Comparative Analysis of the *MAX* Pathway

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Abstract

The pattern of branch outgrowth is a key determinant of the plant body plan. In most angiosperms branching is flexible, as branches are produced from axillary meristems which can either remain dormant or grow out. Strigolactones (SLs), a new class of plant hormones, repress branching in a range of angiosperms, including Arabidopsis, and there is increasing evidence that SLs are regulators of plant development in response to nutrient stress. This study has exploited genetic and physiological methods to investigate the evolution of SL biosynthesis and roles across the four major lineages of vascular plants.

The cytochrome P450 family member *MAX1* in Arabidopsis is required for the synthesis of SLs, and forms part of a signalling pathway containing at least four other genes in Arabidopsis and five in rice. Most other components of the strigolactone signalling pathway are conserved throughout the land plants, but *MAX1* orthologues are absent from the moss *Physcomitrella patens*, which nevertheless produces SLs. Unlike other members of the pathway *MAX1* orthologues have radiated in the angiosperms, particularly in the monocots. By use of complementation analysis this study presents evidence that *MAX1* catalytic function is conserved in lycopodiophytes and gymnosperms, and that it may therefore have been incorporated into the SL pathway before the division of the vascular plant groups. In angiosperms the radiation of *MAX1* gene copies has led to different evolutionary fates, of conservation of catalytic function in monocots, but divergence in dicots. Deletions of *MAX1* orthologues have also contributed to natural variation in shoot architecture in domestic rice. In addition, this study presents evidence that the action of *D27* in the biosynthetic pathway of SLs in rice is conserved in Arabidopsis. These genetic approaches are complemented with physiological investigation of the actions of strigolactones in non-angiosperm species, including spruce, fern and Selaginella species.

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Author's Declaration

Except where otherwise stated, the work presented in this thesis is my own. Identification of *MAX* orthologues was done in collaboration with Drs Céline Mouchel and Richard Challis as noted in Chapter 3, and of *D27* orthologues in Chapter 6 by Dr Richard Challis, as well as all the phylogenetic trees.

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Figure 3-2B in Chapter 3 has been previously published in Crawford *et al.* (2010).

Chapter 1. Introduction

"*On this same view of descent with modification, all the great facts in Morphology become intelligible, - whether we look to the same pattern displayed in the homologous organs, to whatever purpose applied, of the different species of a class; or to the homologous parts constructed on the same pattern in each individual animal and plant."*

Charles Darwin,
On the Origin of Species By Means of Natural Selection (1859)

The brilliant diversity of a tropical rainforest is the result of many hundreds of years of the interlocking growth, death and regrowth of thousands of species from all the kingdoms of life – plant, animal, fungus, bacteria, archaea and many of those strange branches of the life-river that are not readily recognised. Behind each of these species lies millennia of evolution: reproduction, mutation, and selection, so that each species has its particular capacities for survival among the great variety of environments found in just one square foot of a Darwinian tangled bank. Despite this astonishing array of abilities, the molecular tool-kits underlying this explosion of difference are often very similar. The same components are used to build similar modules, which are repeated with subtle differences depending on the genetics of the organism and, to some extent, its environment.

Plants in many ways exemplify this similarity of construction. Like metazoans, fungi and a few others, they are multicellular, an evolutionary innovation that allowed inner subfunctionalisation of the organism into different cell types. These cell types in themselves become repeated modules (tissues), which go together to form organs – structures that in plants particularly may be repeated many times. In flowering plants, roots and lateral roots are repeated to form complex networks, sepals, petals, stamens and carpels are repeated together to form flowers, and leaves, stem segments and axillary meristems are repeated to form the shoot and its branches.

The growth and positioning of cell types, tissues and organs in multicellular organisms are coordinated in the process known as development. Most metazoan species develop into organisms that can move, allowing them to change their environment by moving to a new one. In metazoans most developmental patterning is done early in life, and at the end of embryogenesis most of the major organs and tissues are specified. Although there are some exceptions, such as the change from tadpole to frog in the tetrapods, and the extreme developmental changes of larvae developing into adults in the arthropods, metazoans have one unchanging set of organs throughout – even in those that metamorphose, their final form is fixed as to the number and position of their organs. In organisms such as plants and fungi, which are sessile for most of their lifecycle, growth forms their main source of movement and response to their environment, and changes to developmental patterning continue throughout their lives and are vital to their survival. As a result, plants have evolved suites of mechanisms to sense their environment and to control and coordinate the production of different organs. The evolution of one small part of this coordination mechanism is discussed here.

1.1 Shoot branching

Shoot branching is one of the most recognisable characteristics of plant bodies, as branches provide the architecture from which leaves (the main source of energy) and the reproductive units form. The control of branch production, to allow optimal positioning of organs whose function depends on their local environment (light for leaves, accessibility to pollinators for flowers) is therefore key to determining the survival and reproduction of the plant. The development of branches, as for most other aspects of plant life, is best understood in the angiosperms, the flowering plants. In this group, the embryo is bipolar, with two regions from which the most of the plant will be formed: the root apical meristem and the shoot apical meristem (SAM). Meristems are the tightly coordinated structures of pluripotent cells that generate all post-embryonic plant tissues, including secondary meristems. These secondary meristems include the axillary shoot, lateral and adventitious root, and vascular cambial meristems, and from different inceptions take a number of different forms. Lateral and adventitious root meristems form *de novo* in both root and

shoot from the pericycle for lateral roots, or in the case of adventitious roots also from cambial tissue, and their siting and development is largely defined by hormone signalling (Benková and Bielach, 2010; Rasmussen *et al.*, 2012). The vascular cambium, a layer of meristematic cells within the vascular tissue that allows the secondary thickening of the stem, and is therefore important to the production of wood, is produced during the development and patterning of vascular tissues (reviewed in Baucher *et al.*, 2007). In the shoots of angiosperms axillary meristems form part of a series of repeated modules called phytomers, produced by the SAM, that make up the main stem. The phytomer consists of a section of stem (the internode), a leaf, the petiole of which joins the stem at the node, and between the leaf axil and the stem, an axillary meristem (Figure 1-1) (McSteen and Leyser, 2005).

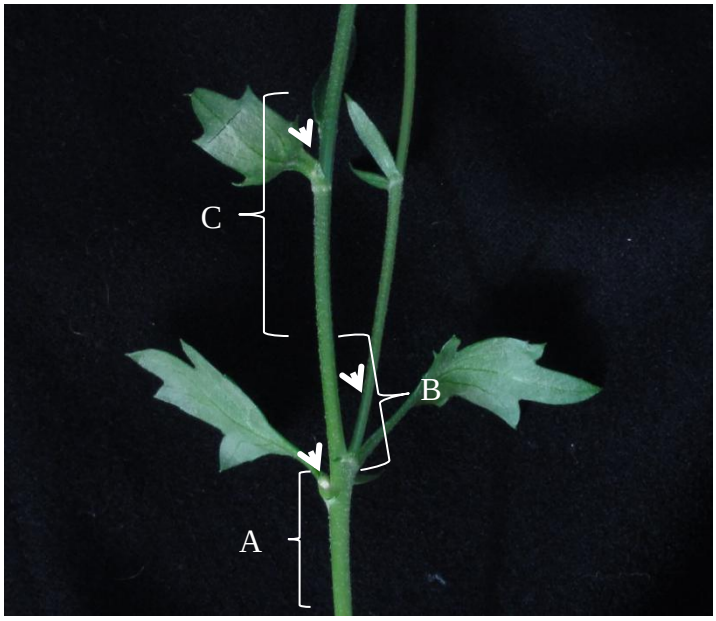


Figure 1-1. Three different phytomers in a chrysanthemum (*Dendranthema grandiflora*) stem – one with a dormant bud (A), one with a branch (B) and one with only an axillary meristem (C), and white arrows indicate bud, branch and axillary meristem (too small to see by the naked eye) respectively.

The relationship between primary and secondary meristems may be one of equilibrium or of varying degrees of dominance in either direction depending on environmental cues such as temperature, light, nutrient content of the soil; and developmental cues such as age and flowering status. Information about any of these factors can be locally produced or transmitted from organs far distant

from their site of influence. In the case of the SAM and subtending axillary meristems the relationship is often one of dominance by the SAM. Axillary meristems can either activate to produce branches or a flowering shoot, go perpetually dormant, or switch between dormancy and active growth. Those that have produced some tissue may also be called axillary buds, which may have the same or different developmental characteristics to those of axillary meristems (reviewed in Bennett and Leyser, 2006). In many angiosperms the primary shoot meristem restricts the outgrowth of axillary meristems and buds lower down the stem, rendering them dormant in a process called apical dominance. Should the primary shoot apex be lost (for example, broken off or eaten by predatory herbivores), axillary meristems will be released to grow out to replace the primary shoot. The long distance signalling required to coordinate the status of multiple meristems, the environment and the plant's developmental status is mediated by a variety of factors, including the movement of proteins and RNA and particularly a dedicated hormone signalling network (reviewed by Domagalska and Leyser, 2011). As a result, the control of shoot architecture in angiosperms consists of at least two interacting and conserved systems, firstly the shoot meristem, and secondly the hormone signalling system.

1.1.1 Shoot meristems

Although the molecular modules controlling the maintenance of shoot and root apical meristems as pluripotent regions contain a number of shared or similar components, only the processes involved in shoot meristem maintenance (and for axillary meristems, their production) will be discussed here. In Arabidopsis as in all seed plants, meristems are multicellular structures, in which more than one cell maintains pluripotency. Within the meristem an area of stem cells called the 'central zone' (CZ) grow and divide slowly, producing daughter cells that are moved by the continued production of cells out of this region of pluripotency to the peripheral zone (see Figure 1-2). In the peripheral zone new organs may become specified. This area of pluripotency is maintained by expression of the homeobox transcriptional repressor WUSCHEL (*WUS*) in the 'organising centre' (OC), a group of cells immediately below the CZ (reviewed in Besnard *et al.*, 2011). *WUS* is a member of the *WOX* family of

plant-specific homeobox transcription factors (TFs) that are implicated in meristem development in both roots and shoots in angiosperms, and are conserved throughout land plants, although the action of *WUS* itself is an angiosperm innovation (Nardmann *et al.*, 2009).

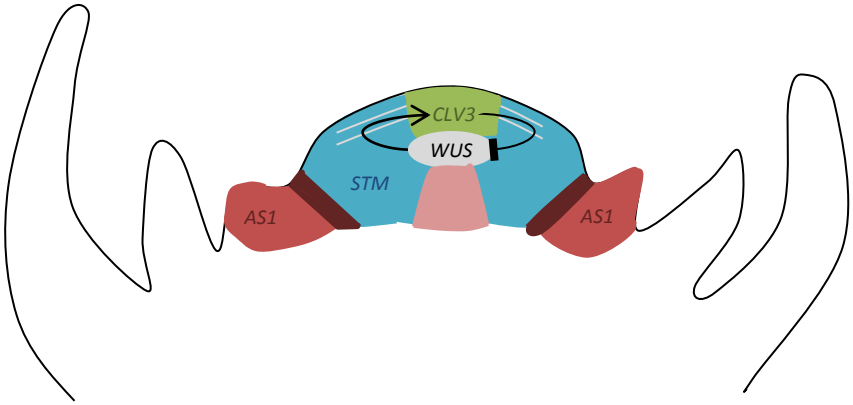


Figure 1-2. Structure of the SAM in Arabidopsis (surrounded by expanding leaves), with the areas of expression of some of the regulatory genes labelled. Blue = area of the meristem, red = differentiating primordia, grey = OC, green = CZ, pink = RZ. Deep red lines represent the organ boundary regions where genes such as *CUC* and *LAS* will be expressed. Adapted from Besnard *et al.* (2011).

The presence of *WUS* is required to maintain stem cell identity in the CZ. In turn, its expression is controlled by the production of a mobile peptide signal, *CLAVATA3*, produced by the CZ cells, which restricts *WUS* expression in the OC below (Katsir *et al.*, 2011). The balance of this interaction contributes to control of meristem activity and is affected by a number of factors, particularly the signalling of the cytokinin group of plant hormones, which are required for stem cell maintenance and which themselves are regulated by *WUS* (reviewed in Durbak *et al.*, 2012). Immediately below the OC the rib zone (RZ) forms the growing stem beneath the meristem, within which the vascular tissues of the stem differentiate. Throughout the CZ and OC and into the peripheral zone another meristem marker, *SHOOT MERISTEMLESS* (*STM*), is expressed. *STM*, like *WUS* is a member of a homeodomain TF family, the *KNOX* genes, which are involved in the specification of meristematic identity and whose actions are partly controlled through interacting with *BELLRINGER* (*BELL*) family homeodomain TFs (reviewed by Hay and Tsiantis, 2010). In angiosperms *KNOX* genes also interact antagonistically with the *ARP* family of genes such as

ASSYMETRIC LEAVES1 of Arabidopsis. *ARP* genes in Arabidopsis are expressed in emerging primordia during organogenesis, where they contribute to the downregulation of meristematic *KNOX* expression to provide determinacy. The sites at which lateral organs are produced in the peripheral zone are defined by the patterning of maxima of the hormone auxin, and auxin signalling contributes to downregulation of *KNOX* homologues. Auxin signalling also interacts with cytokinin signalling (CKs, another hormone group) at the CZ and OC to maintain high CK levels (Zhao *et al.*, 2010) and in turn in young and developing tissues CKs have been shown to upregulate auxin synthesis (Jones *et al.*, 2010). Thus these hormones between themselves, with other hormones (the gibberellins and brassinosteroids especially) and with other transcriptional and gene networks specific to the meristem provide a system of feedback and feedforward mechanisms that maintain the pluripotency of the meristem whilst allowing it to grow and react (Hay and Tsiantis, 2010; Besnard *et al.*, 2011; Durbak *et al.*, 2012).

1.1.1.1 Axillary meristems

The derivation of axillary meristems, whether arising *de novo*, in common with the mechanism suggested for root lateral meristem, or persisting as a detached part of the meristem of the primary meristem, has historically been a matter of debate in plant development. However, it seems that in angiosperms axillary meristems (AMes) are specified as part of leaf development within the phytomer, although due to changes in growth of different regions the AMe may end up on the leaf itself or on the stem some distance from it (this debate has been reviewed by Steeves and Sussex, 1989; and its conclusion reviewed by McSteen and Leyser, 2005). As a result, the correct establishment and placement of AMes is also related to the establishment of polarity in the subtending leaf, a process in which the Class III HD-ZIP family TFs such as *REVOLUTA*, among others, is involved, and to the correct specification of the boundaries of lateral organs, a process involving not only the *KNOX* and *ARP* factors noted above but also the actions of other transcription factors like the *CUP-SHAPED COTYLEDON* (CUC) family (Talbert *et al.*, 1995; Raman *et al.*, 2008; Hay and Tsiantis, 2010).

Axillary meristem specification itself is controlled by a suite of axillary-meristem specific factors in angiosperms, including the R2R3 Myb (TFs) *Blind/RAX1* in tomato and Arabidopsis, the *Ls/LAS/MOC1* GRAS TFs of tomato, Arabidopsis and rice and the *ROX/LAX1/BA1* bHLH TFs of Arabidopsis, rice and maize (McSteen and Leyser, 2005; reviewed in Yang *et al.*, 2012). *LAS* in particular is activated early in the development of angiosperm leaf primordia, though it specifies an area adjacent to the primordia, within the primary meristem region still defined as indeterminate by *STM* expression, and the expression of *LAS* is required for the reactivation of meristem identity later in the development of the leaf-AMe module (Greb *et al.*, 2003).

1.1.1.2 Dormancy control in axillary meristems

The maintenance of dormancy in these meristems is an equally complex process. Dormancy can take more than one form, and be imposed by different environmental and developmental stimuli (Rohde and Bhalerao, 2007). Likewise axillary meristems can adopt diverse fates giving rise to indeterminate shoot branches, determinate flowers and in some species underground storage organs, each of which may be subject to a different set of regulatory factors (Bennett and Leyser, 2006). Many of these factors are hormones, but in the case of branch production the TCP transcription factors *TB1* (in maize) and its Arabidopsis orthologues *BRANCHED1 (BRC1)* and *BRC2*, pea orthologue *PsBRC1* and rice orthologue *FINE CULM1 (FC1)* are important to the read-out of these interactions, to different extents in different species (Doebley *et al.*, 1997; Aguilar-Martinez *et al.*, 2007; Minakuchi *et al.*, 2010). All three have axillary meristem (AMe) specific expression and repress branch outgrowth, and *BRC1* expression closely correlates with axillary bud activity in Arabidopsis (Doebley *et al.*, 1997; Aguilar-Martinez *et al.*, 2007; Minakuchi *et al.*, 2010). Downstream of *TB1*, the class I HD-ZIP *GRASSY TILLERS1 (Gt1*) has recently been identified as also being an important negative regulator in axillary meristem outgrowth, and is also regulated by light, suggesting it forms part of the integration of the shade avoidance response in branching control (Whipple *et al.*, 2011). Upstream of the *Tb1/BRC* family, however, the precise factors regulating the mechanism of their downregulation have yet to be defined, and these may differ between species.

1.1.2 Hormone pathways

The hormones of plants (sometimes termed plant growth regulators), have a history of interest to investigators of plant development and shoot branching in particular going back over a century (possibly first reviewed by Bayliss, 1918). For many years a set of approximately five substances or substance groups were recognised as hormones – the auxins (a group of structures defined by their effect on plant growth, as suggested by its Greek namesake αυξειν, to grow), the cytokinins, the gibberellins, ethylene and abscisic acid (ABA; Santner and Estelle, 2009). More recently, this little population has bloomed, and the brassinosteroids, salicylic acid, jasmonic acid and strigolactone-related compounds have generally been accepted as hormones to some degree (Jaillais and Chory, 2010)**.** Mutants in Arabidopsis suggest the existence of at least one other, as-yet-unidentified and carotenoid derived signal (reviewed in Mouchel and Leyser, 2007; Lee *et al.*, 2012). Several other groups of non-cell autonomous signalling molecules exist, including the short peptide signals such as *CLAVATA3*, reactive oxygen species, mobile RNAs, and some have been proposed to have hormone-like properties and actions, such as *FT,* the mobile protein that is required for photoperiodic induction of flowering in Arabidopsis (the much sought-for 'florigen') and also regulates seasonal dormancy in poplar (Böhlenius *et al.*, 2006; signalling molecules reviewed by Van Norman *et al.*, 2011; Turnbull, 2011). However, the term hormone in plants is usually applied to the small molecules derived from secondary metabolism that can carry long-range signals and are active at low levels (Santner and Estelle, 2009; Jaillais and Chory, 2010).

Several of these hormones have been implicated in the control of shoot branching and dormancy in axillary meristems, including all of the original canonical five at some time, a point perhaps unsurprising given the generally pleiotropic nature of plant hormones. However, of these, auxin was the first identified (Thimann and Skoog, 1933) and is one of the most important in shoot branching, along with cytokinins and the newest group of hormones, the strigolactones.

1.1.2.1 Auxin

The hormone auxin is one of the best characterised signals known in plant development and evolution, and probably the most important. Auxin has a role in a vast array of environmental and internal developmental processes, acting as a morphogen in the establishment of plant body axes, tracing the future lines of vasculature, and regulating the growth rate, positioning and production of organs in both shoots and roots in response to internal developmental and external environmental cues (reviewed by Leyser, 2011). One particular function it performs in many seed plants is the control of shoot branching (McSteen and Leyser, 2005; Cline *et al.*, 2006).

A particular feature of auxin signalling is the importance not only of its presence but of its movement – the polar auxin transport (PAT) mechanism. This mechanism is a unique and specific, self-regulating and self-organising transport system of dedicated plasma-membrane influx and efflux carriers (Benjamins and Scheres, 2008). The self-organising nature of auxin transport is vital to the establishment of the peaks and troughs in auxin concentration that specify the emergence of organs in both root and shoot, and is generated through complex feedback and feedforward mechanisms acting on the placement and action of the influx and efflux carriers. These mechanisms have provided material for a number of elegant mathematical models of plant development (for example, those of Smith *et al.*, 2006; Lucas *et al.*, 2008; and Prusinkiewicz *et al.*, 2009). The production of auxin transport channels – a process known as canalisation – is driven in part by the behaviour of the PIN family of auxin efflux carriers, which export auxin across the plasma membrane, but are continuously cycled from there to internal vesicles, a process necessary for plant development (Paciorek *et al.*, 2005). This endocytotic cycling requires, in the case of PIN1 and PIN7, the action of the ADP ribosylation factor-GTP/GDP exchange factor (ARF-GEF) GNOM, which is involved in the regulation of vesicular trafficking to endosomes, and *gnom* mutants show severe patterning defects from embryogenesis. Constant endocytotic cycling allows changes to the polarity of PIN protein localisation on the plasma membrane, and this localisation is partly controlled by the auxin-regulated protein serine/threonine kinase PINOID through the phosphorylation

status of the PINs (Benjamins and Scheres, 2008). Endocytotic cycling is inhibited by auxin itself, possibly through the action of the *ABP1* auxin receptor, so that auxin self-regulates its own polar transport stream both by stabilising PIN proteins at the plasma membrane, and (via PINOID and other factors) by polarising them in the direction of auxin flow, thus generating directional, self-reinforcing transport (Paciorek *et al.*, 2005; Benjamins and Scheres, 2008; Dhonukshe *et al.*, 2008; Robert *et al.*, 2010). The resulting auxin channels may then differentiate into vascular traces, and so play an important role in the development of the vascular network, and the channels remain in the adult vascular tissue throughout the plant (Sachs, 1981; Baucher *et al.*, 2007).

In the control of shoot branching, the polar transport of auxin, travelling from its point of synthesis in the growing tip and tissues of the shoot, down the stem to its point of action, is key to the maintenance of dormancy in axillary meristems. Removal of the auxin source by decapitation of the growing shoot tip leads to the outgrowth of axillary buds further down the stem, and replacement of this source by exogenously supplied auxin can prevent this outgrowth (Thimann and Skoog, 1933). Disruption of polar auxin transport with inhibitors also allows outgrowth of buds further down (Panigrahi and Audus, 1966; Chatfield *et al.*, 2000). However, the points and mechanism of auxin action in shoot branching are more complex than the simple presence of auxin from the shoot directly repressing outgrowth, as auxin from the polar auxin transport stream does not enter the bud itself (Booker *et al.*, 2003). The presence of one or more second messengers has therefore been postulated (Booker *et al.*, 2003).

1.1.2.2 Cytokinins

The actions of cytokinins (CKs) are likely to form at least part of this second messenger role (reviewed in detail by Muller and Leyser, 2011). CKs are both synthesised locally in the bud and travel upwards from the roots, directly promote meristem activity and can promote bud outgrowth when applied directly to the bud (Muller and Leyser, 2011 and references therein). When basally applied CKs can activate buds even in the presence of apical auxin, and thus they act antagonistically to auxin in apical dominance (Chatfield

et al., 2000). CK production in the nodal stem is downregulated by apical auxin, and this has contributed to a model in which release of CK production from repression by the loss of apical auxin on decapitation promotes bud outgrowth (Tanaka *et al.*, 2006). Cytokinins are implicated in the promotion of meristem identity and outgrowth, partly through their interactions with auxin itself and through direct effects on cell cycling (reviewed in Durbak *et al.*, 2012). However the precise mechanisms of CK promotion of bud outgrowth is likely to be considerably more complicated, as the feedback loops between CKs and auxin act at a number of levels (Muller and Leyser, 2011), some of which are discussed below.

1.1.2.3 Strigolactones

Mutants in a range of species revealed the existence of another factor, acting in concert with auxin and cytokinins (reviewed in Domagalska and Leyser, 2011). In Arabidopsis these mutants were termed the *max* mutants, for *More AXillary growth.* The *MAX* pathway produces and responds to a signal that acts at long-range, is produced in the root and shoot, travels upwards towards the shoot apex in the transpiration stream in the xylem and can act at or near the bud to repress its outgrowth (Booker *et al.*, 2005; Stirnberg *et al.*, 2007; Kohlen *et al.*, 2011). These signal are carotenoid derived and this, along with a defect in the formation of symbiotic relationships with fungi in the mutants in pea, led to their recent identification as being the strigolactone-related (SLs) group of compounds (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008).

Like auxin, the action of SLs in branching control is to repress outgrowth, and so their action is proposed to form part of the ‘second messenger’ function. SL biosynthesis genes are upregulated by auxin (Bainbridge *et al.*, 2005; Johnson *et al.*, 2006; Arite *et al.*, 2007; Foo *et al.*, 2007; Hayward *et al.*, 2009). However, in common with auxin and cytokinins, the precise mechanisms of action of SLs have not been conclusively defined. In one hypothesis of their action, SLs act directly within the bud to maintain dormancy, antagonistically to CKs, with the dormancy regulator *BRC1* in Arabidopsis being a putative target in a more-or-less direct signalling cascade (Dun *et al.*, 2006; Brewer *et al.*, 2009; Dun *et al.*, 2009; Braun *et al.*, 2012). However, in assays using excised

nodes without a natural or supplied auxin source, synthetic SL analogues are incapable of repressing outgrowth (Crawford *et al.*, 2010) – an inability suggesting that interaction with other hormones is key to SL action.

1.1.2.4 Hormone Interactions – the Canalisation Hypothesis

The beginning of the investigation of apical dominance was with auxin, and auxin may yet be its end. Auxin downregulates CK synthesis, upregulates SL synthesis and feedback regulates its own synthesis (Leyser, 2011). Auxin also regulates its own transport, and the transport of auxin from the bud to the main stem has been proposed as key to the outgrowth of dormant buds (Sachs, 1981). In the canalisation hypothesis of branching control, the ability of buds to export auxin to the main stem determines their release from dormancy. This export is a competitive process, with buds competing not only with the primary apical meristem but with buds above and below for a common transport route in the main stem (Bennett *et al.*, 2006; Prusinkiewicz *et al.*, 2009; Crawford *et al.*, 2010; Balla *et al.*, 2011; reviewed in Domagalska and Leyser, 2011). This transport route provides the auxin 'sink' to which auxin transport, via PIN polarisation, will canalise, if the balance between the auxin sources and the 'sink strength' allows (Prusinkiewicz *et al.*, 2009). SLs also influence PIN cycling, as SL addition decreases the amount of PIN protein localised to the basal plasma membrane and SL mutants have increased PIN and increased auxin transport, in antagonism to auxin's own effect on its transport (Bennett *et al.*, 2006; Crawford *et al.*, 2010). In the canalisation hypothesis of bud outgrowth, SL repression of shoot branching is mediated via their dampening effects on auxin transport, thereby increasing the competition between buds and the apical auxin source (Prusinkiewicz *et al.*, 2009; Crawford *et al.*, 2010).

In addition to those discussed here, other hormones such as gibberellins, and factors such as light, also affect bud outgrowth (Bennett and Leyser, 2006). With so many interdependent factors, acting both with the bud and across the whole plant, precise conclusions about the relative importance of the different aspects of hormone interaction are hard to draw, leaving the question of the direct action versus canalisation hypotheses open to further research – the situation, like the hormones, remains in flux. However, whatever their precise

mode of action at (or nearby) the branching node, the identification of SLs as signals involved in branching control has led to their recognition as the newest group of plant hormones, and considerable interest in the investigation of their mechanisms of action, of their synthesis, and in the case of this thesis, of their evolution.

1.2 The MAX pathway and Strigolactones

1.2.1 Discovery

Strigolactones are so named for strigol, the compound first identified as a germination stimulant active at hormonal level for the parasitic plant *Striga lutea* in the 1960s (Cook *et al.*, 1966). SLs are exuded from plant roots, and so their presence acts as a beacon for the proximity of a host species to parasitic species such as those of the *Orobanchaceae* family, the *Striga*, *Orobanche*, and *Alectra* genera (Humphrey and Beale, 2006). Parasitic on a wide range of crops, including legumes and members of the Solanaceae and Brassicaceae, these species cause substantial economic damage and abandonment of cultivation of susceptible species in many countries in the developed world (Humphrey and Beale, 2006; Parker, 2009). However, *Striga* arguably wreaks the most havoc through its effect on cereal crops, particularly maize, pearl millet and sorghum, on subsistence farms in Africa, and the problem of infestation is increasing (Parker, 2009). This has driven considerable research in SLs as potential targets for use in battling these pernicious weeds (Zwanenburg *et al.*, 2009).

A turning point in strigolactone research was the discovery of a role for their exudation from the host plant. After nearly forty years of knowing of their existence, Akiyama and colleagues reported that SLs simulated the branching of hyphae in arbuscular mycorrhizal (AMy) fungi (2005). AMy symbioses have been proposed as key to the success of the land plant as they provide plants with the ability to colonise, and collect nutrient from, larger areas of ground via fine fungal hyphae at a lower cost than would be possible with their own roots (Wang and Qiu, 2006; Parniske, 2008). However, these symbioses do still come with a cost in the form of sugar, and sometimes other nutrients, supplied to the symbiont fungus, so there is a selective pressure to limit symbiosis formation to

when it is most required (Parniske, 2008). The plant side of the initial communications in attracting fungal symbionts now appears largely, though not entirely, to be mediated by the exudation of SLs from their roots, this time as beacon for fungal help (Bouwmeester *et al.*, 2007).

SLs were known to be carotenoid-derived (Matusova *et al.*, 2005) and this was one of the factors that contributed to their matching to the carotenoid-based *MAX* pathway by two groups (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). There were four genes known in the *MAX* pathway in Arabidopsis, identified from the *max* mutants. MAX3 and MAX4 are the carotenoid cleavage dioxygenases (CCDs) that produce a graft-transmissible signal that is subsequently modified by MAX1, a cytochrome P450 family protein in a clade unique to plants (Booker *et al.*, 2004; Schwartz *et al.*, 2004; Booker *et al.*, 2005). *MAX2* forms part of the signal transduction pathway, and is a member of the F-box protein family, which is involved in providing substrate specificity to the proteolytic 26S proteasome pathway, a role conserved in this family in many organisms, including mammals (Stirnberg *et al.*, 2002; Stirnberg *et al.*, 2007). The mutant phenotypes of the Arabidopsis, pea and rice orthologues of *MAX2* are resistant to the addition of synthetic SLs (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). *max2* among the Arabidopsis mutants also has more severe and additional phenotypes, particularly in germination, photomorphogenesis and leaf shape defects (Shen *et al.*, 2007; Stirnberg *et al.*, 2007; Nelson *et al.*, 2011; Waters *et al.*, 2012).

Similar mutants to the biosynthetic *max*es also exist in pea (*ramosus, RMS,* mutants), petunia (*decreased apical dominance, DAD)* and rice (*dwarf, D* and *high-tillering dwarf, HTD*), and have led to the identification of orthologous genes to *MAX2*, *MAX3* and *MAX4* in these species, as well as other components not previously identified in *Arabidopsis*, principally the biosynthetic *D27* and mysterious *D14* components found in rice (see Table 1-1).

Table 1-1. Characterised orthologues of MAX genes in four species. 'Founding member' in bold. References: (3 - Stirnberg *et al.*, 2002; 2 - Sorefan *et al.*, 2003; 1 - Booker *et al.*, 2004; 10 - Ishikawa *et al.*, 2005; 13 - Snowden *et al.*, 2005; 11 - Johnson *et al.*, 2006; 6 - Zou *et al.*, 2006; 7 - Arite *et al.*, 2007; 12 - Simons *et al.*, 2007; 12 - Gomez-Roldan *et al.*, 2008; 8 - Umehara *et al.*, 2008; 9 - Arite *et al.*, 2009; Gao *et al.*, 2009; 5 - Lin *et al.*, 2009; Liu *et al.*, 2009; 14 - Drummond *et al.*, 2012; 4 - Waters *et al.*, 2012)

	D27	***CCD7***	***CCD8***	***MAX1***	***D14***	***MAX2***
Arabidopsis	*AtD27*	***MAX3***[1]	***MAX4***[2]	***MAX1***[3]	*AtD14*[4]	***MAX2***[3]
Rice	***D27***[5]	*D17*[6]	*D10*[7]	No mutants, five orthologues known[8]	***D14***[9]	*D3*[10]
Pea	Unknown	*RMS5*[11]	***RMS1***[3]	Unknown, at least 2 orthologues suspected[12]	Unknown	***RMS4***[11]
Petunia	Unknown	*DAD3*[12]	*DAD1*[13]	*PhMAX1*[14] (not known as mutants, but role established	Unknown	*PhMAX2a* and *PhMAX2b*[14] (not known as mutants, but role established)

1.2.2 Phenotypes and functions

All these mutants lacked the presence of, or ability to respond to, the carotenoid-derived, graft-transmissible signal that would be identified as SL (Leyser, 2008). In terms of phenotype, mutants in strigolactone production, recognition or transduction show increased numbers of branches due to higher proportions of axillary buds breaking dormancy and growing out. In the Arabidopsis mutant phenotype this is mainly noticeable in buds from rosette leaves. Arabidopsis wild type axillary meristems typically activate in a basipetal wave (down the stem) on flowering, and also to a lesser extent in an acropetal wave, from older bud to younger bud up the stem (Hempel and Feldman, 1994). *max* mutants initiate many more of these first order axillary meristems in the rosette, which are normally dormant in the wild type (first order branches are generated from the main stem – higher order branches are produced from branches themselves, and the proportion of these is not affected, Figure 1-3).

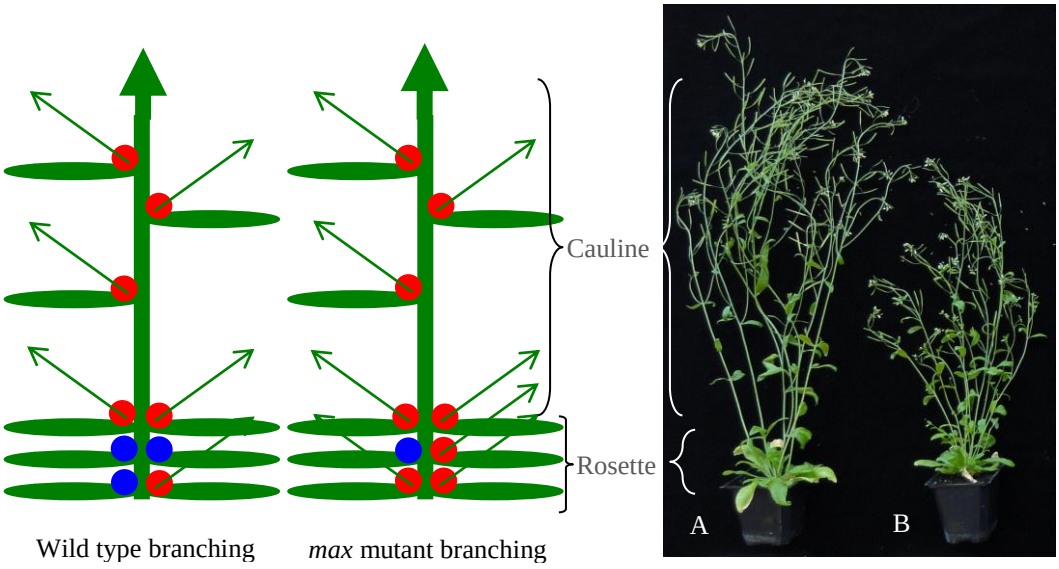


Figure 1-3. Branching pattern in *Arabidopsis thaliana* wild type and *max* mutants. Buds are produced in the axils of leaves made both in the vegetative (rosette leaf) stage and the transitional inflorescence stage - these leaves and nodes are referred to as 'cauline'. Arrows represent active, growing meristems, red circles for buds actively growing out, blue for dormant buds. Plant A) Columbia-0, an ecotype, and B) a Columbia-0 plant carrying a mutation in *MAX1* (allele *max1-1*).

Mutants across all species also display several pleiotropic phenotypes such as reduced height, changes in leaf size and shape and in Arabidopsis, petunia and rice delayed senescence, hinting a wide range of roles for SLs (Woo *et al.*, 2001; Stirnberg *et al.*, 2002; Ishikawa *et al.*, 2005; Snowden *et al.*, 2005; Arite *et al.*, 2007; Yan *et al.*, 2007). Indeed, not only have they been shown to be germination stimulants for parasitic plants, attractants for mycorrhizal fungi, accelerators of senescence, and a missing link in shoot branching control, SLs have recently been implicated in a wide range of other processes (and Xie *et al.*, 2010; reviewed by Tsuchiya and McCourt, 2012). These include; promoting germination in non-parasitic plants (Tsuchiya *et al.*, 2010; Nelson *et al.*, 2011; Toh *et al.*, 2012); light signalling (Kebrom *et al.*, 2010; Mayzlish-Gati *et al.*, 2010; Koltai *et al.*, 2011); promoting nodulation (the formation of symbioses with nitrogen fixing bacteria) in pea (Foo and Davies, 2011); restricting the development of cambial thickening and the production of adventitious roots; and in a concentration dependent manner promoting root elongation and root hair development (Agusti *et al.*, 2011; Kapulnik *et al.*, 2011; Koltai, 2011; Ruyter-Spira *et al.*, 2011; Rasmussen *et al.*, 2012). In cambial and root

development, SL action has also been found to be related to its effects on auxin signalling, as it is for shoot branching (Agusti *et al.*, 2011; Ruyter-Spira *et al.*, 2011; Rasmussen *et al.*, 2012; Kapulnik *et al.*, 2011; Koltai, 2011). This plethora of roles is similar to those of other plant hormones, and marks them as key regulators of plant development.

The phenotypes affected by SLs may be diverse, but several aspects of their function and regulation suggest that there may be a unifying factor to their actions. Their effects on plant growth in the shoot are largely restrictive, but they have promotive effect on root development, especially in phosphate limited conditions, and their exudation promotes the formation of phosphate-supplying AMy symbioses (Bouwmeester *et al.*, 2007; Agusti *et al.*, 2011; Domagalska and Leyser, 2011; Ruyter-Spira *et al.*, 2011). Moreover, SL production, exudation and the expression of SL biosynthesis genes are upregulated in response to phosphate and, in some species, to nitrogen limitation (Yoneyama *et al.*, 2007; Yoneyama *et al.*, 2007; Lopez-Raez *et al.*, 2008; Umehara *et al.*, 2010; Ruyter-Spira *et al.*, 2011; Kretzschmar *et al.*, 2012; Yoneyama *et al.*, 2012). These factors suggest that SLs might be general regulators of development in response to nutrient availability (particularly that of phosphate) and to some extent light availability, although in these actions SLs form a single part of a complex signal integration process with many other inputs, frequently other hormones (for example, as reviewed by Domagalska and Leyser, 2011).

1.2.3 Regulation, signal transduction and transport

The signal transduction of SLs and their own regulation is not yet completely understood, although their mode of transport has been better characterised. Grafting experiments between roots and shoots, and also using epicotyl intergrafts, had previously indicated that the branching inhibitor was upwardly mobile (Beveridge *et al.*, 1996; Foo *et al.*, 2001; Booker *et al.*, 2005; Simons *et al.*, 2007), and SLs have since been identified in xylem sap (Kohlen *et al.*, 2011). A mechanism of exit from the xylem, and also from the roots when exuded, has been supplied by the recent identification of the petunia ABC transporter protein *PhPDR1* as a strigolactone transporter by Kretzschmar *et al.* (2012). PDR1 is required for proper exudation of SLs and for proper shoot

branching control, although the phenotypes are not as severe in the *pdr1* transgenic knock-down as in the *dad1* biosynthesis mutant (Kretzschmar *et al.*, 2012). Consistent with these roles, *PhPDR1* is expressed both in the subepidermal cells of lateral roots, and in the vasculature of the stem above ground, especially near nodes with axillary meristems, perhaps allowing the unloading of SLs from the xylem into the living tissues in which it is likely to act, whether directly or via effect on auxin transport (Kretzschmar *et al.*, 2012).

MAX2, and its homologues in rice, *D3*, and in pea, *RMS4*, are the only confirmed signal transduction components of the SL pathway. They are leucine-rich repeat F-box proteins, which form the part of the SCF complex that interacts directly with the substrate in E3-RING ubiquitin ligases, which mark proteins for destruction via the 26S proteasome by attaching ubiquitin proteins to them (Vierstra, 2009). Several other F-box proteins have been implicated in hormone signalling cascades, such as those of auxin, jasmonic acid and gibberellins (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005; Ueguchi-Tanaka *et al.*, 2005; Katsir *et al.*, 2008). However, as yet there is no receptor for SLs confirmed and nor are there any direct targets for degradation or transcriptional regulation mediated by *MAX2*. Regulators for SLs themselves include auxin, which transcriptionally upregulates the expression of the biosynthetic components *MAX3* and *MAX4* and their orthologues in pea and rice, in a manner dependent on auxin-signalling component *AXR1* in Arabidopsis (Bainbridge, 2005; Foo *et al.*, 2005; Johnson *et al.*, 2006; Zou *et al.*, 2006; Arite *et al.*, 2007; Hayward *et al.*, 2009). This process forms at least part of the negative feedback of SLs on their own biosynthetic genes, reported in all four species in which mutants are known (Foo *et al.*, 2005; Arite *et al.*, 2007; Foo *et al.*, 2007; Simons *et al.*, 2007; Umehara *et al.*, 2008; Hayward *et al.*, 2009). In addition to auxin, upregulation of biosynthetic SL genes on phosphate limitation has also been reported, consistent with the upregulation of SL biosynthesis in the same conditions in a large number of species (Umehara *et al.*, 2010; Kohlen *et al.*, 2011; Yoneyama *et al.*, 2012 and references therein). Finally, recently the GRAS transcription factors *NODULATION-SIGNALLING PATHWAY1 (NSP)* and *NSP2* have also been shown to be required for SL

production and upregulation of *MtD27* and a *MAX1* orthologue in *Medicago truncatula*, a legume (Liu *et al.*, 2011), a finding discussed further in Chapter 5.

1.2.4 Biochemical structure and hormone pathway

SLs are formed of a backbone of four rings, with variation in the degree of saturation on the rings between different compounds (see Figure 1-4, taken from Umehara *et al.* 2008). The three ABC rings form a single lactone and are joined to the fourth 'D' ring, a γ-butyrolactone moiety, by an enol ether bond liable to nucleophilic attacks, such as by water, making most of the SL compounds labile in water and ethanol (Akiyama *et al.*, 2010 and references therein). However, this C-D section is required for the hyphal branching activity of SLs in fungi and to their germination activity in parasitic plants (Zwanenburg *et al.*, 2009; Akiyama *et al.*, 2010).

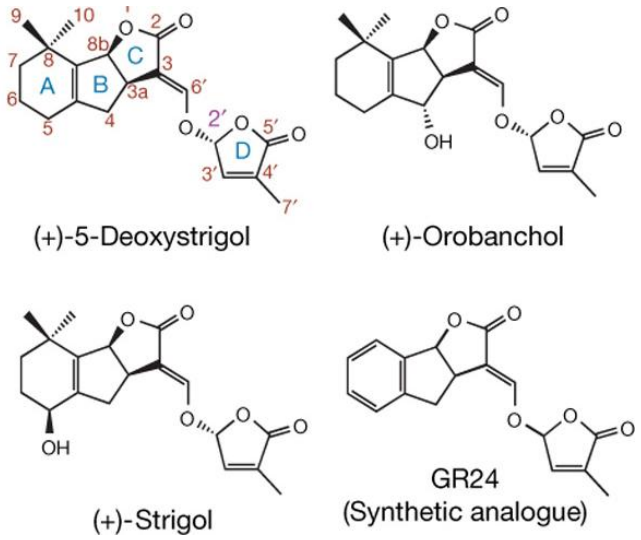


Figure 1-4. Structure of four strigolactones, taken from Umehara *et al.* 2008. 5-deoxystrigol is believed to be the first compound synthesised with activity in shoot branching (Rani *et al.*, 2008), and the predominant SL in rice, while orobanchol is probably the predominant SL in Arabidopsis (Goldwasser *et al.*, 2008; Kohlen *et al.*, 2011). Strigol is the SL founder, and GR24 is a synthetic analogue that has become highly used in studies of plant branching.

A wide range of strigolactones, including strigol, sorgomol, orobanchol and 5-deoxystrigol, have been isolated from plants, of which 5-deoxystrigol has been proposed as a first active compound before elaboration by hydroxylation reactions changes its structure further (Rani *et al.*, 2008). Although the

particular chemical structures active in shoot branching are still unknown, Umehara *et al.* (2008) and Gomez-Roldan *et al.* (2008) demonstrated that a synthetic strigolactone compound called GR24 could rescue biosynthetic, but not signalling, mutants in the MAX (Arabidopsis), RMS (pea) and tillering dwarf (rice) pathways, that these compounds are produced in planta, and that they are absent in the biosynthetic but not the signalling mutants of the pathway. These biosynthetic mutants are discussed further below.

1.2.4.1 D27

D27 was identified from analysis of a group of rice mutants assembled on the basis of their 'tillering dwarf' phenotype – mutants that displayed reduced stature but that produced more tillers (branches) than wild-type plants – by Ishikawa *et al.*, in a study that also identified all the other mutants in the *MAX* pathway known in rice (2005). As well as their higher production of tillers, which could be reduced by the addition of GR24, *d27*, like the other mutants, also had reduced culm length and plant height and increased auxin content and polar transport in the shoot (Ishikawa *et al.*, 2005; Arite *et al.*, 2007; Lin *et al.*, 2009). Interestingly from an evolutionary point of view, when the affected locus was identified, it was found to encode a protein with no previously-characterised family members nor conserved domains. In full-length form D27 binds an iron cofactor, although this was lost in C' terminal truncated polypeptides. The role of *D27* in the SL-related hormone pathway was strongly supported by the reduction in levels of 2'-*epi*-5-deoxystrigol in the mutant and lowered induction of *Orobanche minor* seed germination by mutant root exudates compared to the wildtype (Lin *et al.*, 2009). The protein is plastid localised, like those of *MAX3* (*D17* in rice) and *MAX4* and *D10* (the rice *MAX4* orthologue), and shares similar expression patterns to *D17* and *D10* (Booker *et al.*, 2004; Auldridge *et al.*, 2006; Arite *et al.*, 2007; Lin *et al.*, 2009). The location of the protein and its iron content led to the hypothesis that *D27* catalyses a redox reaction required for SL biosynthesis, either after (Beveridge and Kyozuka, 2010) or before the action of *D17* and *D10*. This hypothesis was confirmed very recently by the findings of Alder *et al.* (2012), which identified D27 as having catalytic activity as a carotenoid isomerase required to convert

all-*trans*-β-carotene into 9-*cis*-β-carotene (discussed further in Chapter 6), the substrate required by the next step in the pathway, CCD7.

1.2.4.2 MAX3 (CCD7) & MAX4 (CCD8)

The CCD proteins belong to a family of non-haem, iron-containing polyene dioxygenases, with nine members in Arabidopsis. Of these nine, five belong to the 9-*cis*-epoxy-dioxgenase (*NCED*s) clade, all of which are involved in synthesis of the phytohormone ABA (Frey *et al.*, 2012). *CCD7* and *CCD8* orthologues each belong to phylogenetically distinct clades and both share more similarity to non-plant orthologues than to plant *CCD*s (such as *NCED9*) outside their own clade (Sorefan *et al.*, 2003; Wang *et al.*, 2011a and pers. comm. R. Challis). Mutants in these genes have been found in all four of the species in which SLs have been characterised mutationally (see Table 1-1 and references therein). In addition, the role of *CCD8* in SL mediated regulation of shoot branching has also been demonstrated in the economically important floristry species chrysanthemum (Liang *et al.*, 2010), as has the role of *CCD7* in tomato (Vogel *et al.*, 2010) and of *CCD7* and *CCD8* in kiwifruit, demonstrating that SLs are active in branching in a woody perennial (Ledger *et al.*, 2010).

The two CCDs had been shown to be required for the production of a mobile substrate, upstream of the action of *MAX1,* and able to sequentially cleave the apocarotenoid all-*trans*-β-carotene *in vivo* to produce 13-apo-β-carotenone (Booker *et al.*, 2004; Schwartz *et al.*, 2004). Around the same time, the work of Matusova *et al.* had indicated that at least part of the SL molecule was derived from carotenoids, and proposed a pathway in which cleavage of the C11-C12 bond of 9-*cis*-β-carotene by a CCD provided the ABC rings of the structure, and the D ring was added later (2005). More recently, the work of Alder and co-workers has confirmed that the production of a putative SL precursor requires the 9-*cis* isomer of β-carotene (2012). However instead of the second lactone (the D ring) being added later, it is formed by the cleavage of 9-cis-β-carotene into 9-cis-β-apo-10´-carotenal (and a second product, β-ionone) by CCD7 and conversion to a novel compound, carlactone, by the action of CCD8 (Alder *et al.*, 2012 and see Figure 1-4, taken from that paper). The carlactone compound already possesses the D ring, and the final steps to the

production of strigolactones include cyclisation to form the B and C rings instead, roles for which *MAX1* may be a candidate (Alder *et al.*, 2012).

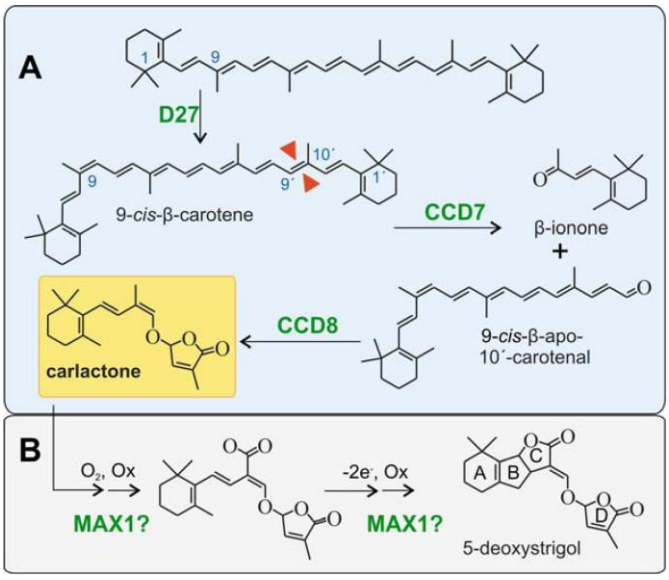


Figure 1-5. Biochemical pathway for SL synthesis taken from Alder *et al.* (2012, supplemental data). A) Steps established by Alder *et al*. B) Steps proposed for the continuation of the pathway.

1.2.4.3 MAX1

Unlike the CCD genes, grafting studies have shown that *MAX1* is not required to be active in the same tissues as *MAX3* and *MAX4* to produce the wildtype branching phenotype (Booker *et al.*, 2005). These results suggested that *MAX1* is downstream of the action of the CCDs within the biosynthetic pathway, and that unlike the CCDs was acting on an upwardly mobile, graft-transmissible substrate. *MAX1* was first identified as a component of the strigolactone pathway via analysis of the *max1-1* mutant in Arabidopsis, an ethyl methane sulphonate (EMS) induced mutation in the Enkheim-2 ecotype background, chosen from the AIS collection because of its many-stemmed phenotype (Stirnberg *et al.*, 2002). The affected gene was identified as *At2g26170*, a member of the cytochrome P450 monooxygenase superfamily (shortened to CYPs; Booker *et al.*, 2005). This enzyme family is almost ubiquitous in living organisms, occurring even in viruses, and its members catalyse a wide range of redox reactions with an equally diverse variety of substrates (Hannemann *et al.*, 2007; Nelson, 2011). These reactions are catalysed through the movement of electrons via a haem cofactor, bound

through a conserved cysteine group, an arrangement that generates the characteristic light absorption at 450nm that gives these proteins their name. This flexibility of CYPs to catalyse such a variety of different reactions has contributed to making identification of *MAX1*'s precise role in the MAX pathway difficult, although it may catalyse hydroxylation reactions downstream of carlactone or even downstream of the first active SL compound.

1.2.4.4 D14

When mutated, *d14* and *Atd14* render rice and Arabidopsis incapable of response to GR24 (Arite *et al.*, 2009; Waters *et al.*, 2012), suggesting a very late biosynthetic step or involvement in signal transduction. As a member of the α/β fold hydrolase superfamily D14 has relatives both with receptor functions in plants in the gibberellin pathway (Ueguchi-Tanaka *et al.*, 2005) and with a wide range of biosynthetic functions. These include that of Salicylic-Acid Binding Protein 2, which is required for production of the plant hormone salicylic acid (Forouhar *et al.*, 2005), or that of AidH, a bacterial protein that hydrolyses the γ-butyrolactone ring of bacterial quorum-sensing signal molecules N-acylhomoserine-lactones (Mei *et al.*, 2010), which share this lactone group with SLs (Tsuchiya and McCourt, 2012). As a result, it is as yet unknown whether D14 represents a late-acting member of the biosynthetic pathway, a putative part of a receptor complex, or a step in the latter signal transduction.

D14 has several paralogues in both the rice and Arabidopsis genomes, which themselves are conserved in many land plants (Waters *et al.*, 2012). *D14* and these sister clades have been shown to have diverged in function and expression to play similar roles in two parallel signalling pathways by the group of Professor Steven Smith at the University of Western Australia. The SL signal transduction component mutant *max2* has phenotypes not shared by the biosynthetic mutants in the MAX pathway, particularly photomorphogenic defects in seedlings (Nelson *et al.*, 2011). In the study by Waters *et al.* (2012) Smith and co-workers found that these phenotypes are in common with mutants in *AtD14like,* which are defective in sensing karrikins, germination stimulants from smoke which show structural similarity to SLs (specifically the 'D' butenolide ring). *Atd14like* mutants do not show SL insensitivity. However,

mutants in *AtD14*, which do not share the seedling dormancy phenotypes, do instead largely share the SL insensitivity of *max2* mutants –residual responses to GR24 being due to a slight redundancy with *AtD14like*. *AtD14like* is the more ancient of the two orthologues, perhaps reflecting an ancient role in promoting germination. The tempting (and tentative) conclusion to draw is that that the duplication of *D14like* has allowed the evolution of parallel pathways, both sensing molecules whose presence predates *in planta* roles (karrikins from smoke, SLs as biologically synthesised compounds whose actions previously occurred outside the plant) and which share structural similarity, whilst retaining an elegant efficiency by sharing downstream signal transduction components.

Such an example of "evolution by molecular exploitation" has been previously reported in the steroid hormone signalling pathway of vertebrates (Bridgham *et al.*, 2006). A predisposition in the ancestral corticoid receptor to aldosterone, a hormone not present in the ancestral vertebrate, was exploited when a modification to the catalytic activity of a cytochrome P450 in the tetrapod lineage produced this new steroid. The corticoid receptors had duplicated much earlier in the vertebrate lineage, and so both the genetic and chemical materials were present for the evolution of a new, yet specific, hormone-ligand interaction (Bridgham *et al.*, 2006). In SL signalling, the predisposition of the receptor to the butenolide lactone ring compound may have provided the ability to receive the structurally-similar karrikin compounds, even before that reception became associated with a specific response.

This story of the evolution of hormone signalling pathway components is a good example of the importance of duplication and subsequent sub- or neo-functionalization to the elaboration of developmental mechanisms, be it *HOX* genes in animals or *KNOX* genes in plants (Gehring *et al.*, 2009; Hay and Tsiantis, 2010). As a new regulator of plant development, analysis of the evolutionary history of SL signalling and synthesis will shed light on the coordination of growth in different species, and the universality of this method of growth control in the plant kingdom.

1.3 Evolution of shoot branching

The land plants are a monophyletic group that is believed to have evolved from the charaphyte group of green algae approximately 470 million years ago (mya, Pires and Dolan, 2012). With these algae they share a number of characteristics important to land-plant development, including multicellularity, apical growth, *PIN*-like orthologues and several other elements of auxin signalling (although not all), and the control of diploid development by *KNOX/BELL* interactions (Lee *et al.*, 2008; De Smet *et al.*, 2011; reviewed in Pires and Dolan, 2012). Land plants possess two multicellular life stages, one haploid, and one diploid, and the degree of dominance and independence of each stage has changed in the successive groups that have emerged through evolution, generally towards elaboration of the diploid sporophyte at the expense of the complexity and independence of the gametophyte. Figure 1-6 shows gives a broad plan of the relationship of the extant land plant groups. In the mosses, liverworts and hornworts (the 'bryophytes') the haploid gametophyte is the dominant phase, and this produces thallus or leaf- and root-hair-like structures on at least one different growth axis, while the diploid sporophyte has a single growth axis (it never – normally, pers. comm. J. Langdale – branches) and is virtually parasitic upon the gametophyte (Bell and Hemsley, 2000). In lycopodiophytes and ferns the sporophytic, the diploid sporophyte stage is dominant, and has a developed vascular system, although the gametophyte is still free-living and independent, if usually tiny (Bell and Hemsley, 2000). In the seed plants, the gametophyte has become the maternal tissue of the seed and pollen, totally dependent on the sporophyte and in the case of angiosperm pollen, reduced to only two nuclei (Willis and McElwain, 2002). Development in gametophyte and sporophyte appear to be differently regulated, with the KNOX and BELLRINGER transcription factors that specify indeterminacy and meristem identity in angiosperms involved in sporophytic but not gametophytic development in mosses, lycopodiophytes and ferns (Harrison *et al.*, 2005; Sano *et al.*, 2005; Singer and Ashton, 2007; Sakakibara *et al.*, 2008).

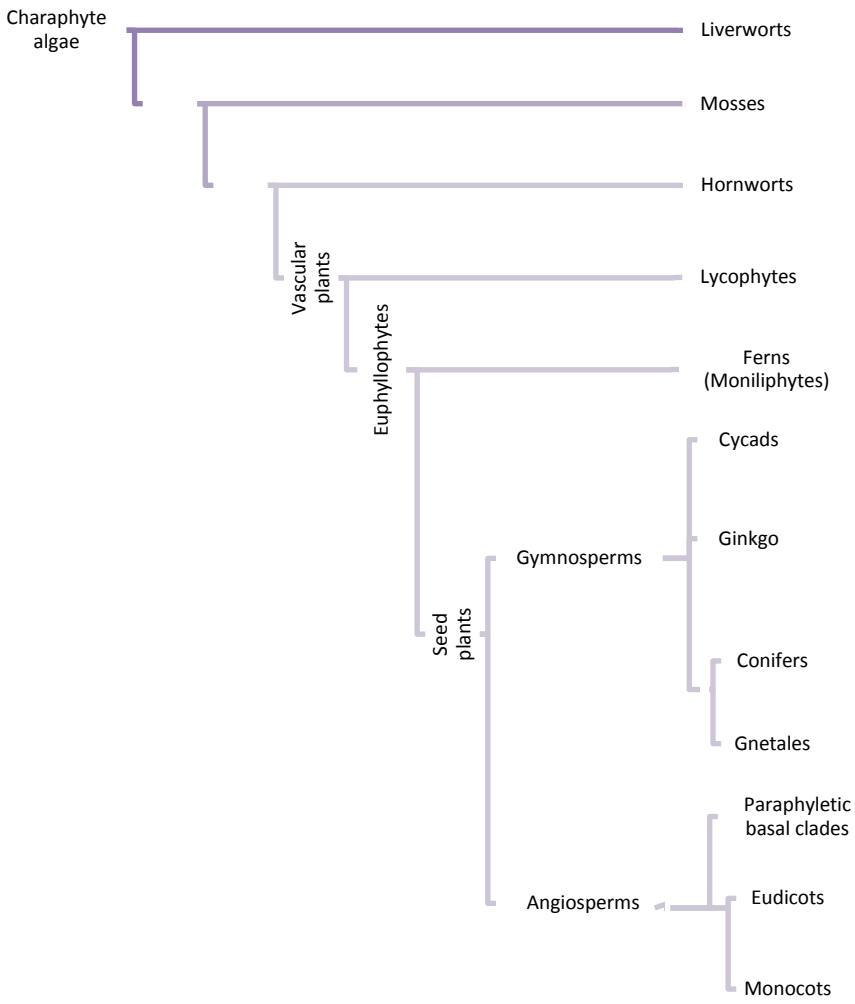


Figure 1-6. Phylogenetic relationships of extant plant groups, adapted from Tudge (2006) and Pires and Dolan (2012) .

1.3.1.1 Telome theory & the evolution of axillary branching

Branching in the different groups of land plants varies greatly, and branching in the vascular plants is discussed further in Chapter 4. In angiosperms, branches develop from axillary meristems, and AMes in turn develop with the leaf. 'True' leaves, or 'megaphylls' are believed to have derived, in evolutionary terms, from indeterminate bifurcations – *i.e*. branches (reviewed in Beerling and Fleming, 2007). Extant bryophytes do not have leaves or branches in the sporophyte at all, but only a single growth axis topped by a determinate structure, the sporangium, although the gametophyte produces both branches and leaf-like structures (Bell and Hemsley, 2000; reviewed in Langdale, 2008). Lycopodiophytes have evolved leaves independently as

'microphylls', structures believed to derive from a single determinate spike or branch and containing only one vascular strand (Tomescu, 2009). Branching in lycopodiophytes, which develop the sporophyte shoot from a meristem of much less complexity than that of angiosperms (frequently a single apical cell) is generally described as occurring only through bifurcation of the shoot tip (Bell and Hemsley, 2000 - but see Chapter 4). Megaphylls are thought to have developed from branches produced by these bifurcations, an idea known as Zimmerman's telome theory. There are three important stages in the telome theory of evolution of branch to leaf: overtopping, or the establishment of dominance of one branch over the other and of determinacy in the overtopped branch; planation, in which subsequent branching of the subordinate branch become flattened into a single plane; and the webbing that produces a laminar structure (Willis and McElwain, 2002; Beerling and Fleming, 2007). However the evolution of 'megaphylls' has occurred at least twice within the 'euphyllophytes' or true leaved plants – ferns and seed plants – and in the case of ferns many aspects of the frond indicate that it retains shoot-like characteristics of iterative development (Tomescu, 2009; Sanders *et al.*, 2011). Nevertheless, very similar developmental modules have been co-opted to regulate the development of all leaves, even where they have evolved separately in different lineages from different origins. The interaction between *KNOX* genes and their downregulation by *ARP* TFs is required in the development of determinate leaf structures in all vascular plants (Beerling and Fleming, 2007; Dolan, 2009; Hay and Tsiantis, 2010). The *KNOX/ARP* interaction, key to the distinction between determinacy and indeterminacy, including in the specification of AMes in angiosperms, may have evolved from controlling meristem bifurcation in the ferns and lycopodiophytes (Harrison *et al.*, 2005) but there is no ARP orthologue in moss, which shows no branching in the sporophyte (Floyd and Bowman, 2006) and these factors do not control the processes of branching and leaf formation in fern or moss gametophytes (Sano *et al.*, 2005). This is despite the presence of a leafy, almost shoot-like structure, the gametophore, in the gametophyte of the model moss *Physcomitrella patens*, but the absence of branching or leaf production in the sporophyte (Sakakibara *et al.*, 2008). The class III HD-ZIP TFs like *REVOLUTA* that govern leaf specification and vascularisation betray a different origin for microphylls, as

they do not act in the same manner in lycopodiophytes as they do in angiosperm megaphylls, but nevertheless they are still involved in similar processes (Floyd and Bowman, 2006).

The role of auxin seems likely to be conserved in many aspects of leaf development, as local auxin accumulation is involved in the specification of the future leaf primordium and vasculature formation in seed plants, both processes with conserved components in leaves between angiosperms to lycopodiophytes. Even the maintenance of dominance of one meristem over another by auxin signalling and polar auxin transport, known in some angiosperms and gymnosperms, may be conserved in apical dominance in some ferns, if not all (Croxdale, 1976; Pilate *et al.*, 1989). Auxin signalling components are present and active in moss development, including in the production of root-hair-like rhizoids, suggesting that the actions of auxin maxima may be universal in land plant development (Poli *et al.*, 2003; Eklund *et al.*, 2010; Jang *et al.*, 2011; De Smet *et al.*, 2011). Whether the conserved aspects of auxin signalling extend to auxin polar transport in moss, and particularly whether it is present in both sporophyte and the dominant gametophyte generation, is still a matter for contention. It has been reported that active (i.e. effected by known inhibitors) auxin transport is present in the sporophyte of mosses and liverworts, and that auxin is important to the axial growth of sporophytes in all three bryophyte groups (Poli *et al.*, 2003; Fujita *et al.*, 2008). Fujita *et al.* in the same study also found that the gametophyte lacked PAT. However, previously an auxin transport mechanism has been reported in moss gametophytes, particularly the rhizoids (Rose *et al.*, 1983; Rose and Bopp, 1983) and the presence of a spatial mismatch in auxin production and reception in developing rhizoids has been more recently reported, perhaps supporting Rose *et al.*'s findings (Eklund *et al.*, 2010). Mosses do possess orthologues of *PIN* proteins, but these belong to the *PIN5* clade that in angiosperms is localised to the endoplasmic reticulum rather than the plasma membrane and regulates intracellular auxin homeostasis, not intercellular transport, and this may be the role of *PINs* in mosses too (Mravec *et al.*, 2009; De Smet *et al.*, 2011).

Axillary meristems themselves then are foreshadowed by some of the

components that mediate their control, specifically polar auxin transport and its regulation of development, and meristem specification. Dormant meristems in the shoot are also present in gymnosperms, ferns and lycopodiophytes and in all three repression of outgrowth has been associated with auxin to some degree (Wochok and Sussex, 1975; White and Turner, 1995; Cline *et al.*, 2006). The question arises whether SLs, as auxin 'second messenger's, are also present.

1.4 Evolution of strigolactones

The presence of a strigolactone control of axillary branching seems well conserved in the angiosperms, with active pathways reported in Arabidopsis, rice, pea and petunia (Table 1-1). However strigolactones are involved in several aspects of plant physiology, and their involvement in mycorrhizal symbiosis in particular may well predate the evolution of axillary meristems. Fossil evidence shows that mycorrhizal symbioses arose at least 460 million years ago, before the evolution of vascular plants, and these symbioses are believed to be among the key adaptations that allowed the land-plant radiation, as they are widespread and frequent throughout all land plant taxa (Wang and Qiu, 2006; Parniske, 2008). The roles of SLs in other parts of plant development may represent the co-option of this substance, which was already produced on nutrient limitation, to a more general role in coordinating developmental responses to that limitation. However, the ancestral role could have equally been developmental, and the mycorrhizal connection a later adaptation. Most extant moss species lack AMy symbioses (Wang and Qiu, 2006) but the moss *Physcomitrella patens*, the genome of which has been fully sequenced, contains orthologues to *CCDs 7* and *8* and *MAX2*. Physcomitrella has been found to exude several SLs, and when SL biosynthesis mutants were generated by knock-out of the moss *PpCCD8* orthologue, the resulting plants had increased branching and extended colony growth, which could be rescued by addition of GR24 (Proust *et al.*, 2011). In Physcomitrella SLs also seem to act like a quorum-sensing signal, limiting growth of not only the original colony but also surrounding ones (Proust *et al.*, 2011). Whether this reflects an ancestral role of colony growth coordination, or one derived during the more than four million years since the emergence of the moss lineage, is a fascinating question. The important role which SL biosynthesis and signalling play in plant

growth and development, at least, appears to be conserved, arguing that this could be conserved in all land plant groups, and making their evolution of great scientific interest for the understanding of plant hormone evolution.

