficit and nematode infection in late-harvested fruit, even though each individual stress had no effect (Figure 4.19). Higher hexose concentrations have frequently been reported in fruits under water deficit or salinity stress, thus contributing to a higher fruit quality due to increased fruit sweetness and flavour (Gao *et al.*, 1998; Auerswald *et al.*, 1999; Veit-Kohler *et al.*, 1999; Yin *et al.*, 2010). Under conditions of water or osmotic stress the sink strength of tomato fruit can be increased

in order to achieve a maintained level of assimilate translocation and accumulation of dry matter (Gao *et al.*, 1998; Veit-Kohler *et al.*, 1999; Yin *et al.*, 2010). To increase the sink strength in fruits of stressed plants, sucrose is hydrolysed more rapidly by the enzymes sucrose synthase and invertase and converted into starch, thus maintaining a sucrose gradient between the leaves and the fruit. During ripening the starch is converted back into the sugars glucose and fructose (Yelle *et al.*, 1988; Wang *et al.*, 1993). Although nematode stress or water deficit alone did not affect the process of sucrose translocation into the fruit, in the jointly stressed plants the reduced plant growth rate due to nematode infection combined with higher sink activity in the fruit due to osmotic stress may have caused a switch of carbohydrate allocation away from vegetative growth, thus channelling a higher level of sucrose into the fruits (Veit-Kohler *et al.*, 1999). Sucrose itself was not detected in the tomatoes. This absence is consistent with earlier studies that have failed to detect sucrose in ripe tomatoes or have found it present only in trace amounts, and indicates that by this stage of fruit development all the sucrose had been converted to starch (Gao *et al.*, 1998; Veit-Kohler *et al.*, 1999). In several reports stress-induced increases in sugar levels are not maintained when expressed as a proportion of dry weight (Mitchell *et al.*, 1991; Zushi and Matsuzoe, 1998), and the effect could be attributed to differing water contents of the fruits. In the current study when the sugar concentration was calculated as a proportion of dry weight, the differences in sugar levels between treatments were reduced as a result of differences in fruit water composition. However, significant differences were individually observed between the nematode-stressed fruits and those under joint stress, suggesting that the results were not merely due to fruit water content. In addition, tomato fruit sugars are most often quantified in terms of the proportion of fresh weight (Zushi and Matsuzoe, 1998; Auerswald *et al.*, 1999; Veit-Kohler *et al.*, 1999).

4.4.5 Concluding remarks

The results of this study highlight the influence of environmental stresses on physiology and tomato fruit parameters and indicate a complex interaction between the environment and the water status, growth and reproduction within the plants. Infection with plant-parasitic nematodes has been shown to disrupt the water status of plants, thus affecting their response to drought stress. It has also been demonstrated that after plants recover from an early abiotic stress there is no lasting disruption of defence and resistance mechanisms against nematode infection.

There is much interest in the possibility of improving the nutritional quality of tomato fruit by adjusting agronomic conditions to incur plant stress (Dorais *et al.*, 2008). Inflicting water stress has previously produced some success in improving levels of carotenoids and sugars (Mitchell *et al.*, 1991; Veit-Kohler *et al.*, 1999; De Pascale *et al.*, 2007; Pernice *et al.*, 2010). This has usually incurred a yield penalty, however. The current study has found that water deficit can furthermore delay flowering and ripening, and may actually diminish the levels of antioxidants such as carotenoids and some flavonoids, whilst having little effect on other nutritional compounds. An interesting comparison can be made with the effect that a biotic stress has on tomatoes: Infection with root-knot nematodes actually had a positive effect on the nutritional qualities of tomato fruits, albeit with greatly reduced yield.

There has been little research into the confounding effect of multiple stresses on nutritional quality in tomatoes, or their impact on a system designed to induce controlled water stress. This study has shown that the simultaneous imposition of biotic and abiotic stress results in a new profile of the levels of nutritional compounds that does not bear close resemblance to that of either stress individually. Certainly the effect of the combined stresses on antioxidant and sugar concentrations was not additive and would have been difficult to predict. In normal growing conditions plants are frequently exposed to more than one stress at any one time, and therefore care should be taken when proposing a set of environmental conditions to try and maximise quality parameters.

Chapter 5. General discussion

5.1 How plants control the response to multiple stresses

This study characterised the response of plants to a combination of drought stress and infection with plant-parasitic nematodes. Molecular and physiological data has suggested that plants respond differently to multiple simultaneous stresses than they do to individual stresses (Haverkort *et al.*, 1991; Rizhsky *et al.*, 2002; Rizhsky *et al.*, 2004; Voelckel and Baldwin, 2004; Luo *et al.*, 2005; Achuo *et al.*, 2006). This is proposed to be an adaptive mechanism whereby plants can save costly resources by sensing the exact set of environmental conditions encountered and responding accordingly (Anderson *et al.*, 2004). The effects of concurrent biotic and abiotic stress are of particular interest, as their signalling pathways are controlled by antagonistic hormones that can suppress alternative responses (Anderson *et al.*, 2004; Asselbergh *et al.*, 2008b; Ton *et al.*, 2009). Despite this, the whole-genome transcriptome response of plants to simultaneous biotic and abiotic stress has not previously been described. This study has confirmed that plants respond differently to multiple stresses compared to individual stresses, activating new programmes of gene expression in response to each stress combination. The accumulation of secondary metabolites and nutritional compounds is also differentially affected. Specific categories of genes have been identified that may be important in controlling this novel stress response, of which several genes can individually affect plant stress resistance. The results support the theory that biotic and abiotic stress responses are antagonistic, and confirm the importance of hormones, particularly ABA, in managing stress responses. Understanding such processes is crucial for addressing the problem of crop productivity in a future of changing climatic conditions and growing population.

