

Mechanisms underlying changes in the morphology of rice plants infected with the parasitic weed *Striga hermonthica*

A thesis submitted by

Rodrigo Antonio Echevoyén Nava

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Department of Animal and Plant Sciences

University of Sheffield

Sheffield, S10 2TN

United Kingdom

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Abstract

Parasitic plants of the genus *Striga* (also known as witchweeds) are root hemiparasites that cause devastating losses in crop production in sub Saharan Africa (SSA). Two of the most important species, *S. hermonthica* and *S. asiatica*, infect the staple cereal crops of SSA including maize, sorghum, millet and rice. These parasitic weeds represent the major biotic constraint to cereal crop production. They cause losses in yield of 40-100% and negatively impact the quality of life of millions of the poorest subsistence farmers. Infection of crop plants by *Striga* causes severe stunting of the host plant, thinning of stems and in plants such as rice, suppression of tillering. In rice the number of tillers produced is very important as grain yield is often correlated with tiller number. At present the mechanisms underlying changes in the morphology and architecture of *Striga*-infected plants are not fully understood and the aim of this thesis was to investigate the potential role of plant growth regulators in the suppression of tillering and the stunting rice plants infected with *S. hermonthica*.

In Chapter 2 a detailed analysis of the changes in growth and architecture of the susceptible rice cultivar IAC165 following infection by *S. hermonthica* was carried out. This revealed that *Striga*-infected rice plants showed severe stunting, decreased biomass, increased root to shoot ratio, thinner stems and suppression of tillering. In addition, this study revealed, for the first time that the suppression of tillering in *Striga*-infected rice plants to an inhibition in the formation and outgrowth of lateral buds.

In Chapter 3 the hypothesis that strigolactones are involved in the suppression of tillering in *Striga*-infected rice plants was investigated by analysing the effect of the parasite on the suppression of tillering in rice plants with altered concentrations of strigolactones (plant growth regulators involved in the regulation of branching in plants). Strigolactone concentration was modified by (a) the use of strigolactone mutants (*d10-1*, a strigolactone biosynthetic mutant and *d3-1* a strigolactone signalling mutant) and (b) the application of a carotenoid biosynthetic inhibitor (fluridone) or a synthetic analogue of strigolactone (GR24). These studies revealed a partial role for strigolactones in the inhibition of tillering. In addition, there was an increase in the expression of CCD8 (encoding a strigolactone biosynthetic enzyme) in the leaf axils of *S. hermonthica* - infected plants, consistent with a role for strigolactones in the suppression of tillering. The effect of *S. hermonthica* on the biosynthesis of strigolactones in infected roots was also investigated using CCD8:GUS transgenic rice plants. These studies revealed increased expression of CCD8 in cells of the vascular bundle as *S. hermonthica* penetrated into the root and initiated fusion of xylem vessels with its host, suggesting a role for strigolactones during the infection process

In Chapter 4 the potential roles of multiple plant growth regulator pathways in the changes in plant morphology of rice plants infected with *S. hermonthica* were investigated by profiling changes in gene expression in the roots, stem and leaves of control and *Striga*-infected plants using Affymetrix microarrays. This is the first study to analyse whole genome expression in the whole plant simultaneously following infection by *S. hermonthica*. This study showed the profound impact of *S. hermonthica* on the regulation of auxin metabolic, signalling and transport pathways of infected plants, revealing the importance of this plant growth regulator in plant parasitism.

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

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Chapter 1

General introduction

1.1 Introduction

Cereal crops play a crucial role in farming systems and the diet of millions of people worldwide. According to the Food and Agriculture Organization (FAO), the forecast production of cereal crops in the year 2012 is predicted to be 2379.7 million tonnes, distributed as 1207.3 million tonnes of coarse grains, 675.1 of wheat, and 488.2 of rice (source FAO, 2012, <http://www.fao.org/worldfoodsituation/wfs-home/csdb/en>). Cereals are the most important staple food crops in the African continent, but their production is largely compromised by abiotic and biotic stresses. The former include climatic, edaphic, agronomic and management problems, while lepidopteran stemborers and the parasitic weeds of the genus *Striga* represent the major biotic constraints to crop production (Ejeta and Gressel, 2007; Khan *et al.*, 2010).

Only about 1% of angiosperms (approximately 4,200 reported species) have evolved the ability to become parasites of other plants (Kuijt 1969; Nickrent and Musselman 2004). Parasitic plants infect host plants through a specialized organ called the haustorium, abstracting nutrients and water (Kuijt 1969; Hibberd and Jeschke 2001; Keyes *et al.*, 2007; Palmer *et al.*, 2009). They can be divided in two major groups: hemiparasitic and holoparasitic. The leaves of obligate hemiparasites contain chlorophyll and have low rates of photosynthesis, but not enough to survive without also requiring some carbon (and all of their nitrogen and water) from the host. Holoparasites do not contain chlorophyll and therefore do not photosynthesize, thus they are completely dependent on their hosts for all of their nutrient requirements (for a review see dePamphilis *et al.*, 1997). Parasitic plants may be obligate (unable to survive in the absence of a host) *e.g.* *Striga* and *Orobanche* species, or facultative like *Tryphysaria* or *Rhinanthus* species. Facultative parasites can be fully independent but will infect a host if possible (Jamison and Yoder 1998). Some species of parasitic plants are economically important, due to their devastating effects on crop (*e.g.* *Striga*, *Orobanche*, *Alectra*, *Cuscuta* species) and forestry (*e.g.* *Arceuthobium* species) production (Parker and Riches 1993; Estabrook and Yoder 1998; Nickrent and Musselman 2004).

1.2 *Striga* (witchweed) species

Commonly known as witchweed, the genus *Striga* contains root obligate hemiparasites of the Orobanchaceae family of plants. The genus *Striga* is thought to originate from Africa and includes over 40 species of which only 11 are of agronomic importance (Mohamed *et al.*, 2001). In Sub-Saharan Africa, *Striga* species represent the major biotic constraint to cereal crop production, especially *S. hermonthica* and *S.*

asiatica. These two species infest more than 50 million hectares of cereals crop land (Ejeta, 2007; Scholes and Press, 2008) and have been estimated to affect the lives and food security of more than 100 million people (FAO, 2012). Figure 1.1 shows the extent of land currently infested with *Striga* seeds. This area stretches from West to East Africa and includes the whole of sub Saharan Africa (Ejeta 2007). Both *S. hermonthica* and *S. asiatica* can be found in all areas but *S. hermonthica* is dominant in West and East Africa. However from Tanzania southwards (including Madagascar) *S. asiatica* becomes the dominant species.

S. hermonthica and *S. asiatica* infect the roots of cereal crops, including upland rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), pearl millet (*Pennisetum glaucum*) and maize (*Zea mays*). They cause losses in yield that range from 30 – 100% and these losses have been valued at more than 7 billion USD every year (Ejeta, 2007). Control of *Striga* is very difficult, partly because of the intimate association of the lifecycle of *Striga* and its host and partly because the soils are so heavily infested with *Striga* seeds.

1.3 The *Striga* life cycle

The life cycle of *Striga* species is illustrated in Figure 1.2. In order to germinate, the tiny *Striga* seeds need a period of conditioning (moist, warm conditions with an optimum temperature of 24 – 30 °C) depending on the species (Hsiao *et al.*, 1981; Kim and Adetimirin, 2001; Mohamed *et al.*, 2001). *Striga* seeds will only germinate in response to chemicals present in the root exudates of host and some non-host species (Bouwmeester *et al.*, 2007; Cardoso *et al.*, 2011; Alder *et al.*, 2012) (Fig 1.2 (2)). The most widely investigated germination stimulants for parasitic plant seeds are known as the strigolactones and have been reported in more than 80% of land plants (Cook *et al.*, 1966; Vail *et al.*, 1990; Müller *et al.*, 1992; Matusova *et al.*, 2005).

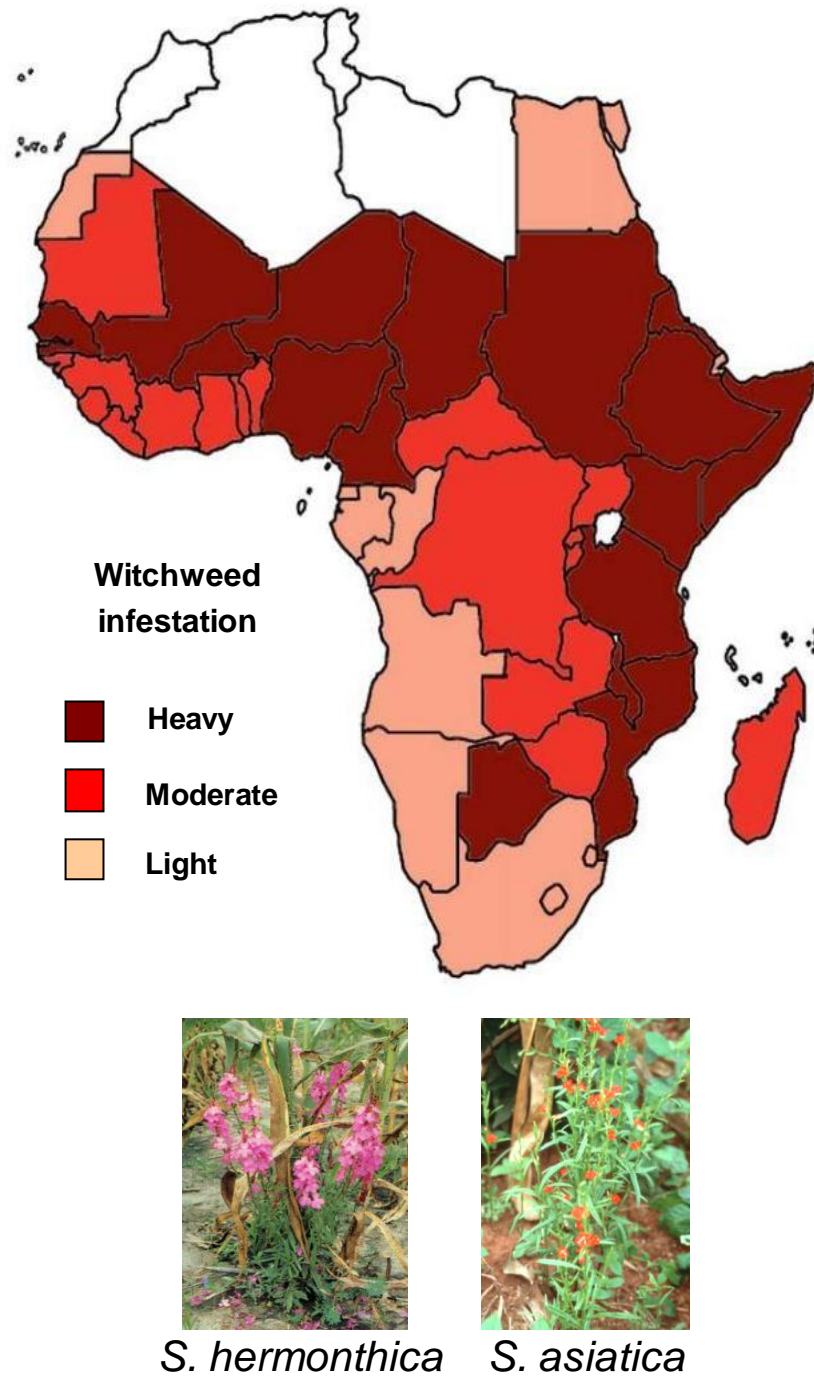


Figure 1.1 Areas of Africa infested with *Striga* seeds (from Ejeta, 2007)

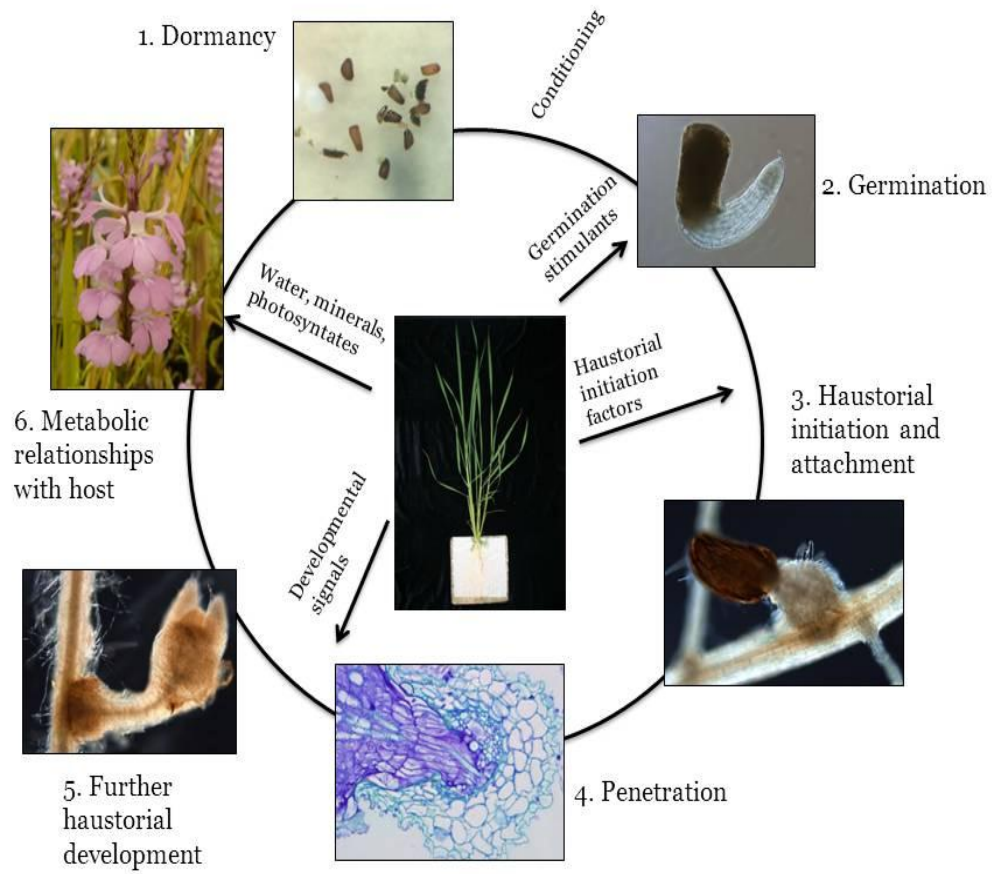


Figure 1.2 Stages of the life cycle of *S. hermonthica*.

Strigolactones are a family of sesquiterpene lactone compounds derived from the carotenoid pathway (Bouwmeester *et al.*, 2003; Matusova *et al.*, 2005). The structure of strigolactones share a common four-ring backbone, but differ in the saturation of rings and the combination of substituents they can carry (Roldan *et al.*, 2008). Strigolactones include compounds such as orobanchol, sorgolactone, alectrol, strigol and most recently carlactone (Figure 1.3) (Cook *et al.*, 1966; Hsiao *et al.*, 1981; Hauck *et al.*, 1992; Müller *et al.*, 1992; Siame *et al.*, 1993; Sun *et al.*, 2008; Alder *et al.*, 2012).

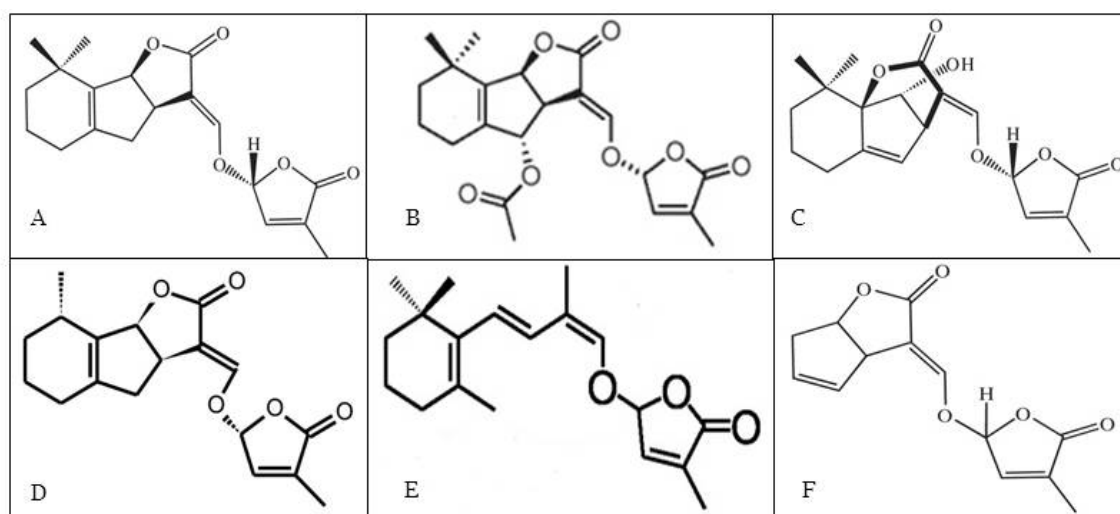


Figure 1.3 Chemical structure of strigolactones. (A) 5-Deoxystrigol, (B) Orobanchol, (C) Alectrol, (D) Sorgolactone, (E) carlactone, and the artificial analogue (F) GR24.

Artificial analogues of strigolactones, such as GR24, induce germination of parasitic plant seeds (Kondo *et al.*, 2007), and provide a useful tool for controlled laboratory assays of *Striga* seed germination. In addition to strigolactones other chemical compounds, such as flavonoids, phenyliminoacetates and phenyliminoacetonitriles have also been shown to stimulate germination of parasitic weed seeds (Bouwmeester *et al.*, 2007; Kondo *et al.*, 2007). Ethylene and gibberellins have also been shown to play a role in germination post stimulation as shown by reduced germination after exogenous application of ethylene and gibberellin inhibitors (Logan and Stewart, 1991; Zehhar *et al.*, 2002; Sugimoto *et al.*, 2003).

After germination, there must be a transition from vegetative to parasitic phase, mediated by the development of the haustorium. A suite of chemical compounds known as haustorial initiation factors (HIFs) which are also derived from the host are responsible for the initiation of haustorium development (Fig 1.2 (3)). These

compounds include 2,6-dimethoxy-p-benzoquinone (2,6 DMBQ) (Chang and Lynn, 1986), antocyanidin and peonidin (Albrecht *et al.*, 1999). HIFs are synthesized in plants via the oxidation of phenylpropanoid precursors (reviewed in Lynn and Chang, 1990), involving H₂O₂-requiring oxidase activity in host and parasite (Chang and Lynn, 1986). The formation of a redox cycle is necessary for the differentiation of the haustorium (Keyes *et al.*, 2007; Palmer *et al.*, 2009). This is achieved by the expression of quinone oxydoreductases (Bandanarayake *et al.*, 2010). In *S. asiatica*, an exposure time to 2,6-DMBQ of at least six hours is required for the transition from vegetative to parasitic phase by haustorium initiation at the root apical meristem. If exposure time is shorter the haustorium differentiation is aborted and the meristem elongation is restored, in which case a second haustorium can be later formed (Smith *et al.*, 1990). However, the formation of a second haustorium has not been reported for *S. hermonthica*. Haustorial formation can be inhibited by cyclopropil-p-benzoquinone (CBPQ) (Smith *et al.*, 1996), however, ectopic application of an artificial haustorium initiation inhibitor (2,3,5-triiodobenzoic acid) six hours after exposure to 2,6-DMBQ, does not present an inhibitory response, indicating there is a no turn back point during the haustorium development (Tomilov *et al.*, 2005). The haustorial differentiation begins by rapid cell cycle arrest, changes in the direction of cell division from longitudinal to radial dimensions of the apical meristem of the radicle, swelling and finally formation of haustorium hairs (Smith *et al.* 1996). When the haustorium has successfully differentiated, penetration of the epidermis and cortex by a wedge-like file of cells occurs 24 to 48 hours after infection (Hood *et al.*, 1998) (Fig 1.2 (4)). The parasite invades through the cortex and endodermal barrier and connects its xylem vessels to the xylem vessels of the host, thus gaining access to water and nutrients from the host (Dörr, 1997; Hibberd and Jeschke 2001). This process takes 3 days in a susceptible interaction.

Once attached to the host roots the parasites grow up through the soil and emerge and flower approximately 6-8 weeks after attachment. *Striga* flowers are pollinated by insects of at least ten different species, including, but not restricted to, butterflies (*Junonia*, *Vanessa*, *Eurema*, *Colotis*), bee flies (*Litorhina*, *Systoechus*), honey bees (*Apis*) and a moth (*Cephenodes*) (Musselman *et al.*, 1983). *S. asiatica* is an in breeding species whereas *S. hermonthica* is an obligate out breeder (Aigbokhan *et al.*, 1998). This means that the genetic diversity of *S. hermonthica* seeds is very high (Gethi *et al.*, 2005; Scholes and Press, 2008; Huang *et al.*, 2012). At the end of its reproductive stage, a single *Striga* plant can produce up to 200,000 seeds, which can potentially remain viable in the soil for as long as two decades. The huge quantities of

seed in the soils across Africa coupled with their longevity and genetic diversity poses enormous problems for control of the parasite (Scholes and Press 2008).

1.4 How is *Striga* controlled?

There are many recent reviews of strategies that are being used, or developed, to control *Striga* (e.g. Oswald *et al.*, 2005; van Ast *et al.*, 2005; Scholes and Press 2008; Hearne *et al.*, 2009) and only a brief over-view is given here. At present the most common strategies employed by farmers to try to control *Striga* consist of agronomic practices. For example, *Striga* plants are still weeded by hand to prevent the build-up of seeds in the soil (Parker and Riches 1993) although by the time *Striga* is above ground their removal does not improve crop yield as the parasite has irreversibly damaged the growth and physiology of the crop by this stage (Gurney *et al.*, 1999). Crop rotations of cereal crops with crops that are not hosts for *Striga* can improve yields particularly if cereal crops are rotated with legumes that improve soil fertility and also reduce the *Striga* seed bank by suicidal germination of *Striga* seeds. However the success of crop rotations depends upon the availability of land (often farmers cannot afford to rotate cereal with non-food crop) and the variable response of host productivity following rotations (Kureh *et al.*, 2006).

It has been known for many years that improving the nitrogen content of the soils will lower the amount of *Striga* infestation on crops as nitrogen lowers the number of parasite attachments and improves the nutritional status of infected plants (Cechin and Press, 1994). However, although this is a simple solution for improving crop yields African farmers rarely have the financial resources to buy fertilizers and their effect is often variable in the field due to climatic and soil variability (Hearne 2009). Intercropping of maize and sorghum with the forage legume *Desmodium uncinatum* or *D. intortum* has been very successful at reducing the amount of *Striga* attachment to the crop plant and improving cereal yields significantly in some areas of Africa (Khan *et al.*, 2002; Khan *et al.*, 2010). *Desmodium* species causes large scale suicidal germination of *Striga* seeds. Their root exudates contain germination stimulants and as *Desmodium* is a perennial plant exudates will be continually produced causing suicidal germination of seeds all year round. In addition, the root exudates are also thought to contain an inhibitor of haustorium formation in *Striga* species (Khan *et al.*, 2002; Hooper *et al.*, 2010). Up to 2010, Khan *et al.* indicated that approximately 30,000 smallholders in East Africa have adopted traditional mixed intercropping of maize with *Desmodium* spp, increasing the average the yield per hectare from 1 to 3.5 tonnes ha⁻¹.

Despite the success of this control method uptake of this technology has been

slow due to the cost of *Desmodium* seeds, the fact that *Desmodium* is a non-food crop and that, in drier regions, it suffers from drought stress.

The problem that parasitic plants represent for crop production has brought together international efforts to identify resistant cultivars of maize, sorghum and rice and to identify Quantitative Trait Loci (QTL) and genes underlying resistance (Hausman *et al.*, 2000; Gurney *et al.*, 2003; Badu-Apraku *et al.*, 2006; Kaewchumnong and Price, 2008; Scholes and Press 2008; Cissoko *et al.*, 2011). *Striga*-resistant cultivars represent an economically sustainable method of control as they require very few inputs from the farmers other than seeds. A great deal of progress has been made over the last 10 years in the identification of germplasm of both cultivated and wild relatives of rice (Gurney *et al.*, 2006; Kaewchumnong and Price, 2008; Cissoko *et al.*, 2011), sorghum (Hausman *et al.*, 2004; Mohamed *et al.* 2003) and interspecific inbred maize line ZD05 (*Zea diploperennis* backcrossed into cultivated maize) (Amusan *et al.* 2008) that show some resistance to different *Striga* species or ecotypes. In addition different phenotypes associated with different types of resistance reactions have been described. For example, some resistance reactions in sorghum are characterised by rapid necrosis at the site of attachment and resemble a hypersensitive reaction (Mohamed *et al.*, 2003). In rice the rice cultivar Nipponbare, Gurney *et al.*, (2006) showed that some resistant reactions were associated with an inability of the parasite to penetrate through the endodermis and make connections with the host xylem vessels. More recently this phenotype has been seen in a range of rice cultivars that exhibit resistance to *S. hermonthica* (Yoshida and Shirasu 2009) including the interspecific NERICA cultivars (Cissoko *et al.*, 2011). Despite the increased understanding of resistance in cereals to parasitic plants we still know little about the genes involved in resistance. To date only one resistance gene in cowpea that provides resistance in cultivar B301 to race SG3 of *S. gesnerioides* (Li and Timko, 2009) has been described and been shown (by silencing of the resistance gene) to function as a resistance gene.

Although cultivars of rice and sorghum have been discovered that have good resistance to *Striga*, none have shown complete immunity *i.e.* have no successful *Striga* attachments. Part of the reason for this, and for the fact that only a few resistant cultivars of sorghum, maize and rice are currently being used by farmers on a regular basis, is the genetic variability of the *Striga* seed bank (Huang *et al.*, 2012). In order to breed crops with durable resistance to *Striga* we need to know more about the relationship between virulence genes in different ecotypes and species of *Striga* and host resistance genes.

At present it is clear that no single control method will protect plants against *Striga* and the best control is achieved by an integrated management strategy. There is a great need to develop new strategies for control of this parasite and a greater knowledge about the mechanisms underlying the devastating effects of *Striga* on host growth, morphology and physiology would underpin such an approach. The main aim of this thesis is to advance our understanding of the biology of a susceptible host-parasite interaction and in particular to elucidate some of the mechanisms underlying the changes in morphology and architecture of plants infected with *S. hermonthica* since these changes contribute to the low yields of *Striga*-infected rice by reducing the number of productive tillers.

1.5 How does *S. hermonthica* alter the morphology and biomass of its hosts?

Many studies of the effect of *S. hermonthica* on the growth and morphology of maize and sorghum (e.g. Press and Stewart, 1987; Gurney *et al.*, 1995; Taylor *et al.*, 1996) and to a lesser extent rice (Cechin and Press, 1994; Watling and Press 2000) have been carried out over the last 20 years. These studies have shown that infection of cereals by *S. hermonthica* or *S. asiatica* causes a suite of characteristic changes to the morphology and architecture of the infected plant which ultimately contributes to the significant losses of grain yield that occur in infected compared to uninfected plants. These characteristic changes include severe stunting of *Striga*-infected plants as internodes do not elongate properly, a thinning of the stems and lower total leaf area as leaves may be smaller or fewer (Cechin and Press, 1994; Gurney *et al.*, 1995; Taylor *et al.*, 1996; Mayer *et al.*, 1997; Frost *et al.*, 1997). In plants that produce a lot of tillers e.g. rice, infection by *S. hermonthica* suppresses tillering (Cissoko *et al.*, 2011) leading to the reduction in stem biomass. All these changes result in a loss of above ground biomass compared to uninfected plants. Infection by *Striga* also lowers root biomass in comparison to uninfected plants but this loss is not as great the loss in above ground biomass resulting in an increase in the root : shoot ratio in infected plants (Cechin and Press, 1994; Gurney *et al.*, 1995; Taylor *et al.*, 1996; Mayer *et al.*, 1997; Frost *et al.*, 1997; Cissoko *et al.*, 2011). These characteristic changes in plant morphology and architecture are illustrated in Fig 1.4 which shows the effect of *S. hermonthica* on the architecture, morphology and biomass partitioning of rice cultivar IAC 165. Although we know how the morphology and biomass of infected plants is altered by *S. hermonthica* our understanding of the mechanisms underlying these changes is less well established. The morphological changes observed in *S. hermonthica*-infected plants

leads to a reduced photosynthetically active leaf area, thus decreased carbon assimilation and reduced growth (Frost *et al.*, 1997; Watling and Press, 2000)

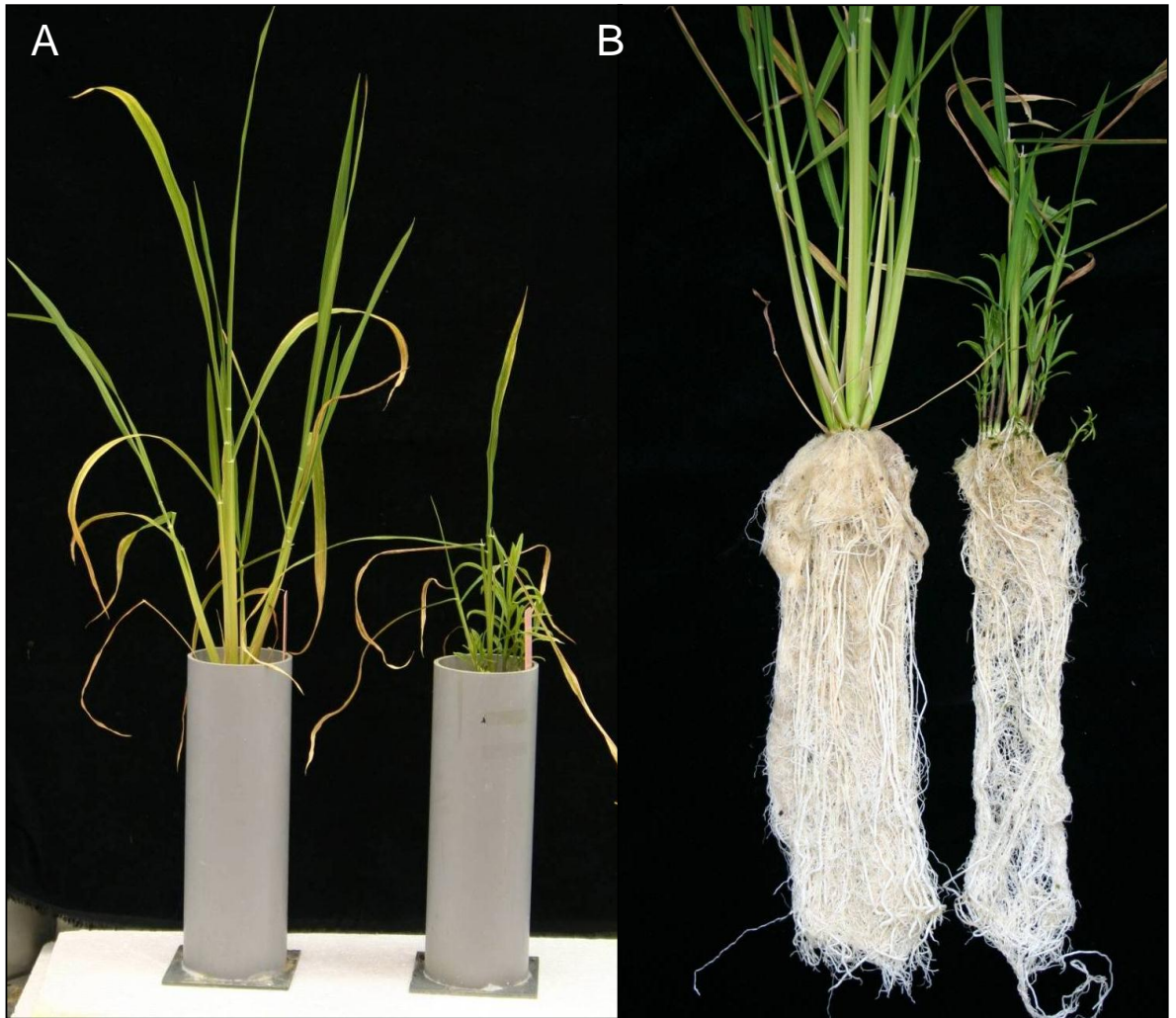


Figure 1.4. Effect of *S. hermonthica* on the morphology and architecture of the rice cultivar IAC165. (A) Uninfected and *Striga*-infected plant growing in pots. The infected plant is stunted and has fewer leaves than the uninfected plant. The infected plant has not produced tillers and has severely reduced above ground biomass compared to the uninfected plant. (B) The root systems of uninfected and *Striga*-infected rice plants. The infected plant has lower root biomass compared to the uninfected plant. (Scholes, unpublished).

1.5.1 Mechanisms underlying alterations in the morphology and biomass of cereals infected with *S. hermonthica*.

It has been suggested that at least some of the loss of biomass of *Striga*-infected plants is due to acquisition of carbon, nitrogen and other solutes from the host plant *i.e.* to a sink effect of the parasite. This is likely to be the case during the later stages of infection when parasite biomass is large but it cannot account for the differences in biomass of infected and uninfected plants during the early stages of infection when parasite biomass is small. Gurney *et al.*, (1999) showed that the growth and yield of sorghum plants infected with *S. hermonthica* was not correlated with the number or biomass of parasites attached to the roots and this effect has also been seen in maize and millet cultivars infected with *Striga*. Fig 1.5 shows the effect of different amounts of parasite biomass on the roots of the sorghum cultivar, CSH-1. There was a large decline in grain yield when parasite biomass was small. As parasite biomass increased the loss of grain yield only increased by small amounts. This suggests that mechanisms, in addition to acquisition of nutrients by the parasite, are involved in the loss of host biomass and yield. One of the approaches of this thesis consists in analysing the role of different plant growth regulators in the characteristic stunting and reduction of tillering in *S. hermonthica*-infected rice plants.

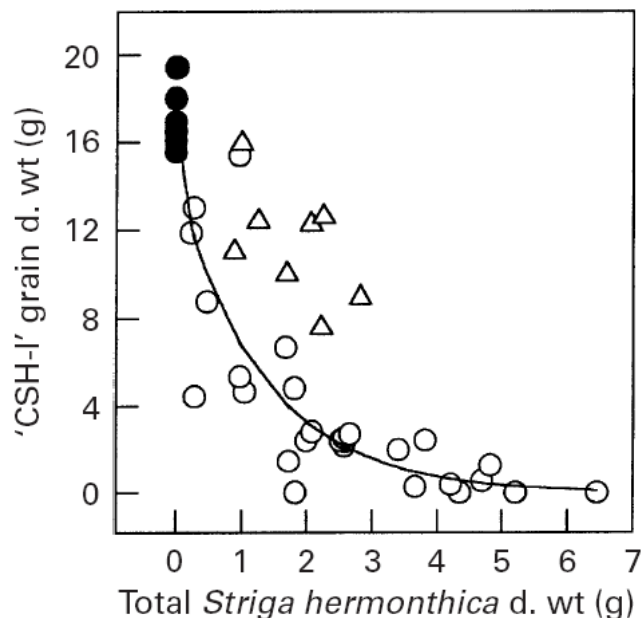


Figure 1.5. The relationship between grain dry weight and the amount of *S. hermonthica* on the roots of sorghum plants (cv. CSH-1). Closed circles represent uninfected plants whilst open circles represent plants infected with *S. hermonthica*. Taken from Gurney *et al.*, 1999.

The loss of biomass of *Striga*-infected plants may also be partly due to the effect of *Striga* on host architecture, which decreases the proportion of photosynthetic tissue to non-photosynthetic tissue and increases self-shading because the plants are stunted (Gurney *et al.*, 1995). In addition, both laboratory and field studies have shown that rates of photosynthesis are usually lower in the leaves of *Striga*-infected plants compared with those of uninfected plants largely due to stomatal closure following infection (Cechin and Press, 1993; Frost *et al.*, 1997; Gurney *et al.*, 1995). In some studies alterations in the amounts of ABA in *Striga*-infected plants have been detected (Taylor *et al.*, 1996; Frost *et al.*, 1997; Shen *et al.*, 2007) and may explain the low stomatal conductance of infected plants. It has also been suggested that alterations in ABA may play a role in regulating plant height. Accumulation of ABA during drought stress has been associated with stunting of plants and reduced leaf elongation (Bacon *et al.*, 1998; 1990; Quarrie, 1982) but this has not been investigated further.

The mechanisms underlying the stunting of plants infected with *S. hermonthica* have not yet been discovered. In 1980 Musselman suggested that the parasite may produce a 'toxin' that moves from host to parasite, but such a metabolite has not yet been identified. Plant growth regulators such as auxins, cytokinins, gibberellins (Salas Fernandez *et al.*, 2009; Hedden, 2008; Yamaguchi, 2008; Finet and Jaillais, 2012; Hwang *et al.*, 2012) and more recently strigolactones (Beveridge and Kyojuka, 2010; Domagalska and Leyser, 2011) are important in regulating plant growth and allometry and may be important in mediating the response of host plants to infection with *Striga*. There is only one study describing the effect of infection with *S. hermonthica* on the amount of different plant growth regulators in the tissues of infected plants (Drennan and El Hiweris, 1979). These authors showed that *Striga*-infected sorghum tissues contained greater amounts of ABA and ethylene and lower amounts of cytokinins and gibberellins when compared to uninfected plants. Clearly there is a need to investigate the potential role of plant growth regulators in the alterations in architecture and allometry of *Striga*-infected plants. There have been great advances in our understanding of plant growth regulator biosynthetic and signalling pathways and of the interactions between different plant growth regulators since the study of Drennan and El Hiweris, (1979). In addition, the availability of mutants in specific components of biosynthetic and signalling pathways allows specific hypotheses to be tested and the availability of genomic technologies (e.g. microarrays for transcript profiling) makes this an ideal time to revisit the hypothesis that *Striga*-induced alterations in plant growth regulator metabolism and signalling underlie some of the alterations in the growth and architecture of infected host plants. This is the aim of this thesis.

1.5.2 Does *S. hermonthica* suppress tillering in rice plants by altering strigolactone or auxin pathways?

Tillering is a complex process in which the expression of many genes must be meticulously regulated. Tillering, also known as lateral bud outgrowth is the result of the formation of axillary buds in the axil of leaves and their subsequent outgrowth. The first described functional gene involved in tillering in rice was MONOCULM1 (MOC1). MOC1 promotes tiller bud outgrowth in rice and *moc1* rice mutants are not able to produce tiller buds, resulting in only one main culm (Li *et al.*, 2003). MOC1 encodes a nuclear protein member of the transcription factor GRAS family. MOC1 is involved in meristem initiation and outgrowth by regulating the expression of the transcription factors *Oryza sativa* homeobox 1 and Os TEOSINTLE BRANCHED1, respectively (Li *et al.*, 2003; Lo *et al.*, 2008).

Dormancy of tiller buds has been historically attributed to the classical theory of apical dominance controlled by auxins and cytokinins (de Saint German *et al.*, 2010; Zhang *et al.*, 2010). Additional to these two hormones, a secondary signal controlling shoot branching had been suggested in studies with plants that exhibited increased shoot branching in various species including *Arabidopsis*, pea, petunia, chrysanthemum and rice (Beveridge *et al.*, 1996; Doebley *et al.*, 1997; Simons *et al.*, 2007). Decapitation of the apical meristem of rice and other plant species results in outgrowth of lateral buds by a reduction of auxin levels (Shimizu *et al.*, 2009; Zhang *et al.*, 2010), however it has been recently shown that polar auxin transport occurs too slowly and auxin depletion following decapitation in pea is not correlated to bud outgrowth (Renton *et al.*, 2012).

Evidence for a novel, mobile/transmissible signal involved in regulating tiller bud outgrowth came from the analysis of a range of branching mutants of *Arabidopsis*, pea, petunia (see Beveridge 2006; Beveridge and Kyoizuka 2010) and rice (Arite *et al.*, 2007; Umehara *et al.*, 2008). Studies performed by Arite *et al.*, (2007) and Umehara *et al.*, (2008) demonstrated that strigolactones are the predicted transmissible signal that negatively regulate tiller bud outgrowth in rice. Strigolactones (SL) are a group of terpenoid lactones that were originally discovered in root exudates because of their role as stimulants of parasitic plant seed germination (Hess *et al.*, 1992; Yokota *et al.*, 1998; Masutova *et al.*, 2005, Humprey and Beale, 2006) and as inducers of hyphal branching in mycorrhizal fungi (Akiyama *et al.*, 2005; Akiyama and Hayashi, 2006).

Strigolactones are synthesised in chloroplasts by breakdown of carotenoids through the action of carotenoid cleavage dioxygenases (CCDs) as shown in Figure 1.6. Nine CCDs have been identified in *A. thaliana*, five of them putatively involved in

ABA biosynthesis, and four of them required for the biosynthesis of strigolactones (Tan *et al.*, 2003; Arite *et al.*, 2007; Sun *et al.*, 2008). The current model for strigolactone biosynthesis (Figure 1.6) indicates that the carotenoid cleavage dioxygenase 7 (CCD7) is the first enzyme involved in the biosynthesis of strigolactones. CCD7 is encoded by the HIGH TILLERING DWARF 1 / DWARF17 (HTD1/D17) gene in rice, MORE AXILLARY GROWTH 3 (MAX3) in *A. thaliana*, RAMOSOUS3 (RMS3) in pea and DECREASED APICAL DOMINANCE 3 (DAD3) in petunia (Johnson *et al.*, 2006; Junhuang *et al.*, 2006; Umehara *et al.*, 2008; Gomez-Roldan *et al.*, 2008). The second enzyme in the biosynthetic pathway is CCD8 that acts downstream from CCD7. CCD8 breaks carotenoid double bonds, generating strigolactone compounds. CCD8 has been described and characterised in *A. thaliana* (MAX4) (Sorefan *et al.*, 2003), rice (D10) (Ishikawa *et al.*, 2005; Junhuang *et al.*, 2006; Arite *et al.*, 2007; Umehara *et al.*, 2008), pea (RMS1) (Beveridge *et al.*, 1996), petunia (DAD1) (Simons *et al.*, 2007) and chrysanthemum (Liang *et al.*, 2010), indicating a common mechanism of biosynthesis across species (Johnson *et al.*, 2006). More recently another gene potentially involved in strigolactone biosynthesis D27 has been discovered in rice (Fig 1.6). This gene encodes an iron-containing protein and is thought to lead to the production of a mobile strigolactone precursor or intermediate. D10, D17 and D27 are expressed mainly in vascular tissues of shoots and roots and their proteins are localised in the plastids.

Evidence for the involvement of these genes in the biosynthesis of strigolactones comes from the analysis of biosynthesis mutants that are defective in CCD7 (*rms5/max3/d17*) or CCD8 (*rms1/max4/d10*). Umehara *et al.*, (2008) analysed the strigolactone content of roots of *d10-1* and *d17-1* (dwarf, high tillering mutants). They found that one of the major strigolactones, 5-epi deoxystrigol was extremely low compared to the concentrations in wildtype plants. In addition, in wildtype rice plants phosphorus starvation caused an upregulation of CCD7 and CCD8 and an increase in 5-epi deoxystrigol. However in the strigolactone biosynthetic mutants the amount of 5-epi deoxystrigol did not alter with altered nutrient status of the soil. In addition, when these authors supplied the roots of 2 week old high tillering *d10-1* and *d17-1* mutants with GR24 (a strigolactone analogue) the number of tillers was reduced in a dose dependent manner. D27 mutants also have reduced amounts of strigolactones in their roots and root exudates and tiller bud outgrowth is inhibited by GR24 (Lin *et al.*, 2009). Once outside the plastids the mobile strigolactone (or precursors) undergo further

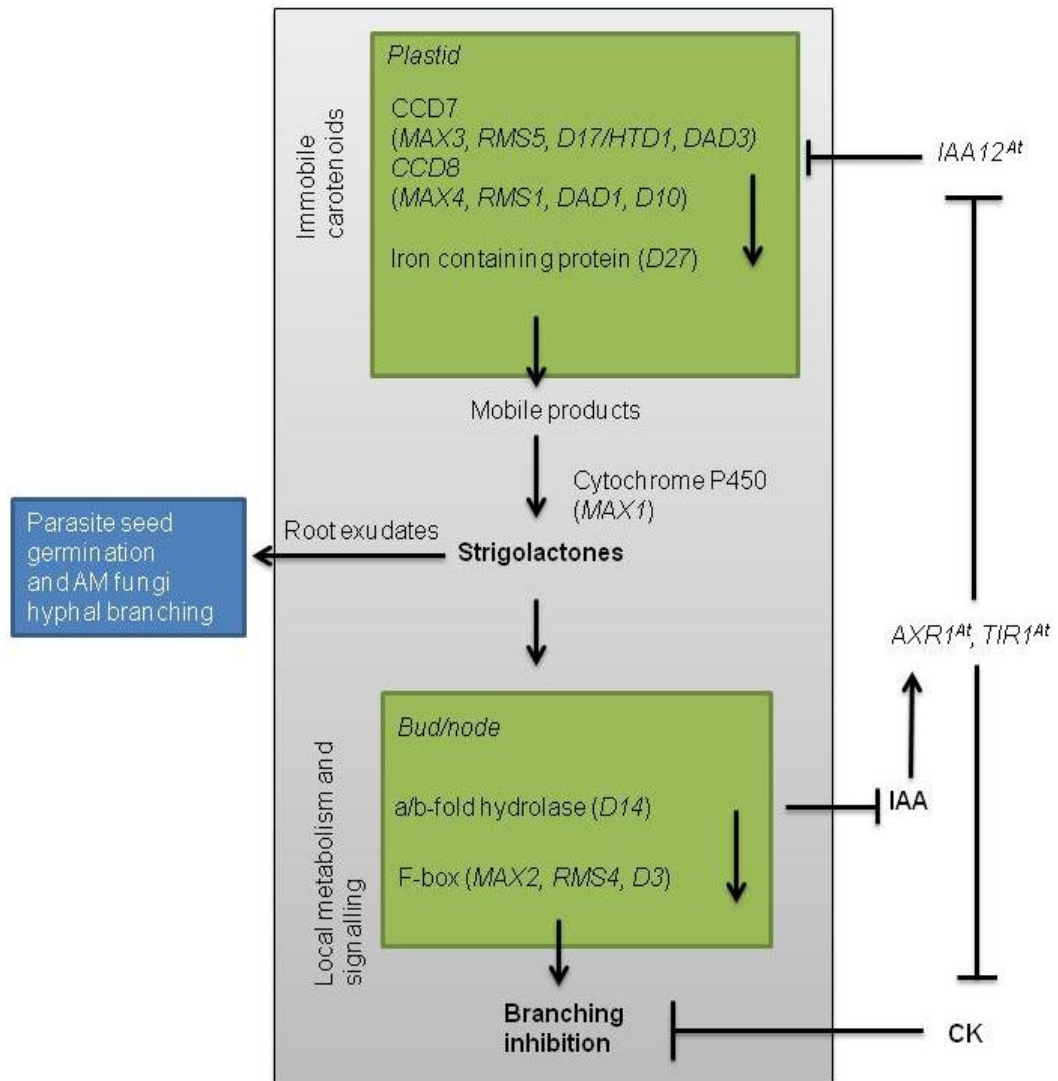


Figure 1.6. Strigolactone biosynthetic pathway and related genes (adapted from Beveridge and Kyozyuka, 2010).

interconversions to convert them into active strigolactones and this involves the action of a cytochrome p450 (known as MAX1).

At present the genes and proteins involved in the perception and signalling pathways of strigolactones are poorly understood. In rice two genes, D14 and D3 encode an alpha-beta fold hydrolase protein and an F-box leucine rich repeat (LRR) protein respectively and are thought to play a role in the signalling or the conversion of strigolactones to their bioactive form (Arite *et al.*, 2009). F-box proteins are often involved in ubiquitin-mediated degradation which suggests degradation of negative regulators may be involved in strigolactone signalling (Beveridge and Kyojuka, 2010) (RMS4 (pea) and MAX2 (Arabidopsis) are homologues of D3) (Fig 1.6). Evidence for the involvement of these proteins in signal transduction or perception comes from the analysis of *d3/rms4/max2* mutants. The high tillering phenotype of these mutants is not rescued by the application of GR24 showing that they are insensitive to strigolactones. Indeed both *d14* and *d3* mutants have elevated levels of strigolactones (Arite *et al.*, 2009).

Strigolactones, auxins and cytokinins are now known to co-regulate lateral bud outgrowth in an intricate hormonal signalling network (Ferguson and Beveridge, 2009). Shoot branching is inhibited by the polar auxin transport stream (PATS) generated by an auxin gradient derived from young leaves and the shoot apical meristem. One model of suppression of lateral bud outgrowth by strigolactones establishes the requirement of auxin transport from dormant buds into the PATS in order for lateral buds to emerge (Bennet *et al.*, 2006; Prusinkiewics *et al.*, 2009; Crawford *et al.*, 2010). The function of auxin is to regulate strigolactone levels by a classical signal transduction pathway (Fig 1.6). Auxin acts by binding to its receptor, the F-box protein transport inhibitor response 1 (TIR1), and the action of the ubiquitin activating enzyme AUXIN RESISTANT1 (AXR1), promoting the degradation of AUXIN / INDOLE-3 ACETIC ACID (Aux / IAA) transcriptional repressors and upregulation of auxin biosynthesis (Leyser *et al.*, 1993; Bennet *et al.*, 2006; Beveridge and Kyojuka, 2010, Xia *et al.*, 2012; Calderon-Villalobos *et al.*, 2012). The feedback mechanisms of auxin on strigolactones involve the stabilization of AUX/IAA12 (one of the transcriptional repressors) (Fig. 1.6). In addition, auxin suppresses biosynthesis of cytokinins, leading to suppression of tillering (Aloni *et al.*, 2006; Shimizu-Sato *et al.*, 2009; Beveridge and Kyojuka, 2010).