Two particularly interesting points in the evolution of strigolactones were identified as the involvement of *MAX1* and *D27*. *D27* was noted to be of interest in that, like *D14*, it is present in duplicate conserved clades in the angiosperms, that appear to have arisen during land plant evolution. These sister clades are also separated by long branch lengths suggesting that different selection pressures have driven divergence. However, the involvement of *MAX1* in particular was even more interesting. Despite being present and active in Arabidopsis as a single copy gene, *max1* mutants remain unreported in other species studied. This may well be due to redundancy, as homology searches in rice have revealed five possible orthologues (Umehara *et al.*, 2010) , two are present in *Medicago truncatula*, and at least two are believed to be present in pea (Gomez-Roldan *et al.*, 2008). Indeed, orthologues are present in all plant genomes searched, frequently in multiple copies in the angiosperms, with the notable exception of moss *Physcomitrella patens* (see Figure 1-7 for a phylogeny of *MAX1* orthologues, Figure 1-6 for a comparable phylogeny of the taxa to which they belong). Nevertheless, orthologues of *MAX2, MAX3* and *MAX4* are all present in moss (and active, in the case of *MAX4)* and generally in all land plants searched (R. Challis, pers. comm.). Does the absence of a *MAX1* in moss suggest its later incorporation into the strigolactone pathway, perhaps coincident with or causative for the development of a role in branching and function as a hormone? As the strigolactone biosynthesis pathway predates branching in the sporophyte generation, at what point did it become incorporated into branching control? The absence of *MAX1* in other species with well-characterised pathways also raised the question of whether its function in the SL pathway is restricted to Arabidopsis and the non-mycorrhizal Brassicaceae group, perhaps due to the release of a symbiotic evolutionary constraint on the signalling molecule. Most particularly, as *MAX1*orthologues are present in other species, do they have conserved effects on the functioning of the SL pathway? This thesis aims to suggest answers some of these

questions, by investigating the role of *MAX1* by complementation analysis, genetics and physiological analysis.

1.5 Aims

This project focused on the complementation analysis of *MAX1* orthologues from a variety of species, with the aim to dissect the influence of changes in biosynthetic enzymes on the pathway as a whole, and in particular to characterise the incorporation of *MAX1* into the biosynthetic pathway (Chapter 3) and contribute to the understanding of its function in other angiosperms, previously undetermined (Chapter 5). In order to provide a context for genes used in complementation experiments that were derived from non-angiosperm species and groups, the role of strigolactones and the control of branching was also investigated in these species (Chapter 4). Finally, in the light of the recent characterisation in rice of D27 and its phylogenetic analysis, investigation of its role and that of its orthologue D27like in Arabidopsis was started, to compare this early evolutionary duplication with the later diversification of *MAX1* (Chapter 6).

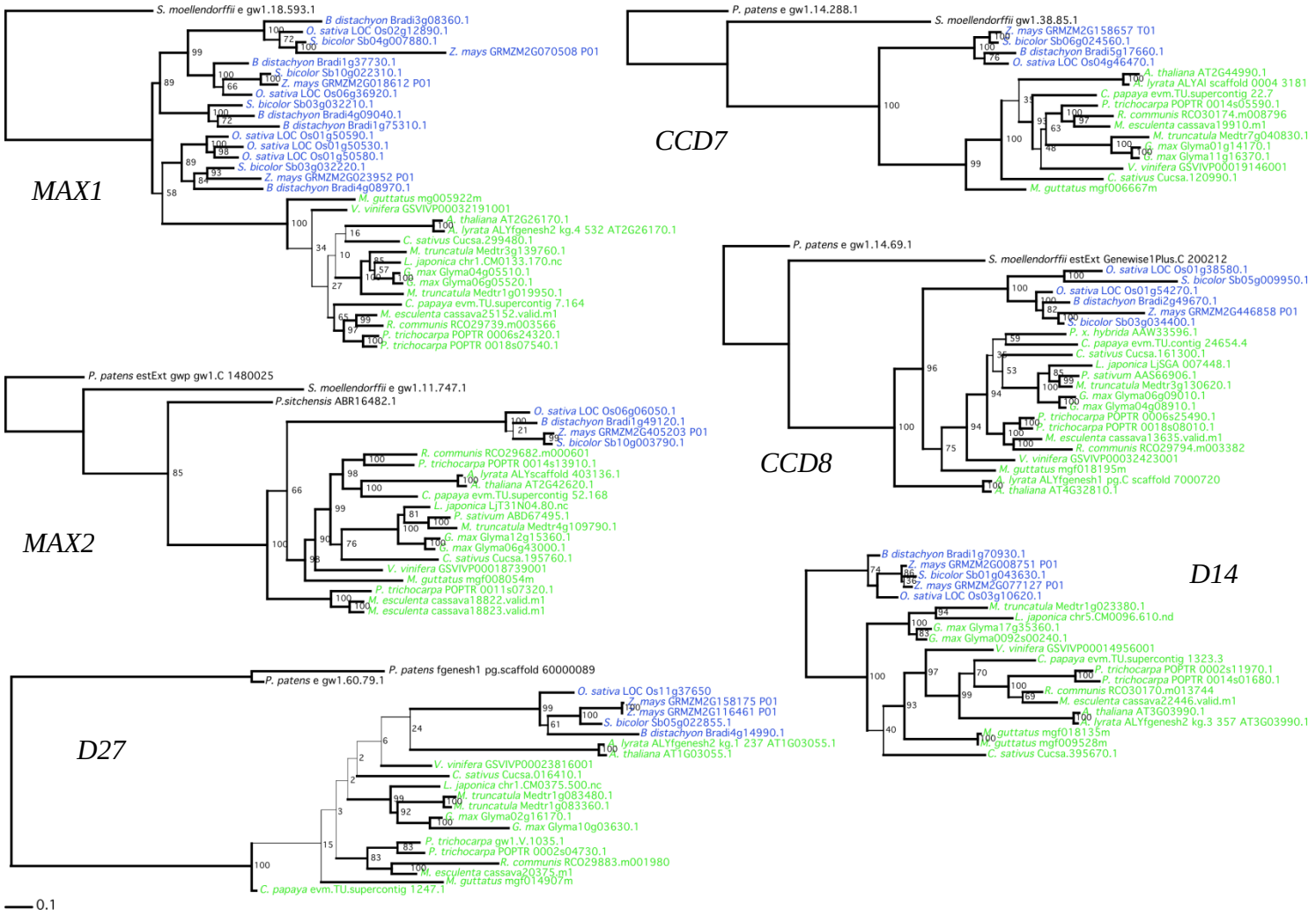


Figure 1-7. Maximum likelihood trees for loci involved in the MAX/strigolactone pathway, showing bootstrap support. Only clades corresponding to the orthologues known to be involved in branching are shown here (for *D27* sister clades, see phylogeny in Chapter 6). Dicotyledons in green, monocotyledons in blue, non-angiosperms in black. Scale bar corresponds to 0.1 substitution per site. Kindly provided by Richard Challis.

Chapter 2. Methods and Materials

2.1 Definition of terms

2.1.1 Nomenclature of duplicated genes

The nomenclature used for genes believed to share descent or function is usually determined by their relationships to each other and to their origin. For example; homologous genes share descent, orthologous genes share a common ancestor and are separated by speciation; paralogous genes are related genes derived from duplication within a genome, and if the duplication were the result of whole genome duplication (WGD) these can be referred to as ohnologues or sometimes homoeologues. Definitions sometimes imply but usually don't require functional similarity. These examples are not exhaustive – for more discussion of these terms see Koonin (2005).

Many of these terms and their variants require knowledge of a gene's history, something not necessarily available, and sometimes also their function, the elucidation of which is the aim of this study. Therefore to save confusion and prevent 'homologuephobia', only two terms are used here. All genes that show sufficient sequence identity to *MAX1* to have been classed as members of the CYP711 clan (and therefore presumed, even though unproven, to share descent) will herein be described as orthologues of *AtMAX1*. Paralogue is used to define the relationship of potential orthologues represented more than once in the same genome as each other, regardless of their duplication mechanism or function. Similar principles apply to *D27, D27like* and its orthologues, and others mentioned here.

2.1.2 Gene and protein naming conventions

Gene names are given in italics, and their protein products are given in regular script. When referring to mutant alleles lower case is used, with the wild-type allele in upper case. As orthologues from a wide number of species are referred to, where available, gene identifiers from genome annotation

projects are provided, if the predicted sequences match well to the cDNA sequences found here.

2.2 Molecular cloning techniques

2.2.1 dH20

dH_2O refers to water micro filtered through a Purelab Ultra lab water system (ELGA, Marlow, UK) and then autoclaved.

2.2.2 RNA extraction

All plant material was ground in liquid N_2 to disrupt the material. For extraction from *Arabidopsis thaliana*, *Oryza sativa* root material, *Medicago truncatula, Ceratopteris richardii* and *Selaginella moellendorffii* the Qiagen RNeasy Plant Mini Kit was used, (www.qiagen.com) with all optional steps included, including the on-column DNaseI digestion. For extraction from *Picea glauca* and *Oryza sativa* shoot the method described by Azevedo *et al.* (2003) was used, adapted according to the amount of material being used, except for Q-PCR for *Picea glauca*. In this case RNA was further purified by starting from point 3 of the plant protocol for the RNeasy Plant Mini Kit (QIAGEN, 2010). RNA quantity and quality were assessed using a Nanodrop™ ND-1000 Spectrophotometer (Thermo Fisher Scientific), and occasionally by gel electrophoresis as well.

2.2.3 DNA extraction from plants

2.2.3.1 For cloning

For cloning and preparation of high quality plant DNA from Arabidopsis, the DNeasy Plant Mini Kit from Qiagen was used according to enclosed instructions. DNA quantity and quality were checked on the Nanodrop® Spectrophotometer.

2.2.3.2 For genotyping

For genotyping the quick protocol described by Edwards *et al.* (1991) was used to extract crude samples of genomic DNA.

2.2.4 cDNA synthesis

cDNA was synthesised from purified total RNA using Superscript™ II M-MLV Reverse Transcriptase from Invitrogen (http://www.invitrogen.com, Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions, using Oligo-$d_{(T)}$ (Invitrogen) as the non-specific primer, except for construction of RACE libraries and cloning *Os06g0565100* from *Oryza sativa*. For cloning this gene, which has a GC-rich hairpin within the coding sequence, and for RACE an adaptation to the manufacturer's instructions for the incubation step was employed. This step is normally just a 50 minute incubation at 42°C with the enzyme, but in an adaption recommended by Dr Dörte Müller the incubation was changed to 40 minutes at 42°C, 10 minutes at 70°C, readdition of the enzyme and 20 minutes at 50°C. Typically 500ng of RNA was used as starting material for RTPCR, where RNA concentration allowed, and no less than 100ng was used for RT-PCR.

2.2.5 3'RACE

3'Rapid Amplification of cDNA ends was used to confirm the stop codon position in *Os01g0701500*, using the protocol as described by Scotto-Lavino *et al.* (2006) and reagents as described for cDNA and PCR.

2.2.6 5'RACE

5'RACE was performed on *Picea glauca* RNA using the protocol described by Sambrook and Russell (2001) and reagents as described for cDNA and PCR.

2.2.7 Sequencing

Sanger sequencing was used to determine the sequences of RACE, and PCR products for cloning and to confirm the sequences of all constructs used to transform plants. Sequencing was performed by the Technology Facility of the University of York using an Applied Biosystems 3130XL machine using primers as described in Appendix A1, and the results analysed using Applied Biosystems Sequence Scanner Version 1.0 (Applied Biosystems, Life Technologies).

2.2.8 PCR

Standard PCRs were used for a variety of purposes, including genotyping plants, cloning with degenerate primers, semi-quantitative reverse-transcription PCR (using gel electrophoresis to visualise differences in cDNA quantity) to check expression of transgenes in Arabidopsis or Medicago genes *in planta*, and colony PCRs for bacterial colony selection. Sample mixes and programmes are given in Table 2-1, (although programmes were adjusted to primers, templates and purposes) and reagents used were from New England BioLabs Inc. (NEB, http://www.neb.com, Massachusetts). Master mixes were used wherever possible. Reactions were carried out using an eppendorf™ Mastercycler (http://www.eppendorf.co.uk), with the recommended programme for PCR products of less than 6kb. The products were visualised using gel electrophoresis.

Table 2-1. PCR conditions for standard PCR

Experiment:	Genotyping plants	Semi-quantitative RTPCR	Colony PCR
Thermopol® buffer	2µl	2µl	1µl
2mM dNTPs	2µl	2µl	1µl
10mM each primer	1µl	1µl	0.5µl
Taq DNA polymerase (5U/µl)	0.05µl	0.1µl	0.05µl
Template	2µl genomic DNA diluted x2	2µl cDNA diluted x4	Colony stab
Final volume made up with dH_2O	20µl	20µl	10µl

Initial denaturing	94°C 2 minutes	94°C 2 minutes	94°C 2 minutes
Cycle – denaturing	94°C 20s	94°C 30s	94°C 20s
Cycle – annealing	Primer Tm 20s	Primer Tm 30s	Primer Tm 20s
Cycle – elongation	72°C 30s-1min	72°C 30s	72°C 30s-1min
Number of cycles	35	25-50	40
Final elongation	72°C 5minutes	72°C 10 minutes	72°C 5 minutes

Table 2-1. PCR conditions for standard PCR (programme).

2.2.9 Error-free PCR

Both proof-reading polymerases *Pfu*Turbo® (Stratagene, Agilent Technologies, Santa Clara, California) or *Pfu* (Promega Corporation, Madison, Wisconsin) were used for error-free PCR for cloning, with the Promega product used for more difficult templates but the Stratagene enzyme for more robust amplification, with mixes and programmes as described in Table 2-2. For templates with a high GC content or low expression, particularly those from *Oryza sativa*, 50mM $MgCl_2$ was added at 1µl to 50µl mix to bring the final concentration of free Mg^{2+} to 3mM, and 10% dimethylsulphoxide (DMSO) for a final concentration of 4%. Reactions were carried out using an eppendorf™ Mastercycler (http://www.eppendorf.co.uk), with the recommended programme for PCR products of less than 6kb. PCR products were then assessed by gel electrophoresis, and for difficult templates (for example, *SmMAX1*, *Os01g0701500* and *Os06g0565100*) the required band was cut out and 1µl from the gel used as template for a further 10-20 cycles.

Table 2-2. PCR reaction mixes and programmes for error-free PCR

Enzyme:	*Pfu*Turbo® (Stratagene)	*Pfu* (Promega)
Buffer	5µl 10x cloned *Pfu* reaction buffer (Stratagene)	5µl 10x *Pfu* reaction buffer (Promega)
2mM dNTPs	5µl	5µl
10mM each primer	2.5µl	2.5µl
DNA polymerase	1µl 2.5U/µl *Pfu*Turbo®	0.4µl 2.5U/µl *Pfu*
Template	0.5-4µl cDNA (undiluted, ~20-50ng)	0.5-4µl cDNA (undiluted, ~20-50ng)
Final volume made up with dH_2O	50µl	50µl
Initial denaturing	95°C 2 minutes	95°C 2 minutes
Add *Pfu* hotstart	N/A	Yes
Cycle – denaturing	95°C 2 minutes	9°C 2 minutes
Cycle – annealing	Primer Tm 25s	Primer Tm 25s
Cycle – elongation	72°C 30s-1min	72°C 2min/kb
Number of cycles	30	30
Final elongation	72°C 10 minutes	72°C 10 minutes

2.2.10 Gel electrophoresis

Gel electrophoresis was carried out using gels made from 0.8 – 3% molecular grade agarose (Sigma Aldrich Corporation, USA) dissolved in 1 x - TBE (0.445M Tris-borate, 10mM EDTA, pH 8) and run in gel tanks (Flowgen, Nottingham) at 2-6V/cm. 1-2µl of SYBRSafe dye (Invitrogen) was added per

100ml of gel, and visualisation carried out with a SafeImager™ (Invitrogen), photographed and analysed with GeneSnap™ software (Syngene, Biocon, Bengaluru, India). Purification from electrophoresis gels and PCR mixes was carried out using the illustra GFX™ PCR DNA and Gel Band Purification Kit from GE Healthcare (Amersham) according to manufacturer's instructions.

2.2.11 PCR Primers

Primers were designed by eye by the author with the assistance of the web based oligonucleotide programs provided by NCBI (Primer Blast www.ncbi.nlm.nih.gov/tools/primer-blast/) and Integrated DNA Technologies Ltd (OligoAnalyzer, eu.idtdna.com/analyzer/Applications/OligoAnalyzer/), except where designed or gifted by others, as noted in Appendix A1. Primers were synthesised by Sigma-Aldrich Corporation (USA).

2.2.12 Q-PCR

Q-PCR was performed on an ABI 7000 QPCR machine (Applied Biosystems) and analysed with the corresponding software. Primers were tested by producing standard curves based on a sequence of 20ng/µl, 2ng/µl, 0.2ng/µl and 0.02ng/µl purified single-stranded cDNA from a tissue presumed to be highly expressing the tested gene, and on the dissociation curves. Reaction mixes used were: 5µl cDNA from a total of 500ng, 250ng or 125ng total RNA depending on sample concentration, 12.5µl SYBR® Green I dye (using the ROX internal passive reference dye, Applied Biosystems), and 5.5µl of a 2mM mix of the primers. Master mixes were always used. Primers used are listed in Appendix A1 and were designed using Primer Express v3.0 (Applied Biosystems). cDNA for Q-PCR was prepared as described above, and for standard curves was purified using the illustra GFX™ kit described in Section 2.2.10 and quantified by Nanodrop™ 1000.

2.2.13 Restriction digestion

Restriction digests were carried out using restriction enzymes (NEB) with appropriate buffers. A typical digest mix would be:

2µl	10x reaction buffer (appropriate buffer chosen from NEB double digest recommendation)
1µg	(at 50-600ng/µl) DNA
0.2µl	100x BSA if required
1µl	Restriction enzyme 1 (NEB) (typically 5-20 units)
1µl	Restriction enzyme 2 (NEB) (if required)
	Distilled, autoclaved water to 20µl

Master mixes were used where possible. Reactions were incubated at 37°C or 28°C as appropriate for 1 hour, and subsequently for single enzyme digests of vectors, 1µl of 5U/µl Antarctic phosphatase (NEB) was added, mixed in, and the reaction mixture was incubated at 37°C for a further 15 minutes. Reactions were heat inactivated for 20 minutes at 65°C or 80°C, as appropriate. Digests were analysed by gel electrophoresis and bands cut out and purified as described above.

2.2.14 Ligation

Ligations were carried out using vector: insert ratios of 4:1, 3:1 or 2:1, depending on insert size and vector determined using the following calculation:

$$\text{Insert fragment (ng)} = \frac{\text{[Vector fragment (ng)] x [Insert fragment (bp)]}}{\text{[Vector fragment size (bp)]}}$$

These were added to the following mix:

2µl	10x reaction buffer (NEB)
10-150ng	Insert DNA (typical amount)
50ng	Vector DNA (typical amount)
1µl	400U/µl T4 DNA ligase (NEB)
	Distilled, autoclaved water to 20µl

The reactions were then incubated for ~24 hours at 14°C, and 10µl of the reaction was used immediately for transformation of *E. coli* or stored at -20°C in case of transformation failure.

Where amenable (i.e. for ligations where the final vector construct sums to less than 10 Mb, both vector and fragment are available in high concentration, and restriction digest was used) a variation was used adapted from a protocol designed by Michael Koelle (pers. comm.) in which digested fragments or blunt end PCR products were run on low-melt gels in 0.75 x TAE (Tris-Acetate EDTA) buffer in a 4°C room (to prevent the gel melting). DNA bands were visualised, cut from the gel, and melted in a 70°C heating block. 5µl vector band and 10µl insert band were then mixed quickly with 2µl dH_2O and 2µl T4 ligase buffer, placed on ice for 1 minute, and 1µ T4 DNA ligase enzyme was added, thoroughly mixed, left on ice for a further minute and then incubated for ~24 hours at 14°C. For *E. coli* transformation, the reactions were melted at 70°C again, diluted with 80µl 0.1M Tris-HCl pH7.3, placed on ice to cool for a few seconds and then 10µl of the reaction was quickly mixed with *E. coli* cells and transformed as normal.

2.2.15 Cloning from PCR products

For products produced by standard PCR the Original TA® Cloning Kit from Invitrogen was used to clone PCR fragments from standard PCR for sequencing. For products produced by error-free PCR for cloning the Zero-Blunt® TOPO® Cloning Kit (also Invitrogen) was used as detailed in Appendix A2. Both were used as per manufacturer's instructions.

2.3 Bioinformatics

2.3.1 Orthologue identification

Orthologues of *MAX1* identified by the author were found by reciprocal BLAST searches using protein sequences of *AtMAX1*, *SmMAX1* and when identified *PgMAX1* against translated nucleotide sequences from different nucleotide sequence collections and different plant taxa on the NCBI and Phytozome websites (Goodstein *et al.*, 2012; NCBI).

2.3.2 Coding sequence prediction

Coding sequences for Medicago, rice and Selaginella were taken from their GenBank or TAIR curated predictions, except where these conflicted with

known *MAX1* gene structure. For these, GeneMark-E* at http://exon.gatech.edu/ (Lomsadze *et al.*, 2005) was used to predict a more likely sequence from genomic sequence surrounding the orthologue. Primers were designed against the longest open reading frame, and sequences were confirmed from the resulting clones, except for the stop codon of *Os01g0701500*, which was confirmed by 3'RACE as above. Coding sequence for *PgMAX1* was identified from cDNA by using 5'RACE based on a resequenced clone from the Arborea project (see Appendix A1 for primer sequence details).

2.3.3 Alignments

Alignments were produced by Neighbour-Joining algorithm in Clustal X 2.0.9 (Larkin *et al.*, 2007) and alignments edited and consensus sequences produced in BioEdit (Hall, 1999).

2.4 Constructs

2.4.1 Overexpression constructs

All overexpression constructs using the CaMV *35S* promoter were created in the pART7 binary vector as described by Gleave (1992), including those donated by Dr Sally Ward. Cloning strategies varied for each gene due to differences in the ease of amplifying full-length coding sequences – details are provided in Appendix A2.

2.4.2 Pre-transcriptional repression construct

An adapted version of the pFGC5941 vector (Kerschen *et al.*, 2004), kindly donated by Dr Louise Jones' lab, in which a constitutive *NOS* promoter drives an inverted repeat of the CaMV *35S* promoter was further adapted to drive an inverted repeat of 426bp of the *AtD27like* (*At1g64680*) promoter from +12 to -413 of the transcriptional start site, by sequentially excising each CaMV 35S repeat and religating with the *AtD27like* promoter PCR fragment, into which appropriate restriction digest sites had been designed for directional cloning.

2.5 Production of Transgenic Organisms

2.5.1 Bacterial selection and growth

Plates (Petri dishes, Sterilin®, ThermoFisher Scientific) were made from LB supplemented with 1% sucrose and 0.8% agar, autoclaved, and after cooling antibiotics were added from stock solutions of 1000 times working concentration added at 1:1000 dilution. Stock solutions were as follows, and filter sterilised:

50mg/µl Kanamycin monophosphate, in dH_2O
50mg/µl Carbenocillin, in dH_2O
100mg/µl Streptomycin, in dH_2O
50mg/µl Gentomycin, in dH_2O

For blue/white selection of colonies (used for pART27), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added at 40µg/ml final concentration to the medium in the same way as the antibiotics. 40µl 100mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was spread on the top of plates just before plating of the bacteria. Bacterial growth plates were grown in incubators. *E. coli* were grown at 37°C overnight, and *A. tumefaciens* at 28°C for 2-3 days.

2.5.1.1 Colony selection and plasmid preparation

After growth on plates colonies were picked into a half-size standard PCR with primers specific for the insert or plasmid, and the results of the PCR used to select colonies. Colonies of *E. coli* were grown in liquid LB culture overnight and plasmids purified using a Spin Miniprep Kit (QIAGEN), and DNA quantity and quality checked on the Nanodrop® Spectrophotometer.

2.5.2 Escherichia coli transformation

Aliquots of 100µl *E. coli* DH5α were prepared using the method of Inoue *et al.* as described by Sambrook and Russell (2001) and stored at -80°C. For transformations, aliquots were placed on ice until they thawed, then for transformation of ligations as described above, 50µl of cells was mixed with 10µl of ligation mix, but for subcloning reactions, as above, 3µl ligation was

added to 50µl cells. The mix was then left on ice for 15 minutes, heat shocked at 42°C for 30 seconds and returned to ice for 2 minutes. 250µl liquid LB was added to each transformation and they were shaken at 37°C for 40 minutes, before being spread on LB plates containing the appropriate antibiotic.

2.5.3 Agrobacterium tumefaciens *transformation*

Chemically competent *Agrobacterium tumefaciens* GV3101 were prepared and transformed by a method modified from Höfgen and Willmitzer (1988). A single-colony from an LB plate was used to inoculate 5ml LB containing gentamycin, which was cultured overnight at 28°C and 250rpm shaking, and then in turn used to inoculate 200ml LB with gentamycin. This culture was incubated for 3-4 hours at 28°C and shaking, before cells were pelleted at 3000g for 20 minutes at 4°C. The supernatant was discarded and cells were washed in 10ml Tris-EDTA buffer at 4°C. Cells were then recentrifuged, resuspended in 20ml LB, and flash frozen in aliquots then stored at -80C.

For transformation 50µl aliquots were left to thaw on ice, 0.2-1µg of the pART27 binary vector plasmid stirred into the aliquot, left on ice for a further 5-10 minutes, flash frozen (cold shocked) in LN_2 for 3-5 minutes, placed in a 37°C water bath for 5 minutes, then 500µl LB was added and cells were incubated at 28°C with shaking at 250rpm. 250µl were then spread on LB plates containing gentamycin and the appropriate antibiotic for the plasmid, and incubated at 28°C for two days.

2.5.4 Plant transformation

Transformation was performed using the floral dip method, adapted from Clough and Bent (1998). *Arabidopsis thaliana* of the appropriate genotype were grown at 2 plant per pot density on soil in long day conditions or for four weeks short day conditions followed by long day conditions until the first siliques had reached maturity. *Agrobacterium tumefaciens* was prepared by picking transformed colonies into 10ml LB media containing gentamycin and the plasmid-specific antibiotic, and incubated with shaking at 250 rpm overnight at 28°C. Of this 10ml, 0.9ml was added to 0.9ml 30% glycerol and flash frozen in liquid N_2 for storage, and 5ml was used to inoculate 400ml of LB with

antibiotics and incubated as before. Half an hour before transformation, 100ml of fresh LB, 5g of sucrose and 20µl of Triton-1000X were added to the culture, which was returned to the incubator until dipping. Inflorescences were dipped in the culture for approximately 1 minute, and plants returned to the greenhouse in clear plastic bags over night. The following day the bags were removed and plants were allowed to set seed.

2.5.4.1 Arabidopsis transgenic selection and establishment of transgenic lines

Transgenic plants were selected by growing seed on ATS plates supplemented with 1% sucrose and 0.8% agar and antibiotics added as for bacterial plates. Stock solutions were as follows:

50mg/ µl Kanamycin monophosphate, in dH_2O
50mg/ µl phosphinothricin (Basta®, Bayer CropScience) in dH_2O
12mg/ µl Sulphadiazine, in dH_2O

For the T_1 generation Basta® resistant plants were also selected by growth on soil and watering with Basta® at 1 and 3 weeks old.

To establish stably transformed lines of Arabidopsis, T_1 seed was screened and 10-25 resistant plants were selected, numbered and allowed to self-fertilise. Seed was collected from these individual plants and the seed screened on plates to check for a 3:1 antibiotic resistant: sensitive segregation, which should indicate a single successful insertion event. For each single insertion T_1 plant 10 resistant T_2 progeny were transferred to soil, numbered, allowed to self and the seed collected. This seed was screened for 100% resistance to discover which parent was homozygous, and for homozygous T_2 plants expression of the transgene in pools of 10 x 10 day old seedlings was tested by semi-quantitative RTPCR. For *max1-1* complemented plants, homozygosity of the *max1-1* allele was also checked by use of an Enkheim CAPS marker that segregates with the *max1-1* mutant mutation – details of this and RTPCR primers are in Appendix A1 and A2. T_3 progeny of T_2 plants homozygous for *max1-1,* the transgene and with good expression of the transgene were selected for phenotyping.

2.6 Plant growth and experimentation

2.6.1 Plant material

Arabidopsis thaliana (L.) Heynh. (Arabidopsis).

All seeds and lines except *AtD27* RNAi 2-1 and 1-12 were sourced from the Leyser group stocks at the University of York. *AtD27* RNAi 2-1 and 1-12 were the gift of Dr Yonghong Wang at the Institute of Genetics and Developmental Biology, Beijing. Other lines used were as follows:

Ecotype Columbia-0 (Col-0, wild type).

Mutants:

max1-1 (EMS point mutation in the Enkheim background, backcrossed 7 times to Columbia-0), ***max2-1*** (EMS mutation, Columbia-0) and double mutant ***max1-1 max2-1*** all described by Stirnberg *et al.* (2002),

max3-9, an EMS mutant (Booker *et al.*, 2004),

max4-1, a T-DNA mutant (Sorefan *et al.*, 2003),

Atd27-1, a T-DNA mutant (GK134E08) from the GABI-Kat collection (Rosso *et al.*, 2003) and described here (Chapter 6).

Transgenic lines:

35S::AtMAX2 max1-1, a *MAX2* overexpression line in the *max1-1* background (Stirnberg *et al.*, 2007)

35S::AtMAX1 max1-1 and ***35S::SvMAX2 max2-1***, overexpression lines for *MAX1* from *A. thaliana* and a *MAX2* orthologue from willow (*Salix viminalis*) in the *max1-1* and *max2-1* backgrounds respectively, both made by Dr Sally Ward.

All Arabidopsis transgenics and mutants are in the Col-0 background except where otherwise stated.

Other species:

Ceratopteris richardii Brongn. (cfern). Spores of homozygous wildtype diploid line Hn-n. (Scott and Hickok, 1987) kindly provided by Dr Heather Sanders, University of Oxford, along with much kind advice on their care.

Medicago truncatula Gaertn. (barrel medic, Medicago). Accessions Jemalong A17 and R108 kindly provided by Dr Michael Schulze, and ParaggioF by Dr Céline Mouchel, both of the University of York.

Oryza sativa L. spp. japonica cultivar Nipponbare rice seedlings were kindly donated by Prof. Dale Sanders' group at the University of York.

Picea glauca (Moench) Voss (white spruce).

- RNA for the cloning of *PgMAX1* was from adult needles of clone WS 1062 at Glencorse clone bank site, UK Forestry Commission Northern Research Centre, Roslin, Scotland (Thanks to Joan Cottrell and Rob Sykes at the UK Forestry Commission).
- Seeds for experimentation were half-sibling family lots F20072140093 and F20072140021from the Tree Seed Centre, with thanks to Dave Kolotelo and Spencer Reitenbach of the Tree Seed Centre, Ministry of Forests, Lands and Natural Resources Operations and Tim Lee of the Vernon Seed Orchard Company, both of British Columbia, Canada.

Selaginella kraussiana (Kunze) A.Braun, (Krauss' spikemoss). Cuttings kindly provided by Dr Younousse Saidi and Susan Bradshaw, University of Birmingham.

Selaginella moellendorffii Hieron. (gemmiferous spikemoss). Bulbils from Plants' Delight (sequenced genotype) kindly provided by Prof. Jo Ann Banks, Purdue University, USA.

2.6.2 Growing conditions

All plants were grown in one of 3 growth rooms or chambers as described below, and watered when necessary by the Horticultural Technicians of the University of York.

Greenhouse: natural light supplemented with artificial light to provide long day (16 hours light) conditions at ~150 μmol $m^{-2}s^{-1}$. Temperatures between -15-24°C.

Growth room:

- Long day – 16 hours light, 8 hours dark, temperatures 19-22°C day, 18-20°C night, light intensity ~60-100 μmol $m^{-2}s^{-1}$.
- Short day - 8 hours light, 16 hours dark, temperatures 19-22°C day, 18-20°C night, light intensity ~80 μmol $m^{-2}s^{-1}$.
- 'Warm' growth room – long day light conditions, but temperatures at 24°C day, 20-22°C night, ~120 μmol $m^{-2}s^{-1}$.

Percival growth cabinet: short day conditions (8 hours light, 16 hours dark) light intensity ~80 μmol $m^{-2}s^{-1}$, temperatures 20°C day, 18°C night.

2.6.3 Hormone treatments

GR-24 was supplied by LeadGen Labs LLC as an equal mix of diastereomers, and dissolved in 100% acetone to make a 10mM stock kept at -80°C.

β-Napthoxyacetic acid (NAA) and indole-3-acetic acid (IAA; heteroauxin) were supplied by Sigma Aldrich Corporation and dissolved in 100% ethanol to make a 10mM and 200mM stocks respectively kept at -20°C.

Unless otherwise stated, all controls in treatments involving hormones were mock treated with the carrier.

2.6.4 Arabidopsis

2.6.4.1 Growth media

Arabidopsis plants were grown on F2 compost pre-treated with Intercept (both Levington Horticulture, Ipswich, UK) in trays supplied by Desch Plantpak (Maldon, UK). P40 4cm pot trays were used except where noted otherwise.

When grown on plates seeds were sterilised as described below and grown on Arabidopsis Thaliana Salts (Lincoln *et al.*, 1990) solidified with 0.8% agar and supplemented with 1% sucrose.

2.6.4.2 Seed sterilisation

Arabidopsis seeds were sterilised by one of two methods:

- Wet method: Up to 2000 seeds in a 1.5ml microcentrifuge tube were shaken in 500µl of 70% ethanol for 1 minute. The ethanol was removed and replaced by 500µl 10% NaClO with 0.01% Triton-1000X (Sigma Aldrich Corporation). This was shaken for 8 minutes for seeds used in analytical experiments, 15 minutes for resistance selections. Seeds were rinsed by three washes with dH_2O, and spread on plates in either water or ATS with 0.05% agar.
- Dry/gas method: Up to 100mg of seed in open microcentrifuge tubes, were placed in a sealed box containing chlorine gas. This was left for 2 hours for seed to be used for experimental purposes, 3 hours for seed only being selected for resistance (e.g. T_1 or T_2 seed). For larger volumes of seed Petri dishes (Sterilin®) with lids half closed were used instead of tubes.

2.6.4.3 Dose response for GR24

Plants were grown in 500ml Weck Jars (Weck, Germany) on ATS medium, 1% sucrose, 0.8% agar, as described by Crawford *et al.* (2010). Stock solutions of 1000 times working concentration GR24 dissolved in acetone was added at 1:1000 dilution to cooled autoclaved medium. 50ml medium was used per jar, and 7 equally spaced seeds were added per jar in sterile conditions. Seeds were sterilised with the wet method (described above) and then stratified for 2 days at

4°C prior to planting. Jars were kept in long-day growth room and were randomised once a week. Rosette branches were scored when bolts had reached the top of the jar and the first siliques had been formed (approximately four-five weeks post germination). Branches were scored as growing out when visible to the naked eye. Treatments and genotypes were colour-coded to 'blind' the test and prevent bias.

2.6.4.4 Short day branching assay

Following and adapting from Greb *et al.* (2003), seeds were stratified for 2 days at 4°C, and grown on soil in the Percival short day condition cabinet for 28 days, then moved to long day conditions in the greenhouse. Plants were grown in blocks of 10 plants which were randomised once every 1-2 weeks. When bolts of a 10 plant block reached 10-15cm, they were decapitated, and branches longer than 0.5cm were counted 10 days after decapitation.

2.6.4.5 Long day branching assay

Seeds were stratified for 2 days at 4°C, and grown on soil in long day conditions in the greenhouse for approximately 6 weeks until the primary inflorescence stem had ceased flowering, at which point rosette branches longer than 0.5cm were scored.

2.6.4.6 Leaf phenotyping

Seeds were stratified for 2 days at 4°C, and grown on soil in P24 trays in the greenhouse for 5 weeks or, for the experiments described in Chapter 5.1.3 for 6 weeks. Leaves were processed as described in Weight *et al.* (2008) and Keiffer *et al.* (2011); cotyledons and adult rosette leaves were removed in phyllotaxic sequence and laid on acetate sheets, pressed within book leaves and scanned using a Scanjet 4370 scanner (Hewlett-Packard, www.hp.com) at 300 dpi resolution. Pictures were saved as .TIF and analysed with LeafAnalyser (Weight *et al.*, 2008). Leaf nodes and tips were corrected by hand, and the coordinates produced by LeafAnalyser were Procrustes fitted using MorphoJ (Klingenberg, 2011) which also produced the centroid size data. LeafAnalyser was then used to produce a PCA eigenvector matrix from a library of 1500 leaves from ten natural Arabidopsis accessions produced by Vera Matser

(Kieffer *et al.*, 2011) and Procrustes-fitted by Joe Vaughan of Dr Richard Waites' group at the University of York. The eigenvector matrix was used to calculate leaf point models scaled to the standard deviations of the natural accession database, using a program written in Python provided by Joe Vaughan and adapted by the author. These leaf point models form the data presented.

2.6.5 Medicago

Seeds for the Q-PCR experiment were removed from pods and scarified by rubbing with sandpaper, then planted in 4cm pots on 50:50 mix of sand and terra-green (Oil-Dri Corporation, Illinois, USA), and fertilised once a fortnight (start of week one, week three and week five) with Phostrogen All Purpose Plant Food (Bayer Garden, Bayer AG, Germany). Plants were grown in the green house for five weeks before harvesting, and tissues cut with razor blades as shown in Figure 5-6 before flash freezing in LN_2.

2.6.6 White Spruce

Seeds were stratified by placing on damp filter paper (Whatman™, GE Healthcare as above), in the dark at 4°C for one week. Plants were then grown in 8cm square pots (Plantpak) on a 50:50 mix of F2 compost (as above) and vermiculite (William Sinclair Holdings Plc., Lincoln, UK) treated with Intercept (as above), at a density of 1-3 plants per pot (>90% of plants were in 1- or 2-plant pots).

2.6.6.1 Excised bud assay

Half-strength Murashige & Skoog (MS) (1962) medium with Nitsche's vitamins (DUCHEFA Biochemie B.V., Haarlem, The Netherlands), 2% sucrose and 0. 2-(N-morpholino) ethanesulfonic acid (MES) buffer was corrected to pH6.5 with 1M KOH was jellified with 0.8% technical agar, autoclaved and 50ml added into 10cm square tissue culture plates (Sterilin). Plates were injected with 1mM IAA or carrier and/or 1mM GR24 or carrier at 1µl per ml. Plates were then left overnight at 4°C to equilibrate. Agar was then cut and arranged to produce plates with a thin central section containing no agar, and 3 plates per treatment as follows: control apical/control basal, 1µM IAA

apical/control basal, 1μM IAA apical/1μM GR24 basal. Sections of stem with one visible dormant axillary bud were cut from 3 month old greenhouse grown plants, surface sterilised in 2% NaClO for 20 minutes, washed 3 times in dH_2O and fitted between the agar blocks. Plates were placed vertically in the long day growth room and photographed every 2-3 days.

2.6.6.2 Initial decapitation assay

Four month old seedlings of lot F20072140021 ('F'21') grown in the greenhouse and with dormant apical buds were decapitated (or left whole in the control experiment). 50μl of 200mM IAA or ethanol carrier was mixed in 1ml liquid lanolin to final concentration of 10μM, and a small dab added to the cut surface of the plants. 10μl of 5μM GR24 in a dH_2O based mixture of 5% acetone, 4% polyethylene glycol and 25% ethanol was added to the lowest point of the stem at which needles started once every three days for one month. Photographs were taken of each plant at each dosing time point to record any outgrowth.

2.6.6.3 SEM

1 year old seedlings of F'21 grown in the greenhouse were decapitated below actively growing apices and left for two weeks. After two weeks plants were inspected visually for outgrowth, photographed and then sections of stem cut and dropped into water. Sections were fixed in 2% glutaraldehyde and 0.05M $NaPO_4$ pH7.2 phosphate buffer for 48 hours, initially using vacuum infiltration and <0.01% Triton-X1000 to assist sinking. Sections were then washed in phosphate buffer twice for 30 minutes each before being dehydrated in an acetone series of 25%, 50%, 70%, 90% and 3 x 100% washes of 30 minutes, with the final wash continuing overnight followed by drying in a critical point drier. The samples were gold-coated and visualised on a JEOL 6490LV SEM.

2.6.6.4 Long-term GR24 dosing experiment

Plants of lot F'21 (90 seeds, 1st replicate, 120 2nd two replicates) were germinated and allowed to grow in the short day growth room for two months (except for the April replicate, which spent an extra month in these conditions)

at which time all apical meristems had formed bud scales, before moving to the greenhouse. After 3 weeks all but 3 of ~60 plants had reactivated growth, and at this point dosing with 100µl of 0, 1 or 10µM final concentration GR24 in 1% acetone in dH_2O was started, with each treatment being balanced for pots with 1 or 2 plants. Doses were applied to the soil at the stem base. Dosing was done approximately every 8 days, at which time several measurements were taken in the first replicate – plant height from the base of the needles (the point at which the cotyledons were formed), number of axillary buds, bud scale formation/activity of apical and axillary buds, and the leaf number subtending the axillary buds (i.e. their position). In the second two replicates only the activity of individual buds (apical and axillary) was recorded as no hint of a difference was seen in the other measures, whereas a possible promotion effect had been seen by addition of 1µM GR24 on apical activity times.

2.6.6.5 Apical dormancy experiment

120 seeds per replicate of lot F'21 were germinated in the warm (24°C), long day growth room and after one month, at which point plants had started to produce axillary buds, they were moved to the cooler, short day growth room to induce dormancy, from which time they were dosed with 0 and 1µM GR24 as for the long-term experiment, but at weekly intervals and formation of apical buds measured once a week until all plants had ceased apical growth.

2.6.6.6 Decapitation experiment

Plants were left in short day conditions for 131 days (just over 3 months) and then returned to the warm growth room. After 2 weeks 80% of the first replicate, 64% of the second replicate and 33% of the third replicate had reactivated apically and several had also actively growing branches. Plants were either decapitated by removal of the apical bud or all the apical growth since this reactivation, or left whole, and dosed once a week with 5ml of 0 or 10µM final concentration GR24 at 1/1000 dilution to each pot, to ensure delivery of the hormone to the roots. The time of bud break of each axillary bud was then measured over three weeks, scoring once every 3 days.

2.6.6.7 Q-PCR experiment 1– high/low phosphate

Plants from lot F20072140093 were stratified as above, germinated on filter paper for 1-4 days, and then grown in individual 4cm pots on 50:50 mix of sand and terragreen (as for Medicago) fertilised once a week with 10ml half-strength Murashige & Skoog (MS) (1962) liquid medium. The medium was corrected to pH5.7-5.8 with 1M NaOH and autoclaved before use. Plants were grown for 2 weeks supplemented with standard medium at 10ml, then the medium supplement was added at 20ml for a further 4 weeks at which point some had produced axillary buds. Before dosing on the 7th week pots were washed by adding 20ml dH_2O and letting it drain through three times. Then plants were fertilised as before, but with media in which 0.625mM of KCL was substituted for the 0.625mM KH_2PO_4 ('no-phosphate medium'). After a week, three plants were dissected into roots, 'shoots' (all tissue above the cotyledons) and 'hypocotyl' (between roots and 'shoots') and the tissues pooled and flash frozen in LN_2. The remaining plants were split into two groups (balanced to have the same number of plants with the same number of axillary buds). Half were dosed with standard media, and the other half with the no-phosphate medium. After a further week plants were dissected and flash frozen as before. RNA was extracted as recorded above.

2.6.6.8 Q-PCR experiment 2– high/low phosphate with or without GR24

Plants of lot F'21 were grown as described for the first Q-PCR experiment but supplemented with 20ml standard medium for the first three weeks, and then moved carefully to new pots of sand and terragreen supplemented with 20ml of no-phosphate medium once a week for 5 weeks. At that point, acetone carrier control was added to the medium at 1/100 concentration. After a week, plants were harvested as described above. For the first replicate plants were divided in half (only 8 plants were available) and one half were treated with standard medium with carrier control, and the second half with standard medium and 1μM GR24. In the second experiment only 4 plants survived at all. In the third experiment plants were split into four groups, with all four combinations of no phosphate/standard medium and GR24/carrier control. After one week plants were harvested as before.

2.6.7 Selaginella kraussiana

Cuttings to be used in experiments were grown 50:50 F2 and vermiculite mix in the long day growth room in P1 or P15 trays with lids maintained in standing water, and shaded with 0.4 neutral density filters (Lee Filters, Andover, Hampshire). For growth on plates, C-fern medium was prepared as described by Hickok and Warne (1998), but 5g/l sucrose was added and the solution corrected to pH7 before autoclaving. For the initial and decapitation experiments (Sections 4.3.1 - 4.3.2 , Figure 4-15 to Figure 4-20) 1% technical agar was used to solidify media, but for GR24 only experiments (Section 4.3.3 , Figure 4-21 - Figure 4-22) 0.8% agar was used and 1ml/l of Gamborg's vitamins 1000X solution (Sigma Aldrich Corporation) was added to the medium to encourage growth and purchase of the rhizophores in the medium. Plates were kept in long-day growth room conditions and shaded with two layers of white muslin.

2.6.7.1 Initial experiments

For the initial experiments explants with one expanded node were cut from the parent plants and were surface sterilised in 15% NaClO for 3 minutes, rinsed three times in dH_2O and placed 3 to a single plate, with GR24 added at 1/1000 to final concentrations of 0, 1 or 10μM, with two plates per treatment. Plants were transferred to new plates and apices counted at 25, 78 and finally at 94 days, at which point lengths of rhizophores long enough to be visible to the naked eye were measured with a ruler, as was the whole explant at its longest point, and plants were also weighed on a microbalance.

2.6.7.2 Decapitation and GR24 experiments

Explants were cut with approximately two expanded nodes and surface sterilised in 2% NaClO for 15 minutes (this method was recommended as more suitable for larger explants by Dr Heather Sanders). Explants were then washed 3 times in dH_2O, allowed to dry for a few minutes and added to plates with GR24 added as before to final concentrations of 0 or 1μM. At the end of the experiments, rhizophore length was measured either by ruler or by dissecting nodes with fine forceps to reveal developing rhizophores under a dissection microscope. Nodes were photographed by microscope-mounted camera and

images analysed using ImageJ (Rasband, 1997). Treatments were randomly assigned numbers to 'blind' the test before data analysis.

For the decapitation experiments, plants were decapitated before sterilisation with a scalpel under the dissection microscope, at the smallest possible node, and the major branch was always chosen. Explants were moved to new plates once a week for three weeks in total before phenotyping. 30 plates were originally set up per treatment, and after plates had been removed due to contamination numbers between 9 – 14 were left, except for a third experiment in which all decapitated plants were lost.

For the GR24 only experiments the protocol was adapted to use plates with less agar and vitamins added as described above, and explants were added without decapitation to plates with 0, 1 or 10μM GR24. Explants were moved to new plates after two weeks, with any explants that were slightly but not seriously contaminated re-sterilised as before, and scored after a further two weeks. 40 plates were used per treatment, of which 20-31 survived.

2.6.8 Ceratopteris richardii

Spores were surface sterilised by the wet method described for Arabidopsis seeds, and spread on Petri dishes (Sterilin) of C-fern medium (Hickok and Warne, 1998) solidified with 0.8% agar and corrected to pH6. Gametophytes were grown for one month in the warm growth room, with 1-2ml dH_2O added every two weeks to encourage fertilisation and growth of sporophytes. When at the five-leaf stage, sporophytes were moved to 25ml liquid Cfern medium with 0.05% agar in autoclaved square Magenta® culture vessels (Sigma-Aldrich Corporation) with 10 sporophytes per vessel. After 2 weeks plants were moved to autoclaved round baby-food jars sealed with Magenta® B-caps (both Sigma-Aldrich Corporation) containing 25ml 'experimental' media at a density of 6 plants per vessel, and grown on these media for four weeks, with media being replaced with fresh media at 2 weeks. 'Experimental media' were:

For the phosphate experiment, 'phosphate sufficient' was standard media, but 'no phosphate' media had the 0.625mM KH_2PO_4 replaced with equivalent molar of KCl. 3 vessels were used per treatment.

For the GR24 experiment, GR24 dissolved in acetone was added at 1µl/ml of media and concentration 0, 0.1, 1 or 10mM to a final concentration of 0, 0.1, 1 or 10µM. 6 vessels were used per treatment.

Plants were then measured using a ruler and counting numbers of sporophylls, roots, measured length of the longest single root, length and width of sporophylls at longest and widest point, and counting leaves with signs of senescent (yellowing) leaves and with pinna divisions with acute angles.

2.7 Statistical analysis and representation of data

2.7.1 Statistical analysis

Statistical analyses were performed using the PASW Statistics 18 program (SPSS, IBM Corporation, New York, US), unless distributions were known to be normal and variances equal, in which case ANOVA or Student's t-test was used in Microsoft Excel 2010 (Microsoft, Washington, US), or in the case of Chi-squared test, done by hand. In PASW the 'Explore' function was initially used on parameters not previously analysed (e.g. the first time branching was analysed) to establish normality of the data. Data in which Shapiro-Wilkes values were less than 0.05 were considered not normally distributed and analysed using the Kruskal-Wallis test, but normally-distributed data was analysed by ANOVA with Levene's test for equal variances. Data identified as equal by Levene's was post-hoc analysed with Tukey's HSD, and data rejected by Levene's at p=0.01 was analysed using Tamhane's T2 or Dunnett's T3 tests. Probability cut-offs were adapted to the experiment and are noted in the text, with P=0.05 used as the uppermost boundary of significance.

2.7.2 Graphs & Thesis

All graphs were produced in Microsoft Excel and all error bars show standard error of the mean. This thesis was written in Microsoft Word™ on an ASUS (Taiwan) U53JC Series laptop with bamboo lid running Windows 7 (Microsoft). Diagrams were produced in Microsoft PowerPoint™.

Chapter 3. MAX1 *Incorporation into the* MAX *pathway*

3.1 Introduction to the evolution of MAX1

MAX1 was designated CYP711A1, as the first member of the CYP711 family and clan (Nelson *et al.*, 2004), with other members of this family presumed to be orthologues of *MAX1*. The CYP711 family is plant-specific, although two sister families from the same clan, CYP743 and CYP744, are known to exist in the green algae. However these are specific to that lineage (in which they represent an astonishing third of all CYPs) and they are not shared in the land plants. Indeed, they may only be long branch attracted to the CYP711 family, as they do not cluster with it in more global trees of CYPs (Nelson and Werck-Reichhart, 2011). Not only are *MAX1* orthologues specific to land plants (embryophytes), they are also absent from the genome of *Physcomitrella patens*, the only bryophyte currently sequenced (Rensing *et al.*, 2008). Despite this, *MAX1* orthologues were present in every complete tracheophyte genome published, including that of the lycopodiophyte *Selaginella moellendorffii* (Banks *et al.*, 2011). In eudicots *MAX1* orthologues are generally present as a single copy, but in monocots several orthologues are present – as many as five in rice, representing three separate clades, each of which is also represented in maize and *Brachypodium distachyon* (Nelson *et al.*, 2008 Challis et al. in preparation, Figure 3-1). This apparent conservation and duplication of *MAX1* in flowering plants compared to its absence from Physcomitrella led to the hypothesis that it had been incorporated into the MAX pathway after the divergence of the moss and tracheophyte lineages, and that its subsequent duplication in the angiosperms has allowed orthologues to diversify functionally. In order to investigate how *MAX1* orthologue function has evolved within the SL biosynthesis pathway, a complementation analysis approach was employed, exploiting the ease of producing transgenics and the mutant collection available in the Arabidopsis model.

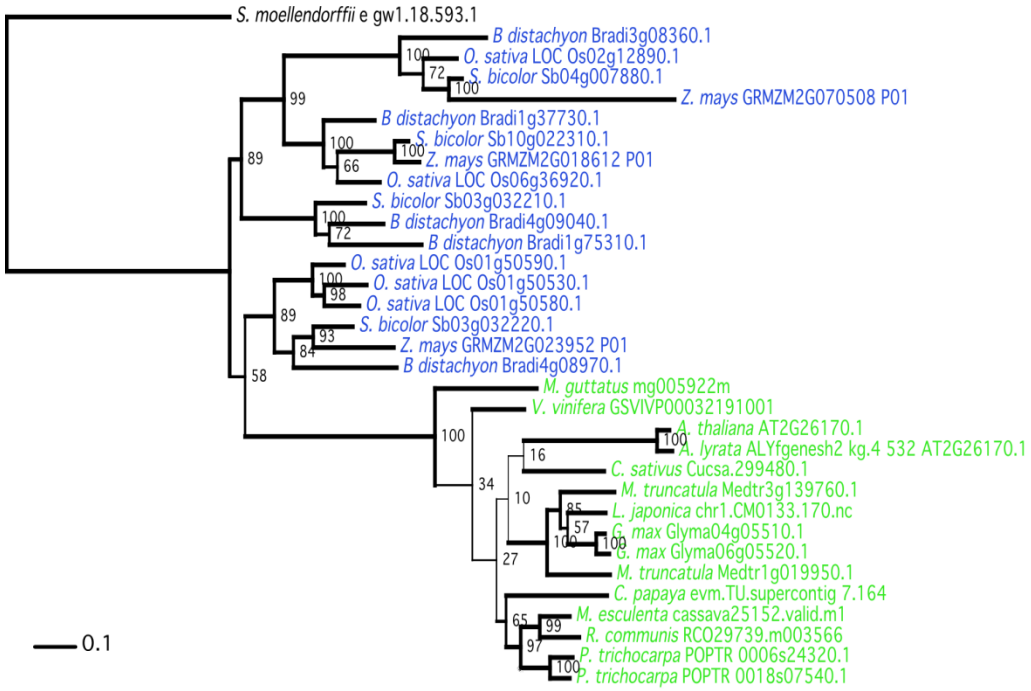


Figure 3-1. Maximum likelihood tree for *MAX1*, showing bootstrap support. Dicotyledons in green, monocotyledons in blue, non-angiosperms in black. Scale bar corresponds to 0.1 substitution per site. Kindly provided by Richard Challis.

3.1.1 Phenotype

In Arabidopsis at least three different mutant *max1* alleles have been described (Stirnberg *et al.*, 2002; Booker *et al.*, 2005; Lazar and Goodman, 2006). All three have phenotypes similar to those of *max2*, *max3* and *max4* mutants, with increased branching in the rosette but with wild-type proportions of higher-order branches, as well as leaves with shorter petioles and shorter and more rounded laminas, and delayed onset of senescence (Stirnberg *et al.*, 2002; Bainbridge, 2005; Booker *et al.*, 2005; Lazar and Goodman, 2006). Unlike *max2* mutants but in common with *max3* and *max4*, *max1* does not show hypocotyl and cotyledonary elongation defects in light (Stirnberg *et al.*, 2002; Shen *et al.*, 2007; Nelson *et al.*, 2011). As a members of the strigolactone biosynthetic pathway, the branching defects of *max3*, *max4* and *max1* can be rescued by addition of strigolactones, as can the tillering defect of the corresponding mutants in rice, *d17* and *d10* (*max3* and *max4* respectively) and that of *d27*, whereas the signal transduction mutant *max2* and the α/β hydrolase mutant *d14* cannot (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008; Arite *et al.*, 2009; Lin *et al.*, 2009). Although the varied capabilities of the CYP family

make the reaction catalysed by *MAX1* difficult to hypothesize with certainty, grafting studies indicate that it acts downstream of *MAX3* and *MAX4*, which produce a mobile, but inactive precursor (Booker *et al.*, 2005). A hypothesis of late action in the pathway has therefore been proposed for *MAX1,* in which it catalyses one of the final steps required for production of active molecules.

3.2 Dose response curves

To further establish the position of *MAX1* in the biosynthetic pathway, and to characterise more closely *max1* phenotypes for comparison to transgenics produced by the complementation analysis, assays were performed to investigate the dose-response curves of the branching phenotype of *max1-1* grown on the synthetic strigolactone GR24, using the method described by Bennett *et al.* (2006). This allowed comparison of the *max1-1* phenotype to that of the *max4-1* phenotype, to check for the possibility of resistance to GR24 in *max1-1*. This resistance would be hypothesised if *MAX1* function were so late in the pathway that it were downstream of the compound that GR24 mimics, and therefore required to produce a GR24-derivative with full shoot-branching activity. However, two experiments revealed no differences in response between *max1-1* and *max4-1* at the concentrations tested, as both showed significant reductions from their growth on the acetone carrier control when grown on 1μM GR24 or higher, but not when grown on 0.1μM GR24 or lower (Figure 3-2). These results infer that GR24 mimics a compound or compounds that are downstream of the action of both *MAX4* and *MAX1*. A further test with all four *max* mutants at 0.5μM, an intermediate concentration between those that did and did not produce a response (Figure 3-3), also showed no difference between any of the biosynthetic mutants, and a significant reduction in branching to levels similar to those of the wildtype control.

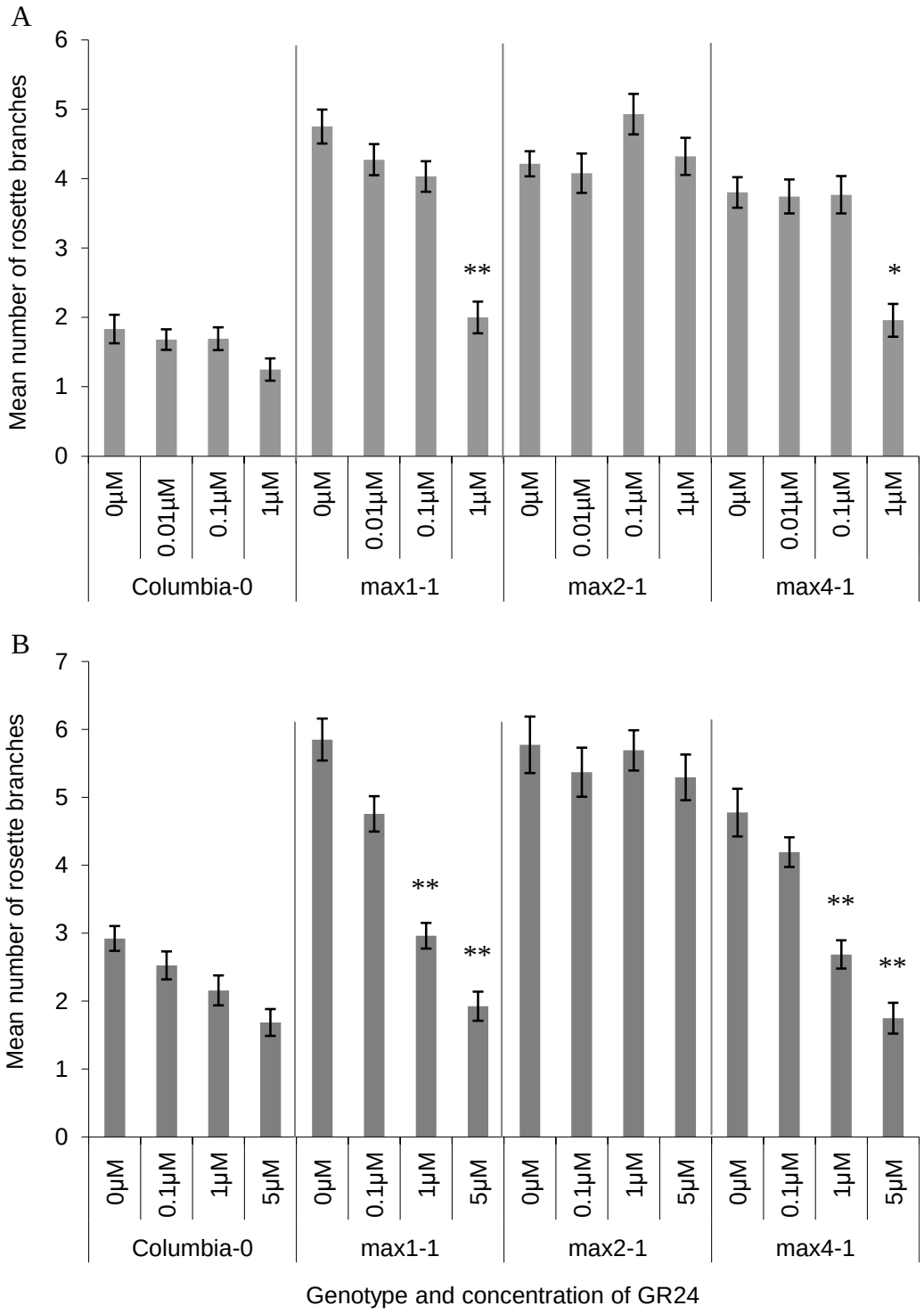


Figure 3-2. Mean number of branches for plants grown on agar containing GR24 dissolved in acetone. A) Experiment 1, B) Experiment 2. Branches were scored after approximately five weeks when the first siliques had formed. Columbia and *max2-1* are controls. Error bars are standard error of the mean. Samples treated with GR24 were compared to the samples of the same genotype treated with acetone, where ** = significant difference to P<0.001, * = P<0.05 in a Kruskal-Wallis test (adjusted for multiple sampling). Figure B is reproduced from Crawford *et al.* (2010).

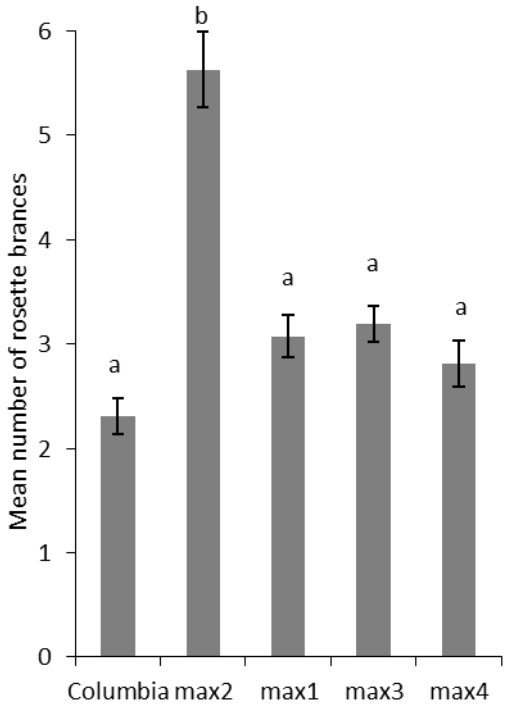


Figure 3-3. Mean number of branches for plants grown on agar containing 0.5µM GR24 dissolved in acetone. Branches were scored after approximately five weeks when the first siliques had formed. Columbia and *max2-1* are controls. Error bars are standard error of the mean. Samples sharing the same letter show no significant difference to P<0.001 in a Kruskal-Wallis test (adjusted for multiple sampling).

3.3 The 'Brassicaceae-specific' hypothesis

With regard to strigolactones, Arabidopsis is unusual among the models studied in two ways; firstly, (like most of the Brassicaceae) it does not form mycorrhizal symbioses, and secondly it is the only model system which currently has a *max1* orthologue mutant. This initially suggested a variation of the hypothesis that *MAX1* was a later incorporation into the biosynthesis pathway; that the absence of the evolutionary constraints imposed by mycorrhizal symbiosis may have allowed the incorporation of *MAX1* into the strigolactone pathway in the Brassicaceae specifically. If this is the case, then there may also have been coevolution of the signal transduction pathway, and particularly the receptor. There is only very limited knowledge of SL signalling at present, with MAX2 as the only confirmed signal transduction component. To test for co-evolution of SL synthesis with the recruitment of MAX1 and SL signalling by modification of MAX2, the ability of MAX2 from a species outside the Brassicaceae, hypothesised not to have *MAX1* in its SL biosynthetic

pathway, was assessed for its ability to rescue an Arabidopsis *max1* mutant. This experiment rested on two assumptions: that *MAX1* catalyses a modification to an SL that is bioactive outside the Brassicaceae, and that *MAX2* was a possible receptor or co-receptor for the compound, with which it would therefore have to coevolve. Two *35S::SvMAX2 max2-1* Arabidopsis transgenics produced by Dr Sally Ward, containing *MAX2* orthologues derived from willow, *Salix viminalis*, (which is in the Salicaceae family, but is also a rosid, like Arabidopsis) under the control of the strong *35S* promoter, which had been found to substantially rescue *max2-1*, were crossed into the *max1-1 max2-1* double mutant reported by Stirnberg *et al.* (2002), which has a similar or slightly stronger rosette branching phenotype than the single mutants. F_2 plants from the cross were then scored for branching by 'long day' branching assay, in which plants are grown in the greenhouse for approximately 6 weeks, until the main stem has ceased producing flowers, and then the number of rosette branches were scored. The 76 plants were found to segregate with a ratio of 48 wild type plants: 29 *max-like*, not significantly different from the 43:33 (9:7 wild type to branchy) ratio expected for no or limited rescue of the *max1* phenotype by the *SvMAX2* construct (not significant at $P \leq 0.05$ in a Chi squared test, see Table 3-1). The 9:7 ratio results from all the plants being homozygous for the *Atmax2-1* mutation, producing a ratio of 9 wild type phenotype plants carrying both the *SvMAX2* transgene and a wild type copy of *MAX1*; 4 plants without the rescuing *SvMAX2* transgene (3 with and 1 without *MAX1*, as the *max2* phenotype is epistatic to the *max1* phenotype); and 3 plants without *MAX1* but with an *SvMAX2* transgene. This was as opposed to the 3 wild-type: 1 *max2-like* segregation expected if the transgene were capable of substantially reducing the *max1* phenotype (the results were significantly different to this ratio at $P \leq 0.05$ in Chi squared test). In addition, it was possible to distinguish differences between the *max-like* plants corresponding to the slight differences between *max1* and *max2* phenotypes, specifically the much stronger leaf shape and curling phenotype in *max2-like* plants. Dividing by these phenotypes gave a ratio of 48 wild type: 17 *max2-like*: 12 *max1-like*, again not significantly different ($P \leq 0.05$ in Chi squared test) from the 43:19:14 ratio expected for no or limited rescue.

Table 3-1. Phenotypic punnet square for expected phenotypes of F_2 plants from the *35S::SALIXMAX2 max2* x *max1max2* cross – note all progeny are homozygous for *max2-1*. Plants carrying both at least one copy of the transgene (so wildtype for the *max2* lesion) and a wild-type *MAX1* copy are in black, those without a rescuing transgene and therefore *max2* phenotype are red, and plants with a transgene but homozygous for *max1-1* are in blue, resulting in a 9:3:3:1 ratio, in which the 1 (lacking both transgene and *MAX1*), is indistinguishable from the those lacking only the transgene.

