

Biosynthesis, regulation and biological role of strigolactones in rice

Catarina S. de Moura Luis Cardoso

Thesis committee

Promotor

Prof. Dr H.J. Bouwmeester
Professor of Plant Physiology
Wageningen University

Co-promotor

Dr C.P. Ruyter-Spira
Researcher, Laboratory of Plant Physiology
Wageningen University

Other members

Prof. Dr A.H.J. Bisseling, Wageningen University
Dr S. Al-Babili, University of Freiburg, Germany
Prof. Dr T.W.M. Kuyper, Wageningen University
Prof. Dr J.J.B. Keurentjes, Wageningen University

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Thesis

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Chapter I

General introduction

Catarina Cardoso

Importance of strigolactone research

Strigolactones are plant produced compounds first identified as germination stimulants of the root parasitic plant species of the genera *Orobancha*, *Phelipanche* and *Striga* (Cook et al., 1972; Yokota et al., 1998). Strigolactones are produced in the roots of the host plant and released into the rhizosphere. The seeds of the parasites germinate when a host root is nearby which the parasite recognizes by the perception of the strigolactones (Bouwmeester et al., 2007).

Upon germination, the radicle elongates towards the host and a haustorium develops that attaches to the hosts root, penetrates the tissues and establishes a xylem-xylem connection (Yoshida and Shirasu, 2012). From this stage onwards, the parasite gets water and photo-assimilates from the host. The seeds of the parasites contain only very few resources to sustain the parasitic plant before they connect with the host's xylem. Hence, spontaneous germination is almost inexistent in these species of parasitic plants and the perception of host-derived strigolactones and other germination stimulants, plays a crucial role for the survival of the plant parasite (Bouwmeester et al., 2007).

The life cycle of parasitic plants benefits from common agricultural practices. One single parasitic plant can produce and shed hundreds of dust-sized seeds on the soil. Ploughing promotes the dispersal of the parasite seeds that remained in the field from the previous season and are ready to parasitize the next crop (Parker, 2009). This results in a fast increase in the soil seed bank, in which the seeds remain viable for a period of 15 years or longer. Once a soil is infested it is extremely difficult to clean it and in many situations this has forced farmers to produce different crops that are not parasitized but less profitable or even to abandon the contaminated fields (Parker, 2009). Crop infestation by species of the *Orobancha* and *Phelipanche* genera is a serious economic problem in areas around the Mediterranean basin, in tobacco, tomato, rapeseed and several legumes. Parasites of the genus *Striga* infest cowpea, sorghum, maize and rice in Asia and sub-Saharan Africa where they pose a threat to food security.

The apparent disadvantage of strigolactone biosynthesis in plants was not understood until recently when several physiological and (additional) ecological

roles were discovered for these molecules. The first evolutionary advantage of strigolactone biosynthesis to be described was their role as signalling compounds for the establishment of symbiosis with arbuscular mycorrhizal fungi (AMF; Akiyama et al., 2005). Plants signal their presence to AMF by producing strigolactones and releasing them into the rhizosphere. Strigolactones induce AMF hyphal branching, increasing the chance of contact with the symbiotic partner and the establishment of root colonization by the symbiotic fungus. In line with this role, strigolactone biosynthesis and exudation into the rhizosphere is highly induced by low-phosphate nutrition (López-Ráez et al., 2008). Under low phosphate, the increased exudation of strigolactones into the rhizosphere promotes the establishment of the symbiosis which helps the plant to retrieve water and nutrients (especially phosphate) from the soil. The plant parasites apparently have evolved a mechanism to intercept this call. Crop infestation is worst in areas where low-input agriculture is practiced and the soils have low nutrient availability a condition that promotes strigolactone biosynthesis (Jamil et al., 2011; López-Ráez et al., 2008; Yoneyama et al., 2007a, 2007b). Because of all these roles, the study of strigolactone biosynthesis and regulation is interesting from an ecological perspective and important to find solutions to fight the parasitic weed problem.

Recent advances in strigolactone research

Biosynthesis and perception

Shortly after the discovery that strigolactones stimulate AMF hyphal branching, these compounds were also found to be plant hormones (Umehara et al., 2008; Gomez-Roldan et al., 2008). This was discovered as a result of efforts to identify strigolactone biosynthetic genes, following the hypothesis that strigolactones were derived from carotenoid cleavage products (Matusova et al., 2005). At the same time, mutants impaired in carotenoid cleavage enzymes had already been extensively studied for their increased branching phenotype in several species such as the *Arabidopsis more axillary growth (max)* mutants; the rice *dwarf (d)* and *high tillering dwarf (htd)* mutants; the *ramosus (rms)* mutants in pea and the *Petunia hybrida decreased apical dominance (dad)* mutants (Stirnberg et al., 2002; Booker et al., 2004; Arite et al., 2007; Bennett et al., 2006; Ishikawa et al., 2005). These mutants lacked the

synthesis or perception of a carotenoid derived mobile compound that could inhibit bud outgrowth. However, the corresponding compound was not yet identified by then. The discovery that strigolactones were the unknown regulatory molecule was a major breakthrough for strigolactone research.

Only quite recently it was demonstrated that strigolactones are derived from all-*trans*- β -carotene (Alder et al., 2012). The first committed step in strigolactone biosynthesis is performed by a carotenoid isomerase, first identified in rice – DWARF 27 (D27) – that isomerises all-*trans*- β -carotene to 9-*cis*- β -carotene. Afterwards, 9-*cis*- β -carotene is cleaved in two consecutive steps by two carotenoid cleavage dioxygenases, first CCD7 (MAX3/RMS5/DAD3 and in rice D17 or HTD1) producing 9-*cis*- β -apo-10'-carotenal, followed by cleavage and a molecular rearrangement performed by CCD8 (MAX4/D10/RMS1/DAD1) producing carlactone. The final biosynthetic step(s) of strigolactone biosynthesis are not yet elucidated. A cytochrome P450 enzyme identified in Arabidopsis (MAX1) acts downstream carlactone to produce strigolactones and might be involved in its conversion (Seto et al., 2014; Booker et al., 2005; Kohlen et al., 2011).

Structure elucidation and detection of strigolactones also have greatly advanced in the past years. The first strigolactone structures to be elucidated in 1972 were strigol and strigyl acetate, isolated from cotton (*Gossypium hirsutum*) and maize root exudates respectively (Cook et al., 1972). In the following years, more strigolactones were isolated and identified such as alectrol from *Vigna unguiculata* (Müller et al., 1992), sorgolactone from *Sorghum bicolor* (Hauck et al., 1992) and orobanchol from red clover (Yokota et al., 1998). Lately, the knowledge on the variety of strigolactone structures and detection power from root exudates or plant extracts have increased dramatically. Currently, there are about 15 different strigolactone structures identified (Xie et al., 2010). Plants produce generally a mixture of several strigolactones and this mixture differs between species.

Strigolactones share a common structure formed by two lactone moieties: a tricyclic lactone whose rings are commonly called A-, B- and C-rings linked by an enol-ether bridge to a butyrolactone D-ring. Strigolactones vary in substitutions that occur in rings A and B and are divided into two groups according to the stereochemistry of the C-ring in α - (orobanchol-like) or β -orientation (strigol-like)

(Zwanenburg and Pospíšil, 2013; Xie et al., 2013). The biosynthetic steps that are responsible for this diversification are not yet elucidated.

The D-ring and the enol-ether bridge are essential features for strigolactone activity with respect to the induction of parasitic plant seed germination, and the inhibition of bud outgrowth. AMF hyphal branching is also dependent on the D-ring but this can also be connected to the C-ring via an alkoxy or imino-ether bond and still induce hyphal branching (Zwanenburg and Pospíšil, 2013; Akiyama et al., 2010; Boyer et al., 2012). Variations of substituent groups on the AB-rings and the stereochemical orientation of the C-ring also have different impacts on AMF hyphal branching and seed germination. AMF were found to have increased sensitivity to certain types of AB-ring decorations but similar sensitivity to both orobanchol- and strigol-types (Akiyama et al., 2010). Also parasitic plant species differ in their sensitivity to strigolactone structures. *Striga hermonthica* and *Orobancha crenata* were found to be more sensitive to strigol-types while *Striga gesnerioides* only germinates in the presence of specific strigolactones and its germination can even be inhibited by some strigolactone variants (Nomura et al., 2013; Sugimoto et al., 1998). A study performed in pea suggests that apolar strigolactone forms are more active at inhibiting axillary bud outgrowth while stereochemistry does not seem to affect activity (Boyer et al., 2012). However, the structure-activity relationship studies done so far on the inhibition of bud outgrowth have not been as extensive as those testing AMF hyphal branching and parasitic seed germination.