5.1.1. The response of plants to multiple stresses is different to that for individual stresses

The results of this study support the hypothesis that when stresses occur in combination the effect on plants is not additive (Rizhsky *et al.*, 2004; Mittler, 2006). The microarray experiment described in Chapter 2 revealed that when *A. thaliana* plants were exposed to drought and nematode stress in combination an entirely new programme of gene expression was activated that was distinct to that observed for either stress individually.

A total of 47 % of dehydration-responsive and 85 % of nematode-responsive genes were no longer expressed when the two stresses occurred concurrently. Furthermore, 2394 novel genes were differentially regulated specifically by the stress combination. Chapter 4 describes changes in nutritional compounds of tomato fruits exposed to single or combined stress. These changes were also non-additive and could not have been predicted from the results obtained for either stress individually. A striking example was the level of fructose and glucose in tomatoes. The concentration was not affected by drought stress or nematode stress alone, but was significantly increased by the stresses in combination. The concentration of phenolic compounds was predominantly affected by nematode infection, whilst carotenoid levels were affected mainly by drought stress. In both cases, the two stresses in combination had a mitigating effect on the changes caused by either stress individually. These findings highlight the necessity to study each stress combination as if it were a new type of stress. This was proposed by Mittler (2006) after the author's examination of the drastically different effect that a combination of drought and heat stress had on US crops between 1980 and 2004 compared to the effect of drought alone. Plants' differential response to multiple stresses at the molecular level may explain why in field conditions the effect of a biotic stress factor may either worsen or improve susceptibility to abiotic stress in crop plants, and *vice versa* (Cockfield and Potter, 1986; Haverkort *et al.*, 1991; EnglishLoeb *et al.*, 1997; Smit and Vamerali, 1998; Audebert *et al.*, 2000; Wiese *et al.*, 2004; Achuo *et al.*, 2006; Xu *et al.*, 2008).

5.1.2. Specific gene categories are induced by different stress combinations

The identification of gene categories differentially regulated by individual or combined stresses in this study provide an insight into the mechanism by which plants control the multiple stress response. Microarray technology is an excellent technique for characterising plants' responses to stress in increasing depth (Denby and Gehring, 2005). The use of online databases has further broadened understanding, as comparison across different expression experiments can improve the power to identify genes crucial to stress responses (Kennedy and Wilson, 2004). A substantial overlap was observed between dehydration-responsive genes identified in this study and those in other studies, confirming the importance of these genes in the response to water deficit (Huang *et al.*, 2008). Cyst nematode parasitism also induced gene categories in common with previous studies, although the differences between microarray studies carried out using varying

time points, tissues, technologies and nematode species means that direct comparisons can be difficult (Szakasits *et al.*, 2009). This study comprised the first analysis of systemic transcriptome changes in leaf tissue as a result of nematode infection. Observed changes indicated the up-regulation of novel signalling pathways involving MAP kinases and WRKY transcription factors. These may be important for transmitting defence signals to distal tissues (Jalali *et al.*, 2006).

In response to a combination of dehydration and nematode infection, genes involved in various key processes were found to be differentially regulated, several of which have been previously associated with plant response to multiple stresses. MYB transcription factors are known to be involved in both biotic and abiotic stress responses, and their prominence amongst multiple stress-induced genes in this study and in the study of combined heat and drought stress by Rhiszky *et al.* (2004) further supports a role for them in controlling crosstalk between pathways (Vannini *et al.*, 2004; Fujita *et al.*, 2006; Vannini *et al.*, 2006). The importance of MYB transcription factors in multiple stress response may be explained by their association with cell wall modification and the production of secondary metabolites such as lignins (Patzlaff *et al.*, 2003; Dubos *et al.*, 2010). Genes associated with both these processes were abundant amongst those up-regulated by joint stress. Both cell wall modification and lignin production can be induced in response to biotic and abiotic stresses (Davin and Lewis, 2000; Vorwerk *et al.*, 2004; Wuyts *et al.*, 2006a; Ithal *et al.*, 2007a; Pelloux *et al.*, 2007; Klink *et al.*, 2010), as they provide physical barriers against pathogens as well as maintaining cell turgor pressure during osmotic stress (Piro *et al.*, 2003; An *et al.*, 2008; Leucci *et al.*, 2008).

Therefore it may be that when plants experience two very different stresses simultaneously, the program of gene expression is switched to a more general defence mechanism that is likely to provide tolerance to a wider range of adverse environmental conditions. A large number of genes were activated by drought and nematode stress in combination, although each may have a small effect as reflected by the relatively low fold changes observed. The extent of this transcriptional response is far reaching, as joint stress-responsive genes were found to be involved in processes as diverse as carbohydrate metabolism, amino acid metabolism, immunity priming, growth inhibition and senescence. In order to control this complex response, many transcription factors,

MAP kinase cascades, hormone-responsive genes, disease resistance proteins and heat shock factors were also employed. These genes may be necessary to tailor stress responses to the exact conditions encountered, an important capability to enable the conservation of valuable resources such as sugars, proteins and secondary metabolites (Anderson *et al.*, 2004; Fujita *et al.*, 2006; Yasuda *et al.*, 2008; Mittler and Blumwald, 2010).