Parental lines	***35S::SvMAX2*** ***MAX1***	***35S::SvMAX2*** ***max1***	**(*max2*)** ***MAX1***	**(*max2*)** ***max1***
35S::SvMAX2 ***MAX1***	*35S:: SvMAX2* *MAX1*	*35S:: SvMAX2* *MAX1*	*35S:: SvMAX2* *MAX1*	*35S:: SvMAX2* *MAX1*
35S::SvMAX2 ***max1***	*35S:: SvMAX2* *MAX1*	*35S:: SvMAX2* *max1*	*35S::SvMAX2* *MAX1*	*35S::SvMAX2* *max1*
(*max2*) ***MAX1***	*35S::SvMAX2* *MAX1*	*35S::SvMAX2* *MAX1*	(*max2*) *MAX1*	(*max2*) *MAX1*
(*max2*) ***max1***	*35S::SvMAX2* *MAX1*	*35S::SvMAX2* *max1*	(*max2*) *MAX1*	(*max2*) *max1*

To quantify the resulting phenotypes in more detail in case of weak effects, a short day decapitation assay was used to compare F_3 plants from three different F_2 parents with the *max1-like* phenotype and homozygous for the transgene. In this assay, to enhance the number of shoot branches for analysis, the method developed by Greb *et al.* (2003) was employed, in which plants are grown in short day conditions for four weeks to delay flowering and increase rosette leaf and axillary bud production. The light period is then lengthened to induce flowering, and when bolting has started the primary meristem is decapitated, to release further buds. This enhances the number of branches even in *max* mutants as although dormancy is reduced in these plants even they retain some dormant buds after growth in short day conditions, and they also retain the decapitation response. As a control a *35S::AtMAX2 max1* line was included in the assay, since this transgene was previously shown partially suppress the phenotype of *max1* (Stirnberg *et al.*, 2007).

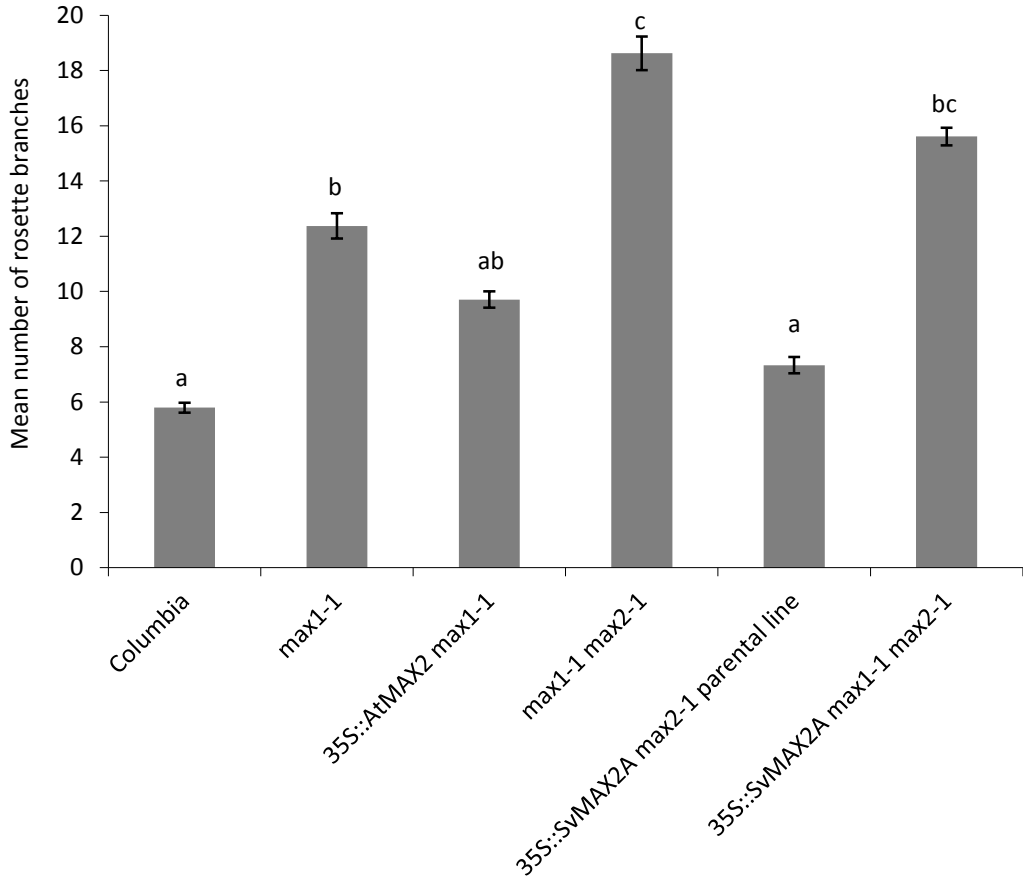


Figure 3-4. F3 plants homozygous for *35S::SvMAX2* but with *max1* phenotype, tested for branching in short day decapitation assay against controls Columbia-0, *max1-1*, *35S::AtMAX2 max1-2*, and parental lines *max1-1 max2-1* and *35S::SvMAX2 max2-1 1-7.3*. Error bars represent standard error of the mean, n=24 for all lines except the *35S::SvMAX2 max1-1 max2-1*, which are the pooled result of 3 separately backcrossed lines. Shared letters indicate no significant difference in Kruskal-Wallis test adjusted for multiple sampling at P≤0.01.

As shown in Figure 3-4, branching in the test lines was found to be intermediate between *max1-1* and *max1-1 max2-1* and not significantly different from either of them (there were also no significant differences between the three test lines). This is similar to the phenotype reported for the *max2-1* mutant (not tested here) which is intermediate between the *max1-1* single mutant and the *max1-1 max2-1* double mutant (Stirnberg *et al.*, 2002). On average the *35S::SvMAX2* construct reduced the branching of the double mutant by three branches on average, a greater reduction than the effect of the overexpression *35S::AtMAX2* construct on the *max1-1* mutant (a 2.6 branch reduction). However, in the double mutant background *35S::SvMAX2* could not significantly reduce the high branching phenotype to either the branch numbers

of the *35S::SvMAX2 max2-1* parent line, nor to less than that of a single *max* mutant. If rescue had occurred, the hypothesis that *MAX1* was a Brassicaceae-specific innovation may have been supported. However, given the number of assumptions required for this experiment, few firm conclusions could be drawn from the lack of full rescue.

3.4 MAX1 complementation by non-angiosperm species

To test more directly the incorporation of *MAX1* into the MAX pathway in other species, the function of *AtMAX1* was compared to that of other orthologues. In this case, it was hypothesized that orthologous proteins from other species capable of catalysing the same reaction as that in Arabidopsis may also act in SL biosynthesis in those species. Therefore if *MAX1* function in the pathway predated the emergence of the angiosperms, non-angiosperm *MAX1* orthologues should be able to act in the Arabidopsis pathway sufficiently well to rescue the mutant phenotype of *max1-1* plants. In collaboration with Dr Richard Challis and Dr Céline Mouchel *MAX1* orthologues from a range of plant species were identified by reciprocal Basic Local Alignment Search Tool (BLAST) searches on the GenBank, TIGR and Phytozome databases (Altschul *et al.*, 1990; Childs *et al.*, 2007; Goodstein *et al.*, 2012; NCBI). *MAX1* orthologues were identified from several angiosperm species, including all those (at that time) with fully sequenced genomes, as well as from *Selaginella moellendorffii*. *S. moellendorffii* represents the lycopodiophytes, the most distantly related group of plants from the angiosperms to possess both vasculature and branching in the sporophyte generation (Willis and McElwain, 2002). Its genome has been fully sequenced, revealing the presence of a single orthologue of *MAX1* (Banks *et al.*, 2011). As the lycopodiophytes are so evolutionarily distant from the angiosperms, and no genomes are available for any taxon between these two, a candidate expressed sequence tag (EST; GenBank accession BT103061) from *Picea glauca* (white spruce, a gymnosperm) was used as the basis for 5'RACE to identify the full length transcript for cloning of the coding sequence, which was used for phylogenetic analysis and complementation of *MAX1* (as '*PgMAX1'*).

ClustalW (Larkin *et al.*, 2007) was used to produce alignments of *MAX1* orthologue proteins, and a 95% consensus sequence (Figure 3-5) and matrix of protein identities were produced in BioEdit (Hall, 1999). This alignment firstly revealed conservation of the PFGxGPRxCxG haem-binding motif, of the PERF motif corresponding to the PxRx of all Arabidopsis CYPs, and a KExMR motif corresponding to the K-helix motif (from the website of Paquette *et al.*, 2009). All three motifs are either known to be involved in haem-binding (the conserved cysteine in the PFGxGPRxCxG motif forms the thiolate bond with the haem) or thought to stabilise the haem-binding pocket (Paquette *et al.*, 2009). However, there are no obvious highly conserved motifs particular to *MAX1*, especially when compared to other CYP711 clan members from the green alga *Chlamydomonas reinhardtii*. The point mutation that abolishes function in the *max1-1* mutant is a C-to-T substitution, predicted to convert Proline-117 to a leucine (Booker *et al.*, 2005), but this proline is not conserved even within other potential *MAX1* orthologues, although it is frequently present in other Arabidopsis CYPs (from the website of Paquette *et al.*, 2009) and forms part of the first Substrate Recognition Sequences proposed by Nelson *et al.* (2008). These alignments also indicate that *SmMAX1* shares very low sequence identity and protein similarity to *AtMAX1* (Table 3-2), as its protein identity is only 38.9%, even less than the 40% normally required to be classified in the CYP711 family. This is in contrast to the similarity of the gymnosperm *PgMAX1*, which shows higher identity to *AtMAX1* than several (although notably not all) monocot genes.

Figure 3-5. Alignment of selected *MAX1* genes, showing consensus sequences from BioEdit (95% threshold identity) and Clustal (complete consensus as '*', 'strong' groups with >0.5 score in the Gonnet PAM250 similarity matrix as ':', 'weak' groups with ≤0.5 score as '.'). The Arabidopsis P-117 that is affected in the *max1-1* mutation is highlighted in grey.

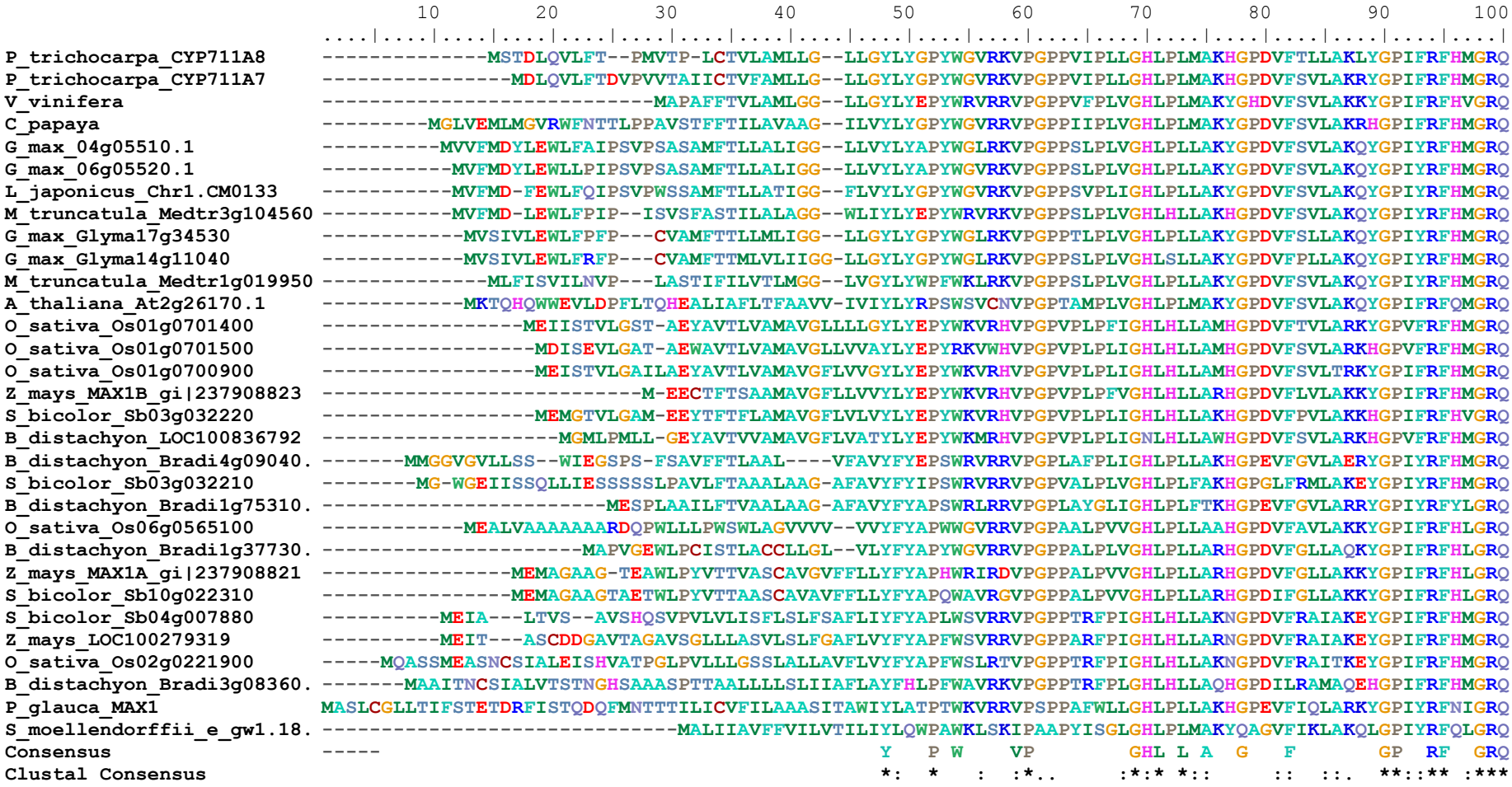
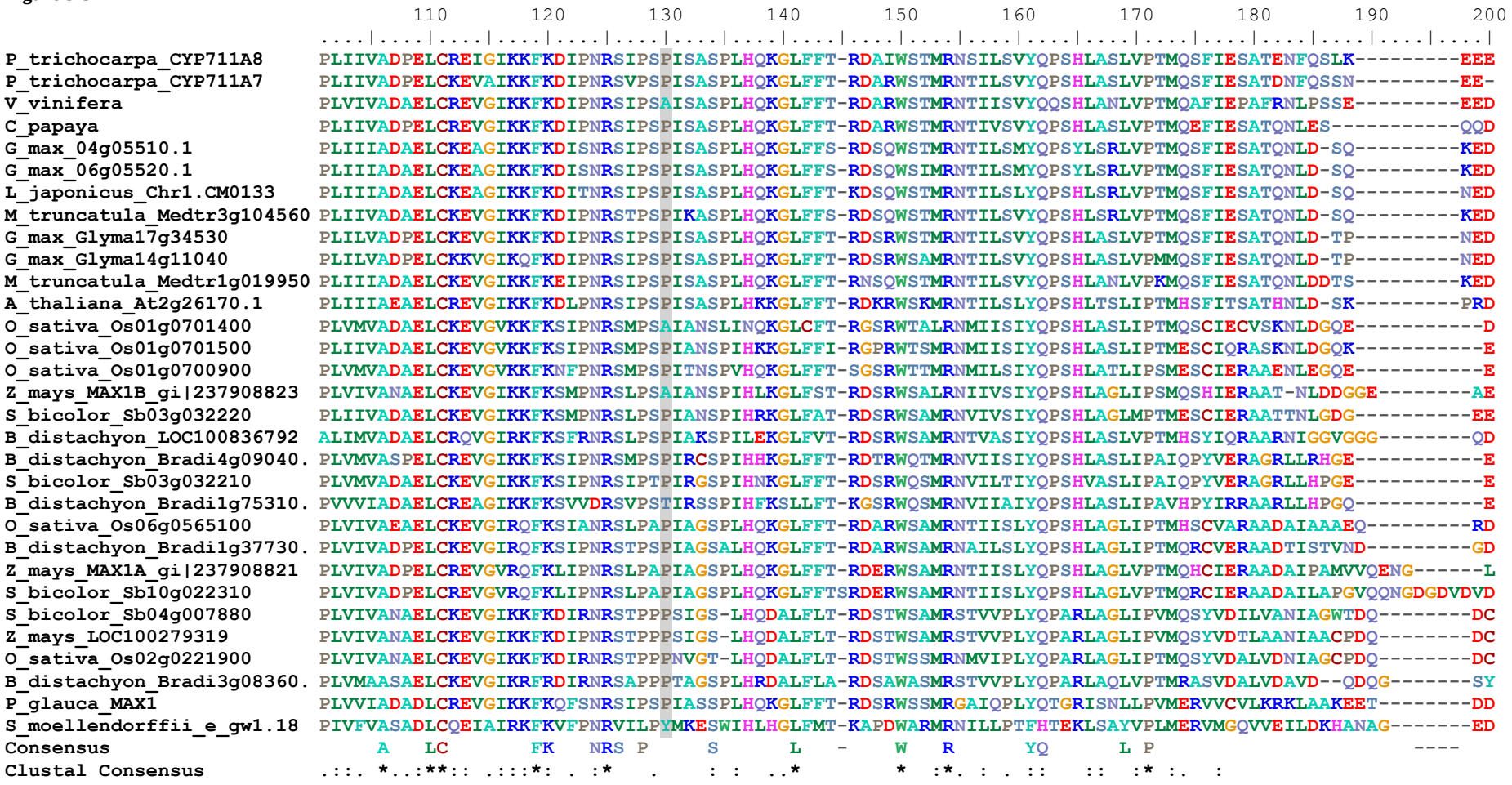


Figure 3-5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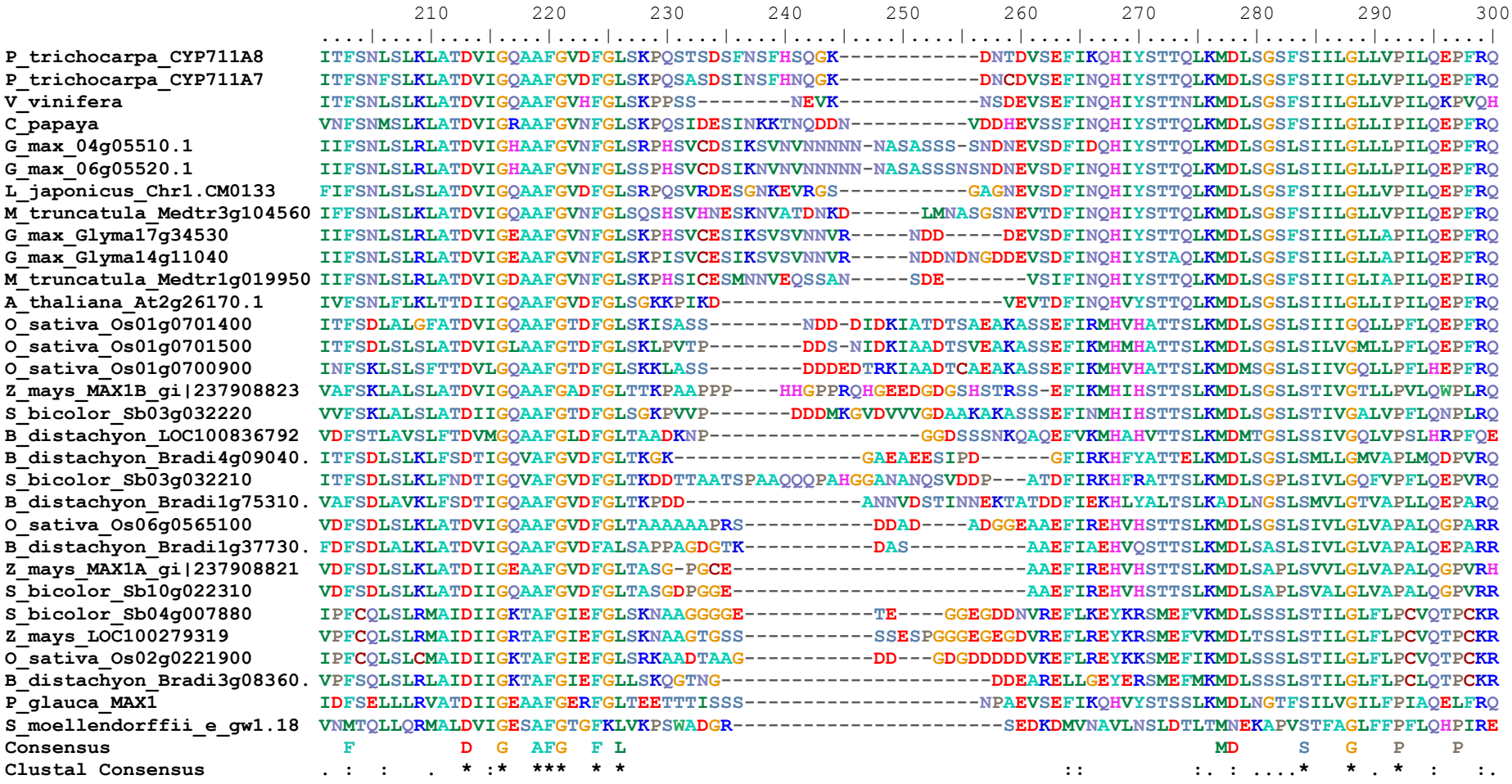
Consensus
Clustal Consensus

Figure 3-5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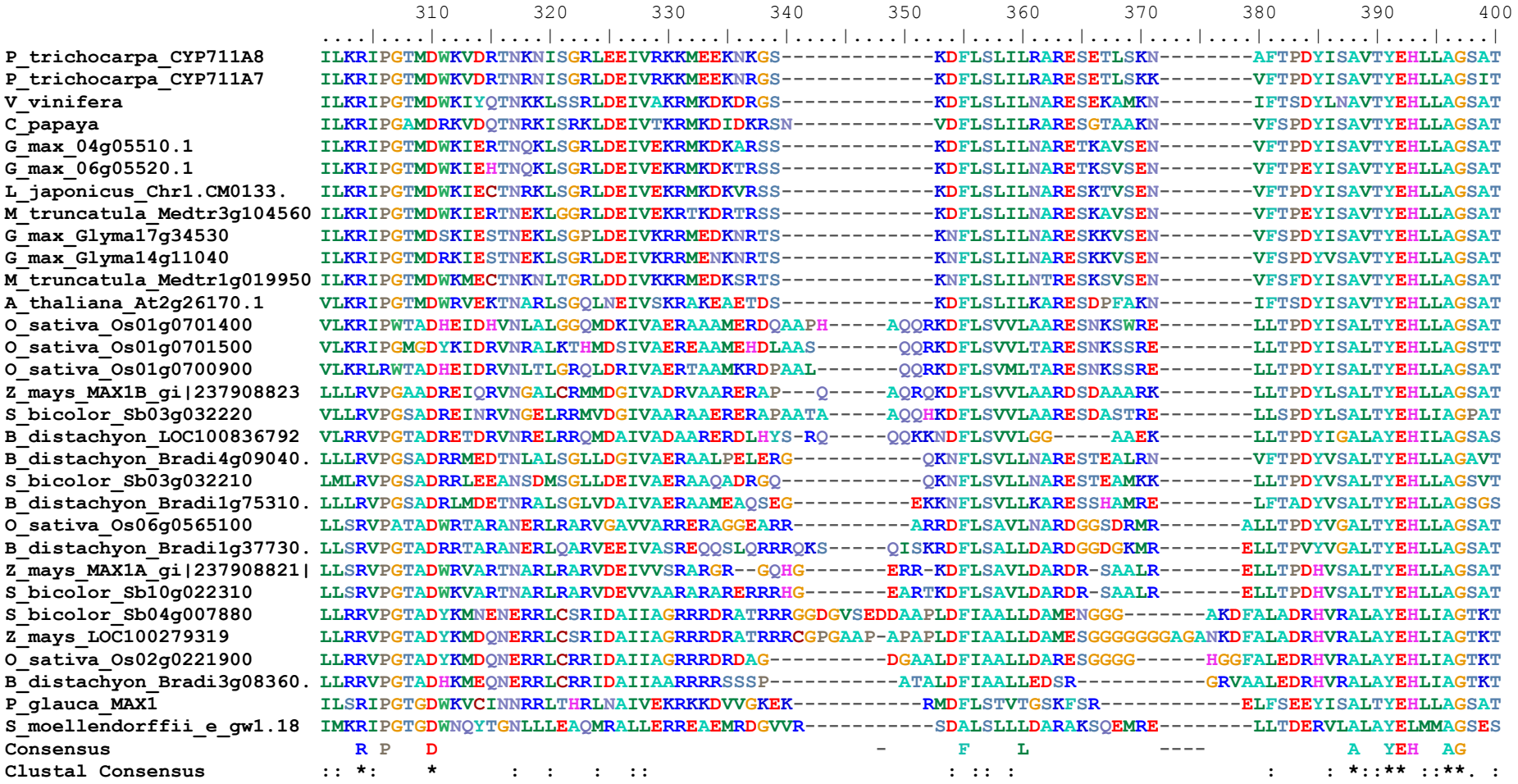
Consensus
Clustal Consensus

Figure 3-5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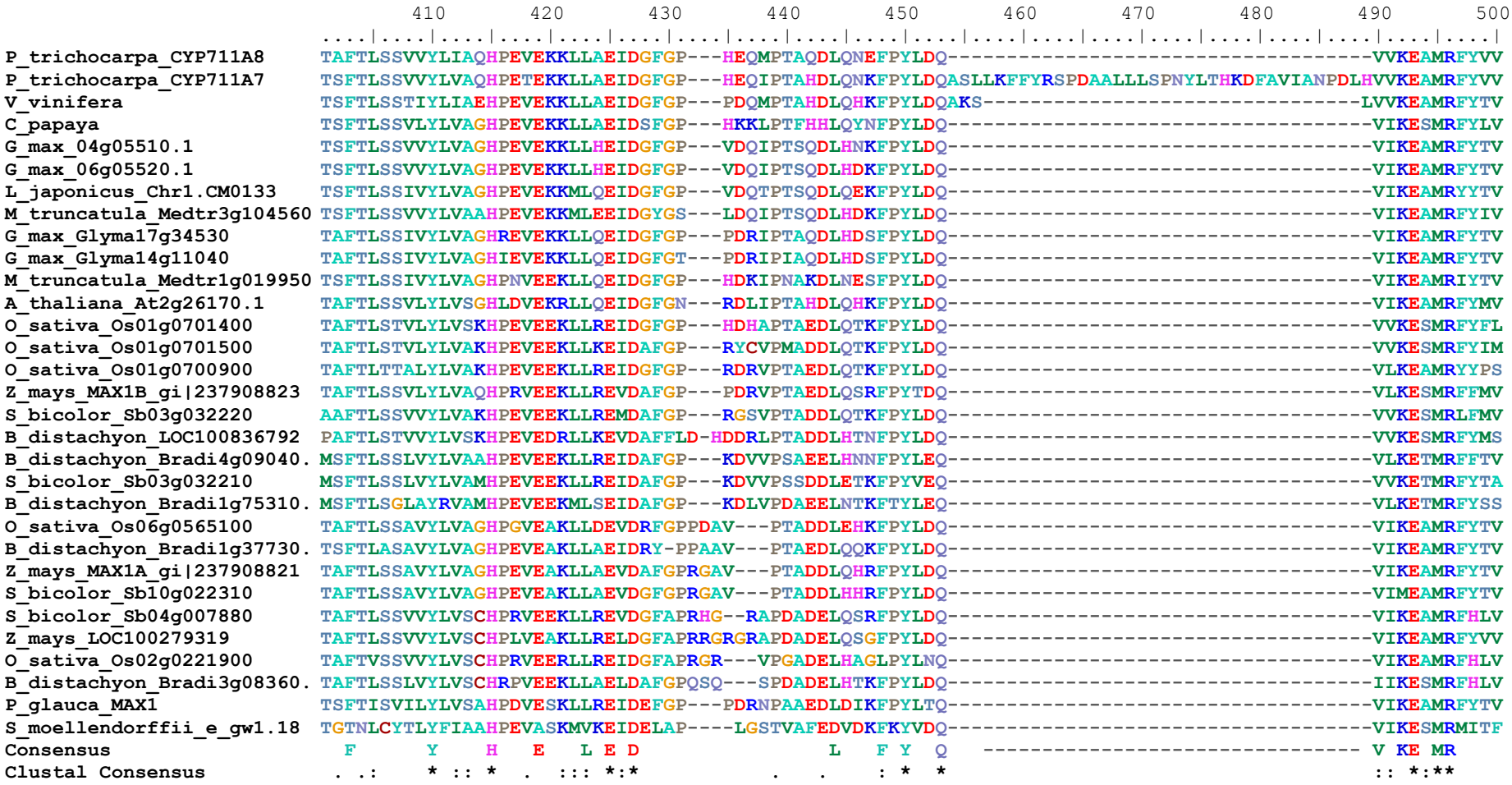
Clustal Consensus :: *: * : : : :: : :: : : * *::** ::**. :

Figure 3-5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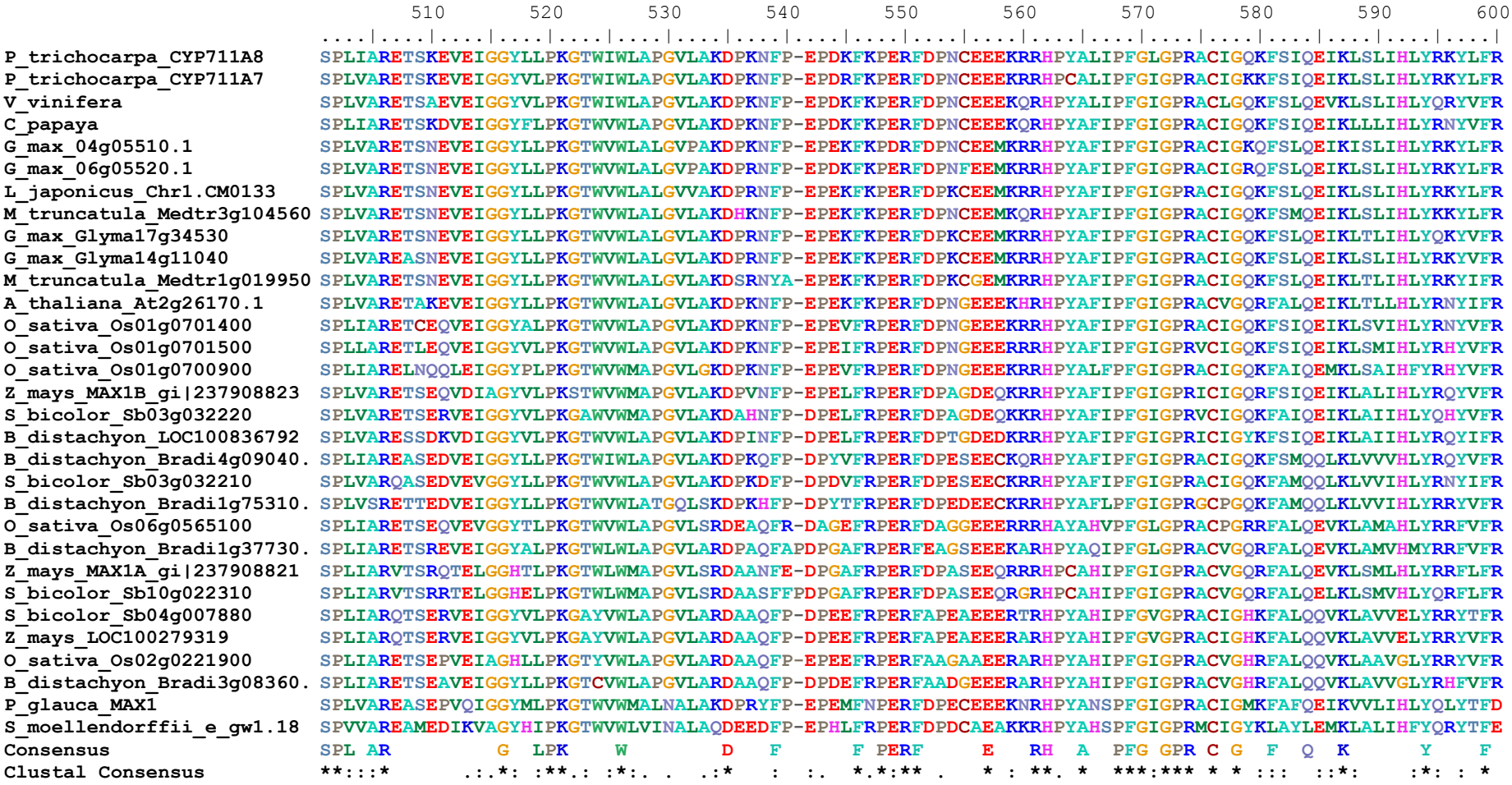
Consensus
Clustal Consensus

Figure 3-5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P_trichocarpa_CYP711A7 SPLVARETSKEVEIGGYVLPKGTWIWLAPGVLAKDPKNFP-EPDRFKPERFDPNCEEEKRRHPCALIPFGIGPRACIGKKFSIQEIKLSLIHLYRKYLFR
V_vinifera SPLVARETSAEVEIGGYVLPKGTWIWLAPGVLAKDPKNFP-EPDKFKPERFDPNCEEEKQRHPYALIPFGIGPRACLGQKFSLQEVKLSLIHLYQRYVFR
C_papaya SPLIARETSKDVEIGGYFLPKGTWVWLAPGVLAKDPKNFP-EPDKFKPERFDPNCEEEKQRHPYAFIPFGIGPRACIGQKFSIQEIKLLLIHLYRNYVFR
G_max_04g05510.1 SPLVARETSNEVEIGGYLLPKGTWVWLALGVPAKDPKNFP-EPEKFKPDRFDPNCEEMKRRHPYAFIPFGIGPRACIGKQFSLQEIKISLIHLYRKYLFR
G_max_06g05520.1 SPLVARETSNEVEIGGYLLPKGTWVWLALGVPAKDPRNFP-EPDKFKPERFDPNFEEMKRRHPYAFIPFGIGPRACIGRQFSLQEIKLSLIHLYRKYLFR
L_japonicus_Chr1.CM0133 SPLVARETSNEVEIGGYLLPKGTWVWLALGVVAKDPRNFP-EPEKFKPERFDPKCEEMKRRHPYAFIPFGIGPRACIGQKFSLQEIKLSLIHLYRKYLFR
M_truncatula_Medtr3g104560 SPLVARETSNEVEIGGYLLPKGTWVWLALGVLAKDHKNFP-EPEKFKPERFDPNCEEMKQRHPYAFIPFGIGPRACIGQKFSMQEIKLSLIHLYKKYLFR
G_max_Glyma17g34530 SPLVARETSNEVEIGGYLLPKGTWVWLALGVLAKDPRNFP-EPEKFKPERFDPKCEEMKRRHPYAFIPFGIGPRACIGQKFSLQEIKLTLIHLYQKYVFR
G_max_Glyma14g11040 SPLVAREASNEVEIGGYLLPKGTWVWLALGVLAKDPRNFP-EPEKFKPERFDPKCEEMKRRHPYAFIPFGIGPRACIGQKFSLQEIKLSLIHLYRKYVFR
M_truncatula_Medtr1g019950 SPLVARETSNEVEIGGYLLPKGTWVWLALGVLAKDSRNYA-EPEKFKPERFDPKCGEMKRRHPYAFIPFGIGPRACIGQKFSLQEIKLTLIHLYRKYIFR
A_thaliana_At2g26170.1 SPLVARETAKEVEIGGYLLPKGTWVWLALGVLAKDPKNFP-EPEKFKPERFDPNGEEEKHRHPYAFIPFGIGPRACVGQRFALQEIKLTLLHLYRNYIFR
O_sativa_Os01g0701400 SPLIARETCEQVEIGGYALPKGTWVWLAPGVLAKDPKNFP-EPEVFRPERFDPNGEEEKRRHPYAFIPFGIGPRACIGQKFSIQEIKLSVIHLYRNYVFR
O_sativa_Os01g0701500 SPLLARETLEQVEIGGYVLPKGTWVWLAPGVLAKDPKNFP-EPEIFRPERFDPNGEEERRRHPYAFIPFGIGPRVCIGQKFSIQEIKLSMIHLYRHYVFR
O_sativa_Os01g0700900 SPLIARELNQQLEIGGYPLPKGTWVWMAPGVLGKDPKNFP-EPEVFRPERFDPNGEEEKRRHPYALFPFGIGPRACIGQKFAIQEMKLSAIHFYRHYVFR
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S_bicolor_Sb03g032220 SPLVARETSERVEIGGYVLPKGAWVWMAPGVLAKDAHNFP-DPELFRPERFDPAGDEQKKRHPYAFIPFGIGPRVCIGQKFAIQEIKLAIIHLYQHYVFR
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B_distachyon_Bradi4g09040. SPLIAREASEDVEIGGYLLPKGTWIWLAPGVLAKDPKQFP-DPYVFRPERFDPESEECKQRHPYAFIPFGIGPRACIGQKFSMQQLKLVVVHLYRQYVFR
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B_distachyon_Bradi1g75310. SPLVSRETTEDVEIGGYLLPKGTWVWLATGQLSKDPKHFP-DPYTFRPERFDPEDEECKRRHPYAFLPFGIGPRGCPGQKFAMQQLKLVVIHLYRRYVFR
O_sativa_Os06g0565100 SPLIARETSEQVEVGGYTLPKGTWVWLAPGVLSRDEAQFR-DAGEFRPERFDAGGEEERRRHAYAHVPFGLGPRACPGRRFALQEVKLAMAHLYRRFVFR
B_distachyon_Bradi1g37730. SPLIARETSREVEIGGYALPKGTWLWLAPGVLARDPAQFAPDPGAFRPERFEAGSEEEKARHPYAQIPFGLGPRACVGQRFALQEVKLAMVHMYRRFVFR
Z_mays_MAX1A_gi|237908821 SPLIARVTSRQTELGGHTLPKGTWLWMAPGVLSRDAANFE-DPGAFRPERFDPASEEQRRRHPCAHIPFGIGPRACVGQRFALQEVKLSMLHLYRRFLFR
S_bicolor_Sb10g022310 SPLIARVTSRRTELGGHELPKGTWLWMAPGVLSRDAASFFPDPGAFRPERFDPASEEQRGRHPCAHIPFGIGPRACVGQRFALQELKLSMVHLYQRFLFR
S_bicolor_Sb04g007880 SPLIARQTSERVEIGGYVLPKGAYVWLAPGVLARDAAQFP-DPEEFRPERFAPEAEEERTRHPYAHIPFGVGPRACIGHKFALQQVKLAVVELYRRYTFR
Z_mays_LOC100279319 SPLIARQTSERVEIGGYVLPKGAYVWLAPGVLARDAAQFP-DPEEFRPERFAPEAEEERARHPYAHIPFGVGPRACIGHKFALQQVKLAVVELYRRYVFR
O_sativa_Os02g0221900 SPLIARETSEPVEIAGHLLPKGTYVWLAPGVLARDAAQFP-EPEEFRPERFAAGAAEERARHPYAHIPFGIGPRACVGHRFALQQVKLAAVGLYRRYVFR
B_distachyon_Bradi3g08360. SPLIARETSEAVEIGGYLLPKGTCVWLAPGVLARDAAQFP-DPDEFRPERFAADGEEERARHPYAHIPFGIGPRACVGHRFALQQVKLAVVGLYRHFVFR
P_glauca_MAX1 SPLVAREASEPVQIGGYMLPKGTWVWMALNALAKDPRYFP-EPEMFNPERFDPECEEEKNRHPYANSPFGIGPRACIGMKFAFQEIKVVLIHLYQLYTFD
S_moellendorffii_e_gw1.18 SPVVAREAMEDIKVAGYHIPKGTWVWLVINALAQDEEDFP-EPHLFRPERFDPDCAEAKKRHPYAHSPFGIGPRMCIGYKLAYLEMKLALIHFYQRYTFE
Consensus SPL AR G LPK W D F F PERF E RH A PFG GPR C G F Q K Y F
Clustal Consensus **:::* .:.*: :**.: :*:. . .:* : :. *.*:** . * : **. * ***:*** * * ::: ::*: :*: : *

Figure 3-5


610
620
630
P_trichocarpa_CYP711A8 HSPHMEKPLELDFGIVLNFRHGVKLRIVKRT------
P_trichocarpa_CYP711A7 HSPTMEKPLEFEFGIVLNFKRGVKLRIIKRT------
V_vinifera HSPNMEKPLELEYGIILNFKHAVKLRAIKRHP-----
C_papaya HSPNMENPIELEYGIVLNFKYGVKLRVIKRT------
G_max_04g05510.1 HSPNMENPLELQYGIVLNFKHGVKLRVIKRTE-TC--
G_max_06g05520.1 HSPNMENPLELQYGIVLNFKHGVKLRAIKRKE-AC--
L_japonicus_Chr1.CM0133.170.nc HSPNMENPLELEYGIVLNFKHGVKVRAIKRTERSC--
M_truncatula_Medtr3g104560 HSADMESPLELEYGIVLNFKHGVKFSVIKRTEMSC--
G_max_Glyma17g34530 HSVDMEKPVEMEYGMVLNFKHGIKLRVIRRT------
G_max_Glyma14g11040 HSLDMENPVEMEYGMVLNFKHGLKLRVIRRT------
M_truncatula_Medtr1g019950 HSLNMEKPVELEYGLVLNFKHGIKLRVIKRT------
A_thaliana_At2g26170.1 HSLEMEIPLQLDYGIILSFKNGVKLRTIKR-------
O_sativa_Os01g0701400 HSPSMESPLEFQYSIVCNFKYGVKLRVIKRHTA----
O_sativa_Os01g0701500 HSPSMESPLEF-------------------------
O_sativa_Os01g0700900 PSPSMESPPEFVYSIVSNFKNGAKLQVIKRHI-----
Z_mays_MAX1B_gi|237908823 HSPSMESPLEFQFGVVLNFKHGVKLQSIKRHKC----
S_bicolor_Sb03g032220 HSPSMESPLEFQFGIVVNFKHGVKLHVIKRHVENN--
B_distachyon_LOC100836792 HSPSMESPLQFQYGVIVNFKHGVKLQVIHRHKE----
B_distachyon_Bradi4g09040.1 HSPNMEAPLQFQFSIVVNFKHGVKLHVIERNA-----
S_bicolor_Sb03g032210 HSPRMEFPLQFQYSILVNFKYGVKVQVIERKN-----
B_distachyon_Bradi1g75310.1 HSPGMEFPLQLEFSIVNNFKHGVKLQVIDREEH----
O_sativa_Os06g0565100 RSPRMESPPELQFGMVLSFRRGVKLTAVERRHAAAA-
B_distachyon_Bradi1g37730.1 RSPRMESPPEFQFGMVLSFRHGVKLRAIKRLTRNEAV
Z_mays_MAX1A_gi|237908821 RSPRMESPPELQFGIVLNFKKGVKLVAVERCAAMPL-
S_bicolor_Sb10g022310 RSPQMESPPELQFGIVLNFKNGVKLVAVERCAAMS--
S_bicolor_Sb04g007880 HSPAMESPLQFDFDLVLAFRHGVKLRAIRRS------
Z_mays_LOC100279319 HSPSMESPIQFDFDLVLAFRHGVKLRAIRRG------
O_sativa_Os02g0221900 HSPAMESPLQFDFDLVLAFRHGVKLRAIKRTNT----
B_distachyon_Bradi3g08360.1 HSPDMESPVEFDFDLVLGFRHGVKLRAIRRTND----
P_glauca_MAX1 HSPAMENPLEFQFGIVVSVKYGIRLRLRHRRAQSPV-
S_moellendorffii_e_gw1.18.593. HSPAMENPLAVRLSIVVRPIHGVKLRVRKREIC----
Consensus S ME P R -
Clustal Consensus * ** * .

Table 3-2. Matrix of protein identities for selected *MAX1* orthologues

Sequence Identity Matrix	A thaliana At2g26170.1	L japonicus	G max 17g34530	G max 06g05520.1	C papaya	G max 04g05510.1	M truncatula Medtr3g104560	P trichocarpa CYP711A8	V vinifera	M truncatula Medtr1g019950	G max 14g11040	P trichocarpa CYP711A7	O sativa Os01g0701400	O sativa Os01g0701500	O sativa Os01g0700900	S bicolor Sb03g032220	Z mays MAX1B	O sativa Os06g0565100	Z mays MAX1A	B distachyon Bradi4g09040.1	S bicolor Sb03g032210	B distachyon Bradi1g75310.1
L japonicus Chr1.CM0133.170.nc	0.700																					
G max Glyma17g34530	0.690	0.804																				
G max 06g05520.1 LOC100808297	0.686	0.868	0.808																			
C papaya	0.685	0.746	0.740	0.726																		
G max 04g05510.1 LOC100797803	0.684	0.867	0.813	0.956	0.731																	
M truncatula Medtr3g104560	0.684	0.837	0.795	0.841	0.720	0.838																
P trichocarpa CYP711A8	0.682	0.746	0.741	0.721	0.762	0.726	0.720															
V vinifera GSVIVT00032191001	0.679	0.732	0.725	0.716	0.733	0.712	0.717	0.762														
M truncatula Medtr1g019950	0.670	0.777	0.825	0.774	0.712	0.774	0.750	0.710	0.698													
G max Glyma14g11040	0.669	0.783	0.940	0.790	0.721	0.794	0.779	0.720	0.698	0.794												
P trichocarpa CYP711A7	0.633	0.703	0.696	0.682	0.723	0.687	0.681	0.841	0.723	0.673	0.676											
O sativa Os01g0701400	0.590	0.593	0.602	0.600	0.597	0.599	0.601	0.609	0.615	0.582	0.591	0.580										
O sativa Os01g0701500	0.568	0.567	0.568	0.566	0.574	0.567	0.575	0.588	0.577	0.554	0.555	0.557	0.794									
O sativa Os01g0700900	0.567	0.575	0.578	0.566	0.569	0.570	0.584	0.585	0.587	0.567	0.565	0.555	0.812	0.762								
Sorghum bicolor Sb03g032220	0.564	0.583	0.575	0.570	0.579	0.571	0.592	0.578	0.589	0.555	0.566	0.552	0.701	0.695	0.692							
Z mays MAX1B	0.556	0.567	0.572	0.567	0.572	0.568	0.576	0.578	0.594	0.558	0.567	0.550	0.686	0.649	0.657	0.767						
O sativa Os06g0565100	0.555	0.548	0.537	0.548	0.538	0.541	0.529	0.546	0.551	0.519	0.532	0.507	0.552	0.533	0.534	0.554	0.570					
Z mays MAX1A	0.551	0.551	0.547	0.541	0.548	0.539	0.542	0.572	0.577	0.528	0.540	0.533	0.549	0.523	0.533	0.569	0.574	0.715				
B distachyon Bradi4g09040.1	0.549	0.562	0.577	0.558	0.581	0.563	0.561	0.564	0.584	0.557	0.568	0.531	0.594	0.561	0.561	0.576	0.581	0.535	0.545			
S bicolor Sb03g032210	0.548	0.569	0.569	0.570	0.564	0.565	0.569	0.557	0.562	0.551	0.567	0.530	0.590	0.555	0.562	0.571	0.567	0.536	0.538	0.752		
B distachyon Bradi1g75310.1	0.547	0.561	0.557	0.555	0.554	0.550	0.547	0.577	0.580	0.537	0.548	0.540	0.562	0.529	0.541	0.560	0.569	0.729	0.701	0.550	0.534	
S bicolor Sb10g022310	0.543	0.541	0.539	0.534	0.538	0.530	0.538	0.554	0.562	0.516	0.529	0.519	0.540	0.509	0.523	0.568	0.563	0.703	0.898	0.539	0.521	0.693

Table 3-2

Sequence Identity Matrix	A thaliana	L japonicus	G max 17g34530	G max 06g05520.1	C papaya	G max 04g05510.1	M truncatula Medtr3g104560	P trichocarpa CYP711A8	V vinifera	M truncatula Medtr1g019950	G max 14g11040	P trichocarpa CYP711A7	O sativa Os01g0701400	O sativa Os01g0701500	O sativa Os01g0700900	S bicolor Sb03g032220	Z mays MAX1B	O sativa Os06g0565100	Z mays MAX1A	B distachyon Bradi4g09040.1	S bicolor Sb03g032210	B distachyon Bradi1g75310.1
P glauca MAX1	0.540	0.549	0.533	0.536	0.526	0.533	0.530	0.519	0.530	0.528	0.522	0.491	0.499	0.481	0.484	0.483	0.478	0.485	0.472	0.484	0.479	0.477
B distachyon LOC100836792	0.534	0.523	0.523	0.515	0.532	0.517	0.535	0.528	0.535	0.503	0.517	0.508	0.635	0.616	0.612	0.661	0.658	0.523	0.529	0.523	0.523	0.521
B distachyon Bradi1g37730.1	0.523	0.536	0.513	0.524	0.521	0.526	0.515	0.510	0.537	0.511	0.509	0.486	0.564	0.539	0.546	0.549	0.550	0.503	0.504	0.691	0.663	0.510
O sativa Os02g0221900	0.474	0.471	0.470	0.472	0.471	0.476	0.468	0.480	0.473	0.462	0.465	0.443	0.472	0.446	0.446	0.476	0.479	0.524	0.504	0.482	0.465	0.518
S bicolor Sb04g007880	0.468	0.471	0.476	0.477	0.472	0.475	0.476	0.481	0.481	0.462	0.476	0.445	0.478	0.451	0.446	0.489	0.490	0.519	0.506	0.479	0.465	0.529
Z mays LOC100279319	0.462	0.467	0.472	0.467	0.480	0.466	0.467	0.477	0.471	0.464	0.476	0.445	0.479	0.451	0.449	0.495	0.489	0.531	0.506	0.475	0.462	0.532
B distachyon Bradi3g08360.1	0.454	0.462	0.473	0.466	0.467	0.458	0.452	0.460	0.456	0.450	0.464	0.425	0.446	0.441	0.420	0.473	0.454	0.510	0.501	0.466	0.451	0.508
S moellendorffii e_gw1.18.593.1	0.389	0.401	0.396	0.391	0.385	0.387	0.393	0.389	0.412	0.392	0.391	0.360	0.382	0.362	0.369	0.376	0.374	0.355	0.353	0.352	0.368	0.366

Sequence Identity Matrix	S bicolor Sb10g022310	P glauca MAX1	B distachyon LOC100836792	B distachyon Bradi1g37730.1	O sativa Os02g0221900	S bicolor Sb04g007880	Z mays LOC100279319	B distachyon Bradi3g08360.1
P glauca MAX1	0.468							
B distachyon LOC100836792	0.516	0.458						
B distachyon Bradi1g37730.1	0.487	0.460	0.521					
O sativa Os02g0221900	0.500	0.425	0.443	0.426				
S bicolor Sb04g007880	0.509	0.438	0.454	0.431	0.778			
Z mays LOC100279319	0.507	0.429	0.451	0.435	0.759	0.864		
B distachyon Bradi3g08360.1	0.505	0.419	0.439	0.413	0.685	0.684	0.657	
S moellendorffii e_gw1.18.593.1	0.349	0.402	0.353	0.378	0.344	0.350	0.337	0.338

To investigate further whether the low similarity between Arabidopsis and Selaginella sequences were reflective of divergent function, and hence the late incorporation of *MAX1*into the SL pathway, the function of *SmMAX1* was tested in Arabidopsis, along with that of the gymnosperm white spruce orthologue. *MAX1* orthologues were cloned from cDNA produced from *S. moellendorffii* and *Picea glauca* (bulbils and seeds respectively kindly provided by J. A Banks, Purdue University, USA, and Spencer Reitenbach and Tim Lee of the Tree Seed Centre and Vernon Seed Orchard Company of British Columbia, Canada) and denoted *SmMAX1* and *PgMAX1*. These clones were placed under the control of the strong promoter CaMV *35S* in order to ensure high levels of expression, so that complementation tested MAX1 function and not the expression of the transgene. The resulting constructs were transformed into *max1-1*, and transgenic lines were selected and brought to homozygosity for phenotypic analysis in the T_3 generation.

3.4.1 Branch phenotype

Increased rosette branching, as the most visible phenotype of SL biosynthesis mutants, was used as a sensitive quantitative measure of rescue. To enhance the number of shoot branches for analysis, the method developed by (Greb *et al.*, 2003) was employed, as described previously. For *SmMAX1* eight independent transgenic lines were assayed, and for *PgMAX1* eleven were assayed, and both *35S::SmMAX1* and *35S::PgMAX1* constructs were found to be capable of complete rescue of *max1-1* (Figure 3-6 and Figure 3-7).

In addition to the branching phenotype, the height of *max* mutants is also reduced, a characteristic suspected to be causally linked to the increase in branching, as the same amount of resources are stretched over a larger number of branches. The heights of the individual transgenic lines were therefore compared with their branch numbers, to assess further any differences between transgenic lines by providing a second dimension of variation (Figure 3-8). Although the individual transgenic lines of *35S::SmMAX1* are more variable in their clustering with the Columbia-0 control, both the Selaginella and spruce constructs show the ability to rescue both height and branching.

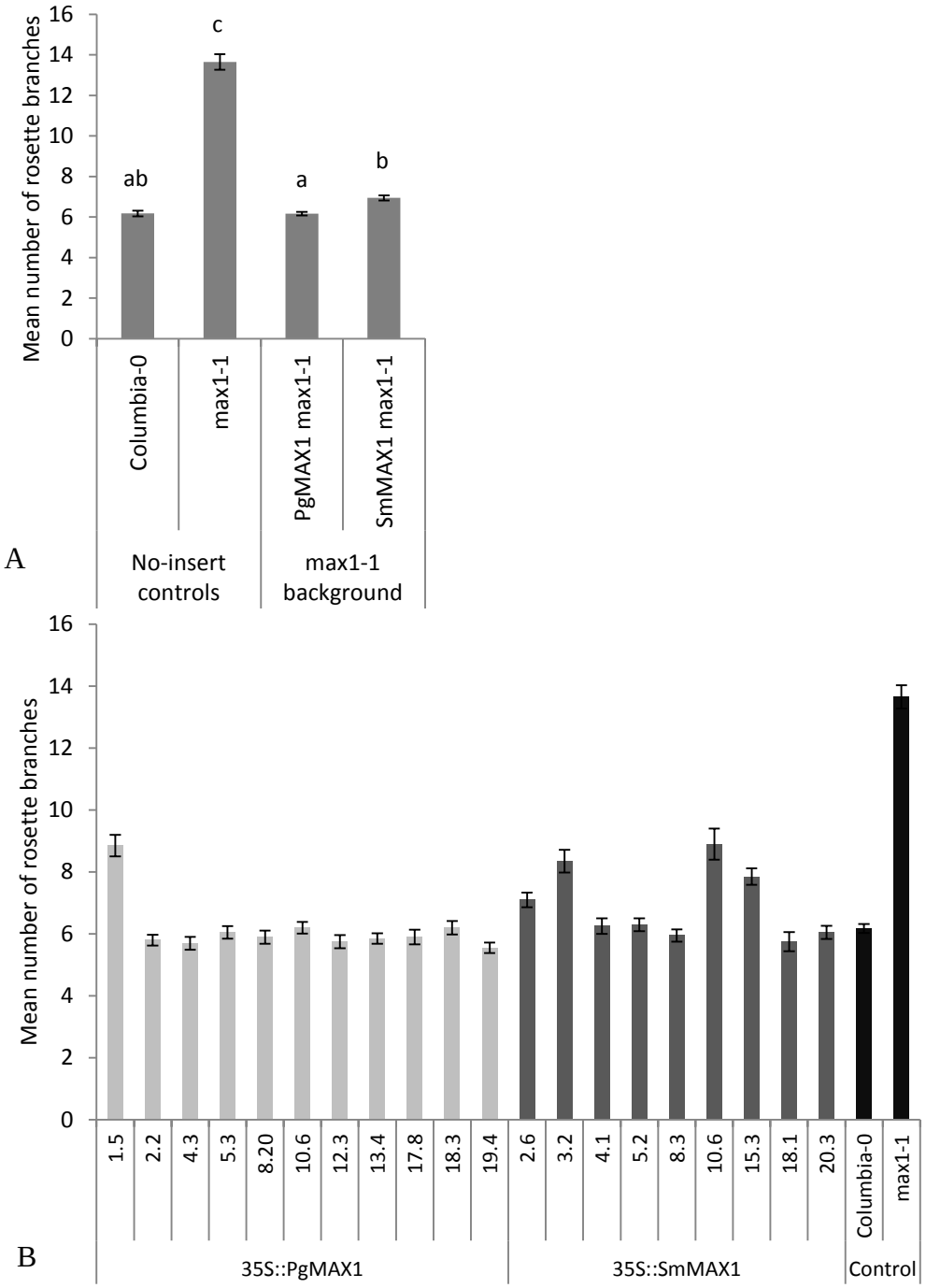


Figure 3-6. Rosette branching of Arabidopsis *max1-1* mutants complemented with *SmMAX1* and *PgMAX1* under the constitutive 35S promoter. Branching was assessed by short-day decapitation assay as described by Greb *et al.* (2003). **Data for constructs are (A) mean averages of independent transgenic lines shown in (B), n for each line = 20, except for Columbia-0 and *max1-1* for which n=40. (A) Shared letters indicate no significant difference in a Kruskal-Wallis test to P≤ 0.001. Error bars show standard error of the mean.**

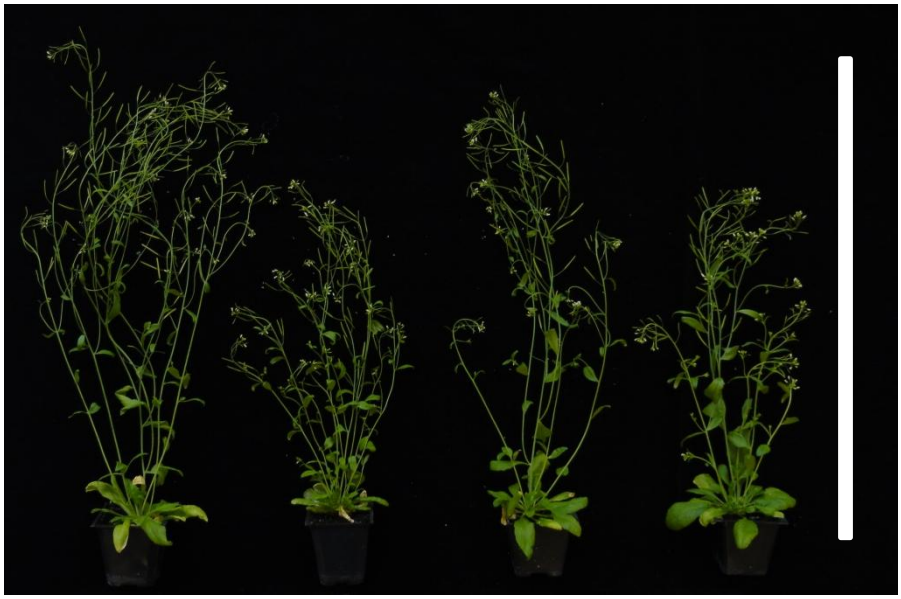


Figure 3-7. Photograph of Columbia-0, *max1-1*, *35S::PgMAX1 max1-1* line 4.3, and *35S::SmMAX1 max1-1* line 8.3, from left to right, with both transgenics showing rescue. White bar = 40cm.

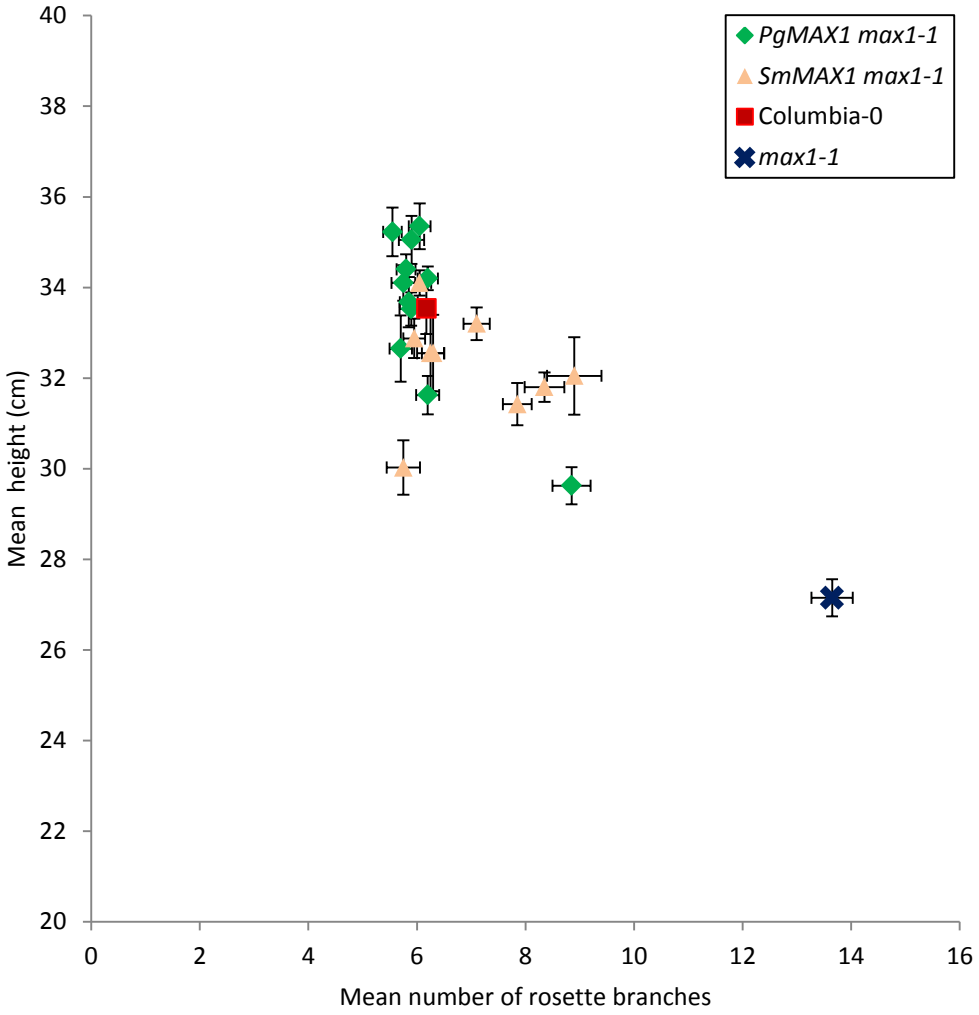


Figure 3-8. Branching plotted against height for individual constructs derived from *Selaginella moellendorffii* and *Picea glauca*. N =20, except for *max1-1* and Columbia-0 where n=40. Height (in

centimetres) of the longest branch was measured the day of scoring for branching. Error bars show standard error of the mean. Note y axis starts at 20cm.

3.4.2 Leaf phenotype

As rosette leaf shape is also affected in the *max* mutants, this phenotype was also used as a measure of rescue for the overexpression transgenics. *max* mutants have rosette leaves with rounder, shorter laminas and shorter petioles than wild-type plants, leading to a smaller rosette diameter (Stirnberg *et al.*, 2002; Lazar and Goodman, 2006). The leaves also curl downwards at the edges, an effect most pronounced in the *max2* mutants. However, while easily recognisable neither of these phenotypes is particularly dramatic.

Leaf shape is a complex phenotype which, if measured by hand, is time consuming, and relatively few dimensions can be measured accurately. However, the development of geometric morphometric approaches - automated imaging techniques combined with multivariate statistics - has allowed analysis of leaf shape to become a sensitive indicator of changes invisible to the naked eye (Langlade *et al.*, 2005; Micol, 2009; Kieffer *et al.*, 2011). Previous work had indicated that the LeafAnalyser approach developed by Weight, Parnham and Waites (2008) could be used to identify differences between wild type and *max* Arabidopsis leaves (V. Matser, pers. comm.). LeafAnalyser is an automated image and data analysis program which identifies the margin of leaves within images via an adjustable threshold, and assigns each leaf node numbers, allowing all leaves from one plant to be analysed from a single image. It then calculates positions for the individual leaf tips and the leaf centres (or centroids) based on this margin, aligns these vertically, and plots a user-defined number of evenly spaced landmarks around the leaf margin. The coordinates of these landmarks can then be exported from the program for further analysis, or fed into the statistical analysis side of the program. In this mode the distances between pairs of landmarks are used in a principal component analysis (PCA), which can be used to generate a leaf shape space in which deviations in form between different leaf groups can be compared (Weight *et al.*, 2008; Kieffer *et al.*, 2011).

For analysis of the complemented *max1-1* mutants, the Columbia wild-type

and *max1-1* mutant plants were grown with two independent transgenic lines each for *35S::PgMAX1* and *35S::SmMAX1* for five weeks, when the adult leaves were removed and scanned to produce images that were analysed with the image analysis mode of LeafAnalyser. The resulting coordinates were Procrustes fitted using the morphometrics program MorphoJ (Klingenberg, 2011). This method produces a calculation of the leaf size based on the centroid – the central point as calculated from the mean distance of all the landmarks – and then fits all the leaves to the same size, allowing size and shape to be analysed separately. LeafAnalyser was then used to run a PCA on a library of 1500 leaves from ten natural Arabidopsis accessions that had previously been produced by Vera Matser (Kieffer *et al.*, 2011) and Procrustes-fitted by Joe Vaughan of Dr Richard Waites' group at the University of York. The eigenvector matrix produced was then used to calculate a leaf point model for each of the leaves from the complementation experiment, which were scaled to the standard deviations of the natural accession database. Ninety-six principal components (PCs) were produced, corresponding to the ninety-six pairs of coordinates (from tip to landmark and centroid to landmark) for the forty-eight landmarks used in creating the leaf data. 85.44% of the total variation was contained in the first five PCs, with a further 6.32% contained in the next five.

In order to determine the salience of the PCs to *max* mutants, each of the first ten PCs were compared to see which differed significantly between Columbia and *max1-1,* and LeafAnalyser was used to produce models of the 'mean leaf' and the 'mean leaf +/- 2 standard deviations' to estimate the type of shape variation they explained (see Figure 3-9 for examples). PCs 1 and 4 appeared to show variance in petiole orientation on a right-to-left axis, while PCs 5, 6, 7 and 8 all seemed to represent differences in petiole thickness, either along the petiole or at its junction with the lamina, but none were different between wild-type and mutant. However, PCs 2, 3, 9 and 10 represented phenotypes significantly affected by the *max1-1* mutation. From the PC space produced by LeafAnalyser (Figure 3-9) PC2, which explains 26.29% of the total variation, appears mainly defined by leaf width at the base of the lamina and its junction to the petiole. PC3, which contributes 13.66% of the variation, seems to reflect the degree to which lamina area is distributed along the length of the

whole leaf, and as a result, the lamina: petiole ratio. Finally, PCs 9 and 10, which reflect only 0.88% and 0.79% of the total variation respectively, describe correspondingly subtle phenotypes. PC9 looks like it varies on a left-to-right axis, showing the roundness on one side of the leaf compared to flatness on the other, whereas PC10 seems to correspond to the length of a vector crossing the lamina diagonally from a proximal left point to a distal right point, contributing to the left-to-right axis and a little to the total length. Taken together, the phenotypes affected by the *max1-1* mutation represent 41.52% of the natural variation in leaf shape out of the 91.80% of variation considered, as well as its effect on total leaf area.