The receptor for strigolactones in plants is probably an α/β -hydrolase - D14 in rice and Arabidopsis; DAD2 in petunia (Arite et al., 2009; Waters et al., 2012; Hamiaux et al., 2012). It has been shown in petunia and rice that DAD2/D14 hydrolyses GR24 and in the presence of this substrate interacts with D3 (MAX2/RMS4 or *petunia hybrida* MAX2a) (Hamiaux et al., 2012; Zhao et al., 2014). MAX2/D3 is an F-Box protein, and is a component of the SCF (SKP1-CULLIN-F-Box) complex involved in post-transcriptional regulation of downstream target proteins through ubiquitination (Vierstra, 2012; Stirnberg et al., 2002, 2; Zhao et al., 2014). One target of this complex has been identified in rice – DWARF53 (D53) – which promotes shoot branching (Zhou et al., 2013; Jiang et al., 2013). A dominant mutation in D53 caused by removal of a few amino acids results in a protein variant that is insensitive to strigolactone mediated degradation via the D14-D3 complex (Jiang et al., 2013).

This protein variant accumulates in the plant and induces extensive tillering and a dwarf phenotype (Jiang et al., 2013). In Arabidopsis, MAX2 was found to interact with BRI1-EMS-SUPPRESSOR 1 (BES1) to mediate its degradation, and also this effect is promoted by GR24 and AtD14 while BES1 negatively regulates shoot branching (Wang et al., 2013). D14 was also found to interact with SLENDER RICE1 (SLR1) which belongs to the family of DELLA proteins that mediate gibberellin responses (Nakamura et al., 2013). The interaction is dependent on the presence of strigolactones but it is still unclear what mechanisms are governed downstream of D14/SLR1 complex formation.

Endogenous roles of strigolactones

The first effect of strigolactones in plants to be described was the inhibition of bud outgrowth. It is not yet fully understood how strigolactones exert their inhibitory effect on axillary buds. Two different mechanisms have been proposed and may occur in parallel. Studies in pea show that strigolactones regulate expression of *Pisum sativum* BRANCHED1 (*PsBRC1*) in a MAX2 dependent manner (Dun et al., 2012; Brewer et al., 2009). *PsBRC1* is homologous to rice FINE CULM1 (FC1); maize TEOSINTE BRANCHED1 and Arabidopsis BRANCHED1 (BRC1) which are TCP (TB1, CYCLOIDEA, PCF domain) transcription factors expressed in buds that repress bud outgrowth. Direct strigolactone application to buds leads to fast down-regulation of *PsBRC1* (Dun et al., 2012). Additionally, *Psbrc1* mutants have increased branching and are insensitive to strigolactone application. This suggests that strigolactones directly suppress bud outgrowth. Earlier grafting studies in Arabidopsis show that production of strigolactones in the shoot or in the roots is sufficient to inhibit shoot branching (Simons et al., 2007; Morris et al., 2001; Sorefan et al., 2003; Booker et al., 2004, 2005). Strigolactones are transported via the xylem (Kohlen et al., 2011), however, it is as yet unclear if they are transported to the buds or if there is local strigolactone biosynthesis in the buds to directly inhibit their outgrowth.

In the other proposed mechanism for bud outgrowth inhibition, strigolactones are thought to regulate auxin transport in the stem (Shinohara et al., 2013; Domagalska and Leyser, 2011; Waldie et al., 2014). Auxin is produced in the leaves and shoot apex and is transported to the roots through the stem. The directional flow of auxin is mediated by PIN-FORMED1 (PIN1), an auxin efflux transporter and

the resulting auxin stream is referred to as the Polar Auxin Transport (PAT) stream. Strigolactones promote PIN1 protein recycling, thus decreasing its polar abundance in the cell membrane and reducing PAT capacity (Shinohara et al., 2013). The reduced capacity to transport auxin in the stem creates a competition for transport capacity between the auxin derived from the apex and the auxin present in the axillary bud. The transport of auxin from the bud to the main PAT stream is proposed to be necessary to develop vasculature tissue that connects the bud to the plant vascular system (Bennett et al., 2014). This connection is in turn required to allow bud outgrowth (Balla et al., 2011; Prusinkiewicz et al., 2009). Hence, strigolactones are proposed to inhibit bud outgrowth by suppressing this mechanism.

Strigolactones also have an effect on root development. The application of strigolactones promotes root and root hair elongation, induces longer root meristems and arrests lateral root emergence (Ruyter-Spira et al., 2011; Kapulnik et al., 2011). The effect of strigolactones on root development is partly explained by changes in auxin levels and transport however the underlying mechanisms by which strigolactones control auxin fluxes in roots are not yet understood (Koltai et al., 2010; Kapulnik et al., 2011; Ruyter-Spira et al., 2011).

The root apical meristem is divided in four zones: the meristematic zone where the cells have a few rounds of anticlinal divisions; the transition zone, where cells stop division and initiate elongation in transition to the following zone - the elongation zone, where cells, more than double their length and the differentiation zone, identified by the presence of vasculature and elongating root hairs. Root development is controlled by auxin fluxes from and to the apical root meristem (Blilou et al., 2005). Shoot derived auxin enters the root meristem via the main PAT stream, facilitated by PIN1. After reaching the Quiescent Centre (QC, a group of constantly dividing cells) auxin is distributed in the columella and lateral root cap and it is directed to the root epidermis. In the epidermal cells, auxin is transported basipetally (towards the hypocotyl). At the elongation zone, part of the auxin transported in the epidermis is redirected inwards to the pro-vascular cells, re-enters the main PAT stream and is transported back to the meristematic zone (Blilou et al., 2005). This reflux movement creates a relatively high auxin concentration in the root tip and maintains an auxin gradient that decreases towards the direction of the differentiated cells. The control of this gradient is proposed to determine the length

of the root meristematic zone and the rate of primary root elongation (Grieneisen et al., 2007). Application of strigolactones to roots stimulates its elongation and leads to longer and less well defined zonation of the meristematic zones which resembles what is predicted in modelling studies of auxin transport where the reflux at the transition zone is diminished (Ruyter-Spira et al., 2011; Grieneisen et al., 2007). This suggests that strigolactones reduce auxin reflux, however, the direct target(s) of strigolactones in the primary root meristem are still unknown (Ruyter-Spira et al., 2011, 2013). Auxin fluxes and homeostasis in the root meristem are mainly controlled by several PIN proteins, each with different cell polarities and tissue localization, hereby directing auxin fluxes (Blilou et al., 2005). In the apical root meristem, the abundance of PIN1, 3, and 7 are reduced by strigolactones but the effect is observed only a few hours after treatment suggesting that this is a secondary effect rather than the targeted PIN1 protein degradation that is observed in the stem (Ruyter-Spira et al., 2011; Shinohara et al., 2013).

The positive effect of strigolactones on root hair elongation is also dependent on auxin transport (Koltai et al., 2010). Moreover, this effect is non-cell autonomous because expression of MAX2 under an endodermis-specific promoter is sufficient to induce root hair elongation upon strigolactone treatment (Koren et al., 2013). Hence, the effect of strigolactones on root hair elongation appears to be an indirect effect of the auxin homeostasis in the epidermal cells.

Strigolactones arrest lateral root development before the emergence of newly formed lateral roots (Ruyter-Spira et al., 2011; Kapulnik et al., 2011). It has been proposed that the reduction of PIN1 protein levels and PAT stream depletes the lateral root primordia of auxin, necessary for the emergence to form lateral roots (Ruyter-Spira et al., 2011; Shinohara et al., 2013). Interestingly, under phosphate limiting conditions, strigolactones have the opposite effect on lateral root development, promoting lateral root emergence (Ruyter-Spira et al., 2013). This is thought to be a consequence of a different auxin status of the plant which is more responsive to auxin under low phosphate (Ruyter-Spira et al., 2013). Indeed, modelling studies have shown that the response to strigolactones changes according to the auxin status/sensitivity of the plant (Ruyter-Spira et al., 2011; Shinohara et al., 2013). The underlying mechanism for the observed response shift is however not yet understood.