The interaction between strigolactones and auxins has been demonstrated by the use of mutant plants in both hormonal pathways. Apically applied auxin is capable of inducing expression of CCD8 and CCD7, resulting in inhibition of bud outgrowth

(Hayward *et al.*, 2009). In contrast, auxin depletion by decapitation of the shoot apical meristem results in decreased strigolactone levels and lateral branching (Foo *et al.*, 2005; Arite *et al.*, 2007; Hayward *et al.*, 2009). Consistently, axillary buds of strigolactone mutants are resistant to auxin treatment and do not show an effect (Sorefan *et al.*, 2003; Bennet *et al.*, 2006), whereas the semi-dominant auxin response mutant with increased branching *bodenlos (bdl)* shows a substantial decrease in the expression of CCD7 and CCD8, thus reducing strigolactone levels and increased branching. However, the wild type phenotype can be restored in the *bdl* mutant by the application of the strigolactone analogue GR24 (Hayward *et al.*, 2009).

One of the objectives of this project was to determine if strigolactones and / or auxins play a role in the morphological changes of rice infected with *S. hermonthica*, based on the hypothesis that the strigolactone and / or auxin metabolic pathways are altered following infection by *S. hermonthica*.

1.5.3 What are the mechanisms underlying the stunting of plants infected with *S. hermonthica*? A role for plant growth regulators?

So far, the mechanisms underlying the severe stunting of *Striga*-infected plants are unknown. It has been suggested that *Striga* may produce a “toxin” that moves from parasite to host (Musselman, 1983) but such a compound has yet to be identified. Alterations in the metabolism of plant growth regulators in plants infected by *S. hermonthica* has also been suggested previously (Drennan and El Huweris, 1979; Frost *et al.*, 1997) but few studies have investigated this hypothesis in detail. Based on the roles that gibberellins play in cell growth and internode elongation, as seen in gibberellin defective mutants (Gale and Marshall, 1973; Behringer *et al.*, 1990; Ueguchi-Tanaka *et al.*, 2000; van der Knapp *et al.*, 2000), this hormone became the first candidate to be suggested to be involved in the stunting observed in *S. hermonthica* - infected plants.

Gibberellins (GAs) are tetracyclic, diterpene compounds based on the ent-gibberellane skeleton. They were first discovered for their effect on stem elongation (Campbell and Cassady 1969; Behringer *et al.*, 1990; Ikeda *et al.*, 2001), and later for functions as seed germination, floral initiation, leaf expansion, trichome development and sex determination (Frantz and Bugbee 2002; Chhun *et al.*, 2007; Jan *et al.*, 2006). Many gibberellins (~125 GAs) have been reported in plants but relatively few of them are biologically active (http://www.plant-hormones.info/gibberellin_nomenclature.htm). GA₁, GA₃, GA₄ and GA₇ are the most common bioactive forms (Yamaguchi 2008). The main target of bioactive GAs is the intercalary meristem (van der Knaap *et al.*, 2000),

which is found at the base of internodes and leaf blades. This meristem is responsible for internode elongation in rice due to effects of GAs (van der Knaap *et al.*, 1997). GA-deficient plants show varying degrees of dwarfism, similar to the stunting effect observed on *Striga*-infected plants.

The discovery of the gibberellin receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1) notably expanded the understanding of GA metabolism (Hedden, 2008). GID1 is a nuclear receptor to which bioactive GA binds in order to recognise the transcriptional regulator DELLA proteins (Murase *et al.*, 2008). After binding to the GA-GID1 complex, DELLA proteins are recruited by an F-box protein in a E3 SKP1-CULLIN-F box complex for polyubiquitylation and consequent degradation by the 26S proteasome (Silverstone *et al.*, 2001; Sasaki *et al.*, 2003; Fu *et al.*, 2004; Dill *et al.*, 2004; Griffiths *et al.*, 2006). The degradation of DELLA proteins by GA-GID1 induction allows expression of GA responsive genes (Murase *et al.*, 2008). *A. thaliana* contains five DELLA proteins, GIBBERELLIN INSENSITIVE (GAI) (Peng *et al.*, 1997); REPRESSOR OF *ga1-3* (RGA) and RGA-LIKE1, 2 and 3 (Silverstone *et al.*, 1998; King *et al.*, 2001; Dill and Sun, 2001; Lee *et al.*, 2002). In rice, SLENDER (SLR1) is the homolog to GAI and RGA in *A. thaliana* and *Reduced height (Rht)-B1b Rht-D1b* in wheat (Ikeda *et al.*, 2001; Pearce *et al.*, 2011). A more detailed description of SLR1 is presented in the paragraphs below.

Biosynthesis of bioactive GAs from geranyl-geranyl diphosphate (GGDP, a precursor for diterpenoids) requires three different classes of key enzymes: plastid-localized terpene synthases (TPS), membrane-bound cytochrome P450 monooxygenases and soluble 2-oxoglutarate-dependent dioxygenases (Zhu *et al.*, 2006). GGDP is converted to *ent*-kaurene by two TPSs: *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS). *ent*-kaurene oxidase (KO) catalyses sequential oxidation on carbon-19 of *ent*-kaurene to produce *ent*-kaurenoic acid, which is converted to GA₁₂ by *ent*-kaurenoic acid oxidase (KAO). Conversion of inactive GAs to active forms occurs through oxidations on C-20 and C-3 of GA₁₂ by GA₂₀-oxidase (GA₂₀ox) and GA₃-oxidase (GA₃ox), respectively (reviewed in Yamaguchi, 2008). Results from various experiments suggest that bioactive GAs are produced at the site of their action (Silverstone *et al.*, 1997; Itoh *et al.*, 1999). In a genomic context, GA biosynthesis genes are clustered on a single chromosome in fungi, whereas they are located on multiple chromosomes in plants (Yamaguchi *et al.*, 1998; Hedden and Phillips 2000). The expression patterns of the GA biosynthetic genes *OsGA₃ox2*, *OsGA₂₀ox2*, the gene encoding the α -subunit of the heterotrimeric GTP-binding protein (*G α*) and the signalling *SLR1* gene, have been determined by the use of promoter: β -

glucuronidase (GUS) reporter expression. Expression of these genes has been found mainly in rapidly elongating and dividing organs and tissues (Kaneko *et al.*, 2003).

An intermediate component of the GA signal transduction pathway in rice is the SLENDER (SLR) gene. This gene presents high homology to the height regulator genes (members of the transcription factor GRAS gene family) in maize (D8), Arabidopsis (GAI and RGA) and wheat (RHT-1Da); it has been demonstrated that GA sensitivity is affected by mutations on these genes (Olszewski *et al.*, 2002). *slender* (slr1-1) mutant in rice is caused by a single recessive mutation and results in a constitutive GA response phenotype, showing no inhibition of stem elongation by exogenous application of GA biosynthesis inhibitors (Ikeda *et al.*, 2001). In contrast, YABBY1 (YAB1), a GA regulatory element in rice that controls the expression of the biosynthetic genes OsGA₂₀ox2 and OsGA₃ox2, causes semi-dwarfism when it is constitutively expressed in transgenic plants, however, the wild type phenotype can be recovered by exogenous application of GAs (Dai *et al.*, 2007). Gibberellins can be deactivated by various mechanisms. 2 β -hydroxylation is the best characterized deactivation mechanism of GAs, catalysed by GA₂-oxidases (GA₂oxs) (Dai *et al.*, 2007; Lo *et al.*, 2008). GA methyltransferases GAMT1 and GAMT2 have provided another deactivation mechanism in Arabidopsis, with a putative role in seed development, resulting in plants with GA deficiency and a typical GA-deficient phenotype (Varbanova *et al.*, 2007).

In addition to gibberellins, other plant growth regulators may also be involved in regulating the height of plants. For example mutants defective in auxin biosynthesis (e.g. the rice tryptophan deficient dwarf mutant (*tdt1*)) or transport (e.g. rice plants over expressing the auxin transport related small auxin up RNA 39 (SAUR39) or both biosynthesis and transport (e.g. rice plants over expressing members of the AUX/IAA transcriptional regulator (OsIAA)) show distinctive morphological characteristics, such as stunting, alterations in branch angle and decreased amounts auxin (Song *et al.*, 2009; Kant *et al.*, 2009; Sazuka *et al.*, 2009).

The auxin transport mutant SAUR39 shows a similar phenotype to *S. hermonthica* - infected plants (Fig 1.7). SAUR39 belongs to a family of auxin-responsive genes, known as small auxin up RNA genes, whose function is largely unknown (Jain *et al.*, 2006; Kant *et al.*, 2009). Over expression of SAUR39 in rice plants resulted in reduced polar auxin transport, lower shoot and root growth, reduced tillering, smaller vascular tissue and lower yield compared to wild type (Kant *et al.*, 2009), resembling *S. hermonthica* - infected plants (Figure 1.7 A and B). Whole genome transcript profiling of transgenic rice plants over-expressing SAUR39 showed

alterations in the regulation of auxin metabolism, photosynthesis, chlorophyll production, senescence, and sugar metabolism (Kant *et al.*, 2009; Jain and Khurana, 2009).

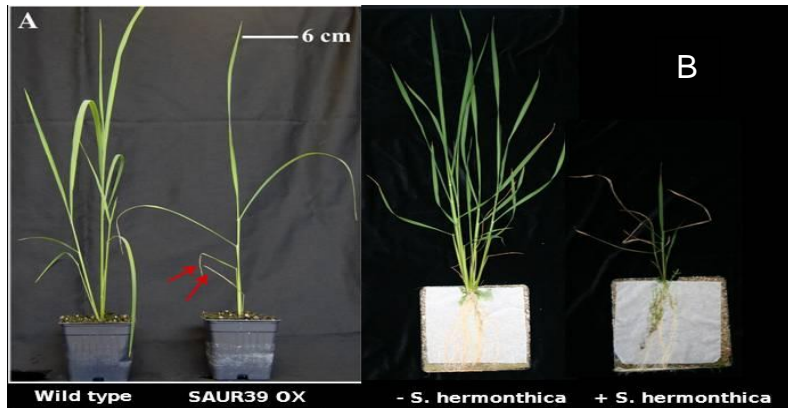


Figure 1.7 Comparison between (A) wildtype and transgenic rice plants over expressing the SAUR39 (a Small Auxin Up RNA gene) that acts as a negative regulator of auxin synthesis and transport in rice (Kant *et al.*, 2009) and (B) rice plants infected with *S. hermonthica*. Red arrows indicate increased leaf angle in SAUR39 overexpressing plants compared to their wild type.

In order to test the hypothesis that changes in the transcription of genes, regulating gibberellins, strigolactone and auxin metabolism and signalling lead to the characteristic stunting, reduced number of tillers and other morphological alterations of *S. hermonthica* - infected plants, respectively (Fig. 1.8), analysis of transcriptional changes in the whole genome profile was performed. This involved profiling changes in gene expression in the roots, stems and leaves of *Striga*-infected plants at different times after infection using whole genome microarrays. This is the first time such an analysis has been undertaken.

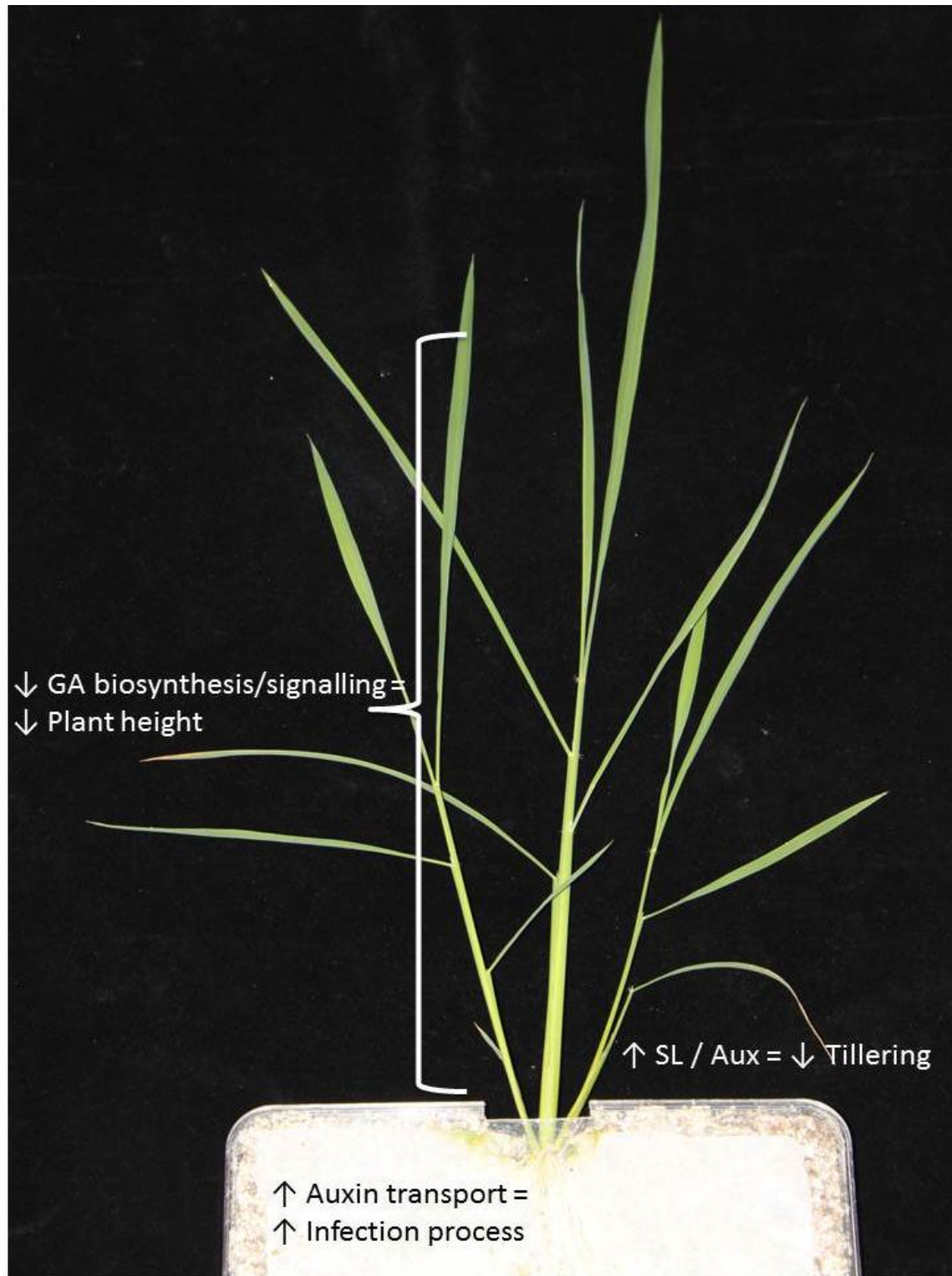


Figure 1.8. Morphological alterations detected in *S. hermonthica* infected rice plants and the proposed responsible plant growth regulators underlying such modifications. Arrows indicate an increase or decrease. GA: Gibberellins; SL: Strigolactones; Aux: Auxins.

1.6. Aim and objectives

The aim of this project was to investigate the mechanisms underlying alterations in the growth and morphology of rice plants infected by the parasitic weed *Striga hermonthica* during a compatible interaction focussing on the potential role of plant growth regulators in the suppression of tillering and stunting of the host plant.

In Chapter 2 a detailed analysis of the changes in growth and morphology of the susceptible rice cultivar IAC165 following infection by *S. hermonthica* was carried out. Specifically the hypothesis that the lower number of tillers in *Striga*-infected plants is due to an inhibition in the formation and emergence of lateral buds was investigated.

In Chapter 3 the hypothesis that strigolactones are involved in the suppression of tillering in rice plants infected with *S. hermonthica* was investigated by examining the effect of *Striga* on the suppression of tillering in rice plants with altered concentrations of strigolactones. Strigolactone concentration was manipulated by (a) the use of strigolactone mutants (D10-1, a strigolactone biosynthetic mutant and D3-1 a strigolactone signalling mutant) and (b) the application of a carotenoid biosynthetic inhibitor (fluridone) or a synthetic analogue of strigolactone (GR24). In addition the effect of *S. hermonthica* on the biosynthesis of strigolactones in the region of the stem containing the tiller buds and in the roots of infected plants was investigated by infecting rice plants containing a CCD8 (strigolactone biosynthetic gene):GUS promoter-reporter construct.

In Chapter 4 the potential roles of multiple plant growth regulator pathways in the changes in plant morphology of rice plants infected with *S. hermonthica* are investigated by profiling changes in gene expression in the roots, stem and leaves of control and infected plants using Affymetrix microarrays.

Chapter 2

How does *S. hermonthica* alter the growth and morphology of rice plants?

2.1 INTRODUCTION

Rice is one of the most important cultivated crops worldwide. In Sub Saharan Africa (SSA) the production of rice increases year upon year partly due to the introduction of the NEw RIce for Africa (NERICA) cultivars (Wopereis *et al.*, 2008). Rodenburg *et al.*, (2010) reported that over the last 30 years the amount of land cultivated by rice has grown by 105% with an increase in production of 170%. However, the production of upland rice is constrained by the parasitic plants *S. hermonthica* and *S. asiatica*, which are a major problem in the African continent, affecting the lives of millions of people (Parker and Riches, 1993; Scholes and Press, 2008). *Striga* species are obligate hemiparasitic weeds that infect the roots of cereal crops, including upland rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), pearl millet (*Pennisetum glaucum*), maize (*Zea mays*) and cowpea (*Vigna unguiculata*). The life cycle of *Striga* is complex and closely linked to that of its host plant. Germination of *Striga* seeds in the soil is triggered by compounds (mostly strigolactones) exuded from the roots of host, and non-host species (Bouwmeester *et al.*, 2003). The radicle attaches to the host root by means of a specialized organ called the haustorium which forms in response to a set of chemicals cues known as Haustorium Inducing Factors (HIFs) present in root exudates (Yoder *et al.*, 2001). Following attachment, the parasite grows through the host root cortex and endodermis, and forms connections between parasite xylem vessels and those of its host (Parker and Riches 1993). *Striga* spp cause negative effects on host growth, morphology, physiology and yield following infection. These include a significant reduction of the photosynthetic rate of its host (Rodenburg *et al.*, 2008; Frost *et al.*, 1997; Gurney *et al.*, 1999; Shen *et al.*, 2007), severe stunting, thinning of cell walls (Gurney *et al.*, 1999; Mayer *et al.*, 1997), drought-like effects (Shah *et al.*, 1987; Frost *et al.*, 1997; Hearne, 2001; Menkir and Kling, 2007) and rapid cessation of root elongation (Baird and Riopel 1985; Tomilov *et al.*, 2005). The grain yield of susceptible cultivars is often 40-100% lower than uninfected plants depending upon host genotype and level of infestation (Ejeta, 2007).

Most studies of the effect of *Striga* species on the growth and morphology of infected plants have focussed on maize and sorghum. In rice, the morphology of *S. hermonthica* infected plants is also altered; they exhibit stunting, thinning of the stems, a decrease in total biomass and a reduction of tillering (Watling and Press, 2000). Tillering, also known as shoot branching, is one of the major factors in determining the yield of rice plants (Li *et al.*, 2003). Each tiller has the capacity to produce a panicle thus contributing to yield. In reality, only tillers formed during the early phases of growth will have grains that fill completely. Those produced at a later stage often have only partial grain fill (Sakamoto and Matsuoka, 2008). Thus, the suppressive effect of

Striga on tillering in rice has a major effect on grain yield yet we know little about the mechanisms underlying the phenomenon. It is not clear whether *S. hermonthica* inhibits the formation of tiller buds, their outgrowth or both in a susceptible interaction.

Shoot branching is a complex process that involves the formation of lateral meristems from the leaf buds in the axils of leaves and its consequent outgrowth (Lauer 1991; Bos and Neuteboom 1998). Tillering is controlled by a fine regulation of environmental and hormonal cues, in which auxins, cytokinins and strigolactones are involved (Ishikawa *et al.*, 2005; Zou *et al.*, 2005). Strigolactones were first discovered as germination stimulants for parasitic plant seeds (Siame *et al.*, 1993), but more recently they have been found to be the missing hormone responsible for shoot branching of rice and other plant species (Umehara *et al.*, 2008). Although strigolactones are known to induce germination of *Striga* seeds, little information is available about the effect of *Striga* spp on strigolactone production post infection. The possible role of strigolactones in the suppression of tillering in *S. hermonthica*-infected rice plants is discussed further in Chapter 3.

Despite many years of research, the mechanisms used by *Striga* species to induce morphological changes in its host at early stages of infection have not been brought to light. In order to investigate these further in rice (Chapters 3 and 4) it is necessary to carry out a detailed analysis of the changes in morphology that occur following infection and to determine whether the *Striga*-induced suppression of tillering is due to an inhibition of tiller bud outgrowth or formation or both.

Therefore, the aim of this study was to carry out a detailed analysis of the changes in growth and morphology of the susceptible rice cultivar IAC165 following infection by *S. hermonthica*. Specifically I focussed on:

1. Determining how *S. hermonthica* altered main stem height and thickness, the number, length, specific leaf area (SLA) and time of leaf emergence of leaves on the main stem, the total number of leaves on the plant and the number and time of emergence of tillers.
2. Testing the hypothesis that, following infection, the lower number of tillers in *Striga*-infected plants is due to an inhibition in the formation and emergence of lateral buds.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

Plants of the susceptible rice cultivar IAC 165 (*O. sativa* spp. *japonica*) were grown in environmentally controlled rooms with an irradiance of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ at plant height, a 12 h photoperiod, a temperature of 28 / 24 °C (day / night, respectively) and relative humidity of 60%. Rice seeds were germinated between two sheets of glass-fibre filter (GFA) paper (Whatman, Maidstone, Kent, UK) supported by a block of moistened horticultural rock wool. After seven days, individual seedlings were transferred to root observation and sampling chambers called rhizotrons (Gurney *et al.*, 2006). These chambers consisted of modified perspex tissue culture dishes measuring 25 x 25 x 2 cm filled with vermiculite (Sinclair, Gainsborough, UK), a layer of rock wool on the bottom to avoid its dispersal, and a fine mesh (100 μm) (Plastok Group, Birkenhead, Merseyside, UK) on top of the vermiculite, onto which the seedlings were placed. Rhizotrons had openings at the top and bottom to allow emergence of the aerial part of the plant and draining of nutrient solution, respectively. A lid was placed on the rhizotron and, rhizotrons were wrapped in aluminium foil in order to prevent light reaching the roots. The use of rhizotrons over pots or field conditions provides the advantage of a more controlled infection and the possibility of temporal evaluation of the root system in order to ensure that *S. hermonthica* attaches successfully to the host. Each rhizotron was watered via an automatic dripper system four times per day, delivering a total of 240 ml of 40% Long Ashton solution containing 2 mM ammonium nitrate (Hewitt, 1966). Ten replicate plants per treatment (control and *Striga*-infected) were established. Seven days after the rice seedlings were transferred into rhizotrons, they were infected with *S. hermonthica* seeds (see section 2.2.3).

2.2.2 *Striga* seed sterilisation, preconditioning and germination

Striga hermonthica seeds collected from a maize host grown in Kibos, Kenya in 1997 were used to inoculate the rice seedlings. Prior to infection of rice plants, *S. hermonthica* seeds were surface sterilised, conditioned and germinated. *S. hermonthica* seeds were sterilised in 10% sodium hypochlorite for 5 min. Seeds (12 mg aliquots) were placed onto Whatman glass fibre filter paper in a funnel with a flask underneath it, and rinsed with 200 mL of distilled water to ensure that all the bleach was removed. Finally the seeds were distributed homogeneously on the filter paper that was placed in a Petri dish. The Petri dish was sealed with parafilm, foiled wrapped and incubated at 28 °C for twelve days to allow seeds to condition. Twelve hours prior to infection of the rice plants, 1.5 ml of an artificial germination stimulant (GR24 (0.1

ppm)) was placed into each Petri dish. The Petri dish was incubated overnight at 28 °C. The following morning, just before infection, the Petri dishes were viewed using a stereomicroscope (Prior, Cambridge, UK) and percentage germination of the seeds was calculated. Percentage germination was always greater than 70%.

2.2.3 Infection of rice seedlings

One week after transfer of seedlings into rhizotrons, the roots of rice plants on the mesh were infected with 12 mg of pre-germinated *S. hermonthica* seeds per plant. Seeds were removed carefully from the filter paper and rinsed with distilled water to remove the GR24 in which they were germinated. A disposable plastic pipette was used to position the seeds as close as possible to the host root system. A fine paintbrush was used in order to position the seeds at a distance approximately 5 mm from the roots. Plants were infected with germinated seeds (rather than relying on the production of host root exudates) to ensure synchronous attachment and growth of the parasites.

2.2.4 Measurements of growth

In order to compare the growth of control and *Striga*-infected plants the following non-destructive measurements were made every other day for 1 month following inoculation of the roots with *S. hermonthica* seeds:

1. The height of the main stem and the length of internodes. Internode length was calculated as the distance between the leaf sheaths of two contiguous leaves. These measurements allowed relative growth rates to be calculated using the formula $RGR = (L2 - L1)/(T2 - T1)$ where L = length and T = time.
2. The diameter of the main stem. This was measured 5 mm above the root crown using digital Vernier callipers (Mitutoyo, England, UK).
3. The total number of leaves on the plant (main stem plus tillers).
4. The number of leaves, their length and their turnover on the main stem. Individual leaf length was measured from the tip of the blade to the sheath.

Plants were harvested 28 days after infection. *S. hermonthica* individuals were removed from the roots of each infected plant and placed in a Petri dish. *S. hermonthica* plants were photographed with a Canon EOS500D digital camera and their number and length were measured using Image J v. 1.43 (National Institute of

Health, Maryland, USA). Petri dishes were then placed inside a drying oven for two weeks at 58 °C to obtain the dry weight of the *S. hermonthica* plants.

Following removal of the *Striga* plants from the roots, total leaf area of leaves of the main stem of rice was measured using a ΔT Area meter MK2 (Delta-T Devices, Burwell Cambridge, England). Each plant was divided into root, leaves and stems and dried in envelopes at 58 °C for two weeks to obtain dry biomass. The leaves of the main stem were dried separately to allow calculation of Specific Leaf Area (SLA) (leaf area / dry weight of the leaf).

2.2.6 Microscopic examination of tiller bud formation

In order to determine how *S. hermonthica* affects the development of the base of the stem, including the formation of tiller buds, stem samples from control and *Striga*-infected plants were collected at 0, 3, 5 and 13 days after infection (DAI) and then embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany), following the manufacturer's procedure. Firstly, the tissue was dissected with stainless steel scalpels (Swann Morton Limited, Sheffield UK) then placed in Carnoy's fixative (3:1, 100% ethanol : acetic acid) and vacuum infiltrated for fifteen minutes. Vacuum was released slowly and the samples were then washed with 100% ethanol two times within the following 24 hours. Samples were transferred to 1:1 Technovit 7100 : 100% ethanol solution for 48 hours, then replaced by 100% Technovit 7100 solution for a further 48 hours. Samples were positioned in eppendorf lids and polymerisation solution (Technovit 7100 : Hardener II; 15:1 proportion) was added until it reached the top of the lid. Polymerisation was left overnight. When the resin had polymerised, 4 cross incisions were made to the eppendorf lid to release the sample. Once released, samples were individually fixed to histomolds (Heraeus Kulzer, Wehrheim, Germany) for mounting on the microtome. Histomolds were prepared by placing the mold upside down on aluminium foil and pouring Technovit 3040 (Heraeus Kulzer, Wehrheim, Germany) into the mold (3:1 powder to liquid proportion), holding firmly until the resin was solidified. Sectioning was carried out using a Leica RM 2145 microtome (Leica Microsystems, Germany). Transverse and longitudinal sections (5 μm in thickness) were made at different positions along a 5 mm section of stem above the root primordia. This region contained the tiller buds. Water drops were positioned on glass slides. The sections were collected carefully with tweezers or needles and put in the water drops. The slide was then positioned on a hot plate at 65 °C to remove the water. The polychromatic stain Toluidene Blue O (TBO) was used for staining the microscopic sections. TBO was prepared as described by Ruzin (1999). A plastic pipette was used

to put TBO on top of the sections. Samples were exposed to TBO for 20 seconds and washed with dH₂O. The slides were placed on the hot plate to dry. Once dried, the sections were fixed with Depex® mounting media on 25 x 75 x 1.00 mm microslides (Menzel – Glaser) with coverslip. Visualisation of samples and image acquisition was made using an Olympus BX51 microscope and the program CellB (Olympus Optical Ltd., London, UK).

2.2.5 Statistical analyses

Developmental parameters were analysed using Student's t-test or ANOVA, as appropriate. All statistical analyses were performed using the statistical package R version 2.10.1.

2.3 RESULTS

2.3.1 The effect of *S. hermonthica* on the morphology of the main stem

The thickness and height of the main stem (MS) of *S. hermonthica*-infected plants was severely reduced in comparison to control plants over a time course of 28 days (Fig. 2.1 A and B, respectively). The thickness of the MS did not differ significantly between treatments up to 5 days after inoculation. However, by day 8 the main stem of *Striga*-infected plants was significantly thinner than that of the control plants ($t = 4.1991$, $df = 14.673$, $p\text{-value} < 0.001$) and this difference became more pronounced with infection. By 15 DAI, the width of the MS of uninfected plants was still increasing, whereas the stem of infected plants had stopped increasing in diameter. By the time plants were harvested, the thickness of the MS of infected plants was only 50% that of control plants (2.1 A).

Sections through the base of the stem at 2 and 5 DAI showed that the thickness of the stem was similar in both control and *Striga*-infected plants (Fig. 2.2 and 2.3, respectively) consistent with the measurements of stem thickness. However, by 13 DAI transverse sections through the stem of *S. hermonthica* infected plants revealed that it was significantly thinner than that of the uninfected plants (Fig. 2.4), reflecting the measurements shown in Figure 2.1 A.

In order to determine whether the change in thickness of the stem of *Striga*-infected plants was due to a change in the number and / or size of cells in the stem the length and number of cortical cells was measured along a transect from the outer cortex to the endodermis in 20 cross sections of the stems of uninfected and infected plants. Both the number and length of cells in the cortical region of the MS were significantly lower in *Striga*-infected compared to uninfected stems 13 DAI ($t = 3.4953$, $df = 11.945$, $p\text{-value} = 0.0045$) (Fig. 2.5). The average number of cells spanning the cortical region was 18.7 ± 0.83 for uninfected plants, compared to 14.2 ± 0.89 in *Striga*-infected plants. The average length of cells this region was $35.66 \mu\text{m} \pm 0.67 \mu\text{m}$ and $17.89 \mu\text{m} \pm 0.62 \mu\text{m}$ for control and *Striga*-infected plants, respectively.

One of the most remarkable characteristics about the phenotype of *S. hermonthica*-infected susceptible plants is the stunting of the host. Plant height to the highest visible ligule of *Striga*-infected rice plants was severely reduced compared to uninfected controls during the evaluation period of 28 days (Fig 2.1B). Initial differences in height became significant by 8 DAI ($t = 3.6863$, $df = 7.488$, $p\text{-value} = 0.007$), when the parasite biomass was still very small. The average height of uninfected and *S. hermonthica*-infected plants was 12.78 ± 0.53 cm and 9.90 ± 0.69 cm, respectively. From 8 to 28 DAI the height of uninfected plants had increased by 90% whereas the

height of infected plants increased by 32%. The height of uninfected plants was nearly two fold that of infected plants by the end of the experiment (24.26 and 13.08 cm, respectively) ($t = 21.166$, $df = 7.367$, $p\text{-value} = 7.23e-08$).

The stunting of the main stem of *S. hermonthica*-infected plants was due to shorter internodes when compared to an uninfected plant (Fig 2.6). For the purpose of this study, nodes were considered as the region of the leaf where ligules emerged. Therefore, an internode was defined as the distance between two contiguous ligules. The number of internodes on the main stem was the same for uninfected and *Striga*-infected plants in early stages of infection up to 10 DAI (3 internodes were present in all plants). By 12 DAI, uninfected plants had one more visible internode (internode 4) compared to infected plants. Interestingly, throughout the whole period until harvest 28 DAI, uninfected plants had only one more visible internode than *S. hermonthica*-infected plants (7 and 6 internodes, respectively).

There was no significant difference in the length of the first 3 internodes in control and *Striga*-infected plants throughout the time course of the experiment (Fig 2.6). By 10 DAI, there was a significant difference in the length of internode 4 in control (3.24 ± 0.19) and *Striga*-infected (1.72 ± 0.2 cm) plants respectively ($t = 6.2054$, $df = 7.983$, $p\text{-value} = 0.00026$). The following internodes (5 and 6) were also significantly shorter in *S. hermonthica*-infected compared to uninfected plants. Emergence of the fifth internode was delayed in *S. hermonthica*-infected plants by 13 days. It was not possible to measure its length until after the emergence of the sixth internode on infected plants that occurred at 25 DAI, since leaves were emerging on top of each other, instead of allowing internodes to elongate (Fig 2.6). At 28 DAI internode 5 was just visible in infected plants but its length was only 10% compared to the same internode in control plants (0.44 versus 4.4 cm, respectively). By 28 DAI the uninfected plants had produced internode 7 (the final internode of the main stem) but this was not visible in *S. hermonthica*-infected plants.

2.3.2 How does *S. hermonthica* alter the total number of tillers and leaves on infected rice plants?

Tillers are arranged in a hierarchical order, *i.e.* primary tillers are those that develop from lateral buds on the main stem; secondary tillers develop from lateral buds formed on the primary tillers at later stages of development and so on (Jaffuel *et al.*, 2005). Infection of rice plants with *S. hermonthica* severely suppressed the production of tillers when compared to uninfected controls by 28 DAI as illustrated in Figs. 2.7 and 2.8. Fig 2.8A shows the total number of tillers on control and *Striga*-infected rice plants

over a 30 day period following infection by *S. hermonthica*. At eleven days after inoculation the number of emerged tillers on *Striga*-infected plants was significantly lower than on uninfected plants ($t = 4.2$, $df = 16.691$, $p\text{-value} = 0.0007$). At this stage 90% of uninfected plants had produced one primary tiller, whilst only 20% of infected plants had one successfully emerged primary tiller (Fig. 2.8 A). Between 17 and 20 DAI, 60% of the infected plants had only produced the first primary tiller. During the same period, uninfected plants had between 2-3 tillers (composed of 2 primary and 1 secondary tiller) (Fig 2.8 A). Emergence of the second primary tiller had occurred in only 10% of the *S. hermonthica*-infected plants by 22 DAI. By 28 DAI uninfected plants had a total of 4 tillers on average, (two primary and two secondary) whilst only 30% of the infected plants had two primary tillers and the rest only one (Fig 2.8). No secondary tillers emerged in infected plants during the experimental period.

Not only was the emergence of tillers delayed in *S. hermonthica* -infected plants, their stems were thinner than those of uninfected plants (Fig. 2.8 B and C). The first primary tiller of *Striga*-infected plants was 0.181 ± 0.26 mm in thickness at 8 DAI compared to 1.32 ± 0.38 in the control treatment ($t = 2.8041$, $df = 13.193$, $p\text{-value} = 0.014$). By 28 DAI the thickness of the primary tiller was 6.51 ± 0.26 and 2.16 ± 0.29 mm for control and infected treatments, respectively ($t = 12.1005$, $df = 17.953$, $p\text{-value} < 0.001$). The second primary tiller appeared in uninfected plants at 13 DAI, but it was not until 22 DAI when it emerged on *S. hermonthica*-infected plants. The thickness of the stem of the second primary tiller was 0.286 ± 0.3 mm in the infected treatment compared to the 4.05 ± 0.26 mm for uninfected plants ($t = 9.9705$, $df = 17.615$, $p\text{-value} < 0.001$). The thickness of the second primary tiller in control plants was ten-fold greater than that of infected plants by the day of harvest ($t = 12.7039$, $df = 17.932$, $p\text{-value} < 0.001$).

The suppression of tillering in *S. hermonthica* - infected plants may be due to suppression of tiller bud outgrowth and / or to a lack of development of new tiller buds. At 2 DAI transverse sections through the base of the stem where tiller buds form revealed that uninfected and *S. hermonthica* - infected plants had similar numbers of tiller buds at the same stage of development (Fig 2.2). By 5 DAI transverse sections through the stem base showed that the first primary tiller had emerged in uninfected plants and that the second primary tiller was just about to emerge. In addition two smaller tiller buds at an earlier stage of development were also visible (Fig 2.3A). In contrast, in *S. hermonthica* - infected plants none of the tiller buds showed signs of outgrowth. By 13 DAI control plants had several emerged tillers and new small tiller buds were continually forming (Fig 2.4A). In infected plants only the first tiller had emerged and those tiller buds that were present 2 DAI were still present. They were

bigger than the newly forming tiller buds in uninfected plants but their outgrowth was arrested (Fig 2.4B). As a consequence few new tiller buds developed in the infected plants during the experimental period.

The lower number of tillers of *S. hermonthica*-infected rice plants directly affected the total number of leaves on the plant (Fig. 2.9 A). Differences in leaf number between control and infected plants became significant by 11 DAI ($t = 3.806$, $df = 18$, p -value = 0.001) and remained significantly different until the day of harvest. By 13 DAI, the total number of leaves was a third lower in *S. hermonthica*-infected plants compared to controls and was due to the lack of emergence of the first primary tiller (and its leaves) in infected plants. By 20 DAI control plants had 3 times as many leaves as *Striga*-infected plants. By 25 DAI, the total number of leaves on *S. hermonthica*-infected plants had begun to decrease as leaves on the infected plants began to senesce and die.

Senescence of leaves occurred in both control and infected plants. The proportion of new emerging leaves compared to those that were senescing differed between treatments (Fig. 2.9 B). By 20 DAI both treatments had a greater number of leaves that were senescing (appeared yellow/brown in colour) compared to those that were dark green. Between 20 and 30 DAI the number of healthy to senescing leaves remained relatively constant in uninfected plants as new leaves were continually emerging either on the main shoot or on the primary and secondary tillers. However, in *S. hermonthica*-infected plants, the lack of emergence of new tillers and leaves, coupled with the fact that older leaves were senescing led to a significant decrease in the ratio of healthy to senescing leaves. By the day of harvest 57% of leaves on *S. hermonthica*-infected plants were dead or senescing, compared to 15% in the control treatment. However when just considering leaves on the main shoot, *S. hermonthica*-infected plants had a greater number of healthy green leaves compared to uninfected plants during the first 20 DAI ($t = 10.3397$, $df = 18$, p -value = 0.0001) suggesting that senescence of leaves on the main shoot was delayed in the infected plants (Fig 2.9C). After 20 DAI some leaves on the main stem of infected plants rapidly withered and died whereas those on the main stem remained green for longer (Fig 2.9C).

In conjunction with the decrease in the total number of leaves on the main stem, infection by *S. hermonthica* altered the length and relative growth (extension) rate of leaves on the MS (Figure 2.10). At the time of inoculation of plants with *S. hermonthica* the first 2 leaves on the main stem were beginning to senesce and were not measured. The emergence of leaves 3 and 4 on the main stem was synchronous in both uninfected and *Striga*-infected plants, but leaves on the infected plants were shorter

and took slightly longer to reach their final length as rates of elongation were slower (Fig 2.10). The fifth leaf on *Striga*-infected plants emerged later and was smaller than on the uninfected plants. The severity of the effect of *S. hermonthica* on leaf length and rate of elongation increased with time, e.g. the seventh leaf of infected plants was less than half the length of the seventh leaf on control plants and its emergence was delayed by 9 days (data not shown).

Alterations in the growth and development of the host due to the presence of *S. hermonthica* resulted in a decrease in the total leaf area (Fig 2.11A) and biomass (Fig 2.11B) of leaves on the main stem of *Striga*-infected compared to control plants. Interestingly, specific leaf area (SLA) was not affected by infection (Fig. 2.11C) indicating that the leaves on uninfected and infected plants were of similar thickness

2.3.3 Alterations in biomass partitioning of rice plants following infection with *S. hermonthica*

Infection of rice cultivar IAC 165 by *S. hermonthica* had a severe effect on the total biomass and partitioning of biomass between roots, stems and leaves (Fig 2.12). The presence of *S. hermonthica* caused a reduction in leaf, stem and root biomass of 79.1 ± 1.26 , 93.1 ± 0.71 and 88.2 ± 1.1 %, respectively compared to uninfected plants. The root to shoot ratio was greater in infected compared to control plants as above ground biomass was altered to a greater extent than root biomass. Infected plants supported approximately 45 *Striga* individuals per plant with an average length of 2.6 cm and an average dry weight of 40.7 mg (Fig. 2.13).

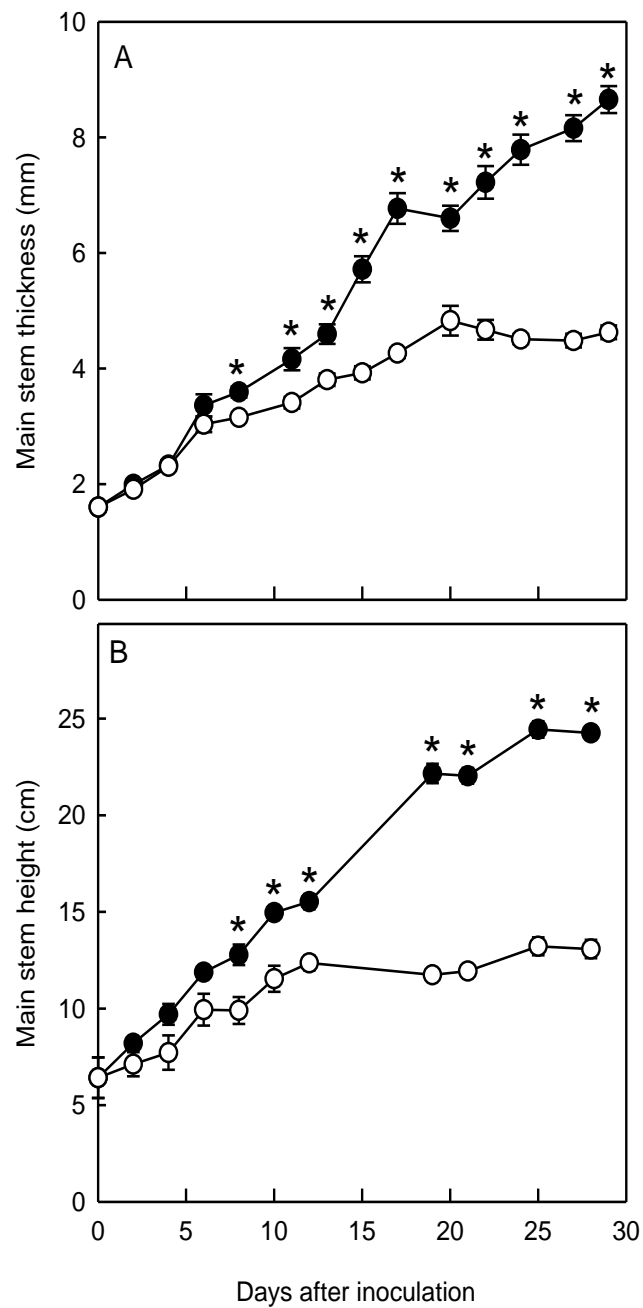


Figure 2.1. The effect of *S. hermonthica* on (A) the thickness and (B) the height of the main stem of uninfected (●) and *S. hermonthica*-infected (○) rice plants (IAC165) over a time course of 28 days. Means + SE are shown, n = 10. (*) indicates that control and *S. hermonthica* infected plants differ significantly (p < 0.01) as assessed by Student's t-test at each time point.

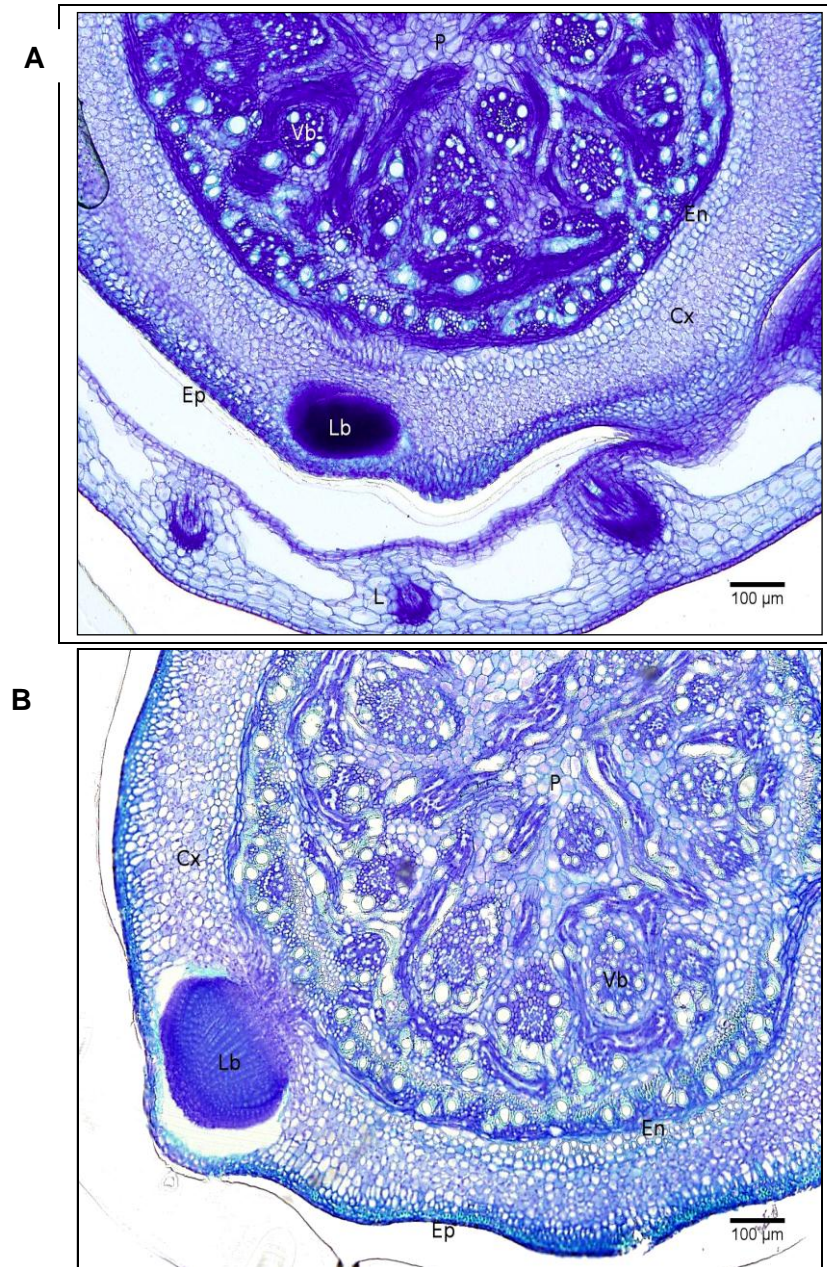


Figure 2.2. Transverse section across the base of the main shoot of (A) uninfected and (B) *S. hermonthica*-infected rice plants (IAC165) 2 days after inoculation showing lateral bud (Lb), vascular bundles (Vb), stem pith (P), cortex (Cx), leaves (L), epidermis (Ep) and endodermis (En).