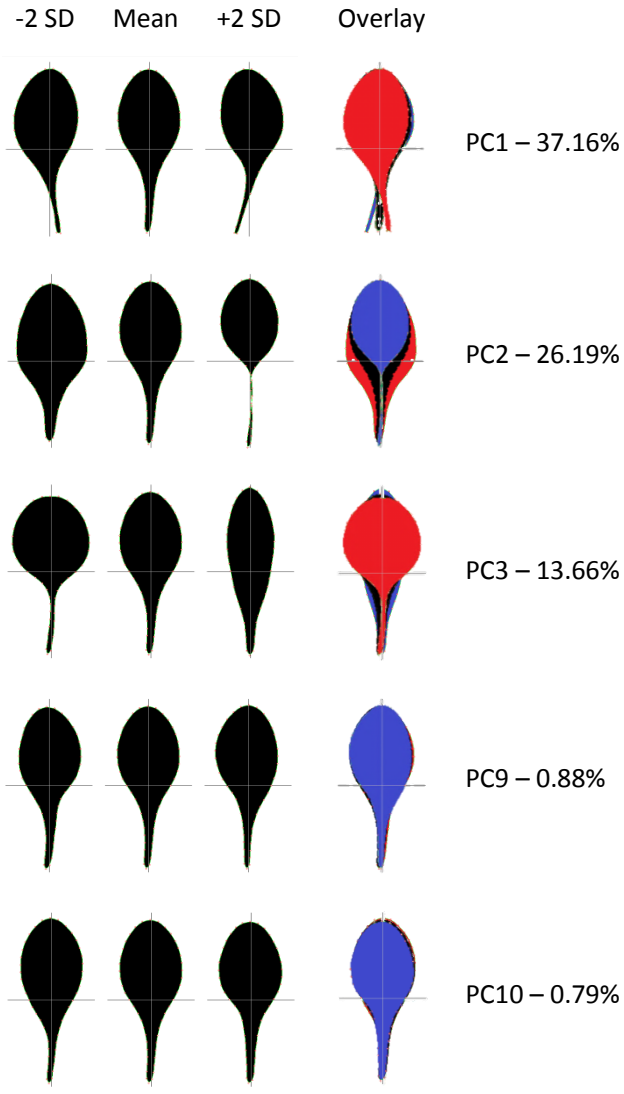


Figure 3-9. Principal components 1-3, 9 and 10: effect on leaf shape and percent of variation each explained. Overlays: red = –2SD, black = mean, blue = +2SD.

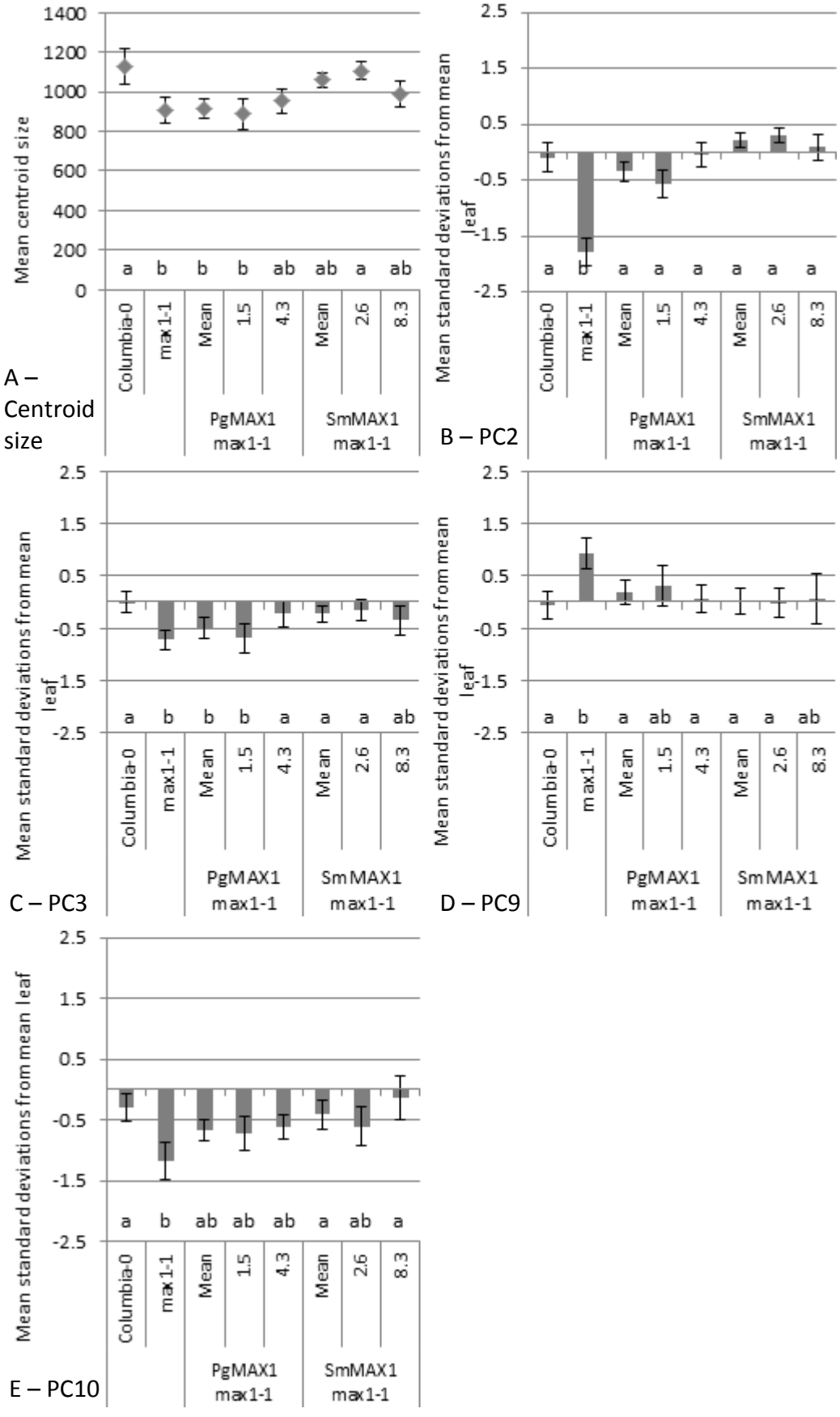


Figure 3-10. Leaf shape analysis for Procrustes-fitted adult leaves four and above from *max1-1* plants complemented with non-angiosperm *MAX1* orthologues. Error bars are standard error of the

mean, calculated on number of plants as n, where n = 15 for controls, and lines 2.6 (n = 10) and 8.3 (n=8) were used for *SmMAX1*, and lines 1.5 (n=7) and 4.3 (n=8) for *PgMAX1*. Shown are mean centroid sizes, which corresponds to leaf size (A) and standard deviations from the mean leaf for PC2 (width at centre, B), PC3 (area distribution, C), PC9 (D) and PC10 (E). Letters indicate non-significance in Tamhane's T2 post-hoc test at P>0.001 (centroid, PC2) or P>0.05 (PC10) and Tukey's Honestly Significant Difference (HSD) at P>0.05 for PC3 and PC9 (which have equal variances).

The leaf phenotypes identified as being affected in *max1-1* mutants were then used to investigate the rescue phenotypes of the *PgMAX1* and *SmMAX1* transgenics (Figure 3-10). As PC3, PC9 and PC10 represent smaller percentages of total variation, the cut-off for significant values was raised from 0.001 to 0.05 to reflect the smaller changes they convey. Generally, and in opposition to their effect on branching, *PgMAX1* lines displayed less rescue over the five phenotypes considered than did *SmMAX1* transgenics. For leaf size (as measured by centroid size parameter produced in MorphoJ, Figure 3-10A), *PgMAX1* showed no rescue at all, and incomplete rescue for PC3 (3-10C) and PC10 (3-10E), whereas *SmMAX1* only showed incomplete rescue for centroid size. However, in terms of PC2 (3-10B) and PC9 (3-10D), both lines rescued.

As the branching results were derived from far more independent lines than used for leaf analysis, these results were broken down to see how differences between transgenic lines related to leaf rescue. The two independent lines used for leaf analysis of *PgMAX1*, 1.5 and 4.3, showed a wide variation in ability to rescue branching (see Figure 3-6), with 1.5 showing the least degree of rescue for all lines of this construct, whereas 4.3 showed more typical complete rescue. Nevertheless, the breakdown of the different lines indicated that the less successful rescue of centroid size and PC10 by *PgMAX1* was due to unsuccessful rescue by both lines, not just that of 1.5, whereas for PC2, PC3 and PC9 the relative patterns of rescue were the same as those of the branching data. For *SmMAX1*, the lines chosen also varied in branch rescue, with 2.6 not being as successful as the fully-rescuing line 8.3, but still less branchy than *PgMAX1* 1.5. However, for leaf phenotypes 8.3 rescued less well than 2.6 for centroid size, PC3 and PC9, but better than 2.6 for PC10. This may indicate that leaf size, PC3 and PC10 phenotypes are more sensitive to MAX1 activity than PC2 and the branching phenotype, requiring a different threshold for phenotypic change. If so, then it would seem that the spruce homologue of *MAX1* is less

capable than the Selaginella one of rescuing Arabidopsis, despite its closer phylogenetic relationship and protein similarity.

3.5 Discussion

The placing of *MAX1* in the strigolactones pathway has been a difficult question both from evolutionary and biochemical perspectives. From the biochemical point of view, as a CYP the possible reactions that *MAX1* might catalyse are diverse. Outside of the CYP711 clan, *MAX1* shows most similarity to the Thromboxane A_2 Synthases (TXAS) of mammals, which carry out two different reactions, an isomerisation and a fragmentation of the hormone Prostaglandin H_2 (Booker *et al.*, 2005). This similarity to TXAS may mean that MAX1 doesn't require molecular oxygen or an electron donor, like the CYP74 family, which also catalyse substrates (allene epoxides) generated by dioxygenases within the plastid. The CYP74s are plastidically localised and act on the dioxygenase products directly, using parts of the substrate itself as the oxygen donor (Booker *et al.*, 2005; Hannemann *et al.*, 2007). However, MAX1 lacks a plastid target-peptide and the precise nature of its substrate is unknown. Grafting studies demonstrated that it is downstream of the mobile precursor that requires *MAX3* and *MAX4*, (Booker *et al.*, 2005), in conjunction with biochemical studies of the SL pathway, which proposed the action of a CYP or CYP-like activities downstream of the carotenoid-derived precursor (Matusova *et al.*, 2005; Rani *et al.*, 2008). Experiments were therefore designed to investigate whether that resulted in any resistance to rescue by a SL analogue, GR24, which is known to be capable of rescuing biosynthetic *max* mutants in rice and Arabidopsis, although only active at much higher concentrations than endogenous SLs such as 5-deoxystrigol (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). *max1-1* is as sensitive to low levels of GR24 as *max4-1*, with growth on GR24 reducing rosette branch numbers at the same concentrations in both mutants (Figure 3-2). *MAX1*, then, appears to be upstream of the synthetic SL GR24 in the pathway, a hypothesis supported by the biochemical and physiological studies of Rani *et al.* (2008) and Kohlen *et al.* (2011). In the Kohlen *et al.* study, Arabidopsis was found to produce 5-deoxystrigol and orobanchyl acetate, as well as orobanchol which had been reported previously (Goldwasser *et al.*, 2008). Both *max1-1* and *max4-1* mutants lacked detectable

levels of orobanchol in root exudates, and root and stem extracts from the mutants showed a reduced ability to stimulate germination of the parasitic plant *Phelipanche ramosa*, a standard assay for SL activity, although 5-deoxystrigol was present in too low a concentration for direct measurement even in Columbia-0. As *max1-1* is required for all SL activities tested (shoot branching, parasitic plant germination, and production of orobanchol), it seems likely that it is upstream of all the active SL structures, of which 5-deoxystrigol has been proposed as the biochemical start point (Rani *et al.*, 2008; Kohlen *et al.*, 2011). However, *max1-1* resistance (compared to *max4*) to the addition of 1.25μM GR24 has been reported from work on the role of SLs on root elongation and lateral root suppression (Ruyter-Spira *et al.*, 2011). The dose response curves generated for primary root extension and root hair elongation found by Ruyter-Spira *et al.* are very different to those found for branching phenotypes – in roots, concentrations of GR24 of 1.25μM and 2.5μM promoted elongation, concentrations of 10μM GR24 inhibited it (2011). Such reversals of effect at different concentrations is not uncommon in plant hormones, but had not previously been reported for SLs, and has not been reported for branching in any species studied. These authors postulate that the resistance of *max1-1* mutants to GR24 may reflect a dual role of *max1-1* in more than one reaction in the production of SL compounds - both reactions necessary to the production of 5-deoxystrigol, and in reactions (such as the hydroxylation reactions proposed by Rani *et al.*, 2008) downstream of this initial compound which enhance the activity of strigolactone structures. Which particular members of the SL compound family are active in shoot branching and root architecture control have yet to be elucidated, and nor have the particular chemical moieties that influence SL effectiveness been found. Indeed, it has been proposed that not only may the different SL species have different purposes, but that the response of different species to SLs may depend on the balances of different strigolactone structures they receive, in a similar manner to pheromone signalling in animals, in which it is the mix of compounds received, rather than any particular compound, that elicits the response (Tsuchiya and McCourt, 2012).

As *MAX1* was (and, based on the results from Ruyter-Spira *et al.*, remains) a

possible late step in the biosynthesis of branching-active SLs in Arabidopsis, the hypothesis was raised that its presence in the pathway was a Brassicaceae-specific event, made possible by the relaxation of selection that would have occurred when the Brassicaceae broke their symbiotic relationship with arbuscular mycorrhizzae. However, the experiment used to test this required *MAX2* to have coevolved with the structure of the active strigolactone, a point only likely if *MAX2* directly interacted with SL as part of the receptor complex. As F-box proteins form receptors in plants for both auxin and jasmonate-isoleucine conjugates (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005; Katsir *et al.*, 2008), this assumption is plausible. However, a *MAX2* homologue from the willow *Salix viminalis* could not rescue the Arabidopsis *max1-1* phenotype. There are a number of possible reasons for this, which include; that *MAX1* does not catalyse a late step in bioactive SL biosynthesis, a probability since *max1* mutants appear to lack 5-deoxystrigol; that *MAX2* is not a receptor and therefore would not influence the reception of the compound detected, and that *MAX1* may be active within the *Salix viminalis* pathway. In addition to *MAX1* and *MAX2*, the identification of *D14* in rice added another component to the later part of the SL signalling pathway, for which either a late biosynthetic role or a signal transduction role may be possible. As well as its role in the SL pathway, *MAX2* is also required for transduction of the karrikin-related signal, compounds found in smoke, which stimulate germination after fire (Nelson *et al.*, 2011). Although the karrikin and strigolactone pathways are separate in most of their actions, they converge at *MAX2*, suggesting that there is another component that provides specificity of response – in the case of the karrikins this is provided by *D14like*, a homologue of *D14* (Waters *et al.*, 2012). There is no evidence (as yet) that this specificity is due to a role for either of the *D14* family orthologues (or *MAX2* for that matter) as a receptor, nor that if *D14* has a catalytic function it affects the same moiety of the active SL as *MAX1*, but both are possibilities and it may be that *D14* is acting in a similar role to that proposed for the 'late action' of *MAX1*, as a near-final step in SL biosynthesis.

Although the *SvMAX2* experiment was inconclusive, the hypothesis that *MAX1* incorporation postdates the emergence of the Brassicaceae group was greatly weakened on the basis of the complementation of *max1-1* by constructs

from both conifers and lycopodiophytes, as well as Dr Ward's finding of rescue of *max1-1* by *35S::SvMAX1* (Sally Ward, pers. comm.). In addition, researchers working on petunia (*Petunia hybrida*), another (angiosperm) model for SL signalling, have found that not only can the *PhMAX1* orthologue rescue Arabidopsis, but that knock down of *PhMAX1* expression causes increased branching in petunia itself, providing the first evidence for *MAX1* function *in planta* in shoot branching control outside of Arabidopsis (Drummond *et al.*, 2012). The ability of *SmMAX1, PgMAX1* and *PhMAX1* to rescue substantially the Arabidopsis *max1-1* branching, height and (for *PgMAX1* and *SmMAX1*) leaf phenotypes shows a conservation of protein function across a wide evolutionary range. Although this does not necessarily reflect a role in SL production *in planta* of the non-angiosperm species, this conservation does suggest that *MAX1* was incorporated fairly early in land plant evolution to the MAX pathway, or even first incorporated and then lost in moss, and that the SL biosynthesis pathway has been substantially conserved throughout that time. This provides an interesting mirror to the Brassicaceae-specific hypothesis for *MAX1*, as most mosses, like the Brassicaceae, have also secondarily lost the ancestral mycorrhizal symbiosis (Wang *et al.*, 2010). The existence of an active role for SLs in development, if not mycorrhizal symbiosis, has been established in *Physcomitrella patens*, despite its lack of a *MAX1* homologue. Proust *et al.* (2011) have demonstrated that the moss homologue of *CCD8/MAX4* is required for production of several strigolactone compounds reported from angiosperms, including orobanchol, a compound which in Arabidopsis requires the activity of *MAX1* for its production (Kohlen *et al.*, 2011). The similarity of the compounds produced by moss to those present in angiosperms could imply that in Physcomitrella a different gene or set of genes has been co-opted to the role of *MAX1* in SL production – and indeed, it may add weight to the possibility that *MAX1* function is a land-plant synapomorphy (possibly even ancestrally required for the AMy symbiosis) that Physcomitrella has subsequently lost over time. However, it is also possible that the reaction catalysed by *MAX1* is connected to the long-distance nature of hormone signalling in vascular plants, but which is less necessary in bryophytes, in which tissues are only a few cells thick - perhaps in the conversion to activity of a more stable precursor better suited to long-distance transport. Although no *MAX1* orthologues have yet been

found in other bryophytes, the sequencing of the *Marchantia polymorpha* genome will contribute to this question, as liverworts are the most basal extant land plants, the only group thought to have diverged from other land plants before the mosses, and they also form AMy symbioses (Willis and McElwain, 2002; Qiu *et al.*, 2006).

The use of both leaf phenotypes and branching/height phenotypes to investigate function of the transgenes in Arabidopsis raised some interesting points, particularly the mismatch in the degree of rescue between different phenotypes. Although both constructs are capable of rescuing *max1-1* completely in terms of branching and height, and although *PgMAX1* shares higher protein similarity with *AtMAX1* than does *SmMAX1*, this construct was less able to rescue the leaf size and shape phenotypes of the leaves. Little is known about the mechanism of SL action in leaf development, and to determine the significance of these effects requires repetition of the leaf experiment, but these results may indicate that leaf phenotypes are influenced to different degrees or by different aspects of MAX pathway than those of branch outgrowth. As leaf lamina size is highly sensitive to incorrect (higher or lower) concentrations of auxin during leaf development (Ljung *et al.*, 2001), this may explain the high threshold requirement for SLs to rescue phenotypes such as centroid size, as this sensitivity may amplify the effects of tiny changes in auxin transport generated by perturbation of SL concentration, which are not sufficient to affect branch outgrowth. Indeed, in the case of centroid size particularly, GR24 treatment itself has been found to reduce leaf size, and to delay vascular development through its effects on auxin signalling (Ruyter-Spira *et al.*, 2011). Further work on leaf shape determinants will help to unravel whether other leaf shape phenotypes are similarly affected, although the general similarity of those measured here with the branching results suggests not. However, in whatever way the hormone they produce may be acting, the ability of both *MAX1* constructs to rescue most Arabidopsis MAX pathway phenotypes implies that protein similarity, in the case of CYPs at least, is not necessarily a good guide to function, but that both lycopodiophytes and gymnosperms may conserve SL signalling and a role for *MAX1* in the biosynthesis of these hormones.

Chapter 4. Roles for Strigolactones in Non-Angiosperm Species

Given the presence of all the known genetic components required for SL synthesis and signalling in vascular non-angiosperm taxa, and the presence of SLs in even more distant taxa (Proust *et al.*, 2011), what of the physiological and developmental roles of SLs in these diverse groups? Of the three extant non-angiosperm lineages of vascular plants the gymnosperm lineage are almost entirely perennial, and most are large trees or shrubs, whereas the extant lycopodiophytes more closely resemble mosses in size and shape, as is reflected in the 'clubmoss' and 'firmoss' names of many species, although extinct members of this group formed the forests of the Carboniferous (Willis and McElwain, 2002). Between these groups, the extant ferns (moniliphytes) span the full range from short-lived, tiny annuals to the impressive perennial structures of tree ferns, some reaching twenty meters in height (Bell and Hemsley, 2000; Willis and McElwain, 2002).

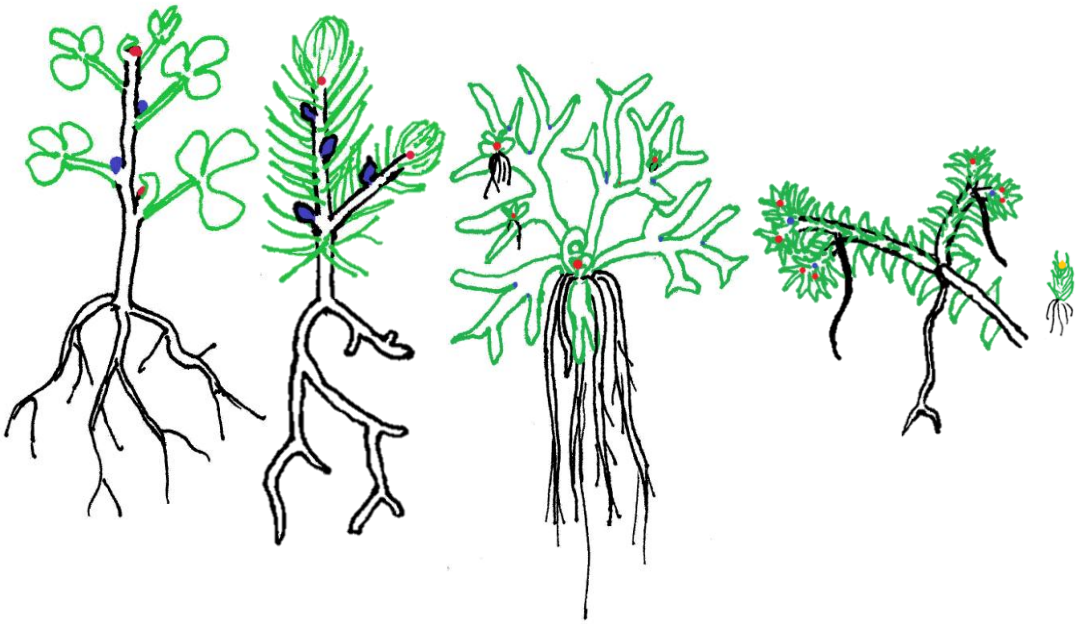


Figure 4-1. Sample body plans of the sporophyte generation of five of the seven major extant land plant groups. From left to right: Medicago seedling (angiosperm), spruce seedling (gymnosperm), young c-fern (moniliphytes), section of *Selaginella kraussiana* (lycopodiophyte), gametophyte of Physcomitrella with sporophyte in orange at tip of gametophore (mosses). Leaf equivalents are shown in green, active meristems in red, dormant meristems (or similar structures) in blue. All diagrams approximately life-size.

Most gymnosperms (particularly conifers and *Ginkgo*) share recognisably similar body plans to angiosperm trees, including determinate, multiveined leaves, indeterminate and iterative shoots producing branches from axillary meristems, and bipolar embryos with roots derived from a root apical meristem (Steeves and Sussex, 1989; Bell and Hemsley, 2000). However, the body plan of the lycopodiophytes is very different to that of angiosperms, as they form branches by the dichotomous division of the shoot tip, and produce 'leaves' which generally have at most one vascular trace (although some *Selaginella spp.* have more than one, bifurcating trace), rather than the ramifying patterns of angiosperm leaves (Willis and McElwain, 2002). Ferns are different again, and as varied as angiosperms in their body plans. The leaf-like fronds of ferns grow in an iterative pattern somewhat like angiosperm shoots, although these fronds may divide dichotomously, and produce a limited number of determinate modules (pinnae) rather than indeterminate branches - except where the fronds may be so indeterminate as to produce entire new plants on the 'leaf' margin. The fronds themselves are produced from an axis that may be above ground or rhizomatous, that in some taxa branches dichotomously, but that can in some taxa produce other indeterminate branches from dormant buds (Bierhorst, 1971; White and Turner, 1995; Bell and Hemsley, 2000). This great variety of vascular plant body plans, moreover, only apply to the sporophyte generation (the dominant generation in all of these groups), and not to the gametophytes, which arguably vary even more between the lineages.

In such a variety of forms, has evolution of SL signalling in branching control taken the same path in each? In moss, SLs are involved in controlling filament branching of the gametophyte and restricting colony extension in a quorum sensing-type manner coordinating the growth of different colonies, but not, of course, of branching of the single-axis sporophyte (Proust *et al.*, 2011). In angiosperms, SLs are not just involved in branch outgrowth control, but play roles in a wide range of developmental processes in the sporophyte– plant height and cambial thickening in the shoot, lateral and adventitious root and mycorrhizal symbiosis formation below ground, germination and photomorphogenesis in seedlings, and are regulated by phosphate and sometimes nitrogen availability (and Xie *et al.*, 2010; Agusti *et al.*, 2011; Foo

and Davies, 2011; and reviewed in Koltai, 2011; Toh *et al.*, 2012; Tsuchiya and McCourt, 2012; Yoneyama *et al.*, 2012). The unifying and conserved factor between the angiosperm and moss processes seems to be coordination of development and restriction of growth, suggesting that this was the ancestral role. However, which particular aspects of plant development are under the influence of SL signalling in the non-angiosperm, sporophyte-dominant vascular lineages is more difficult to hypothesise, except where those processes are clearly analogous to SL-controlled processes in angiosperms. Physiological experiments on *Picea abies* (white spruce), *Ceratopteris richardii*, (c-fern) and *Selaginella kraussiana* were therefore developed to establish systems for studying the effects of SLs on axillary branching (where applicable) and in responses to phosphate limitation across a wide span of plant forms, to enhance the understanding of SL evolution in physiological as well as genetic terms.

4.1 Gymnosperms - Picea glauca

The gymnosperms are the most closely related group to the angiosperms, and with the exception of their reproductive biology (and the stranger species of the Gnetales, particularly *Welwitschia mirabilis*) they appear to share many developmental mechanisms with that group. Conifers in particular share axillary branching patterns with those of angiosperms, including the repressive action of auxin in the maintenance of apical dominance and the promotive effect of cytokinins on production and outgrowth of axillary buds (Cline *et al.*, 2006). Likewise, auxin and its polar transport via PIN family proteins are known to be required in gymnosperms for developmental patterning in embryos, *KNOX* family genes specify meristematic zones, and at least some of the factors governing adaxial-abaxial polarity in leaf formation (important, in eudicots, to the specification of axillary meristems) are also conserved (Sundås-Larsson *et al.*, 1998; Floyd and Bowman, 2006; Larsson *et al.*, 2008; Palovaara *et al.*, 2010; Larsson *et al.*, 2012). Based on these similarities in development the possibility that SL signalling in branch outgrowth control might also be held in common between angiosperms and conifers was explored. White spruce was chosen as a representative of the gymnosperms because it is a commercially important forest tree for which large-scale EST sequencing and genome mapping resources are becoming available (Rigault *et al.*, 2011). Database

searches revealed potential orthologues of several *MAX* genes, including *MAX1*, *MAX2* and *MAX4*, from both white and Sitka spruce (*P. sitchensis*, a close relative of white spruce). Experiments were then designed based on the hypothesis that the axillary meristems of spruce were under similar developmental control as those of angiosperms, and that SLs would therefore be implicated in the outgrowth and breaking of dormancy in axillary buds.

Given the important role that dormancy of apical meristems plays in the development of many temperate perennial species, and that there are aspects of similarity between this process and that of axillary meristem dormancy (Rohde and Bhalerao, 2007) the hypothesis that the control of SLs may in such species extend to control of the apical bud was also investigated. As conifers are mostly trees or shrubs and are all perennial, many also share with angiosperm trees the ability to suspend growth temporarily to survive unfavourable conditions, the phenomenon of seasonal dormancy (Tudge, 2006; Rohde and Bhalerao, 2007). Superficially, this dormancy is often evident from the formation of 'buds' at the meristems – structures containing the meristem, and often the prepatterned primordia that will expand upon reactivation to form the following season's growth, all encased in a protective covering, the bud scale (Sutinen *et al.*, 2009). However, dormancy defined by production of the bud scale is deceptive, as although growth cessation is a prerequisite for dormancy, bud formation is not, and even then buds may reactivate growth if conditions remain or return to being favourable within a certain time, sometimes termed 'second flushing', where 'flushing' is used to describe bud break and active growth (Rohde and Bhalerao, 2007). Dormancy itself has been more usefully defined by Rohde and Bhalerao (2007) as the point at which growth cannot be reactivated by the return of favourable conditions for considerable time (Olsen, 2010). This is sometimes also referred to as 'endodormancy', to distinguish it from ecodormancy, in which dormancy is maintained after the point at which the bud is capable of reactivating due to unfavourable environmental conditions, or paradormancy, in which dormancy is imposed on the bud by other parts of the plant (as reviewed by Rohde and Bhalerao, 2007). The onset of endodormancy is promoted by changes in photoperiod and temperature, as well as endogenous factors such as hormones, including gibberellins, abscisic acid and auxin, and

the requirements for these different factors vary between different plants (and reviewed by Rohde and Bhalerao, 2007; and Olsen, 2010; Baba *et al.*, 2011). At least some of the molecular aspects of photoperiod signalling in connection with growth cessation, the PEPB gene family, are conserved between the angiosperm model tree poplar (*Populus spp.*) and the spruce species *Picea abies* (Norway spruce) and *Picea sitchensis* (Gyllenstrand *et al.*, 2007; and reviewed in Olsen, 2010; Karlgren *et al.*, 2011). A role for SLs has not yet been demonstrated in control of seasonal dormancy or growth cessation in any species, but given its other actions, this possibility was investigated in spruce both as a model conifer and as a model tree.

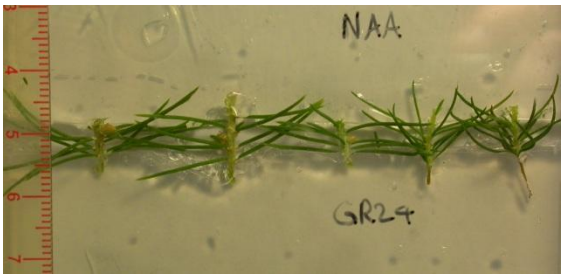
4.1.1 Initial decapitation studies and protocol development

Initial experiments focussed on the establishment of decapitation and hormone application systems similar to those used in Arabidopsis. First-year seedlings of spruce were used for experimentation for two reasons. Firstly this eases the production of sample material, and secondly because spruce is a 'determinate' tree species. For the first few years of growth (and particularly the first year) the patterning and expansion stages of stem and leaf development happen within the same season ('free growth'). In 'indeterminate species' free growth may occur also in older plants, but in older plants of more determinate species patterning and formation of stem units increasingly occurs in the preceding year, with the current year's growth merely being the expansion of these preformed units (Gyllenstrand *et al.*, 2007; Olsen, 2010; El Kayal *et al.*, 2011). The use of seedlings therefore allowed visualisation of any developmental changes within the same season.

To test for an effect on outgrowth of individual buds in spruce, the excised-bud assay developed for Arabidopsis by Chatfield *et al.* (2000) was adapted to investigate the effects of auxin and SL on spruce axillary buds (Figure 4-2A). In these experiments, excised nodal segments carrying a bud were treated with auxin (β-naphthoxyeacetic acid – NAA - a synthetic auxin, apically), with or without GR24 (supplied basally). In Arabidopsis, apical auxin inhibits outgrowth of axillary buds in an apical-dominance-like effect, which is accentuated by the presence of GR24 in the basal medium (Chatfield *et al.*,

2000; Crawford *et al.*, 2010). When GR24 is supplied in the absence of an auxin source, whether natural (e.g. from another bud) or externally supplied, it has no effect on bud growth (Chatfield *et al.*, 2000; Crawford *et al.*, 2010). As the bud scale of spruce axillary buds limits changes in bud length (the measurement used for Arabidopsis experiments) instead the number of buds in which the bud scale split (bud burst) was recorded over time.

A



B

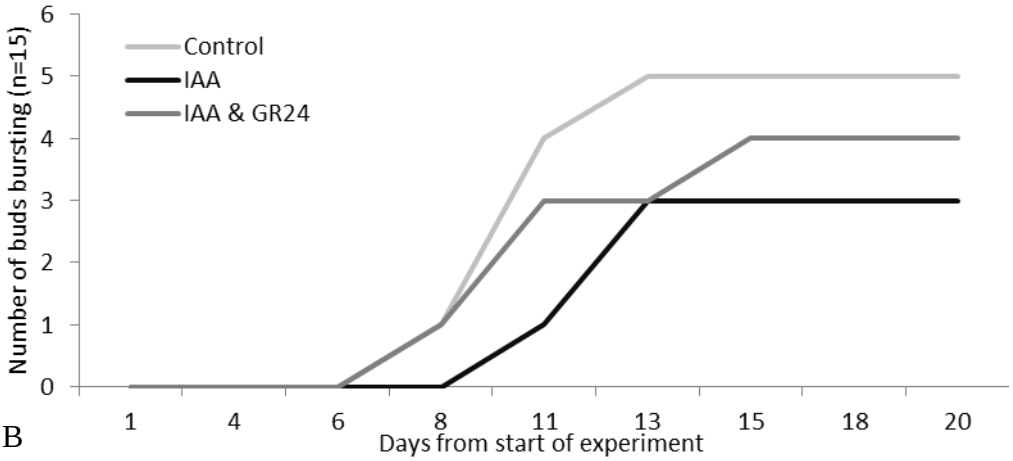


Figure 4-2. Excised bud assay adapted from that described for Arabidopsis by Chatfield *et al.* (2000). A) Sections of stem with well-developed buds from actively growing shoots were excised, surface sterilised and placed between nutrient agar blocks containing the synthetic auxin NAA or ethanol carrier above, and synthetic SL GR24 or acetone carrier below (picture from a different iteration of this experiment to results shown). B) The number of buds showing outgrowth activity (bursting through the bud scale) recorded every 2-3 days, N = 15 for each treatment.

The response from a single replicate (Figure 4-2B) might suggest a pattern of reduction in bud burst in response to auxin, as would be expected from the angiosperm model. However, the difficulty of cleanly excising nodal segments with appropriate buds from stems with such close-set needles, the quick contamination of the agar plates, the slow nature of the growth response in spruce and the large amount of material required for this experiment rendered it impractical to repeat on the larger scale needed for reliable results. A similar

attempt to use larger explants in liquid medium tubes killed most of them before developmental changes were seen, and seeds planted on agar did not germinate.

Given the difficulty of growing the spruce in axenic conditions, experiments with whole plants on soil were attempted. Plants were chosen that had formed dormant apical buds with bud scales (hereafter referred to as apical buds) because this allowed use of plants that were at a similar stage of activation and facilitated the easy removal of the apical meristem (presumably the principle auxin source) without damaging too many of the surrounding leaves (Figure 4-3A). Lanolin containing a natural auxin, 10μM indole-3-acetic acid (IAA), or the ethanol carrier as a control, was applied at the time of decapitation to the cut surface (Figure 4-3B), and 5μM GR24 (or the carrier acetone) was applied to the lower stem once a week in a PEG-based mixture adapted from that used for Arabidopsis bud applications by Gomez-Roldan *et al.* (2008). Outgrowth of tissue was measured from all axillary buds on the plant every 2-3 days for a month. Tissue outgrowth was seen from all treatments within 9 days of the start of the experiment, and within a month all treatments had an equal number of outgrowing branches or buds, however this outgrowth was not from axillary buds such as those shown in Figure 4-3C and D. Even though there were no significant differences seen between treatments at any time point (with the exception of the undecapitated controls, Figure 4-3E), a possible suppression of outgrowth at the 19 day stage by GR24 suggested that further investigation might be warranted. The suppression of outgrowth by apical auxin seen in the split plate assay described above was not repeated. However, outgrowth only occurred from the area of the cut surface, not from previously-formed visible axillary buds on the main stem, suggesting that it was either being produced from preformed axillary bud primordia remaining from the incomplete decapitation of the apical bud, or arose from that tissue as a wound response. As a result of the outgrowth occurring directly from the cut surface, the lanolin applied was therefore also in direct contact with these outgrowing branches, so that the auxin would be supplied directly to the bud, not via the stem as intended, potentially confounding the results (Figure 4-3B). The source of the outgrowth was therefore investigated in order to provide information for the

redesign of the protocol.

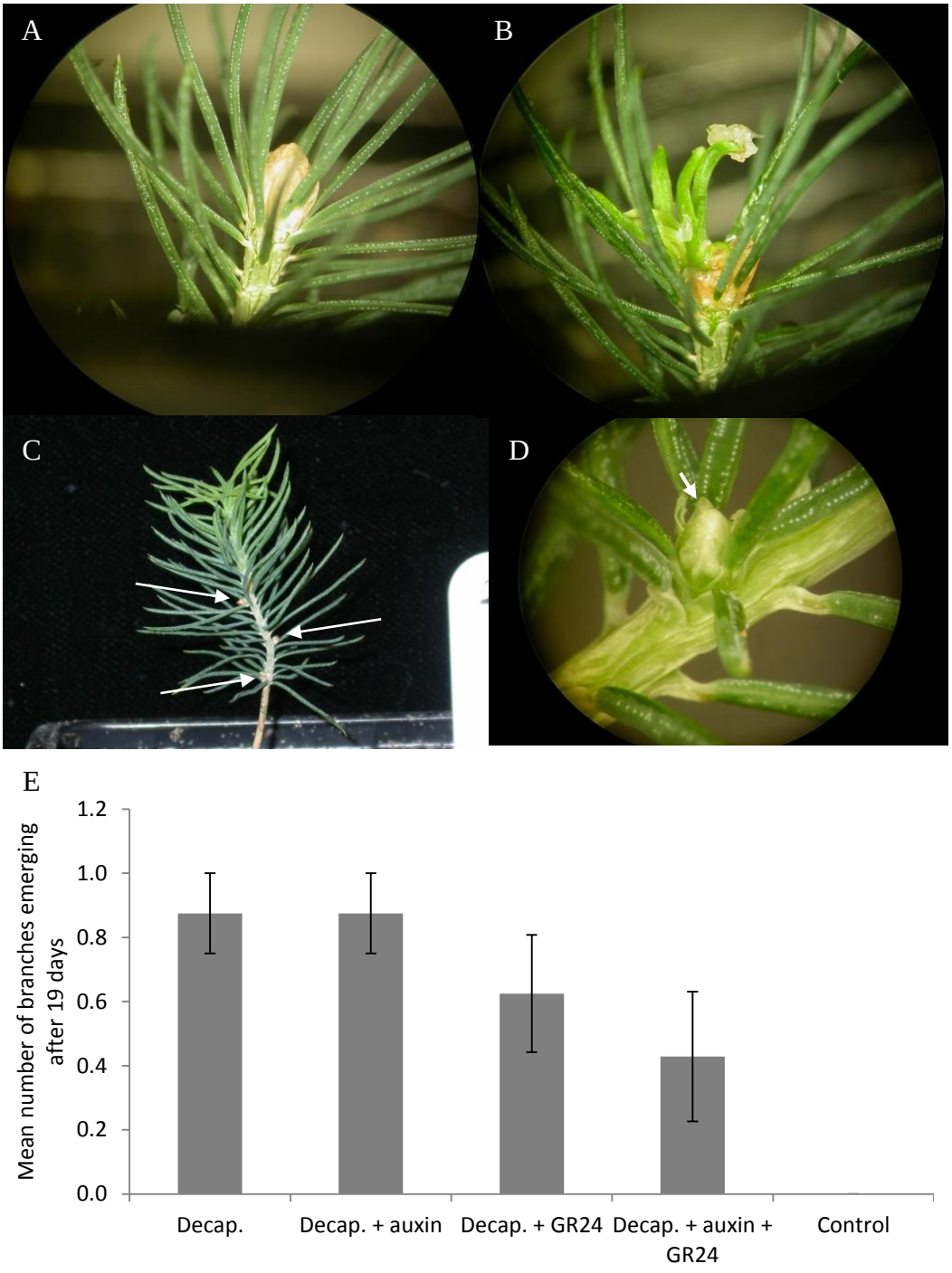


Figure 4-3. Decapitation experiment on white spruce. Branch buds in *Picea glauca* (white spruce). Plants were decapitated, cut surfaces treated with lanolin with or without 10mM IAA (auxin) as indicated. 5µM GR24 (or acetone carrier) was applied to the stem below the first needles every 2-3 days for one month, and plants were photographed at the same time for one month and sporadically thereafter. Controls were not decapitated, although new branch production was scored. A) & B) Dormant apical bud before and after decapitation, with B showing outgrowing branch with lanolin still adhering to the needles. C) & D) Axillary buds (arrows). E) Mean number of branches or new buds produced by 19 days after decapitation, the first point at which different branches could be

discerned from the general outgrowth and healing of the apical tissue and the only point at which means between different treatment showed much difference, although no differences were significant (ANOVA, P≤0.05) Error bars are standard error of the mean.

In order to identify the origin of the outgrowing branches in the decapitation experiment, and to judge whether they were derived from *ab initio* development from the meristematic apical cut surface or from invisibly small axillary buds close to the apex, actively growing plants were decapitated mid-stem for comparison. Two weeks post-decapitation, at which point almost all the apically decapitated plants had produced outgrowth, the actively growing plants showed no sign of new outgrowth close to the cut surface, nor from axils without visible buds close to the meristem, when inspected either by eye or when examined under a Scanning Electron Microscope (SEM, Figure 4-4A-D, n = 6 plants investigated).

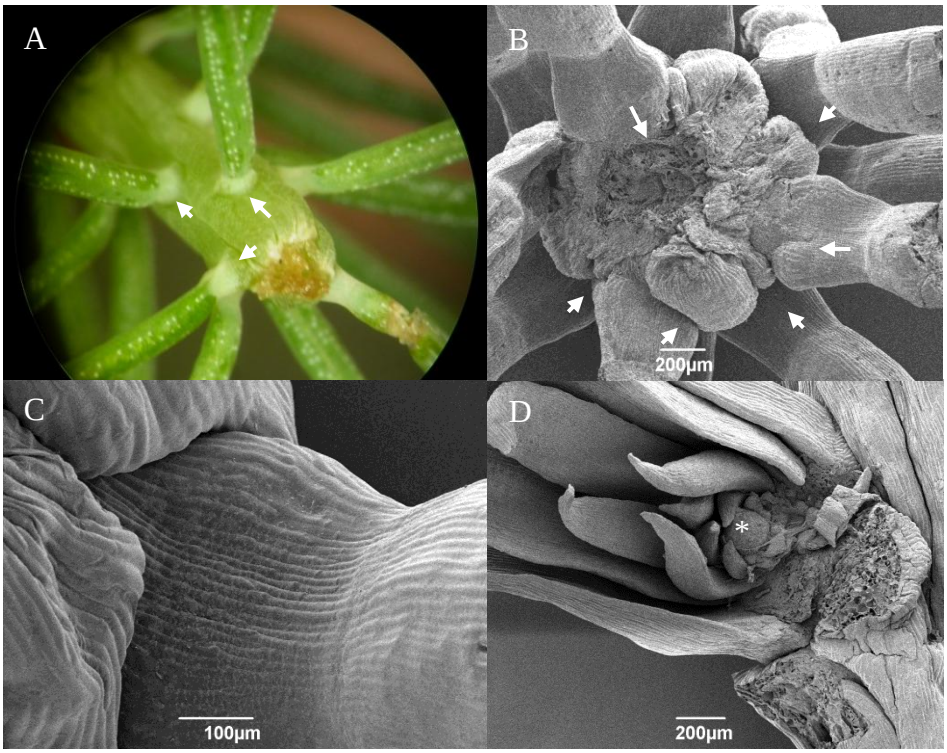


Figure 4-4. Axillary buds in actively growing white spruce that has been decapitated A) Apex, decapitated two weeks previously, under compound microscope, and B) a similar apex under SEM. Arrows show axils (all empty). C) Close up of an empty axil. D) Actively growing apical meristem (star), with some needles removed to reveal it.

This supported the hypothesis that the branches produced in the original decapitation experiment were either wound responses, or activation of axillary

meristems from nodes within the bud scale, or that nodes near the apical bud meristem have a different developmental potential than more mature nodes further down the stem. Thereafter, only plants that were actively growing or about to break dormancy were used for experimentation, as the decapitation response during active growth seemed more likely to be analogous to that of decapitation in annual angiosperm model plants. In addition, in further decapitation experiments efforts were made to decapitate the entirety of the apical bud, while balancing this with avoiding damage as much as possible to surrounding tissues.

4.1.2 Long term effects of SL application

Having determined that plants with dormant apical buds react in a different manner to those actively growing, to investigate the effects of GR24 on active growth an induction system was adapted from that of Little and MacDonald (2003) to synchronise the release of apical buds from dormancy. Seedlings were germinated and grown for 2 months in short day conditions, (although in the April replicate this was extended by one month) so that seedlings formed dormant apical buds immediately. Plants were then moved to long day conditions in the greenhouse to synchronise re-activation. At this point, a long-term experiment was employed to investigate the action of GR24 in the development of undamaged plants. By three weeks after induction almost all plants had reactivated, and from that point 100µl GR24 at 0, 1 or 10µM in 1% acetone was added to the soil at the base of the plant to encourage uptake by the roots (Figure 4-5A). The hormone concentrations were chosen to maximise the possibility of discerning an effect of the GR24. Treatments were applied and the plants were scored for a range of phenotypes once approximately every 8 days for 136 days, at which point in the first two replicates of the experiment, most plants had ceased active growth and formed dormant scaled apical buds. During the experiment several of the apical and (in some cases) axillary buds went dormant and then reactivated in ‘cycles’ of activity, sometimes more than once, although this varied a lot between plants and replicates. However, the ‘final’ dormancy at the 136 day time point was more-or-less collectively reached by

the plants of the first two replicates, with several of the plants having been dormant for several weeks.

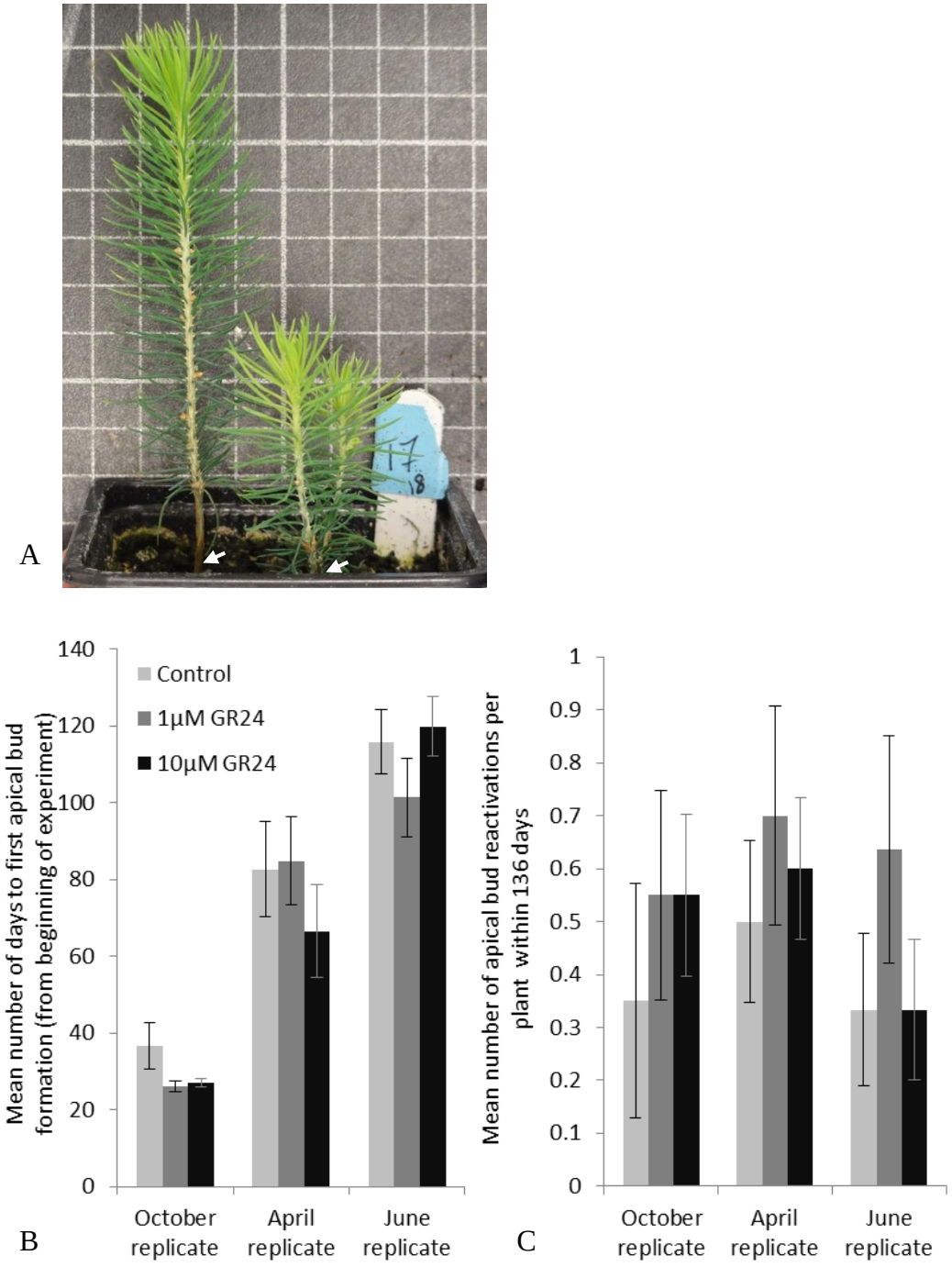


Figure 4-5. Long term GR24 dosing experiment: A) experimental set up, with a pot containing two plants, arrows showing point of hormone application. Plants were planted one or two to a single 8cm pot, with equal numbers of one- and two- plant (and in the April replicate, one three-plant pot each) per treatment. Grid behind is of 10mm squares. B) Time to the first time that the apex forms a dormant bud and C) the number of times that the dormant apical bud then reactivates over the experiment time period, across three replicates at different times of the year (labelled with month of

planting on soil). N = 19-20 for the October and April replicates, and 32-34 for the June replicate. Error bars are standard error of the mean.

In this experiment neither the addition of 1μM nor 10μM GR24 were sufficient to produce consistent or significant effects across three replicates, in either of the apical bud activity phenotypes measured (Figure 4-5B and C) or the axillary bud ones (Figure 4-6). Although the number of reactivations of the apical meristem (after dormant apical bud formation) was consistently greater with application of 1μM GR24 than on acetone application, this was never significant and was not consistently observed with 10μM GR24 (Figure 4-5C). Likewise, the October and April replicates hinted at the possibility of a promotion effect of 1μM GR24 on the length of time axillary branches spent in active growth, followed by a suppression effect at 10μM GR24, a pattern consistent with the action of some hormones, including that of GR24 in Arabidopsis root phenotypes (Ruyter-Spira *et al.*, 2011), but this pattern itself was reversed in the final replicate (Figure 4-6D). No consistent effects were seen in the number of dormant or active buds and branches produced, activated (by number or proportion – proportions not shown), or in when they first became active (Figure 4-6A-C). In addition in the final replicate the height of the main stem and the width of the stem base were measured at the end of the experiment, and again no clear effect of the hormone applications was evident (Figure 4-7).

Considerable variation between replicates was observed. Plants in the first two replicates generally had ceased active growth at least once by the end of the 136 days, whereas plants in the final June replicate took far longer to form dormant apical buds, although once dormant they were just as likely, on average, to reactivate (Figure 4-5B and C). Plants in the June replicate also produced considerably more axillary buds, of which a larger proportion activated and did so more quickly, contributing to the higher mean time spent active by axillary buds in this replicate than for the others, although the second replicate was also more active than the first (Figure 4-6). This increased degree of growth by the second and third replicates may reflect the time of year at which they were planted, as the April and June replicates were moved into the greenhouse in July and September respectively, whereas the first replicate was

induced at the end of December, so that growth for this replicate started at the coldest and shortest natural day length time of the year. Although the temperature in the greenhouse is controlled and the light period supplemented with artificial light, this does not completely disguise the seasonal changes, and plants may have responded to this by limiting their growth. Against these seasonal changes, no consistent effect of GR24 application could be discerned.

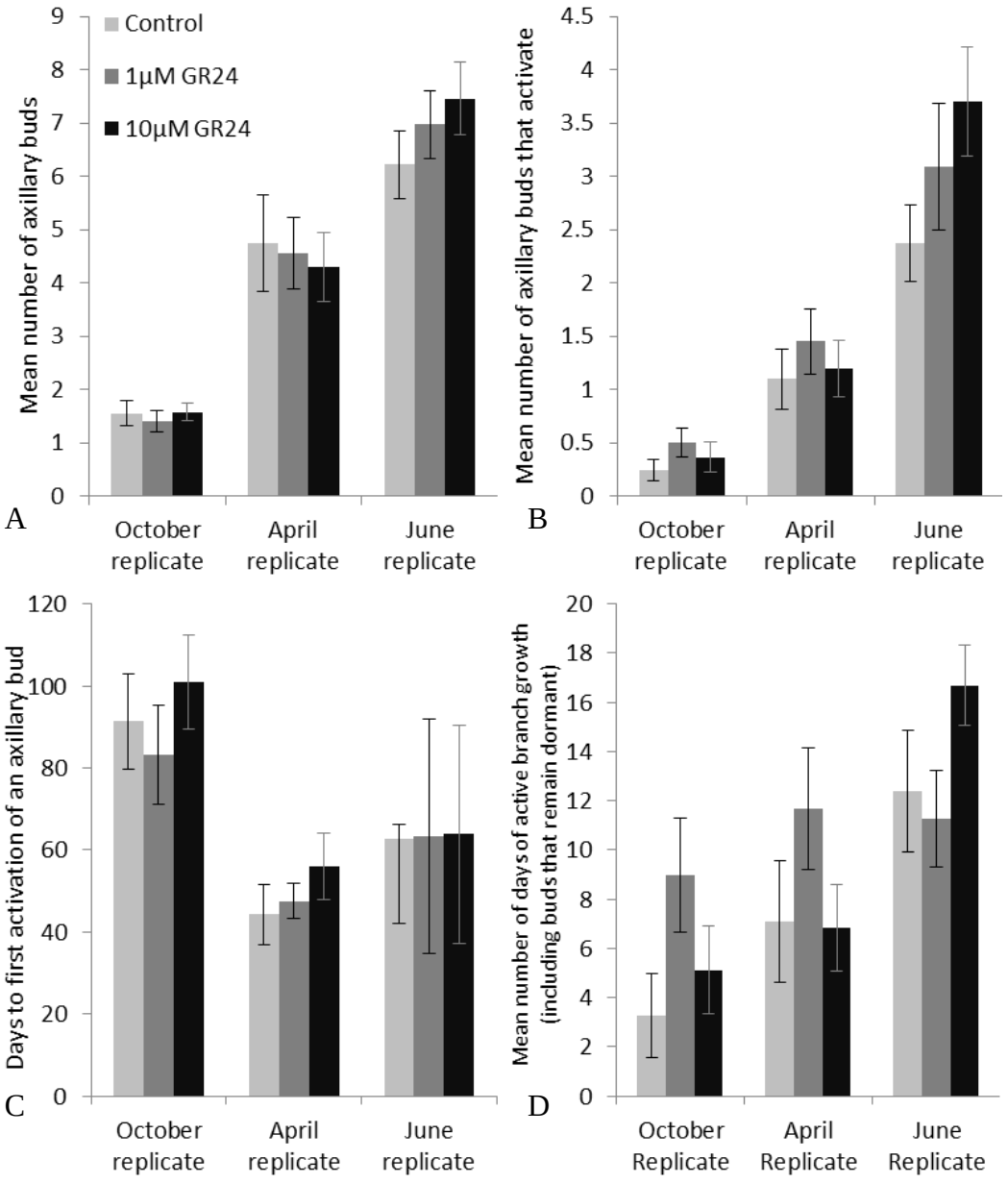


Figure 4-6. Long term GR24 dosing experiment: A) total number of visible axillary buds formed per plant, B) the number of those buds that activated during the experiment, C) time to the first time that an axillary bud activated for each plant, D) the amount of time each bud spent active during a single phase of activity per plant (averaged over several all cycles of activity per bud, where

applicable, with non-activating buds scored as zero). N = 19-20 for the October and April replicates, and 32-34 for the June replicate. Error bars are standard error of the mean.

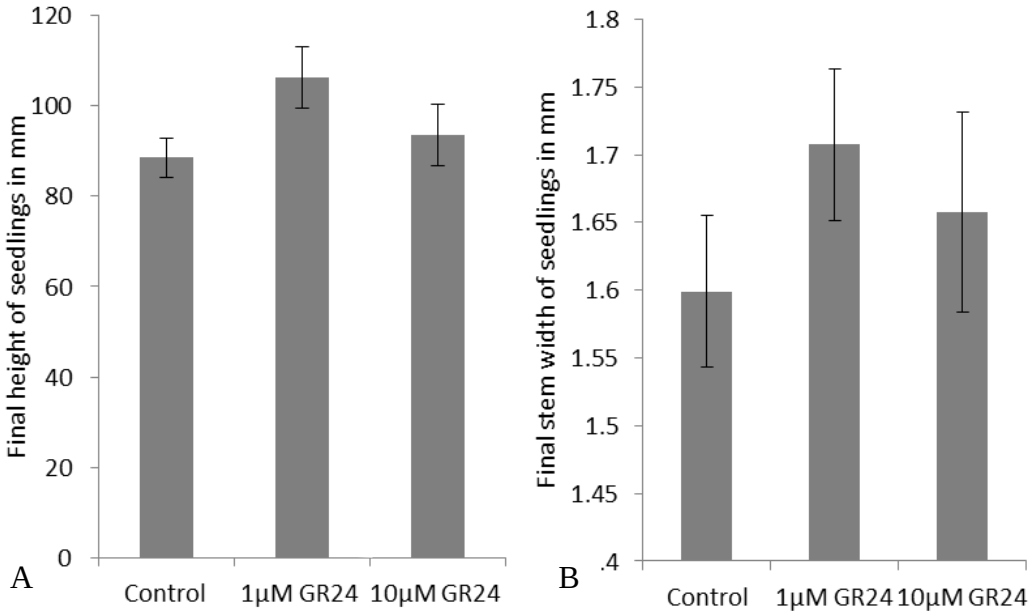


Figure 4-7. Long term GR24 dosing experiment: A) final height and B) stem width for the June replicate. N = 32-34. Error bars are standard error of the mean.

4.1.3 SL effects on dormant apical bud formation

As the long term experiment did not produce any consistent result of application of GR24, the induction system was adapted further to see whether in more environmentally-controlled conditions, slight effects of SL addition could be discerned. Firstly, the hypothesis that SL might be involved in the control of apical growth and particularly the development of dormant apical buds was investigated in more detail. The induction system used in the long-term experiment was employed, but instead of germinating plants in short day (8 hour light, at 15-20°C) to induce dormancy they were instead germinated in warm long day (16 hours light) conditions at 24°C. After one month, when all the plants were actively growing and some had produced visible (although not active) axillary buds, the plants were moved to short day conditions at 20°C to provide conditions conducive to the formation of dormant apical buds. At this point, and once a week thereafter, the plants were dosed as for the long-term experiment, although only with the control and 1µM GR24 concentrations to increase the sample numbers and improve the statistical power of the experiment. 1µM GR24 was chosen for its possible promotive effect on apical

bud reactivation in the first two replicates of the long-term experiment. The plants were also scored more frequently (three times a week) than for the long term experiment to increase the resolution of the timing information. However, even with these measures, no difference was seen in the time taken for the apex to form a dormant apical bud (Figure 4-8) demonstrating that at these concentrations and under these conditions, GR24 has no effect on the cessation of active growth in apical meristems in white spruce.

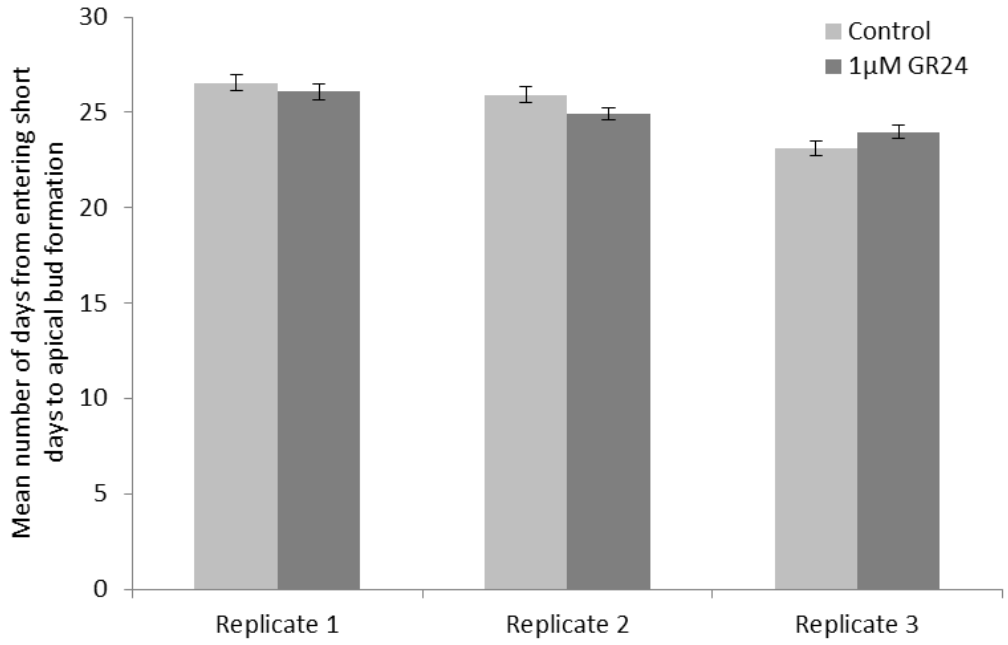


Figure 4-8. Short term apical dormancy experiment: Time taken for apical buds to form in spruce in short day conditions, when dosed with GR24, across three replicates. No differences were significant (Student's t-test). N= 35 for replicates 1 and 2, and N = 48 and 50 (acetone and 1µM GR24 respectively) for the third. Error bars are standard error of the mean.

4.1.4 SL effects on outgrowth after decapitation

Having established that there is no effect of GR24 on apical activity either over a season of growth or in dormancy-inducing conditions, further investigation was made on the effect of SLs in control of apical dominance and branch outgrowth, using the more controlled protocol from the short-term apical bud dormancy experiment. Plants from the control group of the short-term apical bud experiment were allowed to remain in short day conditions for a total of 131 days, and then returned to long day, warm (24°C) conditions. Within two weeks of movement to long day conditions 80% of the first replicate, 64% of

the second replicate and 33% of the third replicate had reactivated at the apex, and of the reactivating plants, some had also actively growing branches. At this point, plants were either decapitated or left whole, and dosed once a week with 5ml of 0μM (for control and decapitated plants) or 10μM GR24 (decapitated plants only) to each pot, to ensure delivery of the hormone to the roots. The time of bud break of each axillary bud was then measured over three weeks, by which time axillary buds on several plants (11 and 9 respectively) in the first two replicates had returned to dormancy, although only two plants had in the final replicate. No consistent pattern was seen in the time taken for axillary buds to break dormancy across all three replicates (Figure 4-9A). However, in the first and second replicates, a reduced percentage of axillary buds activated in the undecapitated plants compared to the mock-treated plants, as shown in Figure 4-9. This effect may be contributed to by the continued growth of the main stem in the control plants, which also produced more axillary buds, although several of these also activated. In the first replicate the application of GR24 appeared to attenuate the effect of decapitation, so that the GR24 plants were, although still statistically similar to the decapitated, mock-treated group, also statistically similar to the control group. Although the effect of decapitation was repeated in the second replicate, the GR24-associated effect was not, and in the final replicate no differences were seen in any treatment.

For these experiments, care was taken to decapitate below the bud scale, to reduce the incidence of the putative wound-induced outgrowth described above. Nonetheless, some outgrowth of this type was seen, which might have re-established apical dominance, confounding the effects of decapitation on more basal axillary buds. When those plants that showed apical outgrowth within 10 days of the start of the experiment were removed from the results, the patterns of outgrowth did not change in first or third replicate (Figure 4-9B), as only a few plants were affected, but in the second replicate, the pattern of response to GR24 from the first replicate reappears, although the sample sizes for this replicate are also then very low (only 5 and 4 for the two decapitated samples). The results from the first two replicates suggest that this experiment may be worth repeating with larger sample sizes (which were particularly low for this experiment due to lack of material), but from this data few conclusions can be

drawn for the effect of GR24 on axillary bud outgrowth after decapitation.

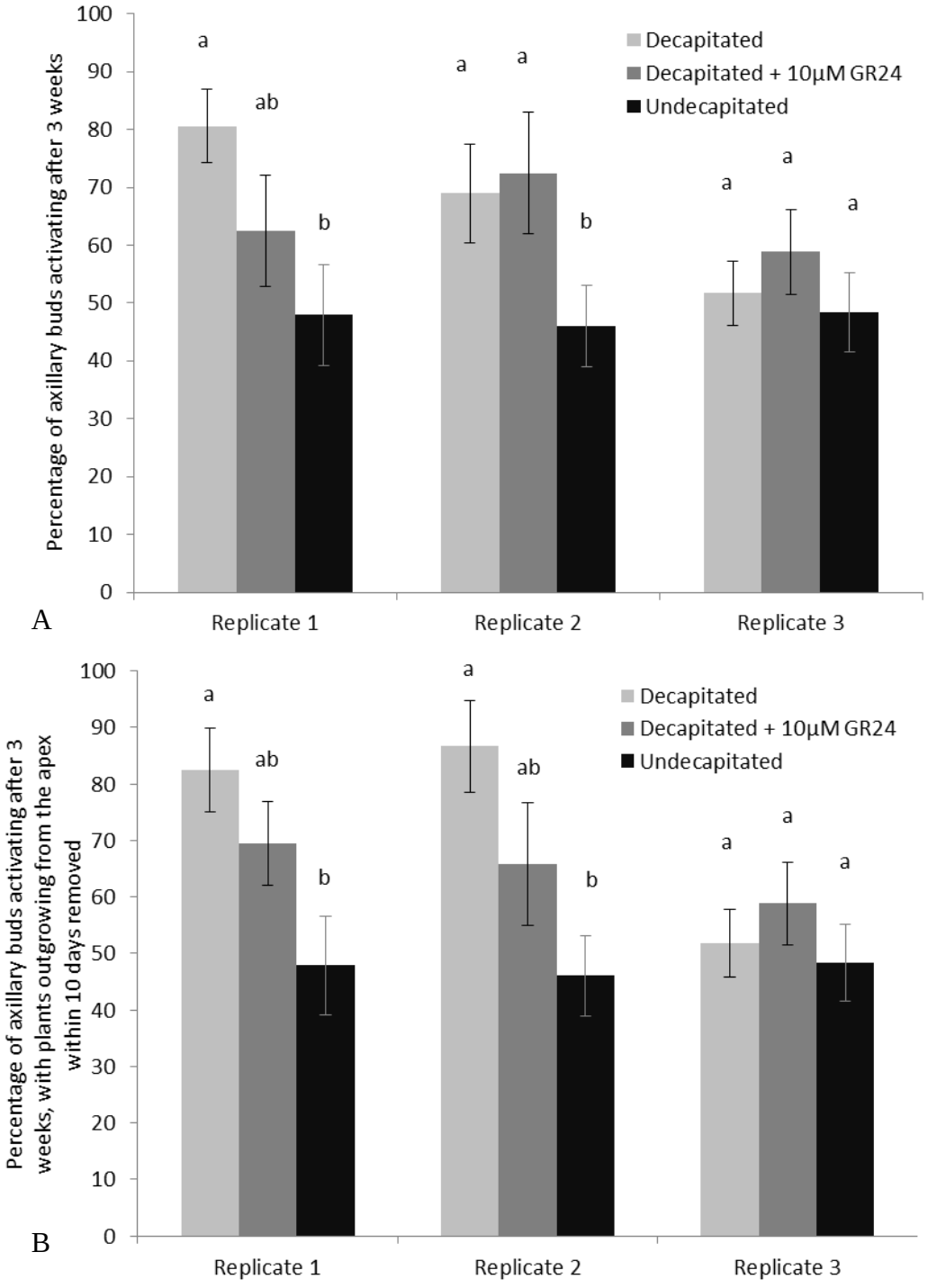


Figure 4-9. Percentage of axillary buds of white spruce activating within three weeks of decapitation of the apex. A) N=10-12 for replicate 1, n=11 for replicate 2 and n=14-17 for replicate 3. B) Same data as A, but with plants showing outgrowth from the apex within 10 days of decapitation removed. N=9, 9 and 12 for decapitated with acetone, decapitated with GR24, and undecapitated respectively for replicate 1, n=5, 4, and 11 for replicate 2 and n=16, 16 and 14 for replicate 3. Same letters indicate non-significance in an ANOVA using Tukey's HSD post hoc test, P≤0.05. Error bars show standard error of the mean.