The biosynthesis of strigolactones is upregulated under low phosphate (López-Ráez et al., 2008; Jamil et al., 2011). It was described above how this regulation is important to promote the symbiosis with AMF that helps the plant to retrieve phosphate from the soil (Akiyama et al., 2005; Umehara et al., 2008). The recent findings on the endogenous effects of strigolactones show that these hormones also mediate the plant's response to low phosphate availability, preparing the plant to optimally deal with the low phosphate availability. During low-phosphate conditions, strigolactones are necessary to reduce the shoot to root ratio, through the inhibition of shoot branching and an increase in lateral root formation (Umehara et al., 2010; Kohlen et al., 2011; Ruyter-Spira et al., 2011). This response shifts the resource usage from the shoots to the roots promoting exploration of scarce nutrients in the soil.

New physiological roles for strigolactones are emerging in recent reports such as promotion of secondary growth, involvement in seed germination, and interaction with light signalling pathways (Tsuchiya et al., 2010; Agusti et al., 2011; Toh et al., 2012).

Strigolactones are also found to be produced by liverworts, mosses and in some algae (Delaux et al., 2012). In the bryophyte *Physcomitrella patens* strigolactones are involved in detection of conspecific individuals and regulation of protonema growth (Proust et al., 2011) whereas in algae, strigolactones stimulate rhizoid elongation (Delaux et al., 2012).

An increasing number of carotenoid derived signalling compounds

The importance of carotenoids in plants is well known for their photo-protective and light-harvesting roles (Cazzonelli and Pogson, 2010). Carotenoids are also precursors of abscisic acid, a hormone that mediates drought stress responses and the recently characterized strigolactones (Cazzonelli and Pogson, 2010).

Additionally, carotenoids are precursors for mycorradicin and two yet unidentified compounds: an inhibitor of shoot development (BYPASS) and the second involved in lateral root initiation (Van Norman et al., 2004; Walter et al., 2010; Van Norman et al., 2014).

Mycorradicin accumulates in roots colonized by arbuscular mycorrhiza and although its role is not yet understood, it seem to influence the senescence of

arbuscules – mycorrhizal organs that develop inside the plant cells and mediate nutrient exchange between the symbiotic partners (Walter et al., 2010). BYPASS is reported to be a graft-transmissible root derived compound that inhibits shoot and leaf development. The evidence for the existence of this compound was found in a loss of function mutant, impaired in a repressor of BYPASS biosynthesis, BPS1 (Van Norman et al., 2004). The last reported carotenoid derived signalling compound, which structure has not yet been identified, is a putative shoot derived signal that triggers lateral root initiation in coordination with auxin gradient oscillations in the apical root meristem (Van Norman et al., 2014).

During the studies on strigolactone biosynthesis, it was found that CCD7 and CCD8 orthologs from rice, *Arabidopsis* and pea produce β -apo-13'-carotenone directly from all-*trans*- β -carotene (Alder et al., 2008, 2012; Schwartz et al., 2004). This apocarotenoid is commonly called d'orenone and is found to block root hair elongation and gravitropic responses in *Arabidopsis* roots and to modulate auxin transport by affecting the auxin efflux transporter PIN2 (Schlicht et al., 2008). The synthesis of d'orenone has so far only been reported *in vitro* or using heterologous expression in *E. coli*. However, the effects of d'orenone on *Arabidopsis* roots suggest that d'orenone or a downstream product could be yet another carotenoid derived signalling compound. The biosynthetic pathways of d'orenone and strigolactones share two enzymes. Hence, d'orenone could potentially interact with the strigolactone biosynthesis pathway by means of substrate competition for an enzyme or by indirect feedback regulation. These are interesting aspects regarding the regulation of strigolactone biosynthesis and will be also investigated in this thesis.

Objectives of this thesis

Upon discovery of the role of strigolactones as promoters of beneficial plant-microbe interactions and modulators of plant architecture, it became evident that development of cultivars with reduced strigolactone biosynthesis could have undesired effects on crop performance. The present work is therefore focused on getting a better understanding of the biosynthesis of strigolactone, its regulation and of the contribution of strigolactone structural variation to parasitism and symbiosis.

To carry out this study we have used rice and its parasite *Striga hermonthica* as models. Rice has the advantage of being a model species for grasses and a crop itself, and like other economically important grass species is affected by parasitic plants of the genus *Striga*. Moreover, rice also establishes symbiosis with arbuscular mycorrhiza which enables us to compare the impact of strigolactone biosynthesis on the interaction with both the parasite and the symbiotic fungus.

A short description of the chapters in this thesis is presented below:

Chapter 2

In this chapter I have reviewed some aspects of strigolactone biology. The life cycle of the parasitic plants of the genus *Striga*, *Orobanche* and *Phelipanche*, the detrimental effects on the host plants, the economic impact and the ecological significance of strigolactones in underground communication is reviewed. This chapter also describes the strigolactone biosynthetic pathway, its regulation and the emerging physiological roles of strigolactones in the host plants. I also discuss how the perception and signalling pathway might have evolved in parasitic plants, and finally how various strategies may lead to possible solutions to control parasitic weeds. This review was published after peer-review in the journal *Plant Science*.

Chapter 3

To have a better understanding of our model system, in this chapter I have profiled rice root exudates using chromatography fractions to identify stimulants of *Striga hermonthica* seed germination and *Gigaspora rosea* hyphal branching. I show how structural differences in the active compounds contribute to different extents to the interaction with both the parasite and the symbiotic partner. This work was been accepted for publication in the peer-reviewed journal *PLOS ONE*.

Chapter 4

In this chapter I describe the detection of a quantitative trait locus involved in strigolactone biosynthesis in rice, using a well characterized RIL population derived from a cross between Bala and Azucena cultivars for which it had previously been

Chapter 1

reported that they exhibit differential tolerance to *Striga hermonthica* parasitism. The two cultivars produce different amounts of strigolactones and this variation can be almost completely explained by a rearrangement in chromosome 1 where two *MAX1* orthologs (*SLB1* and *SLB2*) are deleted. Moreover, this polymorphism was found to be highly associated to the indica/japonica split in the rice population. This work was published in the peer reviewed journal *Proceedings of the National Academy of Sciences of the USA*.

Chapter 5

Here, I investigate a possible interaction between the biosynthetic pathway of strigolactones and d'orenone and their physiological effects. I compare the effects of strigolactones and d'orenone on tillering and root development using mutant lines for strigolactone biosynthesis. I also investigate a possible feedback regulation of d'orenone application on the expression of strigolactone biosynthetic genes. A model is proposed explaining the possible effect of strigolactones and d'orenone on auxin transport and root development.

Chapter 6

In this chapter, I discuss the results obtained in this thesis and the new advances in strigolactone research. I focus on the conservation of strigolactone biosynthesis across Viridiplantae, on how diversification of strigolactones is possibly achieved and the potential evolutionary advantages of structural diversification of strigolactones in a three party co-evolution system between host-plant, AMF and parasites.

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Strigolactones and root infestation by plant-parasitic *Striga*, *Orobanche* and *Phelipanche* spp.

Catarina Cardoso^a,
Carolien Ruyter-Spira^a & Harro J. Bouwmeester^{a,b}

^a *Laboratory of Plant Physiology, Wageningen University, The Netherlands*

^b *Centre for Biosystems Genomics, Wageningen University, The Netherlands*

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Abstract

Strigolactones are signaling molecules that play a role in host recognition by parasitic plants of the *Striga*, *Orobanche* and *Phelipanche* genera which are among the most detrimental weeds in agriculture. The same class of molecules is also involved in the establishment of the symbiosis of plants with arbuscular mycorrhizal (AM) fungi. In addition, the strigolactones are being shown to be involved in an increasing number of physiological processes in plants, such as the regulation of plant architecture and the response to abiotic factors such as nutrient availability and light. These new findings suggest that biosynthesis and perception of strigolactones are conserved throughout the plant kingdom. The structural variation in the strigolactones discovered so far and its possible role in host recognition by the parasites and AM fungi as well as the evolution of strigolactone-dependent-germination in parasitic plants will be discussed. Finally, due to the recent advance in strigolactone research, new insights are emerging on the relation between parasitic and host plants which may result in new strategies to control parasitic plant infestation that will be discussed in this review.