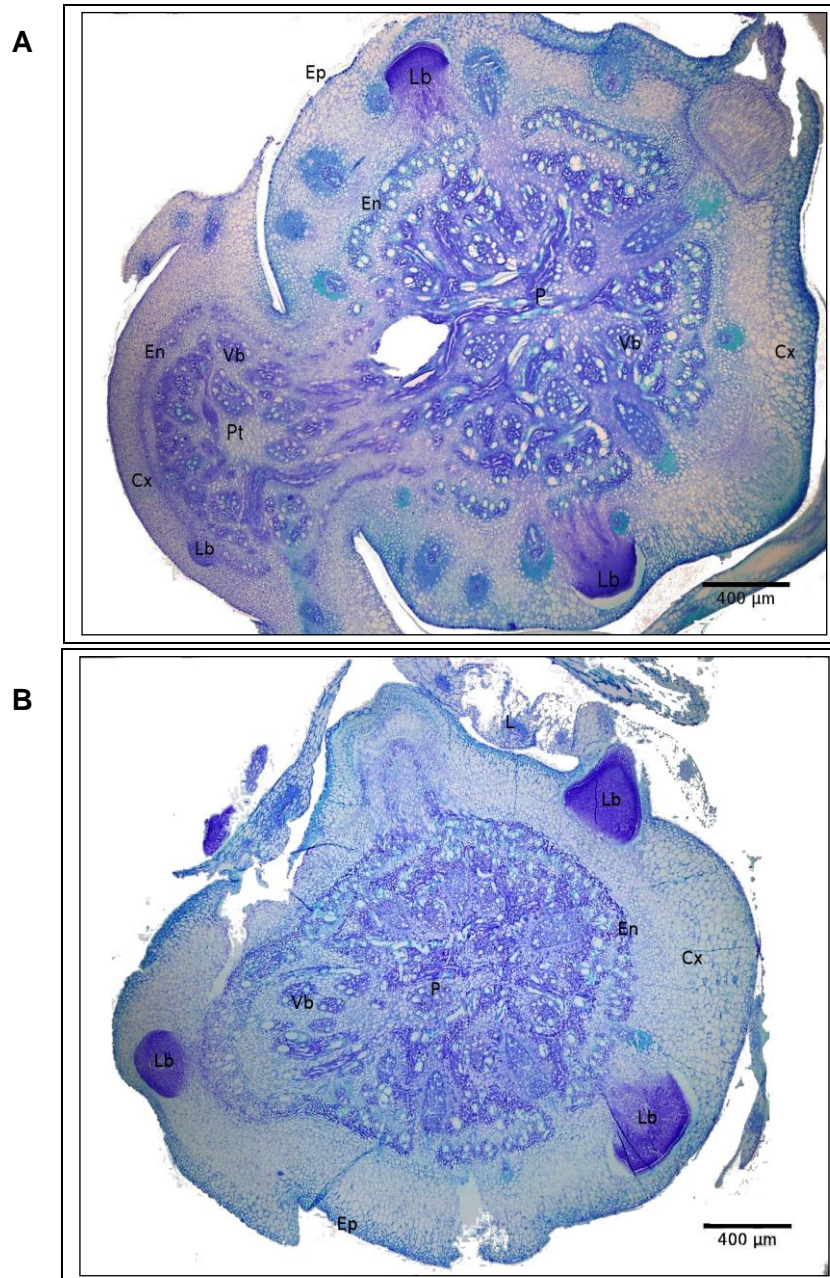


Figure 2.3. Transverse section across the base of the main shoot of (A) uninfected and (B) *S. hermonthica* -infected rice plants (IAC165) 5 days after infection showing primary tiller (Pt), lateral buds (Lb), vascular bundles (Vb), stem pith (P), cortex (Cx), leaves (L), epidermis (Ep) and endodermis (En).

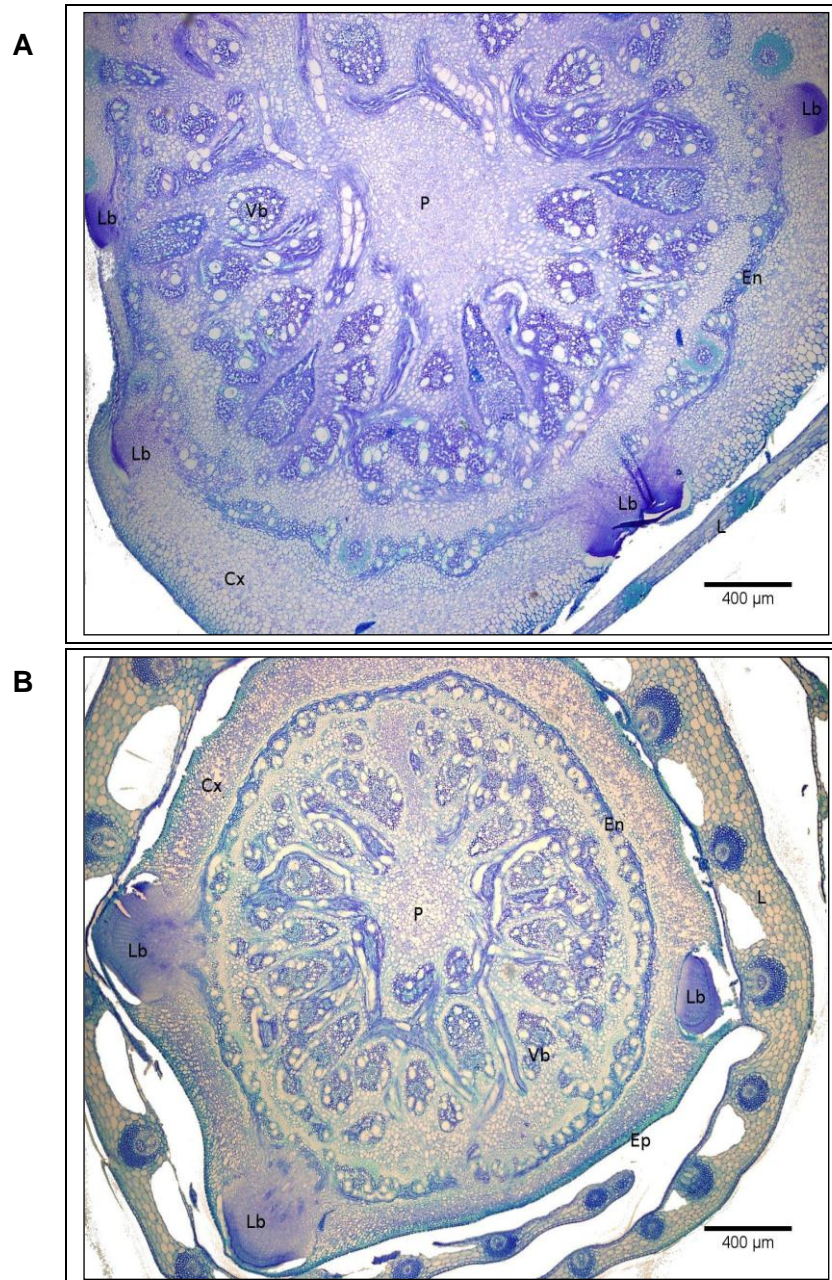


Figure 2.4. Transverse section across the base of the main shoot of (A) uninfected and (B) *S. hermonthica* -infected rice plants (IAC165) 13 days after infection showing differences in lateral bud (Lb) development. Vascular bundles (Vb), stem pith (P), cortex (Cx), leaves (L), epidermis (Ep) and endodermis (En).

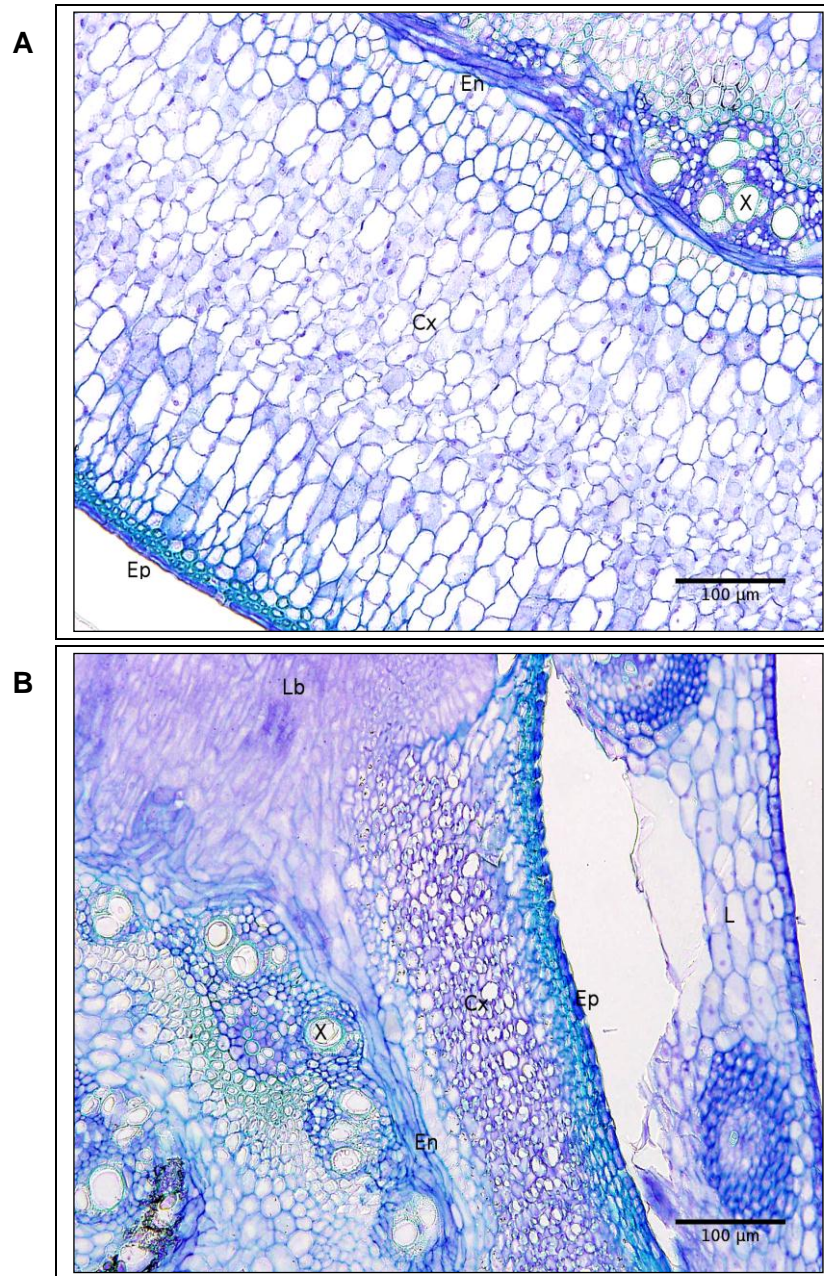


Figure 2.5. Transverse sections across the base of the main shoot of (A) uninfected and (B) *S. hermonthica*-infected rice plants (IAC165) 13 days after infection showing differences in the thickness of the cortex (Cx). Leaves (L), epidermis (Ep) and endodermis (En), xylem (X) and the site of emergence of a lateral bud (Lb).

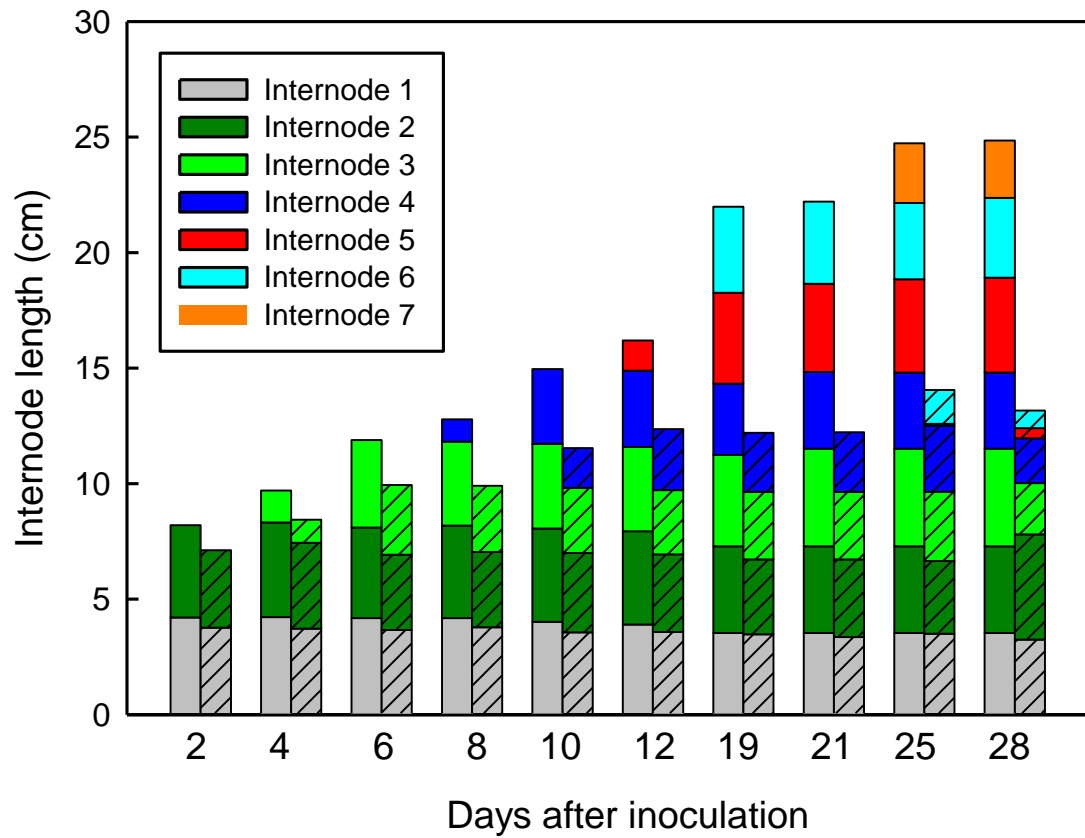


Figure 2.6 The effect of *S. hermonthica* on internode elongation of the main stem of rice cultivar IAC165 over a time course of 28 days. Uninfected plants are shown as open bars and *S. hermonthica*-infected plants as hatched bars. Internode number is the first internode (base of the stem) and internode 7 the last (top of the stem). Mean values are shown, $n = 10$.

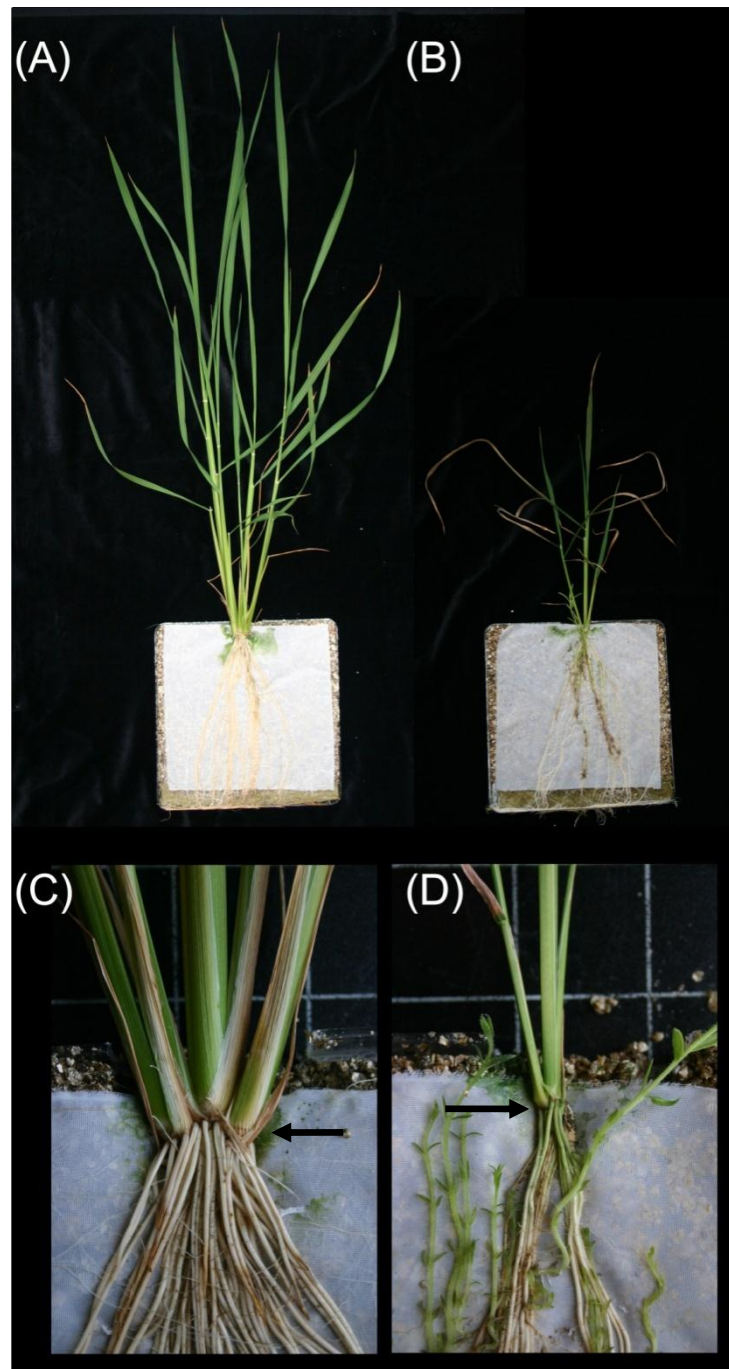


Figure 2.7. The effect of *S. hermonthica* on the morphology of the susceptible rice cultivar IAC165 28 days after inoculation on (A) an uninfected plant and (B) a *S. hermonthica*-infected plant. Close up of the base of the main stem and tillers of (C) uninfected and (D) *S. hermonthica*-infected plants. Black arrow indicates the first primary tiller.

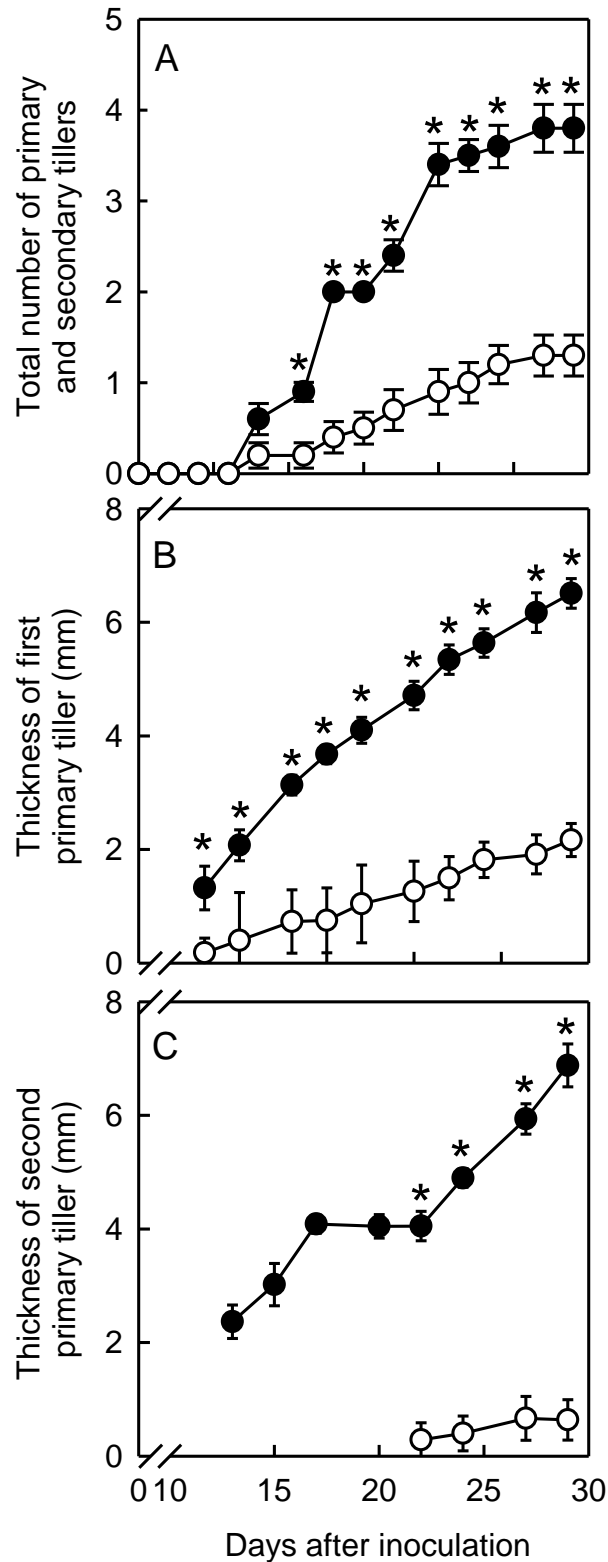


Figure 2.8. The effect of *S. hermonthica* on (A) the total number of tillers per plant and (B) the thickness (mm) of the first and (C) second primary tillers on uninfected (●) and *S. hermonthica*-infected (○) rice plants (IAC165) over a time course. Means + SE are shown, $n = 10$. (*) indicates statistically significant differences ($p < 0.01$). (*) indicates that control and *S. hermonthica* infected plants differ significantly ($p < 0.01$) as assessed by Student's t-test at each time point.

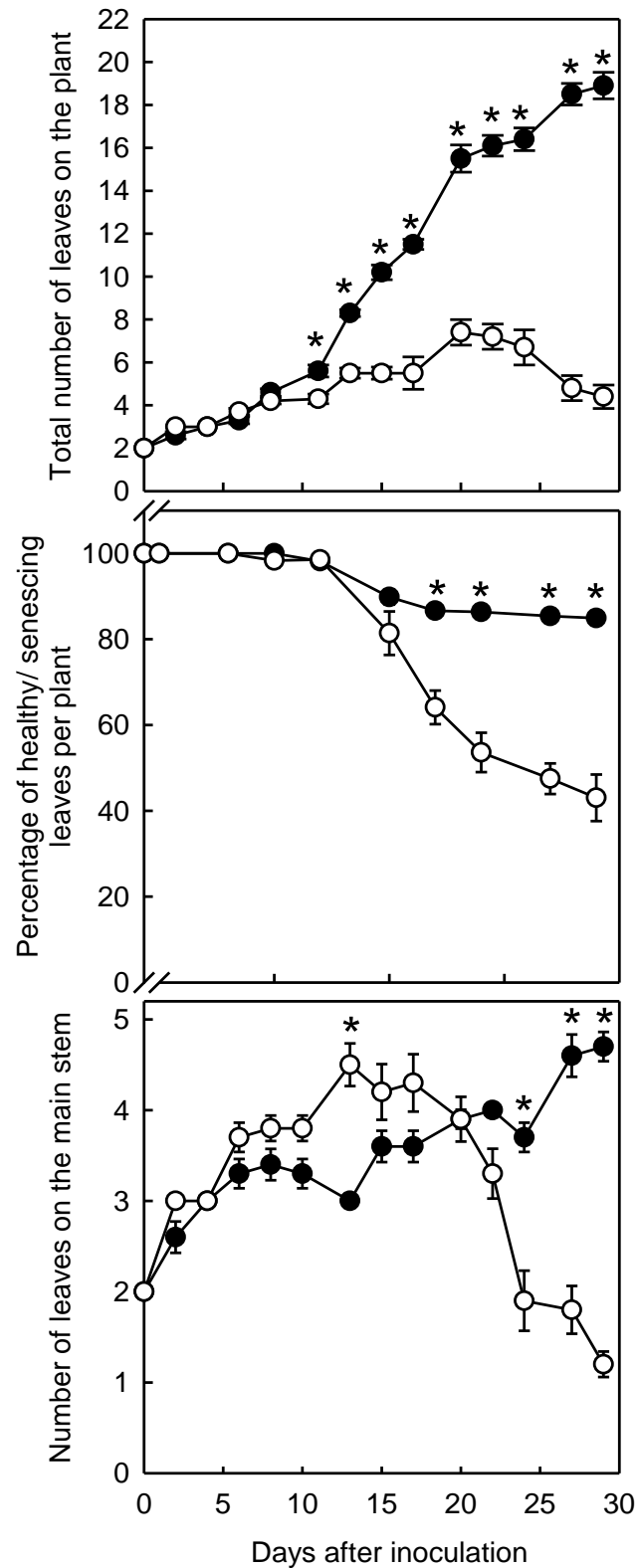


Figure 2.9. The effect of *S. hermonthica* on the total number of leaves (A), percentage of live / dead leaves (B) and the number of leaves on the main shoot (C) of control (●) and *Striga*-infected (○) rice plants over a time course. Means + SE are shown, n = 10. (*) indicates that control and *S. hermonthica* infected plants differ significantly ($p < 0.01$) as assessed by Student's t-test at each time point.

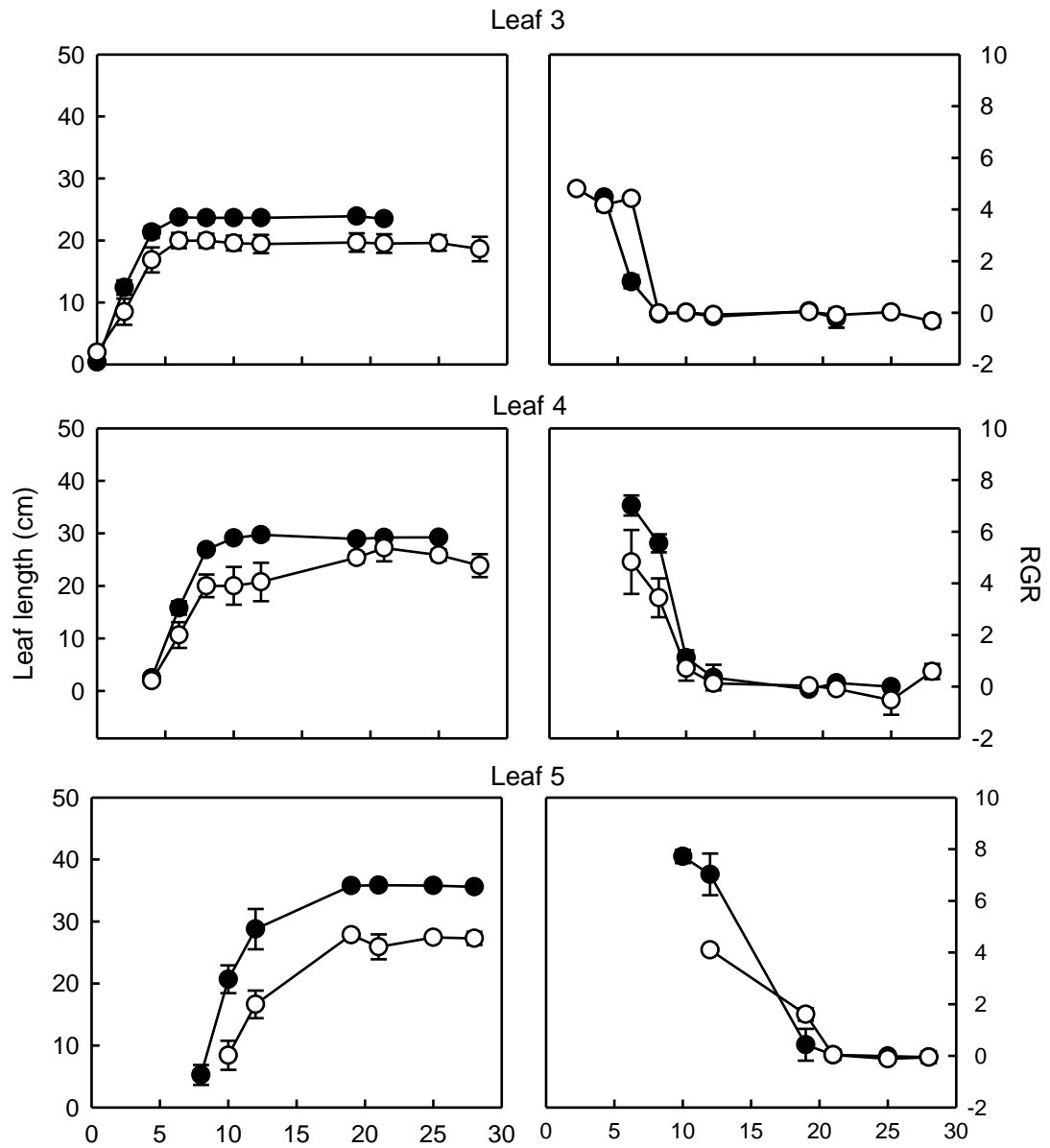


Figure 2.10. The effect of *S. hermonthica* on leaf length and relative growth rate (RGR) of the third, fourth, and fifth leaves on the main shoot of uninfected (●) and *S. hermonthica*-infected (○) rice plants (IAC165) over a time course. Means + SE are shown, n = 10.

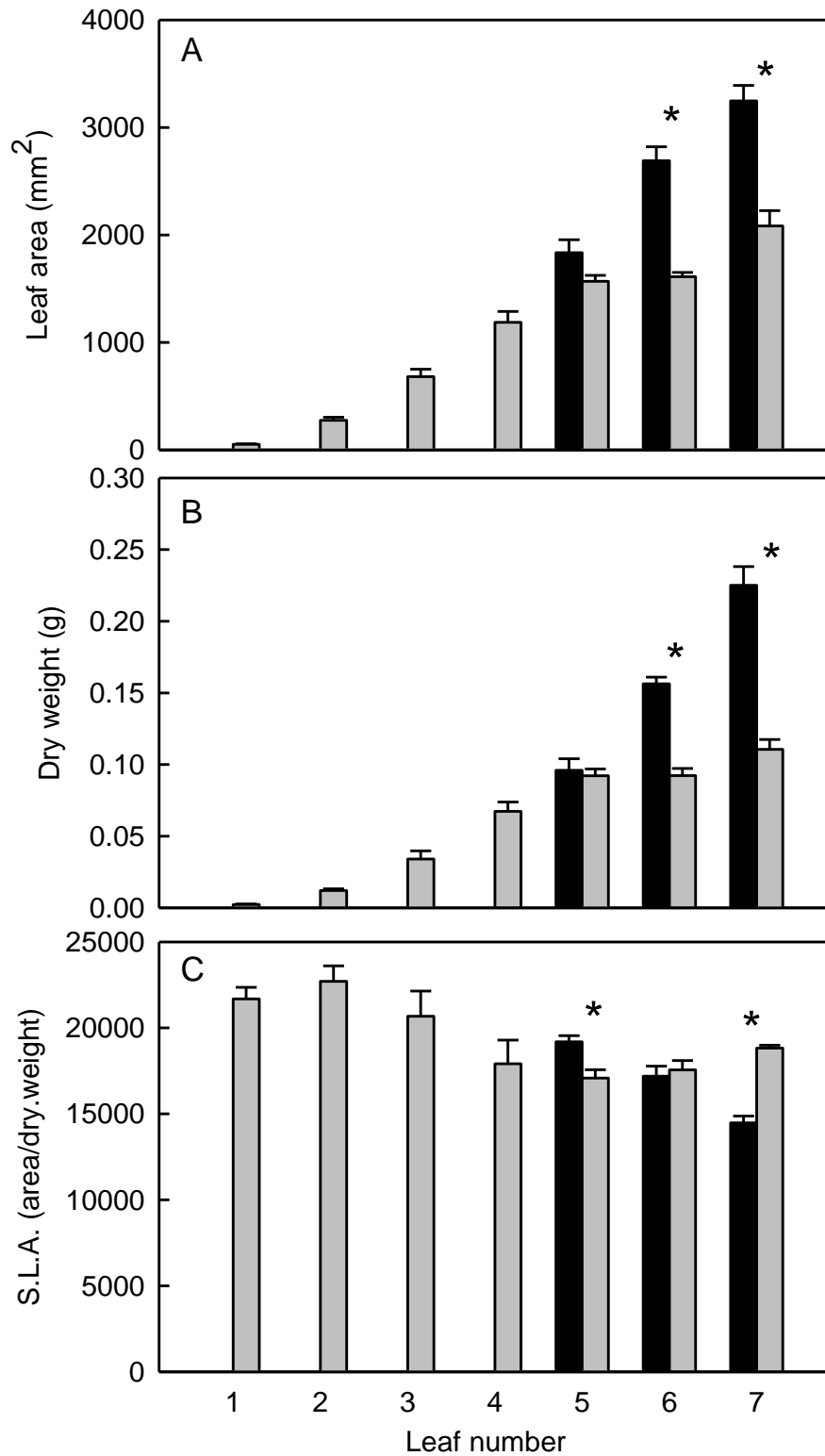


Figure 2.11 The effect of *S. hermonthica* on (A) individual leaf area, (B) dry weight and (C) specific leaf area of leaves on the main stem of uninfected (black bars) and *S. hermonthica*-infected (grey bars) rice plants 28 DAI. N.B. Leaves 1-4 on control plants dried before harvest. Means + SE are shown, n = 10. (*) indicates that control and *S. hermonthica* infected plants differ significantly (p < 0.01) as assessed by Student's t-test.

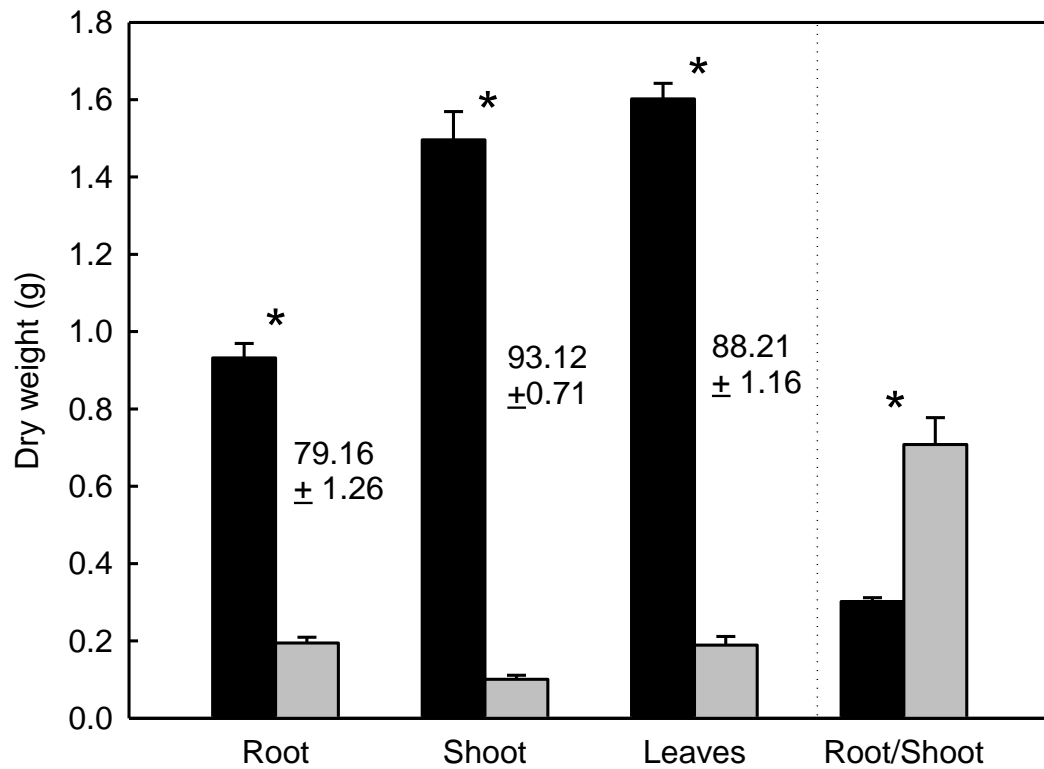


Figure 2.12. The effect of *S. hermonthica* on biomass partitioning in rice plants (IAAC165). Uninfected plants (black bars) and *S. hermonthica*-infected plants (grey bars) at 28 days after inoculation. Numbers next to the bars indicate the % loss of biomass of *S. hermonthica*-infected plants compared to control. The root to shoot ratio is shown on the right side of the graphic. Means + SE are shown, n = 10. (*) indicates that control and *S. hermonthica*-infected plants differ significantly ($p < 0.01$) as assessed by Student's t-test.

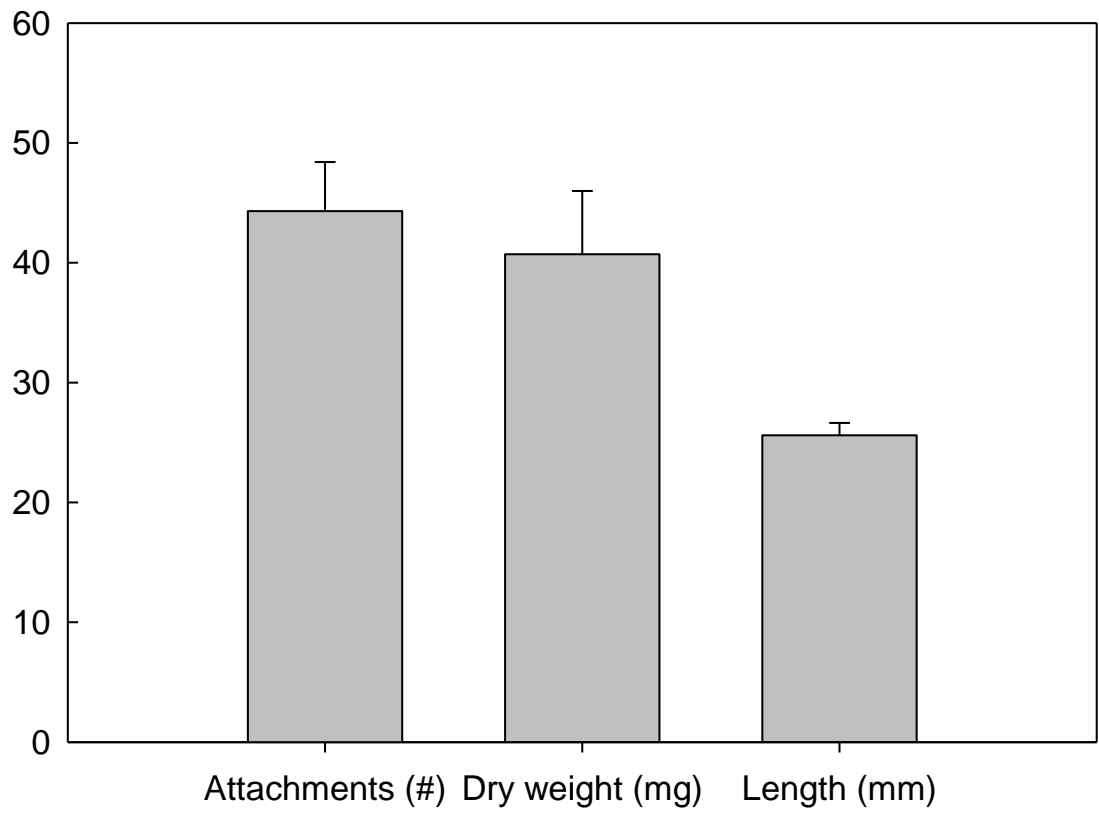


Figure 2.13. The number of attachments, dry weight (mg) and length (mm) of *S. hermonthica* plants attached to the rice cultivar IAC165 28 days after inoculation. Means + SE are shown, n = 10.

2.4 DISCUSSION

2.4.1 How does *S. hermonthica* alter the growth and morphology of its rice host?

Rice is viewed as a key strategic crop in the alleviation of poverty in sub Saharan Africa and the area of land under rice cultivation has increased rapidly due, in part, to the release of the high yielding upland and lowland NERICA cultivars (Rodenburg *et al.*, 2010). In traditional rice growing areas and in areas where farmers are replacing maize and sorghum with rice a key limitation to yield are *S. hermonthica* and *S. asiatica* (Rodenburg *et al.*, 2010). Despite many studies to document the effect of these parasites on the growth and morphology of maize (Clark *et al.*, 1994; Aflakpui *et al.*, 1998) and sorghum (Cechin and Press 1993; Watling and Press, 1997; Frost *et al.*, 1997; Gurney *et al.*, 1999), less is known about their effect on rice. The aim of this study was to carry out a detailed analysis of changes in the growth and morphology of rice plants infected with *S. hermonthica* and to determine whether the suppression of tillering reported for infected plants (Cechin and Press 1993; Cissoko *et al.*, 2011; Jamil *et al.*, 2011) is due to a suppression of the outgrowth of tiller buds or to an inhibition of the formation of tiller buds.

This study showed that infection of rice by *S. hermonthica* resulted in severe stunting of the main stem and a decrease in its width compared to uninfected plants, delayed emergence of leaves on the main stem and a decrease in the number of tillers. The total biomass of uninfected plants was smaller compared to uninfected plants and the partitioning of biomass between stems, leaves and roots was altered resulting in an increase of the root to shoot ratio of infected rice plants. These results are consistent with the findings of Cechin and Press 1994; Watling and Press 1997; Cissoko *et al.*, 2011.

2.4.2 *S. hermonthica* stunts its host by suppressing the elongation of internodes.

This is the first study to show in detail the extent to which internode elongation is altered by *S. hermonthica* in rice plants under a susceptible interaction. The severe stunting of the main stem of rice when infected by *S. hermonthica* was due to a lack of elongation of internodes rather than to a reduction in the number of internodes compared to uninfected plants. The stunting could be observed from early stages of infection, suggesting this is a specific effect on the host, derived from infection by *S. hermonthica*. Additionally, general slowing of growth could be observed in later stages of infection, up to the point where the growth of the plant was nearly null. This effect was most pronounced later during infection; internode 5 showed very little extension

before internode 6 appeared. Several studies have documented the inhibitory effect on internode extension in sorghum and maize as a result of *S. hermonthica* infection. Press and Stewart (1987) found a decrease in the internode elongation, photosynthesis and total biomass of *Striga*-infected sorghum plants. Similarly, Hearne (2001) determined a significant reduction on the height and length of internodes of *S. hermonthica*-infected maize plants. However, despite the research done to understand the underlying mechanisms used by *S. hermonthica* to stunt its host from early stages of infection, this process is not clear to date. Several hypotheses have been formulated regarding this phenomenon including movement of a toxin from parasite to host that affects the aerial part of the host, alterations in source-sink relationships within the infected plant due to removal of nutrients by the parasite and changes in the balance of growth regulatory substances (Musselman, 1980; Press and Stewart, 1987; Musselman and Press, 1995).

Translocation of a toxic compound from *S. hermonthica* to the host has been suggested, since the stunting of the host was detected early after infection, when the parasite biomass was still very small (Musselman and Press, 1995) as well as the fact that *S. hermonthica* is known to contain iridoid glucosides that, if they move into the host may suppress cell division (Rank *et al.*, 2004). However, the so called toxin has not been detected to date, and importantly, active cross-species communication from the parasite into the host has not been conclusively shown in *S. hermonthica*, unlike other parasitic plants that use the phloem rather than the xylem to establish the vasculature connection (Roney *et al.*, 2007, Westwood *et al.*, 2009).

An alternative hypothesis for the stunting of the host is that *S. hermonthica* induces alterations in plant growth regulator metabolism. Many plant growth regulators can affect the height of plants including gibberellins, auxins and cytokinins (Ikeda *et al.*, 2001; Lo *et al.* 2008; for a review see Yamaguchi, 2008). Biosynthesis, signalling or degradation of gibberellins may be altered in a host plant under a compatible interaction with *S. hermonthica*, resulting in a restriction of the biophysical capacity of cells to elongate due to modifications of the cell wall. Gibberellins regulate cell expansion by induction of cell wall loosening enzymes (Kende *et al.*, 1998). Gibberellins can regulate elongation of cells and growth of the whole plant, as shown by the *slender* rice mutant. A single recessive mutation in GA2 oxidase generates tall plants as a constitutive response to gibberellin (Ikeda *et al.*, 2001). The opposite effect is observed in rice plants overexpressing genes responsible for the degradation of bioactive gibberellins, such as GA2 oxidases and DELLA proteins (Dill *et al.*, 2001; Lo *et al.*, 2008). However, unlike *S. hermonthica*-infected plants, these dwarf mutants show increased rather than decreased tillering. Alterations in auxin metabolism can

also mimic the phenotype of *Striga*-infected plants as demonstrated by transgenic rice plants that over express SAUR39, an auxin responsive gene that modulates auxin levels in rice (Kant 2009) (Chapter 1 section 1.5.3) SAUR39 transgenic plants were stunted and exhibited a reduction in tillering. Interestingly the stems of the transgenic plants appeared to be thinner than wildtype plants and the width of the vascular system was smaller. The thickness of the stem of *Striga*-infected rice was significantly smaller than that of control plants in this study. Even though the number of cells and their length was measured in the cortical region of roots it is likely that there was a similar reduction in cell number and cell size in the stem tissue. The hypothesis that alterations in plant growth regulators may be involved in stunting is explored further in Chapter 4 by profiling changes in the expression of plant growth regulator associated genes.

Symbiotic organisms such as arbuscular mycorrhizal (AM) fungi and parasitic nematodes use similar mechanisms to those used by *S. hermonthica* to successfully establish an interaction to their hosts, however there is diversification of the process. Symbionts and nematodes are able to manipulate the host auxin metabolism to their advantage (Akiyama *et al.*, 2005; Grunewald *et al.*, 2009) while *S. hermonthica*, nodulating bacteria and AM fungi use chemical cues derived from root exudates to detect presence of roots known as strigolactones (Foo and Davies, 2011; Akiyama and Hayashi, 2006). It has been suggested that exudation of strigolactones by the host roots was first used by plants as a “cry for help” during phosphate starvation, leading to the recruitment of beneficial organisms (Ruyter-Spira *et al.*, 2012), however parasitic plants use exudation of strigolactones to their advantage. It has not been described to date, however it can be hypothesised that *S. hermonthica* is also able to modify its host auxin metabolism, similarly to AM fungi and nematodes in order to infect its host successfully. The roles of strigolactones and auxin are further explored in the following chapters of this thesis.

2.4.3 Does *S. hermonthica* alter the production and turnover of leaves on the main stem of rice?

The reduction of plant height resulted in the typical phenotype in which the leaves of *Striga*-infected plants emerged on top of each other. However up to 20 days after inoculation *Striga*-infected plants had the same number of leaves on the main stem as uninfected plants (4 leaves) although the length and area of these leaves was smaller than those of the control plants. Itoh *et al* (2005) produced a highly detailed analysis of rice development. In their description the rice embryo had formed three leaves by 8 days after pollination; after this stage they could observe enlargement of organs and maturation of the embryo but no further leaf formation prior to seed germination. Thus, 3 of the 4 leaves on the main stem of *Striga*-infected plants would

have formed prior to infection. After infection leaves continued to develop but their emergence was delayed rather than totally inhibited. It is interesting to note that the 4 leaves on *Striga*-infected plants remained green for longer than those on control plants suggesting a delay in senescence. This phenomenon has also been observed in tobacco plants infected with *O. cernua* (Hibberd *et al.*, 1998). In the latter study the leaves maintained rates of photosynthesis for longer than leaves on control plants. It is not known whether this is also the case in *Striga*-infected rice plants.

The delay in the emergence of the fifth and sixth leaves on the main stem of *Striga*-infected plant could have consequences for the development of the panicle. Sylvester *et al* (2001) showed that six photosynthetically active leaves were required for panicle emergence. In this study plants were harvested before panicles emerged but the panicle was clearly present within the culm of uninfected plants but not in *Striga*-infected plants. It is possible that the lack of panicle formation in *Striga*-infected plants was due to lack of carbon and other nutrients as the parasite is a large sink for host resources.

2.4.4 Formation and outgrowth of tillers are suppressed in *S. hermonthica* infected plants

Uninfected rice plants generated primary and secondary tillers, following the same pattern described by Jaffuel and Dauzat (2005). This consisted of the generation of tillers in a hierarchical order, which resulted from a close link between leaf emergence on the main shoot and development of lateral buds into tillers in early stages of development. In contrast one of the most dramatic effects of *S. hermonthica* on the development of rice plants was the almost complete inhibition of tillering. *S. hermonthica* infected plants produced only 1 or 2 primary tillers. A key aim of this study was to determine whether this lack of tillering was due to inhibition of tiller bud formation and / or outgrowth. This study has shown, for the first time that the low production of tillers in *S. hermonthica* -infected rice plants was due to both inhibition of formation of tiller buds and suppression of tiller bud outgrowth.

The location of tiller buds along the stem of rice plants is determined by the site where leaf formation occurs. There is a transition zone in the stems of monocotyledon plants where root primordia are formed, followed by leaf bud meristems forming from the shoot apical meristem as the plant develops. This normally happens below the first node. Above this region no roots are formed and leaves emerge. The buds at the base of the leaves will later differentiate into tillers if conditions and environment are

appropriate (Umehara et al., 2008). The transverse sections presented in this work show only meristems that correspond to leaf buds. This was achieved by selecting the appropriate section of the stem at the moment of harvesting the tissue. Longitudinal sections could provide more solid evidence that the buds presented in the transverse sections correspond to leaf buds, however, when I attempted to process stem sections longitudinally the samples were too big and the tissue fused in every slice. This was due to the size of the sample that did not allow proper infiltration of the Technovit solution. This could be partially solved by using hand-cut sections, however this technique would not allow cellular resolution as desired, therefore that approach was not taken.

As described in Chapter 1 tiller bud outgrowth is regulated by the interaction of several plant growth regulator pathways, particularly strigolactones and auxins (Arite et al., 2007; Umehara et al., 2008; Arite et al., 2009). Interestingly, strigolactones had been shown to play an important role in plant parasitism as the germination signal for parasitic seeds by indicating proximity of host roots. Much less is known about the effect of the parasite on strigolactone metabolism and signalling post-infection. It is possible that *Striga*-induced alterations in endogenous strigolactones may be responsible for the suppression of tillering in infected plants. This hypothesis was tested by infecting rice mutants with lesions in strigolactone biosynthesis or signalling and examining the alteration in tillering of these plants. In addition, the effect of *Striga* on the biosynthesis of strigolactones in different regions of the infected plant is investigated by infecting transgenic rice plants containing a strigolactone biosynthetic gene promoter (CCD8):GUS construct.

Chapter 3

Are strigolactones involved in the suppression of tillering in *S. hermonthica*-infected rice plants?

3.1 INTRODUCTION

It is widely accepted that alterations in shoot morphology are one of the main responses of susceptible rice plants to infection by *S. hermonthica*. In the previous Chapter it was shown that infected plants had decreased height, reduced stem width, fewer tillers (due to inhibition of formation and outgrowth of tillers buds) and a lower overall biomass when compared with uninfected plants. Ultimately these changes will result in a reduction of crop yield. It is therefore very important to understand how these alterations take place at a mechanistic level as this may allow novel control strategies to be developed. Crop traits such as height and tillering are controlled by several loci (Abe et al., 2012) and knowledge of these loci and the genes and pathways they encode or regulate can lead to breakthroughs in yield improvement. For example, during the green revolution of the 1960's knowledge that plant height was regulated by gibberellins allowed genetic manipulation of this metabolic pathway and the generation of semi-dwarf plants with an improved harvest index and greater grain yield. Until recently these dwarf high yielding cultivars have provided a partial solution to the increase in demand for food by a rapidly increasing population (Peng et al., 1999; Sasaki et al., 2002; Spielmeyer et al., 2002; Sakamoto and Matsuoka, 2004). However, the detailed mechanisms underlying the control of tillering (one of the major impacts of *Striga* on rice plants) have remained elusive and detailed knowledge of the role of strigolactones in the regulation of tillering is only just coming to light (Beveridge and Kyozuka, 2010).