4.1.5 SL genes and phosphate response

The conservation of SL signalling in response to nutrient limitation was investigated by analysis of gene expression. The upregulation of SL biosynthesis in response to limited phosphate availability has been reported for a number of species, including Arabidopsis, pea, rice, tomato, red clover, alfalfa, and wheat (Yoneyama *et al.*, 2007; Lopez-Raez *et al.*, 2008; Umehara *et al.*, 2010; Balzergue *et al.*, 2011; Jamil *et al.*, 2011; Kohlen *et al.*, 2011; Yoneyama *et al.*, 2012). In rice, this upregulation of synthesis was concomitant with upregulation of biosynthetic *MAX* gene orthologues, including three of the five *OsMAX1* orthologues (Umehara *et al.*, 2010). Upregulation of the petunia orthologue of *MAX4* on phosphate starvation has also been reported (Breuillin *et al.*, 2010). In order to establish whether spruce shared this response, quantitative (Q-) PCR was used to measure the effect of phosphate limitation and replacement on mRNA abundance for spruce orthologues of the *MAX* genes.

A phosphate-limited environment was created by growing seedlings on sand and terragreen, supplemented by addition of liquid half-strength Murashige & Skoog medium (1962) once a week. After 6 weeks, when seedlings were established and had started to produce visible axillary buds, the pots were washed three times with dH_2O and subsequently the KH_2PO_4 phosphate source in the medium was replaced with equivalent molar KCl. The plants were allowed to grow without phosphate for one week, and then leaf and root material was collected for analysis ('Day 0'). Phosphate was then added back to the medium, and after one week's growth on phosphate plants were again collected for analysis ('Day 7 Adding Pi'), along with plants that had remained on the no-phosphate treatment as a control group ('Day 7 No Pi'). Identification of *PgMAX1* is described in chapter 3 and spruce orthologues for *MAX2* and *MAX4* were identified from EST collections by reciprocal BLAST searches. The degree of expression of these genes was measured by Q-PCR, and normalised to the expression of two endogenous controls (*PgTUB* and *PgTIF-5α*) previously reported by Abbott *et al.* (2010) and El Kayal *et al.* (2011). Of the three *MAX* genes investigated, only *PgMAX4* was significantly affected by the treatment, and then only in the shoots. The plants remaining on low

phosphate had significantly lower expression of *PgMAX4* than those at the start of the experiment (Figure 4-10). *PgMAX4* was also expressed at significantly higher levels in roots compared to shoots to ($p \leq 0.001$ in Dunnett's T3 test). Despite the non-significance of most of the differences, the pattern of changes between times and treatments of *PgMAX1* and *PgMAX4* in shoots and roots were very similar, while that of *PgMAX2* was different and far less responsive in general (showing no difference between tissue types either). *PgMAX1* and *PgMAX4* both showed indications of downregulation in the roots after a week with phosphate resupply, whereas they did not show much change after the second week without a phosphate source, a pattern that would be consistent with a downregulation of SL production in roots seen in Arabidopsis and rice, and not unlike the pattern reported from rice by Umehara and co-workers in the rice biosynthetic genes orthologues (2008; 2010; Kohlen *et al.*, 2011). In contrast in shoots, both genes showed downregulation after 7 days under either treatment, but here it was the no-phosphate control that was lowest. *PgMAX2* showed very little change between treatments, times or tissues, consistent with the lack of response of the rice orthologue *D3* to changes in phosphate availability, and general ubiquity of expression of *MAX2* orthologues in several species (Johnson *et al.*, 2006; Stirnberg *et al.*, 2007; Umehara *et al.*, 2010; Drummond *et al.*, 2012). These results support the possibility that SL biosynthesis, but not signal transduction, is upregulated in response to phosphate starvation.

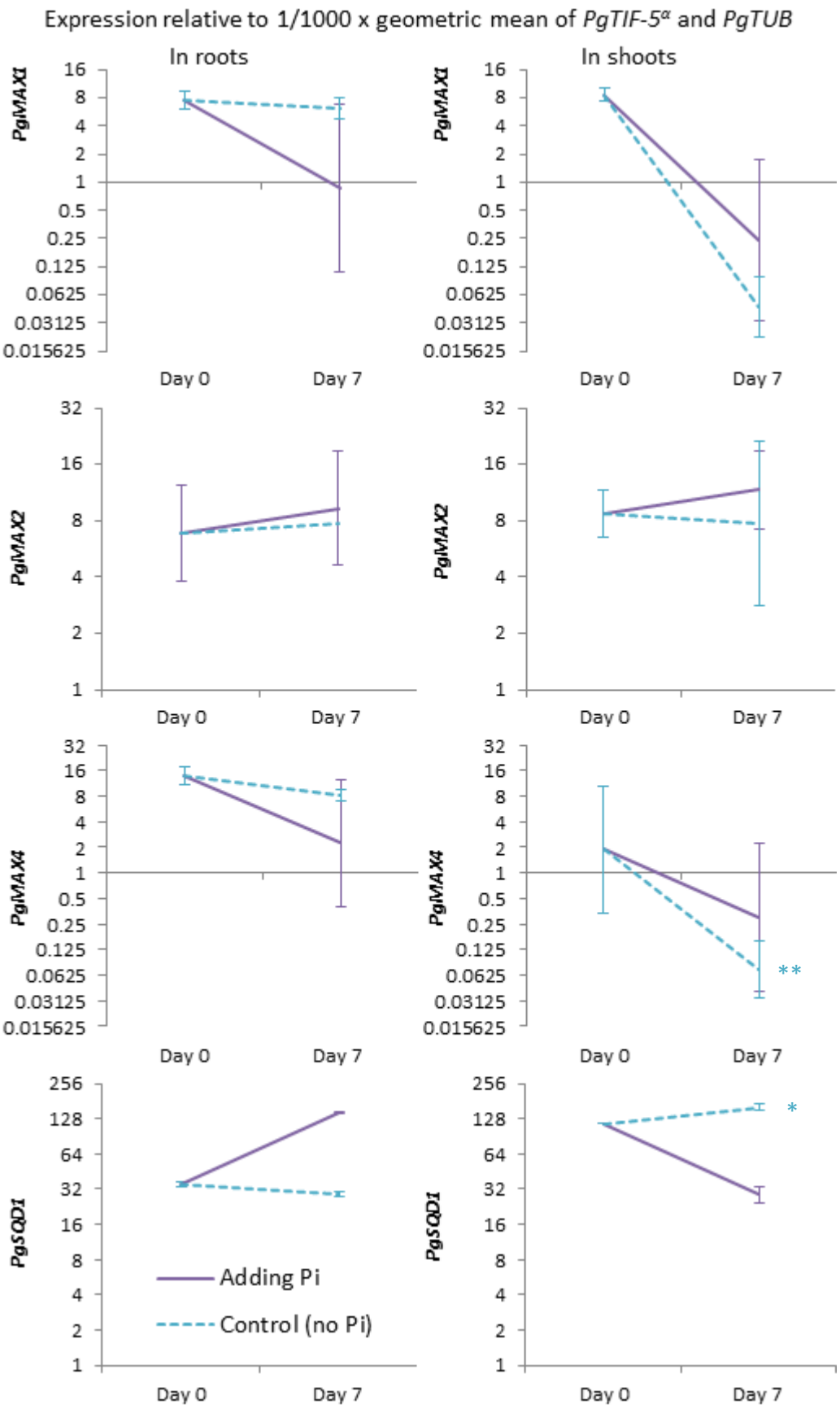


Figure 4-10. Response to limitation and re-addition of phosphate (Pi) in *PgMAX1*, *PgMAX2*, *PgMAX4* and *PgSQD1* gene expression, in roots or shoots (needles, stem and axillary buds). Plants

were starved of phosphate for one week, collected, and then either starved for a further week or had the phosphate source returned, and gene response measured. Expression of test genes is normalised to the geometric mean of two endogenous controls, and the data presented for the *PgMAX* genes are the mean of two biological replicates, each technically replicated 3 times. Data for *PgSQD1* are means of one biological replicate only. * = significant difference to Day 0 sample at P<0.001, ** = P<0.01 and * = P<0.05 in Dunnett's T3 post-hoc test, star colour indicating treatment. Y axis is in log_2, and error bars are standard error of the mean.**

As a positive control of phosphate starvation, for the second biological replicate, a fourth test gene was included – a spruce orthologue of *SQD1* (*At4g33030*). *SQD1* was identified in Arabidopsis as a potential 'smart' indicator gene for phosphate starvation, as it is upregulated specifically in response to withdrawal of phosphate (Hammond *et al.*, 2003). This upregulation is also conserved in the moss *Physcomitrella patens* (Wang *et al.*, 2008), so the potential spruce orthologue was identified by reciprocal BLAST searches and included to gauge the efficiency of the phosphate starvation treatment. Although based on only one biological replicate, unlike the other genes that were based on two, the *PgSQD1* gene did show slight, significant upregulation on the low phosphate treatment compared to the start of the experiment, and more significant upregulation (at p=0.003 in Dunnett's T3 post-hoc test) compared to the high phosphate treatment, although this upregulation only occurred in shoots. In roots, *PgSQD1* expression appeared to be down regulated, although the change was not significant. *AtSQD1* is required for sulpholipid biosynthesis in leaves, and is involved in response to reduced phosphate availability by supporting the replacement of phospholipids in thylakoid membranes of chloroplasts with sulpholipids (Essigmann *et al.*, 1998). Because of this leaf-biased role, the expression of *SQD1* in roots may not relate to plant phosphate status. In support of its leaf-based role, *PgSQD1* was expressed at significantly higher levels in shoots compared to roots (p=0.006, Dunnett's T3).

To investigate further and confirm the phosphate response, and investigate whether the addition of GR24 had a feedback effect on the expression of the *MAX3* and *MAX4* SL biosynthetic genes, as reported for Arabidopsis by Mashiguchi *et al.* (2009) a similar experiment was repeated, but with three changes intended to increase the degree of phosphate starvation (Figure 4-11). Instead of washing the substrate to reduce any adhering phosphate, plants were

moved to a new sand and terragreen mix in clean pots, and this move was done after only three weeks of growth. Plants were then grown on this mix without any phosphate added for 6 weeks before the first samples were taken. Phosphate was then resupplied to the plants as in the previous experiment, and samples taken for analysis the following week. In addition, in this experiment as well as phosphate, half of the plants were also treated with 1μM GR24. As the seedling mortality rate in two replicates of this experiment was quite high, the limited number of plants meant that no-phosphate Day 7 samples were excluded from the first replicate, (the second replicate was lost entirely) so that data for these conditions only derives from a single biological replicate (the third).

In three of the four conditions tested after seven days both *PgMAX1* and *PgMAX4* showed significant upregulation in roots, repeating their pattern of shared expression from the previous experiment (Figure 4-11). Curiously, this upregulation occurred on both the no-phosphate conditions – with or without GR24 – but also when both GR24 and phosphate were added. The only condition with no significant upregulation was that to which phosphate only had been added. Upregulation in a situation which in theory has not changed (other than that plants had gone from six weeks to seven without phosphate) was unexpected - the expected result would be steady-state on no-phosphate and downregulation on sufficient phosphate. However the lack of upregulation in the 'Adding Pi' sample does suggest that this is a phosphate-limitation response, not the inverse, which would be upregulation of the 'Adding Pi' sample when compared to the 'No Pi' control. It may be that the plants had reached a threshold at this age at which phosphate starvation had become acute, causing upregulation of responses. The *PgSQD1* phosphate marker results support this, as *PgSQD1* does not show a consistent change in roots and while it does show a non-significant upregulation on phosphate addition in shoots, akin to the results for *PgMAX1* and *PgMAX4*, *PgSQD1* was even more upregulated (and significantly so) in shoots on the no-phosphate treatments.

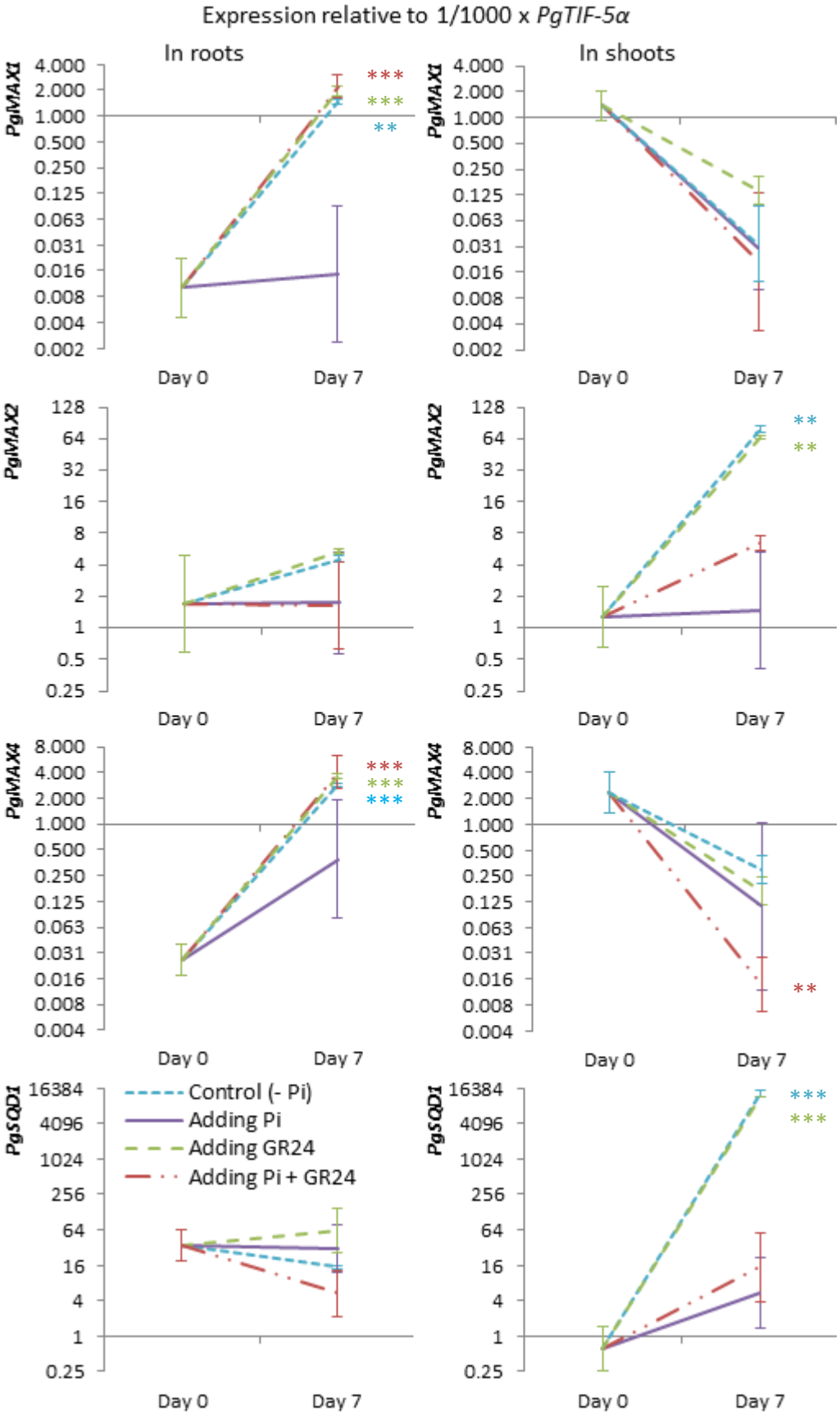


Figure 4-11. Response to limitation and re-addition of phosphate and addition of GR24 in *PgMAX1*, *PgMAX2*, *PgMAX4* and *PgSQD1* gene expression, in roots or shoots (needles, stem and axillary

buds). Plants were starved of phosphate for six weeks, samples taken at Day 0, and remaining plants were then either starved for a further week or had the phosphate source returned, as well as being dosed with 1µM GR24 or the acetone control, and gene response measured. Expression of test genes is normalised to the expression of *PgTIF-5α*. The data presented for all genes for the Day 0 and Added Pi samples (except the Added Pi + GR24 sample in shoots) are the means of two biological replicates, whereas the Added Pi + GR24 shoot and all No-Pi controls (with or without GR24) are a single biological replicate. Each sample was technically replicated 3 times. * = significant difference to Day 0 sample at P<0.001, ** = P<0.01 and * = P<0.05 in Dunnett's T3 post-hoc test, star colour indicating treatment. Y axis is in $\log_2$, and error bars are standard error of the mean.**

The response of *PgMAX1* and *PgMAX4* in shoots is similar to that from the previous experiment, showing downregulation on high-phosphate compared to the Day 0 control, even though this was only significant in the 'Adding Pi and GR24' treatment for *PgMAX4*, whereas in the previous experiment it was the 'No Pi' sample that was significantly downregulated. In this experiment, *PgMAX1* and *PgMAX4* also show higher levels of expression in shoots at the start of the experiment, while *PgSQD1* is expressed at lower levels in shoots compared to roots (and significantly different to p=0.001 in a Dunnett's T3 post-hoc test). Interestingly, the relationships between the high phosphate-and-GR24 treatment and the high-phosphate only data points for *PgMAX4* and *PgMAX1* are very similar to those for the no-phosphate controls (which show no difference of adding GR24). GR24 had been hypothesised to feedback to down-regulate the expression of SL biosynthetic genes, so this apparent mimicking of the phosphate-starvation response is surprising. The responses of *PgMAX2* in the roots, while again not significant, appeared to show more variation between high and low phosphate treatments than in the previous experiment. However, interestingly *PgMAX2* this time does show a response in the shoots, and in a very similar pattern to that of *PgSQD1*, being upregulated in the continued absence of phosphate. As a part of the signal transduction pathway this might be expected, if SL signalling to the shoot is important in phosphate regulation in spruce, as it would presumably increase the sensitivity of the shoots to SLs produced in response to phosphate stress, although as with all the data for the 'No Pi' samples this only based on one biological replicate.

4.2 Moniliphytes (ferns) - Ceratopteris richardii

The leptosporangiate ferns make up approximately ~80% of all non-flowering vascular plant species, having undergone a radiation shortly after (and possibly causally linked to) the angiosperm radiation (Schuettpelz and Pryer, 2009). However, relatively little developmental research effort is currently entrained on the wildly diverse fern taxon, not least because it contains very few species of much economic interest, although historically the ferns have been well studied as models of plant shoot development (and the number of fern examples used by Steeves and Sussex, 1989, gives some idea of this; White and Turner, 1995). As a result, the relationship of different fern organs to those in the angiosperms (whether analogous *et cetera*) is still being elucidated, as is the homology of the molecular events controlling their development (Sano *et al.*, 2005; for example, see Sanders *et al.*, 2011). In terms of their shoot morphology, leptosporangiate ferns have at least one axis of growth that may branch from preformed buds, the outgrowth of which in some species, although not all, is governed by auxin-regulated apical dominance (Croxdale, 1976; Pilate *et al.*, 1989; and reviewed in White and Turner, 1995). However, in some species shoots divide dichotomously, and a very few species branch both dichotomously and laterally (Imaichi, 2008). From the main axis and branches multiveined fronds are produced that are sometimes equated with angiosperm compound leaves, yet share indeterminate, iterative development with angiosperm shoots (Bierhorst, 1971; Bell and Hemsley, 2000; Sanders *et al.*, 2011). The question of whether SL signalling is conserved in ferns was of interest in part because of this different body plan, which has been so evolutionarily successful, if such success can be measured in terms of extant species number or variety of ecological niches occupied.

4.2.1 Experimental species and gene search

As a representative of the ferns, *Ceratopteris richardii*, or c-fern, was chosen for experimentation because it is has emerged, along with *Adiantum capillus-veneris*, as a model for the development of the polypod ferns, with a short lifecycle, a range of mutants available (especially for the study of gametophyte development) and easy growth both in axenic conditions and on

soil (Hickok *et al.*, 1995; Banks, 1999; Chatterjee and Roux, 2000). A BLAST search for fern orthologues of *MAX1, MAX2* and *MAX4* produced one incomplete, putative, EST in *C. richardii* of *MAX2* and another incomplete 5'EST of *MAX1* in *A. capillus-veneris*, but degenerate primers against *C. richardii MAX1* based on the sequence from *A. capillus-veneris* and on other species drew no results, ruling out the possibility of investigating *MAX* biosynthetic gene function directly in this species.

4.2.2 Responses to phosphate limitation

As fern development is very different to that of angiosperms, and little is known about the role of auxin in apical dominance in ferns, identification of shared shoot-developmental modules for investigation was more difficult than for white spruce. Therefore, the putatively evolutionarily ancient role of nutrient-limitation sensing was used as a start point for investigation. Specifically, phosphate limitation responses were used as indication of potential strigolactone-related phenotypes. A test experiment was designed to investigate the effects of phosphate reduction on ferns, and in a parallel experiment the effects of addition of GR24 were measured. To this end, spores were cultured on plates for a one month to generate gametophytes and subsequently sporophytes. When sporophytes had produced approximately five sporophylls (or fronds), they were transferred to liquid media designed for c-fern culture (Hickok and Warne, 1998), and grown for a further four weeks. They were then transferred to a liquid culture containing either the same amount of phosphate as previously, or without any phosphate for 28 days (again, replacing KH_2PO_4 with equivalent molar of KCl). For the GR24 experiment, the same protocol was followed, but maintaining the phosphate concentration and adding GR24 at a range of different concentrations. Depriving the ferns of phosphate unsurprisingly produced fairly dramatic effects – as well as the phenotypes shown in Figure 4-12, the roots had turned from off-white to black in the no-phosphate treatment and plants were visibly smaller. The phenotypes measured in Figure 4-12 were selected either as being likely to show an effect of growth limitation (sporophyll size and number) or because they had a comparable phosphate-limitation effect known in angiosperms (e.g. increased senescence, reduced root length).

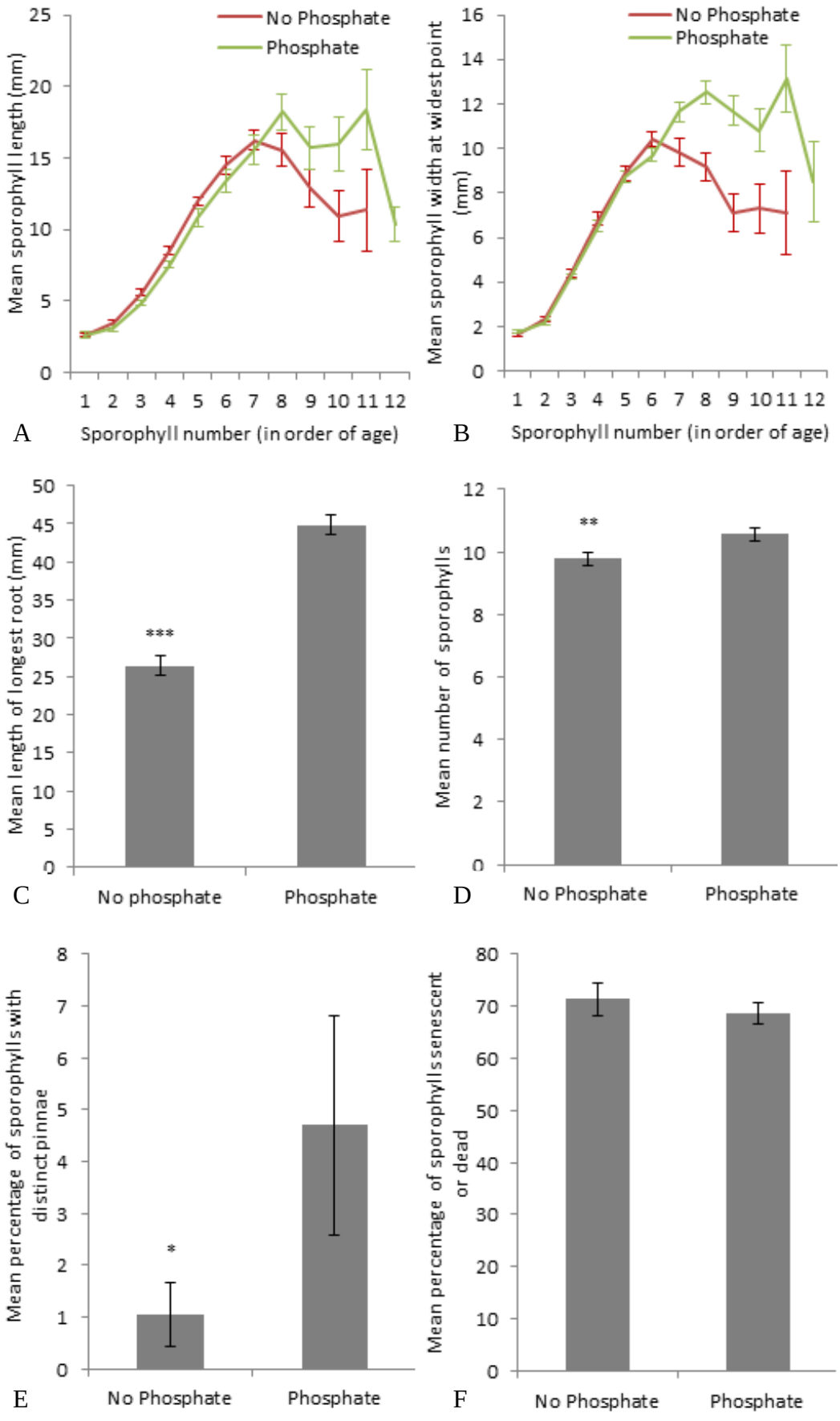


Figure 4-12. Phenotypes of c-fern grown with and without phosphate for 28 days. N = 18 plants. A)

and B) length and width at widest point of sporophylls along a development sequence from the oldest (1) to youngest (~12) sporophyll. C) length of longest root, D) total number of sporophylls produced, E) percentage of sporophylls with distinct pinnae (defined here as the presence of serrations in the leaves with an acute angle) and F) percentage of leaves senescent (yellow) or dead. C-F, mean averages were tested with Student's t-test for unequal variances, and significant differences on no-phosphate indicated by * = P≤0.05, ** = P≤0.01, *** = P≤0.001. Error bars are standard error of the mean.

Root length (measured as the length of the longest individual root, as roots in c-fern are produced from along the shoot below the bases of the sporophylls) was the most clearly affected phenotype, although the number of roots produced was not affected (data not shown). In addition, the total number of sporophylls produced was also slightly decreased, as was the percentage of those sporophylls that had reached the point of producing clearly defined pinnae. The sporophylls produced by c-fern start as undivided flat leaf-like organs with no clear midrib or rachis, but from the eighth leaf become progressively more serrated, until two to three individual pinnae become identifiable (at around the 9^{th}-12^{th} sporophyll in this study). In the angles of the pinnae indentations (the 'sinuses') vegetative buds are produced that can grow to produce entire new plantlets, although no more than one or two of these were seen in this experiment (Hou and Hill, 2002). In older and larger leaves more pinnae are produced, themselves bearing pinnules, so that reproductive sporophylls are highly and iteratively 'branched' Figure 4-13, (Hill, 2001). Possibly as a result of the reduction in total leaf number, fewer such divided sporophylls were found on phosphate-limited plants. However, as the phosphate experiment was an exploratory one and was only carried out once, all data are shown for a single replicate and would need repetition.

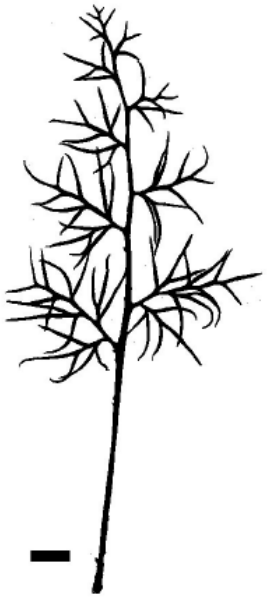


Figure 4-13. Adult reproductive sporophyll of *C. richardii*, showing ramifying iterative pinnules. Reproduced from Hill (2001). Scale bar = 1 cm.

4.2.3 Response to GR24

Having identified root length, sporophyll number and size measurements and the percentage of pinnate-sporophylls as being Pi-responsive, these were measured in two replicates of treatment with GR24, using the same length of time and protocol as the low-phosphate experiment. The size parameters measured for the sporophylls showed no trend of response to GR24 in the first replicate, so were only measured once (Figure 4-14). However, as hypothesized from the phosphate experiment, GR24 did appear, at high concentrations, to have an effect on root length, although much less than the effect of withdrawing the phosphate supply. Addition of 10μM GR24 decreased the length of the longest root significantly (P=0.05 in Student's t-test, borderline significance) in the first replicate by 6.1mm on average compared to the mock treatment control, a reduction of approximately 12%. Lesser concentrations also appeared to have a slight effect. This response was much less than that of the response to phosphate (a 41% reduction).

On the second replicate (Figure 4-14), although the trend of reduction in length on GR24 was repeated by a similar amount (5.7 mm on average) the difference was not significant and the trend was not repeated in the lower concentrations, which were actually longer than the control. Similarly, a (non-significant) trend in reduction of percentage of pinnate sporophylls on 1μM GR24 and 10μM GR24 in the first replicate was replaced, like the root response, by promotion at lower concentrations in the second, although there may still be some reduction on the highest concentration. Unlike for the phosphate treatment, total sporophyll number was not reduced in either replicate, so any reduction in number of divided sporophylls would, if real, be a GR24 specific effect. Larger sample sizes and further replicates might give better resolution and would confirm or refute these trends.

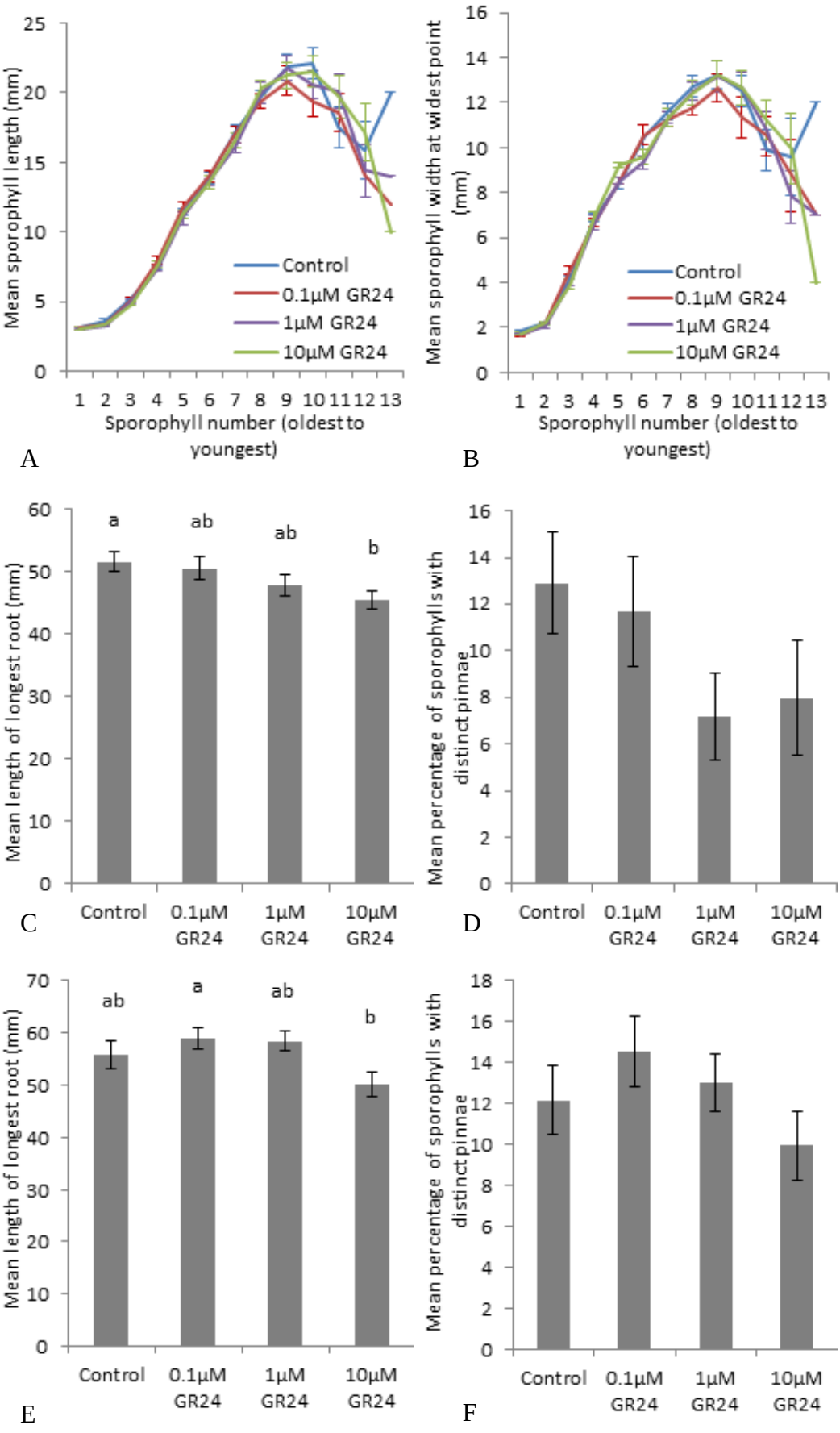


Figure 4-14. Phenotypes of c-fern grown on GR24 or its acetone carrier for 28 days. N = 34-35

plants. A-D) Replicate 1, E- F) replicate 2. A) length of sporophylls, B) width of sporophylls, not replicated as showing no difference or trend. C) and E) length of longest root, D) and F) percentage of sporophylls with distinct pinnae. B-F) Mean averages were tested by ANOVA with Tukey's HSD post-hoc test. Shared letters indicate no significant differences at P≤0.05. Letters not shown for B) and D) as ANOVA results did not reject the no-difference null hypothesis. Error bars are standard error of the mean.

4.3 Lycopodiophytes - Selaginella kraussiana

The extant lycopodiophytes are represented by only six genera, each in their own order – the Selaginales, Isoetales and Lycopodiales. They have leaves of the 'microphyll' type – containing a single, unbranched vascular trace, which are often small and 'stem-hugging', with the long but thin leaves of Isoetes as the exception (Bell and Hemsley, 2000). Microphylls are one instance (and perhaps the first) of several independent evolutions of leaf-like structures, at least three of which share underlying molecular modules controlling their development (Harrison *et al.*, 2005; reviewed in Tomescu, 2009). Although lycopodiophytes are generally described as branching dichotomously (i.e. by equal or unequal division of the growing shoot tip) some members of the Lycopodiales do form branches from lateral meristems, and only one of the *Isoetes spp.* actually branches at all (Bell and Hemsley, 2000; Imaichi, 2008). Selaginella species do branch dichotomously, splitting growth at the apex in two (and in *S. kraussiana* at least does so in a highly predictable manner every six or eight pairs of leaves, Harrison *et al.*, 2007) many species of Selaginella have been shown to have dormant, lateral meristems. These 'angle meristems' are placed at the branch points and usually grow out to produce organs called rhizophores. Although themselves derived from shoots, rhizophores are geotropic and produce root-like organs when they reach the soil, and their relationship to angiosperm roots or shoots has been the subject of much debate (reviewed by Webster, 1992). Recent reports of the expression of shoot meristem marker *KNOX* genes in angle meristems strongly support the growing consensus that the rhizophore is an adapted shoot (Kawai *et al.*, 2010). This interpretation was previously supported by the fact that the angle meristems in some species routinely develop into shoots, and even those that normally develop into rhizophores under certain conditions (particularly loss of the

growing apex) may become specified as branches. Decapitation, inhibitor and hormone addition studies have indicated that the deciding factor for angle meristem fate and outgrowth speed is apical auxin supply (Webster, 1969; Wochok and Sussex, 1973; Wochok and Sussex, 1975). Angle meristems are maintained in a dormant state in part by auxin transported from the apex, and those meristems supplied with high auxin levels specify as rhizophores, whereas those in which auxin supply is reduced, either due to natural differences arising in development (such as the proximity of the apex or vascular traces), removal of the growing apex or experimental intervention (e.g. auxin transport inhibitors) develop as shoots (Webster, 1969; Wochok and Sussex, 1973; Wochok and Sussex, 1975; Jernstedt *et al.*, 1994). The axillary position of this meristem and its delayed outgrowth under the control of apical auxin, led to the hypothesis that this meristem bears developmental and evolutionary similarity to that of the angiosperm axillary meristem. Indeed, even though these meristems are situated in the branch axils rather than the stem-leaf axils, the origin of seed plant leaves is proposed to be from the planation and webbing of dichotomously branched axes (Zimmerman's Telome theory, reviewed in Willis and McElwain, 2002; Beerling and Fleming, 2007). The branching in *S. kraussiana* is unequal, so that when the shoot apex splits, one branch will have two vascular traces and produce four leaves before branching, whereas the minor branch will only have one vascular trace and produce three leaves before dividing (Harrison *et al.*, 2007). This minor branch might have some similarity to the 'overtopped' branch, which corresponds to the leaf, of the Telome theory. Given this hypothesis, the possibility that outgrowth in Selaginella dormant meristems may, like angiosperm branches, be partly under the control of SL signalling was investigated.

4.3.1 Initial studies and protocol development

To select an experimental subject for the effects of SLs, a number of different *Selaginella* spp. were examined for experimentation, including *S. wildenowii* and *S. martensii*, both previous models for branching experiments (Wochok and Sussex, 1975; e.g. Jernstedt *et al.*, 1994), *S. uncinata*, a model for stomatal development ((Ruszala *et al.*, 2011), and *S. moellendorffii*, the sequenced species (Banks *et al.*, 2011). Although *S. moellendorffii* had the

distinct advantage of genomic information available, which would have allowed investigation of endogenous expression of orthologues as for the spruce, this species did not grow reliably in any of the conditions tried. Instead, *S. kraussiana* was chosen for its easy care, quick propagation from cuttings and well-described developmental pattern, similar to that of *S. wildenowii* but smaller and with a much faster rate of growth.

Initial studies focused, like those on c-fern, on developing experimental protocols and establishing phenotypes that might be affected by SL application, although in the case of Selaginella this was done directly, without investigation of the phosphate limitation responses. Instead, investigation initially focussed on the hypothesis that SLs may be acting in a similar manner to their *modus operandi* in angiosperms. SLs have been shown to decrease polar auxin transport in the stems of Arabidopsis, by reduction of PIN auxin efflux proteins at the basal membrane of cells, and this restriction of transport contributes to its increase of the competition between axillary buds and reduction of their outgrowth (Bennett *et al.*, 2006; Prusinkiewicz *et al.*, 2009; Crawford *et al.*, 2010). Selaginella does have conserved PIN orthologues (Křeček *et al.*, 2009) and polar auxin transport associated with the vasculature (Wochok and Sussex, 1973), and there is some circumstantial evidence that in the case of the rhizophore this vasculature may be developmentally related to auxin canalisation. This evidence comes from the report of a distinct file of cells between the angle meristem and the vascular strands of the minor shoot before differentiation of the vascular strand – connection to the minor shoot in particular would be expected if auxin sink strength is implicated in the patterning of vascular strands in Selaginella (Webster and Steeves, 1964). If the action of SLs on auxin transport were conserved in Selaginella SL application might be expected to dampen auxin transport, affect the activity and influence the identity of rhizophores and promote the formation of angle shoots. To this end, for the initial experiment, explants of Selaginella were cut from plants grown on soil, surface sterilised and grown on agar plates containing a medium adapted from that used for the ferns and two different concentrations of GR24 as well as the control. These explants were of course dichotomously branched,

so explants were chosen which had one expanded branch point or ‘node’ (see Figure 4-15 for explanation of the terms used here).

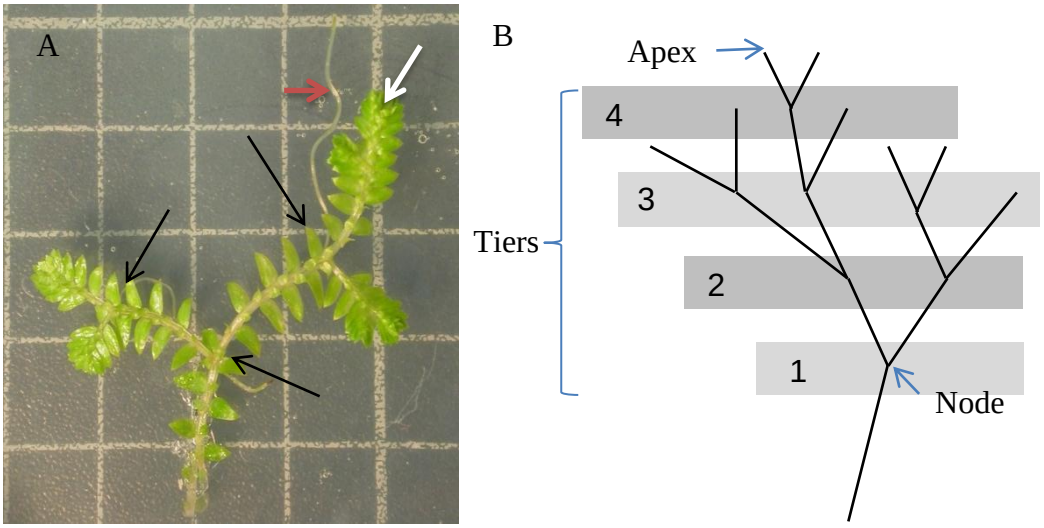


Figure 4-15. A) Selaginella explant with black arrows pointing to ‘expanded’ branch points – nodes - with stems surrounding it expanded, and white arrow showing younger, unexpanded node. All nodes have rhizophores, some of the longer of which are visible here (e.g. red arrow). Grid of 10mm squares. (B) Diagram showing successive levels of dichotomous branching of Selaginella, here referred to as ‘tiers’. Unbranched tips are referred to apices, as labelled.

The plants were grown for 3 months on three GR24 concentrations, transferring to new medium occasionally, during which time the number of apices (branch tips) were counted (Figure 4-16A) and inspected for the formation of shoots instead of rhizophores from the angle meristems. However, the formation of shoots were not observed in any plants, nor on plant material grown on soil**.** At the end of the experiment, plants were weighed and rhizophore and total explant lengths were measured. Although none of the phenotypes differed significantly in this experiment (small sample numbers were used) three phenotypes showed sufficient difference for further investigation – the number of dichotomous branch points (nodes), the length of the rhizophores and the final weight of the explants (Figure 4-16A, C and D).

The number of nodes showed some evidence of reduction on GR24, perhaps consistent with the shoot growth restriction phenotypes of GR24 and reduction in branching in angiosperms. The length of rhizophores, instead of being restricted by GR24 as ‘dormant axillary meristems’, actually seemed to be promoted on high levels of GR24, perhaps consistent with a nutrient foraging

strategy. The weight of the explants was in line with the dichotomous branching data, suggesting a general restriction in growth on both concentrations of GR24.

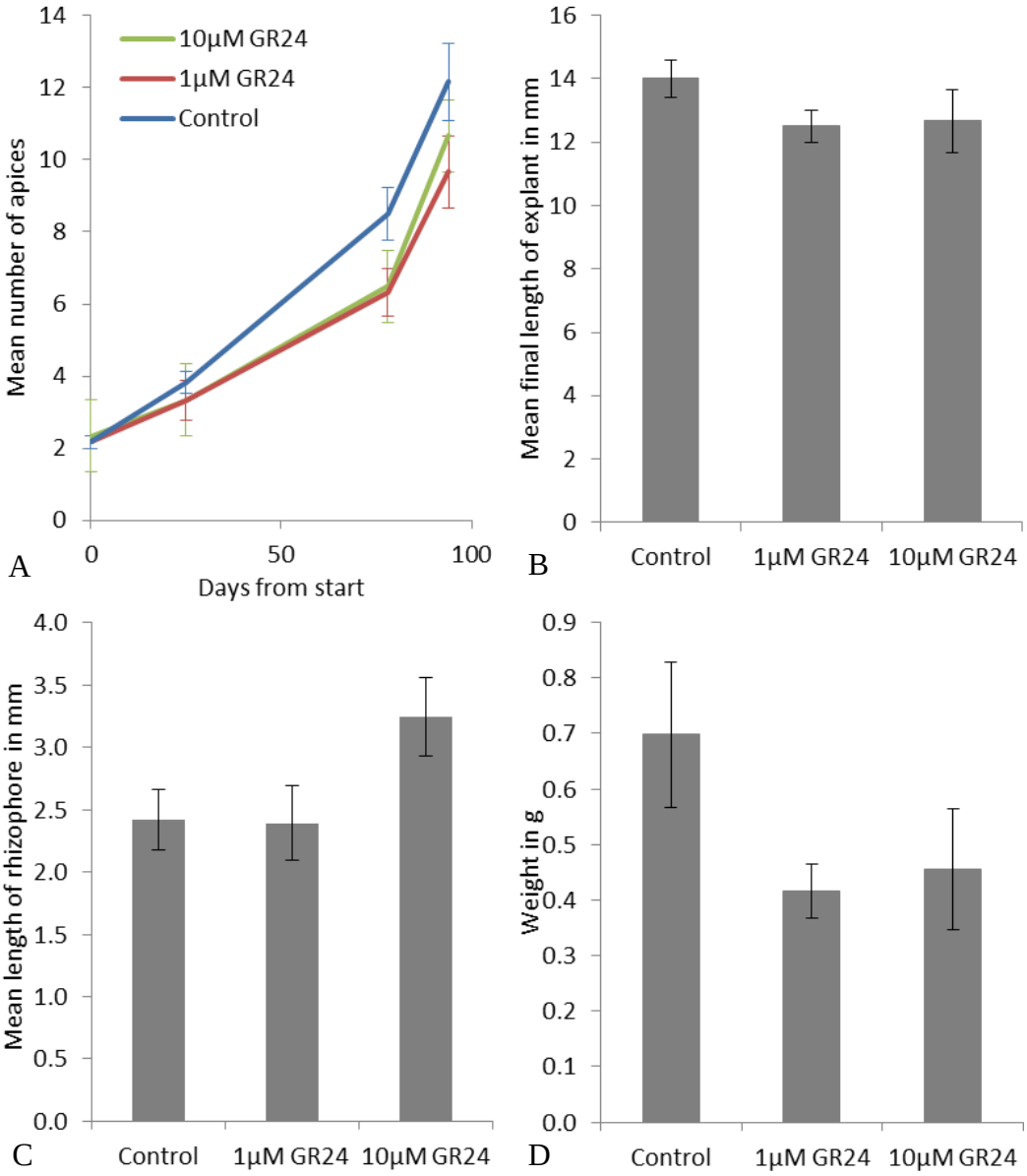


Figure 4-16. Branching and phenotypes of *Selaginella kraussiana* grown on media containing GR24 for 3 months, n=6. A) Number of apices (branch ends) counted at different times over the experiment. B) Length of explants at the end of the experiment, measures from base to longest branch tip. C) Mean length of all rhizophores visible to the naked eye. D) Weight in grams of explants (fresh weight). All phenotypes tested with ANOVA, no significance between any treatment found.

4.3.2 Branching and rhizophore length response to decapitation

To explore further the possibility that the rhizophore meristem is analogous to the seed plant axillary meristem, and that SLs may be acting in a similar manner to their *modus operandi* in angiosperms, a decapitation assay was attempted. This was done to promote the formation of shoots from angle meristems, and to see if this was reduced or further enhanced by GR24 application. In addition, where rhizophores were formed, the assay would allow investigation of whether their outgrowth was delayed by a growth restriction effect of GR24, or as suggested by the initial experiment, promoted. The initial explant protocol was adapted to use more plants, although this time explants were cut with two expanded tiers (like that in Figure 4-15A), and explants were kept on mock treatment or 1µM GR24 for only three weeks. At the start of the experiment explants were either left whole or decapitated by removal of the major branches above the youngest nodes discernible under a dissecting microscope (as shown in Figure 4-16). In the first experiment all suitable nodes were decapitated, and in the second every other node was decapitated. At the end of the three weeks, plants were photographed and easily visible rhizophores were measured directly. The unexpanded apices were then dissected under a microscope and developing angle meristems photographed and their lengths calculated using the image analysis software ImageJ (Rasband, 1997). The rhizophore lengths were then categorised and analysed by the branch tier of the node from which they grew. Explants (which did not necessarily have the same number of tiers) were then compared by normalising tier numbers to either the most basal or the tipmost tier, as shown in Figure 4-18.

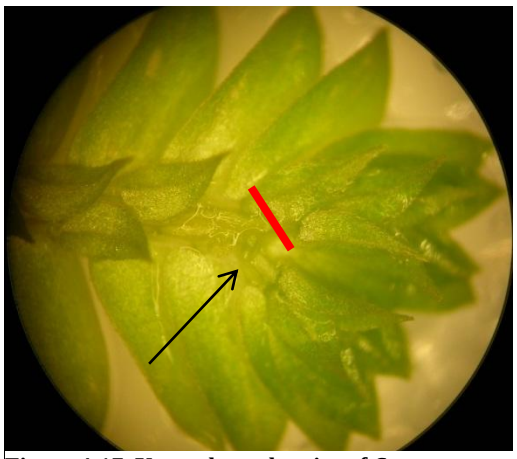


Figure 4-17. Young branch point of *S. kraussiana*, showing developing rhizophore (arrow) and point of decapitation (red line).

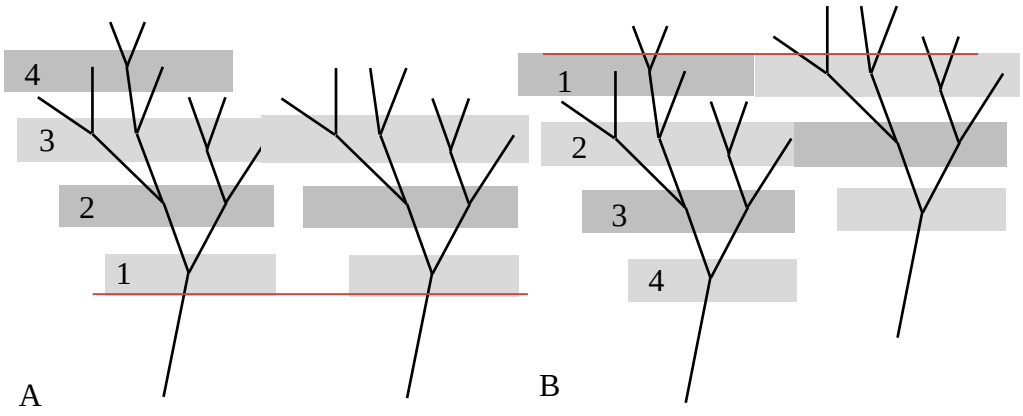


Figure 4-18. Diagram of Selaginella explants normalised to the most basal (A, red line) or apical/tipmost tier (B, red line).

In two similar decapitation experiments, only one angle meristem showed any sign of developing into an angle shoot. As the development of angle shoots when both subtending branches are decapitated occurs within two weeks (Jernstedt, 1985), it was concluded that decapitation of a single branch is insufficient to promote angle shoot formation, with or without GR24. Nevertheless, in both experiments there was a reduction (in one case significant) in the growth of rhizophores on both decapitation and on addition of GR24 on at the tipmost two or three tiers, when tiers were aligned to the basal tier (Figure 4-19). However, when tiers were normalised by the tipmost tier, so that the youngest nodes were compared with each other, decapitation caused only a slight or no reduction in rhizophore growth, but GR24 appeared to promote it, including in explants that had also been decapitated.

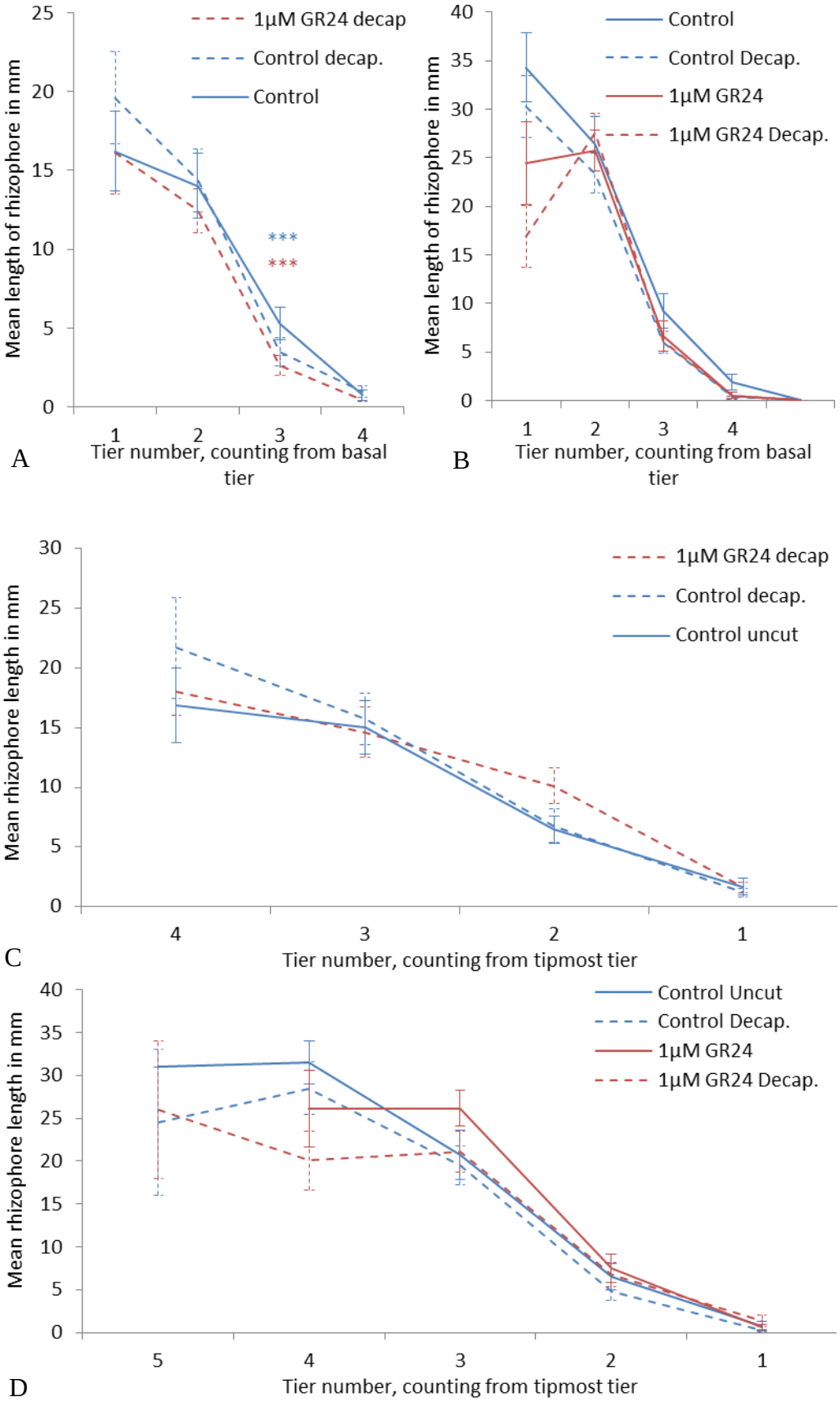


Figure 4-19. Decapitation response of *Selaginella kraussiana* rhizophores length when grown with GR24 for three weeks. Lengths of rhizophores by tier, aligned at basal tiers (A and B) or at tipmost

tiers (C and D). A) and C) experiment 1, n=9-10 for each treatment (n.b. control = undecapitated, grown on acetone carrier). B) and D) n=12-14, except for 1µM GR24 undecapitated where n=9. * = significant difference to undecapitated acetone control at P<0.001 in a Kruskal-Wallis test, star colour indicating treatment. Error bars are standard error of the mean.**

To examine why such different results were gained from different alignments of the same data, the number of nodes actually present at each tier was compared. The major branch tends to grow more quickly than the minor one, and so although in *S. kraussiana* branching is dichotomous, and therefore the number of nodes in an explant would be expected to double at each successive tier, different rates of growth in different branches lead to variable numbers of nodes being produced. When aligned by the basal tier, the number of nodes present in decapitated plants was consistently increased in the third to fifth tiers from the base, although this effect was not significant (Figure 4-20), the first two tiers having been generally completely formed at the beginning of the experiment. GR24, however, appeared to have no consistent effect on node number. Taken together, these results could be interpreted to suggest that on decapitation of a single branch, rhizophore outgrowth is maintained or only slightly decreased, but branching by dichotomous division is increased. This increase in the number of new branches being formed would lead to a larger number of young nodes with shorter rhizophores, creating the reduction in rhizophore length when plants were aligned basally but less so when aligned at the tipmost tier. GR24 in contrast appears to have a promotive effect on rhizophore outgrowth in young nodes, but less effect on dichotomous branch outgrowth. The reduction in rhizophore length when aligned basally may indicate reduced growth of older rhizophores, something possibly supported by results from the older nodes of the tipmost alignments (Figure 4-19). However, attempts to repeat and confirm these experiments in more detail failed due to fungal contamination, to which the decapitated plants are particularly prone, and further repetition would be necessary to confirm the decapitation effects.

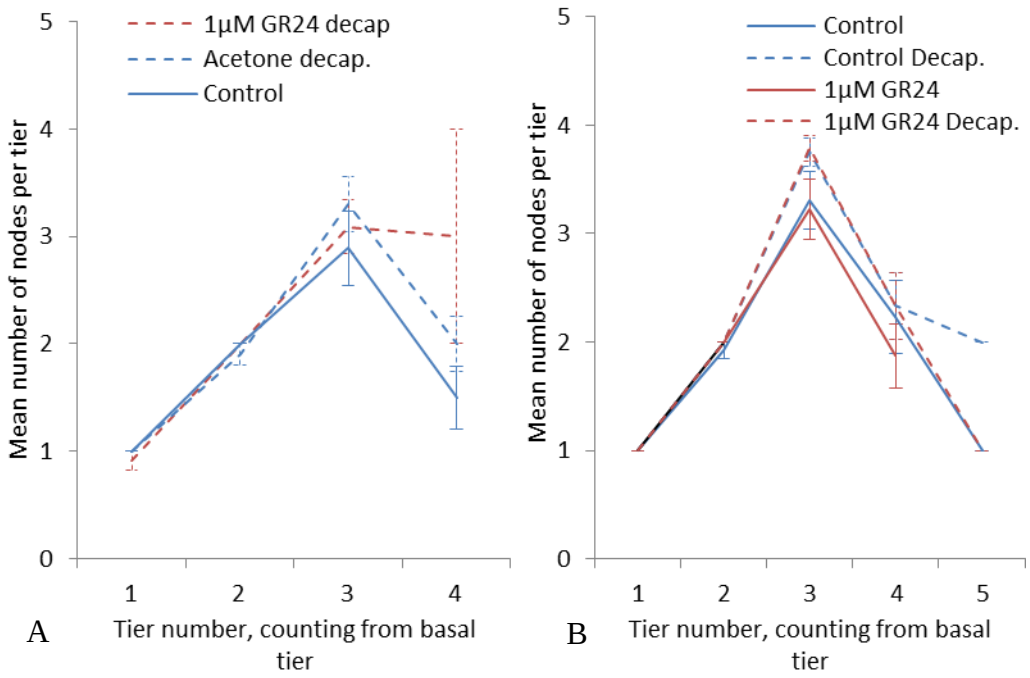


Figure 4-20. Decapitation response of *Selaginella kraussiana* branching when grown with GR24 for three weeks. Number of nodes (branch points) by tier, aligned at basal tiers. Experiments and sample numbers as described previously, no significance found in a Kruskal-Wallis test. Error bars are standard error of the mean.

4.3.3 Branching and rhizophore length response to GR24 and decapitation

To confirm the effect of GR24 identified from the decapitation experiments, the branching and rhizophore length phenotypes were investigated in more detail. The protocol used in the decapitation experiments was extended so plants were kept for four weeks on carrier control, 1µM or 10µM GR24, and were supplemented with vitamins in the growth medium. As seen previously, plants grown on GR24 showed a consistent and often significant elongation of rhizophores at the tipmost branch points compared to the mock treatment (Figure 4-21). In addition a significant reduction in the number of nodes produced at each tier was also seen, in contrast to the results from the undecapitated GR24 treated plants previously (Figure 4-20B). Other experiments carried out using only 1µM GR24 also showed similar results, although the data for these experiments are not shown due to smaller sample sizes and the use of only one GR24 concentration.

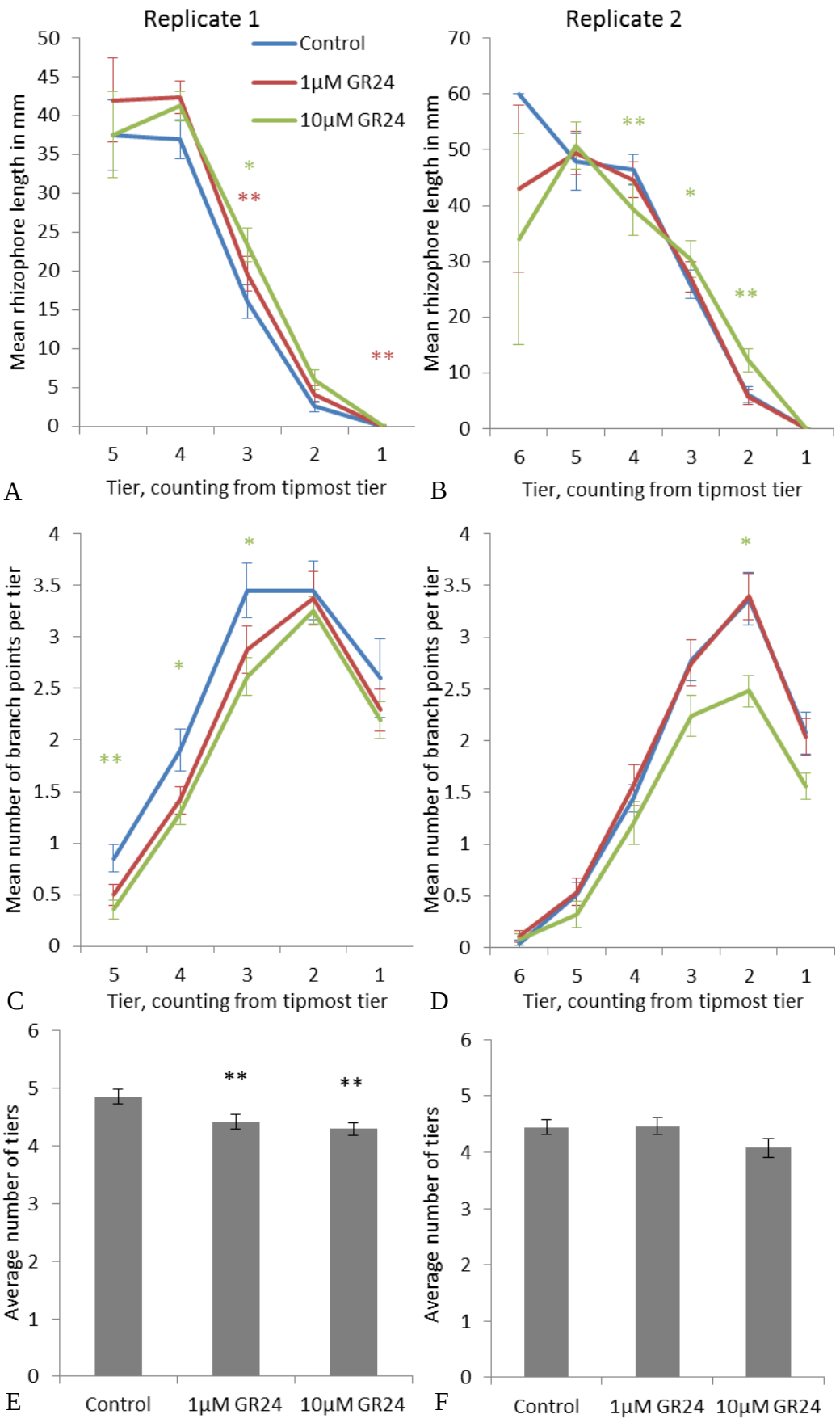

Replicate 1
Control
1µM GR24
10µM GR24
Mean rhizophore length in mm
Tier, counting from tipmost tier
A
Replicate 2
Mean rhizophore length in mm
Tier, counting from tipmost tier
B
Mean number of branch points per tier
Tier, counting from tipmost tier
C
Mean number of branch points per tier
Tier, counting from tipmost tier
D
Average number of tiers
Control
1µM GR24
10µM GR24
E
Average number of tiers
Control
1µM GR24
10µM GR24
F

Figure 4-21. Growth of *Selaginella kraussiana* on GR24 for four weeks. A) and B) Lengths of rhizophores at each tier, and C) and D) number of branch points at each tier, all aligned at tipmost tiers. E) and F) number of tiers of branches on explants. A), C) and E) all first replicate, n=20, 24 and 31 for acetone, 1µM GR24 and 10µM GR24 respectively, and for replicate 2 (B, D and F) n=27, 28 and 25 respectively. * = significant difference to acetone at P<0.001, ** = P<0.01 and * = P<0.05 in a Kruskal-Wallis test, star colour indicating treatment. Error bars are standard error of the mean.**

In one replicate at least (Figure 4-21E) a significant reduction in the total number of tiers produced was also seen, although this effect was less consistent between replicates (including those not shown here). From this it seems that GR24 causes reduced dichotomous branching, in opposition to the possible increase in dichotomous branching in response to decapitation. This restriction of growth was also supported by a significant reduction in the final weights of the explants (only measured in the second replicate, Figure 4-22). However, this effect does not apply to the same extent to rhizophores, in which growth at the tipmost, youngest nodes appears to be maintained or even increased, whereas in older nodes it may be reduced. There is a possibility that the reduction in node production means that the youngest nodes in GR24 grown plants are older (and therefore have longer rhizophores) than those of plants grown without GR24, but even if this is the case it still suggests that rhizophores are not subject to the same growth restriction as node production, or else their growth at the youngest nodes would also be inhibited.