5.1.3. Plants balance stress responses with growth and reproductive requirements

The interconnectedness of abiotic and biotic stress response pathways may be explained by the necessity for plants to conserve resources. The ability of plants to respond to and tolerate stress stems from balancing resources between those needed for growth, and those needed for defence (Herms and Mattson, 1992; Bergelson and Purrington, 1996). This is highlighted by examples where the constitutive activation of a stress-responsive gene confers stress tolerance but at the detriment of growth and yield (Bechtold *et al.*, 2010). For instance, constitutive over-expression of *A. thaliana DREB1A* or rice *DREB1A* confers freezing and dehydration tolerance to *A. thaliana*, but results in severe growth retardation (Liu *et al.*, 1998; Kasuga *et al.*, 1999; Dubouzet *et al.*, 2003). The constitutive expression of a pigeonpea proline-rich protein in *A. thaliana* confers tolerance to osmotic, salt and heat stress but produces plants that are stunted in size (Priyanka *et al.*, 2010). A similar effect was observed in this study in *A. thaliana* plants expressing the 35S::ATMGL transgene. Plants showed resistance to infection with *H. schachtii*, but had a greatly reduced growth rate and seed yield. This negative effect could be prevented by expressing the transgene under the stress-inducible promoter *rd29A*, thus only mobilising plant resources to provide tolerance during times when stress conditions actually occur (Kasuga *et al.*, 1999). Similarly, if aiming to confer nematode resistance by the over-expression of a transgene, a root-specific or feeding site specific promoter would ideally be used (Lilley *et al.*, 2004). When environmental stresses become threatening to the survival of plants, resources are further channelled into reproductive processes and away from growth. Such measures include shortening the life cycle to allow reproduction to occur as quickly as possible (Chaves *et al.*, 2003). This was demonstrated in the current work by tomato plants that had been exposed to nematode stress for an extended period. These produced smaller fruits that ripened significantly faster than their unstressed counterparts.

The cost to plants of defence is reduced if stress-related genes and compounds have several different roles (Herms and Mattson, 1992). We have observed that *RALFL8,* a gene usually expressed exclusively in stamen and pollen maturation, is activated to cause re-modelling of the cell wall in roots to provide tolerance during dehydration and nematode stress. Other flower genes are similarly employed in nematode feeding sites (Karimi *et al.*, 2002; Kanter *et al.*, 2005; Siddique *et al.*, 2009). Antioxidants such as flavonoids are particularly multifunctional, connecting both abiotic and biotic stress responses. Flavonoids protect plant tissues against UV-B and oxidative stress, accumulate as insect antifeedants and act as signalling molecules in the interaction between plants and symbiotic bacteria (Williams *et al.*, 2004; Treutter, 2006; Giuntini *et al.*, 2008). The current work has confirmed in addition that flavonoid production is increased in tomato fruits from plants that have been infected with parasitic nematodes. This substantiates their role as protective molecules that are induced not just locally but systemically following pathogen infection. Chlorogenic acid is similarly utilised in both biotic and abiotic stress responses (Hung and Rohde, 1973; Nicholson and Hammerschmidt, 1992; EnglishLoeb *et al.*, 1997; Pegard *et al.*, 2005; Cle *et al.*, 2008). Induced by drought and nematode infection in tomato fruits, its role as a potent antioxidant and an available precursor in the phenylpropanoid pathway provides benefits to plants experiencing a wide range of stresses. The antioxidant properties of these compounds are sufficiently potent as to be beneficial in human diets (Knekt *et al.*, 1996; Mayne, 1996; Rao and Agarwal, 2000; Bassoli *et al.*, 2008)¸ thus deepening our incentive to understand plant stress responses.

5.1.4. Abiotic and biotic stress responses are antagonistic and controlled by ABA

Several of the results obtained from this study support the theory that under combined biotic and abiotic stress the pathogen defence response becomes down-regulated, a process controlled by the global stress regulator ABA (Anderson *et al.*, 2004; Asselbergh *et al.*, 2008b; Yasuda *et al.*, 2008). Firstly, *A. thaliana* plants undergoing severe drought treatment suffered a higher rate of nematode infection compared to well-watered plants, suggesting that drought stress compromised the resistance to nematode infection. Secondly, a much greater proportion of genes induced in response to nematode infection alone were no longer differentially regulated under combined stress (85 %) than the proportion of dehydration-induced genes (47 %). This suggests that the response to abiotic stress was prioritised over the pathogen response. However, this may

also have been due to the magnitude of stress treatment, as the dehydration treatment was more severe. Thirdly, amongst the genes differentially regulated by joint stress treatment, a large number encoding LRR proteins were down-regulated, whilst few were up-regulated. LRR proteins, which include pathogen R-genes, are involved in pathogen recognition and signalling (Dangl and Jones, 2001; Tameling and Joosten, 2007; Padmanabhan *et al.*, 2009), and may therefore be targeted for down-regulation when stresses occur simultaneously. Fourthly, analysis with the online expression database Genevestigator revealed that a large proportion of the joint stress-induced genes were highly regulated by the hormones ABA, JA, SA or ethylene, suggesting that the observed transcriptome response was strongly coordinated by interaction between these hormones. Most of the candidate genes analysed fell into this category. Expression analysis in hormone signalling mutants showed that *AZI1* and *DUF581* appeared to be repressed by ABA, whilst *ATMGL* was positively regulated. *AZI1* was positively regulated by ethylene, whilst *TCP9* was repressed. Lastly, study of the plant immunity priming gene *AZI1* provided evidence that under conditions of abiotic stress, ABA may antagonise and therefore down-regulate this systemic immunity pathway. In contrast, when constitutively activated the *AZI1* pathway inhibited abiotic stress tolerance. These results together support the evidence for antagonism between biotic and abiotic stress responses in plants, and substantiate the role of ABA as a global stress regulatory hormone.