The potential role(s) of strigolactones in the suppression of tillering in *Striga*-infected plants or in moderating the infection process have not been studied to date. Most studies have focused on the role of strigolactones as germination stimulants of *Striga* seeds and the importance of the amounts and types of strigolactones present in host root exudates. For example, Jamil et al., (2010) used the carotenoid biosynthetic inhibitor fluridone to reduce the amount of strigolactones in exudates of the rice cultivar Shiokari and demonstrated that fewer parasites attached to the roots due to a reduction in seed germination. There are also cultivars that naturally produce low amounts of strigolactones (e.g. some of the NERICA rice cultivars) and exudates from these plants induced low germination of *S. hermonthica* seeds resulting in fewer attachments (Lopez-Raez et al., 2009; Jamil et al., 2011).

As outlined in Chapter 1 section 1.5.1 the regulation and control of tillering is a complex process that involves alterations in the biosynthesis and signalling pathways of the plant growth regulators strigolactones and auxins. The role of strigolactones in

regulating tiller bud outgrowth was elucidated, at least in part, by the analysis of a range of branching mutants of *Arabidopsis*, pea, petunia (see Beveridge 2006; Beveridge and Kyojuka 2010) and rice (Arite *et al.*, 2007 Umehara *et al.*, 2008). In rice there is a range of branching mutants (known as the D mutants) with lesions in biosynthetic and signalling components of the strigolactone pathway as summarized in Table 3.1, Fig 3.1 and as described in section 1.5.1. Research on strigolactones is fairly recent. The biological function of strigolactones as hormonal regulators of branching was determined in rice and *Arabidopsis* in 2008 by Umehara *et al.*, and Roldan-Gomez *et al.*, respectively. Therefore, many genes described in this metabolic pathway are still stated as putative in the existing literature. Wild type phenotype, *i.e.* normal tillering, can be recovered in the high tillering dwarf mutants *d10*, *d27* and *d17*, by exogenous application of the artificial strigolactone analogue GR24 (Ishikawa *et al.*, 2005); suggesting these specific genes are involved in the biosynthesis of strigolactones, whereas wild type phenotype cannot be recovered in the *d3* and *d14* mutants by supplying exogenous strigolactones in the same manner (Ishikawa *et al.*, 2005; Arite *et al.*, 2009). Further characterisation by gene cloning of D3 indicated this gene encodes for an F-box LRR protein, suggesting its involvement in the signalling of strigolactones, as shown by many other F-box LRR proteins involved in signalling of ethylene and other compounds (Ishikawa *et al.*, 2005; Wang *et al.*, 2009). Positional cloning showed that D14 encodes a protein of the α/β -fold hydrolase superfamily, some members of which are involved in signalling, suggesting this gene may be involved in either the activation of inactive forms of strigolactones or their signalling (Arite *et al.*, 2009).

Analysis of the effect of *S. hermonthica* on tillering in selected branching mutants allows us to test the hypothesis that parasite-induced alterations in the biosynthesis of strigolactones and / or signalling pathways is involved in the suppression of tiller bud outgrowth in infected plants.

Table 3.1 Genes involved in strigolactone biosynthesis and signalling.

Gene name	Protein	Reference
DWARF3 (D3)	F-box LRR protein	Ishikawa <i>et al.</i> , 2005
DWARF10 (D10)	Carotenoid cleavage dioxygenase 8 (CCD8)	Arite <i>et al.</i> , 2007
DWARF14 (D14)	α/β -fold hydrolase	Arite <i>et al.</i> , 2009
DWARF27 (D27)	Iron containing protein	Lin <i>et al.</i> , 2009
HIGH TILLERING DWARF1 / DWARF 17 (HTD / D17)	Carotenoid cleavage dioxygenase 7 (CCD7)	Junhuang <i>et al.</i> , 2007

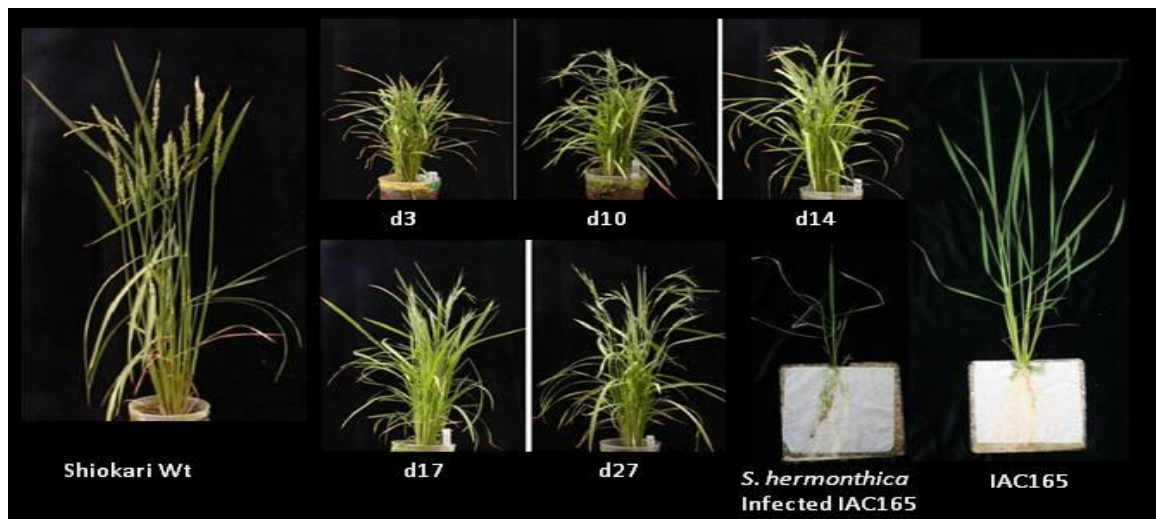


Figure 3.1 Shiohari (wild type) rice and the strigolactone branching mutants 10 weeks after germination (from Ishikawa *et al.*, 2005), and the susceptible rice cultivar IAC165 with and without *S. hermonthica*.

Two of the branching mutants *d3-1* (compromised in the strigolactone signalling pathway) and *d10-1* (a strigolactone biosynthesis mutant) were utilized in this study. Figure 3.2 shows the gene structure and the site of the mutations in D3 and D10 that cause the *d3-1* and *d10-1* rice mutant phenotypes. D3 encodes an F-box leucine rich repeat (LRR) protein which is thought to be involved in strigolactone signalling via ubiquitin-mediated degradation of proteins which suggests that degradation of negative regulators may be involved in strigolactone signalling (Beveridge and Kyojzuka, 2010). D3 comprises four exons and three introns. The protein-coding region is situated in the

first exon. The function of D3 is disrupted due to a transposon insertion in the first exon, thus generating a non-functional transcript in the *d3-1* mutant (Ishikawa *et al.*, 2005). In contrast, D10 encodes the carotenoid cleavage dioxygenase enzyme CCD8 involved in the biosynthesis of strigolactones (see section 1.5.1 for details). D10 is composed of 5 exons and 4 introns. A point mutation consisting of a change of a nucleotide from T to C in the first exon generated a stop codon thus a non-functional product is produced (Arite *et al.*, 2007).

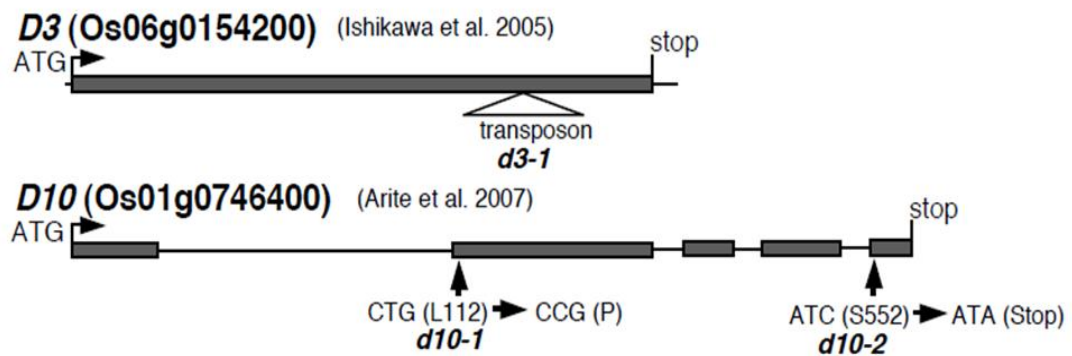


Figure 3.2 Gene structure of the strigolactone signalling and biosynthesis genes D3 and D10, pointing out the sites that generate the mutants *d3-1* and *d10-1* (Ishikawa *et al.*, 2005; Arite *et al.*, 2007).

S. hermonthica causes a suppression of tiller bud outgrowth in rice plants compared to uninfected plants. In this Chapter the hypothesis that *S. hermonthica* causes upregulation of strigolactone biosynthesis following infection leading to an increase in endogenous levels of strigolactones and thus suppression of tiller bud outgrowth, is tested by infecting wildtype Shiokari and *d10-1* rice plants. Specifically I hypothesize that there will be no suppression of tillering in *d10-1* mutants infected by *S. hermonthica* compared to uninfected plants as the biosynthesis of strigolactones in this mutant is compromised by the non-functional CCD8 enzyme (Umehara *et al.*, 2008) have demonstrated that *d10-1* mutants contain very low amounts of strigolactones). Alternatively, if other CCDs are up regulated during infection leading to an increase in strigolactones, tillering could still be reduced in *d10-1*. It is also possible that *Striga* may produce strigolactone-like compounds that are transported into the host and lead to a suppression of tillering. If strigolactones are the only compounds involved in the suppression of tillering in *Striga*-infected plants, infection of the *d3-1* mutant will also have no effect on tillering due to the mutation in the signalling pathway. (*d3-1* mutants have high endogenous levels of strigolactones as their biosynthesis is not regulated due to the lack of perception of the strigolactone signal (Umehara *et al.*, 2008)).

The hypothesis of the involvement of strigolactones in the reduction of tillering in *S. hermonthica* - infected plants was further tested by manipulating the amounts of strigolactones in plants using the strigolactone biosynthetic inhibitor fluridone (Fig. 3.3). Fluridone has been previously shown to reduce the amount of endogenous strigolactones in rice (Jamil *et al.*, 2010). Potentially, this action will reduce endogenous levels of strigolactones simulating the phenotype of the *d10-1* mutant. However, fluridone does not target strigolactones only, instead, it blocks biosynthesis of carotenoids. Such a disruption in the biosynthesis of carotenoids implies possible alterations on other processes that require these compounds; therefore, in order to reduce alterations not specific to strigolactones, low doses of fluridone will be used. Fluridone inhibits the conversion of phytoene into phytoflene by blocking the enzyme phytoene desaturase in the carotenoid biosynthesis pathway (Fig 3.3). The amount of strigolactones in plants was increased by supplying them with the strigolactone analogue GR24. Umehara *et al.*, (2008) supplied Shikari plants with GR24 and showed that plants had fewer tillers and that the number of tillers was related to the concentration of GR24 supplied.

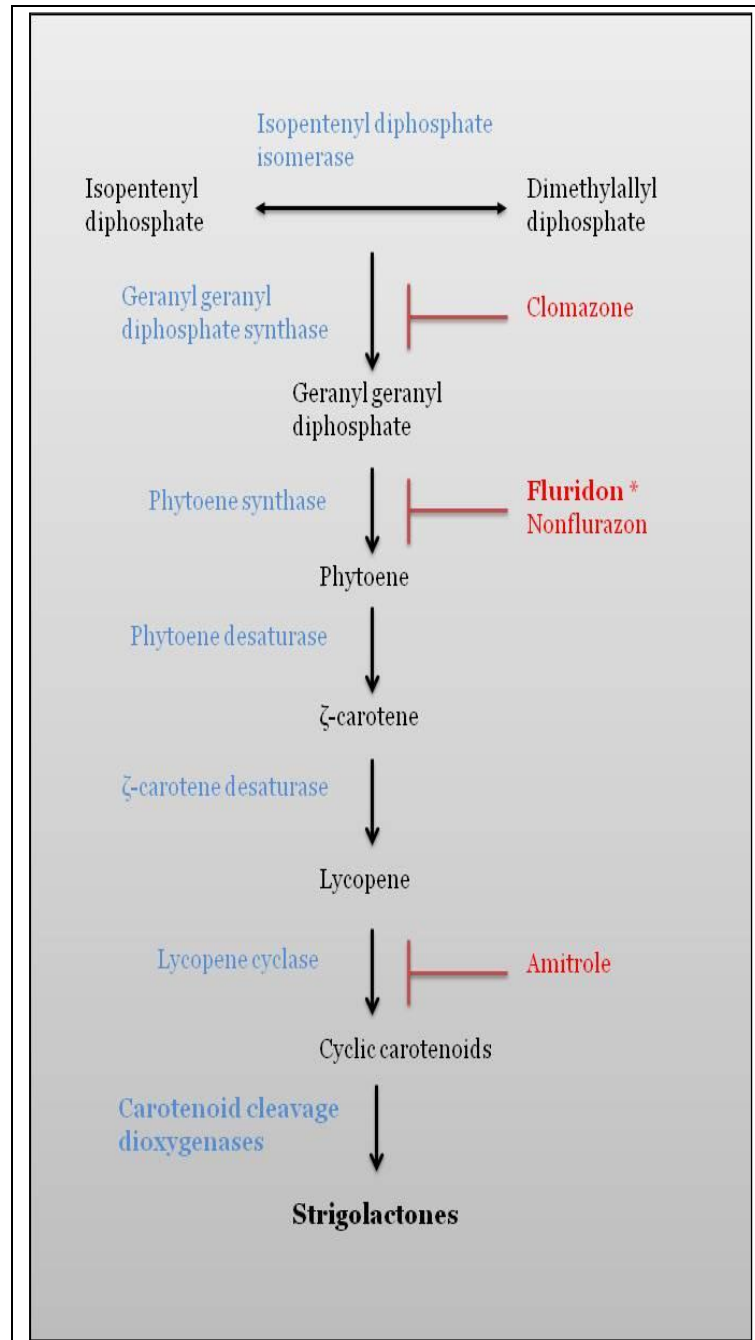


Figure 3.3 Diagram of the carotenoid biosynthesis pathway. Arrows represent enzymatic steps with the enzymes involved in blue letters. Specific inhibitors pointed out in red letters (Modified from Jamil *et al.*, 2010).

In order to monitor changes in the biosynthesis of strigolactones in plants infected with *S. hermonthica* and to determine whether alterations in strigolactone concentration may be involved in modulating the infection process in roots, plants containing a D10 (CCD8):GUS gene promoter reporter fusion were infected with the parasite. The use of plants containing a CCD8:GUS promoter construct allows us to monitor changes in the expression of the CCD8 gene in localized regions of infected

plants at cellular resolution. In order to avoid false positives, plants that did not carry the GUS construct were also subjected to staining. This allowed discrimination of processes that were not specific to the expression of CCD8.

In summary, the main objectives of this Chapter were to determine whether (1) strigolactones are involved in the reduction of tillering in *S. hermonthica* - infected plants by the analysis of strigolactone biosynthesis and signalling rice mutants and the application of the artificial carotenoid inhibitor fluridone or the strigolactone analogue GR24 to manipulate endogenous strigolactone levels and (2) to determine when and where *S. hermonthica* alters the expression of the strigolactone biosynthetic gene CCD8 in infected plants and specifically to determine whether localized alterations in biosynthesis of strigolactones in roots is an important factor during the infection process.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

The rice plant materials used in this Chapter were the strigolactone mutants *d3-1* (F-box LRR protein) and *d10-1* (CCD8) with the respective Shiokari WT line from which they were generated, obtained from Professor Harro Bouwmeester, the University of Wageningen, The Netherlands. Wildtype Shiokari plants were also obtained from the rice stock centre at the International Rice Research Institute (IRRI), in the Philippines (Shiokari WT Accession no. 65761). Transgenic Shiokari plants containing the *DWARF10 promoter:GUS* fusion were also obtained from Professor Kyozyka at the Graduate School of Agricultural and Life Sciences, Tokyo, Japan.

As Shiokari wildtype seeds were obtained from different sources a preliminary experiment was carried out to determine whether they showed the same response to infection by *S. hermonthica* (Sh-Kibos). Wildtype seeds of Shiokari obtained from the Netherlands and IRRI, together with the seeds containing the D10 promoter-reporter fusion (obtained from Japan) were sown in rock wool blocks and grown in rhizotrons as described in sections 2.2.1. Plants were infected 7 days after transfer to rhizotrons to allow proper development of the root on the mesh. Sterilisation and conditioning of *S. hermonthica* seeds was performed as described in 2.2.2. Infection of the rice roots was carried out as described in 2.2.3. In order to determine the effect of *S. hermonthica* on the growth of the Shiokari wild type plants, the total number of tillers, the height of the main stem and the biomass of roots and shoots was measured 21 DAI. The total number of *S. hermonthica* attachments and their biomass was also measured to determine the susceptibility of Shiokari to *S. hermonthica* ecotype from Kibos.

3.2.2 How does *S. hermonthica* alter the growth and morphology of wild type Shiokari and the strigolactone mutants *d10* and *d3-1*?

In order to determine how *S. hermonthica* affects the growth and development of wildtype Shiokari and the strigolactone mutants *d10* and *d3-1* plants were grown in rhizotrons and infected with *S. hermonthica* (Sh-Kibos) as described in Chapter 2 (2.2.1-2.2.3). Ten replicate plants were established for control and infected plants for each treatment. The total number of tillers and plant height to the highest visible ligule were measured every 4 days from inoculation to harvest at 28 DAI. At harvest the number and length of parasites and the dry weight of parasites and host (roots and shoots) were measured as described in 2.2.4. Small sections of root plus *S. hermonthica* attachment were taken from the three different genotypes at 3 and 6 days

after inoculation, to determine whether the strigolactone mutants showed altered susceptibility to the parasite, and photographed using differential interference contrast microscopy using an Olympus BX51 microscope (Olympus Optical Ltd., London, UK).

3.2.3 Manipulation of the concentration of strigolactones in Shiokari using a biochemical inhibitors of the carotenoid pathway and addition of the artificial strigolactone GR24.

In order to manipulate the concentration of strigolactones in Shiokari the carotenoid inhibitor fluridone [1-methyl-3-phenyl-5-(3-trifluoromethylphenyl)-4-(1H)-pyridinone] (Duchefa, The Netherlands) and the artificial strigolactone GR24 were used as the chemical reagents at a working concentration of 0.05 μ M and 1 μ M, respectively.

The experiment had a multifactorial design with two variables, presence or absence of *S. hermonthica* \pm exogenous application of respective reagents. Six replicate plants were established for each treatment. Plants were grown in rhizotrons and infected with *S. hermonthica* as described previously (sections 2.2.1-2.2.3). Fluridone or GR24 were first supplied to the root systems the day before plants were infected by *S. hermonthica* by manually watering each rhizotron with 250 ml of solution. Fluridone and GR24 were then supplied to the roots at 9 am in the morning every 2 days over a 3 week period. To avoid washing of the chemicals out of the rhizotrons by the automated watering system, the supply of nutrient solution was disabled for 24 hours following each application. To assess the effect of fluridone and GR24 on the growth of the plants in the presence and absence of *Striga*, the height of the main stem, number of tillers, dry weight of host roots and shoots and the number and dry weight of *S. hermonthica* individuals was measured 21 DAI.

3.2.4 Does *S. hermonthica* alter the expression of the strigolactone biosynthetic gene CCD8 in Shiokari plants following infection?

In order to determine whether *S. hermonthica* altered the expression of CCD8 (a strigolactone biosynthetic gene) in the roots, stems or leaves of rice plants during the infection cycle, seeds of Shiokari containing the CCD8 (D10):GUS promoter reporter fusion were grown and infected under the same conditions as described in Chapter 2. Three replicate plants per treatment were established. Small pieces of root, stem and leaf tissue were collected at 3, 6, and 9 days after infection and immediately put into ice cold acetone (90%) for 10 minutes to facilitate the penetration of the X-GlcA substrate. The tissues were then washed with pre-chilled 1x phosphate buffer (0.5 M EDTA pH 8,

Triton X-100, 0.5 M sodium phosphate buffer pH7.5 [1 M NaH₂PO₄, 1 M Na₂HPO₄], autoclaved) and then transferred into the X-GlcA-staining solution (X-GlcA dissolved in DMSO, 1X phosphate buffer, 5mM K₄Fe(CN)₆, 5mM K₃Fe(CN)₆). The tissues were vacuum infiltrated with X-GlcA staining solution for 20 min after which the vacuum was released very slowly to avoid disruption of cells. Tissues were incubated in the staining at 37 °C overnight. To remove the staining solution, samples were transferred sequentially to 70 % and 100 % ethanol for 30 minutes each. Finally the samples were placed in 100 % ethanol at 4 °C. In order to determine the spatial distribution of the GUS stain, small pieces of leaf, stem or root tissue ± *S. hermonthica* were viewed under an epifluorescence stereo microscope (Leica UK) and photographed using a CCD camera (Diagnostics Inc).

In order to determine the spatial distribution of GUS stain at cellular resolution samples were embedded in Technovit resin as described in Chapter 2 (2.2.6). Samples were sectioned (5 µm sections) in order to visualize the GUS stain. Sections were mounted in Depex and photographs were taken using an Olympus BX51 epifluorescence microscope (Olympus Optical Ltd., London, UK).

3.2.5 Three dimensional reconstruction of the expression of CCD8 in *S. hermonthica*-infected rice roots

In order to visualize the distribution of CCD8 expression in different regions of roots infected with *S. hermonthica* a three-dimensional reconstruction of serial sections of *S. hermonthica* - infected roots was carried out at 3 DAI using the Free-D program v. 1.07 (Andrey and Maurin, 2005). Approximately 30 images (taken sequentially through the embedded root tissue) were stacked in order. Regions delimiting epidermis, endodermis, xylem and *S. hermonthica* tissue were determined manually as individual objects in each image. Individual cells showing high expression of CCD8 (intense GUS staining) were marked. Three dimensional rendering rendering was carried out using all marked objects.

3.2.5 Statistical analysis

Two-way ANOVA followed by a pairwise multiple comparison Tukey test or a Student t-test were carried out to test for significant differences between treatments as appropriate. Statistical analyses were performed using R software version 2.10.1.

3.3 RESULTS

3.3.1 Does the rice cultivar Shiokari obtained from different sources show the same response to infection by *S. hermonthica* (Sh-Kibos)?

A preliminary experiment was carried out to determine whether *S. hermonthica* affected the growth of the rice cultivar Shiokari, obtained from different sources, in a similar manner and to determine how susceptible wildtype Shiokari and the *d10-1* and *d3-1* strigolactone mutants were to the ecotype of *Striga* from Kibos, Kenya. Fig 3.4 shows the effect of the parasite on the height of the main stem, number of tillers and the biomass of host roots and shoots 21 days after inoculation. The Shiokari plants from Wageningen and IRRI and the plants containing the CCD8:GUS reporter-promoter construct all exhibited a similar response to infection by *S. hermonthica* although the plants obtained from Wageningen were significantly taller (df = 4, F value= 87.53, p < 0.0001) than the other two (Fig 3.4 A and B). The two strigolactone mutants *d10-1* and *d3-1* had a significantly greater number of tillers, were shorter and had less biomass than the wildtype cultivars (Fig 3.4 A and B). There was no significant difference in height, tiller number or biomass between *d10-1* and *d3-1* when infected with *S. hermonthica*.

The number and biomass of *S. hermonthica* attachments on the wildtype Shiokari cultivars was similar but quite low averaging between 5-10 per plant suggesting that this cultivar has some resistance to the *S. hermonthica* ecotype from Kibos, Kenya (although it can still be classed as susceptible) (Fig 3.4 C and D). The two strigolactone mutants *d3-1* and *d10-1* had even fewer attachments (between 2-4 per plant) (Fig 3.4 C and D). The Shiokari cultivar obtained from Wageningen (supplied with the strigolactone mutants) was used in all subsequent experiments to determine the effect of alterations in strigolactone biosynthesis (*d10-1*) and signalling (*d3-1*) on the changes in morphology and tiller number when infected with *S. hermonthica*.

3.3.2 *S. hermonthica* reduces tillering, plant height and biomass of strigolactone mutants *d3-1* and *d10-1*.

A detailed experiment was carried out to compare the effect of *S. hermonthica* on the growth and architecture of wildtype and strigolactone mutants, *d10-1* and *d3-1*, throughout the infection cycle. By the day of infection (two weeks after sowing), both mutants (*d10-1* and *d3-1*) had a higher number of tillers compared to Shiokari wild type (Fig 3.5) and this difference grew steadily more pronounced as time progressed,

especially as the uninfected wildtype cultivar only produced 4-5 tillers during the 28 days of the experiment (Fig 3.5 A). Infection of wildtype Shiokari plants with *S. hermonthica* did not alter the number of tillers produced compared to uninfected plants; by day 28 infected plants also had 4-5 tillers (Fig 3.5 A). In contrast, infection of the *d10-1* and *d3-1* mutants reduced the number of tillers by 20% and 32% respectively when compared to their uninfected controls 28 DAI (Fig 3.5A). The difference in numbers of tillers between control and infected plants of *d10-1* and *d3-1* was significant by 14 DAI ($t = 3.9317$, $df = 16$, $p\text{-value} < 0.001$ and $t = 5.4545$, $df = 13$, $p\text{-value} < 0.0001$, respectively).

As expected, wildtype Shiokari plants were taller than *d10-1* and *d3-1* by the day of infection (2 weeks after sowing). The height of the wild type plants (to the most upper visible ligule) was approximately 20% greater than that of *d10-1* and *d3-1* mutants. By the end of the evaluation period, uninfected wild type plants were significantly taller (73%) compared to both mutants ($p < 0.001$) (Fig. 3.5 B). *S. hermonthica* had a negative effect on the height of wildtype Shiokari plants by 15 DAI ($t = 2.3759$, $df = 14$, $p\text{-value} = 0.03232$) and by 28 DAI uninfected plants were 22 % shorter than uninfected plants. *d3-1* and *d10-1* were significantly shorter than their respective controls by 10 DAI ($t = 3.542$, $df = 14$, $p = 0.003$ and $t = 2.6383$, $df = 16$, $p\text{-value} = 0.01789$, respectively) (Fig 3.5 B) and by 28 DAI they were 29.6 % and 30.7 % shorter than their respective controls.

Although the overall biomass of roots of wildtype Shiokari plants was slightly greater than that of the strigolactone mutants, infection by *S. hermonthica* did not affect the dry weight of the roots at harvest 28 DAI (Fig 3.6 A). However infection by *S. hermonthica* lowered the dry weight of the shoots by 30 % in wildtype Shiokari, 50.5 % in *d3-1* and 31 % in *d10-1* when compared to their respective uninfected controls (Fig 3.6 A).

The number and biomass of *S. hermonthica* attachments on the strigolactone mutants was significantly greater than on the wildtype plants (Fig 3.6 B). Wildtype Shiokari plants supported an average of 5 -10 *S. hermonthica* individuals per plant with a total biomass of approximately 20 mg compared to 15 - 20 attachments on *d3-1* and *d10-1* plants (mean biomass of 50-60 mg). However, the average length of an individual *Striga* plant was similar on all genotypes (Fig 3.6 B). Fig 3.7 shows representative images of the phenotype of the attachments seen on wildtype and strigolactone mutants. On all three genotypes some attachments made connections with the vascular system of the host and went on to develop shoots (Fig 3.7 left panel) whereas other exhibited a resistance reaction where the parasite began to penetrate

the host root but was unable to make connections with the host xylem vessels (Fig 3.7 right panel).

3.3.3 Manipulation of the concentration of strigolactones in Shiokari using a biochemical inhibitor of the carotenoid pathway (fluridone) and addition of the artificial strigolactone GR24.

In order to reduce the concentration of strigolactones in Shiokari plants (to simulate the effect of the genetic mutation in *d10-1*) the carotenoid biosynthetic inhibitor fluridone was applied to the roots as presented by Jamil *et al.* (2010). The growth and development of fluridone-treated plants was slow and defective from the early stages of treatment compared to untreated plants. Plant height, total number of tillers and the dry weight of roots and shoots were significantly reduced compared to untreated controls ($p < 0.001$) (Fig 3.8). The plants also exhibited severe symptoms of fluridone toxicity. After the third fluridone [0.05 μM] application, plants were very chlorotic and had poorly developed root and shoot systems (data not shown) suggesting that the concentration of the chemical (and / or frequency of application) was too high. There was no significant difference in the height, tiller number or biomass of fluridone-treated, uninfected and *S. hermonthica*-infected plants (Fig 3.8). Although the number of *S. hermonthica* attachments on fluridone-treated plants was similar to that on untreated control plants, they were smaller and had a lower total biomass (Fig 3.9).

In order to increase the concentration of strigolactones in the rice plants GR24 was applied to the roots. Application of GR24 had no effect on the height of the main stem of rice compared to untreated plants in the absence or presence of *S. hermonthica*. However *S. hermonthica*-infected plants (\pm GR24) were stunted compared to uninfected plants (Fig 3.8 A). Fewer tillers were produced in both uninfected and infected rice plants treated with GR24 compared to untreated plants, although the effect was not significant. *S. hermonthica*-infected plants (\pm GR24) had significantly fewer tillers than their respective uninfected plants (Fig 3.8 B). The overall biomass of the shoots of plants treated with GR24 was lower than that of the untreated control plants (in the absence of *S. hermonthica*) ($p < 0.01$) (Fig 3.8 C). *S. hermonthica* caused a reduction in shoot biomass of plants with \pm GR24. The reduction in shoot biomass of *S. hermonthica*-infected plants compared to controls (without GR24), was greater than for *S. hermonthica*-infected plants compared to controls (with GR24) due to the greater effect of GR24 on the shoot biomass of uninfected plants (Fig 3.8 C). There was no significant difference in the number, length or dry weight of *S. hermonthica* on rice plants with or without GR24 (Fig 3.9).

3.3.4 How does *S. hermonthica* alter the expression of the strigolactone biosynthetic gene CCD8 in the stems of Shiokari plants following infection?

In order to determine whether *S. hermonthica* altered the expression of CCD8 in the base of the stems sections of tissue were taken from control and infected plants at different times points after inoculation and stained with GUS. At 3 DAI little GUS staining was visible in the stem bases of either control or *S. hermonthica*-infected plants (Fig 3.10 A). By 6 DAI a much darker blue staining could be seen in the stems of *S. hermonthica*-infected CCD8:GUS plants compared to controls indicating an increase in the transcription of CCD8 in stems of the infected plants (Fig 3.10 B). However, the increase in expression of CCD8 in *S. hermonthica*-infected plants was transient as, by 9 DAI, the GUS staining had disappeared and there was no difference in the intensity of GUS staining between control and infected plants (3.10 C). These images were representative of two independent experiments.

Since there were no other GUS gene promoter reporter fusions available, other GUS promoters were not tested in order to compare the procedure. However, in order to make sure that the substrate was able to penetrate the whole tissue, the samples were vacuum infiltrated for twenty minutes, and the vacuum was released very slowly so that it was able to infiltrate into the whole sample. This ensured that the staining solution was present everywhere, as seen by the staining of cells even in the most internal sections of the roots and stems.

Transverse sections through the base of the stems of *S. hermonthica*-infected and control plants 6 DAI showed the cellular localization of the GUS stain (Fig 3.11). The transverse sections through the base of an uninfected (Fig 3.11 A) and *S. hermonthica*-infected (Fig 3.11 B) plant show the vascular bundles and tiller buds. No GUS staining was visible in any of the cells of the control plant but in the *S. hermonthica*-infected plant GUS staining was localized in the leaf axil and in the vascular bundles indicating high expression of CCD8 in these regions. Fig 3.12 shows the vascular bundles in the stems of control and *S. hermonthica*-infected rice plants. There is no intense GUS staining visible in this region of uninfected plants (Fig 12 A) but intense blue staining is visible in the vascular bundles (possibly in proto-xylem cells) of the stems of *S. hermonthica*-infected plants indicating high expression of CCD8 (Fig 3.12 B).

3.3.5 CCD8 (D10) is induced at the site of attachment of *S. hermonthica* to its host.

The expression of CCD8 was evaluated in the roots of uninfected and *S. hermonthica*-infected plants carrying the CCD8:GUS gene promoter reporter construct because it is known that strigolactones are mobilised from the roots to the aerial part of the plant through the xylem, contributing to architectural changes in tillering (Kohlen *et al.*, 2010). Fig 3.13 shows the expression of CCD8 in the root tip of control (Fig 13 A, C and E) and *S. hermonthica*-infected roots (B, D and F) 3, 6 and 9 DAI when roots were 24, 27 and 30 days old. At the first 2 time points GUS staining was clearly visible in the cell expansion zone of the root tip but this had disappeared at the later time point when GUS staining was visible in the vascular system. There was no difference in the intensity or pattern of staining in the roots tips of the control and infected plants but it is important to note that there were no *Striga* attachments in this region of the root (Fig 3.13).

However, at the site of attachment of *S. hermonthica* there was a cell-specific induction of the expression of CCD8. Figs 3.14 – 3.16 show the localization of the expression of CCD8 as the parasite invades the root 3, 6 and 9 DAI. In whole root sections the vascular bundles at sites of *Striga* attachment are stained dark blue compared to uninfected sections of root 3 (Fig 3.14) and 6 (Fig 3.15) DAI. This staining was most intense at the site of attachment but extended for a centimetre or more from the site of attachment before gradually falling to levels seen in uninfected roots. By 9 DAI there was little difference in the intensity of GUS staining in the vascular system of control and infected plants at the site of attachment (Fig 3.16 A, B, D and E). There was no induction of expression of CCD8 in the cortical cells following infection with *S. hermonthica* at point in the infection cycle.

Transverse sections through the root and parasite revealed that CCD8 expression was highly upregulated within the vascular core in cells (possibly protoxylem, phloem and pericycle cells) surrounding xylem vessels as the parasite penetrated through the endodermis into the vascular core (Fig 3.14 F and 3.15 F). However, at 9 DAI, once the parasite had formed connections with the xylem vessels of the host the level of CCD8 expression fell to control levels suggesting that expression of this strigolactone biosynthetic gene was correlated with the fusion of the host and parasite xylem vessels. Three-dimensional reconstruction of a 300 μm section of *S. hermonthica*-infected root 3 DAI clearly showed the localized induction of CCD8 in cells of the vascular core between the central metaxylem and the endodermis closest to the position at which the parasite penetrated through the endodermis (Fig. 3.17). This induction could be observed over a long stretch of root tissue, indicating systemic

expression of CCD8 in a stretch longer than the cells immediately surrounding the site of penetration of the vascular core.

It is interesting to note that CCD8 expression was also high in cells surrounding the xylem vessels when lateral roots emerged through the endodermis (as can be seen in Figs 3.14 B and 3.15 A). Again the expression of this gene is down regulated in these cells in older lateral roots.

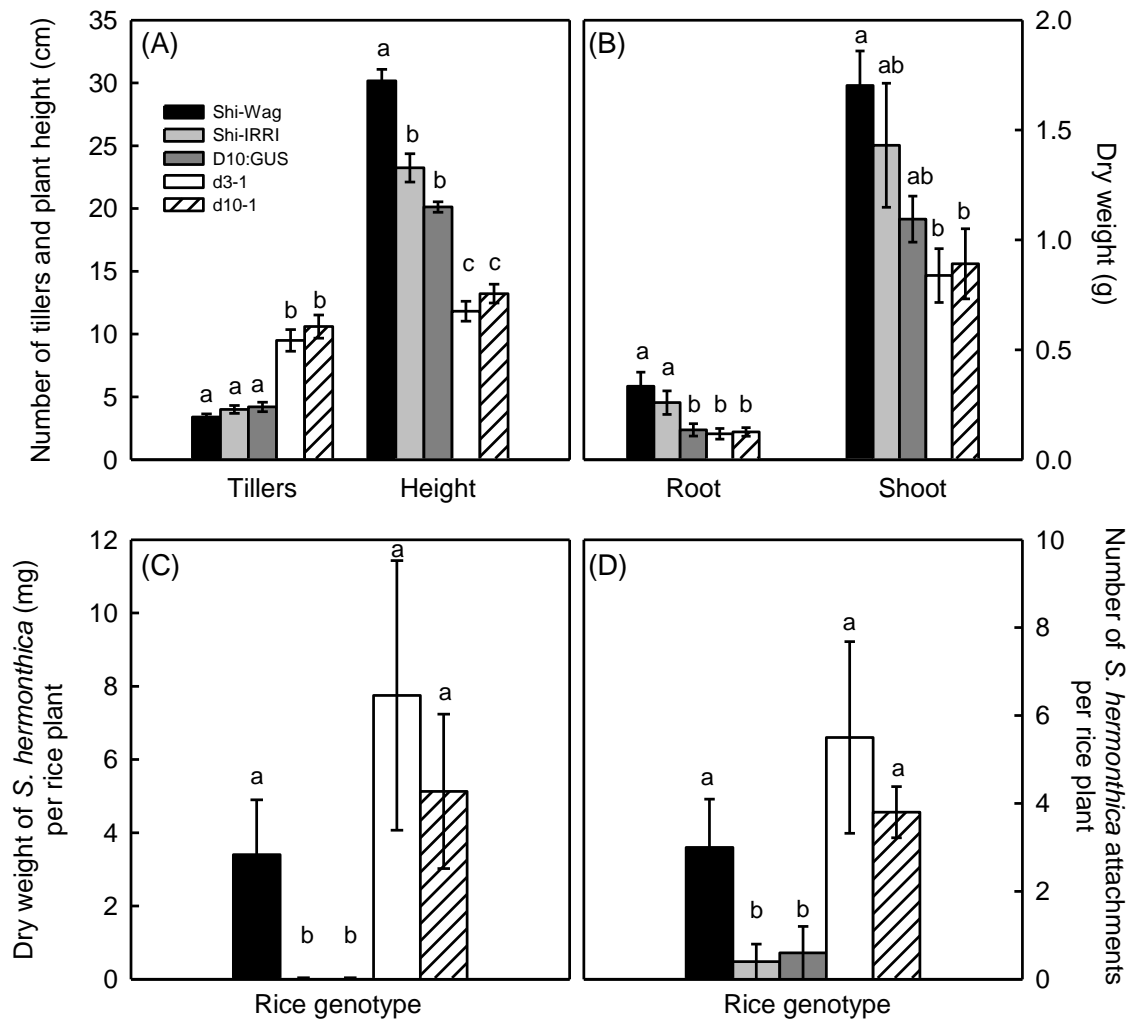


Figure 3.4 The effect of *S. hermonthica* on (A) the total number of tillers and (B) dry weight of Shiokari from the University of Wageningen, The Netherlands (Shi-Wag), the International Rice Research Institute (Shi-IRRI), the CCD8:GUS gene promoter reporter line and the strigolactone *d3-1* and *d10-1* mutants 21 days after inoculation. (C) and (D) show the dry weight and number of *S. hermonthica* attachments per plant.. Data are means \pm SE, n = 10. Columns with different letters differ significantly (ANOVA, and multiple comparison Tukey test p < 0.01).

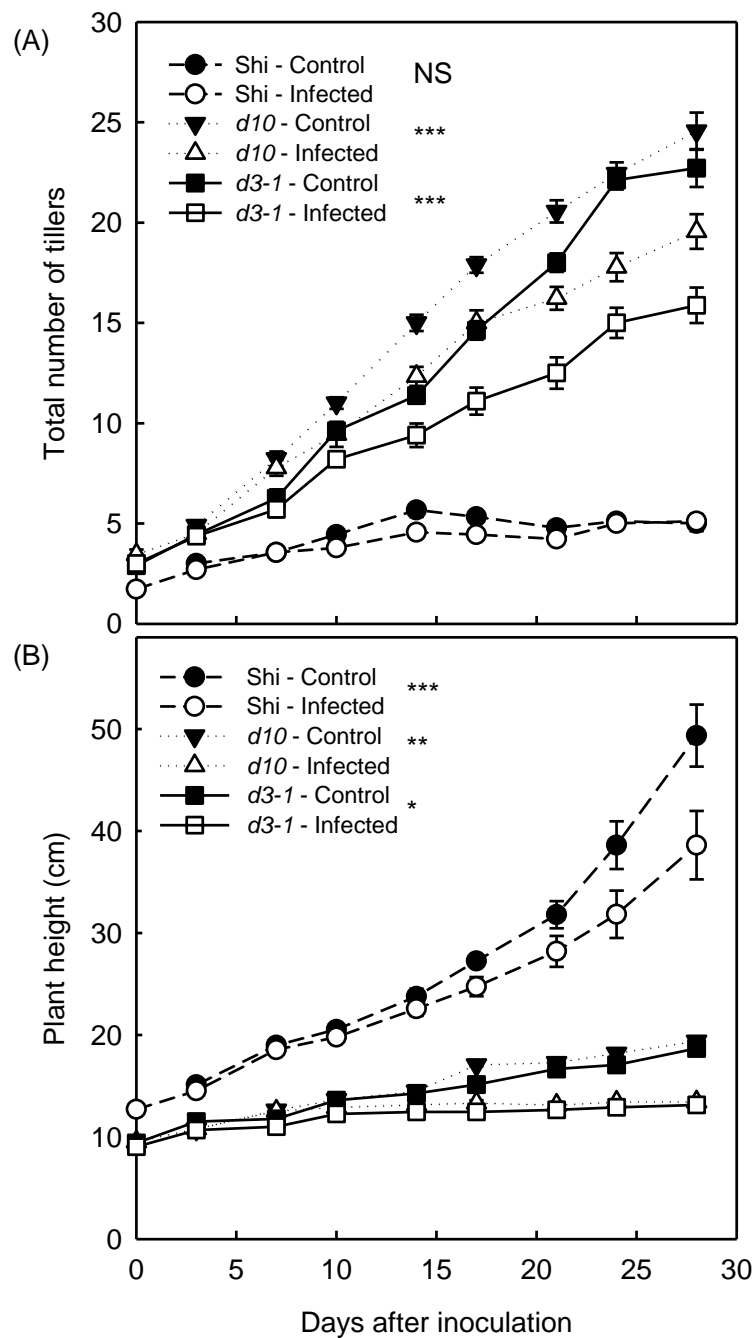


Figure 3.5 The effect of *S. hermonthica* on (A) the total number of tillers and (B) the height of the main stem of wildtype rice cultivar Shiokari and the strigolactone mutants *d3-1* and *d10-1* from the day of inoculation for 28 days. Open symbols represent uninfected plants; closed symbols represent *S. hermonthica*-infected plants. Stars indicate statistically significant differences between control and *S. hermonthica* plants for wildtype *d10* and *d3-1* Shiokari plants (ANOVA, $p < 0.05$ (*), $p < 0.001$ (**), NS = non-significant). Data are means \pm SE, $n = 10$.

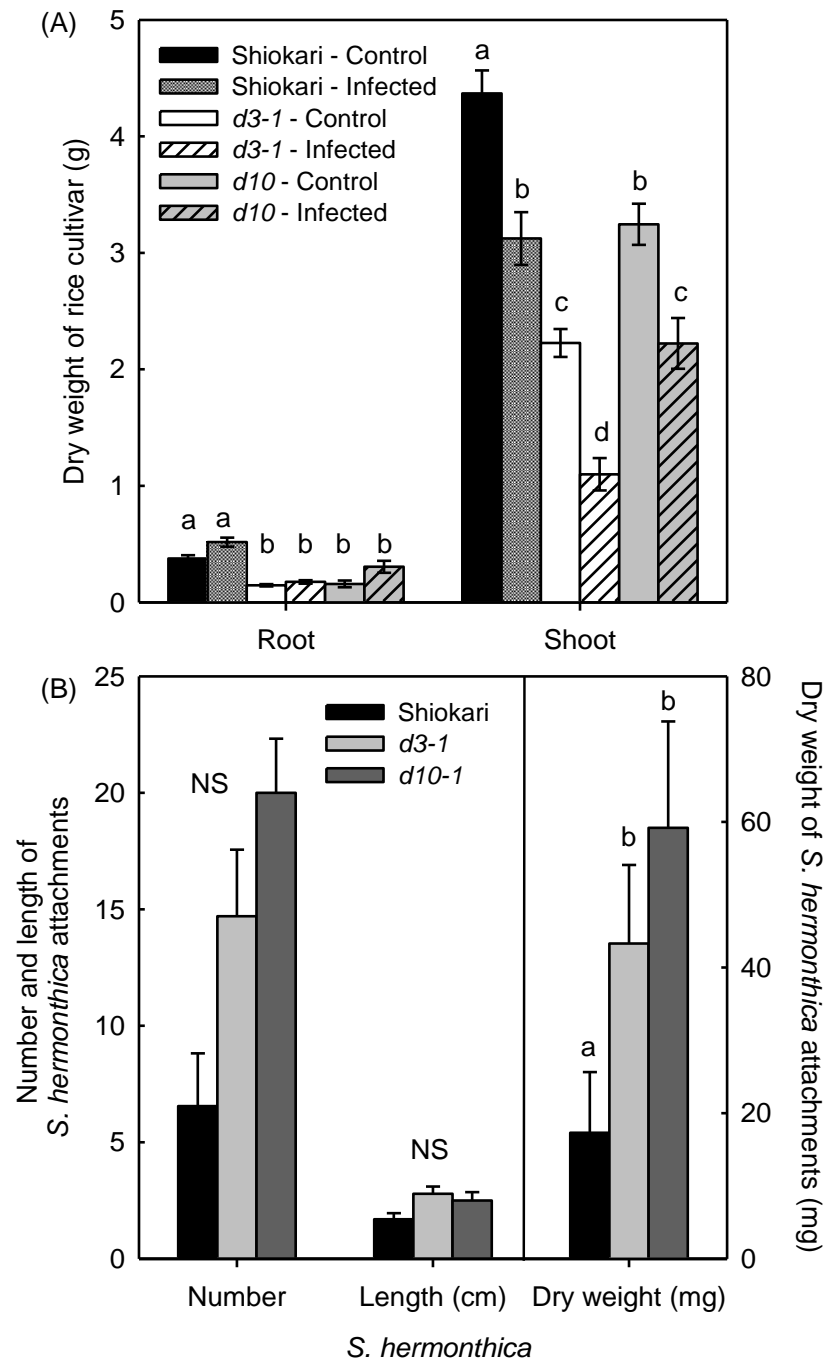


Figure 3.6 Effect of *S. hermonthica* on (A) the dry weight of roots and shoots (g) of control and *S. hermonthica*-infected rice plants (Shiokari wildtype and strigolactone mutants *d3-1*, *d10-1*) 21 days after inoculation. (B) The total number, length (cm) and dry weight of *S. hermonthica* individuals per rice plant. Data are means \pm SE, $n = 10$. Letters on top of columns indicate statistically significant differences (ANOVA and Tukey test ($p < 0.01$)).



Figure 3.7 Representative images of root tissue showing the different phenotypes of *S. hermonthica* attachments to wildtype Shiokari plants and to the strigolactone mutants *d3-1*, *d10-1* at 3 and 6 DAI. Scalebar = 200 μ m

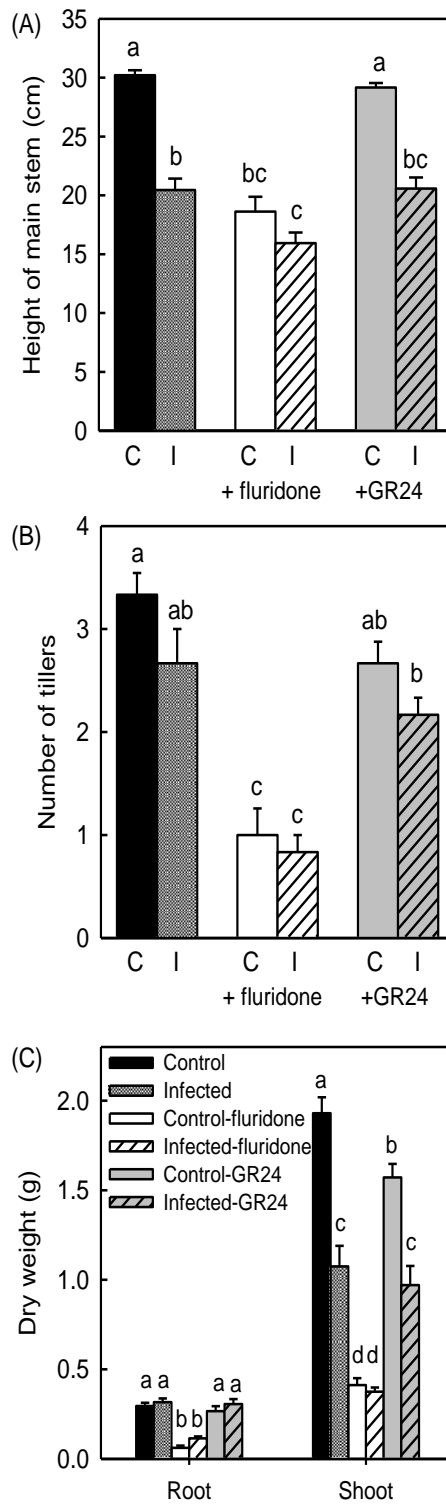


Figure 3.8 The effect of *S. hermonthica* on (A) plant height, (B) total number of tillers and (C) dry weight of (C) uninfected and (I) *S. hermonthica*-infected rice plants (cultivar Shiokari) \pm Fluridone or \pm GR24, 21 days after inoculation. Letters on top of columns indicate statistically significant differences (ANOVA; Tukey multiple comparison test ($p < 0.01$)). Data are means \pm SE, $n = 6$.