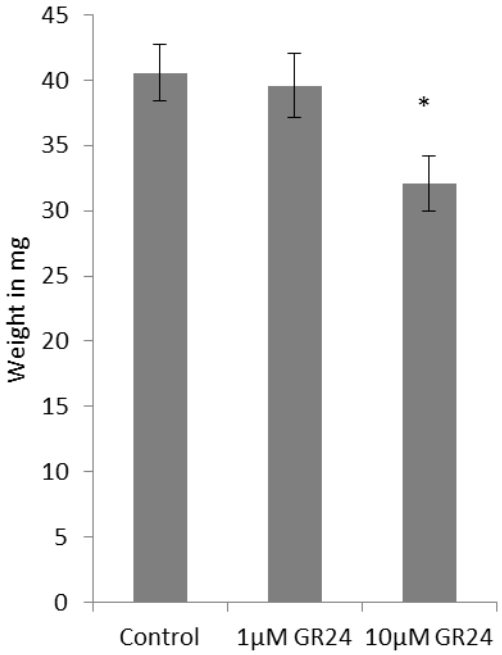


Figure 4-22. Weights of *Selaginella kraussiana* explants grown on GR24 for four weeks, second replicate. N=27, 28 and 25 for acetone, 1µM GR24 and 10µM GR24 respectively, * = significant difference to acetone at P<0.05 in Tukey's HSD test. Error bars are standard error of the mean.

Although these results do not match the hypothesis that GR24 would delay or restrict outgrowth of rhizophores as dormant meristems or promote angle shoot development by affecting auxin transport, they would still support a role for SLs in adaptation of plant development to nutrient limitation as seen in other species. Instead of reducing angle meristem outgrowth, it appears more likely that shoot meristem outgrowth is reduced. If SL signalling in nutrient limitation is conserved in Selaginella, regardless of the particular phenotypes under its control, restriction of growth to a smaller number of axes while maintaining growth in nutrient foraging organs (rhizophores) would make sense.

4.4 Discussion

The conservation of genetic pathways infers a selection pressure to maintain these genes in living species - there is likely to be an adaptive significance, though this itself may not necessarily be conserved. The production and reception of SLs by a largely similar gene set in angiosperms and in moss implies that this pathway has had roles in plant development that preceded the divergence of these groups, close - in geological time at least - to the emergence of the land plants. That both groups have conserved SL signalling also implies that SLs have had an important influence on fitness for both groups for all of the time since that split. As the known biosynthetic and signal transduction gene sets are generally also conserved in lycopodiophytes (closest to mosses) and gymnosperms (closest to angiosperms), the physiological and developmental roles driving their continued presence in these genomes were explored. The moniliphytes, genomic orphans, were included as the major land plant group that falls between these two.

To make the investigation of such a range of different species with, potentially, an equally broad range of SL-related phenotypes, lines of investigation focussed on the specific role of SLs in shoot branching control, and the more general and perhaps ancestrally-unifying concept of SLs as a global coordinator of nutrient limitation responses. This focus on specific hypotheses was all the more important because the tools available for the

confirmation that SLs play endogenous roles in development - tools such as mutants, inhibitors, and in some cases genetic orthologues – are not necessarily available for these species. Without the ability to remove hormones, confirmation of the biological significance of the results of their addition is more difficult. For SLs, the existence of a well-characterised analogue, GR24, makes the experimental addition of SLs possible. However, there remains risk that the effects of GR24 are those of a toxin, rather than reflecting an endogenous role of SLs. Therefore the conclusions presented here must be considered with these caveats in mind, and require further investigation of mechanisms of action and relevance to endogenous events to confirm roles for strigolactone signalling in development in these species.

In gymnosperms, a sufficient number of characteristics of axillary branching were conserved with those of angiosperms that it was considered possible that the roles of SLs in branch outgrowth might be shared with the angiosperms. Given some of the similarities of axillary bud dormancy to apical bud dormancy (Rohde and Bhalerao, 2007) this phenomenon was also investigated with regard to SLs. From the experiments detailed here, there is no clear evidence of an effect of application of the synthetic strigolactone GR24 on the maintenance of seasonally-related growth cessation in spruce apices, and only very tentative evidence in support of a role of GR24 in modulating branch outgrowth, and then only in response to decapitation and at high concentrations (10μM GR24). Even though this decapitation response is slight, it encourages further work to confirm it. In addition, although GR24 has been found to mimic SLs (although not as effectively as endogenous SLs) in angiosperms and in moss, the same may not hold true for gymnosperms, and even if it does, the active concentrations may differ (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008; Proust *et al.*, 2011). Nevertheless, the isolation of natural SLs from gymnosperms (*Pinus* spp.) has been reported (Xie *et al.*, 2010), demonstrating that the biosynthetic pathway is not only present but active in gymnosperms, and although the genes corresponding to that pathway have not been confirmed it appears highly likely that at least some of these are the *MAX* gene orthologues identified here. These orthologues also showed similar patterns of reaction to phosphate limitation to each other and to those in rice (as reported by Umehara

et al., 2010), with the signal transduction gene *MAX2* being largely unaffected by treatment, but the biosynthetic genes showing generally lower expression in phosphate sufficient conditions than in phosphate starvation conditions. The purpose of this upregulation in low phosphate conditions is thought to be both to restrict shoot growth, and by exudation from the roots to encourage the formation of mycorrhizal symbioses, which improve plant phosphate acquisition (Bouwmeester *et al.*, 2007). White spruce, like other gymnosperms, forms mycorrhizal symbioses, but like many in the pine and spruce families these are ectomycorrhizal, rather than AMy type symbioses (Wang and Qiu, 2006). These symbioses have also been shown to improve plant phosphate status in high- and low-phosphate containing soils in the gymnosperm *Pinus pinaster*, maritime pine (Torres Aquino and Plassard, 2004; Tatry *et al.*, 2009). Although SL signalling in ectomycorrhizal symbioses has not yet been reported, convergent evolution of the use of rhizosphere SLs for detection of hosts has been found in AMy fungi and parasitic plants (for example, reviewed in Tsuchiya and McCourt, 2012), so conifer exudation of SLs to attract ectomycorrhizal symbionts would not be surprising. Even if not involved in promoting symbiosis, production of SLs could well be upregulated on phosphate limitation in conifers associated with other nutrient-signalling developmental effects, whether shoot branching related or otherwise, as it is in the non-mycorrhizal species *Arabidopsis thaliana* (Kohlen *et al.*, 2011). In either case, it seems that the nutrient limitation response of the *MAX* pathway is conserved among the seed plants.

The evidence for SL signalling in fern species is considerably less than it is even for the gymnosperms and lycopodiophytes, not least because the absence of a sequenced fern genome, and the very few EST sequencing projects, means that identification of full length *MAX* gene orthologues was not possible, although fragments of sequence available suggest that both *MAX1* and *MAX2* are conserved in some form in ferns. Nevertheless, some experimental evidence was found for a response to GR24 in c-fern root growth and perhaps frond development. These results need to be confirmed and further investigated, but the root length effect in particular was remarkably similar to the effects of GR24 on roots in Arabidopsis, in which primary root growth can be increased

by application of concentrations of GR24 between 1 and 2.5μM in a *max2* dependent manner, but suppressed by concentrations of 10μM GR24 in a *max2* independent manner (Ruyter-Spira *et al.*, 2011). These effects are also controlled by endogenous SLs in Arabidopsis and are dependent on complex interactions between auxin signalling, auxin transport and SLs in Arabidopsis and tomato (Koltai *et al.*, 2010; Ruyter-Spira *et al.*, 2011). Changes in auxin sensitivity have also been proposed to mediate phosphate limitation responses, particularly in development of lateral roots in Arabidopsis, a process in which SL signalling has also been implicated (Perez-Torres *et al.*, 2008; and reviewed in Koltai, 2011; Ruyter-Spira *et al.*, 2011). Auxin is also involved in determining the length of roots in c-fern, although not the initiation of lateral roots (Hou *et al.*, 2004), and in fern preliminary results indicate a strong reduction of root length in phosphate limited conditions. Further investigation of the dose-response to GR24 in c-fern roots is required. However, if the responses are confirmed, investigation of the interactions between GR24 and auxin signalling in the control of root length in the fern might be a way to start the process of confirming that these effects are those of an endogenous plant growth regulator, and not just those of an exogenous toxin.

The combination of the manipulation of endogenous auxin (by decapitation) with GR24 addition was attempted in the experiments on Selaginella, and although the initial hypotheses on the effects of GR24 on rhizophore determination and outgrowth were not supported, the experiments did produce some interesting results. The decapitation experiment indicated that this kind of wounding might promote dichotomous branching, but did not have much effect on rhizophore outgrowth. Although an apical-dominance type release effect had been postulated for the outgrowth of angle shoots, and similar effects along these lines have previously been reported (Webster, 1969; Wochok and Sussex, 1975; Jernstedt, 1985) in this case apical dominance appeared to be operating at the level of the dichotomous division. Shoot division is a very regular process in *S. kraussiana*, occurring every four leaves or three leaves depending on whether the branch is ‘major’ or ‘minor’ (Harrison *et al.*, 2007), and auxin has not been implicated in phyllotaxic patterning of either dichotomous branch or leaves, so whether the apical dominance effects reported here are mediated by auxin is a

fascinating question. However, in the GR24 addition experiment, rhizophore growth appears to be maintained at the tipmost nodes, but nodal branching is decreased, particularly at the higher concentration, consistent with a role for GR24 in increasing competition between branches produced by dichotomous division. Although this may be a toxicity effect, the apparent stimulation of rhizophore growth supports a more specific effect, and perhaps one replicating a nutrient-limitation response – a hypothesis easily tested by investigating the effects of growing plants on low phosphate medium. As for ferns, should these effects be confirmed, further work investigating the effects of GR24 on auxin transport would be interesting, both for the purpose of understanding the developmental mechanisms of Selaginella and for the evolution of the mechanisms of SL signalling.

Although few conclusions can be drawn with confidence from the results of SL action in ferns and lycopodiophytes, these preliminary experiments provide starting points for developing and testing further hypotheses about the conservation of strigolactone signalling across these wide phylogenetic distances. The development of efficient and specific SL signalling inhibitors, towards which steps have already been made (Sergeant *et al.*, 2008; Ito *et al.*, 2010; Ito *et al.*, 2011), will be a boon towards such research, as will the development of mutants and genetic transformation systems in the ferns and lycopodiophytes particularly. If confirmed, the results presented here seem likely to support the hypothesis drawn from the angiosperm and moss phenotypes that SLs are ancestral regulators of development in response to nutrient limitation, whether due to competition from other colonies (as in moss, Proust *et al.*, 2011) or limitation in the soil (Kohlen *et al.*, 2011; Ruyter-Spira *et al.*, 2011).

Chapter 5. MAX1 duplication in Angiosperms

Although the actions of SLs outside the angiosperms are still relatively uncharacterised, the processes in which strigolactones are known to be involved within this group are ever more varied, a phenomenon shared with many other plant hormones. In comparison to its many different roles, the *MAX* genetic pathway is much less diverse, being quite conserved in terms of gene copy number (Figure 1-7), with few gene duplicates present in either monocot or dicot clades, except in soybean in which there was recent whole genome duplication (WGD) only ~13 mya, and in poplar which had a WGD event around 60-65 mya, but has a relatively slow molecular clock (Tuskan *et al.*, 2006; Schmutz *et al.*, 2010). In the angiosperms, WGDs have been unusually frequent, with the ever-increasing number of sequenced plant genomes providing evidence of many paleopolyploidisations, including two events within the cereal lineage, compared to a probable triplication (the 'γ' event) shared by most (if not all) eudicots, and followed by more recent WGDs that are family or genus-specific in both monocots and dicots, such as the β and α events in the eurosid/Brassicaceae lineage to which Arabidopsis belongs (Cannon *et al.*, 2006; Jaillon *et al.*, 2007; reviewed in Paterson *et al.*, 2010; Schmutz *et al.*, 2010; Tang *et al.*, 2010; Argout *et al.*, 2011; Illa *et al.*, 2011; Xu *et al.*, 2011). These recurrent duplications have provided ample opportunity for the genes of the *MAX* pathway to multiply. Yet with the exception of *MAX1,* they generally do not seem to have done so. Although *D14* and *D27* each have duplicate clades, (two in the case of *D14*) these are separated by long branch lengths between clades, suggesting diversifying selection, and there is as yet no evidence that the *D27like* paralogue clade is involved in SL signalling, although *D14like*, which is involved in a parallel signalling pathway in the perception of germination signals from smoke, may retain an ancestral redundancy with SL signalling (Waters *et al.*, 2012). In comparison, *MAX1* has a very different pattern compared to the other genes, for while in the eudicots, orthologues are generally present as a single copy (although along with poplar, and probably pea, *Medicago truncatula* has two) there are multiple copies present in the monocots. Within monocots, three different clades are present, with each containing members from rice, maize, sorghum and Brachypodium (Nelson *et*

al., 2008, Challis, et al.. in preparation). In one clade in particular, further duplications have occurred resulting in five orthologues of *MAX1* in rice. To investigate whether the proliferation of *MAX1* in monocots, and to a lesser extent in dicots, was indicative of subfunctionalisation or diversification at the functional level, the complementation approach was expanded to include paralogous genes from the angiosperms. To compare the evolutionary paths of *MAX1* in monocots and dicots, and in collaboration with Dr Céline Mouchel, two models were selected for complementation analysis, *Medicago truncatula* (a eudicot) and *Oryza sativa* as a monocot.

5.1 Medicago

Medicago truncatula, or barrel medic, is a close relative of the agriculturally important crop *Medicago sativa* (alfalfa), and as a legume is a model for the study of nodulation - a symbiotic relationship with Rhizobia bacteria which shares signal transduction and regulatory components with, and has probably evolved from, the more ancient AMy symbiosis (and reviewed in Parniske, 2008; Maillet *et al.*, 2011). Medicago is a plant with a prostrate growth habit, and little dormancy in its axillary buds, especially in the sequenced accession Jemalong A17 (pers. comm. C. Mouchel). However, the role of SLs in shoot branching control and dormancy are well characterised in pea, a key model for the understanding of these hormones and a close relative of Medicago (Gomez-Roldan *et al.*, 2008; reviewed in Beveridge *et al.*, 2009). *MAX1* homologues and mutants have not been characterised in pea, a point suggested to be due to the presence of redundant copies of *MAX1* (Gomez-Roldan *et al.*, 2008). The investigation of the duplicate *MAX1* orthologues present in Medicago was therefore of interest for understanding the evolution of the MAX pathway in legumes and investigation of redundancy and diversification of *MAX1* in an angiosperm species with a different life history and roles for SLs compared to Arabidopsis.

5.1.1 Branching phenotype

The *MtMAX1* orthologues (Gene Identifiers *Medtr3g104560* and *Medtr1g015860* from the International Medicago Genome Annotation Group, annotated as *Medtr3g139760* and *Medtr1g019950* respectively in Phytozome

notation, which gives the more accurate gene model for *Medtr1g019950*, (Goodstein *et al.*, 2012) were cloned from plasmids kindly provided by Dr Céline Mouchel, originally cloned from the Jemalong A17 cultivar used for the whole genome sequencing project. These were cloned into vectors to create constructs with the *35S* promoter and *nos* terminator and transformed into plants as described for the *PgMAX1* and *SmMAX1* constructs previously. The resulting transgenic lines were then phenotyped and compared to wild-type Columbia-0, the parent *max1-1* and with a single *max1-1* line produced by Dr Sally Ward carrying an *AtMAX1* construct, under the same promoter, as a positive control. Branching and height measurements were carried out as described for the *PgMAX1* and *SmMAX1* transgenics in Chapter 3. Overall comparison of the ability of the two *MtMAX1* constructs to rescue indicated a clear divergence in function. *35S::Medtr3g104560,* like the control *35S::AtMAX1* construct, was able to complement completely *max1-1* in both branching (Figure 5-1) and height phenotypes (Figure 5-2 and Figure 5-3) and this rescue was consistent across all the transgenic lines. Taking all lines together, *35S::Medtr1g015860* did not appear to be capable of rescuing (Figure 5-1A), but some individual lines did show a reduction in branching and an increase in height, indicating a weakly rescued phenotype. In particular branch numbers of lines 14.5 and 2.7 were not significantly different in Kruskal-Wallis tests (adjusted for repeat sampling) from *35S::AtMAX1* across two replicates, and were significantly different from *max1-1* (at $P \leq 0.05$) in the second replicate (14.5 was also not significantly different from Col-0 in terms of height in either replicate). In addition, lines 17.6, 7.2 and 3.9 showed some degree of rescue (no significant difference in between 7.2 and 3.9 and Columbia-0 in branching or heights in replicate 1, or between 17.6 and *35S::AtMAX1* in branching or heights in replicate 2). These lines produce a cluster with an intermediate phenotype between complete lack of rescue and full rescue which can be seen clearly in Figure 5-2. This suggested the possibility that although *Medtr1g015860* had diverged in its functional capability to catalyse the reaction occurring in Arabidopsis, it might have retained a weak ability to do so.

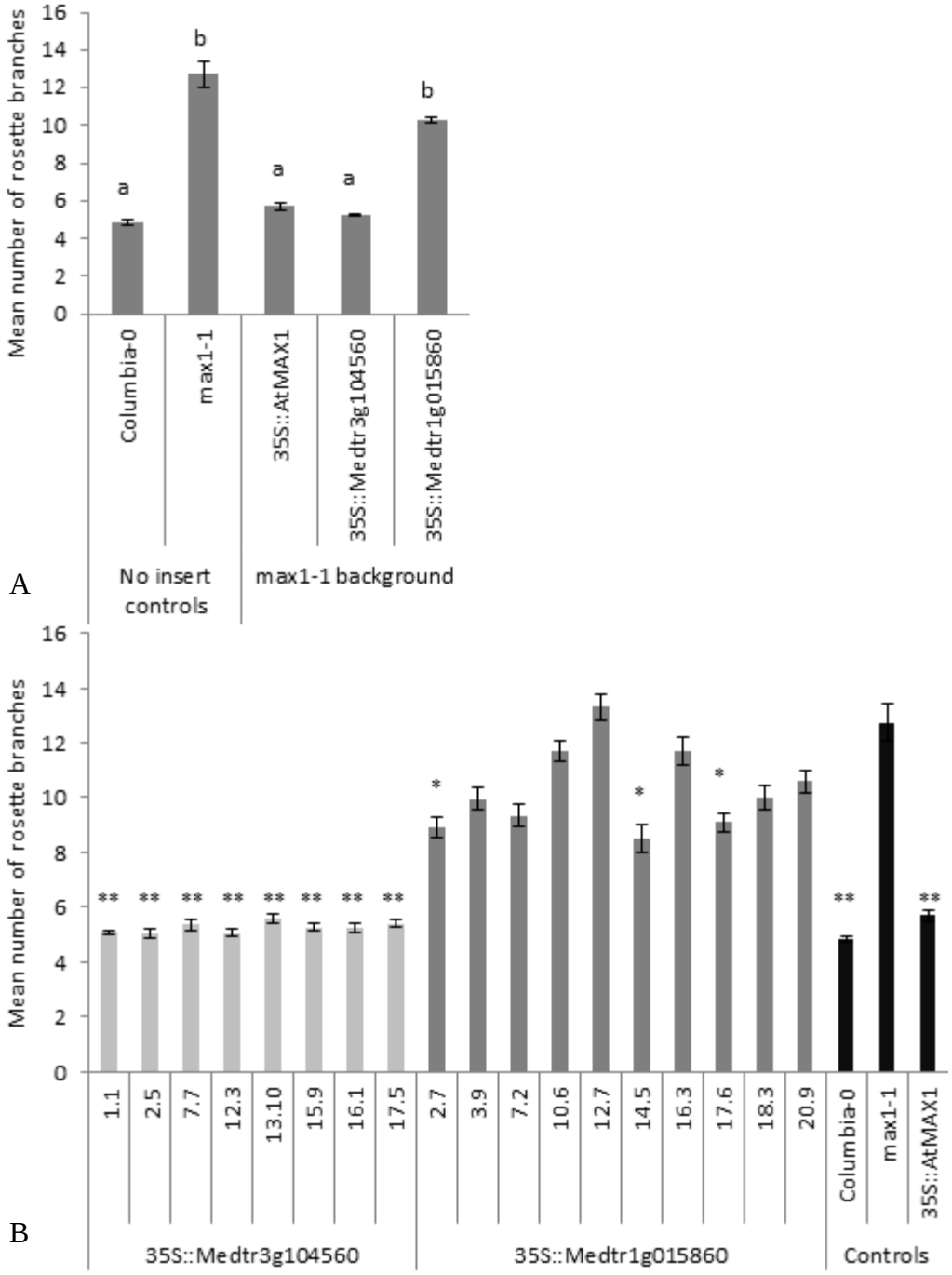


Figure 5-1. Rosette branching of Arabidopsis *max1-1* mutants complemented with *Medicago truncatula MAX1* orthologues under the 35S promoter. Second and representative of two replicates. Branching was assessed by short-day decapitation assay as described by Greb *et al.* (2003). A, shared letters indicates no significant difference in a Kruskal-Wallis test to P≤ 0.001, data are mean averages for independent lines shown in B. B, * = significantly different to *max1-1* at P≤ 0.05, ** at P ≤ 0.001. N for each line = 20, except for Columbia-0, *max1-1* and *35S::AtMAX1 max1-1* for which n=40. Error bars show standard error of the mean.

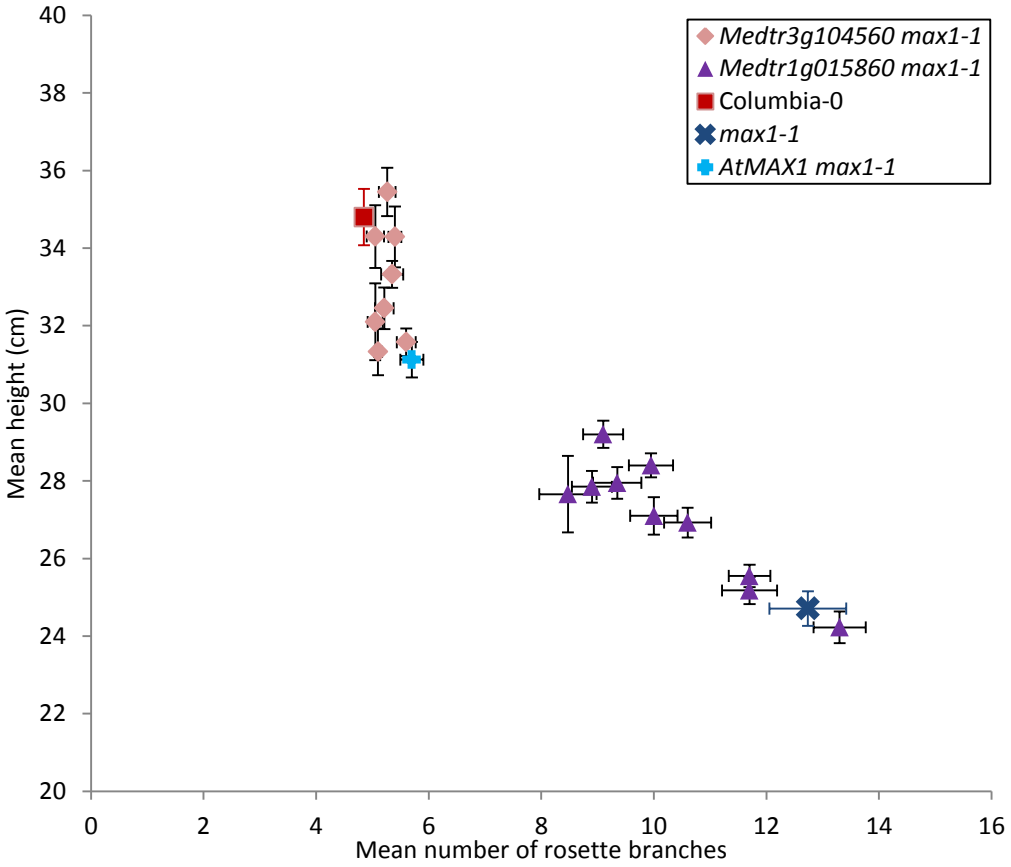


Figure 5-2. Rosette branching plotted against height for individual *MAX1* constructs derived from *Medicago truncatula*. N =19-20, except for *max1-1* and Columbia-0 where n=40. Branching was assessed by short-day decapitation assay as described by Greb *et al.* (2003). **Height (in centimetres) of the longest branch was measured the day of scoring for branching. Error bars show standard error of the mean. Note y axis starts at 20cm.**

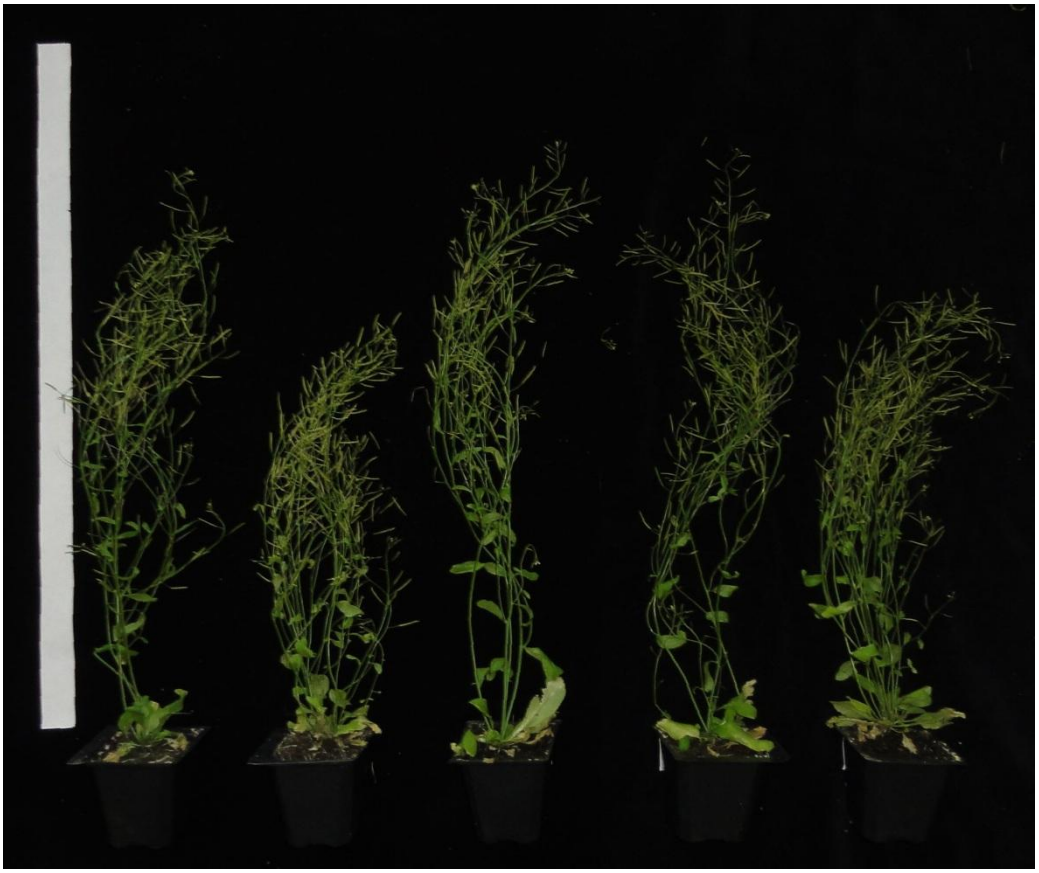


Figure 5-3. Photograph of Columbia-0, *max1-1*, *35S::AtMAX1 max1-1*, *35S::Medtr3g104560 max1-1* line 12.3, and *35S:: Medtr1g015860 max1-1* line 7.2, from left to right, with *AtMAX1* and *Medtr3g104560* transgenics showing rescue and *Medtr1g015860* showing very limited rescue. White bar = 40cm.

5.1.2 Comparison of expression to phenotype

To further explore the possibility that *Medtr1g015860* retained some ability to substitute for *MAX1* in Arabidopsis, branch patterns were compared with the expression of the transgene. Quantitative PCR (QPCR) was used to measure transgene expression in ten day old seedlings, as the CaMV *35S* promoter is considered to be constitutive (Odell *et al.*, 1985; Slater *et al.*, 2007). Expression was normalised to the expression of an endogenous Arabidopsis serine/threonine protein phosphatase 2A gene, *At1g69960*, and an endogenous SAND-related gene, *At2g28390*, both of which had been selected by Dr Malgorzata Domagalska as being developmentally stable.

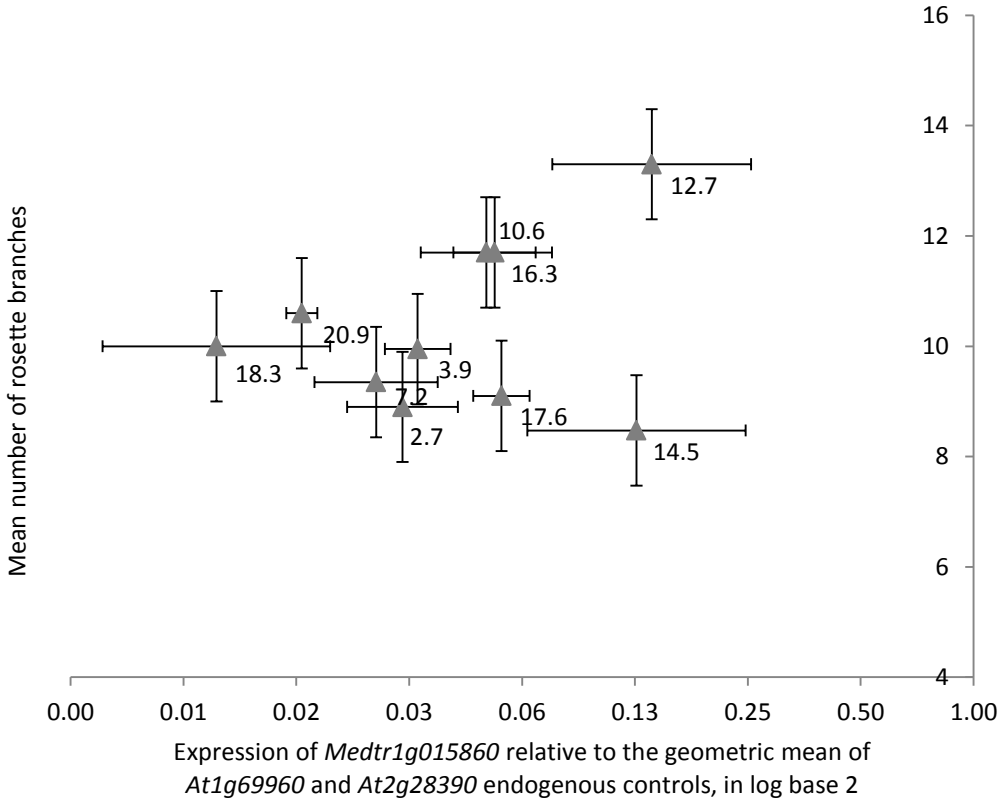


Figure 5-4. Branching rescue from second replicate results against expression for *35S::Medtr1g015860*. Data points are labelled with transgenic line numbers. Numbers of QPCR cycles of *Medtr1g015860* were normalised to the geometric mean number of cycles of *At1g69960* and *At2g28390* as the endogenous controls, and relative expression plotted in $\log_2$. Standard errors are standard error of the mean of two biological replicates, each representing three technical replicates. Note y axis starts at 4 branches.

The resulting estimates of expression were plotted against rosette branch numbers as a measure of rescue, shown in Figure 5-4 where four branches was

set as the zero point on the axis to represent the wild-type phenotype (in the replicate shown here, Columbia-0 had a mean of 4.85 branches, see Figure 5-1). There does not appear to be any relationship between expression and rescue, as the three lines with the highest branch numbers (10.6, 16.3 and 12.7) also have moderate to high expression. Tests with Pearson's coefficient confirmed the lack of correlation, indicating that the inability of the *Medtr1g015860* construct to complement fully *max1-1* is not linked to low expression.

5.1.3 Leaf phenotype

As leaf phenotyping proved of interest in distinguishing rescue ability between *PgMAX1* and *SmMAX1*, leaves for each line of the Medicago constructs were compared to wild-type, mutant and the *35S::AtMAX1 max1-1* control at 6 weeks of age (Figure 5-5) to elucidate further the degree of rescue by *Medtr1g015860*. At this age PC9 showed no significant difference between *max1-1* and Columbia-0, while PC10, although still distinguishing significantly between Columbia-0 and *max1-1*, was unable to distinguish between rescued and non-rescued lines, with almost all lines showing no difference to either wild-type or mutant, and so neither were considered for assessment of rescue. For the three remaining phenotypes, the *35S::AtMAX1* control construct rescued completely, as does *35S::Medtr3g104560*, although lines 2.5 and 13.10 show some variation in rescue, especially in PC3 (Figure 5-5). The *35S::Medtr1g015860* construct failed to rescue the centroid size or PC2 leaf phenotypes as it did branching, although interestingly, as a whole, the construct rescued PC3 to the same degree as *35S::AtMAX1*. As for *35S::Medtr3g104560*, different lines varied in the degree to which they rescued the various phenotypes, but these did not correspond as well to the branching phenotype as might have been expected. 14.5, a line with high expression and low branch numbers in the second replicate, is not rescued at all in its leaves, and nor is 2.7, another low-branching line. However, 7.2 and 3.9, also low-to-mid branching lines, are rescued in terms of PC2 and, along with 17.6, in terms of PC3.

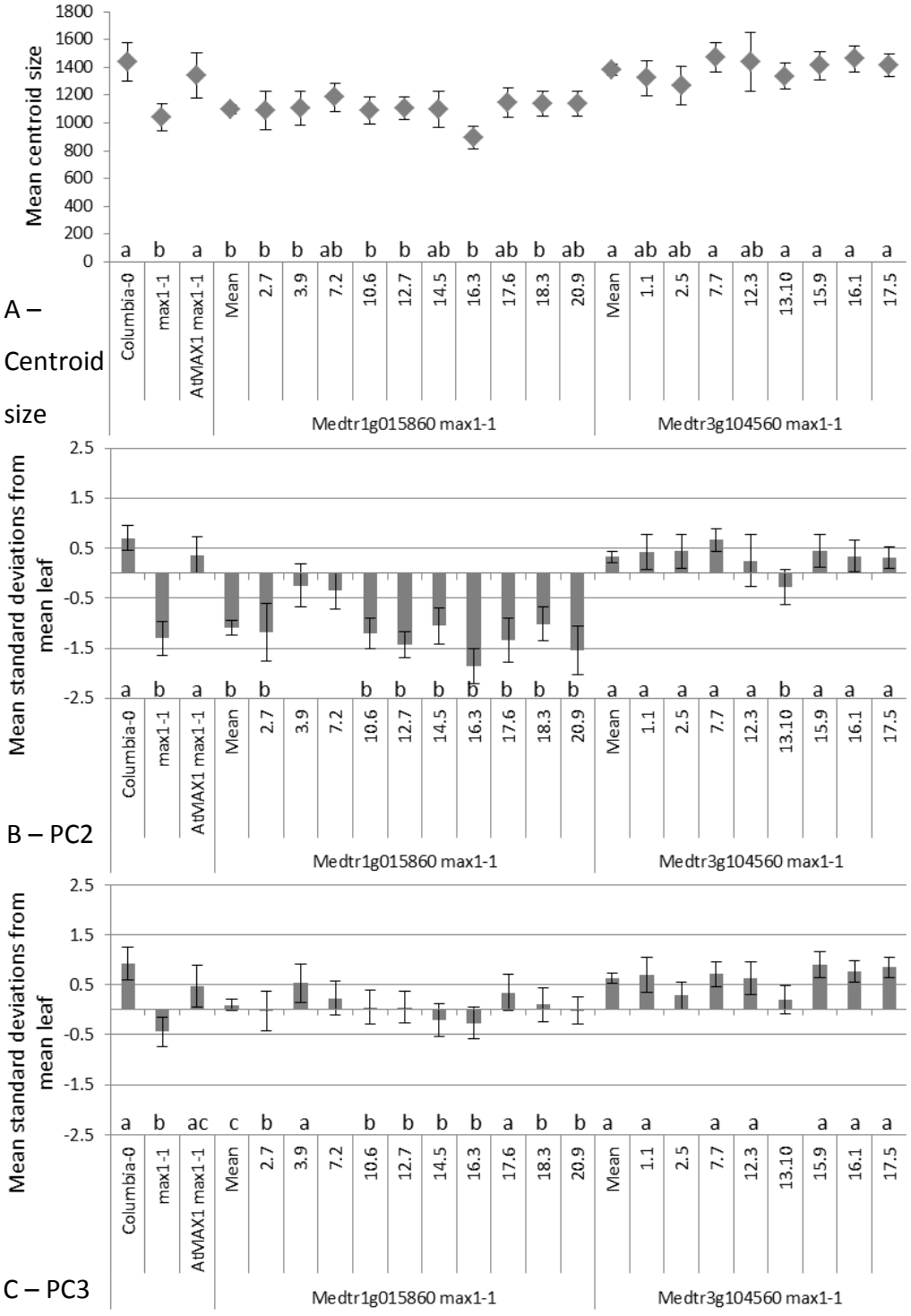


Figure 5-5. Leaf shape analysis for Procrustes-fitted adult leaves four and above from *max1-1* plants complemented with *MtMAX1* orthologues. Error bars are standard error of the mean, calculated on number of plants as n, where n = 6 for Col-0 and *AtMAX1 max1-1*, 9 for *max1-1*, and 6-8 plants for all other lines with the exception of *Medtr1g015960 max1-1* 14.5 (n=5) and *Medtr3g104560 max1-1* 12.3 (n=3). Shown are mean centroid sizes (A) and standard deviations from the mean leaf for PC2 (width at centre, B), and PC3 (area distribution, C). Letters indicate non-significance in Tamhane's T2 post-hoc test at P>0.001 (centroid, PC2) or P>0.05 (PC3).

In the experiment with *PgMAX1* and *SmMAX1*, lines showing rescue in branching were less likely to do so in PC3 or centroid size than in PC2, suggesting that these phenotypes require a higher level MAX1 activity for rescue. A test with Pearson's correlation between average line values for all the leaf phenotypes used here and for branching was significant for all combinations to $p \leq 0.001$. When the test was extended to consider PCs 9 and 10, correlations were also significant at $p \leq 0.001$, except for those between PC9 and centroid size, PC3 and branching, which were p=0.018, p=0.024 and p=0.004 respectively. These generally strong correlations between measures of rescue support that all phenotypes are indeed responding to MAX1 activity of the transgenes, suggesting that variation in rescue between phenotypes does derive from differences in degree, or threshold of response. As a result, although centroid size does not seem to be much rescued by *Medtr1g015860* (although there is some move away from the *max1-1* phenotype in the cases of 7.2, 14.5, 17.6 and surprisingly 20.9), the partial rescue of PC3 and PC2 in some lines probably reflects a weak ability of *Medtr1g015860* to carry out the Arabidopsis *MAX1* function. Nevertheless, the low degree of rescue of all branching, height, leaf phenotypes demonstrate that overall, *Medtr1g015860* function has diverged significantly both from that of *AtMAX1* and *Medtr3g104560*.

5.1.4 In planta *expression of* MtMAX *orthologues*

The expression patterns of the *MAX1* orthologues were explored to see whether they had also diverged. The expression of orthologues to all the MAX pathway genes known in Arabidopsis were compared by semi-quantitative RTPCR to see whether the expression of *Medtr1g015860* differed from that of *Medtr3g104560*, which might indicate that this orthologue had been co-opted to a new role, and whether gene expression patterns in Medicago were similar to those of Arabidopsis. Plants of Jemalong A17 were grown for 5 weeks, at which the most basal node had started to produce a branch, and then tissues were gathered for analysis. The expression of all MAX orthologues followed a similar pattern, with highest expression in the roots, lower stem and some expression in the most basal, branching node, except for *MtMAX2*, which appeared ubiquitous (Figure 5-6). The patterns for *MtMAX2*, *MtMAX3* and to an extent *MtMAX4* are similar to those of their Arabidopsis orthologues, although

MtMAX4 is more highly expressed in leaves and generally in non-root tissues than reported for *AtMAX4* (Sorefan *et al.*, 2003; Booker *et al.*, 2004; Bainbridge *et al.*, 2005; Stirnberg *et al.*, 2007). The *MtMAX1* orthologues, however, do not seem to be expressed as widely throughout the plant as *AtMAX1*, with expression undetectable in the upper stem and most concentrated in the roots, especially for *Medtr3g104560* (Booker *et al.*, 2005). Nevertheless, given the similarity between the patterns of the two *MtMAX1* orthologues, there seems to have been little diversification in the organ-level regulation of these genes at this particular developmental stage, and this study gives no information on differences between cell types, or the responses of the genes to different stimuli.

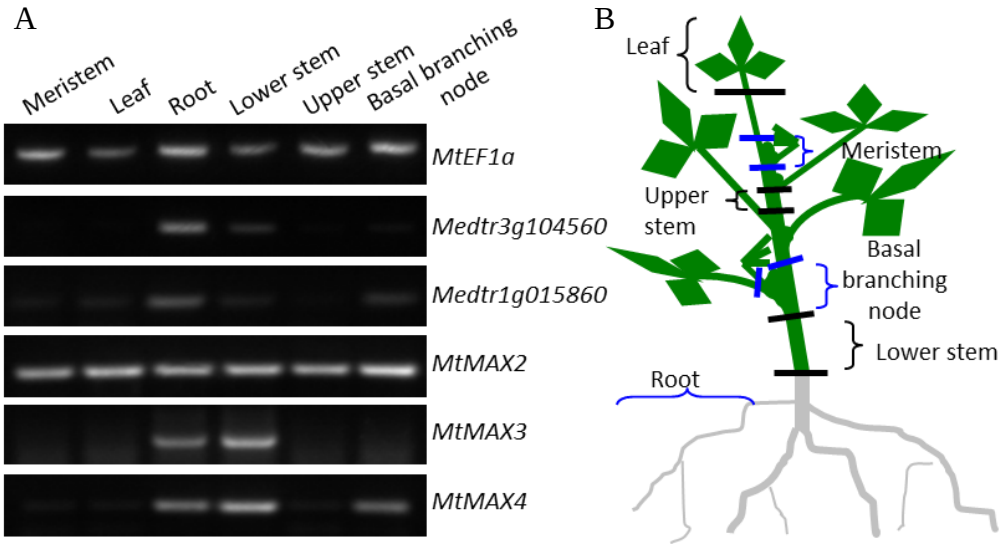
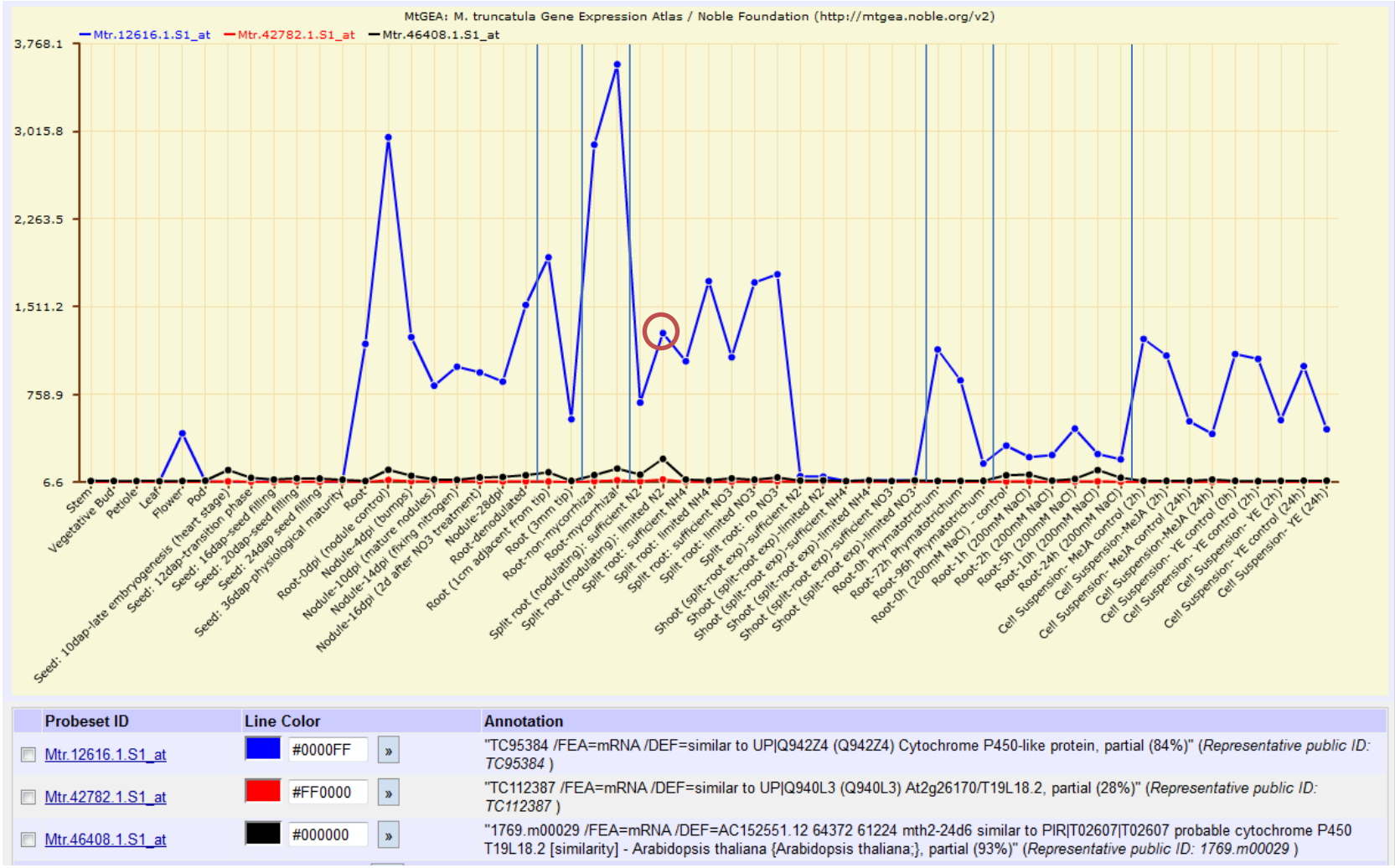


Figure 5-6. Expression of *Medicago truncatula* *MAX* gene orthologues in 5 week old plants. A) Medicago orthologue of Elongation Factor 1α (*Medtr8g014590*) was used as an endogenous loading control. PCR cycles used: *MtEF1α* – 35, *Medtr3g104560* – 40, *Medtr1g015860* – 50, *MtMAX2 (Medtr4g0800200)* – 35, *MtMAX3 (Medtr7g045370)* – 50, *MtMAX4 (Medtr3g109610)* – 45. B) tissues used in RTPCR.

As the RTPCR study was limited in the information it provides on expression, the expression of the *MtMAX1* orthologues was checked in the Medicago Gene Expression Atlas database of publically available results from microarray experiments (Benedito *et al.*, 2008; He *et al.*, 2009). Probesets relating to both orthologues were available, and expression visualised using the Multitranscript Viewer at the Samuel Roberts Nobel Foundation website (http://mtgea.noble.org/v2, He *et al.*, 2009). Two probesets were available for *Medtr1g015860*, which showed very similar expression patterns, although with

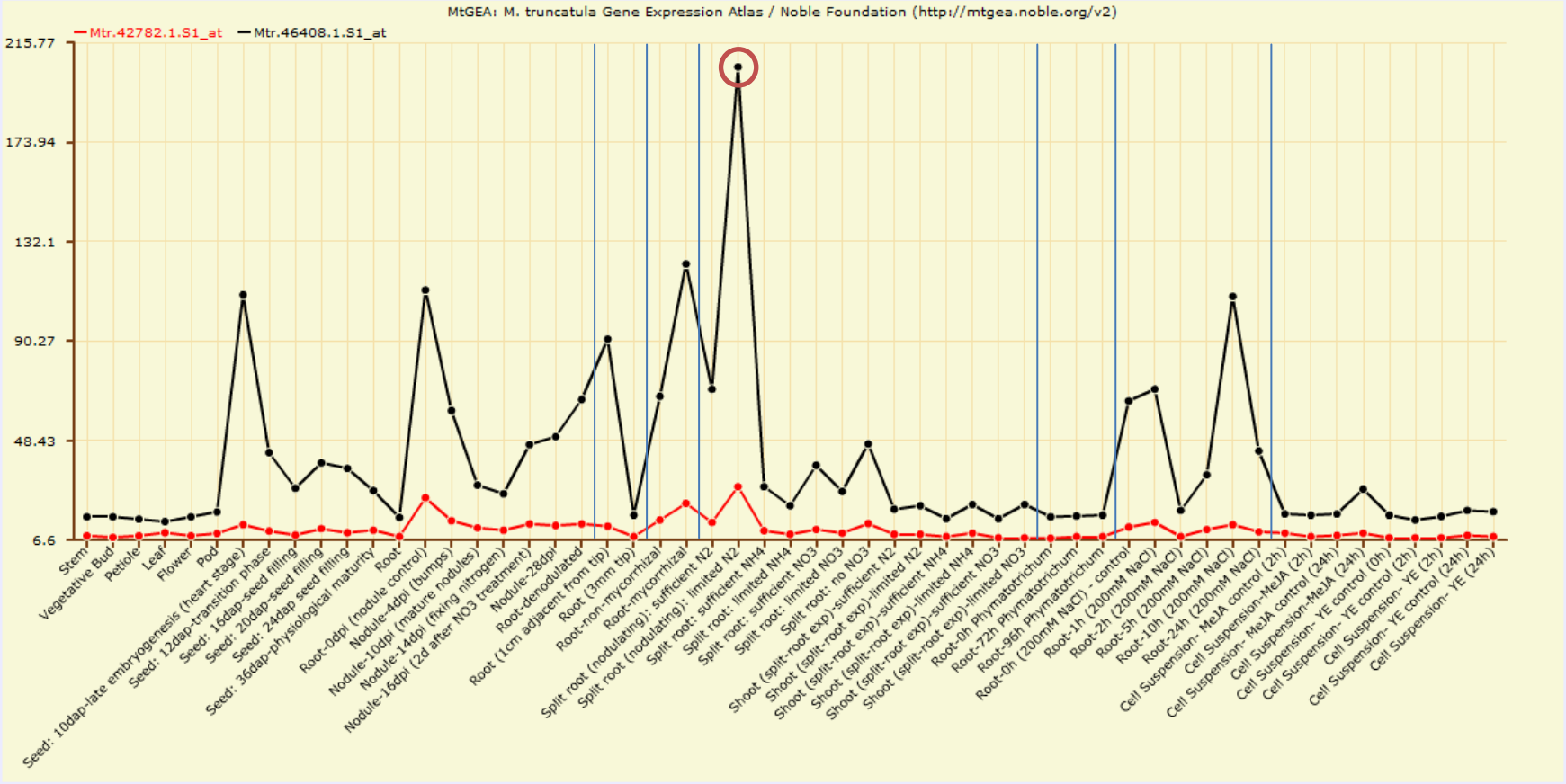
different signal strength. The probeset for *Medtr3g104560* revealed much higher values than those of *Medtr1g015860*, so that plotting on the same graph was impractical (Figure 5-7). Signal strengths are not directly comparable between different probes, and nor are the results of different primer sets in semi-quantitative RTPCR (as they are both influenced by other factors, such as the binding strength of probes and primers), but it is interesting that in both studies *Medtr3g104560* shows the stronger signal of the two, also requiring fewer cycles to amplify in the RTPCR experiment (Figure 5-6). In terms of tissues, *Med3g104560* is very low in shoot tissues with highest expression in the roots, although there is a little in the flowers. In comparison, *Medtr1g015860* seems to be only lowly expressed in roots, in contrast with *Medtr3g104560* and with the RTPCR results. Instead it seems to be only highly expressed in late embryogenesis-stage seeds. Despite these differences in plant-wide relative levels of expression in the root, both genes show similar responses within the roots to nodulation and mycorrhizal symbiosis, with lower expression in roots with nodules than those pre-infection or denodulated, and both increasing in roots with mycorrhizal symbioses, although the relative increase is greater in *Medtr1g015860* (Benedito *et al.*, 2008). There are a few other differences - *Medtr3g104560* may show downregulation responses to biotic stress, as it is slightly reduced both in cell culture in response to yeast elicitors (YE) and in whole roots on infection with the root rot fungus *Phymatotrichum*, whereas *Medtr1g015680* does not seem to respond, but does seem to change on challenge with abiotic (salt) stress, although not with any clear pattern. However, the most interesting difference between the two genes is that found in the experiments described by Ruffel *et al.* (2008), in which split root systems were deprived of nitrate (NO_3^-), (NH_4^-), or for nodulating plants, nitrogen gas (N_2). While it shows relatively little response to NO_3^- or NH_4^- starvation, *Medtr1g015860* is upregulated by a fold change of 3.36 (for probe Mtr.42782.1.S1_at, or 3.46 for probe Mtr.46408.1.S1_at) by nodule deprivation of N_2. Interestingly, in this same set of conditions Ruffel *et al.* found that *MtMAX2* was downregulated by 3.47 fold, although no significant changes to these or any other *MAX* genes (including *Medtr3g104560)* were found. Overall, these data strongly suggest that not only the function, but also the regulation of *Medtr1g015860* has diverged both from that of *AtMAX1* and *Medtr3g104560*.

A



Probeset ID	Line Color	Annotation
Mtr.12616.1.S1_at	#0000FF »	"TC95384 /FEA=mRNA /DEF=similar to UP|Q942Z4 (Q942Z4) Cytochrome P450-like protein, partial (84%)" (*Representative public ID: TC95384*)
Mtr.42782.1.S1_at	#FF0000 »	"TC112387 /FEA=mRNA /DEF=similar to UP|Q940L3 (Q940L3) At2g26170/T19L18.2, partial (28%)" (*Representative public ID: TC112387*)
Mtr.46408.1.S1_at	#000000 »	"1769.m00029 /FEA=mRNA /DEF=AC152551.12 64372 61224 mth2-24d6 similar to PIR|T02607|T02607 probable cytochrome P450 T19L18.2 [similarity] - Arabidopsis thaliana {Arabidopsis thaliana:}, partial (93%)" (*Representative public ID: 1769.m00029*)

B



	Probeset ID	Line Color	Annotation
☐	Mtr.42782.1.S1_at	#FF0000 »	"TC112387 /FEA=mRNA /DEF=similar to UP|Q940L3 (Q940L3) At2g26170/T19L18.2, partial (28%)" (*Representative public ID: TC112387*)
☐	Mtr.46408.1.S1_at	#000000 »	"1769.m00029 /FEA=mRNA /DEF=AC152551.12 64372 61224 mth2-24d6 similar to PIR|T02607|T02607 probable cytochrome P450 T19L18.2 [similarity] - Arabidopsis thaliana {Arabidopsis thaliana;}, partial (93%)" (*Representative public ID: 1769.m00029*)

Figure 5-7. Outputs from the Medicago Gene Atlas (Benedito *et al.*, 2008; He *et al.*, 2009) for probesets Mtr.12616.1.S1_at (*Medtr3g104560*, A, blue line), Mtr.42782.1.S1_at (*Medtr1g015860*, A and B, red line) and Mt.46408.1.S1_at (*Medtrg015860*, A and B, black line). Probeset Mtr.12616.S1_at was removed from B so that the *Medtr1g015860* patterns were visible. Only results from Jemalong A17 are shown, and are sorted by experiment and contributing paper. Blue vertical lines divide data from different papers contributing to the dataset. Red circles highlight equivalent data points from nodulating roots deprived of N_2 from Ruffel *et al.* (2008). dap = days after pollination, dpi = days post inoculation, MeJA = methyl jasmonate, YE = yeast elicitor.

5.2 MAX1 diversity in rice

Monocots are the most agriculturally important group of plants, with production of cereals alone comprising a quarter of global crop production, (2,433 million tonnes) in 2010 (FAO, 2012). The study of the evolution of SL signalling, which has impacts on the agricultural factors of parasitism, symbiotism and branching, the last being a character selected for in domestication of monocots since prehistoric times (Wang *et al.*, 1999) is therefore of clear interest in this phylum. The proliferation of *MAX1* copies within monocots makes it of especial interest for the study of this gene. Three separate, conserved clades within the monocots suggest that *MAX1* may have found three different roles in monocots. Rice is the model monocot for the study of SL biosynthesis, in which all members of the SL pathway but *MAX1* have been identified though mutant phenotype. The exception of *MAX1* implies a high likelihood that at least two of the five *OsMAX1*s are redundantly involved in SL biosynthesis.

5.2.1 Branch phenotype

Of the five orthologues present in rice, two (*Os02g0221900* and *Os06g0565100*, according to the Rice Annotation Project Database – RAP-DB, (Tanaka *et al.*, 2008) - or *Os02g12890* and *Os06g36920* respectively in Rice Genome Annotation Project – RGAP, (Ouyang *et al.*, 2007) – notation,) represent single members of two of the different monocot clades, and were likely produced by whole genome duplication within the monocots, predating divergence of the cereals, as they form part of a syntenic block between chromosomes 2 and 6 dating to the pancereal 'ρ' duplication of approximately 50-70 mya, and are shared in maize and sorghum (Paterson *et al.*, 2004; Salse *et al.*, 2008; syntenic block identified using SyMAP, Soderlund *et al.*, 2011). The

remaining three all belong to the third clade and fall on chromosome one, forming a set of three tandemly repeated genes that may be rice specific, although sorghum also has a tandem pair in this clade at an orthologous position. In the RGAP annotation, these were identified as five loci, designated *Os01g50520* and *Os01g50530* (together forming *Os01g0700900*, RAP-DB), *Os01g50570* and *Os01g50580* (forming *Os01g0701400*) and *Os01g50590*, which corresponds to *Os01g0701500*. Of these, members from all three clades were cloned from *Oryza sativa* Japonica group cultivar Nipponbare, including two, *Os01g0700900* and *Os01g0701500*, from the chromosome 1 tandem repeat. Promoters were added as before and constructs were transformed into *max1-1* plants and phenotyped as described for *PgMAX1*, *SmMAX1* and the *MtMAX1* constructs. As shown in Figure 5-8 - Figure 5-11, three out of the four constructs were capable of rescuing the branching and height of Arabidopsis *max1-1* to at least the same degree as the *35S::AtMAX1* control, with a rescuing orthologue present in each clade. Unlike the case for *Medtr1g015860*, in no lines carrying the single non-rescuing construct, *Os01g0701500* was there any indication of a significant rescue of the *max1-1* phenotype in either branching or height, with nine of ten lines assayed in the first replicate, and four of five in the second, clustering with the phenotype of *max1-1* in Figure 5-11. The odd-one out, line 7.9 (see Figure 5-9) is actually more branchy and even shorter than *max1-1*, suggesting that in this line may have an addition genetic lesion contributing to its phenotype (perhaps caused by the insertion of the transgene at another locus with an effect on branching). Therefore, in the rice orthologues assayed, there appears to be a clear dichotomy in the capability of genes to function in Arabidopsis.

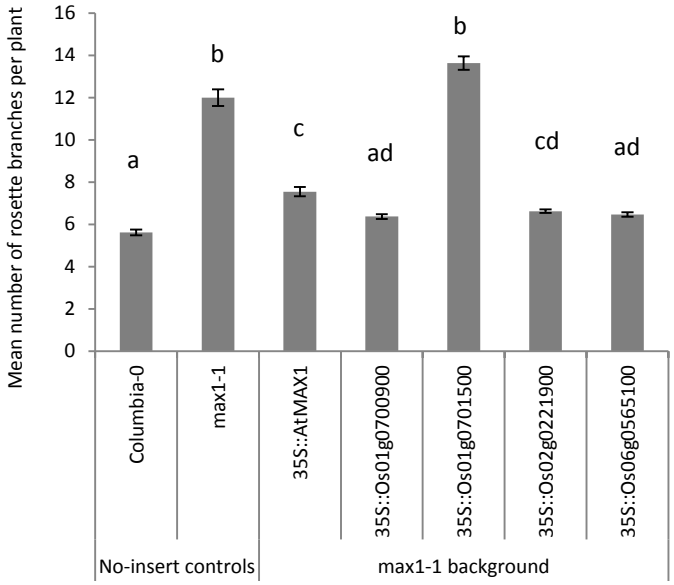


Figure 5-8. Rosette branching of Arabidopsis *max1-1* mutants complemented with *OsMAX1* orthologues under the constitutive 35S promoter. Second and representative of two replicates. Branching was assessed by short-day decapitation assay as described by Greb *et al.* (2003) and shared letters indicates no significant difference in a Kruskal-Wallis test to P≤ 0.001. Data for constructs are mean averages for 10 (*Os01g0700900*), 5 (*Os01g0701500*), 8 (Os02g0221900) and 9 (*Os06g0565100)* independent lines, n for each line = 19-20, except for Columbia-0, *max1-1* and *AtMAX1 max1-1* for which n=40. Error bars show standard error of the mean.

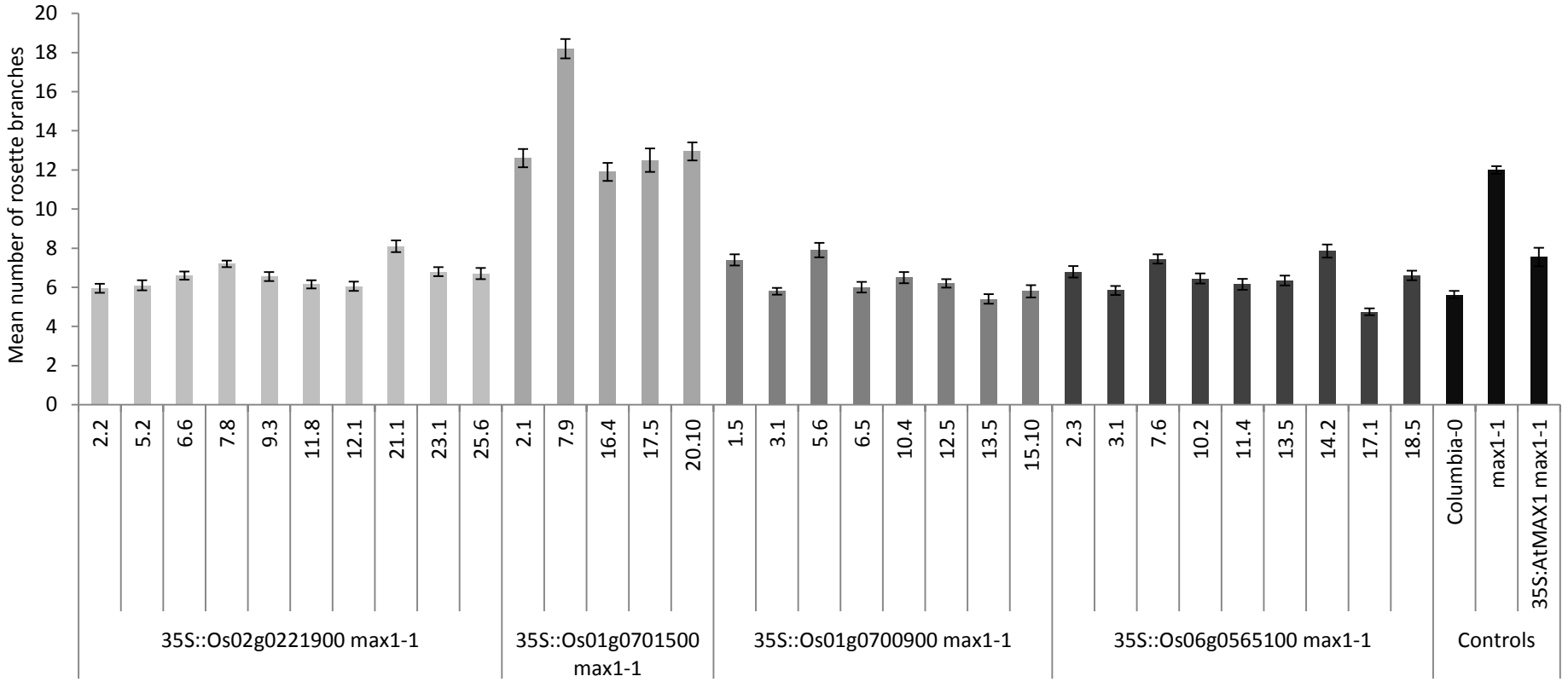


Figure 5-9. Rosette branching of Arabidopsis *max1-1* mutants complemented with *OsMAX1* orthologues under the constitutive 35S promoter, showing independent transgenic lines. Second and representative of two replicates. Branching was assessed by short-day decapitation assay as described by Greb *et al.* (2003). N for each line = 19-20, except for Columbia-0, *max1-1* and *AtMAX1 max1-1* for which n=40. Error bars show standard error of the mean.

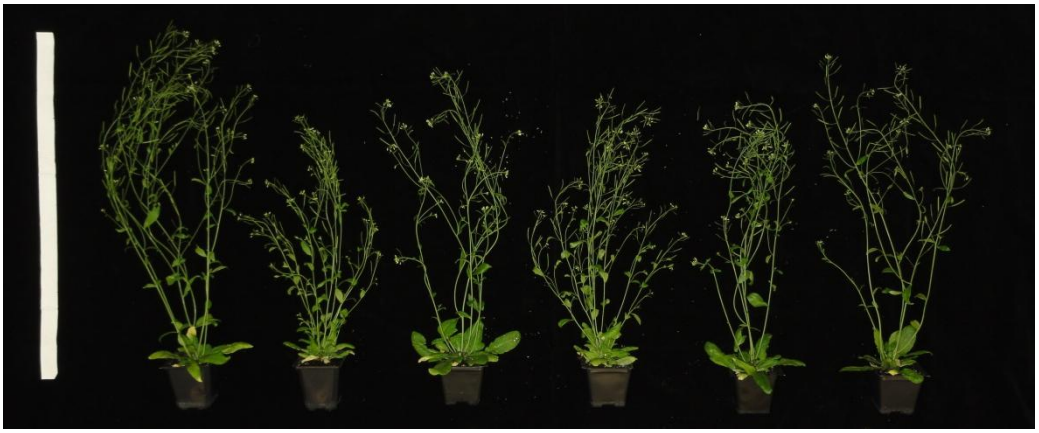


Figure 5-10. Photograph of Columbia-0, *max1-1*, *35S::Os01g0700900 max1-1* line 3.1, *35S::Os01g0701500 max1-1* line 2.1, *35S:: Os02g0221900 max1-1* line 2.2, and *35S:: Os06g0565100 max1-1* line 11.4 from left to right, with all transgenics but *35S::Os01g0701500* showing rescue. White bar = 40cm.