A new model has been proposed regarding the multifaceted role of ABA in pathogen response, whereby the influence of ABA depends on the timescale of infection and also the nature of the attacker (Ton *et al.*, 2009). The model refers to three distinct phases of pathogen infection. In the first, ABA causes stomatal closure to provide penetration resistance to pathogens such as bacteria, thus having a positive effect on the defence response. At this stage ABA antagonises SA, JA and ethylene pathways in order to save resources, as their effects are not yet required. In the second phase, post-invasion defences centre on callose deposition to strengthen cell walls, a process that is aided by ABA during fungal infection but repressed during bacterial infection. During phase three of infection PAMPs (pathogen-associated molecular patterns) induce the hormones SA, JA and ethylene and long distance signals to regulate a broad spectrum of defensive compounds. The ABA-inducible transcription factors *ERD1* and *ATAF1* have been identified as switches which may activate ABA-dependent biotic stress responses

at the expense of abiotic responses (Kariola *et al.*, 2006; Jensen *et al.*, 2008; Ton *et al.*, 2009). However, increased ABA levels arising from abiotic stress conditions may repress the SA, JA and ethylene responses even during phase three. This hypothesis provides a mechanism for the control of ABA over both biotic and abiotic stress signalling, explaining the previous conflicting data (Asselbergh *et al.*, 2008b). It also highlights the need to study stress responses at a range of time points following stress induction. This would have been especially applicable to the current study, in which by necessity the drought and nematode stress had to be imposed consecutively rather than simultaneously.

5.1.5. Individual genes play important roles in multiple stress responses

Plant responses to combined biotic and abiotic stresses are highly complex and involve a variety of interrelated processes, but by elucidating the function of individual genes it is possible to illuminate some of the key mechanisms by which plants control this interaction (Park *et al.*, 2001; Mengiste *et al.*, 2003; Anderson *et al.*, 2004; Fujita *et al.*, 2006; Zhang *et al.*, 2009). One of the key aims of modern plant science is the quest to functionally characterise all the genes in the model plant *A. thaliana* to provide a basis for studying other species (Parinov and Sundaresan, 2000; Kennedy and Wilson, 2004). The results of Chapter 3 of this study will contribute to this pursuit, providing new information on the empirical function of the ten selected candidate genes as well as their involvement in stress responses. Six genes were previously uncharacterised with regard to function, including *RALFL8, ANACO38* and *TCP9*. Of the candidate genes, five were found to affect plant resistance to drought or nematode stress when expression levels were manipulated. Study of *RALFL8* has provided a new insight into links between auxin signalling and stress responses in roots. In order to confirm and further explore the role of these genes in stress resistance, it would be interesting to grow the mutant and over-expression lines under a range of stresses in combination, in conditions as close as possible to field conditions (Mittler and Blumwald, 2010). Genes could be expressed under a stress-inducible promoter to minimise growth and yield loss (Kasuga *et al.*, 1999). A subsequent step would be to identify homologous genes in agriculturally important crop plants and carry out similar analyses.

5.2 A new focus in plant stress research

5.2.1. Environmental pressures and the need for increased crop productivity

The pressure on global crop production is increasing due to climate change and population expansion, presenting the requirement for new stress-tolerant cultivars. Recent predictions based on world population growth indicate that it will be necessary to produce 70 % more food by 2050, requiring an annual increase in food production that is greater than anything achieved so far (FAO, 2009; Tester and Langridge, 2010). Changes in climatic conditions are likely to exacerbate this problem, as the frequency of adverse weather conditions such as drought, high precipitation events, high temperatures and tropical storms is expected to rise (Easterling *et al.*, 2000; IPCC, 2007). In particular, warmer, drier summers in mid-continental regions such as central Europe and central Africa are predicted, along with a reduction in growing season in many regions, extensive salinisation as sea levels rise and a decrease in land suitable for agriculture (Easterling *et al.*, 2000; IPCC, 2007, 2008; Morison *et al.*, 2008). Increases in variability of rainfall and temperature have been shown to put yields at risk as well as adversely affecting nutritional quality of crops (Porter and Semenov, 2005). Crops will be especially at risk during the increasing number of occasions when simultaneous drought and high temperature occur, as this stress combination has been shown to be particularly damaging to agriculture (Easterling *et al.*, 2000; Mittler, 2006; Battisti and Naylor, 2009). The growing population and associated intensification of agriculture will add further strain to global fresh water supplies, the majority of which are already used for irrigation (Shiklomanov, 2000; FAO, 2011). An increase in agriculture has led to the capacity of water resources being exceeded in some parts of China, causing extreme environmental degradation, the drying of major rivers and the abandonment of farmland due to dust storms (Morison *et al.*, 2008). Many parts of the world already have legislative restrictions on water use for agriculture. It is therefore essential that new varieties of crops are produced that can withstand environmental pressures whilst conserving water (Takeda and Matsuoka, 2008).

5.2.2. The importance of improving water use efficiency

The necessity for a change of focus in plant stress research, particularly drought research, has become apparent. Plants that are tolerant to drought stress limit water loss by closing stomata and making physiological and osmotic adjustments. But even