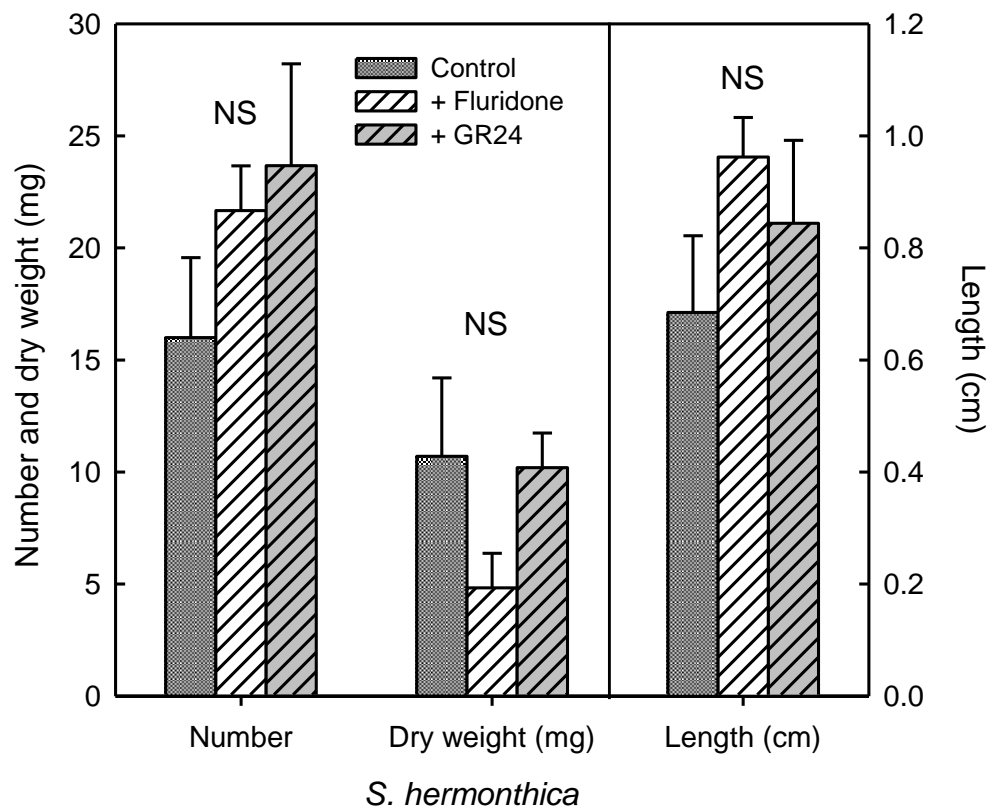


Figure 3.9. Effect of the carotenoid inhibitor fluridone [0.05 μM] and the strigolactone analogue GR24 [1 μM] on the length, total number of attachments and dry weight of *S. hermonthica* individuals, 21 days after inoculation. No differences were detected between treatments. Fluridone had a toxic effect on treated host plants. NS = non-significant. Data are means \pm SE are shown, n = 6.

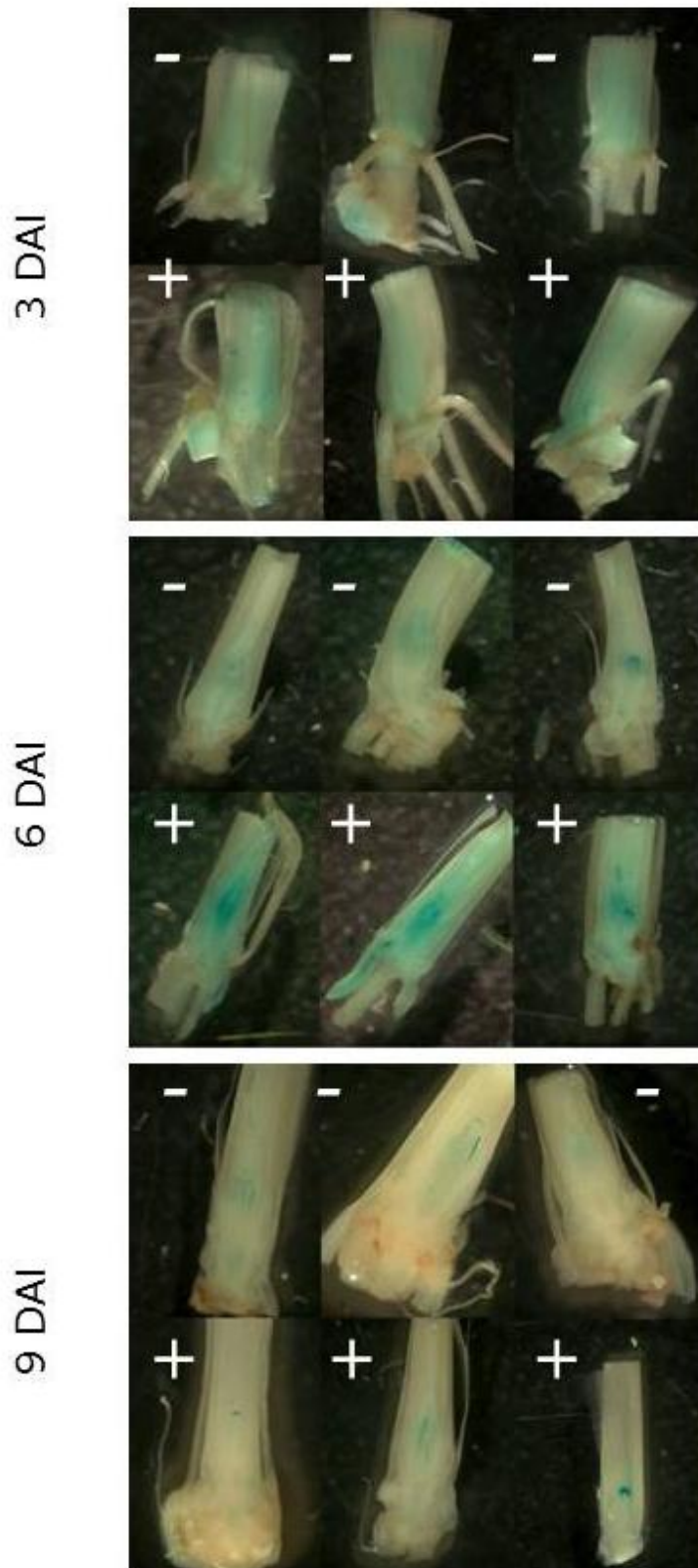


Figure 3.10 Transverse sections through the stems of (-) uninfected and (+) *S. hermonthica*-infected CCD8:GUS gene promoter-reporter fusion plants (cv. Shiokari) showing the location of expression of CCD8 (blue stain) 3, 6 and 9 days after infection. A gradual increase of GUS intensity can be observed by 6 DAI in *S. hermonthica* infected plants, fading away by 9 DAI, with no differences detectable between treatments by 3 and 9 DAI.

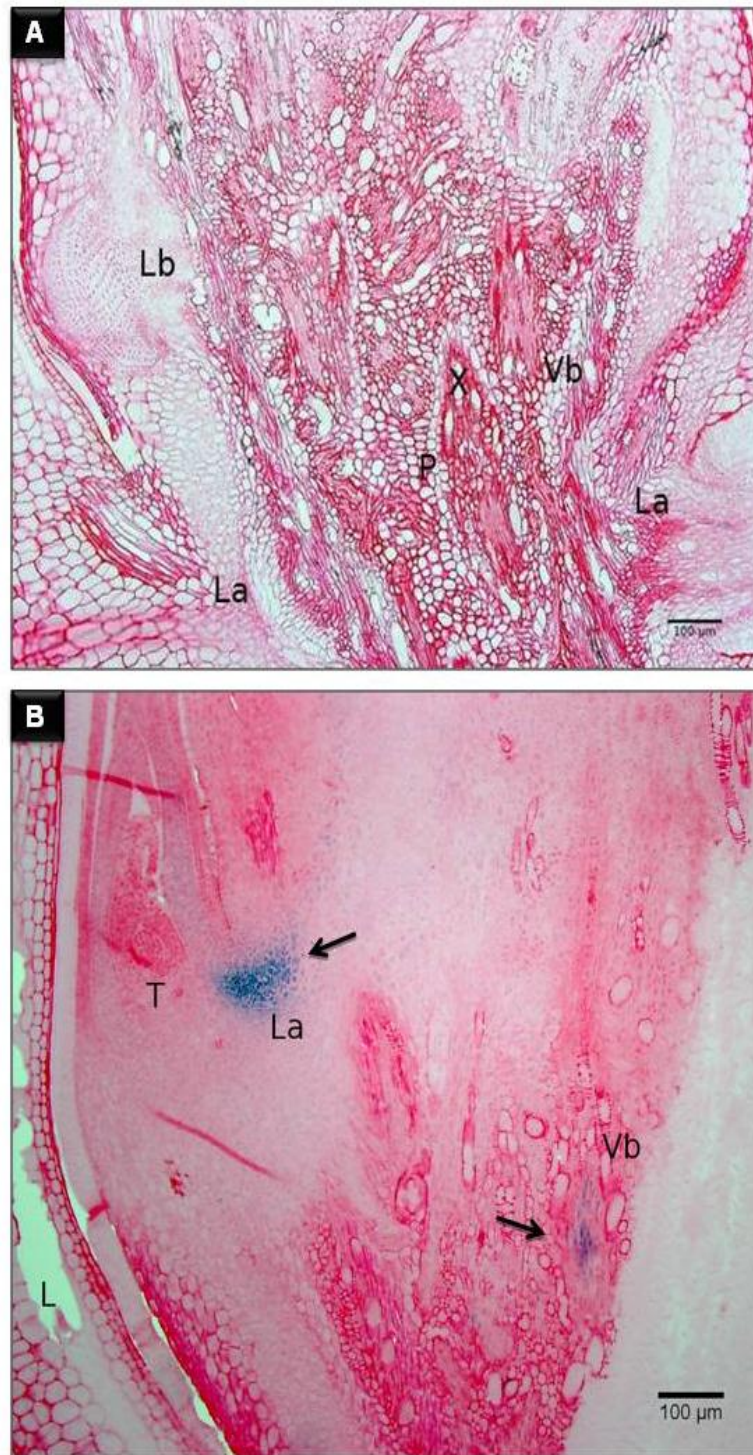


Figure 3.11 Longitudinal sections (5 μm thick) through the base of the stem of (A) uninfected and (B) *S. hermonthica*-infected Shiokari rice plants containing the CCD8:GUS promoter-reporter construct 6 DAI. GUS activity could be detected at the leaf axil of *S. hermonthica*-infected plants compared to controls. Arrows indicate expression of the strigolactone biosynthetic gene CCD8. LB = lateral bud; La = leaf axil; Vb = vascular bundle; L = leaf; T = tiller.

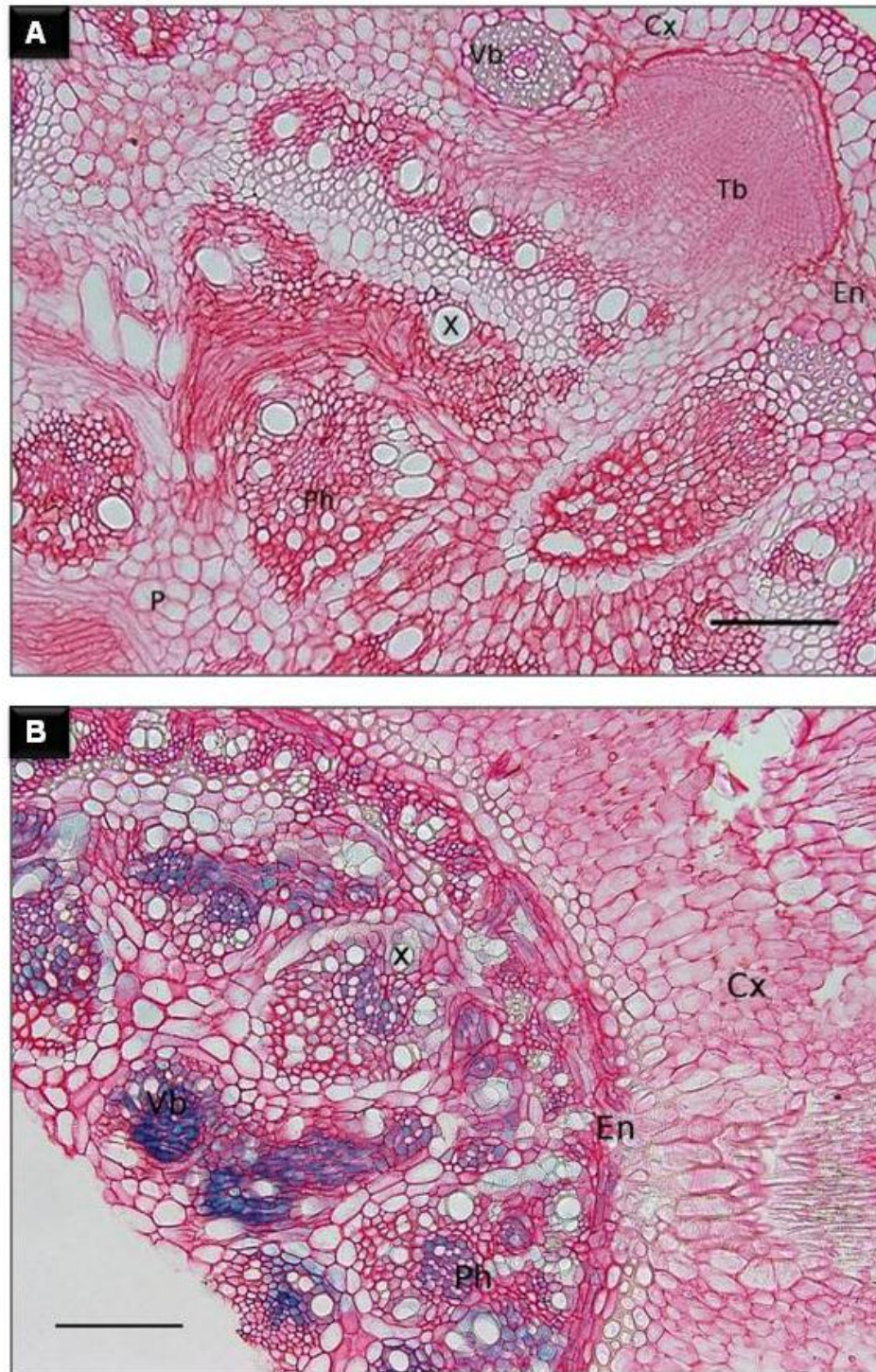


Figure 3.12 Transverse sections (5 μm) through the stem of (A) control and (B) *S. hermonthica* – infected Shiokari rice plants containing the CCD8:GUS promoter-reporter construct 6DAI. Infection by *S. hermonthica* led to induction of CCD8 as shown by blue staining at the vascular bundle of infected plants compared to controls. The sections are counter stained with Safranin-O (red colour). Tb = tiller bud emerging through the stem cortex (Cx) after growing out the endodermis (En); Ph = phloem; X = xylem; Vb = vascular bundle; P = stem pith. Scale bar = 100 μm .

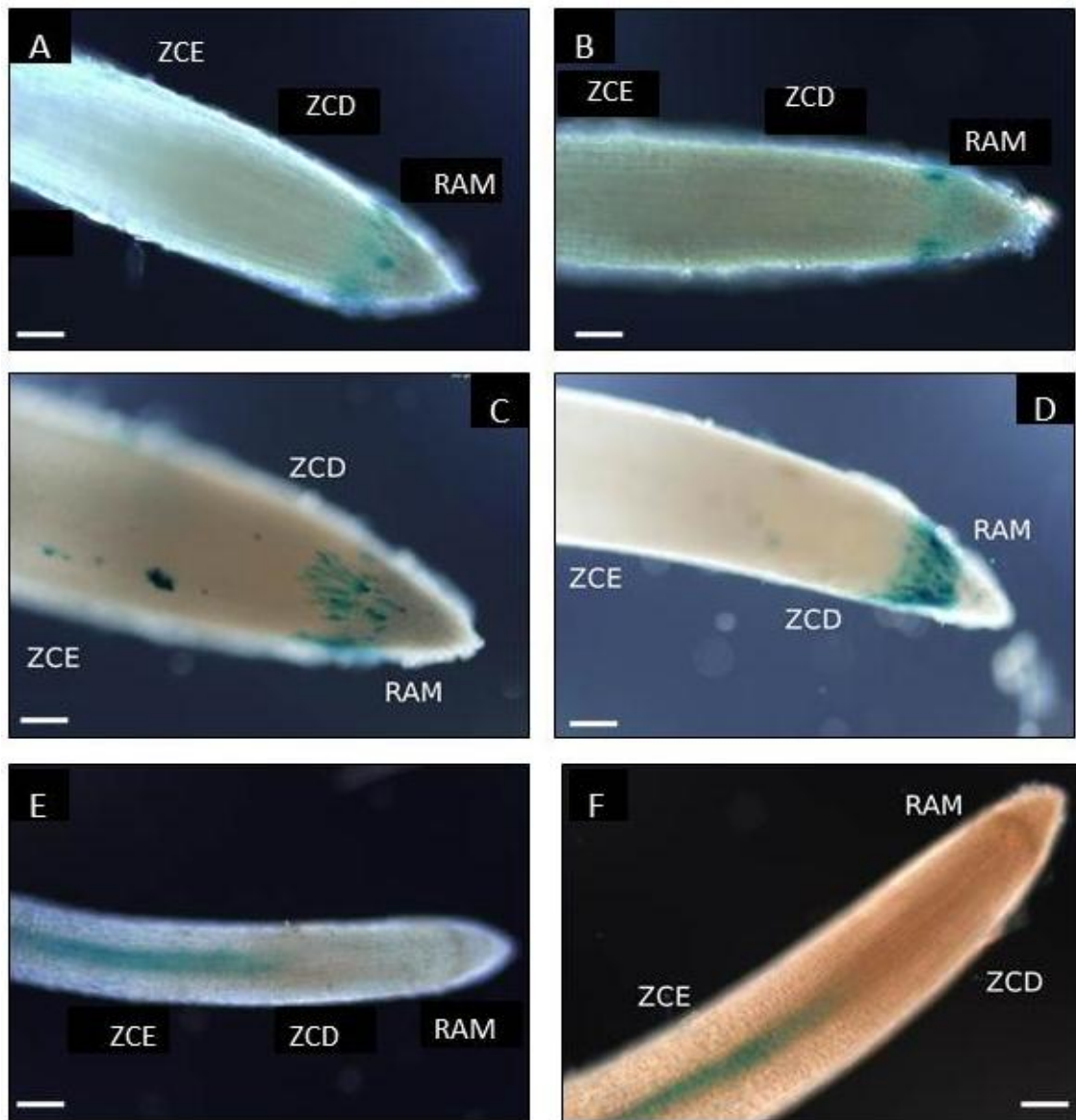


Figure 3.13 Localization of the expression of CCD8 in the root tip of control (left column) and *S. hermonthica* - infected (right column) rice plants (cv Shiokari) containing the CCD8:GUS promoter reporter fusion. (A and B) 3, (C and D) 6 and (E and F) 9 days after inoculation. Expression of CCD8 can be observed by blue staining (GUS) at the root tip during 3 and 6 DAI and at the zone of cell elongation by 9 DAI. RAM = root apical meristem; ZCD = zone of cell differentiation; ZCE = zone of cell elongation. Scale bar = 100 μ m.

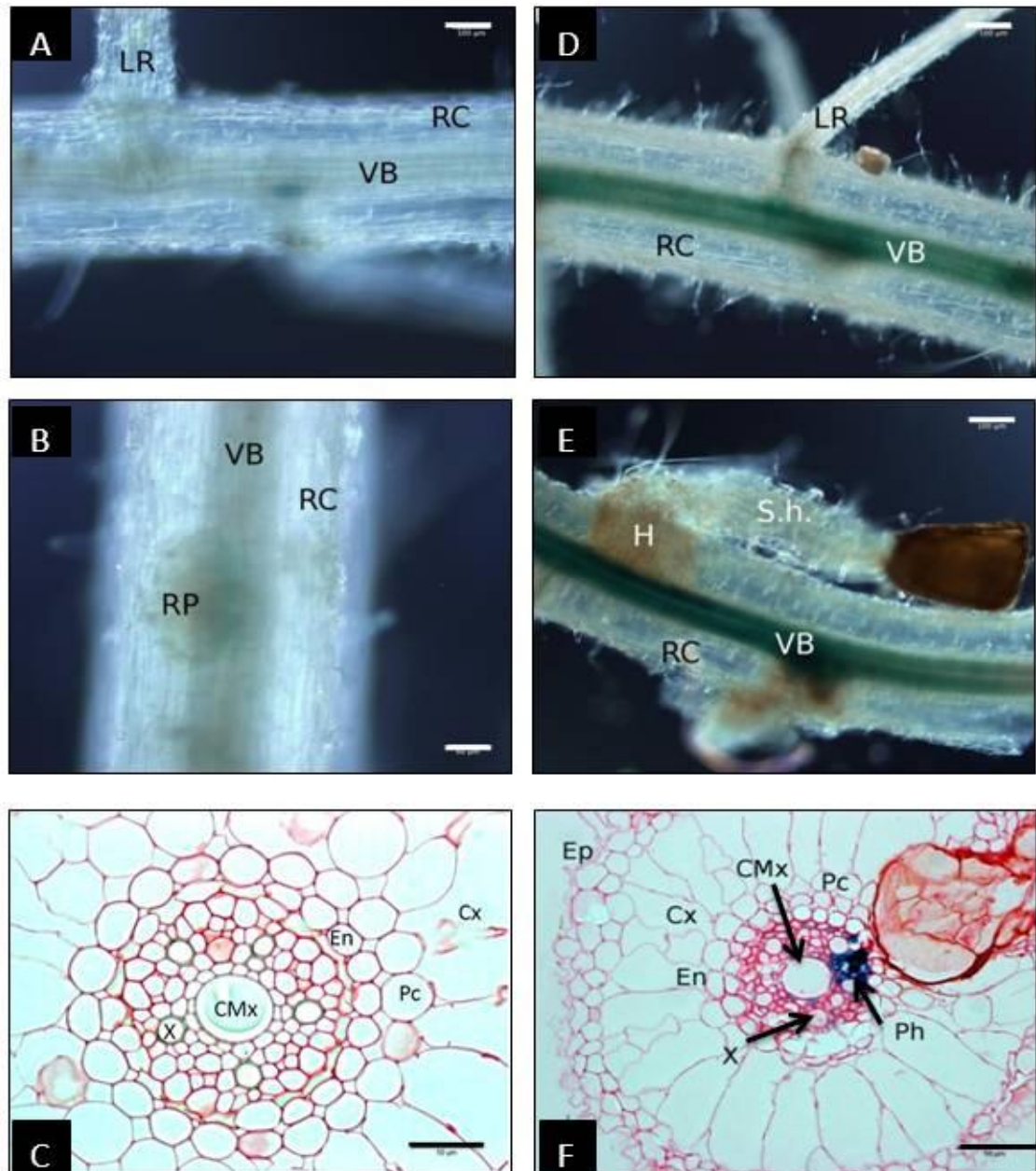


Figure 3.14 Localization of the expression of the strigolactone biosynthetic gene, CCD8 in uninfected (A-C) and *S. hermonthica*-infected (D-F) roots of the rice cultivar Shiokari containing the CCD8:GUS gene promoter reporter construct 3 days after inoculation. Blue staining (GUS) indicates expression of CCD8 in the vascular tissue prior to the fusion of *S. hermonthica* to the xylem vessels of its host. . LR = lateral root; Vb = vascular bundle; Rc = root cortex; Rp = root primordia; H = haustorium; S.h. = *Striga hermonthica*. Ep = epidermis; Cx = cortex; En = endodermis; Ph = phloem; Xy = xylem; CMx = central metaxylem; LR = lateral root. Scale bar = 100 μ m.

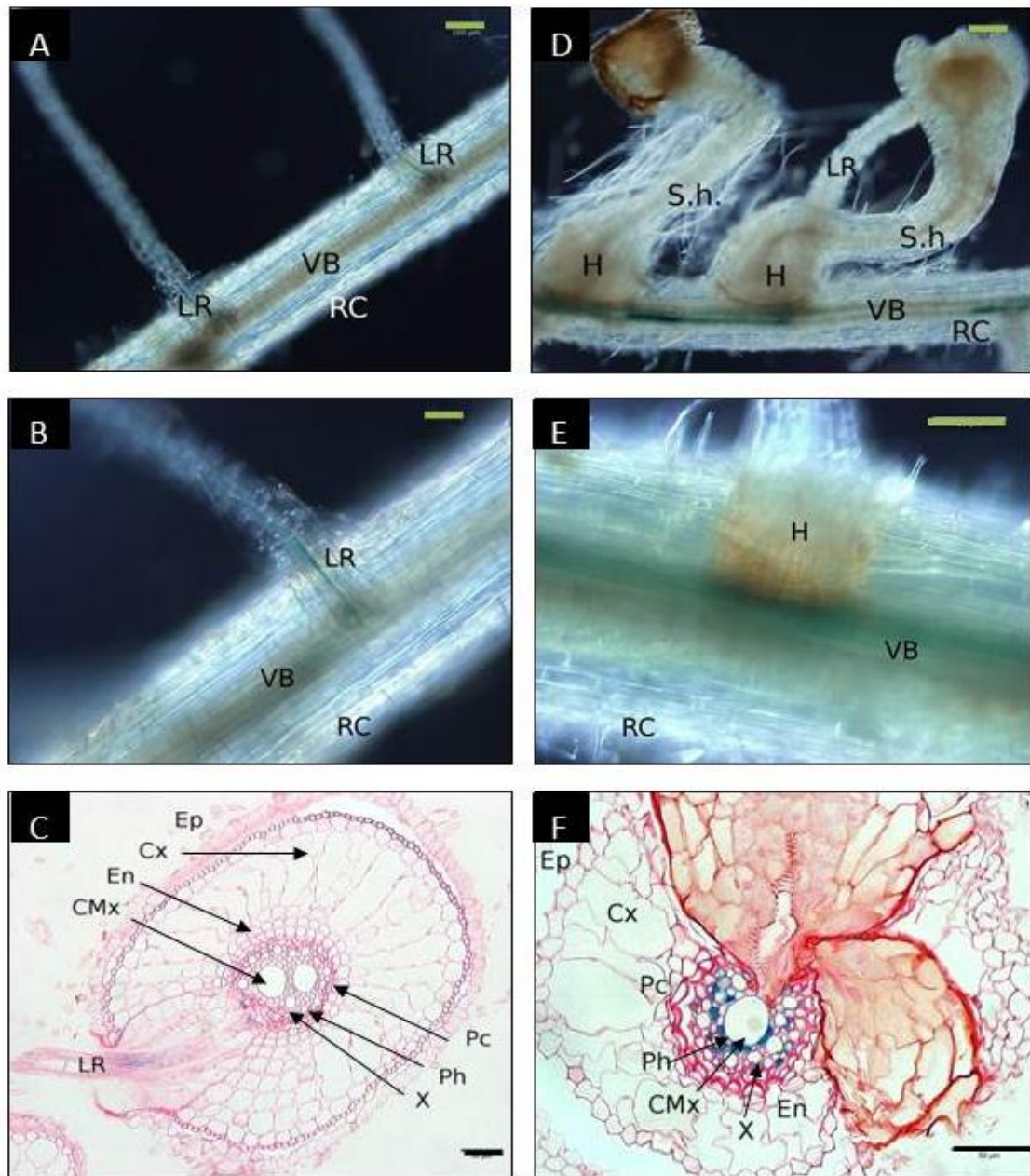


Figure 3.15 Localization of the expression of the strigolactone biosynthetic gene, CCD8 in uninfected (A-C) and *S. hermonthica*-infected (D-F) roots of the rice cultivar Shiokari containing the CCD8:GUS gene promoter reporter construct 6 days after inoculation. Blue staining (GUS) indicates expression of CCD8. LR = lateral root; Vb = vascular bundle; Rc = root cortex; Rp = root primordia; H = haustorium; S.h. = *Striga hermonthica*. Ep = epidermis; Cx = cortex; En = endodermis; Ph = phloem; Xy = xylem; CMx = central metaxylem; LR = lateral root; *Striga hermonthica* in red. Scale bar = 100 μm .

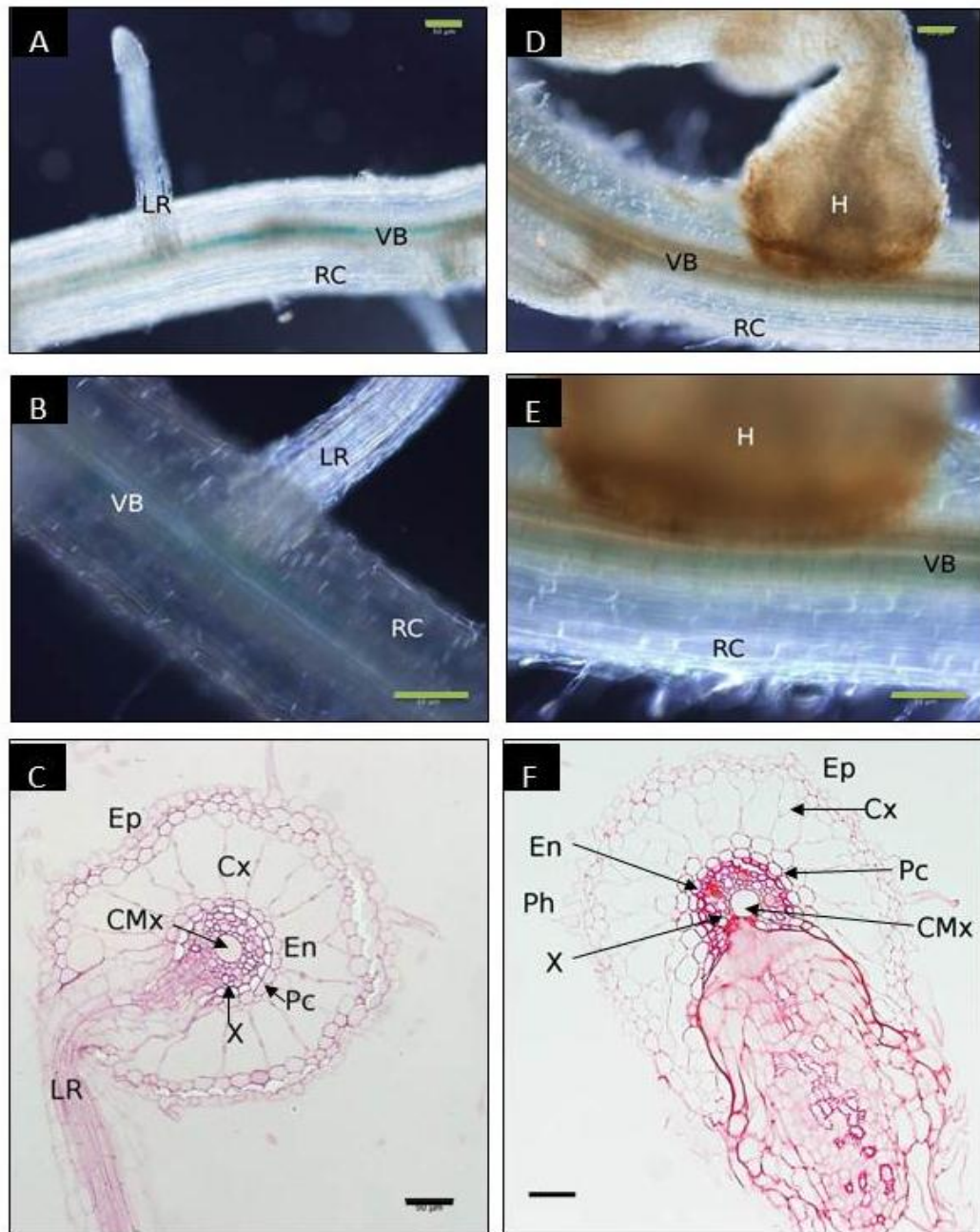


Figure 3.16 Localization of the expression of the strigolactone biosynthetic gene, CCD8 in uninfecting (A-C) and *S. hermonthica*-infecting (D-F) roots of the rice cultivar Shiokari containing the CCD8:GUS gene promoter reporter construct 9 days after inoculation. Blue staining (GUS) indicates expression of CCD8. LR = lateral root; Vb = vascular bundle; Rc = root cortex; Rp = root primordia; H = haustorium; S.h. = *Striga hermonthica*. Ep = epidermis; Cx = cortex; En = endodermis; Ph = phloem; Xy = xylem; CMx = central metaxylem; LR = lateral root; *Striga hermonthica* in red. Scale bar = 100 μm.

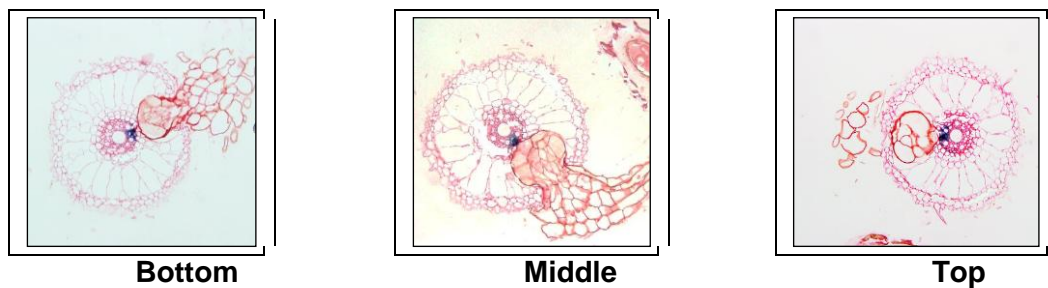
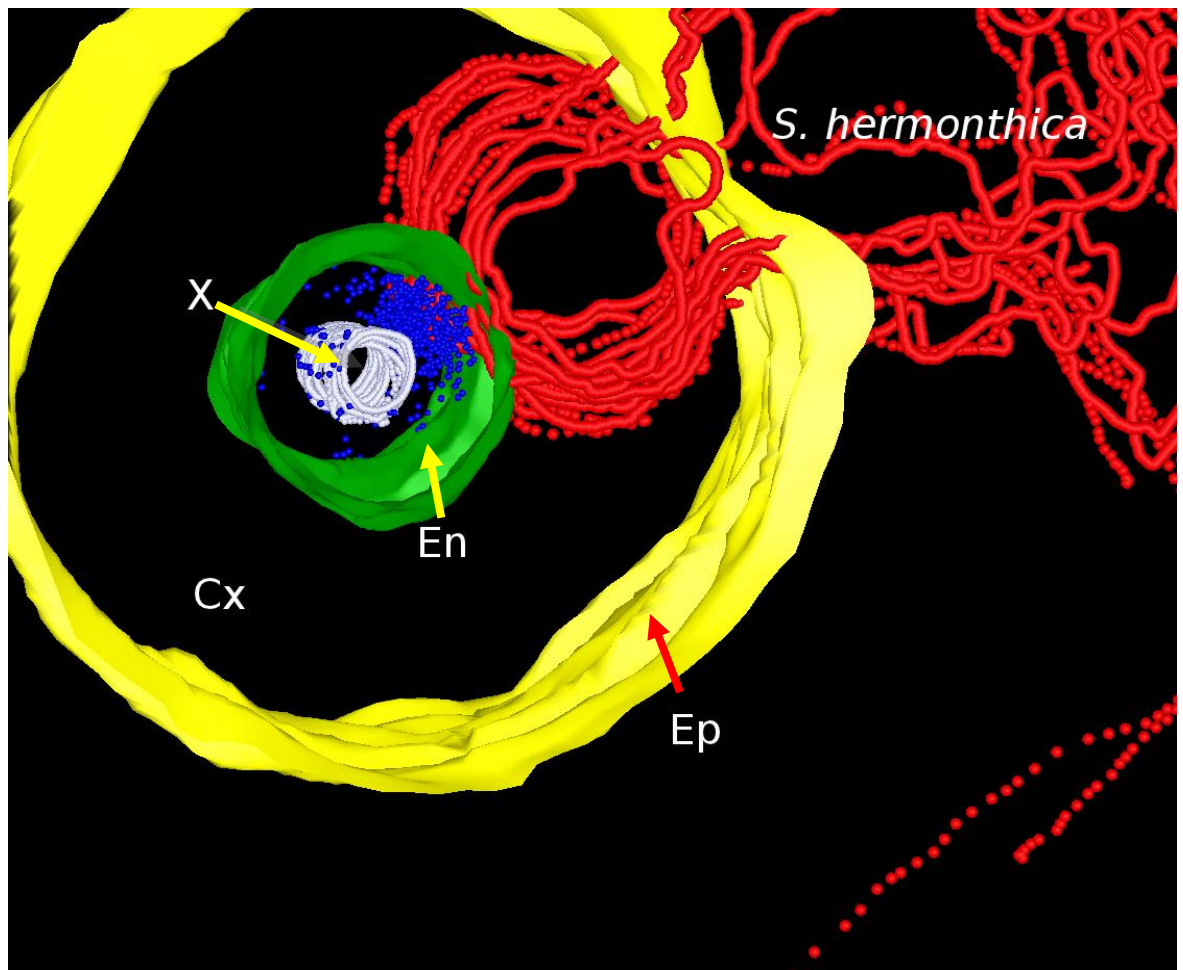


Figure 3.17. Spatial reconstruction of the root of *S. hermonthica* - infected rice plants (cv Shiokari) containing the CCD8:GUS gene promoter reporter construct. 3 days after inoculation. Blue dots represent cells that expressed CCD8. The depth of the image corresponds to the overlaying of serial sections over 300 μm . Example sections in sequential order shown on the bottom. X = central metaxylem (grey); En = endodermis (green); Cx = root cortex; Ep = epidermis (yellow).

3.4 DISCUSSION

3.4.1 Does the rice cultivar Shiokari obtained from different sources show the same response to infection by *S. hermonthica* (Sh-Kibos)?

Rice plants have a vast genetic diversity, as observed in their development and anatomy (Kurata and Yamazaki, 2006). Genomic differences exist not only between the two rice subspecies *indica* and *japonica* (Goff *et al.*, 2002; Yu *et al.*, 2002; Johns and Mao, 2006), and between cultivars of these sub species but also within cultivars. For example, Bryan *et al.* (2000) showed that the difference in susceptibility of the Pi-ta blast resistance gene transformants in the Nipponbare rice cultivar against the fungus *Magnaporthe grisea* was due to a single amino acid substitution from serine to alanine. As we had received seeds of the wildtype cultivar Shiokari from different sources (*i.e.* with the *d10-1* and *d3-1* mutants from Wageningen University, from IRRI and from Japan (plants containing the CCD8:GUS promoter reporter) we carried out a preliminary experiment to make sure that they all showed a similar level of susceptibility to *S. hermonthica* and alterations in morphology.

The three different Shiokari WT lines tested in this experiment behaved in a similar but not identical manner. The genotypes obtained from IRRI and the CCD8:GUS lines were similar to each other but both were slightly shorter and had fewer *S. hermonthica* attachments compared to the genotype obtained from Wageningen. These differences could be due to small changes in the genotype of the cultivars during breeding and bulking of seeds in the different research centres or it may be related to the age of the seeds. However, the results of this experiment show the importance of working with wildtype seeds that are isogenic to mutants that are used in experiments to test the function of specific genes. Since the main aim of this work is to determine whether alterations in strigolactone biosynthesis or signalling is involved in the *S. hermonthica* induced suppression of tillering, the wildtype Shiokari seeds obtained from Wageningen were used in experiments with the strigolactone mutants.

3.4.2 Are strigolactones involved in the suppression of tillering in *S. hermonthica* - infected plants?

The mechanisms through which *Striga* spp cause stunting and suppression of tillering of their hosts are not fully understood to date. With the recent discovery of the role of strigolactones as a key regulator in tillering of rice and the availability of mutants in

different components of the SL metabolic pathway, we were able to test whether these compounds were involved in the suppression of tillering in *S. hermonthica* - infected plants at the biosynthetic or signalling levels.

Unusually in this experiment, the Shiokari wild type plants did not show any inhibition of tillering when infected by *S. hermonthica* by 28 DAI compared to uninfected controls. This may have been due to the low numbers of attachments of *S. hermonthica* to the infected plants. However, the number of parasites attached to the host is not always linearly correlated to the negative effects observed on the host as demonstrated by Gurney *et al.* (1999). It was clear that Shiokari wild type showed partial resistance to *S. hermonthica*. Uninfected and *S. hermonthica*-infected Shiokari WT plants used in this experiment were six weeks old when harvested and both treatments had 5 tillers in average. Shiokari WT is not a particularly high tillering cultivar; it only produces approximately ten tillers by 10 weeks after germination (Ishikawa *et al.*, 2005; Arite *et al.*, 2007). If infected plants had been grown for longer than six weeks, we may have been able to detect suppression of tillering in *S. hermonthica*-infected Shiokari WT plants compared to controls, as uninfected plants had only produced approximately half the number of tillers by the time the last tiller count was performed in this experiment compared to the previously reported number of tillers for this cultivar (Ishikawa *et al.*, 2005; Arite *et al.*, 2009). This needs further investigation focusing on the alterations of the morphology of *S. hermonthica*-infected plants at late stages of infection.

The fact that the initial hypothesis did not work as we predicted, *i.e.* tillering was also reduced in both, the strigolactone signalling and biosynthetic mutants, suggested that strigolactones are not required for the symptom development. It is important to make a clear differentiation of the role of strigolactones between the initial infection process and subsequent phenotype observed, *i.e.* reduced tillering. Both processes are discussed in this work.

In contrast to the wildtype Shiokari, tillering was significantly reduced in *S. hermonthica*-infected *d3-1* and *d10-1* compared to the uninfected controls, which is not consistent with to the initial hypothesis which proposed that these two mutants would not suffer a inhibition of tiller bud outgrowth as a consequence of infection by *S. hermonthica*.

The strigolactone biosynthetic deficient mutant *d10-1* had suppression of tillering due to infection by *S. hermonthica*. *d10-1* is defective in the carotenoid cleavage dioxygenase CCD8 due to a point mutation from T to C in the first intron, leading to a non-functional product, thus reduced levels of strigolactones and increased

tillering (Arite *et al.*, 2007). One of the initial hypothesis proposed that suppression of tillering was due to an increase in the strigolactone levels in *S. hermonthica* - infected plants. The suppression of tillering observed in *S. hermonthica*-infected *d10-1* plants suggested that an increase in the levels of endogenous strigolactones in rice plants is not the main reason for the suppression of tillering, since this mutant is not able to increase its strigolactone contents, at least by the mechanisms described for strigolactone biosynthesis so far. Alternatively, *S. hermonthica* could be the source of strigolactones into the host, translocating strigolactones through the xylem and inhibiting tillering, consistent with the idea that root derived strigolactones are transported from the roots to the shoot in order to regulate branching, as shown in *A. thaliana* plants (Kohlen *et al.*, 2011). If *S. hermonthica* is a source of strigolactones and these are transported into the host to be translocated to the shoot, resulting in suppression of tillering, then the strigolactone signalling mutant *d3-1* should not present differences of tillering between uninfected and *S. hermonthica*-infected plants due to the higher content of strigolactones.

Strigolactones were expected to be up regulated in *S. hermonthica*-infected plants, leading to a more intense suppression of the existing tiller buds. However, the use of strigolactone mutants did not provide the expected results. It is known that auxin plays a role in the emergence of lateral buds and tillering. This process has been better described in *A. thaliana*, whereas in rice more research needs to be done to fully understand the feedback mechanism between auxins and strigolactones in the emergence of tillers, since many genes are yet to be described, as pointed out by Beveridge and Kyojuka (2010). Based on the work that has been reported for *Arabidopsis*, auxin and strigolactones co-regulated each other in a feedback mechanism. Auxin-regulated strigolactone depletion is a major cause of branching after decapitation (Brewer *et al.*, 2009), and auxin depletion causes a significant decrease in the expression of strigolactone biosynthetic genes in several species (Bennet *et al.*, 2006; Arite *et al.*, 2007; Foo *et al.*, 2005; Hayward *et al.*, 2009). Thus it could be hypothesised that either strigolactones or auxin are having a later role in the inhibition of branching observed in *S. hermonthica* infected plants.

Similarly to *d10-1* infected plants, the *S. hermonthica*-infected *d3-1* mutant plants also had suppression of tillering compared to uninfected plants. *d3-1* is defective in the signalling of strigolactones due to a transposon insertion in the coding region of an F-box LRR protein, putatively involved in the sensing of strigolactones (Ishikawa *et al.*, 2005). The suppression of tillering in *d3-1* suggested that strigolactone signalling is not necessary for the suppression of tillering in *S. hermonthica* - infected rice plants.

We could observe that the proportion in which *d3-1* and *d10-1* were affected in the suppression of tillering by *S. hermonthica* was the same, perhaps suggesting that the observed inhibition of tillering is caused by a mechanism different to the strigolactone biosynthesis and signalling pathway. A feedback mechanism consisting on the regulation of auxin transport by strigolactones in order to control shoot branching has been described, (Brewer *et al.*, 2009; Crawford *et al.*, 2010). Auxin metabolism may play a role in the inhibition of tillering, since *d3-1* and *d10-1* are defective in strigolactone but not auxin metabolic pathways. This hypothesis is tested in the following Chapter.

In order to further analyse the role of strigolactones in the inhibition of tillering of *S. hermonthica* - infected plants, the endogenous levels of strigolactones in Shiokari WT plants were altered. The carotenoid biosynthesis inhibitor fluridone was used to reduce strigolactone biosynthesis, whereas exogenous application of GR24 was used to increase strigolactone levels. The fluridone dose used in this experiment was based on the doses reported in experiments testing inhibition of biosynthesis of carotenoids in rice grown in pots without altering other plant metabolic pathways (Jamil *et al.*, 2010), while the GR24 dose used in this experiment was based on doses that inhibited tillering in Shiokari WT rice (Umehara *et al.*, 2008).

The fluridone-treated plants were severely affected in their growth due to a toxicity effect, as observed in the poor condition of these plants, with and without the presence of *S. hermonthica*. Carotenoid inhibitors such as fluridone are used as herbicides in high doses, causing photo bleaching of chlorophyll and death of plants, since they are critical elements in photosynthesis and photoprotection (Demmig-Adams *et al.*, 1999; Pascal *et al.*, 2005; Cazzaniga *et al.*, 2012; Boger and Sandmann, 1998). The fluridone-treated plants in this experiment presented chlorosis, suggesting chlorophyll degradation and excessive alterations in carotenoid production. The chemicals were supplied directly in the root system, as reported by Jamil *et al.* (2010), however we used a rhizotron based system, whereas they grew rice plants in sand-filled pots, therefore reducing the amount of fluridone taken up by the roots. In order to obtain reliable results, repeating this experiment using a lower dose of fluridone and decreasing the light intensity in the growth cabinets to avoid photobleaching should provide a better effect in the inhibition of biosynthesis of carotenoids, thus strigolactone production, mimicking in a more precise way the effects observed on the strigolactone mutant *d10-1*.

Unlike the fluridone-treated plants, GR24-treated plants were comparatively healthy. Albeit no statistically significant differences, GR24 – treated uninfected plants

had slightly fewer tillers than untreated uninfected plants, suggesting that the used doses of GR24 not high enough to provide the desired effect on suppression of tillering, therefore, higher doses of GR24 could induce a more intense effect in suppressing the outgrowth of lateral buds. GR-24 - treated infected plants had reduced tillering compared to untreated uninfected plants and further suppression of tillering compared to untreated infected plants, suggesting at least a partial role of strigolactones in the inhibition of tillering in *S. hermonthica* - infected plants.

Further evidence supporting a role for strigolactones in the suppression of tillering in *S. hermonthica* - infected plants was detected by infecting the D10:GUS reporter. Transient expression of D10 seen in cells at the leaf axil area of transgenic rice plants containing the D10:GUS gene promoter reporter fusion infected with *S. hermonthica* suggested a very specific role of SL biosynthesis in the inhibition of tillering. More studies need to be performed to determine whether the expression levels of D10 in the *S. hermonthica* - infected mutants *d3-1* and *d10-1* increase, leading to higher inhibition of tillering by mechanisms yet unknown. By measuring the endogenous levels of strigolactones in uninfected and *S. hermonthica* - infected dwarf rice mutants, it should be possible to determine to a greater extent the degree of involvement of strigolactones in the inhibition of tillering. In addition, more studies are needed to determine whether *S. hermonthica* is capable of synthesising its own strigolactones.