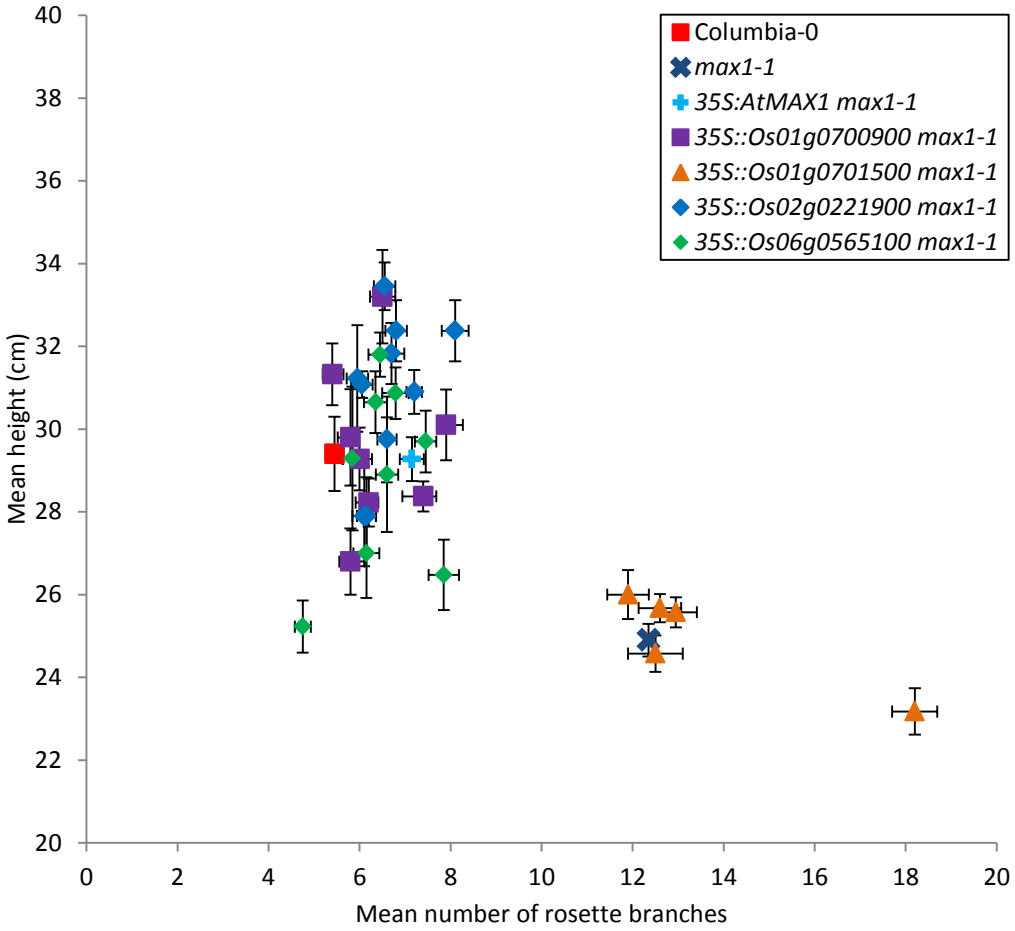


Figure 5-11. Rosette branching and heights of Arabidopsis *max1-1* mutants complemented with *MAX1* orthologues from *Oryza sativa* under the constitutive 35S promoter. N =19-20, except for *max1-1* and Columbia-0 where n=40. Height (in centimetres) of the longest branch was measured the day of scoring for branching. Error bars show standard error of the mean. Note y axis starts at 20cm.

5.2.2 Leaf phenotype

The dichotomy of rice orthologues in rescue capability was also tested in their effect on leaf shape and size (Figure 5-12 and Figure 5-13). As for the non-angiosperm constructs, only two lines were used to explore this phenotype, with information from the first replicate of the branching assay (which did not include *Os06g0565100*, or three of the *Os02g0221900* lines) being used to select lines that showed the least and the most rescue, to reflect the full spread of the phenotypes generated. However, these differences were generally very small in terms of branch rescue, and translated into no significant differences between lines of the same construct for PC3 and PC10 leaf phenotypes. For *Os01g0701500, Os02g0221900* and *Os06g0565100* leaf phenotypes largely mirrored those of branching phenotypes for all PCs, although for the two rescuing constructs, centroids were not completely returned to wild-type size, as was found to be the case for *SmMAX1*. Interestingly, although in the branching assay overall *Os02g0221900* only showed rescue to the level of the *AtMAX1* construct line, rather than to wild-type, the leaf experiment shows full rescue to the Columbia-0 phenotype. However, *Os01g0700900* proves less capable of correcting centroid size, PC3 and PC10 than the others, despite matching their branching activity, suggesting that like *PgMAX1,* it either has been biased by a particularly poorly-rescuing line (which seems less likely here than for *PgMAX1*), or that it perhaps lacks some capability that specifically relates to leaf shape. If this were to be the case, it would be a further example of divergence in function among the rice *MAX1s*, which despite multiple different clades seem to share a high degree of function.

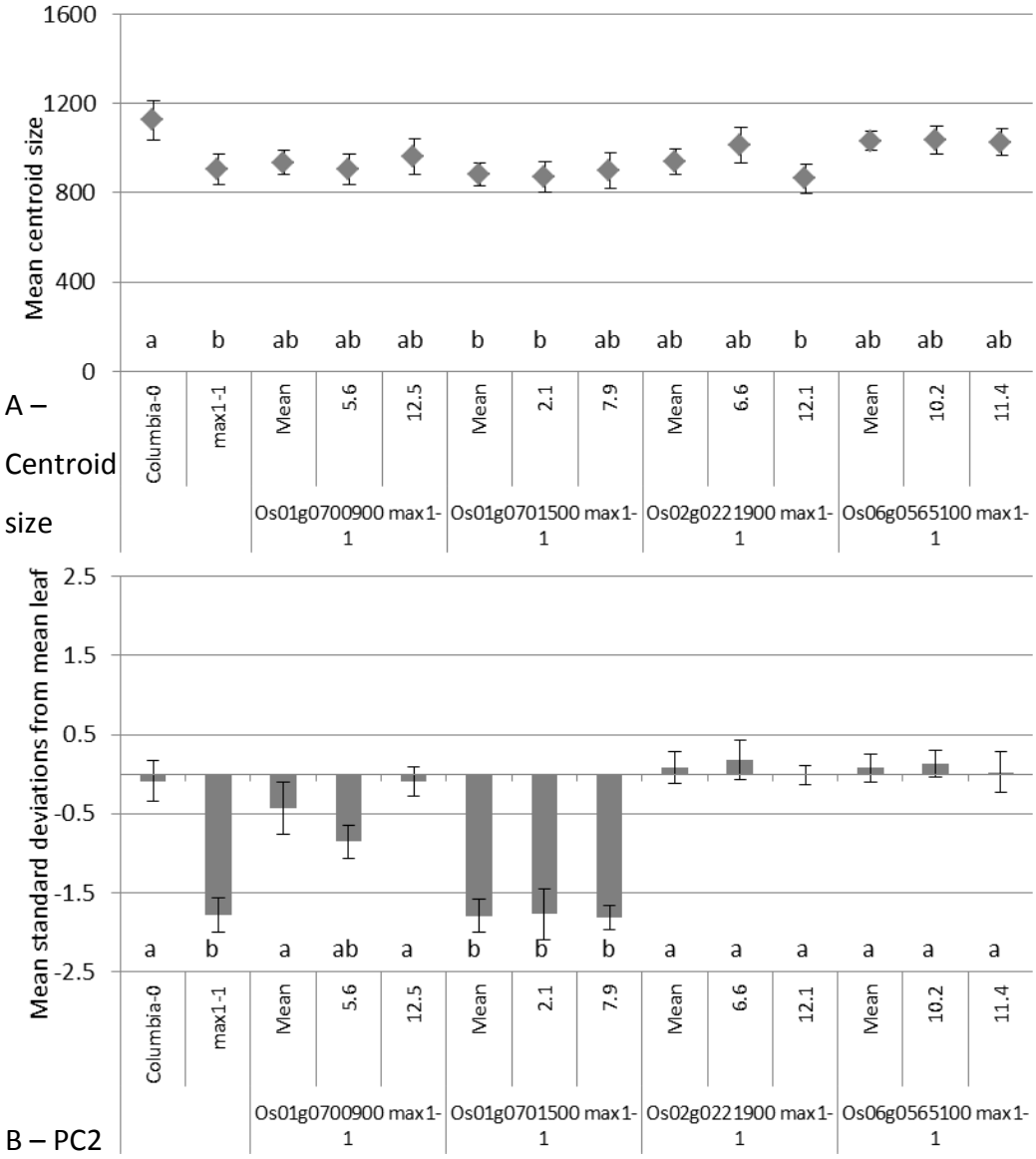


Figure 5-12. Leaf shape analysis for Procrustes-fitted adult leaves four and above from *max1-1* plants complemented with *OsMAX1* orthologues. Error bars are standard error of the mean, calculated on number of plants as n, where n = 15 for controls, and n=6-9 plants per individual transgenic line. Shown are mean centroid sizes (A) and standard deviations from the mean leaf for PC2 (width at centre, B). Line breakdowns are given for phenotypes in which lines of the same construct showed differences in degree of rescue. Letters indicate non-significance in Tamhane's T2 post-hoc test at P>0.001.

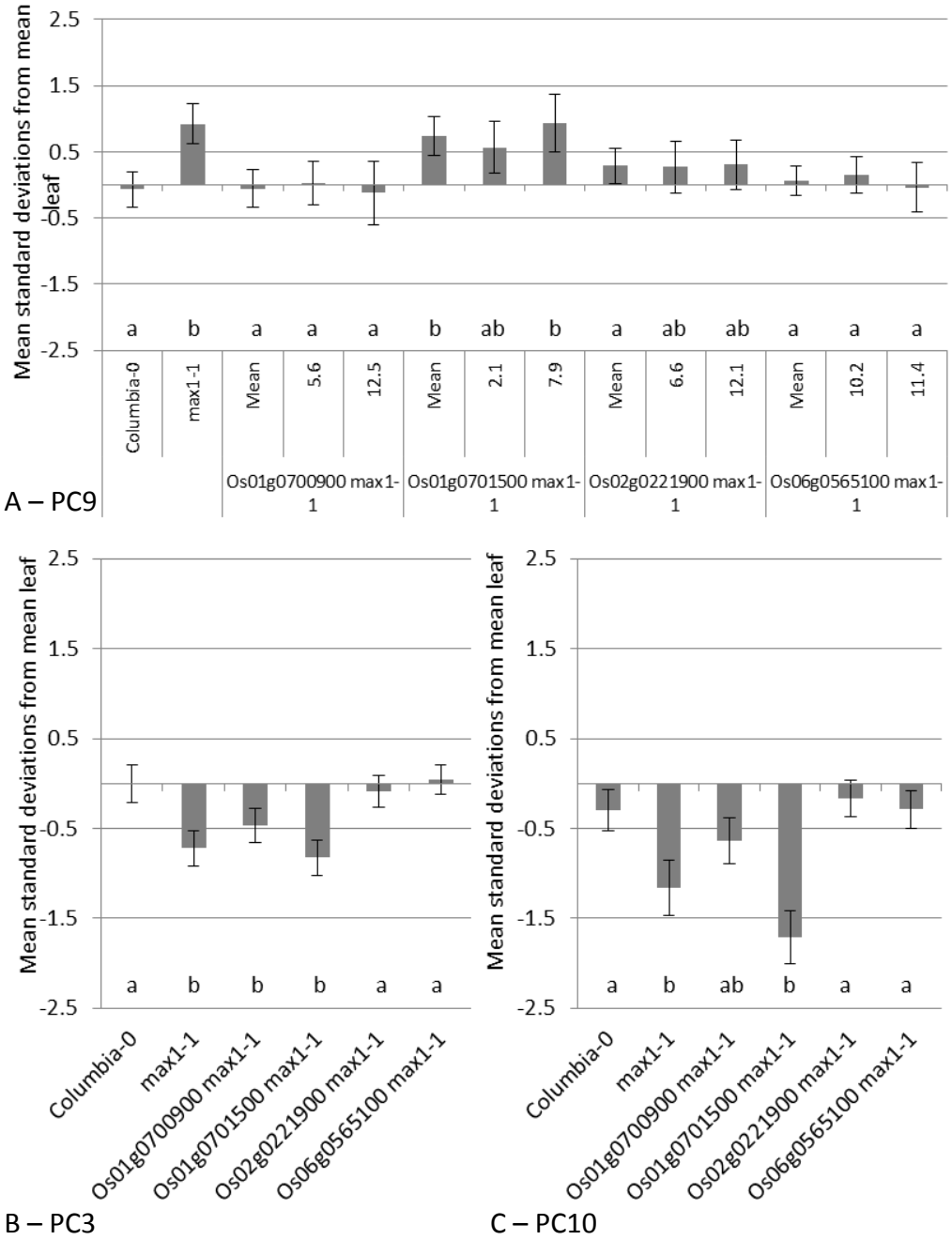


Figure 5-13. Leaf shape analysis for Procrustes-fitted adult leaves four and above from *max1-1* plants complemented with *OsMAX1* orthologues. Error bars are standard error of the mean, calculated on number of plants as n, where n = 15 for controls, and n=6-9 plants per individual transgenic line. Shown are standard deviations from the mean leaf for PC9 (A), PC3 (B) and PC10 (C). Line breakdowns are given for phenotypes in which lines of the same construct showed differences in degree of rescue. Letters indicate non-significance in Tamhane's T2 post-hoc test P>0.05 (PC10) and Tukey's Honestly Significant Difference at P>0.05 for PC9 and PC3.

5.2.3 In planta *expression of* OsMAX *orthologues*

As at least three of the *OsMAX1* orthologues were capable of complementing *Atmax1-1* almost completely, publically available expression databases were again explored for signs of differential expression. The Rice Expression Profile Database (RiceXPro, http://ricexpro.dna.affrc.go.jp) uses the Agilent 44k microarray platform, one of only two with probesets for all five orthologues, and holds data from studies investigating anatomy, leaf development and root development of sequenced cultivar Nipponbare in the field (Sato *et al.*, 2010; Sato *et al.*, 2011). Data from the anatomy and leaf development series were visualised as heatmaps (Figure 5-14) using the meta-analysis database at the Rice Oligonucleotide Array Database (Jung *et al.*, 2008) and do reveal expression differences between orthologues. *Os02g0221900* stands out as being particularly expressed in the leaf blade, increasing over time, but also with some presence in stems and inflorescences. In comparison, *Os06g0565100* is principally expressed in roots and stems, with perhaps some leaf expression. The three chromosome 1 genes show very similar patterns of expression in roots and a little in ripening stems, but do so in a clear series of decreasing overall expression from *Os01g0700900* as the most highly expressed, through the weaker, but almost identical pattern of *Os01g0701400* to *Os01g0701500*, which barely shows expression even in roots. Analysis of the root tissue dataset (not shown as heatmaps were not available) indicated little spatial or developmental differences in expression within the roots for *Os01g0701500* or *Os02g0221900*. In comparison *Os06g0565100* was principally expressed in the endodermal, pericycle and stele tissues and down regulated in the root cap and division zone relative to its expression in the rest of the root, whereas both *Os01g0700900* and *Os01g0701400* showed greater expression in the cortex, and in the developmental series were highest in the elongation zone and younger parts of the maturation zone.

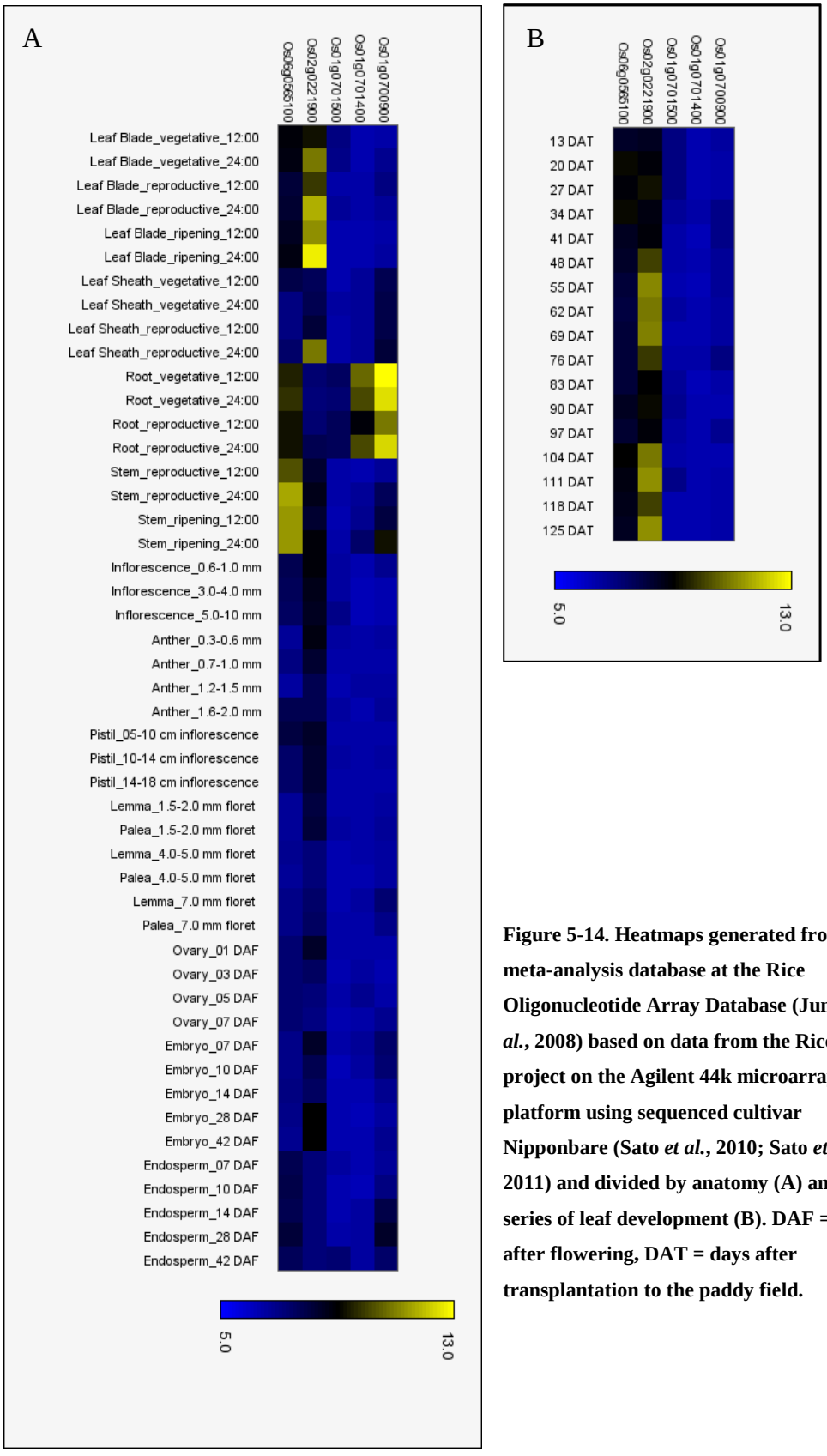


Figure 5-14. Heatmaps generated from the meta-analysis database at the Rice Oligonucleotide Array Database (Jung *et al.*, 2008) based on data from the RiceXPro project on the Agilent 44k microarray platform using sequenced cultivar Nipponbare (Sato *et al.*, 2010; Sato *et al.*, 2011) and divided by anatomy (A) and series of leaf development (B). DAF = days after flowering, DAT = days after transplantation to the paddy field.

5.3 Relating function to gene structure

The functional information from the complementation analysis provided the opportunity to explore links between function and underlying genetic structure in the orthologues. Protein similarities showed no correlation with functional capabilities, as *SmMAX1*, with 34.9% identity to *AtMAX1* rescues, whereas *Medtr1g015860*'s capability is much reduced despite sharing 67% identity to *AtMAX1*. Therefore protein sequence alignments, shown in Figure 5-15, were inspected more closely to discover whether there were any residues that may have contributed to the incapability of *Os01g0701500* and the reduced ability of *Medtr1g015860* to complement Arabidopsis.

In the case of *Os01g0701500* a deletion at the 3' end of the sequence presents a strong candidate for the explanation of its complete loss of *MAX1-like* function, as 19 residues have been lost compared to *AtMAX1*, including two highly conserved lysine residues (K), an otherwise completely conserved glycine (G) that is also found in many other cytochrome P450s from Arabidopsis (Paquette *et al.*, 2009) and an arginine (R) residue that represents the end of the consensus sequence and which is conserved in all other *MAX1* orthologues (see Figure 3-5). This deletion appears to have arisen from a mutation resulting in a premature TAG stop codon, possibly by C-to-T transition from the codon for tryptophan, the amino acid present at this position in *PgMAX1*, *Os06g0565100*, and *Os01g0701400*, the last being the closest relative *Os01g0701500*. Given the number of residues deleted, and their conservation not only in *MAX1* sequences but in other CYPs (indeed, no annotated CYP in Arabidopsis has so few residues at the 3' end after the haem-binding motif), it is quite possible that *Os01g0701500* has not only lost *MAX1* function, but all function, and is becoming a pseudogene.

For *Medtr1g015860*, no deletion of conserved residues was found, but comparison of consensus sequences calculated with and without the two non-*MAX1*-function orthologues revealed only two residues in *Medtr1g015860* different to the consensus (highlighted in yellow in Figure 5-15). These are a change from an aspartic acid (D-286 in *AtMAX1*) to an asparagine and of a phenylalanine (F-431) in Arabidopsis) to a tyrosine. Of these two, D-286 is on

the edge of the fourth Substrate Recognition Sequence defined in Nelson *et al.* (2008), and might be a candidate for the change in function. However, there are several differences compared to the other sequences in non-conserved regions, which may also contribute to affect function. As the mutation(s) that have changed the *MAX1-like* function in *Medtr1g015860* do not seem to have abolished that function, without information on the structure of the CYP711A enzymes and their substrates, the sequence changes leading to function change in *Medtr1g015860* are much less easy to identify than those of *Os01g0701500.*

Figure 5-15. Alignment of protein sequences for all constructs transformed into transgenics. Consensus sequences (100% identity threshold) were generated in BioEdit, both for all sequences, and for only those that showed the capability to completely rescue branching in Arabidopsis *max1-1*.

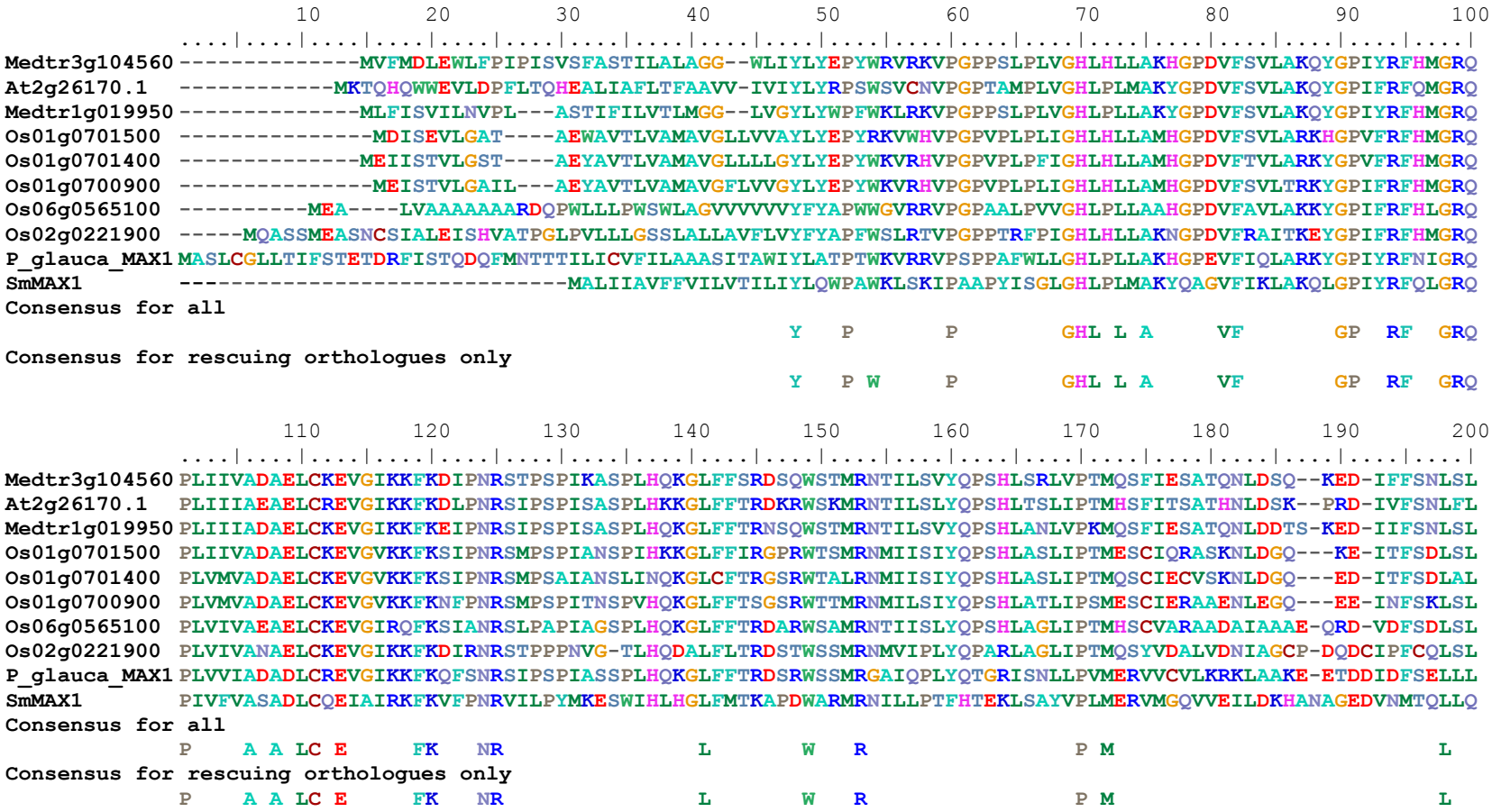


Figure 5-15

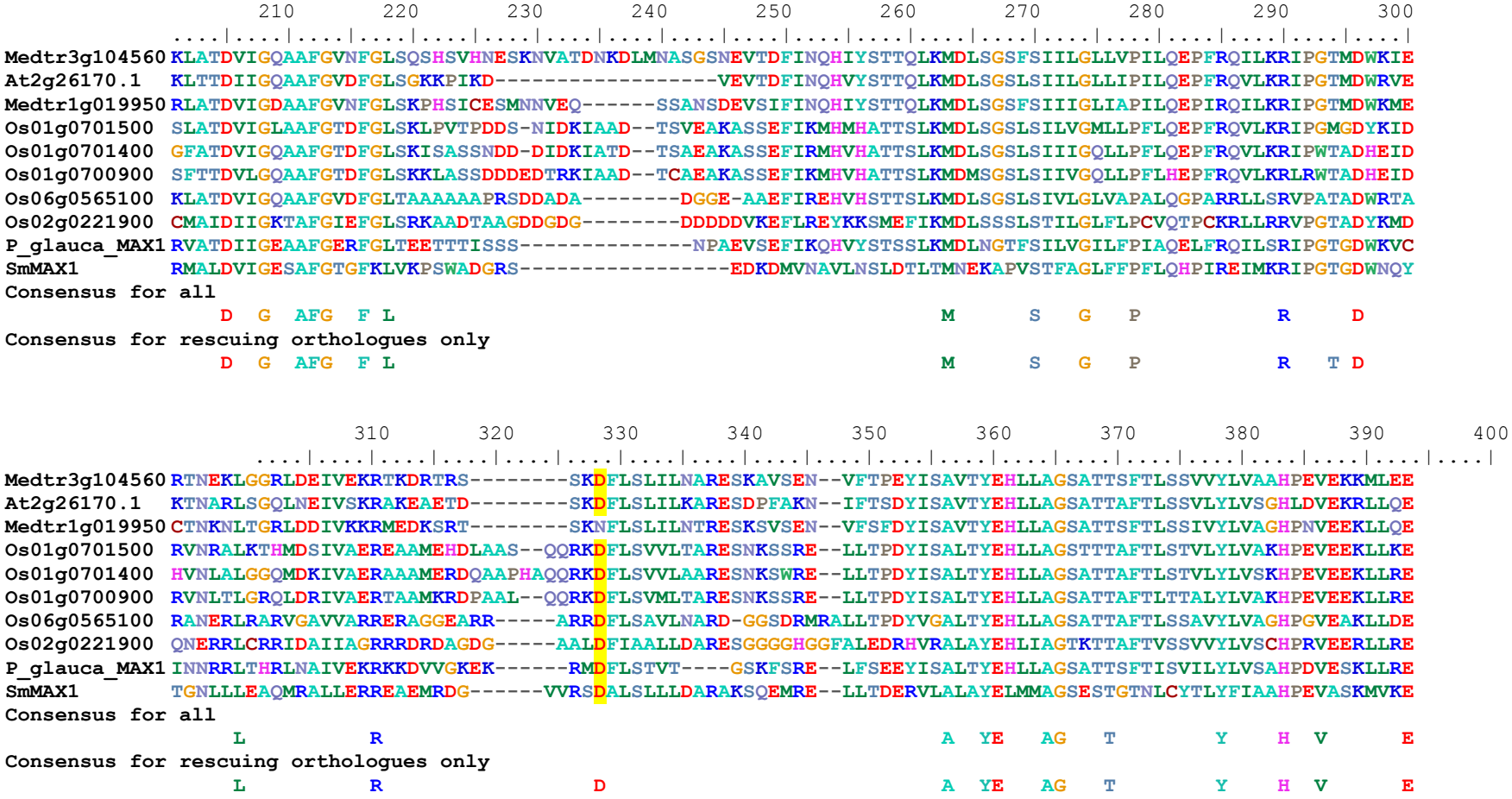
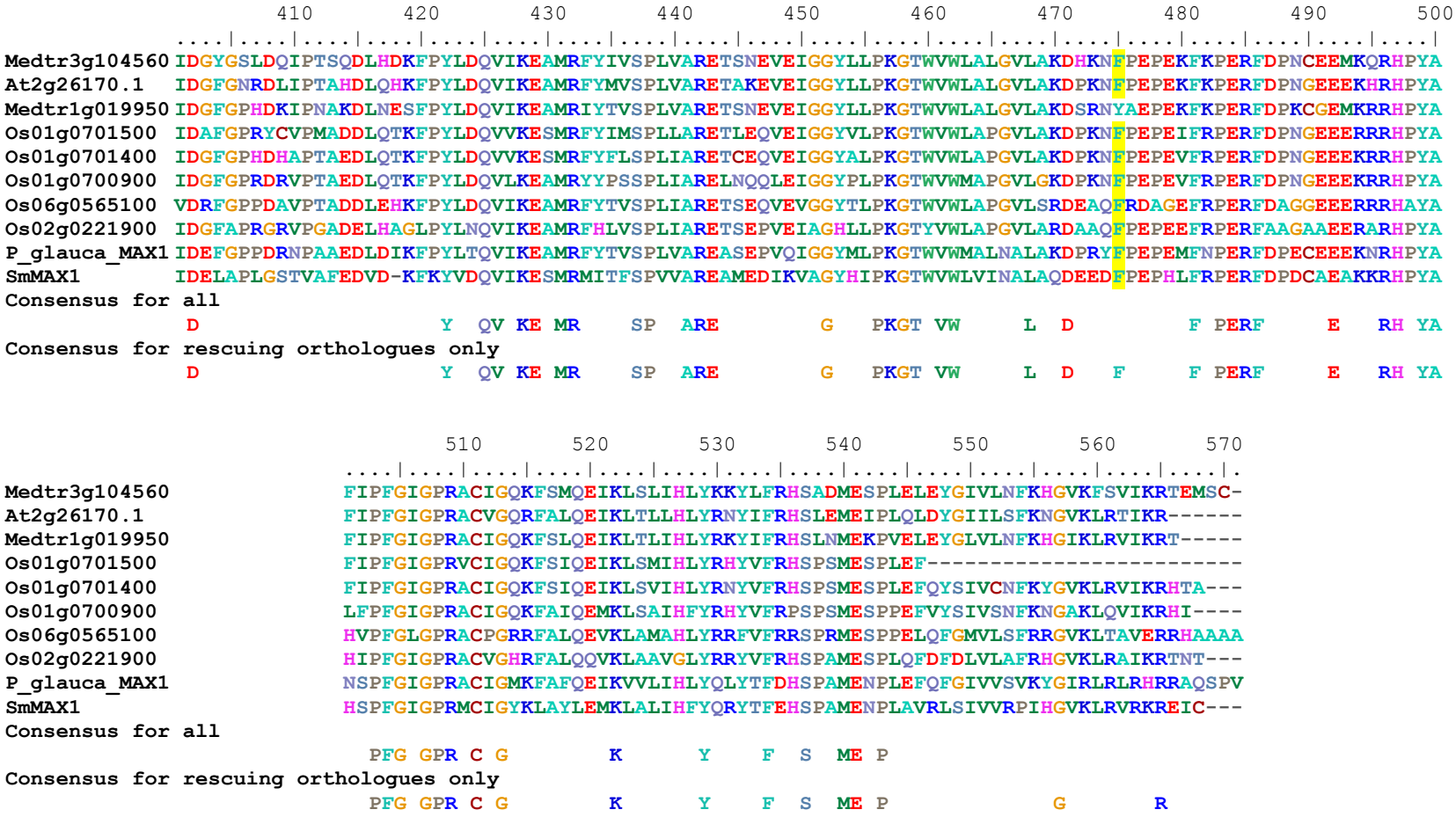

210 220 230 240 250 260 270 280 290 300
Medtr3g104560 KLATDVIGQAAFGVNFGLSQSHSVHNESKNVATDNKDLMNASGSNEVTDFINQHIYSTTQLKMDLSGSFSIILGLLVPILQEPFRQILKRIPGTMDWKIE
At2g26170.1 KLTTDIIGQAAFGVDFGLSGKKPIKD----------VEVTDFINQHVYSTTQLKMDLSGSLSIILGLLIPILQEPFRQVLKRIPGTMDWRVE
Medtr1g019950 RLATDVIGDAAFGVNFGLSKPHSICESMNNVEQ------SSANSDEVSIFINQHIYSTTQLKMDLSGSFSIIIGLIAPILQEPIRQILKRIPGTMDWKME
Os01g0701500 SLATDVIGLAAFGTDFGLSKLPVTPDDS-NIDKIAAD--TSVEAKASSEFIKMHMHATTSLKMDLSGSLSILVGMLLPFLQEPFRQVLKRIPGMGDYKID
Os01g0701400 GFATDVIGQAAFGTDFGLSKISASSNDD-DIDKIATD--TSAEAKASSEFIRMHVHATTSLKMDLSGSLSIIIGQLLPFLQEPFRQVLKRIPWTADHEID
Os01g0700900 SFTTDVLGQAAFGTDFGLSKKLASSDDDEDTRKIAAD--TCAEAKASSEFIKMHVHATTSLKMDMSGSLSIIVGQLLPFLHEPFRQVLKRLRWTADHEID
Os06g0565100 KLATDVIGQAAFGVDFGLTAAAAAAPRSDDADA--------DGGE-AAEFIREHVHSTTSLKMDLSGSLSIVLGLVAPALQGPARRLLSRVPATADWRTA
Os02g0221900 CMAIDIIGKTAFGIEFGLSRKAADTAAGDDGDG--------DDDDDVKEFLREYKKSMEFIKMDLSSSLSTILGLFLPCVQTPCKRLLRRVPGTADYKMD
P_glauca_MAX1 RVATDIIGEAAFGERFGLTEETTTISSS----------NPAEVSEFIKQHVYSTSSLKMDLNGTFSILVGILFPIAQELFRQILSRIPGTGDWKVC
SmMAX1 RMALDVIGESAFGTGFKLVKPSWADGRS----------EDKDMVNAVLNSLDTLTMNEKAPVSTFAGLFFPFLQHPIREIMKRIPGTGDWNQY
Consensus for all
D G AFG F L M S G P R D
Consensus for rescuing orthologues only
D G AFG F L M S G P R T D
310 320 330 340 350 360 370 380 390 400
Medtr3g104560 RTNEKLGGRLDEIVEKRTKDRTRS--------SKDFLSLILNARESKAVSEN--VFTPEYISAVTYEHLLAGSATTSFTLSSVVYLVAAHPEVEKKMLEE
At2g26170.1 KTNARLSGQLNEIVSKRAKEAETD--------SKDFLSLILKARESDPFAKN--IFTSDYISAVTYEHLLAGSATTAFTLSSVLYLVSGHLDVEKRLLQE
Medtr1g019950 CTNKNLTGRLDDIVKKRMEDKSRT--------SKNFLSLILNTRESKSVSEN--VFSFDYISAVTYEHLLAGSATTSFTLSSIVYLVAGHPNVEEKLLQE
Os01g0701500 RVNRALKTHMDSIVAEREAAMEHDLAAS--QQRKDFLSVVLTARESNKSSRE--LLTPDYISALTYEHLLAGSTTTAFTLSTVLYLVAKHPEVEEKLLKE
Os01g0701400 HVNLALGGQMDKIVAERAAAMERDQAAPHAQQRKDFLSVVLAARESNKSWRE--LLTPDYISALTYEHLLAGSATTAFTLSTVLYLVSKHPEVEEKLLRE
Os01g0700900 RVNLTLGRQLDRIVAERTAAMKRDPAAL--QQRKDFLSVMLTARESNKSSRE--LLTPDYISALTYEHLLAGSATTAFTLTTALYLVAKHPEVEEKLLRE
Os06g0565100 RANERLRARVGAVVARRERAGGEARR-----ARRDFLSAVLNARD-GGSDRMRALLTPDYVGALTYEHLLAGSATTAFTLSSAVYLVAGHPGVEAKLLDE
Os02g0221900 QNERRLCRRIDAIIAGRRRDRDAGDG-----AALDFIAALLDARESGGGGHGGFALEDRHVRALAYEHLIAGTKTTAFTVSSVVYLVSCHPRVEERLLRE
P_glauca_MAX1 INNRRLTHRLNAIVEKRKKDVVGKEK------RMDFLSTVT----GSKFSRE--LFSEEYISALTYEHLLAGSATTSFTISVILYLVSAHPDVESKLLRE
SmMAX1 TGNLLLEAQMRALLERREAEMRDG------VVRSDALSLLLDARAKSQEMRE--LLTDERVLALAYELMMAGSESTGTNLCYTLYFIAAHPEVASKMVKE
Consensus for all
L R A YE AG T Y H V E
Consensus for rescuing orthologues only
L R D A YE AG T Y H V E

Figure 5-15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At2g26170.1 IDGFGNRDLIPTAHDLQHKFPYLDQVIKEAMRFYMVSPLVARETAKEVEIGGYLLPKGTWVWLALGVLAKDPKNFPEPEKFKPERFDPNGEEEKHRHPYA
Medtr1g019950 IDGFGPHDKIPNAKDLNESFPYLDQVIKEAMRIYTVSPLVARETSNEVEIGGYLLPKGTWVWLALGVLAKDSRNYAEPEKFKPERFDPKCGEMKRRHPYA
Os01g0701500 IDAFGPRYCVPMADDLQTKFPYLDQVVKESMRFYIMSPLLARETLEQVEIGGYVLPKGTWVWLAPGVLAKDPKNFPEPEIFRPERFDPNGEEERRRHPYA
Os01g0701400 IDGFGPHDHAPTAEDLQTKFPYLDQVVKESMRFYFLSPLIARETCEQVEIGGYALPKGTWVWLAPGVLAKDPKNFPEPEVFRPERFDPNGEEEKRRHPYA
Os01g0700900 IDGFGPRDRVPTAEDLQTKFPYLDQVLKEAMRYYPSSPLIARELNQQLEIGGYPLPKGTWVWMAPGVLGKDPKNFPEPEVFRPERFDPNGEEEKRRHPYA
Os06g0565100 VDRFGPPDAVPTADDLEHKFPYLDQVIKEAMRFYTVSPLIARETSEQVEVGGYTLPKGTWVWLAPGVLSRDEAQFRDAGEFRPERFDAGGEEERRRHAYA
Os02g0221900 IDGFAPRGRVPGADELHAGLPYLNQVIKEAMRFHLVSPLIARETSEPVEIAGHLLPKGTYVWLAPGVLARDAAQFPEPEEFRPERFAAGAAEERARHPYA
P_glauca_MAX1 IDEFGPPDRNPAAEDLDIKFPYLTQVIKEAMRFYTVSPLVAREASEPVQIGGYMLPKGTWVWMALNALAKDPRYFPEPEMFNPERFDPECEEEKNRHPYA
SmMAX1 IDELAPLGSTVAFEDVD-KFKYVDQVIKESMRMITFSPVVAREAMEDIKVAGYHIPKGTWVWLVINALAQDEEDFPEPHLFRPERFDPDCAEAKKRHPYA
Consensus for all
D Y QV KE MR SP ARE G PKGT VW L D F PERF E RH YA
Consensus for rescuing orthologues only
D Y QV KE MR SP ARE G PKGT VW L D F F PERF E RH YA
510 520 530 540 550 560 570
Medtr3g104560 FIPFGIGPRACIGQKFSMQEIKLSLIHLYKKYLFRHSADMESPLELEYGIVLNFKHGVKFSVIKRTEMSC-
At2g26170.1 FIPFGIGPRACVGQRFALQEIKLTLLHLYRNYIFRHSLEMEIPLQLDYGIILSFKNGVKLRTIKR------
Medtr1g019950 FIPFGIGPRACIGQKFSLQEIKLTLIHLYRKYIFRHSLNMEKPVELEYGLVLNFKHGIKLRVIKRT-----
Os01g0701500 FIPFGIGPRVCIGQKFSIQEIKLSMIHLYRHYVFRHSPSMESPLEF------------------------
Os01g0701400 FIPFGIGPRACIGQKFSIQEIKLSVIHLYRNYVFRHSPSMESPLEFQYSIVCNFKYGVKLRVIKRHTA---
Os01g0700900 LFPFGIGPRACIGQKFAIQEMKLSAIHFYRHYVFRPSPSMESPPEFVYSIVSNFKNGAKLQVIKRHI----
Os06g0565100 HVPFGLGPRACPGRRFALQEVKLAMAHLYRRFVFRRSPRMESPPELQFGMVLSFRRGVKLTAVERRHAAAA
Os02g0221900 HIPFGIGPRACVGHRFALQQVKLAAVGLYRRYVFRHSPAMESPLQFDFDLVLAFRHGVKLRAIKRTNT---
P_glauca_MAX1 NSPFGIGPRACIGMKFAFQEIKVVLIHLYQLYTFDHSPAMENPLEFQFGIVVSVKYGIRLRLRHRRAQSPV
SmMAX1 HSPFGIGPRMCIGYKLAYLEMKLALIHFYQRYTFEHSPAMENPLAVRLSIVVRPIHGVKLRVRKREIC---
Consensus for all
PFG GPR C G K Y F S ME P
Consensus for rescuing orthologues only
PFG GPR C G K Y F S ME P G R

5.4 Discussion

The concept that the duplication of genetic material provides the substrate for evolutionary novelties, through subfunctionalisation or neofunctionalisation, has a very long history (reviewed in Taylor and Raes, 2004). However, the majority of duplicates are lost through degeneration, and selection is required to maintain the duplicates in the population. Given the plethora of WGDs known in the angiosperm lineage, if every copy of the *MAX* paralogues deriving from the ancestral angiosperm had been conserved, Arabidopsis would by now have at least 12 parallel SL pathways (from the α and β duplications, and the γ hexaploidisation), and rice would have 4 (from the ρ and σ duplications) and both of these numbers exclude the contribution of local duplications to individual genes. However, the paralogues of most members of the pathway have degraded beyond recognition in most lineages, even those of *MAX2*, which as an F-box LRR protein is one of the more conserved members of a family with an otherwise high gene birth rate (Xu *et al.*, 2009). The exceptions are the triplet- and twin-clades of *D14* and *D27*, and *MAX1*, and within the *D14* and *D27* clades copy-numbers are conserved within the angiosperms (R. Challis et al., in preparation, and Waters *et al.*, 2012). Even in *MAX1*, the most notable duplications have persisted principally in the monocot lineages, to which both WGDs and local tandem duplications have contributed. There are several driving forces for the maintenance of duplicates – subfunctionalisation, neofunctionalisation or functional buffering (redundancy), the likelihood of each being influenced by many factors, including the mechanism of duplication and their original function (Chapman *et al.*, 2006; reviewed in Lynch, 2007, Chapter 8; Wang *et al.*, 2011b). Complementation and expression pattern analyses were therefore used to investigate why the duplicates of *MAX1* present in some eudicot species and all monocot species had been retained. Three possibilities were considered – that they had diverged in function or expression, providing added flexibility of control or new functions, that they were contributing to redundancy in the pathway, or that they were in the process of becoming non-functional.

For the two paralogues of *AtMAX1* in Medicago, divergence of function by one paralogue is clear both in terms of function and regulation (factors that may

not necessarily coevolve). These paralogues are part of a small region of microsynteny between chromosomes 1 and 3 remaining from a WGD estimated to have occurred between the emergence of the legumes and the papilinoid subfamily, to which both Medicago and pea belong, about 58 million years ago (Young *et al.*, 2011). *Glycine max* (soybean) is also in the Papilinoideae group and shares this region of synteny on three chromosomes, having undergone a second, *Glycine* specific WGD approximately 13 mya, resulting in four *MAX1* orthologues (Schmutz *et al.*, 2010). This syntenic area is also shared with a region on chromosome 4, also containing a *MAX1* orthologue, of the grapevine *Vitis vinifera*, regions on poplar chromosomes 6 and 18 (again, the locations of the *PtMAX1* orthologues), and with the Arabidopsis chromosome 2 site of *AtMAX1* itself. The legumes, poplar and Arabidopsis are all fabids (or belong to the eurosid I group), whereas grapevine does not belong to either of the eurosid groupings (although it is a rosid), and unlike the others is not thought to have undergone any WGD events since the γ hexaploidisation shared by all eudicots studied (Jaillon *et al.*, 2007; Argout *et al.*, 2011; Young *et al.*, 2011). This suggests that this apparently shared locus is the ancestral site for a single-copy rosid *MAX1*. If, as the results from *PgMAX1* and *SmMAX1* suggest, and results from Drummond *et al.* suggest for a *MAX1* orthologue for petunia (2012), the *AtMAX1* function corresponds to that of the ancestral *paleoMAX1* function, then it would appear that the *Medtr3g104560* paralogue has conserved this enzyme capability, whereas *Medtr1g015860* has diverged away from it. In either case, it implies that the functional redundancy supplied by *Medtr1g015860* to SL production is likely to be limited, raising the question of whether the mutants in the putative orthologues in pea are missing from SL mutant collections because they are hidden by *MAX1* functional redundancy, or are currently just plain missing, like the pea equivalent of *D27*. It may be that the *MAX1* functions of the pea orthologues have not diverged to the same extent or fate as those of *Medtr1g015860* and *Medtr3g104560* mutant, as peas and medics belong to different tribes of the Papillionoideae subfamily. Indeed, given the apparent conservation of the whole syntenic region of the eudicot *MAX1* locations, it may be that the *MAX1s* have been shielded from the degradation, and especially the rearrangement particularly common in angiosperms, by the close presence of some other, particularly critical factor to eudicot fitness, so that for some of

evolutionary time their own fate has been or still is more dependent on that of another gene than on their own contribution to plant survival.

Whether the functional divergence in *Medtr3g015860* is neo- or subfunctionalisation cannot be judged from the plant-scale phenotype rescue, as CYP450s may carry out several different reactions on the same or different substrates, and although *Medtr1g015860* appears to have lost most capacity to catalyse the ancestral reaction, it retains some. It may thus just have evolved to be optimally adapted to one of the different reactions within a subset that may have originally been catalysed by the ancestral protein. More detailed enzyme kinetic analysis would be required to resolve this point. The expression data, however, may support a hypothesis of subfunctionalisation at the level of regulation, as although the paralogues share very similar patterns, *Medtr1g015860* is specifically upregulated in response to the starvation of nodulating roots of N_2 (Ruffel *et al.*, 2008). Both legumes and non-legumes are known to increase SL exudation in response to phosphate starvation (likely to increase AMy symbiosis), and there is increasing evidence that several plants also upregulate SL synthesis in response to nitrogen (N) starvation. The divide between those species that do or do not exude SLs on N limitation does not correspond to the legume/non legume divide – for example, *Medicago sativa* (alfalfa) is among the legumes that do not increase SLs in response to N limitation (Yoneyama *et al.*, 2012). However, SLs in pea roots have been implicated in promoting the formation of nodules, and this appears to be through an effect on plant development, rather than signalling to the rhizobia, as GR24 does not induce calcium signalling or *nod* gene expression in these bacteria (Moscatiello *et al.*, 2010; Soto *et al.*, 2010; Foo and Davies, 2011). Nevertheless, the production of SLs in *Medicago truncatula* was recently demonstrated to require NODULATION SIGNALLING PATHWAY1 (NSP1) and NSP2, transcription factors from the GRAS family (Liu *et al.*, 2011). *NSP1* and *NSP2* are involved in both the nodulation- and AMy symbiotic signalling pathways in *Medicago truncatula* and *Lotus japonicus*, although *NSP1* is nodulation-specific and the impact on mycorrhizal symbiosis in *nsp2* mutants is only a 41% reduction in colonisation in *M. truncatula* (Maillet *et al.*, 2011). However, these genes are widely conserved throughout the angiosperms and are

functionally conserved at the protein level (from complementation experiments similar to those used here) in both non-leguminous eudicots and in monocots, suggesting that their ancestral purpose, if symbiotically related at all, is more likely to be in the more ancient and wide-spread AMy symbiosis, similarly to SL signalling (Heckmann *et al.*, 2006; Parniske, 2008; Yokota *et al.*, 2010). Liu and co-workers investigated global gene expression in each of the *M. truncatula nsp1* and *nsp2* single mutants, identifying the *MtDWARF27* orthologue and *Medtr3g104560* (termed '*MAX1*' in that study) as being downregulated in both. Their subsequent investigations demonstrated that *NSP1* is required for the production of SLs in Medicago, as well as for high expression and response to phosphate in *MtD27*. *nsp2* still maintains some, lowered *D27* expression and actually has upregulated production of orobanchol, although it is required for the production of didehydro-orobanchol, the major SL in *M. truncatula*. Likewise in rice, double knock-down of the single rice orthologues for *NSP1* and *NSP2* caused reduced expression of *D27*, reduced production of SLs, and like other SL deficient mutants, increased tiller numbers. Although shoot branching was not increased in the corresponding *M. truncatula* mutants, this does not rule out a role for SLs in Medicago shoot branching control, as the Jemalong A17 cultivar used does not show significant dormancy of axillary meristems, the branching phenotype under SL control, although some other *M. truncatula* accessions do, and both these and A17 respond to exogenously applied SLs (C. Mouchel, and O. Leyser, pers. comm.s). Interestingly, the authors also conclude that *NSP1* and *NSP2*-dependent regulation of SLs is not affected by loss of other signal transduction elements in this pathway, and although their data on *MtD27* expression in the corresponding mutants shows no downregulation, they may show some upregulation. This may be of interest, as if SLs are acting to promote nodulation, a lack of fungally- or rhizobially-initiated signalling might indeed be expected to upregulate SL production. If so, the specific upregulation of *Medtr1g015860* under nodulation stress may reflect this, perhaps, for example, as a result of a restriction to regulation by only the nodulation-specific *NSP1*, whereas *Medtr3g104560* is regulated by both *NSPs* more generally in response to symbiosis. If so, it would be fascinating to know whether the changed catalytic function of *Medt1g015860* reflected an adaptation to a nodulation-specific role. The mechanism (direct, indirect &c.) of

NSP regulation of SL genes is still not known, and nor is the regulation of *Medtr1g015860* in response to *NSP1*, as Liu *et al.* investigated only genes jointly regulated by both *NSPs*, but it might represent a hypothesis worth testing.

Although the enzymatic functions of the legume *MAX1* duplicates have not completely diverged, the ancestral *MAX1* function of the rice orthologue *Os01g0701500* has clearly been entirely lost from *Oryza sativa* cultivar Nipponbare. Unlike the two *MtMAX1* genes the time of the duplication arising in *Os01g0701500* is less easy to estimate, as it is at one end of the three-gene tandem repeat on chromosome one, one of which may originally derive from the first σ pancereal duplication. This set of repeats is probably rice specific, but sorghum also has a tandem pair in this clade at an orthologous position, and *Brachypodium distachyon*, a closer relative to rice in the same Pooideae subfamily, has two orthologues that are closely linked in this clade, perhaps suggesting a predilection to local duplication at the ancestral location. Although its age is in doubt, *Os01g0701500*'s evolutionary fate seems fairly clear – losing at least 19 residues from the conserved 3' end would probably destine it to join the majority of duplicates in losing their function and degrading beyond recognition. However, *Os01g0701500* still has an interesting role in the recent evolutionary history of *Oryza sativa*. A collaboration between the group of Dr Harro Bouwmeester at Wageningen University in the Netherlands and that of Dr Adam Price at the University of Aberdeen has identified quantitative trait loci (QTL) for tiller and strigolactone production in a cross between the high-tillering, low SL producing Bala cultivar of the *Indica* group and the low-tillering, high SL producing *Japonica* cultivar Azucena (Cardoso *et al.*, in review). This QTL centres on the *MAX1* tandem repeat, which is present in the Azucena cultivar, but has been rearranged in the Bala cultivar, deleting *Os01g0700900* and *Os01g0701400* and repeating *Os01g0701500* twice. In collaboration with these groups, both *35S::Os01g0700900* (as detailed here) and *35S::Os01g0701400* (by Yanxia Zhang at Wageningen) have been found to be capable of complementing Arabidopsis *max1-1* branching phenotypes fully, suggesting that their deletion in Bala is the cause of the variation in tillering and SL production phenotypes. Further investigation by the groups in Wageningen

and Aberdeen on the presence of the deletion allele in the RiceHapMap cultivars have found that it consistently associated with low SL and high tillering phenotypes, and is far more frequent among cultivars of the Indica group (in 126 of 133 tested) than those of the Japonica group (34 of 190 tested, with 31 of those in the 94 tropical japonicas). The rearrangement seems closely associated with the Indica/Japonica divide, itself probably reflecting different domestication events, and perhaps the reduced need in wetland cultivars for SLs to signal to AMy, as phosphate is much more mobile in water than soil.

However, the duplicated copies of *Os01g0701500* in the sequenced Indica cultivar, 93-11, at least, do not have the 3' premature stop codon found in the Japonica Nipponbare genome sequence, instead having a tryptophan residue (as presumed to be mutated in the Japonica allele) followed by another 21 residues, including the conserved glycine, lysine and arginine residues. Whether these orthologues are active or not in SL production is unknown, as no complementation analysis has been carried out on them. Cultivars carrying the Bala/Indica allele do still produce SLs, and it is possible that the *Os01g0701500* paralogues contribute to these (although clearly less efficiently than the *Os01g070900-Os01g0701400* haplotype). Equally this role could be carried entirely by the *Os02g0221900* and *Os06g0565100* paralogues, both of which are capable of completely rescuing Arabidopsis *max1-1*, in the case of some leaf phenotypes even more efficiently than the *Os01g0700900* paralogue presumably also involved in Japonica. The rescue capability of *Os02g0221900* in particular was somewhat surprising, as this clade has the longest branch length of any of the cereal *MAX1* orthologous clades, but on detailed inspection the main signatures of selection on *Os02g0221900* were indeed found to be of purifying selection (R. Challis, pers. comm.). In terms of functional capability, it would therefore appear that plants carrying the Azucena/Japonica haplotype have four orthologues with strong similarity and functional competence to catalyse the *AtMAX1* function, and that plants carrying the Bala/Indica haplotype have at least two and possibly as many as four, although these are less competent *in planta* for SL production as those of the other haplotype. In summary, the Indica deletion story demonstrates that *MAX1* orthologues in rice are contributing to SL production, and that these alleles are contributing to the

domestic selection, (if not necessarily the natural selection) of an important cereal (Cardoso *et al.*, in review).

If divergence in the roles of these orthologues has occurred, then it seems likely that these roles are defined by differences in regulation. Expression analysis of responses to phosphate limitation by all five Japonica genes in the Japonica cultivar Shiokari by Umehara *et al.* (2010) did reveal some regulatory differences. All but *Os01g0701400* and *Os06g0565100* were upregulated in the roots in response to phosphate starvation in similar patterns seen in the other SL biosynthesis genes, although only *Os02g0221900* was upregulated in shoots, like the other biosynthesis genes. In fact, only *Os01g0701500, Os06g0565100* and *Os02g0221900* were detectable in shoots at all, and *Os02g0221900* was the only one expressed at comparable (even greater) level in shoots as in roots (Umehara *et al.*, 2010). This compares well with the data from RiceXPro, in which *Os02g0221900* was mainly leaf specific whereas the other genes were root- or –stem expressed. Put together, the information from RiceXPro and Umehara *et al.* build a pattern of differential characteristics for the expression of all the orthologues; the expression patterns of *Os06g0565100, Os02g0221900* and the chromosome 1 clade are largely spatially defined, while within the chromosome 1 clade differentiation is provided between *Os01g0700900* and *Os01g0701400* by phosphate response, and between *Os01g0701500* and everything else by its generally low level. However, although these data provide some evidence for subfunctionalisation, there is clearly a great deal of functional redundancy available in the cereal lineages, probably contributing to the lack of *MAX1* orthologue mutants identified in rice.

Chapter 6. D27 *and* D27like

The identification of the loci affected in the *dwarf14* and *dwarf27* rice mutants added new genes to the *MAX* pathway (Ishikawa *et al.*, 2005; Arite *et al.*, 2009; Gao *et al.*, 2009; Lin *et al.*, 2009; Liu *et al.*, 2009). Phylogenetic analysis in the studies of Arite *et al.* and Lin *et al.* identified these genes as also being of interest for evolutionary study. Unlike *MAX1*, *D14* and *D14like* family genes have not multiplied in copy number specifically in the angiosperms or monocots, but do show duplications early in land-plant evolution, leading to two clades being present in vascular plants as well as a third in angiosperms (Arite *et al.*, 2009; Waters *et al.*, 2012). The genetic locus affected in the *d27* mutant is a novel protein, with no conserved domains that have any functional annotation (Lin *et al.*, 2009). Despite this, by BLAST searches Lin and co-workers found that potential orthologues of *D27* were found throughout the land plant kingdom, but not outside of it, suggesting that this may be a plant specific protein family. Further phylogenetic analysis by Dr Richard Challis found two land-plant clades, *D27* and *D27like*, which appear to have diverged early in land plant evolution, perhaps between the emergence of the lycopodiophytes and the emergence of the gymnosperm clades. These clades are joined by long branch lengths (Figure 6-1) suggesting duplication was followed by sub- or neofunctionalisation.

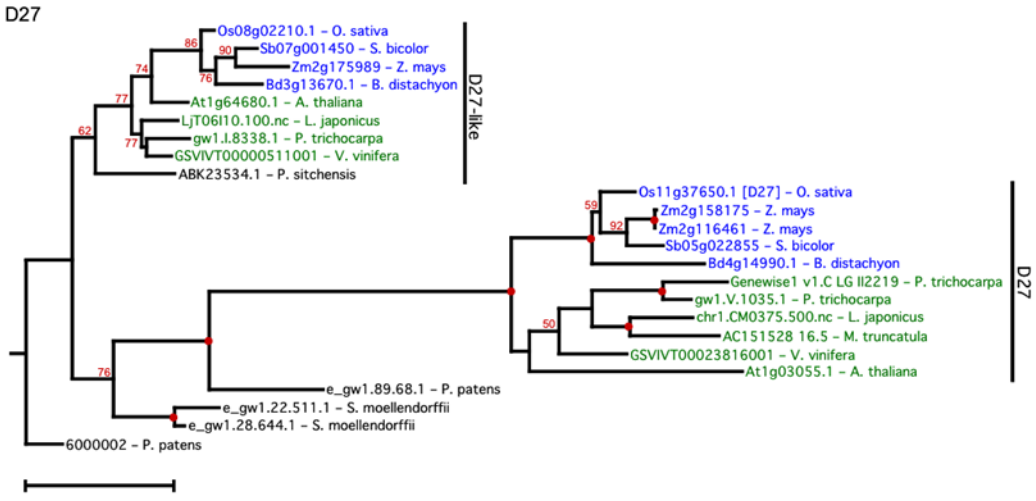

Figure 6-1. Maximum likelihood tree for *D27*, showing bootstrap support. Dicotyledons in green, monocotyledons in blue, non-angiosperms in black. Scale bar corresponds to one substitution per site. Kindly provided by Richard Challis.

As *D27*, like *MAX1*, is a biosynthetic component of the pathway (*D14* being an uncertain case due to the GR24 resistance of its mutants) investigation of the contribution of its divergence to the production of SL-related hormones through evolution seemed very promising.

Two genes in Arabidopsis show homology to *D27*; these are *At1g03055*, which corresponds to *D27*, although it shares only 36% sequence identity at protein level (Table 6-1); and *At1g64680*, which is much more similar (68% protein identity) with '*D27like'*. (The rice genes are annotated *Os08g02210* (RGAP) or *Os08g0114100* (RAP-DB), both for *D27like*, but there is no accurate annotation extant for *D27* save that of the paper reporting it, although it was Os11g37650 in RGAP release 5). As a role for *D27* in SL biosynthesis or shoot branching had not yet been shown in Arabidopsis, the functions of both genes were explored in Arabidopsis, and the hypothesis was raised that the divergence of the *D27* clade from the *D27like* indicated that while *D27* had either retained a role in, or been co-opted into, the SL production pathway (a role it would share in Arabidopsis), the *D27like* clade was involved in a different, non-SL related role.

Table 6-1. Matrix of protein identities for *A. thaliana* and *O. sativa D27* and *D27like* orthologues.

Sequence Identity Matrix	OsD27	AtD27	OsD27like
AtD27	0.361		
OsD27like	0.235	0.300	
AtD27like	0.211	0.292	0.678

6.1 Expression of AtD27 and AtD27like

Existing databases of gene expression were explored to investigate whether the Arabidopsis orthologues of *D27* and *D27like* were expressed in similar patterns both to each other and to *D27*. Expression analysis from Genevestigator (Hruz *et al.*, 2008) and AtGenExpress (Schmid *et al.*, 2005) found that, although *AtD27* signal was rarely significantly identified on the

microarray chip, where registered both orthologues were expressed in cotyledons, rosette and cauline leaves (particularly *AtD27like*), with some expression also in stems, within the sepals of flowers, and in the endosperm of seeds. In all of these tissues, except endosperm, *AtD27like* showed high expression compared to elsewhere in the plant, whereas *AtD27* merely showed slightly more expression than otherwise. However in endosperm, this pattern was reversed, as *AtD27* was particularly highly expressed whereas *AtD27like* showed slightly higher expression compared to elsewhere. Neither gene was expressed highly, if at all, in roots. This compares well with the expression of *OsD27like* in rice, as seen in data from RiceXPro, in which *OsD27like* is highly expressed in leaves, less so in stems and the lemma and palea of florets, and a little in endosperm. However, the expression seen in *AtD27* appears to match less well to that of *OsD27* as reported by Lin *et al.* (2009), possibly because the panicles, shoot bases, axillary meristems, and the vascular tissues, in which *OsD27* was found to have highest expression are rarely dissected out for microarray analysis, with the exception of panicles (inflorescences in Arabidopsis), and so precise data for these tissues for *AtD27* is unavailable.

Exploring databases also contributed to identifying the subcellular location of the proteins encoded by *AtD27like*. Although no information was available for *AtD27*, the proteomics database AT_CHLORO indicates that protein fragments corresponding to the predicted product of *At1g64680* have been identified in fractions purified from chloroplast envelopes and thylakoid membranes, a localisation for *AtD27like* which would match that of *OsD27* (Lin *et al.*, 2009; Ferro *et al.*, 2010).

6.2 Function of D27 and D27like

A genetic approach was used to investigate whether *AtD27* and *AtD27like* had functions in the Arabidopsis SL pathway. Mutant collections in the Columbia-0 ecotype background were searched for insertions associated with either of *AtD27* or *AtD27like* (*At1g03055* and *At1g64680* respectively, in the annotation of The Arabidopsis Information Resource). An insertion line, 134E08 from the Gabi-KAT collection (Rosso *et al.*, 2003) was identified for *AtD27*. This line also carries a T-DNA insertion in *At1g79110*, but this was

easily segregated from the *At1g03055* insertion and plants carrying the single insertion were then backcrossed twice to the Columbia-0 wildtype background. The insertion is within the fifth exon, but right at its beginning (at the 3rd base pair of the exon, see Figure 6-2) according to sequence results using gene-specific and T-DNA primers from the GABI-Kat database. This site is upstream of the site of the mutation in the rice *d27*, and is likely to create a premature stop codon. Neither full-length genomic nor cDNA sequence could be amplified from the mutant using RTPCR, therefore the insertion likely results in complete loss of function of *AtD27*. Only in one out of five plants could transcript be amplified from the mutants, and only then at ten PCR cycles greater than that needed to bring the wildtype gene to plateau phase (Figure 6-3A). This line was therefore designated *Atd27-1*. In addition, seed for two RNAi lines targeting *D27* in Arabidopsis were kindly donated by Dr Yonghong Wang of the Institute of Genetics and Developmental Biology, Beijing. Despite the incomplete knock-down of the *AtD27* transcript in these lines (Figure 6-3) Dr Wang's group had found these lines to show increased rosette branching, although only to a fraction of the *max* mutant phenotype.

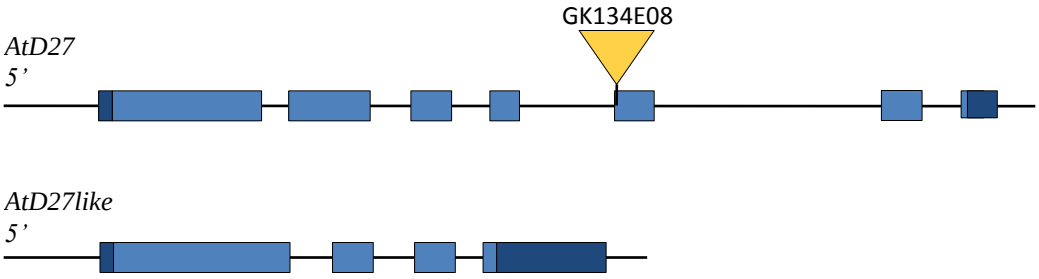

Figure 6-2. Exon structure for *AtD27* and *AtD27like*, with untranslated regions in dark blue, and insertion point of GABI-Kat line 134E08.