Introduction

Strigolactones have recently entered into the spotlight of plant biology research. These plant metabolites were for many years known to play a role in host recognition by parasitic plants of the genera *Striga*, *Orobanche* and *Phelipanche* (Cook et al., 1966; Bouwmeester et al., 2003). The recent discovery of their signaling function in the symbiosis of plants with arbuscular mycorrhizal (AM) fungi (Akiyama et al., 2005; Besserer et al., 2006), and the regulation of shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008) have strongly increased the effort of plant biologists to investigate strigolactones and their role in plant physiology. In this review we will discuss the role of strigolactones in plant–plant parasitism, considering the newly discovered roles of strigolactones. The evolution of the strigolactone signaling pathway in the transition of plant species to obligatory parasitism and the use of our new knowledge for improvement of parasitic weed control and breeding of resistant crop varieties will be discussed.

Root parasitic plant species and their life cycle

Plant species of the genera *Striga*, *Orobanche* and *Phelipanche* are root parasites that affect many important food crops. These three genera, belonging to the Orobanchaceae family, are among the parasitic plant species considered to cause highest negative impact on food production (Parker, 2009). The witchweeds from the genus *Striga* are obligate hemi-parasites. Although they retain some photosynthetic capacity, *Striga* species have an absolute host requirement to develop and complete their life cycle (Aly, 2007). *Striga* spp. occur in tropical and subtropical regions, with special incidence in Sub-Saharan Africa infecting mainly cereal crops. The most damaging is *Striga hermonthica*, followed by *Striga asiatica* and *Striga gesnerioides*.

The broomrapes, of the genera *Orobanche* and *Phelipanche*, cause similar damage to food production as the *Striga* spp. Some species of the genus *Orobanche* had their classification recently altered to the genus *Phelipanche* (Joel, 2009). *Orobanche* and *Phelipanche* spp. are obligate holo-parasites, lacking the ability to photosynthesize, and rely completely on their host for nutritional needs. The *Orobanche* and *Phelipanche* spp. can infect many plant species mostly Solanaceae

and Fabaceae. In some cases, the high infestation levels in the soil have forced farmers to abandon their fields or change crops (see Parker et al. 2009 for additional data on world-wide crop infestation by these parasitic plants).

The life cycles of *Striga*, *Orobanche* and *Phelipanche* spp. share many similarities. All of them produce minute seeds that remain viable in the soil for up to fourteen years (Bebawi et al., 1984). Once germination has been triggered, the radicle protrudes from the testa, elongates towards the host root and develops a haustorium, an organ that can attach to and penetrate the roots of the host plant. In *Striga* spp., the haustorium establishes a xylem–xylem connection with the host from where it can withdraw water and nutrients. *Phelipanche* and *Orobanche* spp. form connections with both phloem and xylem. Given the low transpiration rates observed in these latter species most nutrient exchange between host and parasite probably occurs via a phloem continuum provided by interspecific plasmodesmata (Shen et al., 2006). In contrast, the high transpiration rates observed in *Striga* spp. – that have permanently open stomata – suggest that most of the photoassimilates are obtained from the host via the xylem–xylem connection. After the establishment of the connection with the vascular bundles, the parasite starts taking up water and nutrients from the host, develops shoots and flowers and produces new seeds that will increase the existing seed bank in the soil (Bouwmeester et al., 2003). The fast development of the parasite contrasts with the stunted growth of the host. In *Orobanche* and *Phelipanche* spp. the reduction in biomass of infected hosts can be largely explained by the biomass accumulation of the parasite. However, the strong depression of the host growth caused by *Striga* spp. is not correlated with the increase of parasite biomass. The negative impact on host growth in *Striga* infected plants can already be observed even before the parasite has emerged from the soil, suggesting that *Striga* spp. have a pathological or phytotoxic effect on the host plant. The extent of the damage caused by *Striga* parasitism differs among different host species and also within the same species. Different cultivars may differ in their capacity to tolerate the physiological and pathological effects caused by *Striga* parasitism finally resulting in milder or stronger impacts on crop yield (Swarbrick et al., 2008). The nature of the *Striga* toxicity is not yet understood but some studies show that *Striga* infection reduces transpiration and photosynthesis of the host

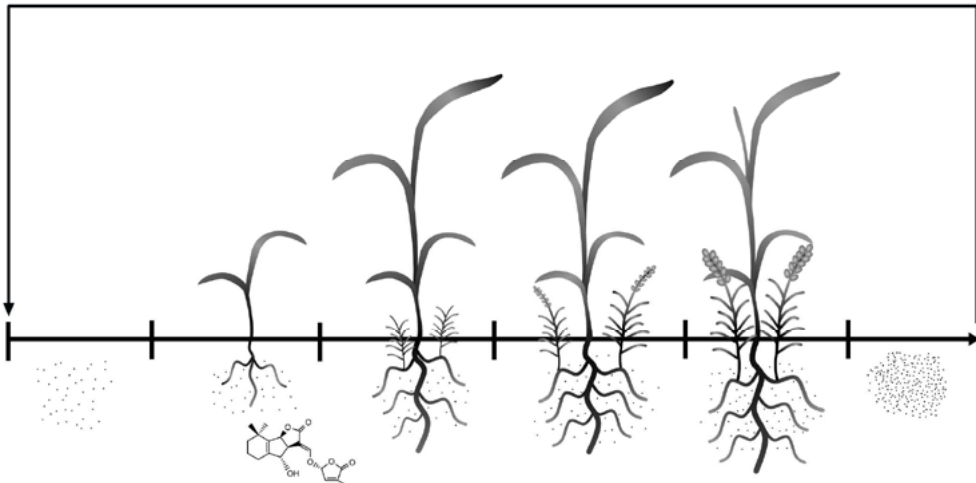


Figure 1. Life cycle of the parasitic plants of the *Striga*, *Orobanche* and *Phelipanche* genera. A potential host plant grows in a soil infected by parasitic plant seeds and triggers parasitic plant seed germination by producing germination stimulants, particularly strigolactones. The radicle of the germinating seed attaches to the host root and forms a haustorium. The parasite develops, flowers and produces a large amount of seeds that further increase the seed bank existing in the soil.

plant which is accompanied by increased levels of ABA (Hibberd et al., 1996; Frost et al., 1997; Gurney et al., 1999).

One of the most important aspects of the life cycle of *Striga*, *Orobanche* and *Phelipanche* spp. is the induction of germination (Figure 1). The seeds of these parasites are tiny and their resources can sustain the germinated seedling only for a very short period of time until it reaches a host root. Hence, germination needs to be precisely timed to assure the presence of a host root nearby. This is accomplished by perceiving compounds produced by the host plant that are secreted into the rhizosphere and stimulate the germination of the seeds.

Before germination can occur, seeds need to undergo warm stratification to become responsive to the germination stimulants (Matusova et al., 2004). Only after this conditioning process, parasitic plant seeds will germinate if exposed to specific compounds produced by the host plants. The duration of conditioning and factors such as temperature, moisture and exposure to light influence the sensitivity of the seeds to the germination stimulants (Chae et al., 2004; Matusova et al., 2004). Little is known about the biological mechanisms acting during conditioning of parasitic plant seeds except for a reported accumulation of cAMP and gibberellins during

preconditioning of *Orobanche minor* (Uematsu et al., 2007). Stratification is a common process in many plant species required to break dormancy and allow seeds to germinate, but in the special situation of *Striga*, *Orobanche* and *Phelipanche* spp. the requirements for germination after stratification are different from other species (Matusova et al., 2004). That is, the stratified seeds of the root parasites also need to be exposed to a germination stimulant. Indeed, in germination bioassays only a very low rate of spontaneous germination (in water, without the presence of a germination stimulant) can be observed in *Orobanche* and *Phelipanche* spp. (1–3%) and in *Striga* spp. spontaneous germination rarely occurs. If conditioned seeds are not exposed to a germination stimulant and germination does not occur, their sensitivity gradually decreases again and the seeds enter into secondary dormancy (Matusova et al., 2004). Parasitic plant seeds remain viable for up to fourteen years in the soils probably undergoing changes in dormancy (which equals sensitivity to germination stimulants) repeatedly during that period of time (Bebawi et al., 1984).