The primary shoot apex can inhibit the outgrowth of lateral buds, this process is known as apical dominance. In rice, the term of apical dominance can also be applied as shown by Arite *et al.* (2007), who performed decapitation experiments once the stem elongated. The main inconvenience with rice when determining apical dominance is that the stem elongates only when it has gone from the vegetative to the reproductive stage. It is possible that *S. hermonthica* interrupts branching in infected plants by similar mechanism to the limitation of tiller bud outgrowth by apical dominance, regulated by auxin flow. However, this potential increased apical dominance, does not benefit the main shoot as it should if the mechanism was solely based on apical dominance.

These finding support the hypothesis of mechanisms different to strigolactones in the characteristic phenotype of *S. hermonthica* - infected plants. The induction of biosynthesis of D10 at the leaf axil of *S. hermonthica* reflected one of the components involved in the inhibition of tillering in infected plants. The interaction between strigolactones and other plant growth regulators such as auxin or ethylene could provide a possible explanation. Auxin has not been tested in a compatible parasitic

interaction; however, it is responsible for apical dominance, thus inhibition of the emergence of formed tiller buds in rice.

3.4.2 Are strigolactones involved in the infection process?

Strigolactones were discovered because of their effect on the positive germination of parasitic plants, a critical step in the life cycle of *Striga* spp (Müller *et al.*, 1992; Humphrey and Beale, 2006; Zwanenburg *et al.*, 2009). This is the first study focused on the role of strigolactones in plant parasitism after germination of *S. hermonthica* seeds in the root system of infected plants. The results obtained by the use of the transgenic rice plant containing the D10:GUS gene promoter reporter fusion suggest a role of strigolactones in the infection process.

Induction of biosynthesis of CCD8 in cells surrounding the xylem vessels in the vascular bundle of *S. hermonthica* - infected roots was detected very strongly as soon as the parasite reached the endodermis by 3 DAI. The intensity of the GUS signal declined by 6 DAI and it spread to cells opposite to the site of attachment, suggesting cellular communication in the vascular bundle cells and a decrease in the transcript levels of D10. By 9 DAI very little or no GUS signal was detected in the cells of the vascular bundle of infected plants, at the same cell localisation where a strong signal could be seen previously. This coincides with the timing when *S. hermonthica* fuses to the xylem vessels of its hosts. These results suggest a fundamental role of strigolactones in processes leading to the fusion of host and parasite xylem vessels, however, the specific role of strigolactones in xylem maturation and differentiation processes is not known to date and needs further investigation.

In addition, even though not statistically significant, *d3-1* and *d10-1* had a higher number of attachments compared to Shiokari WT, suggesting that strigolactone mutants are more susceptible to *S. hermonthica* and may be involved in the infection process. Since Shiokari WT had an unusual resistance to this particular ecotype of *S. hermonthica* (collected in Kibos, Kenya during 2009), future investigations studying this phenomena should consider using a more aggressive ecotype of *S. hermonthica* that allows a better comparison among the three different infected cultivars.

Since we also detected infection by *S. hermonthica* on the SL biosynthesis deficient mutant *d10-1*, we propose that strigolactones interact with alternative mechanisms during the infection process. The spatial distribution of D10 in roots of *S. hermonthica* - infected transgenic D10:GUS rice plants is analogous to the expression pattern of the auxin influx carrier LAX3 in roots of transgenic *A. thaliana* containing the LAX3:GUS reporter fusion during lateral root formation from cells in the pericycle

(Swarup *et al.*, 2008; Peret *et al.*, 2009). Therefore we hypothesize that strigolactones and auxin act together in the infection process during the formation of the xylem to xylem vessels connections.

In summary, these results indicated that strigolactones may play a role in the inhibition of tillering and also in the infection process of *S. hermonthica* - infected plants, however, they cannot be held responsible for the complete process. The results showed a discrepancy between the results obtained with the mutants, which suggested strigolactones are not essential for either the infection process or consequent inhibition of tillering of *S. hermonthica*-infected plants, and the results obtained with the D10:GUS gene promoter reporter which indicated a transient expression of the strigolactone biosynthesis gene. Therefore we hypothesised that other plant growth regulators are involved in the suppression of tillering and infection process, potentially auxin. To address this question, microarray analysis of whole plants was undertaken in a susceptible interaction and the role of auxin and other plant growth regulators in plant parasitism is presented in the following Chapter.

Chapter 4

Analysis of transcriptional changes in *S. hermonthica* infected plants throughout infection: an integrative perspective.

4.1 INTRODUCTION

Over the last 10 years there have been many technological advances that now allow us to profile changes in the expression of many genes simultaneously, especially for species whose genomes have been sequenced (for example rice). Microarrays are small slide slides containing DNA sequences unique to individual genes within a genome. Hybridization of mRNA transcripts to such arrays provides a way to obtain high throughput information on the regulation of many biological processes simultaneously. At present only a few studies have been carried out to examine changes in gene expression in root tissues of host plants undergoing susceptible or resistant interactions with parasitic plants. In 2003 Vieira Dos Santos and colleagues profiled changes in the roots of *A. thaliana* during a resistant interaction with *Orobancha ramosa* and showed that genes associated with defence pathways such as ethylene, phenylpropanoid and jasmonate biosynthesis were up regulated. Dita *et al.*, (2009) profiled changes in the expression of genes in roots of *Medicago truncatula* that exhibited different types of resistance responses to invasion by *Orobancha crenata* infection and showed that different defence related transcripts were activated in each interaction. Finally Swarbrick *et al.* (2008) characterized the global gene expression patterns in the roots of two rice cultivars, Nipponbare (resistant interaction) and IAC 165 (compatible interaction) following infection with *S. hermonthica* and demonstrated that large number of transcripts were up- or down-regulated) by parasitism. This is the only study to date that has examined changes in transcription in the roots of a host plant during a compatible interaction. This study showed that a large number of genes were down regulated during a compatible interaction including those associated with some aspects of plant growth regulator metabolism, biogenesis of cellular components and cell division whilst genes in other categories were up regulated e.g. those associated with nutrient transport and amino acid metabolism.

As demonstrated in this study parasitism of rice by *Striga hermonthica* (compatible interaction) causes severe alterations in the morphology, metabolism, and genetic regulation of susceptible host plants from the moment of penetration of the host root until the end of its life cycle. These morphological alterations consist of a negative impact on the growth of the host, reflected by a significant reduction of plant biomass, height, stem thickness and tillering in rice (as described in Chapter 2). The aim of this Chapter is to profile changes in gene expression in the roots, stems and leaves (simultaneously) of rice cultivar IAC 165 following infection by *S. hermonthica*. Analysis of changes in the regulation of plant growth regulator pathways will allow some of the

hypotheses relating to the role of PGRs in the morphological alteration in *Striga*-infected plants to be investigated.

Gibberellins are the major class of plant growth regulators involved in regulating the height of the plant, as described in Chapter 1 section 1.5.4. A study which analysed the changes in gene expression in the roots of *Striga*-infected plants suggested that many genes involved in the biosynthesis of gibberellins were down-regulated (Swarbrick *et al.*, 2008). However, the current knowledge of alterations in gene expression of gibberellins and other plant growth regulators in the stems and leaves of *S. hermonthica* infected plants is scarce. In addition, auxins can also impact the height of plants as demonstrated by the significant reduction of plant height of transgenic plants overexpressing SAUR39, a gene involved in polar auxin transport (Kant *et al.*, 2009). Transcript profiling of SAUR39ox revealed alterations in the chlorophyll, anthocyanin, sugar and abscisic acid contents as a result of reduced auxin transport (Kant *et al.*, 2009; Kant and Rothstein, 2009). Because of the similarity between the phenotype of SAUR39ox and *Striga*-infected plants, we hypothesised that similar alterations to those observed in the gene expression of auxin metabolism of SAUR39ox may be responsible for the changes in the morphology of *S. hermonthica*-infected plants.

In Chapter 3 it was determined that strigolactones may be involved to some certain extent in the suppression of tillering caused by *S. hermonthica*, as shown by the reduction of tillering in GR24-treated rice plants after infection with *S. hermonthica* and the induction in the expression of the biosynthetic gene CCD8 in the leaf axil area of *S. hermonthica* infected plants. Allometric studies suggest that the control of the root to shoot ratio is determined by the balance between auxin and strigolactones levels (Ruyter-Spira *et al.*, 2010), providing further evidence suggesting that alterations of these two hormones could lead to the characteristic shift in the root to shoot ratio of *Striga*-infected plants (Watling and Press, 2000; Gworgwor and Webber, 2003; Rank *et al.*, 2004). The knowledge and understanding of how strigolactones are involved in the reduction of tillering in *S. hermonthica* infected plants was limited by (1) the use of only two mutants: a signalling (*d3-1*) and a biosynthetic (*d10-1*) mutant, leaving a gap in the remaining components of the strigolactone metabolic pathway (for details on strigolactone metabolism refer to Chapter 1 section 1.5.2), and (2) the regulation of branching derived from the interaction between strigolactones, auxin and cytokinins. Strigolactones may play a role in conjunction with other plant growth regulators controlling the suppression of tillering in *S. hermonthica* infected plants, as outlined in Chapter 3. However, the specific role of strigolactones is not fully understood. We therefore examined changes in the expression of genes involved in biosynthesis,

regulation and degradation of strigolactones, auxins and cytokinins, particularly in the stem bases of *S. hermonthica*-infected plants to understand the role of these plant growth regulators in the inhibition of *Striga*-infected plants.

4.1.2 Is auxin involved in the infection process of *S. hermonthica*?

As described in the section 1.3 of the general introduction Chapter, *S. hermonthica* is a root hemiparasite that infects host plants through a specialised organ called the haustorium. Auxin is important in haustorial formation and development of parasitic plants. In *Triphysaria versicolor*, haustorial formation is induced by exogenous application of auxin in doses ranging from 0.1 to 1 μM of 2,4-dichlorophenoxyacetic acid, 1-naphthalene acetic acid and indole-3-butyric acid after exposure to the haustorial inducing factor DMBQ (Tomilov *et al.*, 2005). In contrast, reduced infection due to a putative role of auxin in the infection process to their hosts was achieved by inhibiting locally auxin transport at the site of infection of *Orobancha aegyptiaca* to *A. thaliana* by application of naphthalenacetic acid, a specific inhibitor of indole-3-acetic acid (IAA) transport (Bar-Nun *et al.*, 2008). In addition, the use of gene promoter reporter transgenic plants fused to the auxin biosynthesis IAA2 gene showed transient expression and localised accumulation of auxins in the root tips of *T. versicolor* (Tomilov *et al.*, 2005). It has not been determined to date whether *S. hermonthica* produces its own auxin compounds, however, *T. versicolor* and *S. hermonthica* hold a close phylogenetic relationship (Westwood *et al.*, 2010; Wickett *et al.*, 2011) and it has been recently shown that the hemiparasite *Santalum album* shows increased levels of auxin and cytokinins when successfully attached to its host *Kuhnia rosmanifolia* Vent. (Zhang *et al.*, 2012), suggesting that *S. hermonthica* might also produce auxins.

Auxin accumulation at specific sites induces localised growth, as seen in haustorium development, lateral root formation and organ primordia development (Bar-Nun *et al.*, 2008; Peret *et al.*, 2009; Ni *et al.*, 2001; Nakayama *et al.*, 2012). This growth is regulated by cell wall extensibility, in which cell wall loosening is activated by auxins (Rayle and Cleland, 1992; Cosgrove 2005). Auxin-stimulated cells present substantial modifications in xyloglucan, the primary hemicellulosic cell wall polysaccharide in dicotyledons, leading to auxin-induced cell expansion (Catalá *et al.*, 1997).

The accumulation of auxin in specific sites is dependent on auxin biosynthesis, transport, signalling and degradation (Peret *et al.*, 2009; Ubeda-Tomas *et al.*, 2012). Auxin biosynthesis can occur at the site where it is needed or can be transported from the shoot apical meristem and young tissues by the polar auxin efflux carrier PIN1 (Bhalerao *et al.*, 2002; Ljung *et al.*, 2001; Ljung *et al.*, 2005; Delker *et al.*, 2008;

Bainbridge *et al.*, 2008). The transport of auxin generates an auxin gradient known as the polar auxin transport stream (Prusinkiewicz, 2009, Hayward *et al.*, 2009). Lateral root formation starts with the formation of a new meristem within the mother root body, where the PIN auxin efflux carriers are critical to determine the site where auxin concentration will be higher (Szymanowska-Pulka *et al.*, 2012). This is followed by transport of auxin into specific cells by the AUXIN / LIKE AUXIN (AUX1/LAX) genes involved in auxin influx carrier in order to generate an auxin gradient, promoting lateral root emergence (Swarup *et al.*, 2008). Such auxin gradients allow the non-disruptive growth of pericycle cells through the root cortex and the epidermis by cell wall modifications, leading into the formation of lateral roots (Perez *et al.*, 2009; Meng *et al.*, 2010; Sabatini *et al.*, 1999; Gookin *et al.*, 2003). We hypothesised that *S. hermonthica* alters auxin levels during the infection process, either via an induction of auxin biosynthesis or transport from the host, or from *S. hermonthica*-derived auxins, creating an auxin gradient that facilitates penetration, by using the auxin signalling pathway. This hypothesis was tested by evaluating how auxin-related genes were expressed throughout roots, stems and leaves of *S. hermonthica* infected plants.

In summary, the aim of this Chapter is to profile global changes in gene expression in the roots, stems and leaves of rice plants infected with *S. hermonthica* using Affymetrix microarrays with a specific focus on plant growth regulator pathways to allow some of the hypotheses relating to the role of PGRs in the morphological alteration in *Striga*-infected plants to be investigated.

The proposed experiments tested to what extent the expression of auxin-related genes and that of other plant growth regulators is altered in *S. hermonthica* infected plants compared to uninfected controls. This provided the first insight of the role of plant growth regulators in plant parasitism.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Seeds of the cultivar IAC165 were sown in rock wool blocks for 5 days, transferred to rhizotrons and grown in controlled environment chambers for two weeks as described in Chapter 2 section 2.2.1. *Striga* seeds were sterilised and conditioned as described in section 2.2.2. One week after transfer of rice seedlings into rhizotrons, roots were infected with 25 mg of germinated *S. hermonthica* (Kibos ecotype) seeds as described in section 2.2.3.

4.2.2 Harvesting of plant material

Plant material was harvested for analysis of changes in gene expression 6 and 14 DAI. Rhizotrons were removed from the growth chamber one at a time and tissues harvested immediately. The lid of the rhizotron was removed carefully to avoid any mechanical damage to the plants. Plants in rhizotrons were photographed with a Canon EOS 550D SLR camera to record the condition of each individual plant. Heavily infected sections of root were cut using a scalpel and placed in a Petri dish containing sterile distilled water. Roots sections were gently brushed to remove *Striga* seeds and young attachments in order to minimize contamination of host root with *Striga* tissue. The roots were then immediately blotted dry and 100 mg of root placed in a 2 ml eppendorf tube and snap frozen in liquid nitrogen. Tissues from the base of the stem (defined as the region above the root tissue containing the first node and tiller bud area) and from the youngest fully expanded leaf (100 mg samples) were then harvested and placed in 2 ml eppendorf tubes and frozen in liquid nitrogen. Uninfected control plants were treated in an identical manner. It took less than 5 min to harvest tissue from a single plant. Six control and six *Striga* infected plants were harvested at each time point.

4.2.3 RNA extraction and preparation of samples

Total RNA was extracted from the six independent biological replicates from each treatment using the Qiagen RNeasy plant kit (Qiagen, West Sussex, UK), following manufacturer's procedure. A 5 mm stainless steel ball bearing was put into each tube containing the tissue sample with 400 µl of lysis buffer, avoiding thawing of the sample. Leaf, stem and root tissue were disrupted with the Qiagen Tissuelyser MM301 (Qiagen, West Sussex, UK) using a frequency of 20 shakes per second for 2 minutes. The

Qiagen RNeasy plant kit protocol was followed as indicated by the manufacturer. In the last step of the protocol, total RNA was eluted in 50 μl of RNase free water (supplied in kit) and quantified using a microvolume UV-Vis spectrophotometer (Nanodrop ND8000, Thermo Scientific, Surrey, UK). To visualise the integrity of the RNA 5 μl of each sample were loaded into wells of 2% agarose gels and run at 100 V for 60 min. Gels were stained with ethidium bromide, then visualised and recorded under UV light in a Epi Chemi II darkroom gel transilluminator (UVP Laboratory Products, Cambridge UK).

In order to generate three independent biological replicates equal amounts of RNA from 2 individual plants were combined. This strategy can reduce inherent plant to plant variability. The concentration of the each biological replicate was standardised to 100 $\text{ng } \mu\text{l}^{-1}$. Each concentration of RNA in each biological replicate was re-quantified using the Nanodrop ND8000 and RNA integrity was checked by running on a 3% agarose gel. RNA samples (20 μl at a concentration of 100 ng per sample) were sent to the European Arabidopsis Stock Centre in Nottingham, UK (NASC) for hybridization to the commercially available rice genome array (Affymetrix Inc, USA). Preparation of cDNA, cRNA and hybridization to the arrays, were carried out by NASC. Following hybridization of the cRNA to the arrays data files were then sent to Sheffield University. Two samples (replicate 1 –uninfected root tissue – 6 DAI and replicate 2 – *Striga*-infected – 6DAI) were not successfully hybridized to arrays due to a leak in the seal on the arrays. Hybridization of these samples was repeated at a later time.

4.2.4 Analysis of microarray data

Data analyses were carried out using the open source R-based program Robin (Lhose *et al.*, 2010). Background-corrected expression data from each of the CEL files was imported into Robin. The first step of the analysis consisted of performing quality control checks in order to make sure that RNA had not degraded and that cRNA had hybridized properly to all the arrays. Box plots of unnormalized expression values on each chip provide an overview of the distribution of signal intensities. If hybridization has been successful all chips should have a similar distribution of signal intensities. Fig 4.1 A shows the distribution of signal intensities of control and infected samples from roots stems and leaves. These were very similar indicating that the chips were of a high quality.

Once we determined proper quality of the microarray chips we proceeded to normalisation of the data (Fig. 4.1). Normalisation was carried out by Robust Multi-array Analysis (RMA) (Irizarry *et al.*, 2003). RMA does background correction under the assumption that the background signal has normal distribution, whereas the real probe

signal is exponentially distributed. The background-corrected data is quantile normalised, assuming nearly identical gene abundance across the microarray chips. A reference distribution is generated by the pooled probe intensity of replicates in the same treatment. Finally, to normalise each microarray chip, the original value of each probe is transformed to its average value across all microarray chips. Fig 4.1 B shows normalized unscaled standard error (NUSE) plots. These plots visualize the distribution of standard errors for each individual chip. Chips showing an increased standard error are of lower quality. Most samples have very low standard error distributions with the exception of replicate 1 –uninfected root tissue – 6 DAI (Fig 4.1 B).

Following normalisation of the data, Pearson correlation hierarchical clustering was performed to make sure that the microarray chips clustered according to the treatment applied (Fig 4.1 C). For stem and leaf tissues the replicates within each treatment and time point clustered together. However, root samples showed an abnormal clustering. Replicate 1 –uninfected root tissue – 6 DAI (which showed a higher standard error distribution) did not cluster with the other 2 control samples and was one of the samples that had to be re-hybridized to an array. This sample was eliminated from the data analysis. The second sample that was re-hybridized to an array (replicate 2 – *Striga*-infected – 6DAI) also showed an abnormal clustering (although the standard error distribution was low). This sample was also from the data analysis. Thus only two replicates were used for analysis of changes in gene expression in the roots at 6 DAI.

Fig 4.2 shows scatter plots of normalized expression values of different replicates plotted against each other (control versus control, infected versus infected and control versus infected) for roots, stems and leaves. Scatter plots allow us to assess whether two replicates show similar behaviour, *i.e.* genes show similar levels of expression (\log_2 expression) on each replicate chip. If they do values should fall on a perfect diagonal line. Fig 4.2 A shows scatter plots of \log_2 expression values of control replicates plotted against each and Fig 4.2 B infected replicates plotted against each other. It can be seen that the expression values fall on a diagonal lines indicating good biological replication. Fig 4.2 C shows scatter plots of expression values of control versus infected replicates. If there are differences in gene expression between control and infected samples the expression values should be scattered above and below the diagonal line. This can clearly be seen for the control and *Striga*-infected samples in Fig 4.2. Those values that fall above the diagonal line indicate upregulated gene expression and those below the line, down regulation of gene expression.

To detect differentially expressed genes, the linear model analysis $E [y_j] = X\alpha_j$ was used, where y_j contains the expression data of each gene, X is the design matrix describing the systematic part of the data and α_j represents the response level for gene j on chip g . (Smyth G., 2004). The comparison consisted of contrasting the expression levels of “*S. hermonthica*-infected” minus “uninfected” treatments. This design showed positive and negative values for up and down-regulated genes in *S. hermonthica* infected compared to uninfected plants, respectively. Only those sequences that were statistically different and presented a fold change greater than 2 were considered. False discovery rate (FDR) (Benjamini and Hochberg, 1995; Benjamini and Hochberg, 2000) was performed to eliminate false positive error signals with an expected p-value < 0.05 cut-off.

Differentially regulated sequences were annotated automatically to the most recent rice genome annotation file available at (http://www.affymetrix.com/products_services/arrays/specific/rice.affx/). Validation of the correct sequence annotation for genes of interest was carried out by comparing the AffyID against the Gramene database (<http://www.gramene.org/>). Repetitive gene annotations were eliminated. Differentially expressed genes were categorised by biological function using the gene bins classification of Goffard *et al.*, (2007). This allowed comparison of changes in gene expression in the different tissues (roots, stems and leaves) of *Striga*-infected and uninfected plants at different times after infection. Lists of differentially expressed genes were imported into Mapman (Thimm *et al.*, 2004) to allowed visualization of changes in gene expression in different metabolic pathways. Multi Experiment Viewer (MeV) version 4.9 was used to generate heat maps and clusters of genes in specific metabolic pathways (Saeed *et al.*, 2003). The log₂ values of the fold change were used for clustering by the Euclidean distance method, as suggested for log₂ data clustering (D'haeseleer, 2005).

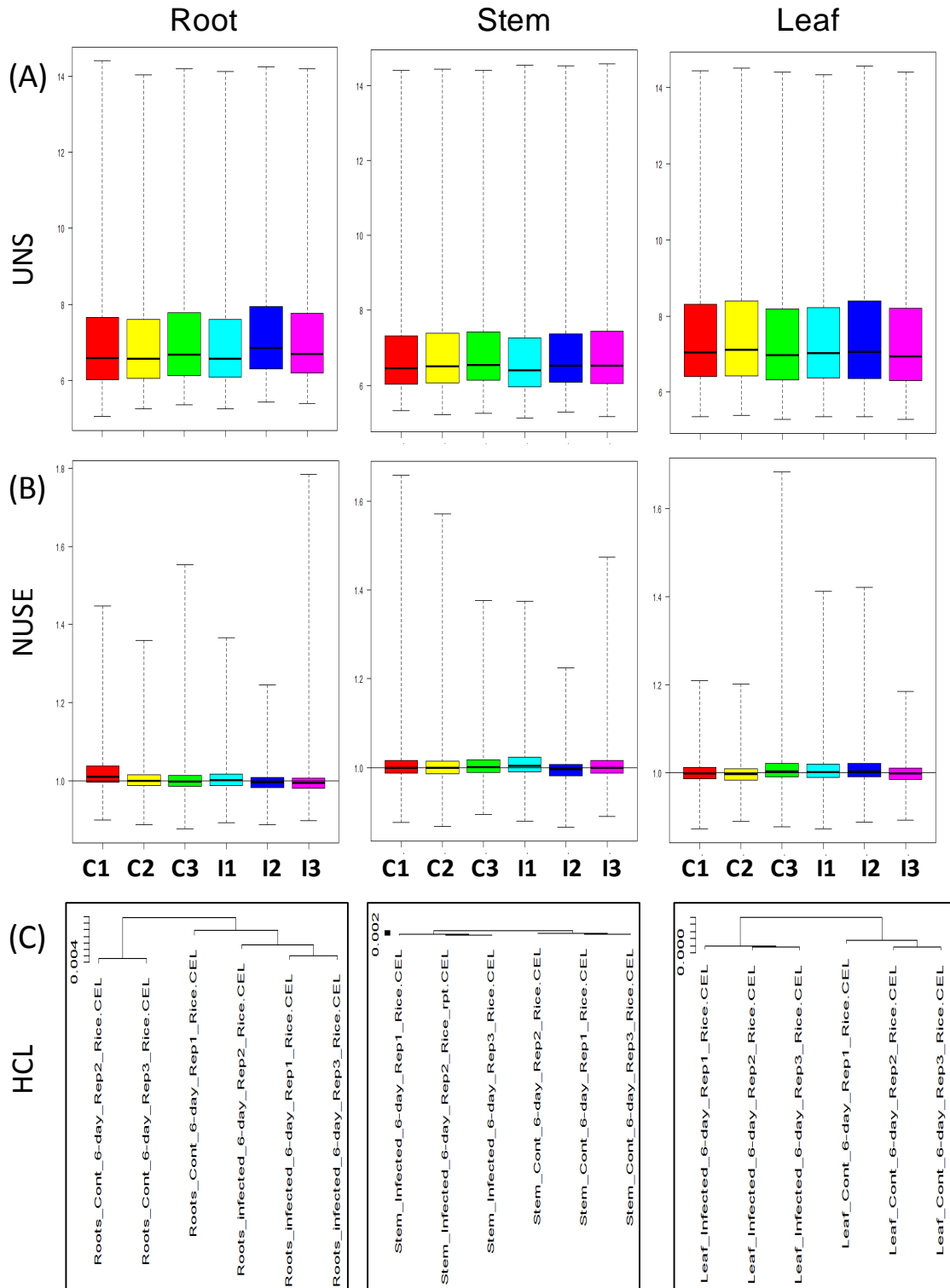


Figure 4.1 Quality checks for Affymetrix microarray chips of uninfected and *S. hermonthica*-infected roots, stems and leaves 6 DAI. (A) Representative box plots of unnormalized (UNS) global signal intensity and (B) normalized un-scaled standard error (NUSE) intensities of root, stem and leaf microarray chips for uninfected (red, yellow and green) and *S. hermonthica* infected (light and dark blue and purple) samples. (C) Hierarchical clustering of samples.

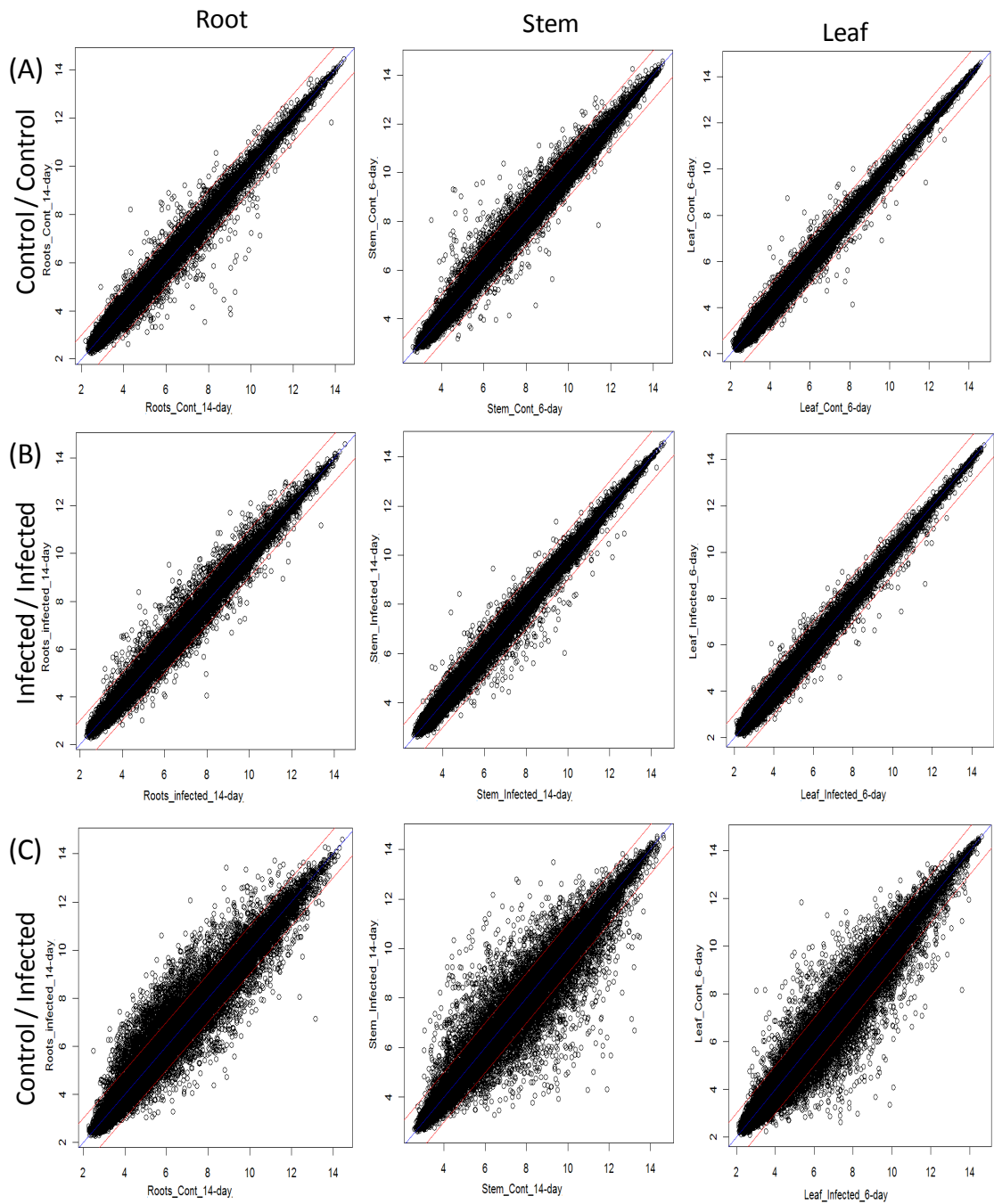


Fig. 4.2 Representative scatter plots of normalized expression values of combinations of two microarray chips for root, stem and leaf tissue. The red line indicates a log₂-fold difference of 1.

4.2.5 Validation of differential expression of genes of interest

In order to validate the data obtained from the microarray analysis, tissue was harvested for qPCR analysis in an independent experiment using the susceptible rice cultivar IAC165 and *S. hermonthica* from Kibos, Kenya collected in 2009.

Growth conditions of rice, conditioning of *S. hermonthica* seeds, and the infection process were performed as described in 4.2.1. Harvesting of roots, stems and leaves was performed as described in section 4.2.3. Tissue was collected from 4 independent plants per treatment (control and *Striga*-infected plants replicates per treatment. RNA extraction, quantification and quality controls were carried out as described in section 4.2.3.

Total RNA was extracted as using the Qiagen RNeasy plant kit (Qiagen, West Sussex, UK), quantified and quality checked as described in section 4.2.3. RNA from each sample was treated with Turbo DNase (Ambion, Paisley, UK) following manufacturer's procedure. The reaction consisted of 1 μl of Turbo DNase (2 units μl^{-1}), 2 μl of 10x Turbo DNase buffer and 13 μl of total RNA per sample up to a volume of 20 μl . The reaction was incubated at 37 °C for 30 min. Then, 2 μl of DNase inactivation reagent were added to the reaction, mixing well by flicking the tube. Finally, the reaction was incubated for 5 min at room temperature, flicking 2 to 3 times. The tubes were centrifuged at 10,000 rpm for 1 minute and the RNA was transferred to a fresh tube.

To synthesise cDNA, SuperScript III reverse transcriptase (Invitrogen, Paisley, UK) was used following manufacturer's procedure. Five μg of total RNA were used for cDNA synthesis for the stem samples, whereas 3.5 μg were used for the root samples. The first step of the 20 μl reaction indicated adding 1 μl of oligo(dT) (50 μM), the RNA template and 1 μl of 10 uM dNTP mix up to 13 μl . The mixture was incubated at 65 °C for 5, and then incubated in ice for 1 min and briefly centrifuged. After centrifugation 4 μl of 5x first-strand buffer, 1 μl of 0.1 M DDT, 1 μl RNaseOUT recombinant RNase inhibitor (40 units μl^{-1}) and 1 μl of Superscript III rt (200 units μl^{-1}) was added. The contents were mixed by pipetting and incubated at 50 °C for 60 minutes. The reaction was inactivated by heating the samples at 70 °C for 15 min. The whole procedure was performed in a Techne TC-5000 thermocycler for optimal temperature control.

Oligonucleotides for 23 genes involved in hormonal processes (Table 4.1) were designed and analysed for hairpin formation, self and hetero dimerization with online tools available at <http://www.idtdna.com>. Additionally, oligonucleotides encoding genes

of interest from relevant publications were also used (Swarbrick *et al.*, 2008; Kant *et al.*, 2009) (Table 4.1). Oligonucleotides were used at a working concentration of 10 μM . Oligonucleotide specificity was tested against cDNA synthesised from total RNA extracted from the stem of control and *S. hermonthica* infected plants by 6 DAI and genomic DNA extracted from the stem of control plants by 21 DAI in a semiquantitative polymerase chain reaction (PCR). Reaction conditions were 95 °C for 2 min, followed by 30 cycles at 95 °C, 58 °C and 72 °C for 30, 30 and 20 seconds, respectively and finally 72°C for 2 minutes. Oligonucleotides that amplified products with a single band of the correct size were used to in quantitative real time PCR (qPCR). To determine the optimal concentration of cDNA to use per qPCR reaction, we tested five dilutions, 1, 1:5, 1:10, 1:50 and 1:100.

Three step qPCR with melting curve was performed in a Corbett Life Science Rotorgene Thermocycler RG6000 (Qiagen, West Sussex, UK). A volume of 10 μl was used per reaction. Each reaction consisted of 5 μl of SYBRgreen mix, 2 μl distilled water, 1 μl for each primer (forward and reverse) and 1 μl of the template in a 1:10 dilution. Actin and presenilin were used as reference genes for stem and root tissue, respectively as these were shown to have similar levels of gene expression in control and infected root tissues (Swarbrick *et al.*, 2008). Reference genes were run alongside each qPCR reaction to ensure proper quantification and standardisation by cDNA input of the gene of interest. Reaction conditions were 95 °C for 10 min, followed by 40 cycles at 95 °C, 60 °C and 72 °C for 10, 15 and 20 seconds, respectively and finally melting of the products from 72 to 95 °C for 1 minute.

The comparative quantification method was used to evaluate differences in the transcript levels of the genes of interest. This method is based on the PCR efficiencies and the mean crossing point deviation between treatments (Pfaffl *et al.*, 2002). T-tests Statistical analyses comparing control and *S. hermonthica* infected root and stem tissue were performed with the package R version 2.12.1 at both time points.

4.3 RESULTS

4.3.1 Analysis of global changes in gene expression in root, stem and leaf tissues of the susceptible rice cultivar IAC165 following infection by *S. hermonthica*

The Affymetrix rice microarray chip contains 51,279 transcripts representing approximately 48,564 transcripts from the *japonica* and 1,260 for the *indica* cultivars. The total number of differentially regulated sequences was 6250 for the whole plant, *i.e.* 12.18% of the total number of probes on the array (Figure 4.3 A). Some genes were only differentially regulated in one of the tissues whilst others, some were differentially regulated in two tissue types and 130 genes were differentially regulated in roots, stems and leaves of infected compared to control tissues (Figure 4.3). The highest number of differentially regulated genes were found in the leaves (3197) followed by roots (2124) and stems (2083). Of the 3197 genes differentially regulated in the leaves of *Striga*-infected plants (compared to uninfected plants) 1771 and 1108 genes were unique to 6 and 14 DAI respectively with another 318 genes that were common to both time points (Fig 4.3 B). In the stem tissue 951 and 1002 genes were unique to 6 and 14 DAI respectively with another 130 genes that were common to both time points (Fig 4.3 B) and in the roots, 616 and 1297 genes were unique to 6 and 14 DAI respectively with 211 genes that were common to both time points (Fig 4.3 B)

Fig 4.4 shows the number of genes that were significantly up or down regulated by more than 2 fold ($p < 0.05$ BH FDR) in the roots, stems and leaves of infected compared to control plants at 6 and 14 DAI. At 6DAI approximately half the genes in roots and leaves were upregulated and half were down regulated whilst there was a greater number of genes down regulated in the stem tissue at this time point. By 14 DAI there was a significantly greater number of up regulated genes in the roots of infected compared to control plants compared to 6DAI (Fig 4.4) and over both time points there were a times as many up compared to down regulated genes. In leaves where there were a greater number of up regulated genes at 6 compared to 14 DAI. In stem tissue more genes were down compared to up regulated at 6 DAI but this situation was reversed at 14 DAI (Fig 4.4).

4.3.2 Functional classification of differentially expressed genes in *S. hermonthica* infected plants

Figure 4.5 shows the proportion of up and down regulated genes in the roots, stems and leaves of *Striga*-infected compared to control plants at 6 and 14 DAI, classified by biological functional (according to the gene bin classification proposed by Goffard *et al.*

2007). The greatest proportion of differentially regulated genes in the roots, stems and leaves (between 30 and 40%) are not yet annotated. Approximately 10 % of the total number of up and down regulated genes in roots, stem and leaves were classified as being involved in carbohydrate metabolism and about the same number (again in all tissue types) were involved in signal transduction (Fig 4.5). Other differentially regulated genes were classified as being involved in energy, lipid and amino acid metabolism, biosynthesis of secondary metabolites, cell growth and death and biodegradation of xenobiotics (Fig. 4.5).

4.3.3 Alterations in the expression of genes associated with different plant growth regulator pathways in *S. hermonthica* infected compared to uninfected plants.

Differentially expressed genes involved in the auxin, gibberellin, ethylene and cytokinin pathways were classified using Mapman. The inconvenience of depending upon automated annotation is that the information obtained might be incomplete or imprecise; therefore the genes of interest were double checked manually using the Gramene database. Out of the large amount of “unknown” sequences, there might be some that passed undetected under this analysis. As our knowledge of full metabolic pathways and the association to their gene accession numbers increases, as well as discovery and characterisation of new genes moves forward, this analysis should be repeated in order to reduce the number of “unknown” sequences that resulted significantly differentially regulated. The expression of genes associated with strigolactone biosynthesis or signalling were manually extracted from the microarray data since this hormonal pathway was not available in Mapman (as of March, 2012). Following classification, the percentage of significantly differentially regulated genes associated with each group of plant growth regulators is shown in Fig 4.6. Over half of the differentially regulated genes were associated with auxin biosynthesis, metabolism or signalling followed by ethylene and cytokinins (18 and 17% respectively) and then gibberellins and strigolactones (8 and 5% respectively) Fig 4.6.

4.3.3.1 What are the biological functions of differentially regulated auxin – related genes?

Auxin metabolism was greatly altered in the roots, stems and leaves of *S. hermonthica*-infected compared to uninfected plants (Fig 4.7). In general there was an upregulation of genes associated with auxin biosynthesis, degradation, transport (including efflux carriers and hydrogen symporters) and response/signalling in all tissues.

Primary auxin response genes include members of the IAA, GH3 and auxin up RNA (SAUR) multigene families. IAA genes encode AUX/IAA proteins and GH3 genes encode indole-3-acetic acid-amido synthetases that convert active auxin (IAA) to an inactive form. These gene families are auxin inducible and are involved in negative feedback of the auxin response (Tomas and Perrot-Rechenmann 2010). The function of the many different SAUR genes is still to be discovered. Transport of auxin into and out of cells is controlled by influx (e.g. AUX and LAX) and efflux (e.g. PIN and ABC transporters).

In roots of rice plants infected by *S. hermonthica* a number of different GH3, AUX/IAA and SAUR gene family members and auxin efflux carrier components were up regulated at 6 and even more strongly at 14 DAI (Fig 4.7 A). In the stems of *Striga*-infected plants an even greater number of genes of the GH3, AUX/IAA and SAUR family were up regulated together with auxin symporters and auxin efflux proteins. These genes were highly upregulated by 6 DAI. The expression of some of these genes had begun to fall by 14 DAI whilst others, particularly the SAUR genes maintained high levels of expression (Fig 4.7 B). Again many of the same gene family members were up regulated in the leaves of *Striga*-infected plants (Fig 4.7 C). One of the most highly upregulated genes in the roots, stems and leaves of *Striga*-infected plants was SAUR39 (Fig 4.7).

Interestingly, the ubiquitin ligases SINAT4 and SINAT5 were up regulated in roots and leaves. These ubiquitin ligases are involved in the degradation of Aux/IAA repressor proteins, which in turn inhibit the biosynthesis of auxin via targeting of the NAC1 transcription factor (Xie *et al.*, 2002). Even though NAC1 was not among the differentially regulated genes in *S. hermonthica* infected plants, NAC5 presented down regulation in the root tissue by 6 DAI, consistent with the up regulation of SINAT5 (Fig 4.7). A phylogenetic analysis study showed the similarities between NAC1 and NAC5 (Ooka *et al.*, 2003), indicating their biological function might be rather similar.

The phenotype of *S. hermonthica* infected plants consists of general stunting of the host plant, defined by reduction of the internode length, as well as thinning of the stems and all the phenotypical characteristics described in Chapter 2. Differential regulation of genes in either the leaves or roots would suggest mobility of compounds all along the plant, either basipetally, such as it is the case with auxins, or acropetally, as it happens with strigolactones, as shown by the influence of strigolactones produced in the roots affecting plant architecture (Kohlen *et al.*, 2011).

4.3.3.2 How similar are changes in gene expression in *S. hermonthica* infected rice plants and SAUR39 overexpressing mutants?

As outlined in Chapter 1 the phenotype of rice plants overexpressing SAUR39 (Kant *et al.*, 2009) was remarkably similar to rice plants infected with *S. hermonthica*. Both sets of plants are severely stunted with lower shoot and root growth and reduced tillering, and yield (this thesis; Kant *et al.*, 2009). Therefore a comparison of the changes in expression of genes in the leaves of both sets of plants was carried out by comparing the expression patterns of the genes highlighted in Kant (2009).

Fig 4.8 and 4.9 show a comparison of up and down regulated genes (respectively) in *Striga* infected (14 DAI) and SAUR39 overexpressing plants. There was a remarkable similarity in the up regulation of specific genes involved in auxin response, senescence, phenyl propanoid metabolism, sugar synthesis and signalling, calmodulin binding and calcium transport, and stress related genes (Fig 4.8). Twenty one of the genes were significantly up regulated in both treatments. These were two GH3 auxin response genes (GH3 genes), 1 ABA responsive gene, 5, 2 genes encoding proteins involved in synthesis of trehalose, and 3 sugar transporters, 3 in calmodulin and calcium binding and transport processes, 2 in anthocyanin biosynthesis (4-coumarate-CoA ligase 1 and phenylalanine ammonia-lyase), and 8 involved in biotic and abiotic stress responses (Fig 4.8).

There was also similarity between the expression patterns of down-regulated genes in both sets of plants although the correlation was not as good as for the up regulated genes (Fig 4.9). Nevertheless it was clear that genes encoding photosynthetic components (e.g. chlorophyll a/b binding proteins), trehalose signalling (TPP) and ethylene signalling (EIN3) were down regulated in both sets of plants (Fig 4.9). Out of 125 genes involved in auxin processes, stress response, photosynthesis, carbohydrate metabolism and development analysed by Kant *et al.* (2009), 89 of them were significantly up regulated and 36 down regulated in leaves of plants infected with *S. hermonthica* (Figs 4.8 and 4.9).

4.3.3.3 How is the metabolism of ethylene altered in *S. hermonthica* infected plants?

A number of genes involved in ethylene biosynthesis and signalling were differentially regulated in rice plants infected with *S. hermonthica* compared to uninfected plants (Fig 4.10). In contrast to the expression patterns of auxin-related genes, ethylene-related genes were largely down-regulated in stem and root tissue. In the leaves larger number of genes were upregulated (Figure 4.10).

Key enzymes in the ethylene biosynthetic pathway were differentially regulated in *S. hermonthica* infected plants. 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase) gene was upregulated in stem and leaf tissue, whereas the 1-aminocyclopropane-1-carboxylate synthase (ACC synthase) was down regulated in stems. Ethylene responsive elements and the ethylene insensitive3-like protein were also down regulation in stem and leaf tissue (Fig. 4.10 B and C). Most changes observed in the leaf tissue occurred at 6 DAI. By 14 DAI significant down regulated genes in the ethylene pathway were the ethylene insensitive3 like protein and a transcription factor of the ethylene responsive factor group (Fig. 4.10 C). No differential regulation was detected in roots at either time point for ACC synthase, ACC oxidase or ethylene receptors (Fig. 4.10 A).

4.3.3.4 Is the expression of cytokinin-related genes altered in rice plants infected with *S. hermonthica*?

Cytokinin-related genes were mostly down-regulated in roots, stems and leaves of *Striga*-infected rice plants (Fig 4.11). In leaf tissue down regulation was most intense at 6 DAI (Fig 4.11 C) whereas in the roots and stems there was a progressive down-regulation of gene expression with time (Fig 4.11 A and B). Only two cytokinin biosynthesis related genes were differentially expressed: one cytokinin synthase gene was transiently upregulated at 6 DAI and then down regulated at 14 DAI in root tissue and cytokinin isopentenyltransferase 1 biosynthetic gene (AtIPT1, loc_os05g47840) (involved in cambial differentiation in leaves) was downregulated in leaves at 14 DAI (Fig. 4.11 A and C). Down regulation of cytokinin dehydrogenase precursors was seen in root, stem and leaf tissue (Fig. 4.11). Type-A response regulators (RR) genes were mostly down regulated in leaves and stems, with the exception of OSRR1, which was up regulated in leaf tissue.

4.3.3.5 How is the expression of strigolactone-related genes altered in rice plants infected with *S. hermonthica*?

The changes that were detectable in the microarray data included increased expression of CCD8-like, an homologue of CCD8 in the leaf tissue by 14 DAI, whereas in the root tissue no changes were detectable. The lack of changes in the expression of strigolactone related genes is in accordance with the results obtained from the strigolactone signalling and biosynthetic mutants d3 and d10, which suggested that an increase in strigolactone content is not the cause for reduced tillering in *S. hermonthica*-infected plants. Relating the expression of CCD8 and other strigolactone

genes to the experiments presented in Chapter 3, there seems to be discrepancy in the data with little evidence to support the putative role of strigolactones in a) the reduction of tillering in *S. hermonthica*-infected plants at early stages of infection and b) the infection process itself.

Figure 4.12 A shows the expression profile of strigolactone-related genes in the roots, stems and leaves of *Striga*-infected plants. There was no significant differential regulation of any of the genes involved in strigolactone biosynthesis, perception or signalling in roots infected with *S. hermonthica* compared to uninfected roots (Fig 4.12 A). However there was an upregulation of strigolactone-related genes within stem and leaves. There was a strong up regulation of one of the two transcripts encoding carotenoid cleavage dioxygenase (CCD8) (designated D10-like) in the stems and leaves of plants infected with *S. hermonthica* consistent with the increase in biosynthesis of this gene reported in Chapter 2 (using CCD8:GUS promoter-reporter plants). There was no upregulation of CCD7 in any tissues (Fig 4.12 A). The gene encoding D3, the F-box protein was again strongly up regulated in the stems and leaves of plants infected with *S. hermonthica* (Fig. 4.12 A). Interestingly, the gene encoding the hydrolase, alpha-beta fold domain containing protein (D14), putatively a component of hormone signalling or an enzyme that converts strigolactone to its bioactive form (Arite *et al.*, 2009), was up regulated in stem tissue at 6 and 14 DAI and in the leaf tissue at 6 DAI (Fig. 4.12 A).

4.3.3.6 Is the expression of gibberellin-related genes altered in rice plants infected with *S. hermonthica*?

Figure 4.12 B shows the expression profiles of gibberellins-related genes in roots, stems and leaves of *Striga*-infected plants. Very few genes involved in gibberellin metabolism signalling were differentially regulated in plants infected with *Striga* at 6 and 14 DAI. Genes encoding Ent-kaurene oxidase (KO) and synthase (KS) were down regulated in root tissue at 6 DAI. KO was also down regulated in roots at 14 DAI, (Fig 4.12B). This gene was also up regulated at 14 DAI in stem tissue (Fig 4.12 B). Two different gibberellin 20 oxidases (GA20ox) were differentially regulated in the roots; GA20ox1 was down regulated, whereas GA20ox2 was up regulated 6 DAI. By 14 DAI, 2 gibberellin stimulated transcript precursors (GAST1 and GAST2) were up regulated in the roots and GAST 2 was upregulated in stem tissue at 14 DAI (Fig. 4.12 B).

4.3.4. Modifications in the cell walls of *S. hermonthica* infected plants.

Many genes involved in cell wall modification were up regulated in the roots of *S.* infected plants at 6 and 14 DAI with expression increasing with time after infection (Fig 4.13). Genes encoding cellulose synthases, alpha and beta expansins, pectin esterases, xyloglucan-related genes and polygalacturonase precursors were up highly up regulated in root tissue by 14 DAI (Fig 4.13A). The cell adhesion molecule fasciclin arabinogalactan precursor was up regulated in roots by 6 DAI.