No insertion lines were available for *AtD27like* that showed an effect on transcript levels or a clear phenotypic effect, so a transcriptional knockdown approach was used (as opposed to post-transcriptional or 'RNAi' approach), in which the promoter is targeted for methylation by use of an antisense hairpin construct. The vector used is an adaptation of the pFGC5941 vector (Kerschen *et al.*, 2004), developed by Dr Louise Jones' lab, in which a constitutive NOS

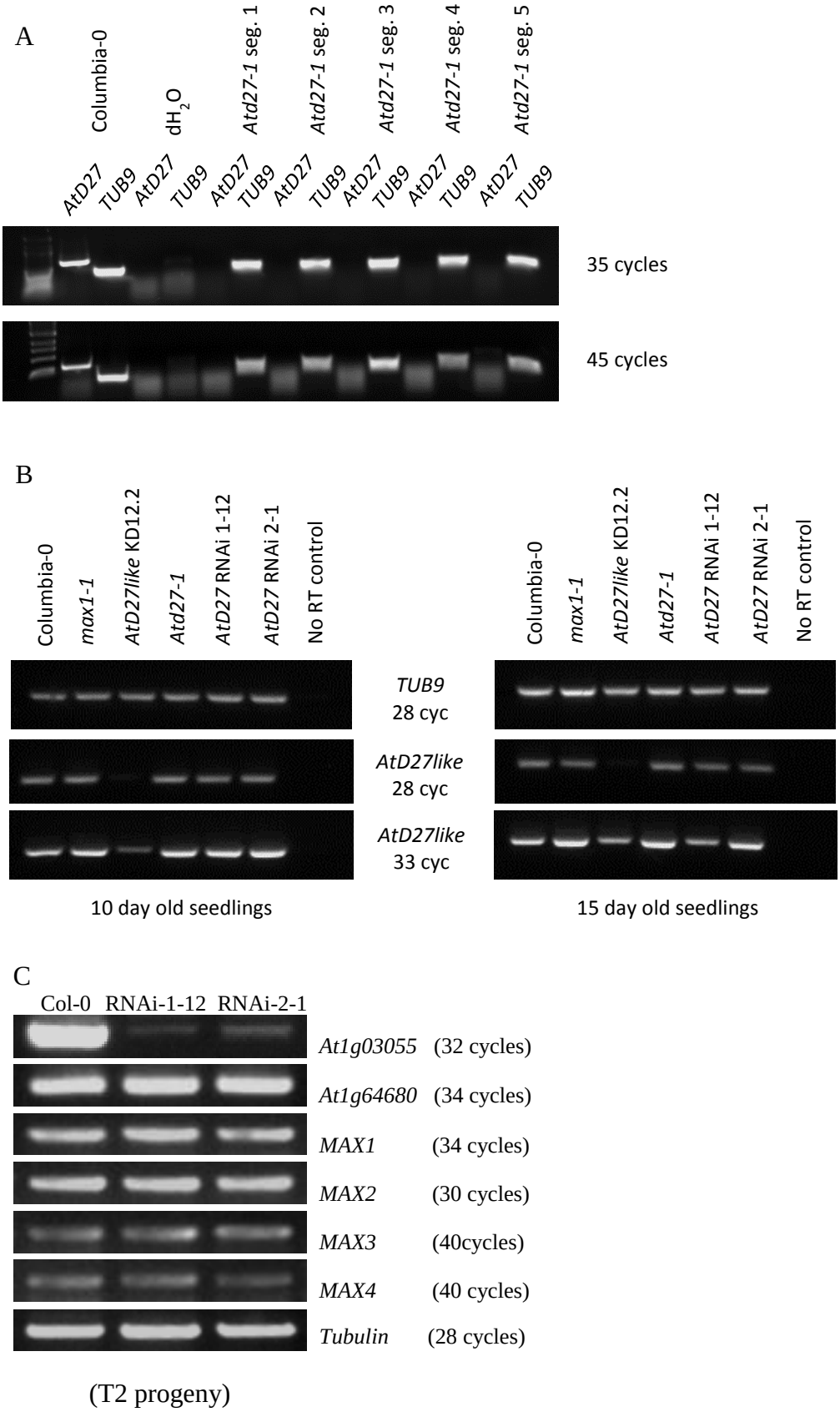

Figure 6-3. Expression of *MAX* pathway genes in mutants and knockdown lines. A) Expression of *AtD27* in adult rosette leaves of *Atd27-1* (5 individual single-insertion segregants from the GABI-Kat

line 134E08 carrying an insertion in *AtD27* only) compared to Columbia-0, B) expression of *AtD27like* in 10 and 15 day old seedlings (pooled RNA from 10 seedlings each) of the *AtD27like* knockdown line and *Atd27* mutant and knockdown lines, and C) expression of *MAX* genes in *AtD27* RNAi knockdowns. Figure C kindly courtesy of Yonghong Wang. Tubulin (TUB9) was used as loading controls. 'No RT' (no reverse transcriptase) and dH_2O were used as no-template controls.

promoter (replacing the 35S promoter in the original vector) drives an inverted repeat of the target promoter to be silenced, with the repeats separated by an intron from the chalcone synthase gene. This approach was employed as it leaves open the possibility of re-complementation by the wild-type gene under the control of a different promoter (e.g. CaMV35S). A single line, KD12.2, showing substantial downregulation of the gene was obtained (Figure 6-3). Given that at least four extra cycles are required to produce a comparable band in the semi-quantitative RTPCR for the knockdown mutant compared to the wildtype, the knockdown may be estimated (assuming primer efficiency to be reasonable) to be approximately 16-fold lower than the wildtype expression level (i.e. less than 10% of it). Therefore, in the absence of any more efficient knock-out or knockdown, this line was used for phenotypic analysis.

6.2.1 Branching

The branching of the knockdown lines and the insertion mutant was assessed as previously described for the *max1* complementation transgenics. In the short day decapitation assay (Figure 6-4 and Figure 6-5) none of the three knock-down lines, *AtD27* RNAi 1-12 and 2-1 and *AtD27like* KD12.2, were different to the Columbia-0 control. This is in contrast to the results seen by Prof. Wang's group for the RNAi lines in the T3 generation. However, a previous replicate, using the RNAi lines, of the experiment shown in Figure 6-4 gave the same result, suggesting that either the phenotype is too weak to be seen in this assay, or possibly that in the generation used by this author (T4) the RNAi construct itself had silenced. The *Atd27-1* mutant, with a mean number of rosette branches of 9.2, was far less branchy than *max1-1* (which had a mean of 13.7 rosette branches – see also Figure 6-6), although it still has on average 3 more branches than Columbia-0, and was significantly different to it at P=0.002 in a Kruskal-Wallis test. *Atd27-1* therefore seems to display an intermediate phenotype to that of *max1-1* and Columbia-0. The branching phenotype does

not seem to be mirrored in the height phenotype (Figure 6-5), but height may be less sensitive to SL depletion. However, these phenotypes clearly show that *AtD27* has a role in shoot branching control in Arabidopsis.

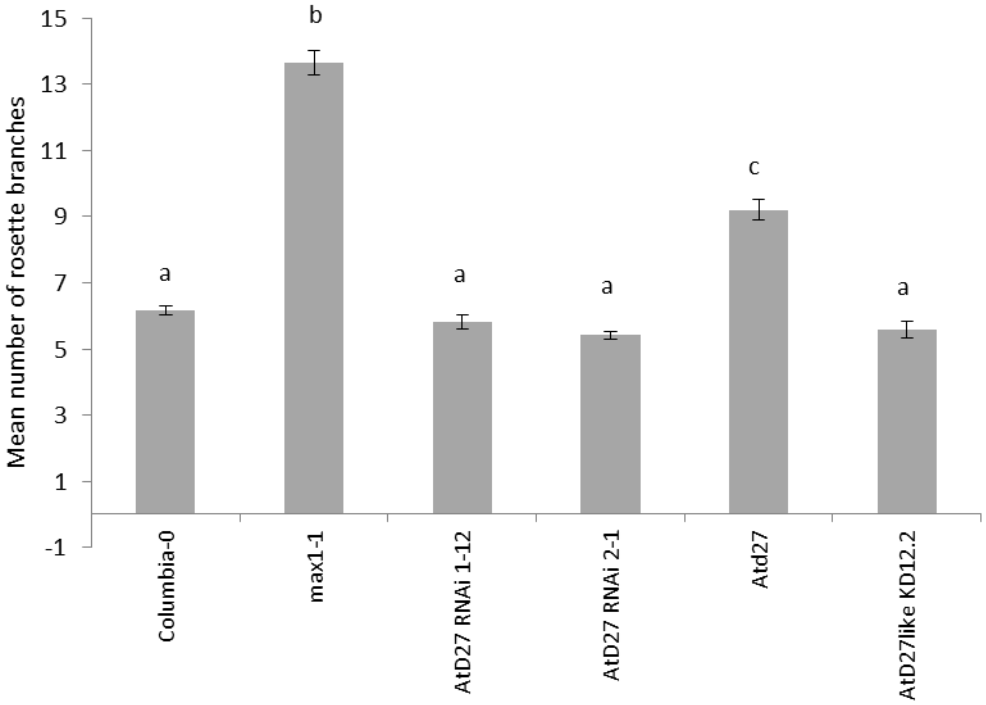

Figure 6-4. Rosette branching of *AtD27* and *AtD27like* knockdowns and mutants compared to wildtype and *max1-1*. Branching was assessed by short-day decapitation assay as described by Greb *et al.* (2003). **N for Columbia-0 *max1-1* and *Atd27* = 40, for knockdown lines n = 20. Shared letters indicate no significant difference in a Kruskal-Wallis test to P≤ 0.001, except letter 'c' which indicates no significant difference to P≤ 0.005. Error bars show standard error of the mean.**

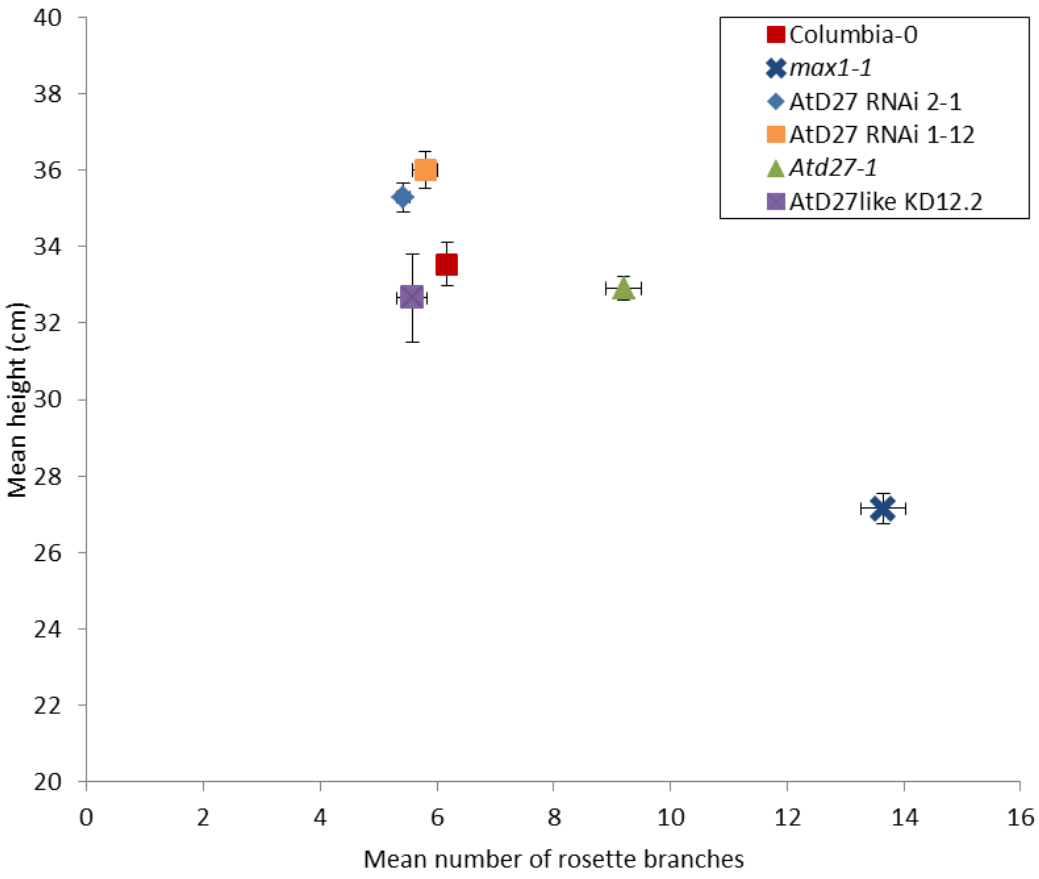

Figure 6-5. Rosette branching plotted against height for *AtD27* and *AtD27like* knockdowns and mutants compared to wildtype and *max1-1*. Branching was assessed by short-day decapitation assay as described by Greb *et al.* (2003). **N for Columbia-0 *max1-1* and *Atd27* = 40, for knockdown lines n = 20. Height (in centimetres) of the longest branch was measured the day of scoring for branching. Error bars show standard error of the mean. Note y axis starts at 20cm.**

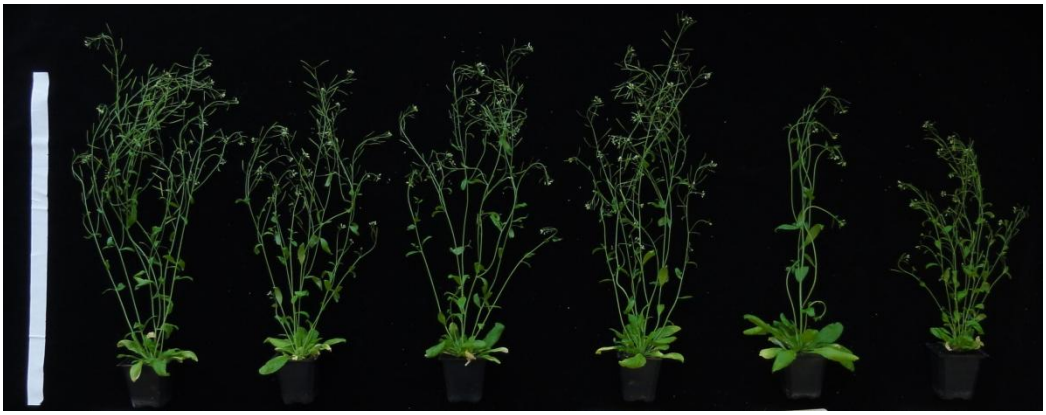

Figure 6-6. Photograph of *AtD27* and *AtD27like* knockdowns and mutants compared to wildtype and *max1-1*. Right to left, Columbia-0, *Atd27-1*, *AtD27* RNAi 1-12, *AtD27* RNAi 2-1, *AtD27like* KD12.2, *max1-1*.

If the *Atd27* mutation results in decreased biosynthesis of strigolactones, it would be expected that supplementing *Atd27* plants with strigolactones would reduce their more-branchy phenotype. Therefore a GR24 dose response assay was carried out on the *Atd27-1*, *AtD27* and *AtD27like* knockdown lines. In this assay the number of rosette branches in all genotypes is reduced, which rendered the differences between *Atd27-1* and Columbia-0 too small to be significant. Although no differences in branching between lines, or between different treatments of the same line were significant, except for the *max1-1* control, across two of the three replicate experiments all lines, including that of Columbia-0, did show some reduction in branching on 1μM GR24 to levels the same or below that of the Columbia-0 acetone-carrier-treated control, suggesting that what little branching phenotype *Atd27-1* possesses, it is not resistant to SL.

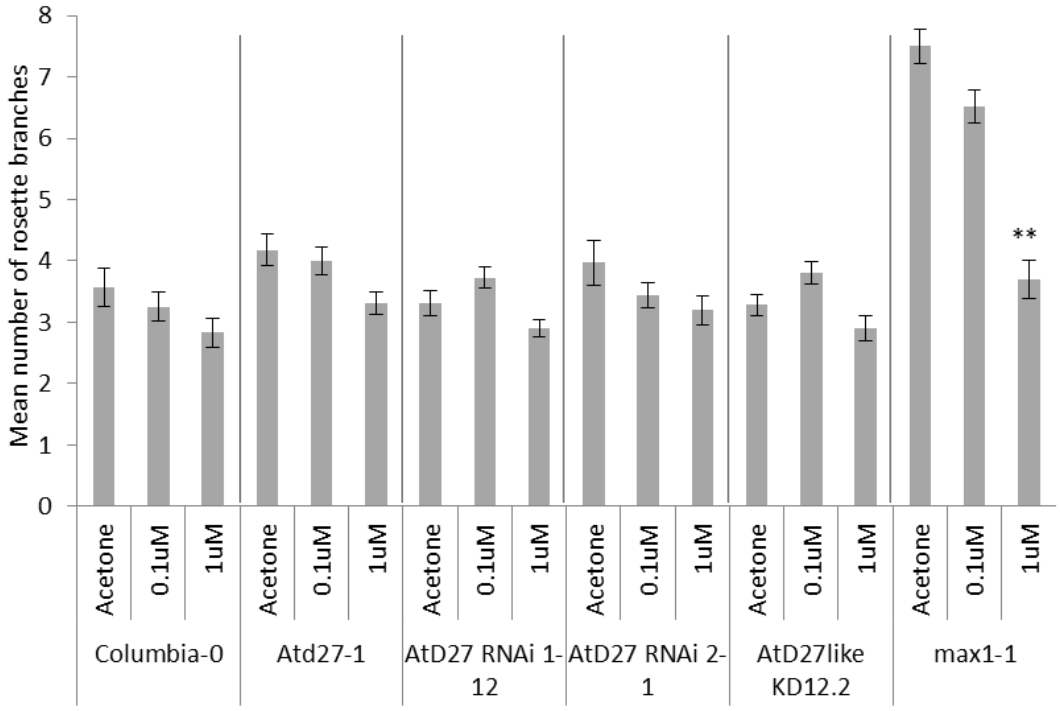

Figure 6-7. Mean number of branches for plants grown on agar containing GR24 dissolved in acetone, with the acetone carrier as a control. 3rd and representative of 3 replicates. Branches were scored after approximately five weeks when the first siliques had formed. Columbia and *max1-1* are controls. Error bars are standard error of the mean. Samples treated with GR24 were compared to the samples of the same genotype treated with acetone, where ** = significant difference to P<0.001 in Kruskal-Wallis test (adjusted for multiple sampling).

6.2.2 Leaf phenotype

To establish if the *Atd27-1* mutant shared any other *max* phenotypes, the leaf shapes of *Atd27-1* and *AtD27like* KD12.2 were measured and compared with wildtype and *max1-1* as for the complementation lines. As found for the branching phenotype, *Atd17-1* generally appears to show an intermediate phenotype between Columbia-0 and *max1-1*, although as seen before for the complementation lines, centroid size and PC3 seem more affected by the proposed reduction in SL signalling, while PC9 is less affected. Equally, as seen for the branching phenotype, *AtD27like* KD12.2 shows no significant difference to Columbia-0, although in the case of the centroid size it is also statistically similar to *max1-1*. Although this reduced centroid size could be interpreted as a sign of a very weak SL-related defect that is only visible in the phenotype most sensitive to SL-change, given the number of otherwise completely rescuing lines that have produced low centroid sizes, and the possibility that overproduction of SLs could have the same effect, the opposite interpretation is more parsimonious with the data. The intermediate effect of the *Atd27-1* lesion therefore also affects non-branching SL-related phenotypes, whereas there is still no evidence of a role for *AtD27like* in SL synthesis.

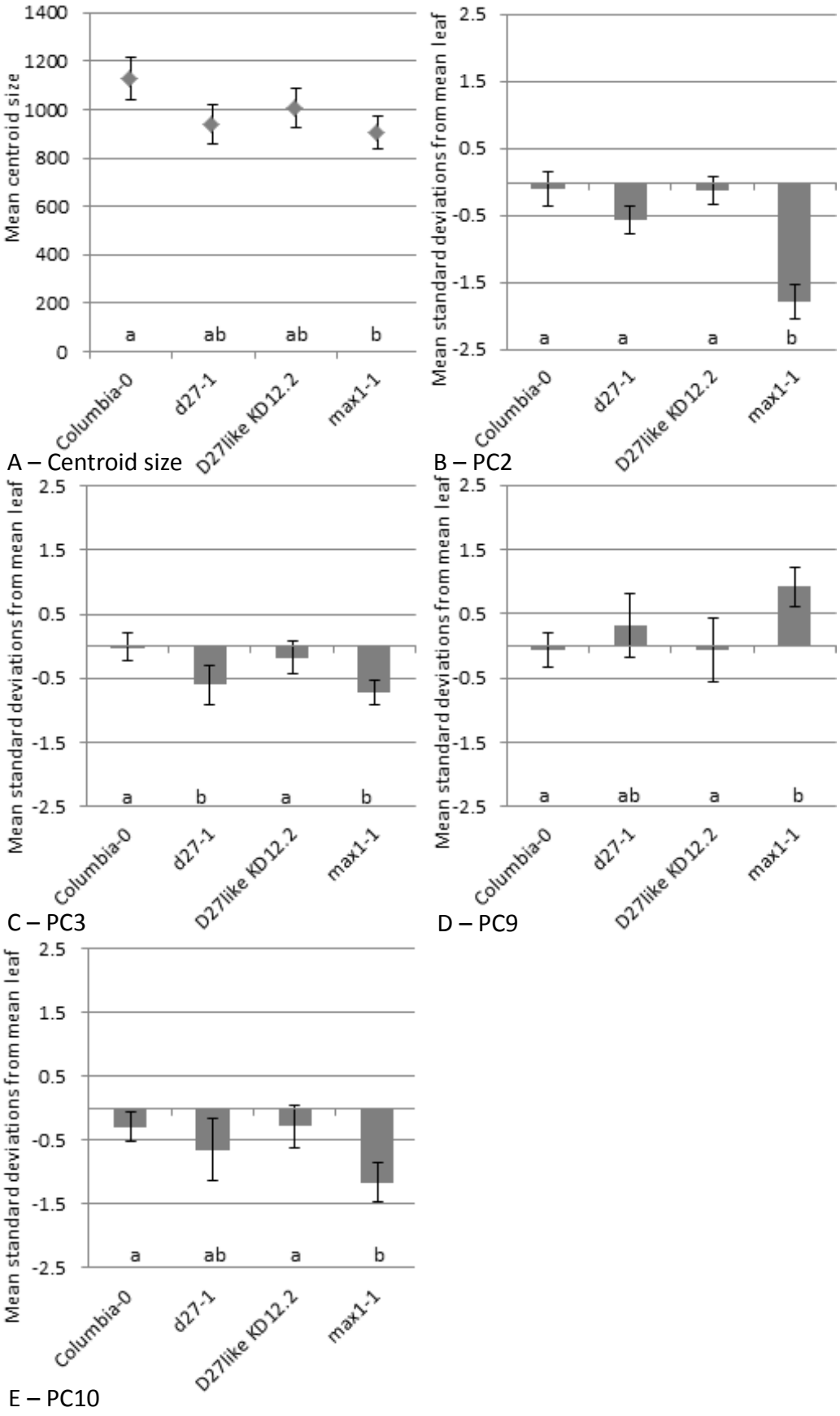

Figure 6-8. Leaf shape analysis for Procrustes-fitted adult leaves four and above from *max1-1* plants

complemented with non-angiosperm *MAX1* orthologues. Error bars are standard error of the mean, calculated on number of plants as n, where n = 15 for controls, and n=7 for *Atd27-1* and *D27like* KD 12.2. Shown are mean centroid sizes (A) and standard deviations from the mean leaf for PC2 (B), PC3 (C), PC9 (D) and PC10 (E). Letters indicate non-significance in Tamhane's T2 post-hoc test at P>0.001 (centroid, PC2) or P>0.05 (PC10) and Tukey's Honestly Significant Difference at P>0.05 for PC3 and PC9.

6.3 Discussion

The duplication of the *D27* clade near the base of the land plant lineage, the long branch lengths between the two clades (indicating substantial change in the *D27* clade compared to the *D27like* clade) and the apparent conservation of copy number within each clade of these unusual proteins in angiosperm genomes all indicate an interesting history for these genes within land plant evolution.

To determine if a story of functional divergence might be indicated by the *D27* duplication, expression and functional analysis of the Arabidopsis orthologues were compared, and some indication was found that they have diverged in expression at least. Although both were generally expressed in shoot tissues, *AtD27like* seems to be most highly expressed in leaves, whereas *AtD27* shows its highest expression in endosperm. OsD27 protein is localised to the plastid, the same subcellular localisation as MAX4/CCD8 and MAX3/CCD7 in Arabidopsis, and possibly that of AtD27like (Booker *et al.*, 2004; Auldridge *et al.*, 2006; Lin *et al.*, 2009; Ferro *et al.*, 2010). Neither of the gene expression patterns for *AtD27* or *AtD27like* initially appear very similar to those of *MAX4* and *MAX3*, which are highly expressed in roots particularly, but both *CCDs* are also expressed in shoot tissues so there is still considerable overlap between them and the *D27* family (Sorefan *et al.*, 2003; Booker *et al.*, 2004; Bainbridge *et al.*, 2005; Auldridge *et al.*, 2006; Mashiguchi *et al.*, 2009). The match between the shoot- and vascular-associated expression patterns of the rice orthologues for *MAX4/CCD8* and *MAX3/CCD7*, *D10* and *D17*, and the expression pattern of *OsD27*, is even clearer than that for the Arabidopsis genes (Zou *et al.*, 2006; Arite *et al.*, 2007; Lin *et al.*, 2009). However, despite the overlap in expression and subcellular locations with each other and with known

MAX pathway components, there is sufficient difference between the two *D27* family orthologues to suggest that their regulation reflects subfunctionalisation at the level of expression, regardless of their catalytic activity.

In terms of function, the case for divergence between the orthologues is much less clear, due to the possibility that the knockdown of *AtD27like*, although it showed no distinct effect on the SL-affected phenotypes tested, may simply be insufficient to produce an effect. Although reduced, *AtD27like* transcript was still visible in the knockdown line, and given the weak phenotype of *Atd27-1* and the lack of phenotype (in this author's hands) of the *AtD27* RNAi lines it remains possible that *AtD27like* plays a role in SL signalling, albeit with a different expression pattern and thus perhaps a different (sub)function to that of *AtD27*. The weakness of the *Atd27-1* phenotype may itself reflect a 'leaky' allele that retains some function, perhaps due to incomplete knockdown. When compared to the other mutants in the SL pathway in rice, *d27* also had the weakest phenotype in respect to tillering and culm length or height of the all mutants in all three studies in which they were compared (Ishikawa *et al.*, 2005; Arite *et al.*, 2007; Lin *et al.*, 2009). This is despite the fact that the mutation in *d27* causes a premature truncation of translation, producing protein that can no longer bind its iron cofactor, and which would therefore be predicted to be a null mutation (Lin *et al.*, 2009). Consistent with this, the exudation of *epi*-5-deoxystrigol is undetectable and the induction of germination of *Orobanche minor* seeds by exudates is abolished by the mutation (Lin *et al.*, 2009), although given the difficulty of measuring SLs it remains possible that an undetectable amount of a SL, which shows more activity in branching suppression than in parasitic plant germination, is still produced in *d27.* As the *Atd27-1* insertion is predicted to be upstream of the point of the *d27* mutation, and probably to cause disruption to the coding sequence of the gene as well as its expression, it is also predicted to be a null, but this has not been confirmed. It also remains possible that the weakness of the *d27/Atd27* phenotype reflects some redundancy for its role, particularly in the case of Arabidopsis.

This redundancy may be supplied by the members of the *D27like* clade, a

hypothesis that could be tested by introducing the *AtD27like* knockdown into the *Atd27* background. However, there are at least two other possibilities – that the enzyme downstream of D27 has some flexibility in its use of substrate, or that the substance produced by D27 is also produced at low levels by another mechanism, such as a non-*D27* family enzyme. The function of the D27 protein from rice has recently been revealed to be that of a carotene isomerase, which reversibly catalyses the isomerisation of the all *trans* configuration of β-carotene, the only isomer produced by lycopene-β-cyclase *in planta* due to its own stereospecificity of substrate, to that of 9-*cis*-β-carotene, the substrate of CCD7 enzymes (Yu *et al.*, 2011; Alder *et al.*, 2012). Alder and colleagues (2012) investigated the function of CCD7 proteins of rice, pea, and key to this study also of that of Arabidopsis, and all showed specificity for the 9-*cis* form of β-carotene, and indeed the 9-*cis* configuration is required for the subsequent production of the putative strigolactone intermediate carlactone by CCD8. This specificity for 9-*cis*-β-carotene in SL synthesis argues against a flexibility in substrate use for SL synthesis providing the weak phenotype of *Atd27-1*, and indicates that the function of a carotene isoöerase is likely to be important in Arabidopsis as well as rice. Although it remains possible that in Arabidopsis another enzyme overlaps the role of *AtD27*, it is more likely that *Atd27-1* is simply a weak allele.

Despite the weak phenotypes of the mutant, the effects of *Atd27-1* on shoot branching and leaf phenotype support the hypothesis that, whether or not they share a conserved catalytic function with the *D27like* clade, *D27* clade orthologues have a conserved role in SL signalling *in planta* in Arabidopsis.

Chapter 7. General Discussion

The understanding of hormone evolution is becoming ever more elaborated in the post-genomics era, as the contribution of genomes to identifying orthologous components allows the complementation of early studies on physiological effects with genetic evidence of the regulatory pathways affecting and affected by hormone action. This study has attempted to exploit both genetic and physiological means to inform on the roles of SLs and their biosynthesis across the four major lineages of vascular plant taxa.

Of the genes involved in the strigolactone signalling pathway, the presence of a conserved function of *MAX1* in lycopodiophytes and gymnosperm lineages, presented in this study, lends weight to the hypothesis that it is a shared element of SL synthesis in these plant groups. Its presence in the pathway would therefore date either to a time before the emergence of vascular plants (approximately 440 mya) or even to before the divergence of the mosses and lycopodiophytes, if the lack of the *MAX1* gene is a derived, rather than anciently conserved, characteristic in mosses. More sequence information from the basal land-plant groups – other mosses, the liverworts and the hornworts – will contribute to answering the question of *MAX1's* evolutionary incorporation to the SL biosynthetic pathway. The sequencing of the genome of *Marchantia polymorpha* in particular will be valuable, but even EST sequencing projects can provide evidence of the presence of orthologues (if not their absence), as found in this project for ferns and spruce.

In the context of the evolution of other hormones, SLs are present and active in the development of moss, as are the auxins with which they interact in growth control in angiosperms, and the cytokinins, which promote the formation of the buds that grow into the leafy gametophores in Physcomitrella, a process also promoted by auxin but restricted by SLs (von Schwartzenberg *et al.*, 2007; Eklund *et al.*, 2010; Proust *et al.*, 2011). Thus the three phytohormones with most control over shoot branching in angiosperms were almost certainly already present in some of the earliest land plants and were acting on plant development. However, SLs predate the evolution of gibberellin signalling in developmental control, which appears to have evolved in a step-

wise manner through the evolution of the ability to interact between different gibberellin signal transduction components at different times in evolutionary history and in different groups, with GA control of plant development perhaps only becoming established in the fern-seed plant ancestor (Vandenbussche *et al.*, 2007; Yasumura *et al.*, 2007). If the presence of *MAX1* is an innovation of the vascular plants, as would be most parsimonious, the story of the incorporation of *MAX1* into the SL pathway may reflect a similar story of co-option of a component, this time in the biosynthetic pathway, perhaps reflecting a selection pressure for a different hormone structure. *MAX1* is not required in moss for the production of a spectrum of SL compounds not unlike those of angiosperms (Proust *et al.*, 2011). Therefore one candidate for a *MAX1*-incorporation selection pressure is the evolution of vasculature. Strigolactones are unstable in water, and the long-distance signalling in the xylem stream of larger plants may require a more robust intermediate, for which *MAX1* is required, either for production or for conversion back into an active substance. Indeed, a study on the presence of SLs in xylem sap in Arabidopsis found an unidentified compound that had parasitic-plant germination activity, that was upregulated in response to phosphate limitation but that was reduced by the *max1-1* and *max4-1* mutations (Kohlen *et al.*, 2011). This compound was absent from roots, making it a strong candidate for a shoot-specific SL, and interestingly it is highly polar compared to other SLs, suggesting a hydroxylation reaction in its production, such as *MAX1* could catalyse (Kohlen *et al.*, 2011).

The results of Ruyter-Spira *et al.* are also interesting from this point of view, because in their study the *max1-1* mutant showed resistance to low levels of directly applied GR24 in some root phenotypes, but none were seen at similar levels in shoot branching in this study, in which GR24 was also applied to the roots, but acts in the shoots (Booker *et al.*, 2005; Ruyter-Spira *et al.*, 2011). This would seem to argue against a key role for *MAX1* in long-distance transport, as in that case the shoot phenotype would be the one expected to be more affected by loss of *MAX1* function, except for the fact that GR24 is considerably more stable than the natural substances it mimics. The half-life of GR24 in water is 10 days, as opposed to the 1.5 days of 5-deoxystrigol, for

instance (Akiyama *et al.*, 2010). Thus it may be that the higher concentrations of GR24 arriving at the shoots is sufficient to obscure its slightly lower efficacy in *max1-1* when it reaches its point of action. In this theory, the concentrations required to effect a developmental change in roots would be much higher than those needed in shoots, which corresponds well with the higher production and concentrations of SL concentrations in roots (Xie *et al.*, 2010). In support of this idea, when directly applied to the xylem stream GR24 suppressed branching of the *rms1* biosynthetic mutant of pea at concentrations a hundred fold lower than reported here, although admittedly in a different organism (Gomez-Roldan *et al.*, 2008; Kohlen *et al.*, 2011). However, *MAX1* is still required for the production of several active SLs from the roots, suggesting that it might, as Ruyter-Spira *et al.* suggest, have more than one role in the pathway.

The capacity for plants to have more than one *MAX1* gene with a role in SL synthesis has also been demonstrated in this study, through comparative analysis of the functions of *MAX1* paralogues in rice and Medicago. Although the initial fate of duplicate genes is redundancy, for many genes this does not provide a sufficient driver for maintenance, and subsequent retention is often a result of either subdivision of the original gene function or the development of novel function due to *"the escape from the ruthless pressure of natural selection"* for the original function (quotation from Ohno, 1970; Lynch, 2007). The importance of these mechanisms in the evolution of genome architecture is the subject of continuing research at the genome level. In the case of the *MAX1* paralogues tested here, subfunctionalisation of expression appears to hold sway over neofunctionalisation of catalytic action in rice. Of the five paralogues in this species, (the four that were tested in this study, and the fifth tested by Yanxia Zhang of Wageningen), all but one was capable of catalysing the Arabidopsis reaction to full phenotypic rescue, although they may yet be producing slightly different compounds with more varied effects in rice (perhaps further tuning the active compounds, *sensu* Ruyter-Spira *et al.*). However, these paralogues do show a variety of expression patterns suggesting that their duplication has allowed fine-tuning of their regulation (Umehara *et al.*, 2010). The deletion of two paralogues has led to major shoot architectural change in rice, the corresponding deletion being split roughly along subspecies

and ecological boundaries, indicating that variation in *MAX1* duplicates continue to be important to the adaptation and domestication of angiosperms (Cardoso *et al.*, in review). Further work, such as the complementation studies as used herein, on the actions of the two orthologues of *Os01g0701500* in the Indica group would be promising to follow the evolution of this tandem clade and its effects on rice plant architecture. In wider terms, orthologues corresponding to each of the three clades present in rice are also represented in several cereal genomes, suggesting that *MAX1* may play similar roles in these crops. Maize, sorghum, and rice are all staple foods for some of poorest in the world. Further work to understand the interaction of *MAX1* orthologues and their specificity in these species will hopefully contribute to the generation of crops with greater pre-attachment resistance to parasites and perhaps more efficient phosphate use, processes already begun (Cardoso *et al.*, in review; Jamil *et al.*, 2011).

In Medicago, unlike in rice, *MAX1* has undergone a change in its catalytic activity. It is unknown whether this is due to pseudogenization of *Medtr1g015860* (as is highly likely for *Os01g0701500*) or to a change in its role. However, combined with the upregulation of *Medtr1g015860* specifically in response to nodulation stress, and the indications that SLs have a role in the promotion of nodulation (Foo and Davies, 2011), this difference makes *Medtr1g015860* an interesting target gene for further study of the mechanism and evolutionary co-option of SL signalling into the plant development of nodules, a symbiosis with importance in agriculture.

The discovery of the catalytic function of *MAX1*, recently advanced by the work of Alder *et al.* (2012) in identifying a new SL intermediate, will further inform understanding of the different roles of *MAX1* in various phenotypes and plant groups, as well as its incorporation into SL biosynthesis. In concert with the work presented here it will allow a more detailed comprehension of the molecular changes influencing the action of cytochrome P450s. Results presented here also provide some support to the presence of the biosynthetic pathway described by Alder and co-workers in Arabidopsis, by providing evidence of a role for *AtD27* in shoot branching control. Whether the *D27like*

clade also act redundantly in this pathway is still unknown, but the genetic resources produced here could provide a beginning to understanding this – for example by incorporation of the *AtD27like* knock-down construct into the *Atd27-1* mutant.

The gold standard for confirmation of a role for *MAX1* in SL biosynthesis in different species is the presence of SL deficiency in the orthologous mutants, and for confirmation of SL roles in development the standard would be specific developmental changes in those groups. In rice this has been demonstrated (Cardoso *et al.*, in review), in Medicago and pea the hunt for such mutants is underway, and in petunia knockdown constructs have been used to the same effect (Drummond *et al.*, 2012). In Selaginella and spruce however this is unlikely to be achieved for some time, if ever, and in the absence of such mutants complementation studies like those used here for *MAX1* are very valuable. In judging the degree of rescue for these studies the LeafAnalyser approach to leaf morphometrics has also proven to be an easy-to-use and quantitative measure of rescue, and the leaf phenotype of the *max* mutants is in itself a worthy target of further work. Indeed, the effects of SLs on auxin transport might make a combination of *max* biosynthetic mutants and the application of GR24 a tool for understanding the effects of auxin concentration and transport in leaf development.

In the absence of mutants and with limited genetic resources an attempt has also been made here to identify physiological roles for SLs in three major plant lineages, the lycopodiophytes, the ferns and the gymnosperms. Although these groups are ‘genomic orphans’ (with the notable exception of *Selaginella moellendorffii*) they include ecologically and economically important species, with ferns filling a vast array of ecological niches and gymnosperms, as forest trees, filling vast tracts of the planet. Identification of roles for SLs in such species contributes to understanding of the differences between host and non-host taxa in the battle against parasitism, and provides further information on the twin developmental and symbiotic roles of these exuded communication signals in multi-species ecological contexts. Not least, physiological data from such species fills a scientific requirement for the understanding of hormone

evolution, as noted by Pires & Dolan in a recent review on the evolution of plants:

" most of the evidence used to infer the evolutionary origin of signalling pathways is based on the genomic identification of homologues of known biosynthetic enzymes, receptors or signal transducers; it is possible that independent plant lineages have evolved slightly different signalling pathways, and it will take more than comparative genomics to identify these mechanisms."

Nuno Pires & Liam Dolan (2012)

The results presented here provide indications that SLs may have conserved functions in phosphate signalling responses in gymnosperms and conserved roles in the coordination of shoot and root-like organs in Selaginella. These findings warrant further study, especially those in Selaginella, which may provide the opportunity to study the early evolution of the interaction of SLs with auxin transport mechanisms, shedding light on the evolution of not just one hormone, but a complex hormone interaction and a new mechanism in plant development.

"As buds give rise by growth to fresh buds, and these, if vigorous, branch out and overtop on all sides many a feebler branch, so by generation I believe it has been with the great Tree of Life, which fills with its dead and broken branches the crust of the earth, and covers the surface with its ever branching and beautiful ramifications"

Charles Darwin,
On the Origin of Species By Means of Natural Selection (1859)

Appendix A1

Table A1. Primers with target, sequence, purpose and any acknowledgements due.

Primer name	Gene target	Sequence	Purpose	Source
Sequencing/identifying endogenous genes				
PgMAX1F	*P. glauca* clone GQ0205_O16	CGCGAGGTGGGTATTAAGAA	Amplifying cDNA to identify *P. glauca MAX1*	
PgMAX1R	*P. glauca* clone GQ0205_O16	Tcgtcggtgtcgaagtcgaa	Amplifying cDNA to identify *P. glauca MAX1*	
PgMAX1F2	*P. glauca* clone GQ0205_O16	TGCGGTTCTACACAGTGTCT	Amplifying cDNA to identify *P. glauca MAX1*	
PgMAX1R2	*P. glauca* clone GQ0205_O16	CGAGACGAGGTAGAGTATGA	Amplification/5'RACE to identify *P. glauca MAX1*	
PgMAX1TCFseq	*P. glauca* clone GQ0205_O16	ATCGCGTTCAATCTGTGAGT	Sequencing cDNA to identify *P. glauca MAX1*	
PgMAX1TCRseq	*P. glauca* clone GQ0205_O16	GACATCGACTTCTCAGAGCT	Sequencing cDNA to identify *P. glauca MAX1*	
PgMAX1gbFseq	*P. glauca* clone GQ0205_O16	AAGGGTACGTGGGTGTGGAT	Sequencing cDNA to identify *P. glauca MAX1*	
PgMAX1gbRseq	*P. glauca* clone GQ0205_O16	CGAAACCACAATCCCAAACT	Sequencing cDNA to identify *P. glauca MAX1*	
PgMAX1Fseq	*P. glauca* clone GQ0205_O16	TCATACTCTACCTCGTCTCG	Sequencing cDNA to identify *P. glauca MAX1*	
PgMAX1 R3	*P. glauca MAX1*	tcgcgtaagggtgtctattc	5'RACE to identify *Picea glauca MAX1*	
PgMAX1 RACE 5' 3	*P. glauca MAX1*	TCGGCAGCGTGTAGCCTATCTG	5'RACE touchdown to identify *P. glauca MAX1*	
dtadaptor primer	Adaptor primer	gactcgagtcgacatcgatttttttttttttttt	for 5'RACE library cDNA synthesis	as from Sambrook & Russell (2001)
adaptor primer	Adaptor primer	gactcgagtcgacatcg	for 5'RACE library cDNA synthesis	as from Sambrook & Russell (2001)
OsMAX1aF	Attempted cloning of *Os01g0701400*	gggggaattcatggagatcatcagcacagtg	Cloning *Os01g0701400*cds, with EcoRI site	Kind gift of Dr Céline Mouchel
OsMAX1aR	Attempted cloning of *Os01g0701400*	ggggtctagactatgcagtgtgcctcttgat	Cloning *Os01g0701400*cds, with XbaI site	Kind gift of Dr Céline Mouchel

Table A1

OsMAX1a test F	Test *Os01g0701400*	ATTCTCCGATCTCGCTCTC	Testing for mRNA presence	
3'RACE Qt	Adaptor primer	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTT	3'RACE library cDNA synthesis	as from Scotto-Lavino *et al.* (2006)
3'RACE Q0	Adaptor primer	CCAGTGAGCAGAGTGACG	Amplifying from 3'RACE	as from Scotto-Lavino *et al.* (2006)
3'RACE Q1	Adaptor primer	GAGGACTCGAGCTCAAGC	Nested amplifying from 3'RACE	as from Scotto-Lavino *et al.* (2006)
OsC 3'RACE 1	*O. sativa Os01g0701500*	GCTAGCCAGGGAAACACTTG	Amplifying from 3'RACE for *Os01g0701500*	
OsC 3'RACE 2	*O. sativa Os01g0701500*	ACCTCTACCGCCATTACGTG	Nested amplifying from 3'RACE for *Os01g0701500*	
- degenerate primers				
Cfern deg F	*C. richardii MAX1*	GCATATTCATTCTACGACACAACTGaaratggayht	Degenerate primer for *C. richardii*, designed using program from R. Challis	
Cfern deg R1	*C. richardii MAX1*	CAGATCCTGCAAGCarrtgytcrta	Degenerate primer for *C. richardii*, designed using program from R. Challis	
Cfern	*C. richardii MAX1*	GGNCACCTBCCCTTGHTGGSNAWG	Degenerate primer for *C. richardii*, based on an EST from *Adiantum capillus-veneris*	
Cfern	*C. richardii MAX1*	CCRAANGCNGYYTSCCCDATCACRTC	Degenerate primer for *C. richardii*, based on an EST from *Adiantum capillus-veneris*	
SkMAX2 deg F	*S. kraussiana/C. richardii MAX2*	TTCTAYTGCTGGRCCGAGGA	Degenerate primer for *C. richardii* and *S. kraussiana*	
SkMAX2 deg R	*S. kraussiana/C. richardii MAX2*	CAHGABCDGCWCKCATCTCDGTG	Degenerate primer for *C. richardii* and *S. kraussiana*	
SkMAX1 deg F1	*S. kraussiana/C. richardii MAX1*	GGSCCMRTYTWCAGRTTCCA	Degenerate primer for *C. richardii* and *S. kraussiana*	
SkMAX1 deg F2	*S. kraussiana/C. richardii MAX1*	TTCCABHTBGGMAGRCARCC	Degenerate primer for *C. richardii* and *S. kraussiana*	
SkMAX1 deg R1	*S. kraussiana/C. richardii MAX1*	CCAMACCCAHGTDCCCTTTGG	Degenerate primer for *C. richardii* and *S. kraussiana*	
SkMAX4 deg F	*S. kraussiana/C. richardii MAX4*	TTGGGVGAYGGRMGAGTGGT	Degenerate primer for *C. richardii* and *S. kraussiana*	
SkMAX4 deg R	*S. kraussiana/C. richardii MAX4*	GGATTVATGSTGYWCATRTC	Degenerate primer for *C. richardii* and *S. kraussiana*	

Table A1

Selmo high conserved F	*S. kraussiana MAX1*	CCAAACCCAAGTTCCCTTTGGAA	Primers designed against conserved sequences of SmMAX1 to use on *S. kraussiana*	
Selmo high conserved R	*S. kraussiana MAX1*	GGGCCAATTTACAGGTTCCAG	Primers designed against conserved sequences of SmMAX1 to use on *S. kraussiana*	
Cloning				
SmMAX1F2	*S. moellendorffii* GI: XM_002972009	GAA TTC ATG GCG CTG ATC ATC GCA GTT TTC TTT GTG	Cloning *SmMAX1* cds, with EcoRI site	
Sm1bR	*S. moellendorffii* SELMODRAFT_96541	atcagcatatctcgcgcttc	Cloning *SmMAX1* cds, with EcoRI site	
PgMAX1 F KpnI	*Picea glauca MAX1*	ATTAGGTACCATGGCGTCTCTATGCGGACT	Cloning *PgMAX1*cds, with KpnI site	
PgMAX1 R HindIII	*Picea glauca MAX1*	CACTAAGCTTCTACACTGGCGATTGC	Cloning *PgMAX1*cds, with HindIII site	
MtMAX1bsubF	*M. truncatula Medtr1g015860*	agtgtaatcttaaatgttcctttgg	Subcloning *Medtr1g015860* cds with EcoRI	
MtMAX1bsubR	*M. truncatula Medtr1g015861*	cttgataccatgcttgaagt	Subcloning *Medtr1g015860* cds with XbaI	
MtMAX1asubF	*M. truncatula Medtr3g104560*	ttagcagctcatctctgttc	Subcloning *Medtr3g104560* cds with EcoRI	
MtMAX1asubR	*M. truncatula Medtr3g104561*	gttcatggatttggaatggttg	Subcloning *Medtr3g104560* cds with XbaI	
OsMAX1cF	*O. sativa Os01g0701500*	gggggaattcatggacatcagcgaggtgctg	Cloning *Os01g0701500*cds, with EcoRI site	Kind gift of Dr Céline Mouchel
OsMAX1cR	*O. sativa Os01g0701500*	ggggtctagactagaactcgagaggggactc	Cloning *Os01g0701500*cds, with XbaI site	Kind gift of Dr Céline Mouchel
OsMAX1DF	*O. sativa Os02g0221900*	ggggctcgagatggaggcaagcaattgctcc	Cloning *Os02g0221900*cds, with XhoI site	Kind gift of Dr Céline Mouchel
OsMAX1DR	*O. sativa Os02g0221900*	ggggtctagatcaggtgttggtcctcttgat	Cloning *Os02g0221900*cds, with XbaI site	Kind gift of Dr Céline Mouchel
OsMAX1eFEcoRI	*O. sativa Os01g0700900*	gggggaattcATGGAGATCAGCACAGTG	Cloning *Os01g0700900*cds, with EcoRI site	
OsMAX1eRClaI	*O. sativa Os01g0700900*	ggggatcgatTTATATATGCCTCTTGATGACCTG	Cloning *Os01g0700900*cds, with ClaI site	
OsMAX1b insert F Blp1	*O. sativa Os06g0565100*	cggctgcgagccgcgtcccggcgac	Cloning *Os06g0565100* cds, with BlpI site	
OsMAX1b insert R Blp1	*O. sativa Os06g0565100*	cgccgcgcctgaagctgagcacc	Cloning *Os06g0565100* cds, with BlpI site	

Table A1

OsMAX1b F2	*O. sativa Os06g0565100*	GTGTGAATTCATGGAGGCTCTAGTGGCG	Cloning *Os06g0565100*cds, with EcoRI site	
OsMAX1b R2	*O. sativa Os06g0565100*	GTGTATCGATCAGGTGATCTGCGCTTGTCT	Cloning *Os06g0565100*cds, with ClaI site	
D27 cloning F Kpn1	*A. thaliana At1g03055*	GTGT GGTACC ATGAACACTAAGCTATCACTTTCTC	Cloning *AtD27* cds, with KpnI site	
D27 cloning R Cla1	*A. thaliana At1g03055*	GTGTATCGATCTAATGCTTCACACCGTAGC	Cloning *AtD27* cds, with ClaI site	
D27like pro F Nco1	*A. thaliana At1g64680*	TTTT CCATGG GAGTTTAGGTTCTTAGCCGAAAGTTGG	Cloning *AtD27like* promoter, with NcoI site	
D27like pro R Swa1	*A. thaliana At1g64680*	CCCC ATTTAAAT CCCTACCACCATCATCTCATACTCTGC	Cloning *AtD27like* promoter, with SwaI site	
D27like pro F Xba1	*A. thaliana At1g64680*	CCCG TCTAGA GAGTTTAGGTTCTTAGCCGAAAGTTGG	Cloning *AtD27like* promoter, with XbaI site	
D27like pro R BamH1	*A. thaliana At1g64680*	TTT TGG ATC CCC CTA CCA CCA TCA TCT CAT ACT CTG C	Cloning *AtD27like* promoter, with BamHI site	
PgMAX4F	*P. glauca MAX4*	ATGGCGGCTGCTTCTTCTTCTTCG	Cloning *PgMAX4*cds to confirm sequence	
PgMAX4 R	*P. glauca MAX4*	TCA GTG AAA TGG AAC CCA GCA G	Cloning *PgMAX4*cds to confirm sequence	
PgMAX2 F	*P. glauca MAX2*	ATGACGATGGAGTTTGGGGACGTTGG	Cloning *PgMAX2*cds to confirm sequence	
PgMAX2 R	*P. glauca MAX2*	GCTCTAGTTGGTCGTGGATTTACTGACTGA	Cloning *PgMAX2*cds to confirm sequence	
Sequencing to check clones				
pART7 F	Vector pART7	gatgacgcacaatcccactatc	Sequencing insertions in the pART7 vector	Kind gift of Dr Lynne Armitage
pART7 R	Vector pART7	cataggcgtctcgcatcatctca	Sequencing insertions in the pART7 vector	Kind gift of Dr Lynne Armitage
Os1c R seq	*O. sativa Os01g0701500*	tccttgagcaacttctcctc	Middle primer for sequencing *Os01g0701500*	
NOSp IR Sequencing CHSA F	CHSA intron from pFGC5941	CACTTACTTACACTTGCCTTGGAG	Sequencing the reversed promoter from the CHSA intron for NOSp vector pFGC5941	

Table A1

Semi-quantitative RTPCR: - of transgenes in Arabidopsis

OsMAX1C RTPCR F	*O. sativa Os01g0701500*	AAAGCTGCCAGTCACACCTG	Semi-Q RTPCR of transgene in *Arabidopsis thaliana*	
OsMAX1C RTPCR R	*O. sativa Os01g0701500*	TTGTTAGACTCCCTCGCCGT	Semi-Q RTPCR of transgene in *Arabidopsis thaliana*	
OsMAX1D RTPCR R	*O. sativa Os02g0221900*	CCTCAACCAGGTCATCAAGG	Semi-Q RTPCR of transgene in *Arabidopsis thaliana*	
OsMAX1D RTPCR F	*O. sativa Os02g0221900*	GAGTGGCGGAACACGTAGC	Semi-Q RTPCR of transgene in *Arabidopsis thaliana*	
OsMAX1E RTPCR R	*O. sativa Os01g0700900*	TCTTCACAAGTGGTTCGAGGTG	Semi-Q RTPCR of transgene in *Arabidopsis thaliana*	
OsMAX1E RTPCR R	*O. sativa Os01g0700900*	CGACGATCCTGTCAAGCTGT	Semi-Q RTPCR of transgene in *Arabidopsis thaliana*	
OsMAX1b qPCR F	*O. sativa Os06g0565100*	GGGATCAGGCAGTTTAAGAGCAT	Semi-Q RTPCR of transgene in *Arabidopsis thaliana*	Designed using Primer Express
OsMAX1b qPCR R	*O. sativa Os06g0565100*	CAGCGAGATGATCGTGTTCCT	Semi-Q RTPCR of transgene in *Arabidopsis thaliana*	Designed using Primer Express
SmMAX1a RTPCR R	*S. moellendorffii MAX1*	GGTGGCGTCAAAGATGGTCA	Semi-Q RTPCR of transgene in *Arabidopsis thaliana*	
SmMAX1a RTPCR F	*S. moellendorffii MAX1*	CTCAAACGTGTAGCGCTGGT	Semi-Q RTPCR of transgene in *Arabidopsis thaliana*	

Table A1 - of Arabidopsis genes in Arabidopsis

TUB 9 F	*A. thaliana TUB9 At4g20890*	GTACCTTGAAGCTTGCTAATCCTA	Loading control primers for Semi-Q RTPCR	Designed by Dr Tobias Seiberer
TUB 9 R	*A. thaliana TUB9 At4g20890*	GTTCTGGACGTTCATCATCTGTTC	Loading control primers for Semi-Q RTPCR	Designed by Dr Tobias Seiberer
AtD27 RTPCR F	*AtD27 At1g03055*	GTGGCTTAGATAGACGCTCAA	Semi-Q RTPCR of *AtD27*	Designed by Dr Y.H Wang's group
AtD27 RTPCR R	*AtD27 At1g03055*	GGCTCCCGACCAAACAT	Semi-Q RTPCR of *AtD27*	Designed by Dr Y.H Wang's group
AtD27like RTPCR F	*AtD27like At1g64680*	GCCGTGAGGGAGGTTCTT	Semi-Q RTPCR of *AtD27like*	Designed by Dr Y.H Wang's group
AtD27like RTPCR R	*AtD27like At1g64680*	GGAGGTGCTTGCCCGTAT	Semi-Q RTPCR of *AtD27like*	Designed by Dr Y.H Wang's group
- of Medicago genes in Medicago				
MtMAX4qF	*M. truncatula Medtr3g109610*	ggtaatctccataatcagtgagaaaaa	Semi-Q RTPCR in *Medicago truncatula*	Kind gift of Dr Céline Mouchel
MtMAX4qR	*M. truncatula Medtr3g109610*	atgcaacccatatggaagtccataa	Semi-Q RTPCR in *Medicago truncatula*	Kind gift of Dr Céline Mouchel
MtMAX3qF	*M. truncatula Medtr7g045370*	atctctatgctgcaaccacctta	Semi-Q RTPCR in *Medicago truncatula*	Kind gift of Dr Céline Mouchel
MtMAX3qR	*M. truncatula Medtr7g045370*	aagacaacatctttgcattgaggta	Semi-Q RTPCR in *Medicago truncatula*	Kind gift of Dr Céline Mouchel
MtMAX1bqF	*M. truncatula Medtr1g015860*	ttggaataggtccaagggcatgta	Semi-Q RTPCR in *Medicago truncatula*	Kind gift of Dr Céline Mouchel
MtMAX1bqR	*M. truncatula Medtr1g015861*	ttgaagttaagaactaaaccatattcaa	Semi-Q RTPCR in *Medicago truncatula*	Kind gift of Dr Céline Mouchel
MtMAX2qF	*M. truncatula Medtr4g0800200*	ccttccggccaattggattt	Semi-Q RTPCR in *Medicago truncatula*	Kind gift of Dr Céline Mouchel
MtMAX2qR	*M. truncatula Medtr4g0800200*	tcctctggttcacatcctcatctt	Semi-Q RTPCR in *Medicago truncatula*	Kind gift of Dr Céline Mouchel
MtMAX1aqF	*M. truncatula Medtr3g104560*	gcaagagatcaagctttcacttatt	Semi-Q RTPCR in *Medicago truncatula*	Kind gift of Dr Céline Mouchel
MtMAX1aqR	*M. truncatula Medtr3g104561*	accatgcttgaagttgaggactatt	Semi-Q RTPCR in *Medicago truncatula*	Kind gift of Dr Céline Mouchel
MtEF1dqF	*M. truncatula Medtr8g014590*	agaatgagcccaaattcctgaagaa	Loading control for Semi-Q RTPCR	Kind gift of Dr Céline Mouchel

Table A1

MtEF1dqR	*M. truncatula Medtr8g014590*	gacgtatgtctctgacagcaaaa	Loading control for Semi-Q RTPCR	Kind gift of Dr Céline Mouchel
MtMAX1bqF2	*M. truncatula Medtr1g015860*	gcacccttatgcattcataccattt	Semi-Q RTPCR in *Medicago truncatula*	Kind gift of Dr Céline Mouchel
MtMAX1bqR2	*M. truncatula Medtr1g015861*	aaccatattcaagttctacaggttttt	Semi-Q RTPCR in *Medicago truncatula*	Kind gift of Dr Céline Mouchel
Q-PCR:				
- of transgenes in Arabidopsis				
MtMAX1b q Taqman F	*M. truncatula Medtr1g015860*	CCAGAGAGGTTTGACCCAAAAT	Q-PCR of transgene in *Arabidopsis thaliana*	Designed using Primer Express
MtMAX1b q Taqman R	*M. truncatula Medtr1g015861*	ACATGCCCTTGGACCTATTCC	Q-PCR of transgene in *Arabidopsis thaliana*	Designed using Primer Express
MtMAX1a q Taqman F	*M. truncatula Medtr3g104560*	TCCTAGAGCTTGCATTGGTCAG	Q-PCR of transgene in *Arabidopsis thaliana*	Designed using Primer Express
MtMAX1a q Taqman F	*M. truncatula Medtr3g104561*	GCTTGAAGTTGAGGACTATTCCATACT	Q-PCR of transgene in *Arabidopsis thaliana*	Designed using Primer Express
At2g28390 for2	*A. thaliana At2g28390*	tgcctatgtccacttctttgatga	Endogenous control for Q-PCR in *A. thaliana*	Kind gift of Dr Malgorzata Domalgalska
At2g28390 rev2	*A. thaliana At2g28390*	ggcgtaccctgcaatctttg	Endogenous control for Q-PCR in *A. thaliana*	Kind gift of Dr Malgorzata Domalgalska
PP2A QPCR for	*A. thaliana At1g13320*	catcaaatttaacgtggccaa	Endogenous control for Q-PCR in *A. thaliana*	Kind gift of Dr Malgorzata Domalgalska
PP2A QPCR rev	*A. thaliana At1g13320*	gccgtatcatgttctccacaa	Endogenous control for Q-PCR in *A. thaliana*	Kind gift of Dr Malgorzata Domalgalska
- of Picea genes in Picea				
PgMAX1 qPCR F	*Picea glauca MAX1*	ATCCTCGCGGGAATTCTGT	Q-PCR in *P. glauca*	Designed using Primer Express
PgMAX1 qPCR R	*Picea glauca MAX1*	TGCGGCTCAGGATCTGTCT	Q-PCR in *P. glauca*	Designed using Primer Express

Table A1

PgMAX2 qPCR F	*Picea glauca MAX2*	TTGTTGGACCGAGGACATACC	Q-PCR in *P. glauca*	Designed using Primer Express
PgMAX2 qPCR R	*Picea glauca MAX2*	TGAGCAAGTTGAGGCTTGACA	Q-PCR in *P. glauca*	Designed using Primer Express
PgMAX4 qPCR F	*Picea glauca MAX4*	CAAAGAACTGGTACGAGGAAGGA	Q-PCR in *P. glauca*	Designed using Primer Express
PgMAX4 qPCR R	*Picea glauca MAX4*	CCTCGGCCTCCGGTCTA	Q-PCR in *P. glauca*	Designed using Primer Express
PgTUB qPCR F	*Picea glauca Tubulin 9*	TATGATGCCCAGTGATACGTCG	Loading control for Q-PCR in *P. glauca*	Taken from El Kayal *et al.* (2011)
PgTUB qPCR R	*Picea glauca Tubulin 9*	ATGGAAGAGCTGCCGGTATGC	Loading control for Q-PCR in *P. glauca*	Taken from El Kayal *et al.* (2011)
PgTIF-5a qPCR F	*Picea glauca TIF-5*α	TCGGCGGTGGCAGAGT	Loading control for Q-PCR in *P. glauca*	Taken from Abbott *et al.* (2010)
PgTIF-5a qPCR R	*Picea glauca TIF-5*α	TCCCCACAACTACGAAATCTCA	Loading control for Q-PCR in *P. glauca*	Taken from Abbott *et al.* (2010)
PgSQD1 qPCR F	*P. glauca SQD1*	gcatctctcaaacagaggctctcaaag	Phosphate stress marker for Q-PCR in *P. glauca*	Designed using Primer Express
PgSQD1 qPCR R	*P. glauca SQD1*	gcccaagctgttggtcaaa	Phosphate stress marker for Q-PCR in *P. glauca*	Designed using Primer Express
Genotyping				
MAX1 SNP F	*A. thaliana At2g26170*	GACAAGAAGTCTTTTGAGTC	Genotyping *max1-1* - product from *max1-1* allele is cut by AluI	Thesis of Barbara Willett (2005)
MAX1 SNP R	*A. thaliana At2g26170*	TGAAGAGGATACCGGGAACA	Genotyping *max1-1* - product from *max1-1* allele is cut by AluI	Thesis of Barbara Willett (2005)
GABI-KAT LB	Left border of GABI-Kat pAC161	CGA TCG ATG CCT TGA TTT CG	Left border outward primer for pAC161	From GABI-Kat, Rosso *et al.* (2003)
GABI_114A05 RP	GABI-Kat line 114A05	GGATACGGCAACTAGGGTTTC	Genotyping *D27* insertion mutant GK114A05 and GK134E08	
GABI_114A05 LP	GABI-Kat line 114A05	CCCGACCAAACATCATTTTAC	Genotyping *D27* insertion mutant GK114A05 and GK134E08	

Appendix A2

Table A2. Cloning strategies for constructs

Construct	Primers	Cloning strategy
PgMAX1	PgMAX1 F KpnI PgMAX1 R HindIII	Amplified and cloned into Zero-Blunt TOPO kit, then digested with sites in primers and directionally cloned into pART7
SmMAX1	SmMAX1F2 Sm1bR	Amplified and cloned into Zero-Blunt TOPO kit. Digested with EcoRI and cloned into pART7, correct orientation checked by digest and sequencing
Medtr3g104560	MtMAX1asubF MtMAX1asubR	Amplified then digested with sites in primers and directionally cloned into pART7
Medtr1g015680	MtMAX1bsubF MtMAX1bsubR	Amplified then digested with sites in primers and directionally cloned into pART7
Os01g0700900	OsMAX1eFEcoRI OsMAX1eRClaI	Amplified and digested using sites in primers and cloned directly into pART7
Os01g0701500	OsMAX1cF OsMAX1cR	Amplified and cloned into Zero-Blunt TOPO kit, then digested with EcoRI sites in pCR4 vector and directionally cloned into pART7
Os02g0221900	OsMAX1DF OsMAX1DR	Amplified with primers and cloned into Zero-Blunt TOPO kit, then digested with sites in primers and directionally cloned straight into pART27 binary vector due to NotI site in cds.
Os06g0565100	OsMAX1b F2 OsMAX1b R2 OsMAX1b insert F Blp1 OsMAX1b insert R Blp1	Amplified most of the gene with OsMAX1b F2 and OsMAX1b R2 cloned into Zero-Blunt TOPO kit, but hairpin caused deletion in this clone near 3' end. Used special high-temperature reverse transcription described in section 2.2.4, and high temperature primers OsMAX1b insert F Blp1 and OsMAX1b insert R Blp1 to amplify the hairpin. Cloned hairpin into Zero-Blunt TOPO kit, then into full-length clone using BlpI sites in gene and in primers. Chose correct orientation by sequencing, then transferred ful length complete clone into pART7 via digest and directional cloning using sites in pCR® 4.
AtD27like Knockdown	D27like pro F Nco1 D27like pro R Swa1 D27like pro F Xba1 D27like pro R BamH1	Amplified promoter as two sections: D27like pro F Nco1 and D27like pro R Swa1, and D27like pro F Xba1 D27like pro R BamH1. Cloned each into Zero-Blunt TOPO kit. First digested with sites in primers and directionally cloned into adapted pFGC5941 (from Dr Jones's lab) the Xba1-BamH1 fragment. Then digested that clone with NcoI and SwaI for second section.

Abbreviations (including gene name abbreviations)

In addition to the abbreviations noted below, standard notation is used for chemical formulas (e.g. N = nitrogen, NO_3^{2-} = nitrate group), amino acids (e.g. P = proline) and nucleic acid bases (A – adenosine) *et cetera.* Which notation is in use is indicated by the context.

ABA – abscisic acid

AHL – N-acyl-homoserine lactone

AMe – axillary meristem

AMy – arbuscular mycorrhizae

ANOVA – analysis of variance test

ARP – ASYMMETRIC LEAVES1/Rough sheath2/PHANTASTICA family

ATS – *Arabidopsis thaliana* salts

BA1 – BARREN STALK1

bHLH – basic helix-loop-helix

BLAST – basic local alignment search tool

BRC# - BRANCHED gene

CCD – carotenoid cleaving dioxygenase

cds – coding sequence (open reading frame of mRNA)

CKs – cytokinins

CUC# - CUP-SHAPED COTYLEDON gene

CYP – cytochrome P450 haem-thiolate protein

CZ – central zone

DAD – Petunia *Decreased Apical Dominance* gene

DNA - deoxyribonucleic acid

D# – Rice *DWARF* gene

EDTA - Ethylenediaminetetraacetic acid

EMS – ethyl methane sulphonate

EST – expressed sequence tag

g – gravity

HD-ZIP – Homeodomain-leucine zipper

HTD# – Rice *HIGH TILLERING DWARF* gene

IAA – indole-3-acetic acid

Kb – kilo base pair of nucleic acid

LAX1 – LAX PANICLE1

LB - Luria Bertoni broth

LN_2 – liquid nitrogen

Ls/LAS – LATERAL SUPPRESSOR

MAX – *more axillary growth*

Mb – million base pair of nucleic acid

MOC1 – MONOCULM1

Mya – million years ago

NAA – β-naphthoxyacetic acid

NCBI – National Centre for Biotechnology Information (Bethesda, USA)

NSP# – NODULATION SIGNALLING PATHWAY gene

OC – organising centre

PAT - polar auxin transport

PC – principal component

PCA – principal component analysis

PCR – polymerase chain reaction

PEG – polyethylene glycol

Pi – inorganic phosphate

PZ – peripheral zone

QPCR – quantitative PCR

QTL – quantitative trait locus/loci

RAP-DB – Rice Annotation Project Database

RAX1 - REGULATOR OF AXILLARY MERISTEMS1

RGAP – Rice Genome Annotation Project

RMS# – pea *RAMOSUS* gene

RNA – ribonucleic acid

ROX - REGULATOR OF AXILLARY MERISTEM FORMATION

rpm – rotations per minute

RZ – rib zone

(Semi-Q) RTPCR – (semi quantitative) reverse-transcriptase PCR

SAM - shoot apical meristem

SCF – SKP1/Cullin/F-box complex

SEM – scanning electron microscopy

SL(s) – strigolactone-related hormone(s)

STM – SHOOT MERISTEMLESS

TB1 - TEOSINTE BRANCHED1

TCP – TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR

T-DNA – transfer DNA from *Agrobacterium tumefaciens*

TF – transcription factor

Tukey's HSD – Tukey's Honestly Significant Difference post-hoc test

TIGR – The Institute for Genome Research

U – enzyme units

WGD – whole genome duplication

WUS - WUSCHEL

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