The first germination stimulant for *Striga* that was identified was called strigol and it was isolated from cotton (*Gossypium hirsutum* L.) root exudates (Cook et al., 1966). Since then, many other compounds with similar structure and with germination stimulatory properties were discovered in root exudates of host plant species (Xie et al., 2010). These compounds, classified as strigolactones, stimulate germination of parasitic plant seeds in minute amounts (Joel, 2000; Bouwmeester et al., 2003). Host plants also produce strigolactones at extremely low rates and it is very laborious to isolate these compounds in sufficient quantities for their analytical characterization. Parasitic plant seeds also germinate upon the perception of other plant-produced compounds such as dihydrosorgoleone, sesquiterpene lactones, kinetin, coumarin, jasmonate; and fungal metabolites (fusicoicins, cotylenin) (Xie et al., 2010). However, the sensitivity to these compounds is generally much lower and not always shared by all three parasitic plant genera, making the strigolactones by far the most relevant compounds for the regulation of parasitic plant seed germination (Bouwmeester et al., 2003).

Ecological significance of strigolactones

As we have reviewed above, strigolactones were initially discovered as germination stimulants of the parasitic *Striga*, *Orobanche* and *Phelipanche* spp. and are exuded into the rhizosphere by the roots of their host plants. It was at that time unclear why plants would produce these compounds considering that they promote plant parasitism. The solution to this dilemma came when strigolactones were reported to induce hyphal branching of AM fungi (Akiyama et al., 2005; Besserer et al., 2006). AM fungi establish a symbiotic interaction with the vast majority of land plants. In the symbiosis, the fungus supplies water and nutrients, which it can obtain very efficiently from the soil, to the plant. In exchange, the plant provides photoassimilates to the fungus. Phosphate is one of the most important elements in this symbiotic interaction. Plants can only assimilate this mineral in the inorganic form, orthophosphate, which is very immobile in soils. Consequently the area surrounding the roots is usually quickly depleted. AM fungi extend their hyphae far beyond the root rhizosphere zone and explore the soil with a fine network of mycelium that can absorb not only inorganic but also organic forms of phosphate. When plants are subjected to a shortage in the available phosphate the production and release of strigolactones into the rhizosphere are increased (Yoneyama et al., 2007b; López-Ráez et al., 2008). AM fungi perceive this signal and respond with extensive hyphal branching. This process increases the chance of encountering the roots of the host plant and hence assists in establishing the symbiosis. The *Striga*, *Orobanche* and *Phelipanche* spp. have likely evolved a mechanism to hijack this communication signal and turn it into a germination inducing signal to respond in the presence of a suitable host. Nitrogen is also traded in the symbiotic interaction and its deprivation also triggers an increase in the production of strigolactones in sorghum (Yoneyama et al., 2001, 2007a).

Field experiments show that AM symbiosis delays the emergence and reduces the number of *Striga* parasites on sorghum (Lendzemo et al., 2005). Root exudates of sorghum plants colonized by AM fungi have less germination stimulatory activity (Lendzemo et al., 2007). In tomato, the decrease in parasitism by *Phelipanche ramosa* upon AM colonization also correlated with a lower induction of germination of seeds of this parasite by the root exudates. Subsequent LC-MS analysis showed that the

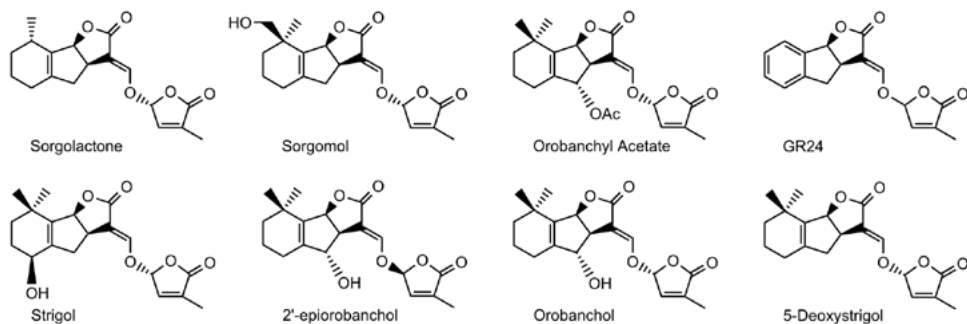


Figure 2 Chemical structure of strigolactones. Strigolactones share a common ABCD ring signature and displays variation in the decorations of the A and B rings and sometimes stereoisomerism. The figure shows several naturally occurring strigolactones and the synthetic strigolactone analog GR24.

root exudates of colonized plants indeed contained lower amounts of strigolactones (López-Ráez et al., 2011). These results suggest that AM fungal colonization likely induces resistance to plant parasitism by reducing the exudation of strigolactones although uptake of the strigolactones by the AM fungi cannot be excluded as yet. It is not yet clear if AM fungi down-regulate the strigolactone biosynthetic pathway directly via the symbiotic interaction or indirectly, as a consequence of a better nutritional status of the host plant. Some new data point at a direct effect of AM colonization on strigolactone biosynthesis. Walter et al. (2010) show that an intermediate of the strigolactone biosynthetic pathway can also be converted into mycorradicin, an apocarotenoid that accumulates in mycorrhized roots. Possibly, these two biosynthetic pathways compete with each other, which could result in reduced strigolactone production upon AM colonization. The development of *Striga* on sorghum plants colonized by AM fungi was also shown to be delayed (Lendzemo et al., 2005) suggesting also AM fungi-induced post-attachment resistance. Colonization by AM fungi can also improve the host plant defense against other pathogens such as pathogenic fungi and herbivores (Pozo and Azcón-Aguilar, 2007; Wehner et al., 2010).

Strigolactones and host specificity

Strigolactones share a common structure, consisting of an ABC ring-system connected to a butyrolactone group (the D ring) via an enol ether bridge (Figure 2). While all

strigolactones have this common structure, there are many chemical variations consisting of substituents on the cyclohexyl A-ring and various combinations of hydroxyl and/or acetate substituents on the A- and B-rings. The C- and D-rings are the same in all known strigolactones with the exception of the 2'-epi orientation of the enol-ether bridge. To date about twelve different strigolactones have been structurally characterized and for some more the existence was demonstrated with GC-MS and/or LC-MS but without unambiguous structural proof using NMR (Xie et al., 2010).

One single plant species can produce several different types of strigolactones and in different quantities (Awad et al., 2006; Xie et al., 2007; López-Ráez et al., 2008). Within the same species, different varieties may also produce different combinations of strigolactones. The structural variants of the strigolactones may have different germination inducing activity on different parasitic plant species. For example, 2'-epiorobanchol is much more active than strigol and solanacol in eliciting *O. minor* and *P. ramosa* germination, while orobanchol has intermediate activity (Xie et al., 2007). Also, *S. gesnerioides* is highly sensitive to orobanchyl acetate whereas its germination is not stimulated by the synthetic strigolactone analogue GR24, which is active in most other species of the three parasitic plant genera (Yoneyama et al., 2009). There are also differences in sensitivity within the same parasitic plant species to exudates of different hosts (Matusova and Bouwmeester, 2006).

Just as for parasitic plant seed germination, the structural differences between strigolactones also have an influence on the AM fungal branching response (Akiyama et al., 2010). In a study on rice, different HPLC fractions of root exudates were assayed for their parasitic plant seed germination and AM hyphal branching stimulatory activities (Cardoso et al., unpublished results). Some fractions were very active in stimulating seed germination but had low AM hyphal branch inducing activity and vice versa, showing that the structural features required for hyphal branching and germination are not the same.

An intriguing question is which factors have led to the development of such a large structural variation in the strigolactones. From an evolutionary point of view, the production of a strigolactone with reduced germination stimulatory activity for parasitic plants but with high hyphal branching stimulatory activity would confer an advantage to a host plant. However, the out-crossing nature of many of the parasitic

plants and the huge genetic potential in the seed banks, may allow for fast adaptation of the parasites to new strigolactone structures due to the strong selection pressure. New strigolactone variants or a novel combination of strigolactones with very low germination stimulatory activity, will only induce germination of a very small percentage of seeds resulting in low levels of parasitism. However, parasitic plants produce an enormous amount of seeds and few successful infections are sufficient to quickly enrich the existing seed bank with a genotype that is more sensitive to the new strigolactone combination. Furthermore, the large seed quantities confer a much faster adaptation potential to the parasite than the host can adapt to produce yet another strigolactone structure. This arms race potentially results in a higher plasticity of the parasite to perceive host exudates, compared with the hosts' specialization to attract AM fungi but not their parasites. Evolutionary change in the sensitivity to exudates of a specific host was for example observed in *P. ramosa* (Matusova and Bouwmeester, 2006). Seeds of *P. ramosa* parasitizing rapeseed were much more responsive to exudates of *Arabidopsis* than *P. ramosa* seeds collected from tobacco and vice versa. In contrast, the latter seed batch was much more responsive to tomato than to *Arabidopsis* root exudates.