The leaves of *S. hermonthica* infected plants showed a similar pattern in the expression levels of expansin precursors, fasciclin-like proteins and polygalacturonases as observed in roots (Fig. 4.13 C). In the stems a greater number of genes encoding components of the cell walls were severely down regulated including xyloglucan and cellulose synthase-related genes. Xyloglucan and cellulose make up about two thirds of the dry weight of primary cell walls (Eckardt, 2004). The stems of *S. hermonthica* infected plants were brittle compared to uninfected plants, supporting the transcriptional modifications observed in the down regulation of xyloglucan and cellulose metabolism. Further measurements of stem flexibility were not performed; however, they would provide useful information on the degree to which *S. hermonthica* alters the cell wall properties at a macroscopic level.

4.3.5. Changes in genes encoding biotic stress response proteins in plants infected with *S. hermonthica*

The expression pattern of biotic stress-related genes in *Striga* infected plants is shown in Fig 4.14. A number of genes encoding pathogenesis-related proteins (including basic and acidic endochitinases, thaumatin-like precursors), dirigent protein pDIR17, disease resistance response proteins (rust resistance-like protein) and xylanase inhibitor proteins were up regulated at 6 and more strongly at 14 DAI (Fig. 4.14 A). Some genes associated with biotic stress responses were significantly down regulated in roots infected with *S. hermonthica* compared to controls. These included genes encoding verticillium wilt resistance protein and a number of WRKY transcription factors including OsWRKY21, OsWRKY11 and OsWRKY28. These genes were significantly down regulated at 14 DAI (Fig. 4.14 A). OsWRKY28 is a negative regulator of basal resistance to the fungus *Magnaporthe oryzae* (Delteil *et al.*, 2012), while OsWRKY11 is positively correlated to tolerance to drought and heat stress (Wu *et al.*, 2009). The biological function of OsWRKY21 is unknown at present.

It is interesting to note that many pathogenesis-related genes, homologues of disease resistance proteins including MLO, dirigent-like proteins and xylanase and

polygalacturonase inhibitor proteins were also up regulated in the leaves of plants infected with *Striga* suggesting a systemic response to infection of the roots. It is important to highlight the up regulation of polygalacturonase inhibitor precursor proteins (PGIP) in root tissue as they are part of the plant response to fungal pathogens that degrade cell walls via cellulases, xylanases and polygalacturonases (Albersheim and Anderson, 1971; Prabhu *et al.*, 2012). OsWRKY30 was up also regulated by 6 DAI in leaves. This gene is responsive to salicylic and jasmoic acid treatment in rice (Ryu *et al.*, 2006). In the leaf tissue, some genes were also down regulated including OsWRKY7, OsWRKY28, OsWRKY24 and OsWRKY71 at both 6 and 14 DAI. These transcription factors have been described in responses to pathogen attack (Ryu *et al.*, 2006; Delteil *et al.*, 2012) (Fig 4.13 B).

4.3.6. Changes in genes encoding abiotic stress response proteins in plants infected with *S. hermonthica*

Differential regulation of abiotic stress related genes was detected in roots and leaves of *S. hermonthica* infected plants. The drought responsive protein DREPP2 and the dehydration stress protein RXW8 were up regulated significantly in roots by 14 DAI (Fig. 4.15 A). In contrast, leaf tissue showed down regulation of RXW8, but significant up regulation of the early responsive dehydration proteins ERD3 and ERD4 (Fig. 4.15 B). Transcripts encoding heat shock proteins (HSP), DNA chaperones and binding protein genes were differentially regulated in root and leaf tissues at 6 and 14 DAI (Fig. 4.15). A heat shock binding protein and the HSP cognate 70 kDa protein were down regulated in roots by 6 DAI but up regulated in leaf tissue at 6 and 14 DAI (Fig. 4.15).

Wound response transcripts were significantly down regulated in root tissue at 6 DAI, while they were up regulated in leaves at 6 and 14 DAI. Similarly, the osmotic stress response ankyrin protein kinase-like showed the opposite behaviour between roots and leaves with the same pattern as wound responsive genes (Fig. 4.15).

4.3.7. Changes in genes encoding development-related proteins in plants infected with *S. hermonthica*

Changes in the expression of genes encoding proteins involved in senescence, ripening, cell longevity and enlargement, nodulation factors, embryogenesis and flowering were detected in roots, stems and leaves at 6 and 14 DAI (Fig. 4.16). In the root tissue, senescence, nodulin factors, a protein induced by tuberization and two flowering T locus genes were up regulated by 6 DAI. By 14 DAI a legumin-like protein,

and a ripening-related protein precursor were up regulated, (Fig. 4.16 A). Down regulation of the hair root formation related protein SEY1, dyacylglycerol (DAG) protein, a chloroplast precursor and the flowering related GIGANTEA could be observed in roots by 6 DAI (Fig. 4.16 A).

The patterns of gene expression showed consistency between stem and leaf tissue at 6 and 14 DAI (Fig. 4.16 B and C). Phytosulfokine precursors, proteins involved in cell longevity and growth, were up regulated in leaves and stems. The flowering gene GIGANTEA was down regulated in stem and leaves at 14 DAI. In stem tissue, nodulin, senescence and ripening genes were up regulated at 6 and 14 DAI. Genes were mostly up regulated in leaf tissue, with the exception of a mediator or ABA (MARD1), embryogenesis and cytochrome C proteins at 6 DAI and DAG, and two flowering T locus genes at 14 DAI (Fig. 4.16 C). In the leaf tissue the transcript for the protein caleosin was up regulated at 6 and 14 DAI with log₂fc values of 1.6 and 3.0, respectively (Fig. 4.16 C). This gene is responsible for stomatal control, transpiration and drought tolerance (Aubert *et al.*, 2010).

4.3.8 Validation of microarray data by qPCR analysis of selected genes

The expression levels of some of the genes that showed differential regulation in the microarray study were evaluated by real time quantitative PCR (qPCR). The genes analysed included SAUR39, AUX1, IAA2 and PIN6 (Fig. 4.16). The expression profiles of these genes were very similar when assessed by microarray transcript profiling and qPCR (Fig 4.17). SAUR39 was again highly upregulated in the stems of *Striga*-infected plants following infection. Differences in the expression of this gene in infected and control tissue was highly significant from 6 DAI ($t = 3.2917$, $df = 3.975$, $p\text{-value} = 0.03$). By 14 DAI, the auxin efflux carrier PIN6 was significantly down regulated in the roots of *S. hermonthica*-infected plants to uninfected plants. In stems PIN6, showed no significant differences between treatments at individual time points.

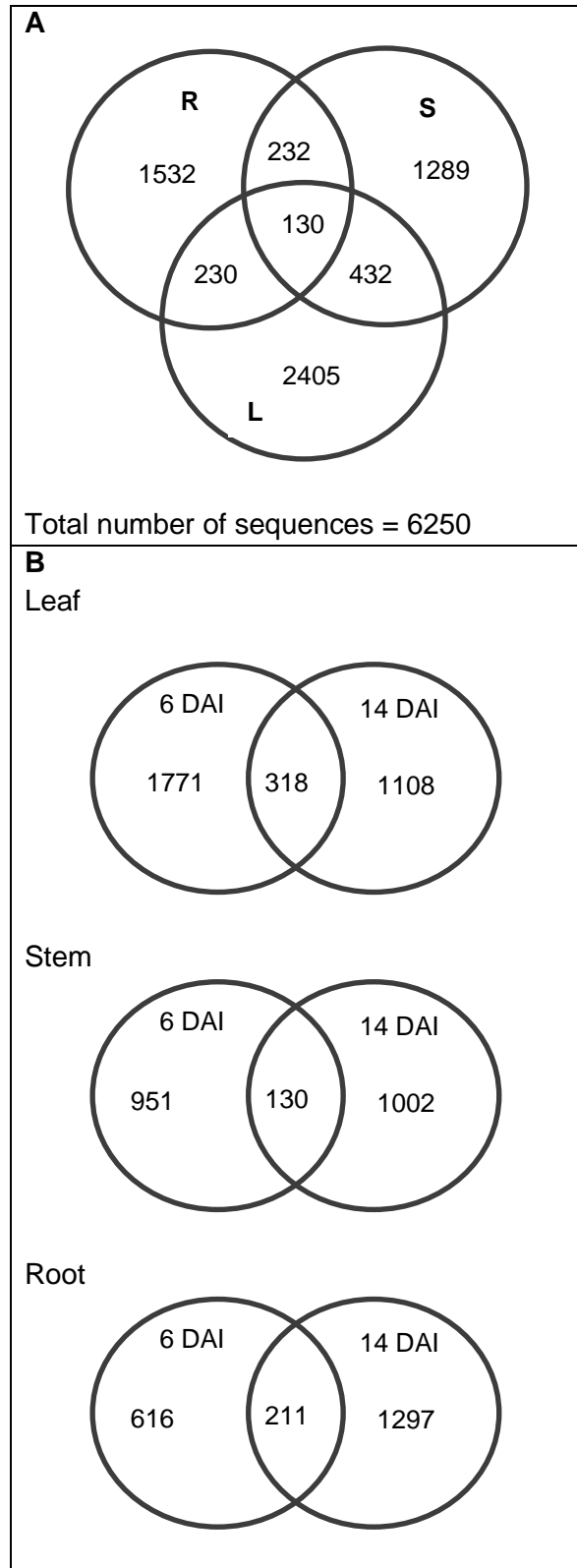


Figure 4.3 (A) Total number of genes that were differentially regulated in the leaves stems and roots of *Striga*-infected compared to uninfected rice plants (IAC 165) and (B) the number of genes differentially regulated in the leaves stems and roots of *Striga*-infected compared to uninfected plants at 6 and 14 days after inoculation. All genes were significantly differentially regulated by more than 2 fold in infected compared to control plants ($p < 0.05$ BH FDR).

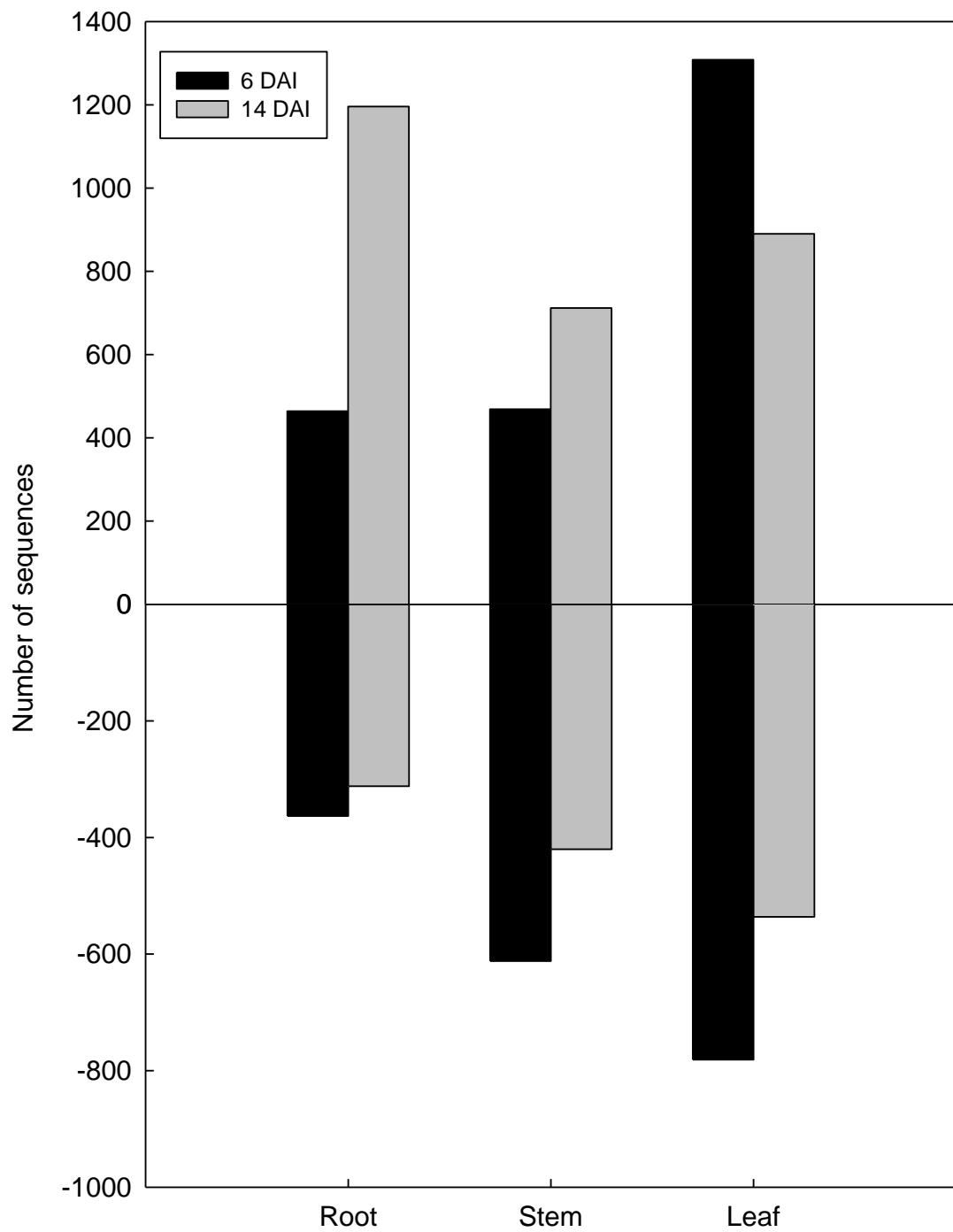


Figure 4.4. The number of genes that were up or down regulated in the roots, stems and leaves of *Striga*-infected, compared to uninfected rice plants (IAC 165) at 6 and 14 DAI. All genes were significantly differentially regulated by more than 2 fold in infected compared to control plants ($p < 0.05$ BH FDR).

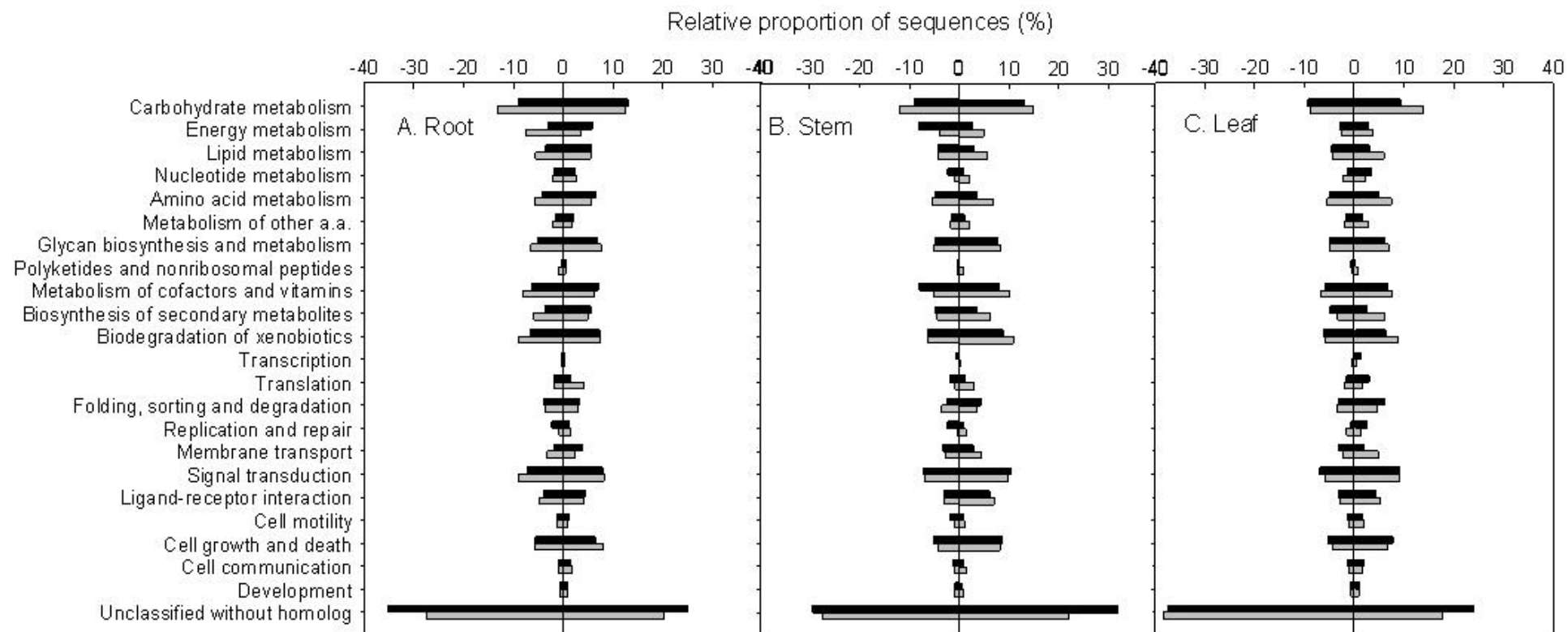


Figure 4.5. Relative proportion of up and down regulated sequences sorted by biological function in A) root, B) stem and C) leaves of the rice cultivar IAC165 following inoculation with *S. hermonthica* at 6 (black bars) and 14 (grey bars) DAI. All genes were significantly up or down regulated ($p < 0.05$, BH FDR) ≥ 2 fold change in *S. hermonthica* – infected tissue compared to control plants.

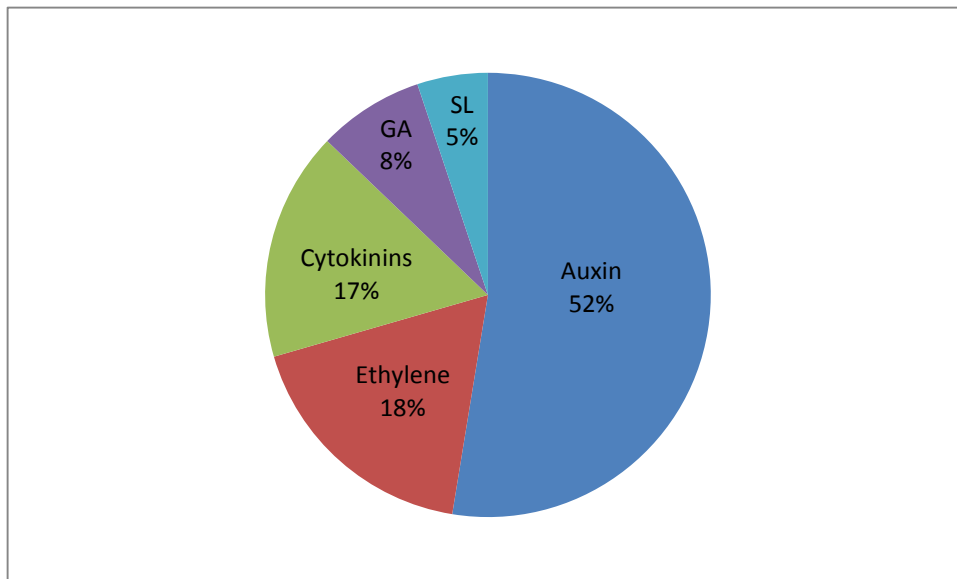


Figure 4.6 The percentage of significantly differentially regulated genes associated with different plant growth regulator pathways in *S. hermonthica*-infected rice plants of the cultivar IAC165 compared to uninfected plants. GA = gibberellins, as indicated by the output of Mapman; SL = strigolactones, defined manually.

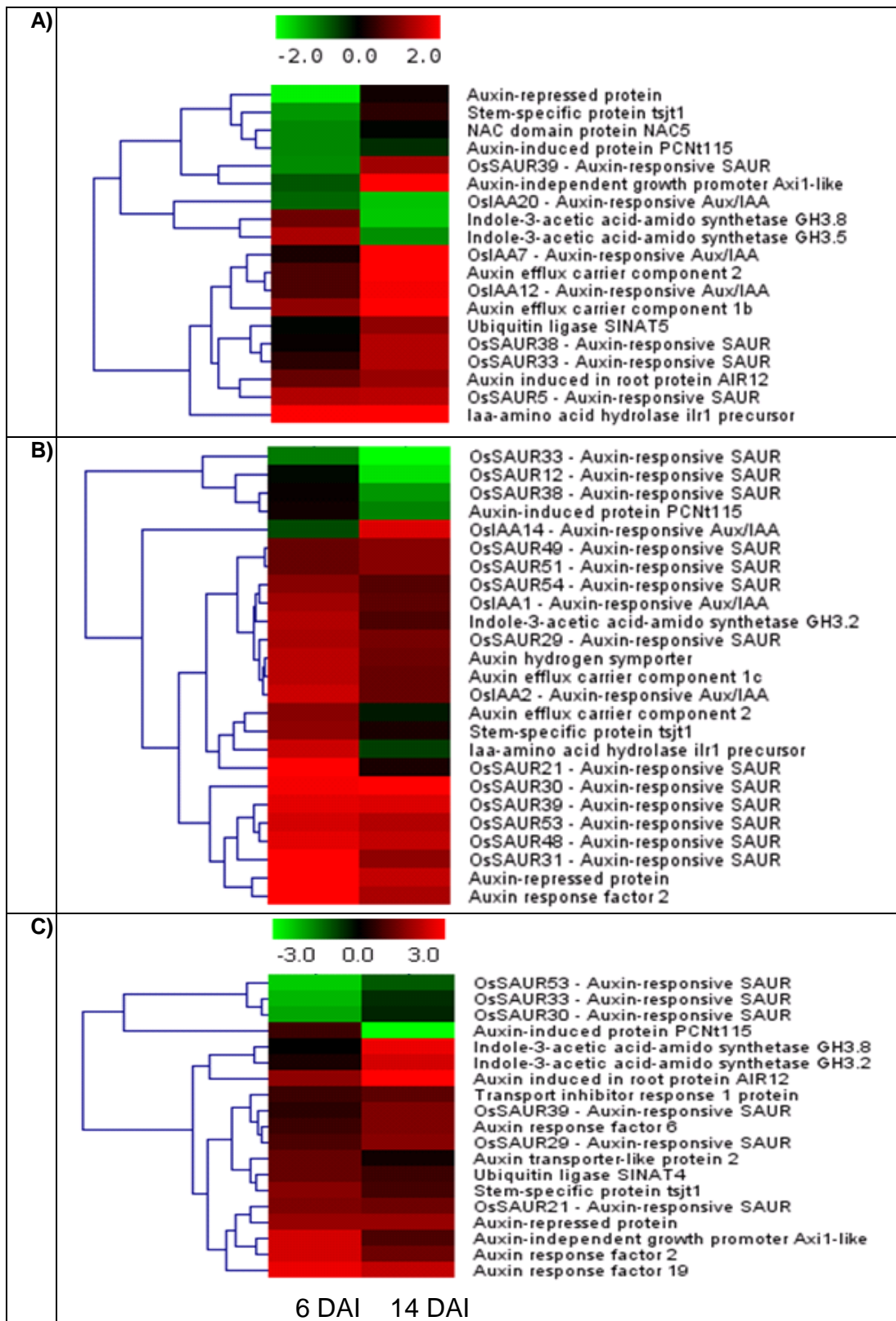


Figure 4.7. Hierarchical clustering and heat map to show up (red) and down (green) regulated auxin-related genes in the roots, stems and leaves of *Striga*-infected compared to control tissue at 6 and 14 DAI. Scale indicates log₂ fold expression values.

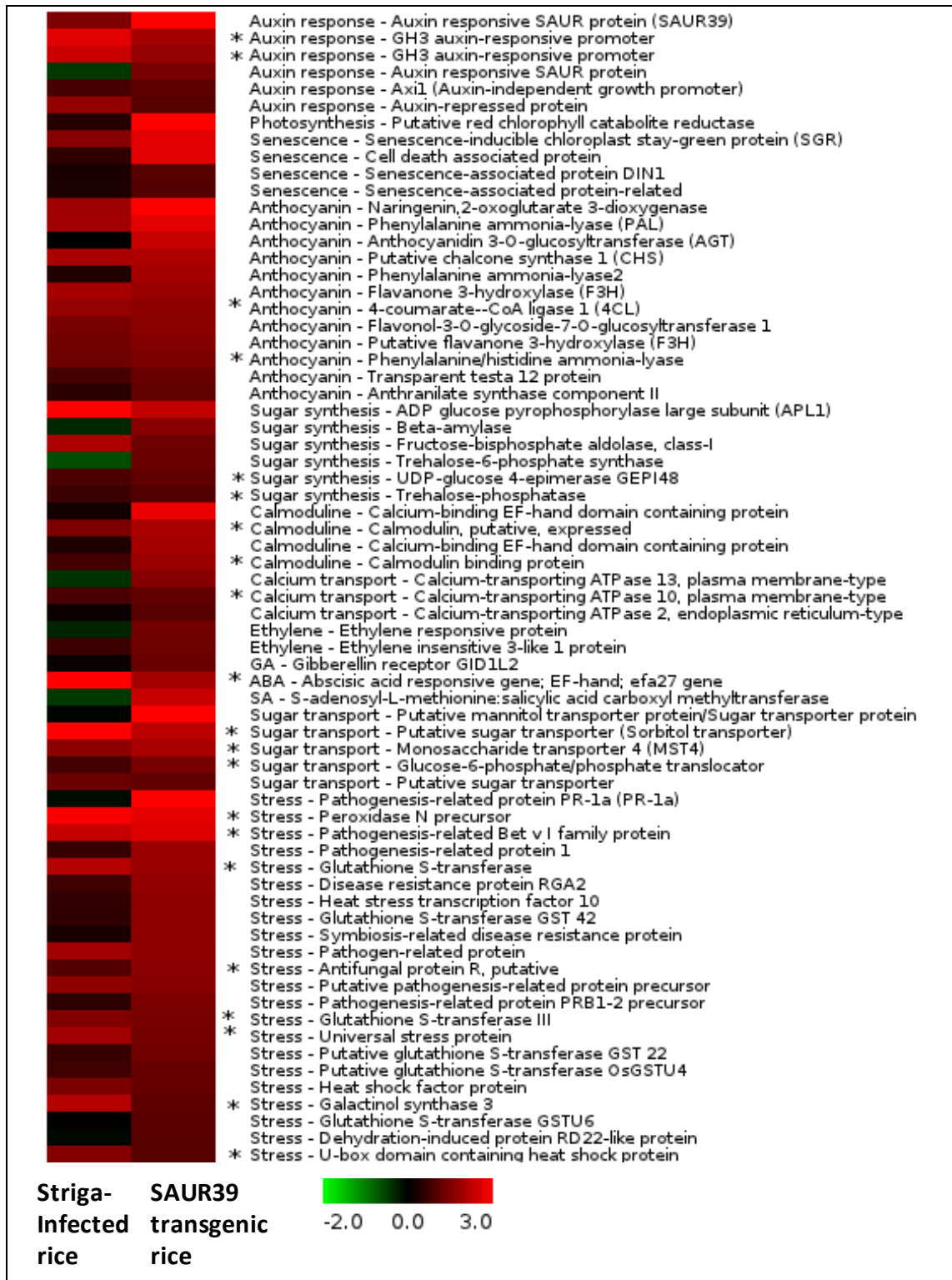


Figure 4.8 Comparison of upregulated genes in the leaves of transgenic rice plants over-expressing SAUR39 (Kant *et al.*, 2009) and leaves of *Striga hermonthica* – infected rice plants 14 DAI. Star symbols (*) indicate differentially regulated genes in *S. hermonthica* infected plants compared to controls. Scale indicates log₂ fold expression values.

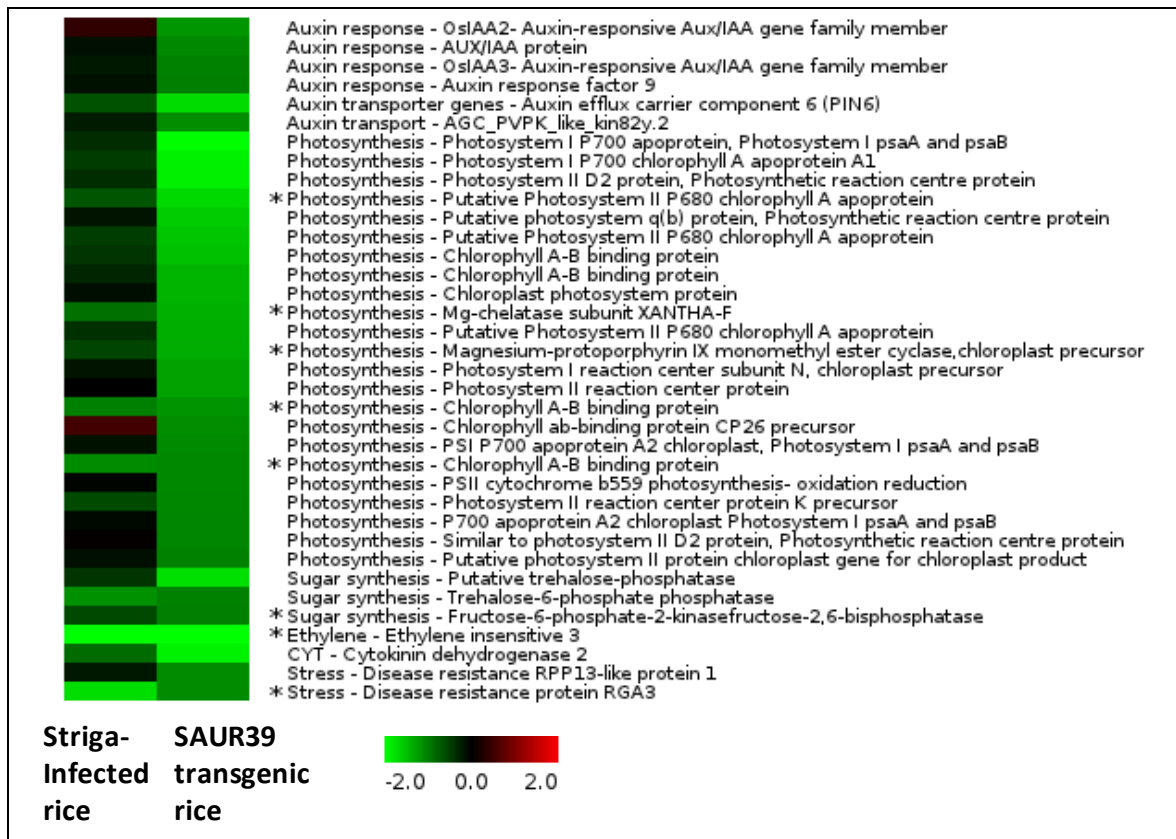


Figure 4.9. Comparison of down regulated genes in the leaves of transgenic rice plants over-expressing SAUR39 (Kant *et al.*, 2009) and leaves of *Striga hermonthica* – infected rice plants 14 DAI. Star symbols (*) indicate differentially regulated genes in *S. hermonthica* infected plants compared to controls. Scale indicates log₂ fold expression values.

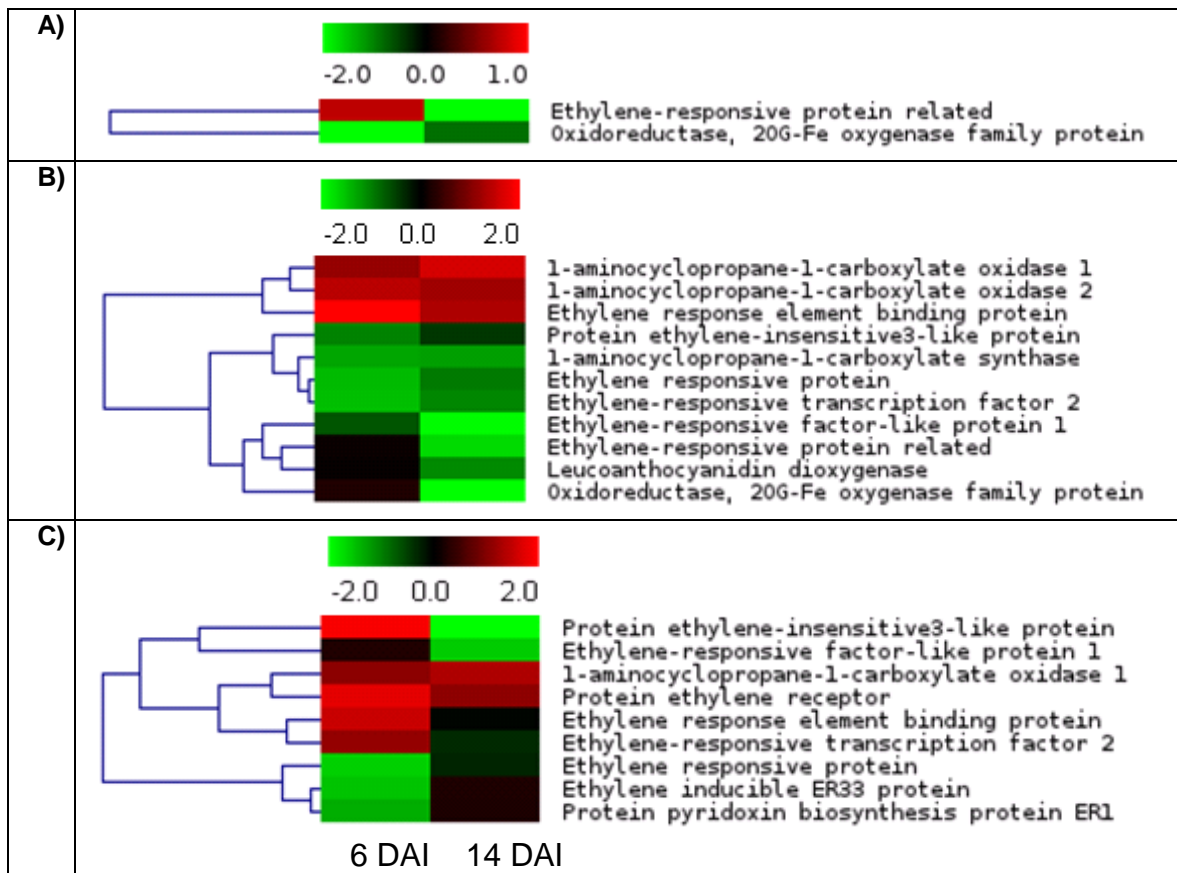


Figure 4.10. Hierarchical clustering and heat map to show up (red) and down (green) regulated ethylene-related genes in the roots, stems and leaves of *Striga*-infected compared to control tissue at 6 and 14 DAI. Scale indicates log₂ fold expression values.

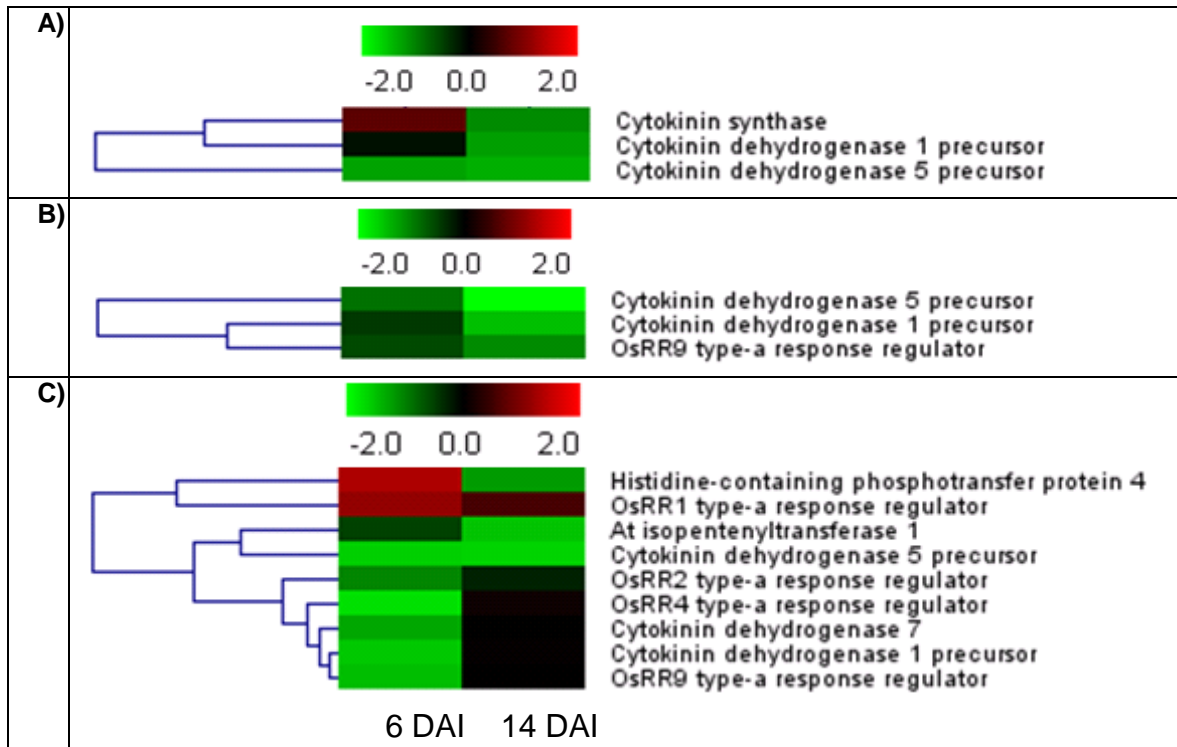


Figure 4.11. Hierarchical clustering and heat map to show up (red) and down (green) regulated cytokinin -related genes in the roots, stems and leaves of *Striga*-infected compared to control tissue at 6 and 14 DAI. Scale indicates log₂ fold expression values.

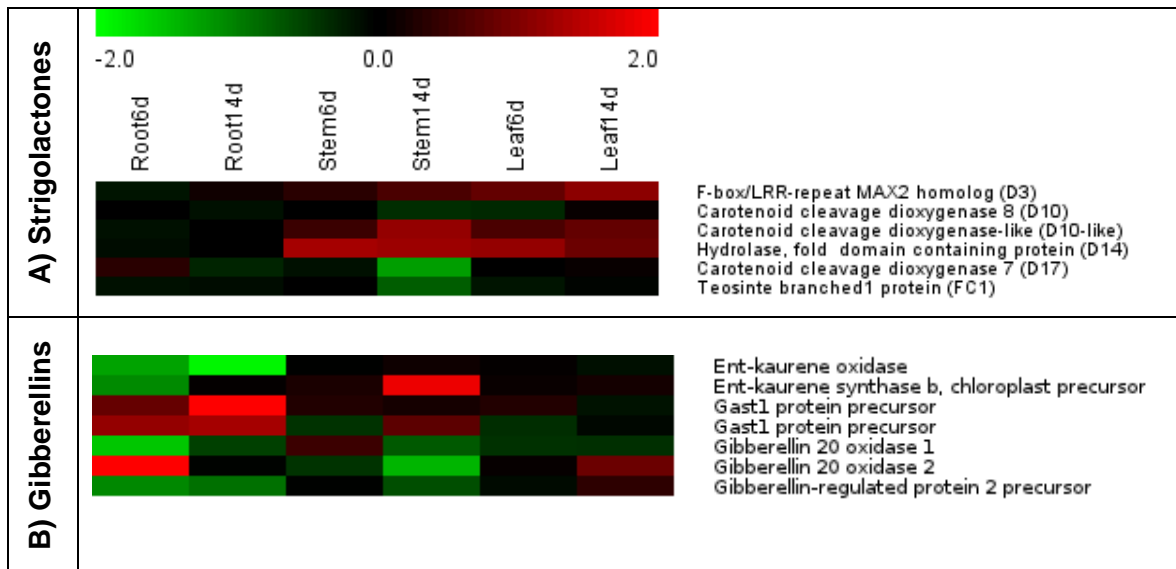
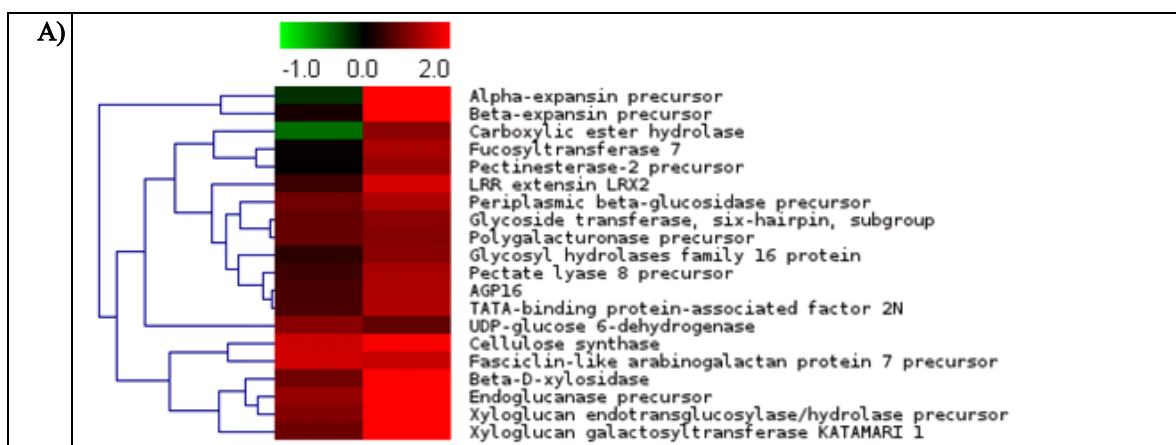


Figure 4.12. Hierarchical clustering and heat map to show up (red) and down (green) regulated strigolactone and gibberellin-related genes in the roots, stems and leaves of *Striga*-infected compared to control tissue at 6 and 14 DAI. Scale indicates log₂ fold expression values.



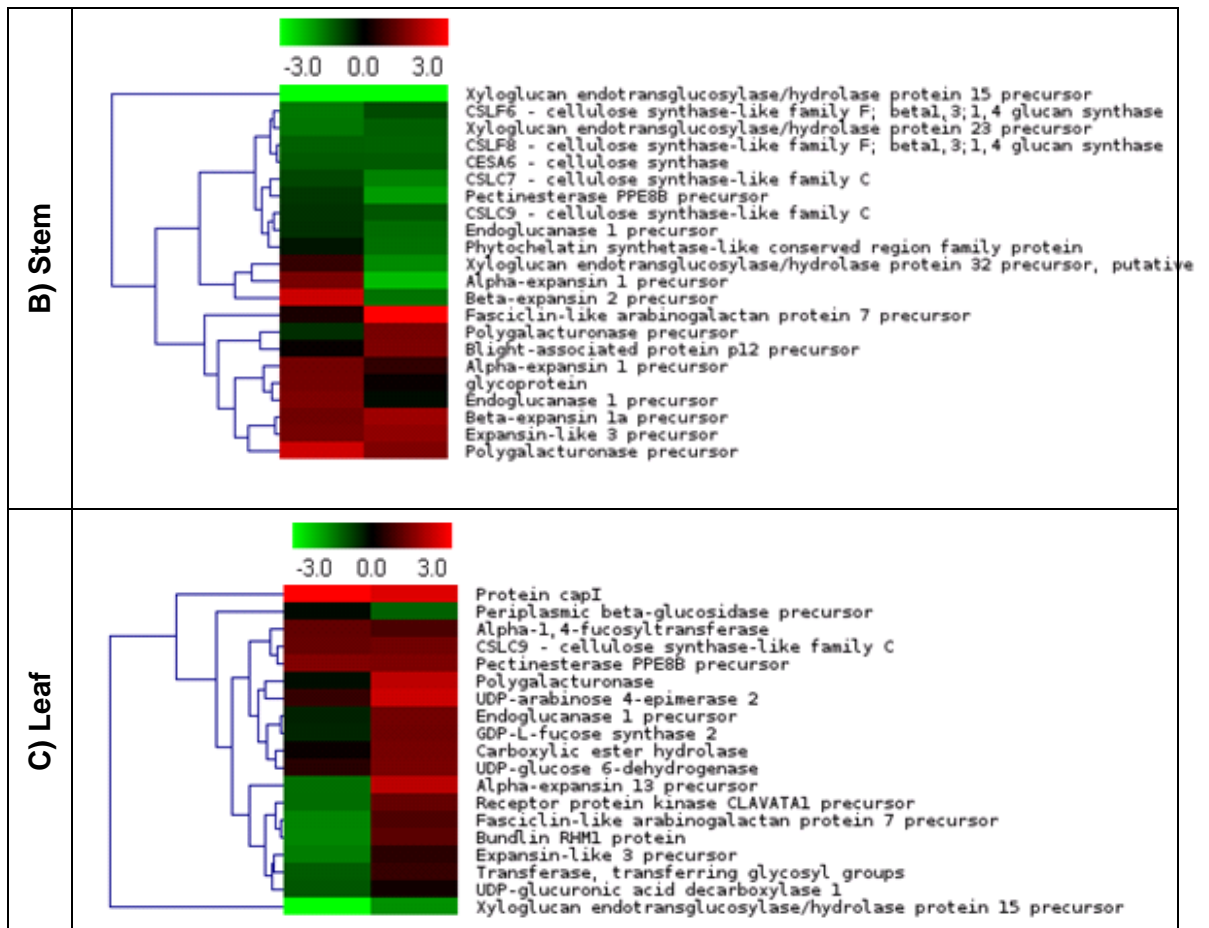


Figure 4.13. Hierarchical clustering and heat map of the expression of cell wall modification-related genes in the roots, stems and leaves of *Striga*-infected compared to control tissue at 6 and 14 DAI. Scale indicates log₂ fold expression values.

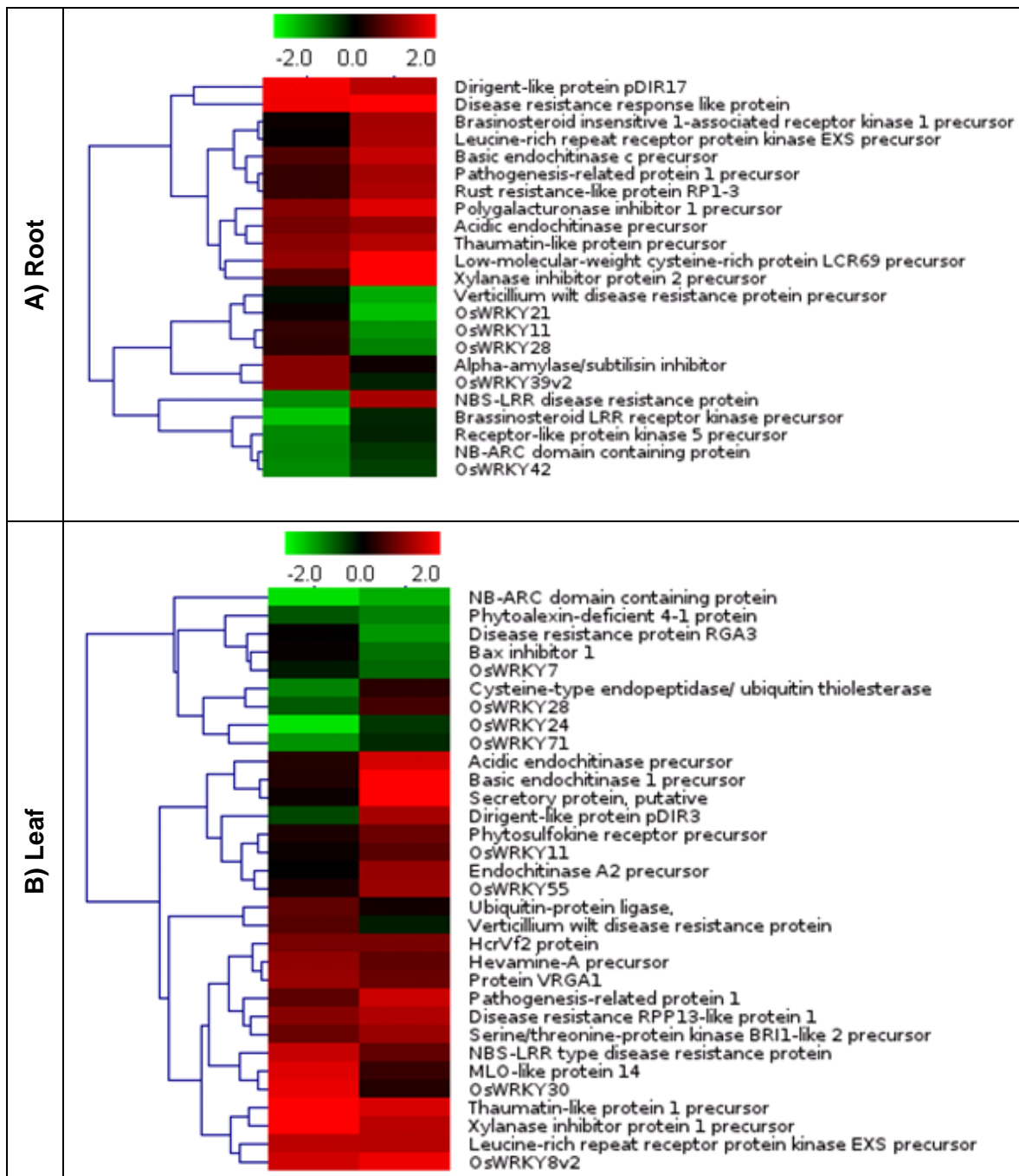


Figure 4.14. Hierarchical clustering and heat map of the expression of biotic stress-related genes in the roots, stems and leaves of *Striga*-infected compared to control tissue at 6 and 14 DAI. Scale indicates log₂ fold expression values.