moderate drought stress responses limit photosynthesis and divert resources away from growth, negatively affecting yield (Morison *et al.*, 2008). Rather than developing crops that can survive extreme drought events, which are still relatively rare in commercial agriculture, it may be more beneficial to produce crops which have increased water use efficiency but which maintain high photosynthesis and yield (Condon *et al.*, 2004; Morison *et al.*, 2008; Bechtold *et al.*, 2010). The aim would thus be to reduce the amount of water needed for a given unit of yield (Passioura, 2006). Molecular studies that have aimed to improve drought tolerance by the over-expression of a single gene involved in the immediate response to short term dehydration treatment have been criticised, as these manipulations are unlikely to improve the water use efficiency of plants over their life-cycle, and may even confer adverse effects on yield (Kasuga *et al.*, 1999; Passioura, 2006; Priyanka *et al.*, 2010). Instead, advantageous traits for water use efficiency tend to be complex and active over the course of plant life cycle, characterised by increased photosynthetic activity under stress conditions (Passioura, 2006). An example of this is the development of new aerobic rice varieties. These have been bred to combine the drought tolerance of upland rice varieties with high yield characteristics of lowland varieties, allowing an increase in water use efficiency of between 32–88% (Bouman *et al.*, 2005). New non-destructive tools have been developed that may facilitate screening for water use efficiency phenotypes. Chlorophyll fluorescence is easily measured and provides a direct indication of CO_2 assimilation rates, useful for determining differences in drought response (Baker and Rosenqvist, 2004; Morison *et al.*, 2008). In addition, thermal imaging techniques reveal differences in stomatal behaviour and transpiration, as plants with open stomata transpire more and are therefore cooler (Merlot *et al.*, 2002). When screening plants for increased water use efficiency, the yield under water deficit conditions should be the main factor analysed rather than the ability to survive an extreme stress (Bechtold *et al.*, 2010; Mullineaux *et al.*, 2011). Quantitative trait loci (QTL) are polymorphic regions of the genome that are highly associated with variability in particular traits. The analysis of QTL has allowed identification of genomic regions responsible for biotic and abiotic stress, enabling breeding for improved water use efficiency (Collard *et al.*, 2005; Morison *et al.*, 2008; Mittler and Blumwald, 2010). For example, QTL analysis in sorghum has revealed four genomic regions that account for almost all the phenotypic variance in the delay of leaf senescence under water-limiting conditions, a trait that allows greater grain filling (Harris *et al.*, 2007; Takeda and Matsuoka, 2008). Progress

in sequencing of the sorghum genome should allow subsequent identification of the relevant genes (Takeda and Matsuoka, 2008). Selection for crops that use less water should be used alongside water management techniques, as run-off and soil evaporation from irrigated crops means that only 13-18 % of irrigated water is actually transpired by plants (Morison *et al.*, 2008). These may include mulching, minimum tillage and improved irrigation scheduling (Jones, 2004; Costa *et al.*, 2007). In addition, improved water use efficiency has been achieved by partial root-zone drying (PRD), a method whereby each half of a plant's root system is watered alternately. Long distance signals from the non-watered roots control stomatal regulation to reduce water loss, whilst the watered roots supply sufficient water to maintain yield development (Kang and Zhang, 2004). This method has been demonstrated to improve water use efficiency whilst maintaining yield (Morison *et al.*, 2008). It is worth noting that resource-poor agricultural systems such as subsistence farming may benefit more from crops bred to allow survival of severe drought conditions (Morison *et al.*, 2008).

5.2.3. Progress in developing nematode resistance

The search for crops that are resistant to nematode infection has also intensified over recent years (Atkinson *et al.*, 2003). This is partially due to the restrictions on traditional nematicides such as DCMP and methyl bromide due to environmental and health concerns (UNEP, 2000; Atkinson *et al.*, 2003; Fuller *et al.*, 2008). In addition, changes in climatic conditions may alter the infective range and population dynamics of various nematode species. For instance, a small temperature increase would allow the nematode *Radopholus similis* to infect a much greater area of banana cultivation in the East African Highlands (Nicol *et al.*, 2011). Meanwhile the drive to reduce water use in rice paddy cultivation in Asia may allow greater infection levels by *Meloidogyne graminicola*, a nematode which cannot survive in continually flooded conditions (De Waele and Elsen, 2007). In Britain the potato cultivar Maris Piper which contains the R-gene *H1* for resistance to *Globodera rostochiensis* has traditionally been grown. This has led to the concomitant rise in pathogenesis by *G. pallida* (Starr *et al.*, 2002). Thus far, the transfer of genes conferring resistance to this nematode into potato has been unsuccessful (Sobczak *et al.*, 2005). Other transgenic techniques may however provide possibilities for engineering nematode resistance. The expression of cysteine proteinase inhibitors in plant roots has provided resistance to several types of nematodes in crops including potato (Urwin *et al.*, 2001), cavendish bananas (Atkinson *et al.*, 2004) and

rice (Vain *et al.*, 1998). Resistance has also been demonstrated by transgenic expression of the plant defence compounds lectins (Burrows *et al.*, 1998), by the expression of a synthetic chemodisruptive peptide (Liu *et al.*, 2005) and through the use of *in planta* RNAi-mediated silencing of nematode genes (Fuller *et al.*, 2008). Simpler physiological traits such as increased root dynamics may provide better opportunities for combining nematode resistance with abiotic stress tolerance, however. The Cara cultivar of potato is tolerant to infection by the nematode *G. rostochiensis*, a trait attributed its ability to produce extra roots when attacked, thus maintaining top growth and resulting in a greater tuber yield than other cultivars (Trudgill and Cotes, 1983; Trudgill *et al.*, 1990). Its deeper root system also confers drought tolerance, and plants are tolerant to high salt levels (Elkhatib *et al.*, 2004). Analysis of ABA levels and stomatal activity suggest that Cara has a higher basal level of ABA than other cultivars and therefore an increased stomatal sensitivity to environmental changes (Fatemy *et al.*, 1985). However, because Cara is tolerant rather than resistant to nematodes, its cultivation allows the build-up of nematode populations in soil.