Physiological role of strigolactones

Recent findings have given strigolactones even greater importance in plant biology. Besides their ecological relevance as rhizosphere signaling molecules, strigolactones are also involved in the regulation of shoot branching. These findings emerged from studies with mutants of *Arabidopsis*, rice and pea that are impaired in the inhibition of axillary bud outgrowth, which gives them a bushy and sometimes also dwarf phenotype. These mutants are impaired in the biosynthesis and perception of strigolactones or a close derivative (Gomez-Roldan et al., 2008; Umehara et al., 2008). Application of strigolactones recovers inhibition of axillary bud outgrowth in biosynthetic mutants but fails to complement the perception mutants. Highly branched or tillered mutants are described in several species: in *Arabidopsis* the *max* mutants (*more axillary growth*), *dwarf* or *htd* (*high tillering dwarf*) in rice, *dad* (*decreased apical dominance*) in petunia and *rms* (*ramosus*) in pea (Rameau, 2010). Strigolactones had already been postulated to originate from the carotenoid pathway

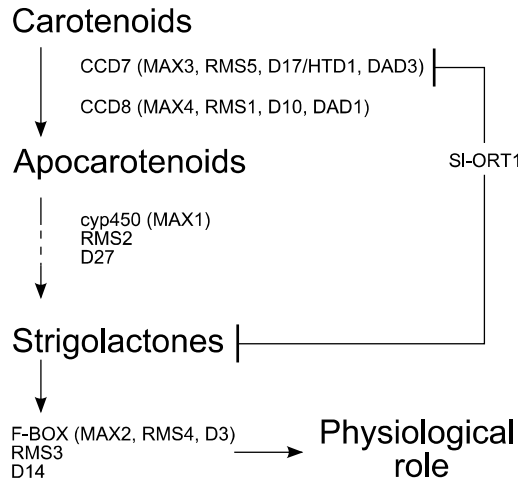


Figure 3. Proposed model for the strigolactone biosynthesis and signaling pathway. The scheme shows the genes (and corresponding mutants in each species) so far known to participate in the strigolactone biosynthetic and signaling pathway finally leading to a physiological response. Strigolactones are derived from the carotenoids through two subsequent enzymatic cleavage steps performed by the carotenoid cleaving dioxygenases CCD7 and CCD8 described in Arabidopsis (MAX), Rice (D/HTD), pea (RMS) and petunia (DAD). For further conversion into strigolactones, a cytochrome P450 enzyme (cyp450) has been reported to be required in Arabidopsis (MAX1) while two other proteins, identified in rice (D27) and pea (RMS2) whose functions have not yet been clarified, must also be involved in strigolactone biosynthesis. In the tomato mutant SI-ORT1, in which the mutation is still unidentified, strigolactone biosynthesis and CCD7 expression are both downregulated. The perception of strigolactones and/or the downstream signaling pathway are mediated by an F-box leucine rich protein (MAX2, D3, RMS4), an α/β -hydrolase found in rice (D14) and a protein of unknown function in pea (RMS3).

(Humphrey et al., 2006; Matusova et al., 2005) and characterization of the branching/tillering mutants confirmed that strigolactones are predominantly produced in the roots by cleavage of carotenoids by the carotenoid cleavage dioxygenases, CCD7 and CCD8 (MAX3 and MAX4 in Arabidopsis, RMS5 and RMS1 in pea, D17/HTD1 and D10 in rice; see Figure 3) (Gomez-Roldan et al., 2008; Umehara et al., 2008). The precursor carotenoid and the apocarotenoid resulting from cleavage have not yet been identified, and the subsequent steps of the biosynthesis not yet fully elucidated but several other enzymes that must be involved in strigolactone biosynthesis and strigolactone perception/downstream signaling have been identified, such as MAX1, RMS2 and D27 (Figure 3). Genes involved in downstream signaling are MAX2, RMS4, D3 (encoding an F-BOX protein), RMS3 and D14 (Figure 3).

Strigolactones also seem to be involved in the inhibition of shoot branching under phosphate starvation. Arabidopsis and rice mutants impaired in strigolactones biosynthesis fail to reduce bud outgrowth in response to phosphate starvation (Umehara et al., 2010; Kohlen et al., 2011). The physiological role of strigolactones in the regulation of shoot branching can also explain why the biosynthetic pathway of strigolactones is maintained in the non-mycorrhizal Arabidopsis. Indeed, strigolactones have been detected in Arabidopsis root exudates and xylem sap (Kohlen et al., 2011; Goldwasser et al., 2008). Under phosphate starvation, the abundance of strigolactones in the xylem sap is increased which correlates with the reduction in shoot branching. Curiously, the amount of strigolactones in Arabidopsis root exudates is also increased by phosphate starvation (Kohlen et al., 2011). In mycorrhizal species the increase of strigolactone biosynthesis and active exudation into the rhizosphere is anticipated to increase the chances to establish symbiosis with AM fungi. In the case of the non-mycorrhizal Arabidopsis the ecological relevance of strigolactone exudation into the rhizosphere – if any – is not yet understood.

Recently, a number of reports have shown that strigolactones are likely to be involved in an increasing number of other plant physiological processes. The application of GR24 to tomato roots increased root elongation through enhanced cell elongation (Koltai et al., 2010a). Higher concentrations of GR24 induced asymmetric root growth and inhibition of root hair elongation. In Arabidopsis, application of GR24 also affects root elongation and lateral root development (Ruyter-Spira et al., 2011). Another recent study showed the possible involvement of strigolactones in regulating light harvesting mechanisms (Mayzlish-Gati et al., 2010). Also MAX2 (the Arabidopsis *max2* mutant is insensitive to strigolactones) seems to be involved in light signaling pathways and has delayed leaf senescence (Shen et al., 2007; Woo et al., 2001).

Evolutionary aspects of strigolactone biosynthesis and the strigolactone signaling pathway

Putative homologs of strigolactone biosynthetic genes *CCD8* and *CCD7* are also found in Bryophytes and algae. This suggests that the strigolactone biosynthetic pathway is conserved throughout the whole plant kingdom. The symbiotic interaction between angiosperms and AM fungi in which strigolactones participate is thought to date

back over 400 million years and is believed to have played an important role in the migration of plants from water to land (Harrison, 2005). Therefore, the strigolactone biosynthesis and signaling pathway are probably ubiquitous throughout the plant kingdom. When the genera *Striga*, *Orobanch*e and *Phelipanche* evolved parasitism, they must have adapted the existing strigolactone biosynthesis and signaling pathway according to the needs of their new life cycle. They have probably evolved by adapting existing perception and signaling mechanisms to use strigolactones as an (exogenous) germination trigger (Westwood et al., 2010). This adaptation could have occurred by temporarily suspending or down-regulating their own strigolactone biosynthesis in the seeds. It is not clear if strigolactones are involved in seed germination of other plant species, but strigolactones can break dormancy of *Lactuca sativa* and *Avena fatua* (Westwood et al., 2010) and the *max2* mutant in *Arabidopsis* is less sensitive to red light induced seed germination (Shen et al., 2007). Under normal germination conditions, however, there are no clear differences in the germination phenotype of the *max* mutants if compared to their wild type backgrounds Col-0 and Ler (Tsuchiya and McCourt, 2009). Nevertheless, it is argued that these accessions have mild seed dormancy and perhaps this obscures possible effects of strigolactones on seed dormancy/germination (Tsuchiya and McCourt, 2009). If in fact strigolactones are not normally involved in seed germination, then parasitic plants may have adapted existing mechanisms to break seed dormancy/induce germination to enforce strigolactone dependency. Intriguingly, forest-fire succession plant species germinate only after exposure to plant derived smoke compounds, the karrikins, which partially resemble strigolactones (Chiwocha et al., 2009; Daws et al., 2008). This fact supports the hypothesis that adaptation of the signaling pathway to break dormancy to respond to different compounds (such as strigolactones or smoke derived compounds) may have occurred several times in evolution. Understanding the mechanisms of strigolactone perception in parasitic plants – that is likely based on the signal perception present in non-parasitic plants (Figure 3) – is of interest to gain more insight in the mechanisms of host recognition and to understand how *Striga*, *Orobanch*e and *Phelipanche* species have evolved towards strigolactone dependency in seed germination.