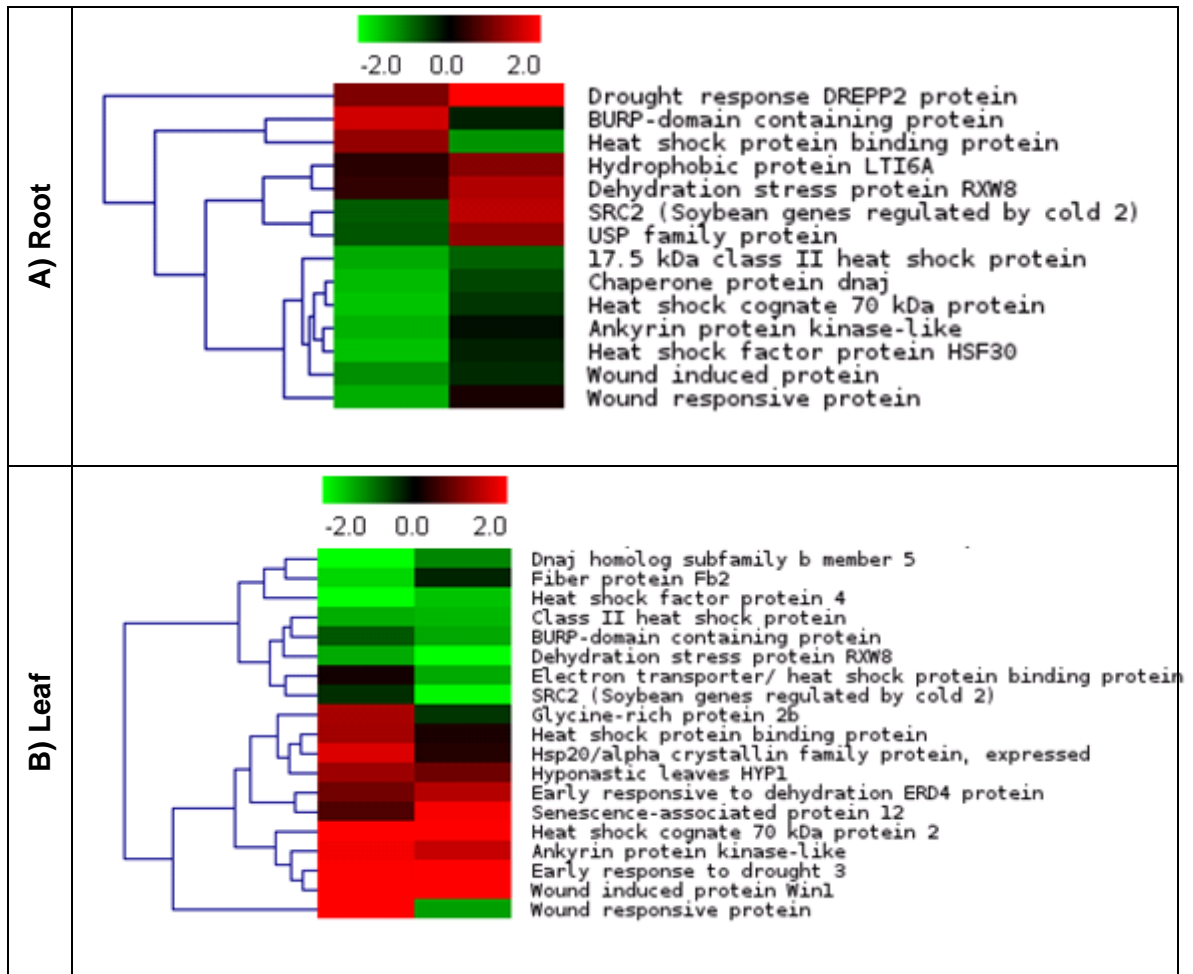


Figure 4.15. Hierarchical clustering and heat map of the expression of abiotic stress-related genes in the roots, stems and leaves of *Striga*-infected compared to control tissue at 6 and 14 DAI. Scale indicates log₂ fold expression values.

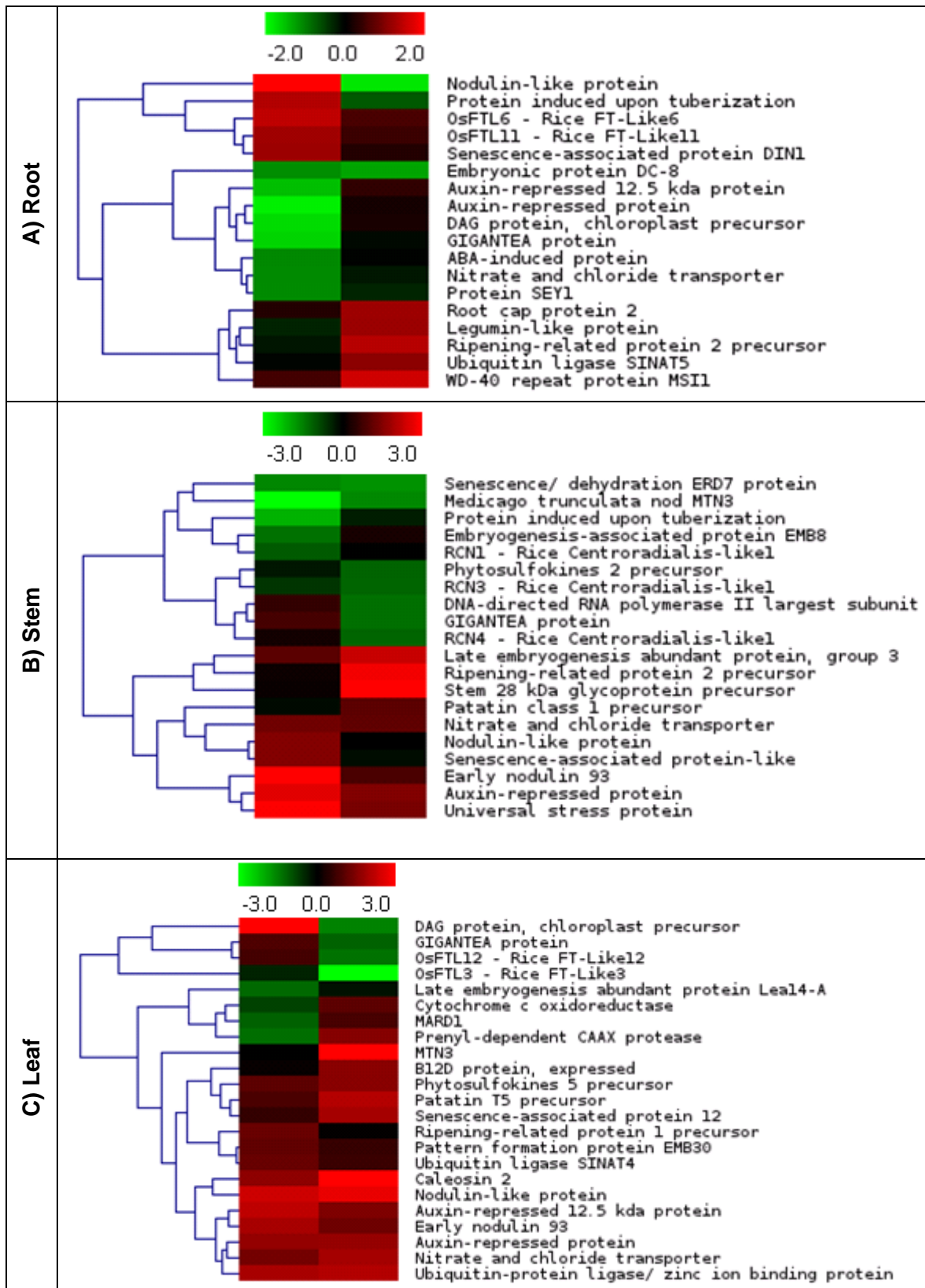


Figure 4.16. Hierarchical clustering and heat map of the expression of development-related genes in the roots, stems and leaves of *Striga*-infected compared to control tissue at 6 and 14 DAI. Scale indicates log₂ fold expression values.

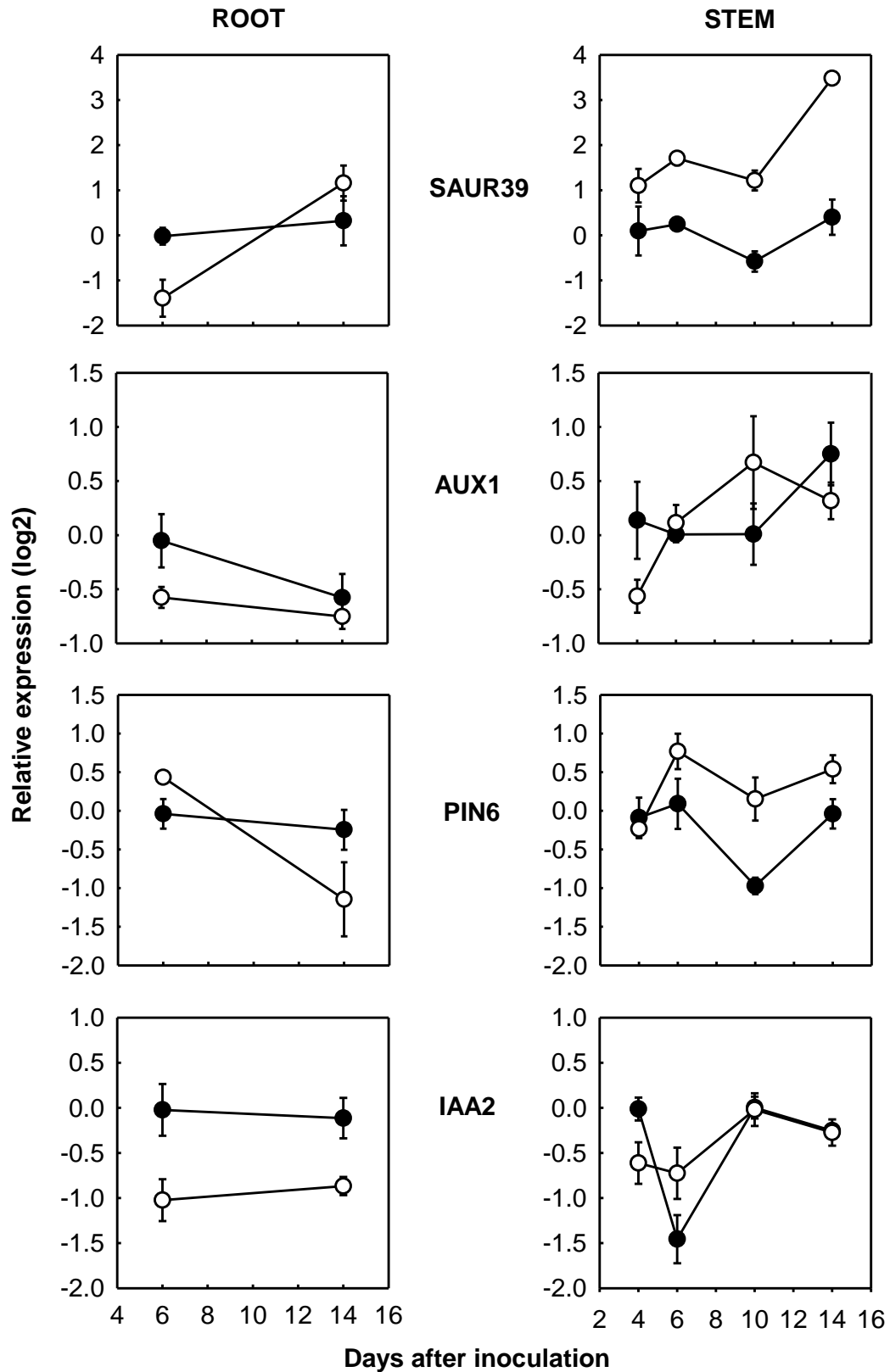


Figure 4.17. Real time qPCR analysis to validate expression of genes of interest in root and stem tissue of control (●) and *S. hermonthica*-infected (○) IAC165 rice plants. Means \pm SE are shown (n=3). Data normalized at 6 and 4 DAI for root and stem, respectively.

4.4 DISCUSSION

S. hermonthica severely affects the architecture and yield of the plants that it infects. Infected plants are stunted, stems are thin and they have low above ground biomass compared to uninfected plants yet we still know relatively little about the mechanisms underlying these changes. The aim of this study was to investigate the hypothesis that parasite-induced perturbations in plant growth regulator pathways underlie the changes in the architecture of infected plants by profiling changes in gene expression in the roots, stem and leaves of *S. hermonthica*-infected plants and uninfected plants.

4.4.1 What are the mechanisms underlying the morphological alterations of *S. hermonthica* infected rice plants?

We hypothesized that alterations in the biosynthesis, signalling and/or degradation of gibberellins, strigolactones and auxins lead to the characteristic phenotype of *S. hermonthica* infected plants, i.e. reduced plant height and tillering. In accordance with this hypothesis differential regulation of genes involved in the metabolic and signalling pathway of the three plant growth regulators were detected. The role of jasmonic and salicylic acid has been related to resistance response (Smith *et al.*, 2009). This thesis was focused to the susceptible response of rice to *S. hermonthica* rather than resistance mechanisms. Therefore, the main scope was focused to plant growth regulators that are known to regulate the developmental processes affected in *S. hermonthica*-infected plants rather than a resistance response. e.g. gibberellins, auxins and strigolactones for stunting and reduction of tillering, respectively. Thus jasmonic and salicylic acid were not analysed. In order to analyse resistance mechanisms of rice to *S. hermonthica*, resistant cultivars, such as Nipponbare or some of the NERICA lines should be used.

As described previously in Chapter 1 section 1.5.3, gibberellins are the major class of plant growth regulators controlling plant height. There were few alterations in the transcription of genes involved in gibberellin metabolism in *S. hermonthica*-infected plants. Genes were differentially regulated in *Striga*-infected plants included *ent*-kaurene oxidase (KO), gibberellin stimulated transcripts (GAST) and gibberellin oxidases (GA20ox) and *ent*-kaurene synthase. *ent*-kaurene oxidase (KO) catalyses sequential oxidation of *ent*-kaurene to produce *ent*-kaurenoic acid in early stages of gibberellins biosynthesis, while GA20 oxidases (GA20ox) are responsible for the conversion of GA53 into the bioactive form GA1 (Curtis *et al.*, 2000; Spielmeyer *et al.*, 2002; Sakamoto *et al.*, 2004). Lesions in the KO enzyme, or the suppression of GA20ox results in reduced plant height (Li *et al.*, 2011; Itoh *et al.*, 2004). The results presented

in this study showed down regulation of KO transcripts in root tissue at both 6 and 14 DAI, being particularly stronger at the latter stage. This suggested a decrease in the amount of available substrate to generate bioactive gibberellins as infection by *S. hermonthica* progressed, however endogenous levels of gibberellins in *S. hermonthica*-infected plants were not measured. GA20ox1 was down regulated in root tissue by 6 DAI whereas GA20ox was up regulated in root tissue by 6 DAI but down regulated in stem tissue by 14 DAI. The differential expression of GA20ox in stems and roots did not show a clear consistent pattern in order to indicate lower concentration of bioactive gibberellins in *S. hermonthica*-infected plants. This suggested that conversion of inactive gibberellins to their bioactive form by GA20ox is not a critical process in the stunting effect observed in *S. hermonthica* infected plants. We were not able to detect alterations in genes involved in signalling of gibberellins in *S. hermonthica* infected plants, in contrast to the results presented by Swarbrick *et al.* (2008) who reported a significant down regulation of the gibberellin receptor GID1. However, the absolute number of gibberellin-related genes differentially regulated in a compatible interaction between *S. hermonthica* and the susceptible rice cultivar IAC165 reported by Swarbrick *et al.* (2008) was the same as the number of differentially expressed genes presented in this thesis (7), which in accordance to his study, were mostly differentially regulated in root tissue.

Additional to the role that gibberellins have in internode elongation, recent discoveries have found that mobile gibberellins from the shoot stimulate xylogenesis in the hypocotyl of *A. thaliana* (Ragni *et al.*, 2011). Whether gibberellins are involved not only in the stunting of *S. hermonthica* infected plants, but also in the formation of the xylem fusions by inducing xylogenesis between parasite and host via the GA signalling pathway requires further investigations, since the only evidence we could find was the up regulation of *ent* kaurene synthase in the stems at 14 DAI. By this time the parasite had already established a successful connection. All the evidence indicated that gibberellins do not play a role in the stunting effect observed in *S. hermonthica* infected plants.

Transcripts of the gene encoding for the strigolactone biosynthetic enzyme CCD8-like (D10-like) were up regulated in the aerial part of *S. hermonthica* infected plants, particularly in stem tissue by 14 DAI. Differential expression of transcripts of another gene (probe sequence) encoding for CCD8 (D10) was not detected. The difference between these genes in terms of the proteins they encode for is unknown at present. However, if the CCD8-like gene is involved in strigolactone biosynthesis the up regulation in the expression of this gene is consistent with the hypothesis that strigolactones play a role in the suppression of tiller bud outgrowth in *S. hermonthica*-

infected plants, as shown in Chapter 3 by the increase of the CCD8:GUS signal intensity detected in the leaf axil of *Striga*-infected plants and the suppression of tillering in GR24-treated plants infected with *S. hermonthica* (Chapter 3). These results suggest that the increase in the biosynthesis of strigolactones in *S. hermonthica*-infected plants could be carried out by homologues of CCD8, such as CCD8-like, or other enzymes involved in carotenoid cleavage that have not been found and described to date.

Up regulation of genes involved in the strigolactone signalling pathway was detected in the expression profile of *S. hermonthica*-infected plants. Increase of the transcript levels of the α/β hydrolase, fold domain containing protein D14 could be detected in stems and leaves at 6 and 14 DAI. In addition, the F-box LRR protein D3 was significantly up regulated in leaf tissue at 14 DAI. The putative role for D3 is the signalling of strigolactones, whereas D14 has been suggested to be involved in either signalling or the conversion of strigolactones to their bioactive form (Ishikawa *et al.*, 2005; Arite *et al.*, 2009). The up regulation of these genes in the aerial part of *S. hermonthica* infected plants further supports a role for strigolactones in the suppression of tiller bud outgrowth in *S. hermonthica* plants. In this case, the role of strigolactones in the suppression of tiller bud outgrowth of infected plants would not be derived from an increase in the concentration of endogenous strigolactones, but in the perception of bioactive strigolactones in the leaves, and particularly stems of *S. hermonthica* infected plants. By testing all the strigolactone mutants against *S. hermonthica* it would be possible to obtain the whole scenario of where and when the strigolactone metabolic and signalling pathway is affected under infestation by *S. hermonthica*. Some evidence presented in this thesis points to a potential role of strigolactones in the inhibition of tillering of *S. hermonthica* infected plants. However, the lack of increase in the expression levels of strigolactone-related genes, (as shown in the microarray analysis,) and the analysis of the *d3-1* and *d10-1* mutants do not support this hypothesis, suggesting that they do not play a role in the inhibition of tillering

We also proposed that auxin might be involved in the inhibition of tillering in *S. hermonthica* infected plants, since it is widely known that auxin is responsible for apical dominance and lateral bud emergence by polar auxin transport, in interaction with cytokinins and strigolactones (Xu *et al.*, 2005; Zhang *et al.*, 2010; Crawford *et al.*, 2010). Consistent with this hypothesis, auxin was the group of plant hormones that had the highest number of differentially expressed genes in *S. hermonthica*-infected plants according to the microarray dataset, suggesting that auxins are very important in the biology of rice plants infected with *S. hermonthica*.

A large number of auxin-related genes were differentially regulated in roots, stems and leaves of *S. hermonthica*-infected plants at 6 and 14 DAI. The most severely affected groups of auxin genes were involved in auxin transport and auxin-responsiveness. In particular, the small auxin up RNA (SAUR) gene family was extensively altered in the three tissues analysed at 6 and 14 DAI. The early auxin-responsive SAUR gene family consists of 58 members in rice, however their functions are still largely unknown (Jain *et al.*, 2006). Five SAUR genes were down regulated in either leaf or stem tissue at 6 and 14 DAI, respectively. These were OsSAUR33, OsSAUR12, OsSAUR38, OsSAUR30 and OsSAUR53, whose biological function has not been described to date. In addition, SAUR5 and SAUR33 were previously reported by Swarbrick *et al* (2008) as two of the most significantly down regulated genes in the roots of *Striga*-infected rice at 11 DAI under a compatible parasitic interaction.

A large number of up regulated SAUR genes was detected in roots, stems and leaves of *S. hermonthica* infected plants. SAUR39 was highly up regulated and was the only SAUR gene to be up regulated in the three tissues at both time points, (with the exception of root tissue at 6 DAI). SAUR39 negatively regulates polar auxin transport, as observed in rice transgenic plants over expressing SAUR39 (Kant *et al.*, 2009). Under normal conditions, transient changes in auxin levels result in the temporary induction of SAUR39, which after a few hours is suppressed, restoring polar auxin transport and normal plant growth (Kant and Rothstein, 2009). In contrast, plant growth and yield are reduced in the constitutive SAUR39ox transgenic rice plant due to a decrease in auxin biosynthesis and polar auxin transport. The decrease of auxin biosynthesis and transport in SAUR39ox resulted in transcriptional changes of genes involved in photosynthesis, senescence, chlorophyll production, anthocyanin accumulation, sugar synthesis and transport as analysed by a microarray analysis (Kant *et al.*, 2009). The current model of action of SAUR39 points out a putative increase in sugar and anthocyanin content and a significant decrease in photosynthesis when SAUR39 is constitutively expressed (Kant and Rothsten, 2009). These alterations are reflected in reduced plant height, less leaves and tillers and lower yield compared to wild type plants (Kant *et al.*, 2009). The phenotype of SAUR39ox rice plants is very similar to the phenotype of *S. hermonthica* infected plants.

The up regulation of SAUR39 in *S. hermonthica*-infected plants was the first indication that SAUR39-mediated auxin transport could be involved in the negative responses on growth that *S. hermonthica* inflicts on its hosts. The gene expression of SAUR39 was analysed by real time quantitative PCR analysis (qPCR) in roots and stems of *Striga*-infected plants. The qPCR results validated the significant up regulation of this gene in roots and stems of *Striga*-infected plants compared to uninfected plants,

consistent with the microarray data. Interestingly, the expression of SAUR39 did not decrease in *S. hermonthica*-infected plants as infection progressed, i.e. expression of SAUR39 was higher at 14 compared to 6 DAI. This suggested 'constitutive' expression of SAUR39 in *Striga*-infected plants, at least during the period analysed, similar to SAUR39ox. In order to determine whether the same metabolic perturbations reported in 4 week old shoots transgenic SAUR39ox rice lines (described above) occur in leaves of *S. hermonthica* infected plants at 14 DAI (4 week old leaves) a detailed comparison of the two studies was performed. The up regulation of SAUR39 was not linear throughout the period analysed in the stem tissue. From the early stages analysed, i.e. 4 to 6 DAI, the expression of SAUR39 increased only slightly, followed by an apparent decrease from 6 to 10 DAI. In contrast, from 10 to 14 DAI there was a significant increase in the relative expression of SAUR39. The function of SAUR39 is to control auxin transport (Kant *et al.*, 2009). Such an increase of SAUR39 from 10 to 14 DAI suggested that auxin transport was altered in *S. hermonthica*-infected plants. When compared to the previous analyses performed, by this time, *S. hermonthica* should have established successful xylem fusions, suggesting that the up regulation of SAUR39 has more to do with development of the haustoria rather than the initial infection process itself.

Up regulated genes in both SAUR39ox. and *S. hermonthica*-infected leaves were similar (in the GH3 auxin responsive genes, sugar metabolism (synthesis and transport), stress-related genes and anthocyanidin metabolism). This suggested that similar responses were triggered in *S. hermonthica*-infected plants compared to SAUR39ox. However, only a few genes that were down regulated in SAUR39ox were also down regulated in the leaves of *S. hermonthica*-infected plants at 14 DAI, since many of the genes that were down regulated in SAUR39ox showed no differential expression in *Striga*-infected plants. Most of the genes that were down regulated in both SAUR39ox and *S. hermonthica*-infected leaves 14 DAI were involved in photosynthetic processes. Lower rates of photosynthesis of *S. hermonthica*-infected hosts have been reported in sorghum, and maize and rice (Frost *et al.*, 2007; Gurney *et al.*, 1995; Watling and Press 2000). These low rates of photosynthesis have been attributed to a decrease in stomatal conductance possibly due to an increase in ABA. However, it is also possible that some of the decrease may also be due to changes in auxin transport as a result of the up regulation of the expression of SAUR39.

Additionally, at a microscopic level, the number of sieve tube element cells in the vasculature of SAUR39ox was smaller, suggesting reduced auxin transport (Kant *et al.*, 2009). As described in Chapter 2, the reduction in the thickness of *S. hermonthica* infected plants was due to a lower number of cells in the cortical region; however, the

number of sieve tube element cells was not measured. Therefore, further investigations need to be carried out in order to determine if the number of cells in the vascular bundle of *S. hermonthica* infected plants is reduced, as those in the SAUR39ox and whether this relates to auxin transport.

These results suggested that polar auxin transport might be decreased in *S. hermonthica* infected plants via the up regulation expression of SAUR39. This may be responsible for the morphological changes observed in the growth and plant architecture of *Striga*-infected rice plants. However, further auxin-transport-based investigations need to be carried out in *S. hermonthica*-infected plants.

4.4.2 Are changes in auxin biosynthesis and signalling important during infection of roots by *S. hermonthica*?

Auxin does not only play a role in shaping plant shoot architecture but it is also critical in many aspects of root development, for example during the formation and emergence of lateral roots (Laskowski *et al.*, 1995; Swarup *et al.*, 2008) and for the elongation of root hairs (Kapulnik *et al.*, 2011). It is interesting to note that root hairs adjacent to *Striga* attachments are longer than those on uninfected plants (data not shown).

Recently it has been hypothesized that *S. hermonthica* may utilize the auxin signalling pathway that regulates lateral root emergence in order to penetrate through the root tissue and establish xylem-xylem connection (Scholes, unpublished). The process by which *S. hermonthica* penetrates the different cell layers is highly regulated. There is no damage to the host cells, the host cell walls appear to part with high spatial and temporal co-ordination to allow the *Striga* endophyte to push through. This is very similar to the way in which a lateral root emerges from the pericycle, through the endodermis, cortex and epidermis, a process regulated by an auxin signal transduction pathway. Swarup *et al.*, (2008) showed in *A. thaliana* that the hormone auxin, originating from the developing lateral root, acts as a local inductive signal which reprograms adjacent cells, causing cell separation and tissue remodelling in advance of developing lateral root primordia.

In this model auxin derived from the primordium causes the degradation of the IAA3/SHY2 repressor protein in adjacent endodermal cells resulting in the up regulation of selected cell wall remodelling enzymes leading to separation of the endodermal cells. In the adjacent cortical cells the auxin gradient induces the expression of LAX3 (which encodes a high affinity auxin influx carrier). The LAX3-dependent accumulation of auxin triggers the up regulation of cell wall remodelling enzymes. This process is repeated in epidermal cells adjacent to the emerging lateral

root. As the expression of LAX3 is highly localised only to cortical and epidermal cells, the up regulation of cell wall degrading enzymes is also highly localised thus ensuring that the integrity of the root is not compromised. A number of cell wall remodelling enzymes whose transcript abundance was upregulated by this auxin signalling pathway include a subtilisin-like protease (AIR3), a polygalacturonase (PG), a pectate lyase (PLA2), a glycosyl hydrolase (GLH17) an expansin (EXP17) and a xyloglucan:xyloglucisyl transferase (XTR6) (Swarup *et al.*, 2008; Péret *et al.*, 2009).

In the root tissue of *S. hermonthica* infected plants, auxin responsive elements, auxin influx and efflux carriers were up regulated as the parasite the root. In addition, there was an extensive up regulation of genes encoding enzymes involved in cell wall modification including expansins (known to act in plant cell separation (Marin-Rodriguez *et al.*, 2002; Roberts *et al.*, 2002; Patterson, 2001) polygalacturonases, xylanases and cellulases, all involved in cell wall modification. The gene encoding AIR12 was also up regulated, being more highly expressed at 14 compared to 6 DAI. AIR genes were first discovered by being induced during root differentiation and formation in cell cultures of *A. thaliana* (Neuteboom *et al.*, 1999). Later characterisation of the AIR12 gene revealed that it encoded an extracellular plasma membrane protein which was expressed during lateral root formation in *A. thaliana* (Laskowski *et al.*, 2006; Preger *et al.*, 2009).

The extensive up regulation of cell wall modification enzymes as the parasite penetrates the roots is consistent with a parasite-induced up regulation of host enzymes. It is possible that this the up regulation of the cell wall modifying enzymes is due to localised changes in auxin as it is known that a closely related parasitic plant, *Triphysaria versicolor*, accumulates a high concentration of auxin at the tip of the radicle in response to the haustorial initiation factor DMBQ (Tomilov *et al.*, 2005). Whether *S. hermonthica* produces auxin in cells as they invade the host root and if they do, whether the auxin is transported into the host is unknown and requires further investigation. In order to further test this hypothesis rice transgenic plants that carry gene promoter reporter fusions (promoters from cell wall modifying enzymes or components of the auxin signal transduction pathway) could be infected with *S. hermonthica*. In addition, the amount of auxins in germinated radicles of *S. hermonthica* could be measured following and modifying the procedure described by Lewis and Muday (2009) in seedlings of *Arabidopsis* labelling radioactively auxin probes.

It is interesting to note that genes encoding polygalacturonase inhibitor proteins (PGIPs) are also induced in *S. hermonthica*-infected rice roots in a similar manner to

the responses observed against fungal pathogens that degrade cell walls (Prabhu *et al.*, 2012; Albersheim and Anderson, 1971). Whether the increase in the levels of PGIPs of *S. hermonthica* infected plants reflects increased resistance to fungal pathogens needs further investigation.

4.4.3 How does infection by *S. hermonthica* alter the expression of genes encoding biotic and abiotic stress proteins?

Differential regulation of genes involved in biotic stress in *S. hermonthica* - infected plants indicated that a large number of genes encoding for pathogenesis related proteins were up regulated in the roots and leaves tissues at 6 and 14 DAI. The characterization of these genes included dirigent proteins, rust resistance proteins, polygalacturonase inhibitors, thaumatin precursors, endochitinases. Members of the WRKY transcription factor gene family were also up regulated. It has been shown that these genes are involved in resistance to many fungal and bacterial pathogens (Delteil *et al.*, 2012; Albersheim and Anderson, 1971; Liu *et al.*, 2007; Ryu *et al.*, 2006). The up regulation of defence genes observed in this experiment was more intense by 14 compared to 6 DAI, suggesting that *S. hermonthica* infected plants triggered inefficient defence responses due to the late activation of these genes, i.e. after *S. hermonthica* has established successfully. Up regulation of genes associated with a defence response were also reported in roots of the rice cultivar IAC 165 infected with *S. hermonthica* (compatible interaction) (Swarbrick *et al.*, 2008). Evidence indicates that PR proteins are induced much earlier during resistant reactions, as shown by up regulation of β -glucanase in roots of pea infected by *O. crenata* (Perez de Luque *et al.*, 2006) or the induction of genes encoding enzymes involved in defence-related secondary metabolism in the roots of rice cultivar Nipponbare infected with *S. hermonthica* (Swarbrick *et al.*, 2008) suggesting that the timing of the activation of these genes by the host is critical for a successful resistance response to *S. hermonthica*. This study has also shown, for the first time, that many genes encoding defence-related transcripts are also up regulated in the leaves of rice plants infected with *S. hermonthica*. This indicates a systemic up regulation of defence transcripts. The activation of genes encoding for PR proteins suggest that *S. hermonthica*-infected plants may have enhanced resistance to other pathogens following infection, however, this needs further investigation.

A range of genes encoding abiotic stress proteins were also up regulated. Two genes involved in early responses to drought and dehydration in leaves and the drought response DREPP2 protein in roots were up regulated following infection of rice

plants with *S. hermonthica*. It has been suggested that the drought response in *S. hermonthica* infected plants is due to stomatal closure as levels of ABA increase with infection (Taylor *et al.*, 1997), however, Swarbrick *et al* (2008) suggested transient drought response that was not maintained at a transcriptional level attributable only to ABA concentration. Our results show that transcription of drought and dehydration related genes is increased by 14 DAI, suggesting that the drought response is more intense at later stages of infection, however, no extensive alterations were detected in ABA metabolism, in accordance with the results reported by Swarbrick *et al.* (2008).

In addition, transcripts encoding for heat shock protein components were up and down regulated in root and leaf tissue of *S. hermonthica* plants at 6 and 14 DAI. Heat shock proteins and chaperones are involved in protein protection by the capacity of acting as a shield, folding or unfolding substrates in a context dependent manner (Verghese *et al.*, 2012). The differential expression of HSPs and chaperones suggested modifications in the protein structure of *S. hermonthica* infected plants. However, further studies are needed to determine whether post-translational modifications take place in *S. hermonthica* infected plants and which proteins are specifically targeted by HSPs and chaperones.

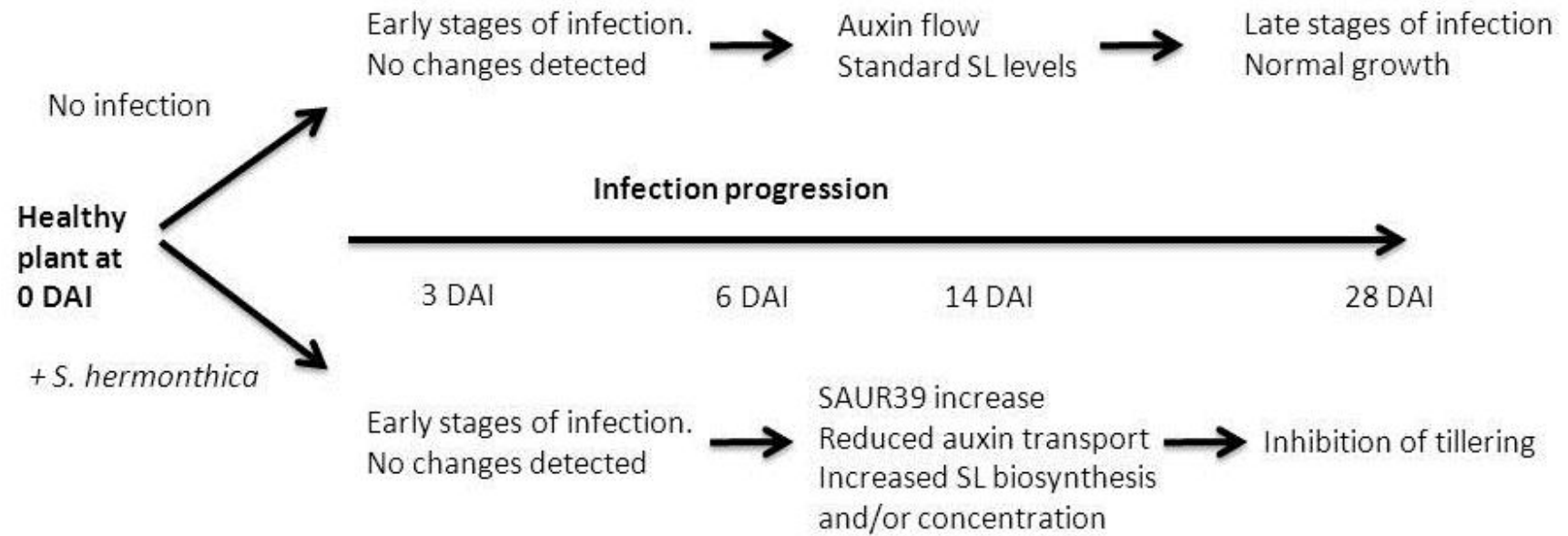


Figure 4.18 Proposed mechanism of action of *S. hermonthica* to reduce tillering of infected rice plants.

Chapter 5

General discussion

5.1 Mechanisms underlying the morphological alterations of rice infected by *Striga hermonthica*

Parasitic weeds of the genus *Striga* represent the major agricultural biotic constraint in sub-Saharan Africa, affecting the lives of millions of people every year (Scholes and Press, 2008). The reduction of yield in *Striga*-infected rice plants is determined by the degree of detrimental effect on the growth of infected plants and architectural changes, importantly suppression of tillering. Tillering, also known as lateral bud outgrowth, largely determines grain production in rice (Sakamoto and Matsuoka, 2008). Therefore we set out to investigate the mechanisms underlying the alterations observed in the morphology of rice plants infected by *S. hermonthica*.

S. hermonthica altered the morphology of infected rice plants in a similar manner to other cereals such as sorghum and maize. The rice plants infected by *S. hermonthica* in the experiments carried out in this thesis showed severe stunting, decreased biomass, increased root to shoot ratio, thinner stems and suppression of tillering (Gurney *et al.*, 2002; Vasey *et al.*, 2005; Kaewchumnong and Price, 2008). However, tillering is not a main component of grain yield in either sorghum or maize due to their intrinsic plant architecture (Zhou *et al.*, 2011; Lukens and Doebley, 2001), but it can be a critical agronomic trait in rice (Gao *et al.*, 2009; Wang and Li, 2011; Belefant-Miller *et al.*, 2012). Therefore it was crucial to understand the mechanisms underlying the morphological alterations in *S. hermonthica*-infected rice plants, mainly the characteristic inhibition of tillering caused by *S. hermonthica*. A key objective was to determine whether *S. hermonthica* suppressed tiller bud formation and / or tiller bud outgrowth in infected rice plants. The detailed growth analysis presented in the second Chapter of this thesis allowed us to demonstrate for the first time that both processes were affected in *S. hermonthica* - infected rice plants from early stages of development.

Following the discovery of inhibition of formation and outgrowth of tiller buds in *S. hermonthica*-infected rice plants, it was necessary to focus on the mechanisms underlying these alterations. Strigolactones, a 'new' plant growth regulator that has recently been identified to control tiller bud outgrowth in rice and branching in other species (Umehara *et al.*, 2008; Gomez-Roldan *et al.*, 2008) was the first candidate. In order to understand whether strigolactones were involved in the suppression of tillering in *S. hermonthica*-infected plants, two approaches were taken. Firstly rice mutants deficient in strigolactone biosynthesis and signalling were infected with *S. hermonthica*. I hypothesised that *S. hermonthica* would not suppress tillering in either of the mutants if alterations in strigolactone biosynthesis or signalling were responsible for the

suppression of tiller bud outgrowth. Secondly, I artificially modified the concentration of strigolactones in *S. hermonthica*-infected wild type plants, using a biochemical approach. This consisted of reducing the amount of strigolactones with the carotenoid biosynthetic inhibitor fluridone, or increasing them by the exogenous application of the synthetic analogue of strigolactones GR24. Fluridone does not only target biosynthesis of strigolactones, but also of many other compounds involved in photosynthesis and carotenoid-derived products, therefore interpretation of these data is difficult. The findings revealed that tillering in both mutants was suppressed by the parasite even with a low number of attachments of *S. hermonthica* individuals, refuting our initial hypothesis. However, a decrease of tillering in *S. hermonthica* - infected GR24-treated plants was observed compared to the uninfected treatment, suggesting that strigolactones may play some role in the inhibition of tillering in *S. hermonthica* - infected plants, but that they do not account for the whole suppression of tillering observed.

Auxin has long been known to control lateral bud outgrowth by apical dominance mechanisms (Leyser, 2003). Recent publications have also shown the close relationship between auxin and strigolactones in controlling tiller bud outgrowth and plant height (Beveridge *et al.*, 2009; Ferguson and Beveridge, 2009; Crawford *et al.*, 2010), suggesting that auxin is an obvious candidate to explain the inhibition of tillering and possibly stunting in *S. hermonthica* - infected plants possibly in conjunction with strigolactones. This hypothesis is supported by the analysis of transcriptional changes of genes involved in plant growth regulator metabolism in the roots, stems and leaves of *S. hermonthica*-infected plants (Chapter 4) which is the first to show the importance of auxin in the morphological alterations observed in susceptible rice infected by *S. hermonthica*.

The comparison between the alterations in transcription in *S. hermonthica*-infected plants and transgenic plants over-expressing the small auxin up RNA 39 gene (SAUR39ox), involved in auxin polar transport (Kant *et al.*, 2009), revealed that very similar alterations in the expression of genes in a range of different metabolic pathways. In particular, in both *S. hermonthica*-infected rice plants and in SAUR39ox transgenic plants there was an increase in auxin transcripts, (including SAUR39) in roots, stems and leaves, and a down regulation of the same sets of photosynthetic genes. It is well known that plants infected with *S. hermonthica* exhibit lower rates of photosynthesis, partly due to stomatal closure (Gurney *et al.*, 1995; Frost *et al.*, 1997; Watling and Press, 2000; Rodenburg *et al.*, 2008) but some of the decrease in the rate of photosynthesis may also be due in part to changes in auxin biosynthesis, transport and signalling, a hypothesis that requires further investigation.

It is clear that further experiments are required to fully understand the role of auxins and strigolactones in the suppression of tillering in *Striga*-infected plants. Such experiments could include the use of mutants in auxin signalling of auxin and morphogenesis such as *OslAA1* (Song *et al.*, 2009), as well as the generation of strigolactone / auxin double mutants, which do not exist to date. Alternatively, the combined use of mutant plants and biochemical approaches could be used, however, determining the proper concentration of the biochemical compounds to be used and the right conditions to grow the plants without altering additional processes in the host plants proved to be complex, as shown in Chapter 3. In order to determine the relative amount of bioactive compounds, mass spectrometry provides a useful tool to quantify the endogenous levels of strigolactones and auxins, complementing the transcript profiling of auxin and strigolactone related genes presented in this thesis (Sato *et al.*, 2005; Kohlen *et al.*, 2011). However, the challenges of mass spectrometry are diverse, for example, it would be necessary first to determine whether the strigolactone levels are detectable in *S. hermonthica*-infected plants. According to Bouwmeester (personal communication) strigolactone levels can be detected in phosphorous-starved *Arabidopsis* and rice roots. The experimental procedure in the plants used in the series of experiments used in this thesis were not deficient in phosphorous, therefore the detection of strigolactones could not be guaranteed. In addition, the use of imaging-matrix laser desorption ionization mass spectrometry (I-MALDI) was attempted in haustorial sections of successfully attached *Striga* individuals, however no positive results were obtained since no distinction between parasite and host could be observed in the root sections. This approach could be reconsidered if performed in an adequate lab with the right standards and enough expertise to overcome the difficulties that might arise. In addition, the use of laser microdissection for gene expression profiling should be investigated.

In addition, the use of gene promoter reporters of auxin related genes would allow us to localise spatially and temporally the distribution of auxin in the plant. Adaptation of the transport assay designed to measure auxin transport in roots, hypocotyls and inflorescences in *A. thaliana* by radiolabelled auxin or the movement of an auxin-induced gene expression signal (Lewis and Muday, 2009) could be adapted to the roots of *S. hermonthica* - infected rice plants. Based on the data in Chapter 3 and the strong co-regulation between auxins and strigolactones, we could hypothesise that the spatiotemporal distribution of auxin related genes would correlate to the patterns described for CCD8. However, this statement needs further investigations.

5.2 The role of strigolactones and auxin in the infection process

One of the most novel discoveries of this project was that strigolactones and most likely auxins play a role in the infection process of *S. hermonthica*. It is important to discriminate the role of these two plant growth regulators between the initial infection process and subsequent symptoms. Initially I proposed that an increase in the endogenous strigolactones levels were responsible for the characteristic inhibition of tillering in *S. hermonthica*-infected plants. However, strigolactone biosynthetic and signalling mutants did not support this hypothesis. The D10:GUS gene promoter reporter plants had a transient signal at the tiller bud area region, however, this could probably not be enough to decrease tiller bud outgrowth. Interestingly, an increase of activity of the promoter of the strigolactone biosynthetic gene D10 was detected at the site of attachment. Even though no auxin gene promoter reporter fusions were tested, a large number of auxin-related genes were differentially regulated in the root, stem and leaves of *S. hermonthica*-infected plants including those involved in transport, degradation and auxin response. In addition, induction of the expression of CCD8 (an enzyme involved in the biosynthesis of strigolactones) was detected in specific cells of the vascular bundle at the site of attachment of *S. hermonthica*. As the parasite penetrated the root and reached the endodermis, cells surrounding the host xylem vessels showed intense GUS staining indicative of high rates of transcription. The intensity of the staining increased further as the parasite penetrated the vascular core and fusion of host and xylem vessels took place from 3 to 6 DAI. After the infection process was complete, CCD8 gene expression was switched off, as shown by cross sections of the site of attachment by 9 DAI. It would be interesting to know at what point during the infection process cell specific up regulation of CCD8 occurs, *i.e.* from the very first events of penetration of the host root, when *S. hermonthica* breaks through the root epidermis, up to the point when parasite and host fuse their xylem vessels.

Expression of CCD8 could not be detected in cells outside the vascular bundle, *i.e.* the root cortex and epidermis at any of the three time points analysed. By collecting and processing *S. hermonthica* - infected root tissue of the same CCD8:GUS gene promoter reporter line over a much more detailed time scale, it would be possible to determine if cells in the vascular bundle or root cortex cells show transient expression of this gene before the parasite breaks through the endodermal barrier. If expression of CCD8 occurs in the vascular bundle of infected plants within the 24 hours after inoculation, before the parasite reaches the host endodermis cells, it would suggest that *S. hermonthica* induces a systemic response of strigolactone biosynthesis in its host before establishing successful xylem to xylem connections. The up

regulation of CCD8 in a cell specific manner, very close to parasite cells may be due to the existence of a mobile signal that is translocated from the parasite to the host. Fig 3.17 (3-D reconstruction of a segment of root as the parasite penetrated through the endodermis) clearly showed that upregulation of CCD8 expression occurred in host cells closest to parasite. Strigolactones or auxins could act as parasite effector molecules triggering alterations in amounts of plant growth regulators and / or the expression of genes involved in plant growth regulator plant metabolic and developmental pathways. It is has been known for many years that some fungal and bacterial pathogens produce plant hormones which act as effector molecules in host plants (Seilaniantz *et al.*, 2011). For example, the fungal pathogen *Gibberella fujikuroi* causes infected rice plants to grow very tall due to the production of fungal gibberellins which move into the host plant. However, in order to determine whether *S. hermonthica* produces plant growth regulator effector molecules it is necessary to show that the parasite is able to synthesise strigolactones and auxin and that they move into the host.

There is an increasing amount of evidence that parasitic plants do produce a range of plant growth regulators. Seedlings of the facultative parasitic plant *Tryphysaria versicolor* show localised auxin accumulation in the tips of radicles shortly after germination (Tomilov *et al.*, 2005), in a manner similar to that observed in the tips of lateral roots. Similarly, *Orobanch* spp. produce indole-3-acetic acid (IAA) during the infection process of *A. thaliana* seedlings, as demonstrated by disrupting auxin transport at the site of infection by localised application of compounds that influence activity or transport of IAA, resulting in decreased infection of the host roots (Bar-Nun *et al.*, 2008). Specifically this study revealed that *O. aegyptiaca* tubercles contained high levels of auxin after successful attachment to their host, although the significance of auxin is yet to be established. Most recently, it was reported that the hemiparasitic plant *Santalum album* (L.) has high concentrations of auxin and other plant growth regulators in attached haustoria during haustorial development as the parasite invades its host *Kuhnia rosmarnifolia* Vent (Zhang *et al.*, 2012). It is also interesting to note that, parasitic nematodes and growth promoting rhizobia bacteria also use the auxin signalling pathway to establish highly specialized feeding sites and nodules, respectively (Goverse *et al.*, 2000; De Meutter *et al.*, 2005; Grunewald *et al.*, 2009a; Grunewald *et al.*, 2009b). Further experiments are required to investigate whether *S. hermonthica* is able to synthesise its own plant growth regulators and whether these are transported into the host as effector molecules.

At present it is not known whether molecules move from *Striga* to the host at any stage during the parasite life cycle. There are two possible pathways through

which compounds may move from parasite to host. The first involves symplastic movement of compounds from parasite to host via plasmadesmata which may form at the host parasite interface within the root. The formation of secondary plasmodesma at the contact area of host-parasite cell walls in *Pisum sativum* infected by *S. gesnerioides* has been described (Dorr, 1996; Ehlers and Kollmann, 2000). The formation of plasmodesmata between parasite and host suggest that *S. hermonthica* might be able to develop such connections in the contact area to its host, leading to bidirectional flow of molecules. In addition, Taylor (2001) injected *S. hermonthica* plants attached to sorghum roots with dyes that move apoplastically (Calcofluor white and Lucifer yellow) or symplastically through plasmadesmata (5(6)-carboxyfluorescein) and demonstrated that all the dyes could be found in the sorghum host. If compounds move from *Striga* into the host plant apoplastically as the xylem vessels of host and parasite are connected this may only occur during the early stages of infection before the parasite emerges above ground. Once above ground, parasite transpiration is very high and, as host stomatal conductance is reduced following infection, high fluxes of water and nutrients occur from host to parasite perhaps making flow in the other direction very difficult. This is an area of parasitic research in which more sophisticated biochemical and microscopy-based studies are needed.

In conclusion, this project is consistent with the idea that plant growth regulators play a critical role in plant parasitism. We showed that changes occur early during infection and become accentuated in later stages of infection. However, we still do not have conclusive evidence that *S. hermonthica* induces changes in the regulation of components of the host plant growth regulator metabolic pathways and more research needs to be done in order to understand this. Once we understand how *S. hermonthica* triggers these responses on the host we should be able to counteract the negative effect of this noxious parasitic weed, leading to a solution of this problem by alleviating the detrimental alterations in the morphology of crops and increasing yield, therefore improving the life quality of millions of people in the African continent.

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