5.2.4. New potential for developing broad-spectrum stress tolerant crops

The ultimate goal of creating stress-tolerant crops either transgenically or through conventional breeding has pervaded almost all aspects of plant science, and is pursued by both public and private sector researchers. Success has already been achieved in many areas (Wang *et al.*, 2003; Umezawa *et al.*, 2006; Bhatnagar-Mathur *et al.*, 2008; Fuller *et al.*, 2008; WARDA, 2008). However, research programs aimed at developing tolerance to a particular stress do not necessarily test resistance to other biotic or abiotic stresses. This oversight can have damaging consequences. For example, a variety of cassava has been bred that is resistant to cassava mosaic virus (CMV), a pandemic causing huge losses across Central and East Africa. The variety was later found to be a greater target for another cassava pest, the whitefly *Bemisia tabaci* (Otim *et al.*, 2006). Studies examining yield failure in new drought-tolerant aerobic rice varieties found that the plants were particularly susceptible to nematode infection, perhaps due to their increased rooting length (Kreye *et al.*, 2009). Furthermore, transgenic cotton plants expressing the *Bacillus thuringiensis* (Bt) insecticidal protein *Cry* showed a reduction in the level of the protein during periods of high temperature, elevated CO_2 levels or drought, leading to decreased resistance to pests (Chen *et al.*, 2005; Dong and Li, 2007). The results described in this thesis confirm that plants respond differently to multiple

environmental stresses than they do to individual stresses. Therefore in order to develop crops that thrive and maintain a high yield in field conditions, an integrated approach should be adopted whereby resistance traits are tested under a range of stress treatments (Mittler and Blumwald, 2010). This would involve imposing both stresses simultaneously and measuring yield compared to non-resistant genotypes. This type of screening was described in Section 3.2.8. and Figure 3.26. In wild type plants, the imposition of combined drought and nematode stress reduced the seed yield to 85 % of the normal unstressed value. In contrast, when the *jaz7* mutant was subjected to the same stress combination, 106 % of the normal yield was produced, highlighting a potentially useful trait. Although abiotic and biotic stress response pathways act antagonistically, this study has indicated that under threat from two very different stresses, plants may activate a generalised tolerance mechanism to protect themselves from a wider range of stresses. The enhancement of this generalised mechanism could therefore provide a useful target for conferring broad-spectrum tolerance in crops. A similar theory has been suggested by Mullineaux *et al.* (2011). They hypothesise that plants have a network of genes controlling a basal stress response, regulated by heat shock factors (HSF), redox and hydrogen peroxide signalling. This network protects plants from low levels of stress and maintains a normal level of photosynthesis and growth up to a certain threshold. Once the threshold is crossed, plants activate a different more drastic stress response mechanism which enables survival in severe stress conditions, but leads to a loss in productivity. This drastic response includes the well-characterised stress pathways such as those controlled by the hormones ABA and SA and involves genes such as *DREB* transcription factors. The theory is supported by the finding that in *Arabidopsis HSFA1b* regulates over 500 genes, controlling tolerance to drought, extreme temperature and a range of biotrophic pathogens, as well as being a determinant of seed yield (Mullineaux *et al.*, 2011). Amongst the 500 downstream genes, none are *DREBs* or ABA regulatory genes, confirming that the observed response is different to that discovered previously. The *HSFA1b* gene is not differentially expressed during stress, but is post-transcriptionally regulated, perhaps providing an explanation for its late discovery in plant stress responses. This also adds fuel to the argument that transcriptomic studies alone are not sufficient to fully understand plant stress responses. The targeting of this newly discovered basal stress response provides exciting possibilities for crop improvement, as when *HSFA1b* was over-expressed in oilseed rape it conferred stress resistance phenotypes as well as

improving yield productivity (Mullineaux *et al.*, 2011). Another example of plants tolerant to both biotic and abiotic stresses was shown in the C24 genotype of *Arabidopsis* (Bechtold *et al.*, 2010). This accession has constitutive expression of SA-induced defences, but unlike other mutant genotypes with constitutive SA responses, C24 suffers no yield loss. It also has a greater water use efficiency, allowing protection from drought stress. This finding further demonstrates that it is possible to create plants with broad spectrum stress tolerance without affecting yield, and further studies are being carried out to determine the genetic basis for this beneficial trait (Bechtold *et al.*, 2010). The challenge for plant scientists in the 21st century will be to develop these traits in agriculturally important crop plants in order to improve stress resistance and productivity to feed an increasingly hungry world. To generate these new varieties, researchers will need to take advantage of a range of the latest technologies in crop improvement, including marker-assisted selection, QTL analysis, expression of transgenes and TILLING (Targeting Induced Local Lesions IN Genomes), a procedure in which new genetic variation is introduced through chemical mutagenesis (Henikoff *et al.*, 2004; Bhatnagar-Mathur *et al.*, 2008; Morison *et al.*, 2008; Mittler and Blumwald, 2010). These should be used alongside conventional selective breeding methods, which can be particularly useful in incorporating novel genetic material from crop wild relatives (Hajjar and Hodgkin, 2007). Wild species tend to be highly adapted to their environments and contain valuable sources of genetic variation (Hawtin *et al.*, 1996). In particular, the integration of genes derived from species already adapted to extreme environments may equip crop plants with traits that enable them to survive the challenges presented by a changing climate.

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List of Abbreviations

ABA	Abscisic acid
cDNA	Complimentary DNA
CPK	Calcium-dependent protein kinase
DNA	Deoxyribonucleic acid
DREB	Dehydration response element binding protein
DW	Dry weight
FW	Fresh weight
GO	Gene ontology
HPLC	High-performance liquid chromatography
HSF	Heat shock factor
HSP	Heat shock protein
J2	Juvenile nematode
JA	Jasmonic acid
LC-MS	Liquid chromatography / mass spectrometry
LEA	Late embryogenesis abundant
LRR	Leucine-rich repeat
MAPK	Mitogen-activated protein kinase
mRNA	Messenger RNA
MS	Murashige and Skoog
NASC	Nottingham Arabidopsis Stock Centre
PAMP	Pathogen activated molecular pattern
PCR	Polymerase chain reaction
PPR	Pentatricopeptide repeat
PR	Pathogenesis-related
qRT-PCR	Quantitative real time PCR
QTL	Quantitative trait loci
RMA	Robust multi-array averaging
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription PCR