Control of parasitic plant infestation

The parasitic plant problem can be approached from several sides, such as control methods based on soil management, biological control and breeding for resistant cultivars. Each measure can alleviate *Striga* and *Orobanche/Phelipanche* damage only to a limited extent. To recover crop yield to similar levels as on non-contaminated soils, several strategies must be implemented in a coordinated manner. It was demonstrated and discussed earlier in this review that colonization by AM fungi can also decrease infestation by parasitic plants (Lendzemo et al., 2007). This control method also has other advantages because acquisition of water and soil nutrients is more efficient in colonized plants, an important aspect in the agricultural areas with few resources where parasitic plants are most prevalent. One of the mechanisms by which AM fungi reduce the infection of crops by parasitic plants is likely the improved phosphate availability and resulting lower strigolactone exudation (Jamil et al., 2011). If farmers can afford to buy fertilizers, direct application of particularly phosphate would be an alternative strategy to reduce strigolactone exudation and hence parasitic weed infection.

Efforts have also been directed at developing new crop varieties that are less susceptible to parasitic plants. Different varieties of the same host species may have different tolerance to parasitic plants. In tolerant varieties the yield is less affected by parasitism (Gurney et al., 2006). Some varieties of host species like rice and sorghum have post-attachment resistance mechanisms that arrest penetration of the parasite by the haustorium preventing parasitism (Yoder and Scholes, 2010). However in many other crop species such resistance mechanisms have never been observed. For instance, there is no report of post-attachment resistance in any maize variety although such resistance was identified in the wild maize relative *Tripasacum dactyloides* and teosinte (*Zea diploperennis*) (Hearne, 2009; Yoshida and Shirasu, 2009). Non-host resistance occurs when parasitism is not established between a potential host species and the parasitic plant. For example, *S. hermonthica* parasitizes rice, maize and sorghum but cannot infect *Phtheirospermum japonicum*. Although its germination is stimulated, *S. hermonthica* fails to develop a haustorium, attach and penetrate *P. japonicum* roots (Yoshida and Shirasu, 2009). While post-attachment resistance mechanisms can be broken down by new parasite races, non-

host resistance is durable and hence understanding the mechanisms involved in this incompatibility is of great interest for resistance breeding.

Another approach to the development of resistant cultivars is by reducing the induction of parasitic plant seed germination. As germination is the first step in the parasitic plant lifecycle, interfering in this process would be attractive. It has indeed been shown that plants producing lower amounts of germination stimulants are less infected by parasitic plants. In sorghum, for example, wild and cultivated varieties were screened for production of germination and haustorium formation stimulants (Rich et al., 2004; Ejeta, 2007). The study revealed that there is a large variation in the germination inducing and haustorium formation capacity among the different accessions with some having very low stimulatory activity. These traits seem to be heritable and controlled by just a few genes. Strigolactone biosynthetic mutants in rice and tomato display reduced infection by parasitic plants (Umehara et al., 2008; Koltai et al., 2010b). However, manipulation of the biosynthesis of strigolactones in order to reduce parasitism is not without risk. Strigolactones have other roles in plant physiology and the number of physiological processes in which strigolactones seem to be involved is increasing at fast pace. Alterations of strigolactone biosynthesis may have implications for the regulation of plant shoot architecture and as a consequence affect total leaf area and leaf area distribution as well as nutrient and water demand. Effects on root architecture may also interfere with efficiency of nutrient and water uptake especially during phosphorus-limiting conditions. A possible strategy to overcome this problem would be to modulate the transport of strigolactones into the rhizosphere instead of regulating its biosynthesis. Strigolactones are produced mainly in the roots and are transported along at least two routes: they are released into the rhizosphere or transported via the xylem (Kohlen et al., 2011) to the shoot to inhibit branching/tillering. So far, it is not known if the secretion of strigolactones into the rhizosphere is an active or passive process. Understanding the mechanism that regulates the transport of strigolactones to the shoot or into the rhizosphere may help to develop new cultivars with reduced parasite germination induction but normal root and shoot architecture.

Of course, the ability to establish a symbiotic interaction with AM fungi may be compromised if the release of strigolactones into the rhizosphere is suppressed. Parasitic weeds are generally most damaging in areas where soils are poor in nutrients

and low input agriculture is practiced (Rubiales et al., 2009). In these cases, AM fungal symbiosis is likely to play an important role in enhancing crop productivity. To our knowledge, cultivars selected for low induction of parasite germination have not been tested for their ability to establish AM fungal symbiosis. However, mycorrhizal colonization is somewhat compromised in strigolactone mutants or transgenic strigolactone knock-down lines of several plant species (Gomez-Roldan et al., 2008; Umehara et al., 2008; Koltai et al., 2010b).

As discussed above, the biological activity of strigolactones differs according to the biological process in which they are acting and the structural variations in the general ABCD-ring backbone (Akiyama et al., 2010). Therefore, host plants that stimulate low seed germination may not necessarily be producers of low amounts of strigolactones. It is possible that a different combination of strigolactones is produced instead, with low germination stimulatory activity but still inducing sufficient AM fungi hyphal branching and having normal shoot and root architecture.

Concluding remarks

The study of strigolactone signaling in the interaction between parasitic plants and their host has been of interest for many years due to its potential to find new ways to reduce the parasitic weed problem. Host plants with low production of the parasitic weed seed germination inducing strigolactones are potentially more resistant to parasitism. However, we now know that strigolactones are not only signaling molecules for root parasites. They also play a role in the rhizosphere interaction of plants with AM fungi and an increasing number of endogenous physiological functions has been assigned to the strigolactones, such as the control of shoot and root architecture and its response to phosphate deficiency. The discovery of the new strigolactone functions in non-parasitic plants suggests that the biosynthesis and signaling pathways for these compounds regulate physiological mechanisms conserved among all plant species, including parasitic plants. During the transition from autotrophy to parasitism, the latter have probably evolved variants of the strigolactone signaling pathways so they could use strigolactones as an endogenous trigger for seed germination. Considering the multitude of recently discovered new biological roles for the strigolactones, breeding for increased resistance to parasitic

plants by developing cultivars with low production of strigolactones may have several undesired side-effects, for example on plant architecture or the interaction with beneficial AM fungi. However, considering our increasing insight in the biology of the strigolactones we suggest that new strategies that involve modification of strigolactone composition or manipulation of strigolactone transport within the plant and to the rhizosphere could be viable alternatives. This stresses the necessity for a thorough study of the relation between structure and biological activity and the elucidation of the biosynthetic steps that are responsible for this structural variation.

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Differential activity of *Striga hermonthica* germination stimulants and *Gigaspora rosea* hyphal branching factors in rice and their contribution to underground communication

Catarina Cardoso¹, Tatsiana Charnikhova¹, Muhammad Jamil^{1,2}, Pierre Marc Delaux^{3,4}, Francel Verstappen^{1,5}, Maryam Amini^{1,6}, Dominique Laressergues^{3,7}, Carolien Ruyter-Spira^{1,8}, Harro Bouwmeester^{1,9}

¹Laboratory of Plant Physiology, Wageningen University, the Netherlands

²current address: Department of Biosciences, COMSATS Institute of Information Technology, Islamabad, Pakistan

³CNRS, Castanet-Tolosan, France

⁴current address: University of Wisconsin – Madison, Department of Agronomy, Madison, WI 53705, USA.