RWC	Relative water content
SA	Salicylic acid
SAR	Systemic acquired resistance
SNK	Student-Newman-Keuls test
TAIR	The Arabidopsis Information Resource
T-DNA	Transfer DNA

Appendices

Appendix 1. PCR Conditions and Reagents

A

Reagent	Volume (µl)	Final Concentration
10 x Taq Buffer	2.5	1 x
dNTP mix	2.5	0.2 mM
$MgCl_2$	0.75	1.5 mM
Primers (forward + reverse)	1	0.2 µM each
Template DNA	0.5	As required
DNA polymerase	1	1 U
Sterile, distilled water	To 25	-

B

Step	Temperature (°C)	Time	
Initial denaturation	94	3 mins	
Denaturation	94	30 secs	x 25 cycles
Annealing	55	60 secs	
Extension	72	40 secs	
Final Extension	72	10 mins	

C

Reagent	Volume (µl)	Final Concentration
5 x HF Buffer	10	1 x
dNTP mix	5	1 mM
Primers (forward + reverse)	5	0.5 µM each
Template DNA	1	As required
DNA polymerase	0.5	1 U
Sterile, distilled water	To 50	-

D

Step	Temperature (°C)	Time (s)	
Initial denaturation	98	30	
Denaturation	98	15	x 30 cycles
Annealing	55	30	
Extension	72	30	
Final Extension	72	8 mins	

A) General volumes and concentrations of reagents used in RT-PCR. Reactions were carried out using BIOTAQ Red DNA Polymerase (Bioline).

B) General PCR cycling conditions used in RT-PCR. After 25 cycles the product increase was still in its exponential phase, thus relative quantities of cDNA could be compared semi-quantitatively.

C) Volumes and concentrations of PCR reagents for cloning. The whole coding sequences of genes were amplified using the proof-reading Phusion DNA Polymerase (NEB).

D) PCR cycling conditions used for proof-reading PCR using Phusion DNA Polymerase.

Appendix 2. Primer Sequences

Gene Name	Gene Accession	Primer Type	Forward Primer	Reverse Primer	Product Size(bp)
Chapter 2					
ACTIN2	AT3G18780	PCR	CTCAGGTATCGCTGACCGTA	GAGATCCACATCTGCTGGAAT	156
DREB1A	AT4G25480	PCR	AACATTTCAAACCGCTGAGA	AACAAACTCGGCATCTCAAA	312
DREB2A	AT5G05410	PCR	CAGCAGGATTCGCTATCTGT	CAGTCGTTGTGGGATTAAGG	368
PR-1	AT2G14610	PCR	GCTCAAGATAGCCCACAAGA	GGCTAAGTTTTCCCCGTAAG	177
MIOX5	AT5G56640	PCR	GACCTCGACGAACCACAAAT	CACACCCAACAGGAAATGTG	191
	AT1G61340	qRT-PCR	GTGATGAGATGGAGGATTCG	CCCACAGATAATCCTCACCA	140
	AT1G22190	qRT-PCR	AACCGTCGCAGTGAAACTAC	ATCTCCTCCTCCGTATCACC	135
CBF4	AT5G51990	qRT-PCR	CCCAGACTCGTTTCTCTCAA	ACGAAGAGCTAAAGCAGCAA	280
RD26	AT4G27410	qRT-PCR	TTATTGGAAAGCAACGGGTA	TCGTCAAGCTGTGATGAAGA	309
DREB1A	AT4G25480	qRT-PCR	AACATTTCAAACCGCTGAGA	AACAAACTCGGCATCTCAAA	312
DREB2A	AT5G05410	qRT-PCR	CAGCAGGATTCGCTATCTGT	CAGTCGTTGTGGGATTAAGG	368
	AT1G52800	qRT-PCR	ATCAAAAGACGGTGAATGGA	CATTGCTCCAACCCATAGAG	89
CYP71B2	AT1G13080	qRT-PCR	AAGGCAATTGTCATGGATGT	GACCCAGAGTGGTTCGAATA	136
MYB45	AT3G48920	qRT-PCR	GCAAAGGAAGGGATTATGGT	CAATCCAGCTTGAAGAGGAA	106
PYL4	AT2G38310	qRT-PCR	AGAGATCTCCGCTCCAATCT	AGAGACGACGTGGACTTGAC	148
	AT5G54040	qRT-PCR	ATGCTTATGACTCGCGGTAG	TTGTAATGCTTGTGGTGTGG	108
ACTIN2	AT3G18780	qRT-PCR	CTTGCACCAAGCAGCATGAA	CCGATCCAGACACTGTACTTCCTT	68
Chapter 3					
SALK LBb1.3	T-DNA left border primer		ATTTTGCCGATTTCGGAAC		
DIR14	At4g11210	T-DNA	TCCACCAAAGATCTCTCATGTG	TTAATATCAAGCTTGACGCGG	1254
AZI1	At4g12470	T-DNA	ACCCCTAAAAACCGAATCATG	AAGCACATTGGAAACCAGATG	1126
F2H15	At1g17970	T-DNA	ATTTTGGCGTGACTTTCTTTG	GGGAAAGAAGGAAGACATTGC	1145
ANACO38	At2g24430	T-DNA	AATATCCTGTATTCGTCGCCC	ATGAACGGAAGTCATGCATTC	1025

Appendix 2 (continued). Primer Sequences

Gene Name	Gene Accession	Primer Type	Forward Primer	Reverse Primer	Product Size(bp)
Chapter 3 (continued)					
JAZ7	At2g34600	T-DNA	GGTACACCGCGGATTAAAATC	ACCCATTTTAGGAGACCGTTG	1062
TCP9	At2g45680	T-DNA	CTCTTTCTGGTCGGTCGTATG	TGATGGTTAGATCAACGGCTC	1018
ATMGL	At1g64660	T-DNA	AGACTGAACATTGGCCACATC	TCCTTCGTTGACATAACGGAC	1012
DUF581	At5g65040	T-DNA	TAAATGTCACGATGATGGCAG	TCCGCACTAACTTTTGTCATG	1119
DIR14	At4g11210	Cloning	ACATCTAGAAACAATGGCAAACCAAATCTAC	ACATCTAGAAACAATGGCAAACCAAATCTAC	555
AZI1	At4g12470	Cloning	ACATCTAGAAACAATGGCTTCAAAGAACTCA	ACAGAGCTCTCAAGCACATTGGAAACCAGA	486
F2H15	At1g17970	Cloning	ACAGGATCCAACAATGTCTTCTACAACAATC	ACAGGTACCTTAAGGCTTGCCATATGCTG	1107
ANACO38	At2g24430	Cloning	ACAGGATCCAACAATGGAACAAGGAGATCAT	ACAGGTACCTCAATAAGATGGCCAGTATC	951
JAZ7	At2g34600	Cloning	ACATCTAGAAACAATGATCATCATCATCAAA	ACAGAGCTCCTATCGGTAACGGTGGTAAG	447
TCP9	At2g45680	Cloning	ACATCTAGAAACAATGGCGACAATTCAGAAG	ACAGGTACCTCAGTGGTTCGATGACCGTGCT	1071
ATMGL	At1g64660	Cloning	ACATCTAGAAACAATGGCTCATTTCCTCGAG	ACAGGTACCTTACATTCTGAGGAATGCTTTC	1326
DUF581	At5g65040	Cloning	ACATCTAGAAACAATGGTGTTAGGAAAGCGT	ACAGAGCTCCTAAATACGAATTGGTTTCT	342
RALFL8	At1g61563	Cloning	ACATCTAGAAACAATGGGGATGTCTAAAAGT	ACAGAGCTCTTAGGTGGGCTTTGGACCT	250
DIR14	At4g11210	qRT-PCR	CGTTTATGGAGCCGACTAGA	GCTTGACGCGGAAATACTTA	123
AZI1	At4g12470	qRT-PCR	CATTGGAAACCAGATGGAAG	TCTGAGGGCTAACGTTCTTG	99
F2H15	At1g17970	qRT-PCR	TCTTACCGAGATGAGGATGC	CCAAGCTCAAGAAGTTGCTC	126
ANACO38	At2g24430	qRT-PCR	CCATTCTTTTCCTCCCATTT	CGGATGAGGAGCTAATCTCA	143
JAZ7	At2g34600	qRT-PCR	GGCACATGTGTGTTTCTTCA	CCGTCTGAACTTCTCAAGGA	109
TCP9	At2g45680	qRT-PCR	CGTCGGATTTGTGACCTAAC	AGGTTGAAGGAAGAGGGAGA	92
ATMGL	At1g64660	qRT-PCR	AACACACGCTTTGCTCTCTC	TGTGTCCTACCCTCAACGAT	114
DUF581	At5g65040	qRT-PCR	TCTCGTCCAGCTTAATTTGC	GAAGTCTCTGCCATCATCGT	96
RALFL8	At1g61563	qRT-PCR	TCTTCTTGCAGGTGTTAGGG	TGAGGCCTCCGTAAGATACA	95
MYB4	At4g38620	qRT-PCR	AACAAATGGTCGCTTATTGC	GCTGATGATTCTTGGATTGG	143

Appendix 2 (continued). Primer Sequences

Gene Name	Gene Accession	Primer Type	Forward Primer	Reverse Primer	Product Size(bp)
Chapter 4 – Tomato Primers					
PR1b	DQ159948	PCR	GCATCCCGAGCACAAAACTAT	CAACACATTGGTTGGTAGCGTAG	170
PR1a2	Y08844	PCR	GAGCGGGTGATTGTAACTTG	CATTTTTCCGCTAACACAT	152
PR2b	M80608	PCR	CCAATTGTTGGGTTTTTGAG	TTCCTATATTGACGCGATCC	158
PR3	Z15141	PCR	ACCCTGATTTAGTTGCGACA	TTGGTAATGACACCGTACCC	172
EIF3	TC231903	PCR	GAGCGATGGATGGTGAATCT	TTGTACGTGCGTCCAGAAAG	149

Key to primer type:

Chapter 2 Primers (*A. thaliana*)

PCR. Primer sequences used to analyse transcript levels of genes by semi-quantitative RT-PCR. *ACTIN 2* was used as a normalising gene.

qRT-PCR. Primer sequences used in qRT-PCR validation of microarray results. *ACTIN2* was used as the normalisation gene. Primers were designed where possible to span exon boundaries to prevent the amplification of genomic DNA.

Chapter 3 Primers (*A. thaliana*)

T-DNA. Primer sequences used to detect presence of T-DNA insertion in mutant lines and confirm homozygosity. Forward = left primer (in gene flanking region 5' of insertion). Reverse = right primer (in gene flanking region 3' of insertion). SALK LBb1.3 = left border (region within T-DNA insertion in 5' -3' orientation). The combined use of the forward and reverse primers amplify the wild type allele whilst SALK LBb1.3 and the reverse primer amplify the T-DNA insertion allele.

Cloning. Primers used for amplification of the entire coding region for cloning. Each primer has the sequence of a restriction enzyme site within it to allow cloning into the 35S over-expression vector.

qRT-PCR. Primers used to detect the transgenic lines expressing the highest level of the transgene using qRT-PCR.

Chapter 4 Primers (tomato)

PCR. Primer sequences used to analyse transcript levels of genes in tomato by semi-quantitative RT-PCR. Elongation Initiation Factor 3 (*EIF3*) was used as a normalisation gene.