⁵Centre for Biosystems Genomics, Wageningen, the Netherlands

⁶current address: Syngenta Seeds B.V., Enkhuizen, the Netherlands

⁷Surfaces Cellulaires et Signalisation chez les Végétaux, Université de Toulouse, France

⁸Plant Research International, Bioscience, Wageningen, the Netherlands

⁹Centre for Biosystems Genomics, Wageningen, the Netherlands

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Abstract

Strigolactones (SLs) trigger germination of parasitic plant seeds and hyphal branching of symbiotic arbuscular mycorrhizal (AM) fungi. There is extensive structural variation in SLs and plants usually produce blends of different SLs. The structural variation among natural SLs has been shown to impact their biological activity as hyphal branching and parasitic plant seed germination stimulants. In this study, rice root exudates were fractionated by HPLC. The resulting fractions were analyzed by MRM-LC-MS to investigate the presence of SLs and tested using bioassays to assess their *Striga hermonthica* seed germination and *Gigaspora rosea* hyphal branching stimulatory activities. A substantial number of active fractions were revealed often with very different effect on seed germination and hyphal branching. Fractions containing (-)-orobanchol and *ent*-2'-*epi*-5-deoxystrigol contributed little to the induction of *S. hermonthica* seed germination but strongly stimulated AM fungal hyphal branching. Three SLs in one fraction, putative methoxy-5-deoxystrigol isomers, had moderate seed germination and hyphal branching inducing activity. Two fractions contained strong germination stimulants but displayed only modest hyphal branching activity. We provide evidence that these stimulants are likely SLs although no SL-representative masses could be detected using MRM-LC-MS. Our results show that seed germination and hyphal branching are induced to very different extents by the various SLs (or other stimulants) present in rice root exudates. We propose that the development of rice varieties with different SL composition is a promising strategy to reduce parasitic plant infestation while maintaining symbiosis with AM fungi.

Introduction

Parasitic plants of the genus *Striga* are economically important species that parasitize the dicotyledonous cowpea, and cereal crops such as rice sorghum and maize (Parker, 2009). In the most affected areas, parasitic plants constitute a major constraint to food production and efficient control methods are scant. *Striga* seeds will only germinate after exposure to host derived molecules, called germination stimulants that the parasite uses to detect host presence. The first phases of root parasitism occur underground and the presence of the parasite is difficult to diagnose until the emergence of its shoots. However, crop yield is already compromised at that stage making timely control of this pest even more difficult (Cardoso et al., 2011; Scholes and Press, 2008). It is therefore important to develop control strategies that act before infection is initiated, for example by avoiding or reducing germination of the parasites' seeds. Strigolactones (SLs) are the best described class of germination stimulants and a reduction in the production of these compounds indeed resulted in reduced *Striga* infection (Jamil et al., 2011a, 2011b, 2012). However, SLs are also signaling compounds for the establishment of symbiosis with arbuscular mycorrhizal (AM) fungi and are plant hormones that modulate plant architecture (Akiyama et al., 2005; Besserer et al., 2006; Gomez-Roldan et al., 2008; Umehara et al., 2008; Ruyter-Spira et al., 2011; Kapulnik et al., 2011) and, therefore, non-discriminate reduction of their production would likely have negative side effects. The symbiotic AM fungi perceive SLs and respond with extensive pre-symbiotic hyphal branching, thus increasing the efficiency of root colonization. In this symbiotic interaction, the fungus takes up nutrients (especially phosphate and nitrogen) and water from the soil and supplies them to the plant in exchange for carbon assimilates (Nadal and Paszkowski, 2013). Plants under phosphate starvation increase production and release of SLs into the rhizosphere to promote the symbiosis (Yoneyama et al., 2007a, 2007b; López-Ráez et al., 2008). In soils contaminated with seeds of the parasitic plants, low phosphate availability results in increased levels of infestation by parasitic plants (Jamil et al., 2011a).

Adaptation responses to low phosphate such as reduced shoot branching and root system expansion are mediated by SLs (Umehara et al., 2010; Kohlen et al., 2011; Ruyter-Spira et al., 2011). SL biosynthetic mutants suffer, to some extent,

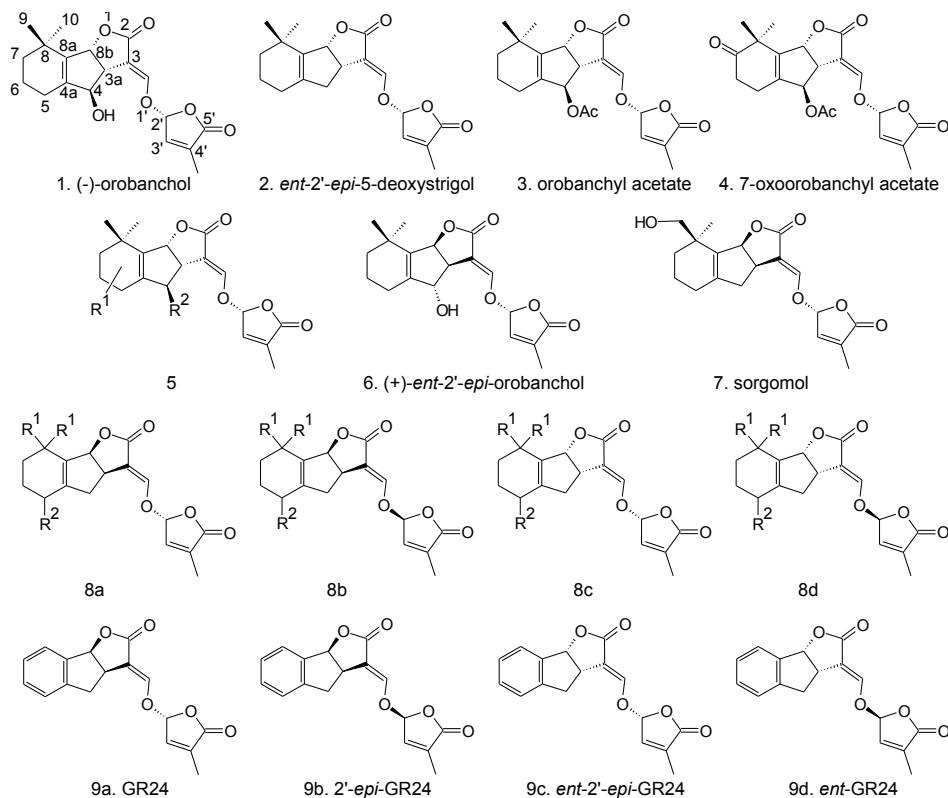


Figure 1. SL structures present in rice root exudates or tested in a germination or hyphal bioassay. (1) (-)-orobanchol; (2) *ent*-2'-*epi*-5-deoxystrigol; (3) orobanchyl acetate and (4) proposed structure of 7-oxoorobanchyl acetate (Xie et al., 2013); (5) proposed structure of methoxy-5-deoxystrigol isomers (R¹=OMe; R²=H) or the methyl ether of orobanchol (R¹=H; R²=OMe); (6) (+)-*ent*-2'-*epi*-orobanchol; (7) sorgomol; (8a-b) stereoisomers of strigol (R¹=CH₃; R²=OH); 5-deoxystrigol (R¹=CH₃; R²=H), stereochemical configurations 8a and 8c are natural (strigol-type and orobanchol-type, respectively) while configurations 8b and 8d are not naturally occurring; (9a-c) stereoisomers of SL analogue GR24.

from reduced symbiosis with AM fungi and exhibit altered plant shoot and root architecture which may negatively affect crop yields (Gomez-Roldan et al., 2008; Kohlen et al., 2012; Koltai, 2011; Ruyter-Spira et al., 2011; Umehara et al., 2008).

SLs are derived from all-*trans*- β -carotene that is isomerized into 9-*cis*- β -carotene by β -carotene isomerase D27 (DWARF27) followed by two consecutive cleavage steps by CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7; HIGH TILLERING DWARF1 - HTD1/DWARF17 - 17 in rice) and CAROTENOID CLEAVAGE DIOXYGENASE 8 (CCD8; DWARF10 - D10 in rice) resulting in the production of carlactone (Alder et al., 2012). The biosynthetic steps that convert

carlactone to SL are not yet elucidated. SLs are a reasonably large class of natural compounds consisting of over 15 structural variants, most of which differ only by having one instead of two methyl groups on the cyclohexenyl A-ring or by having various combinations of hydroxyl or acetoxy substituents on the A- and B-rings (Yoneyama et al., 2009). SLs occur in two distinct stereochemical configurations and the stereochemistry of some SLs was recently revised (Xie et al., 2013). SLs from the orobanchol-like family have an *ent* oriented C-ring (Figure 1, structures **1-5** and **8c**). In the strigol-like family the C-ring has the opposite chirality of the orobanchol-like family (Figure 1, structures **6;7** and **8a**) (Xie et al., 2013). Plants produce a mixture of SLs that differs between and sometimes even within species (Awad et al., 2006; Xie et al., 2007; López-Ráez et al., 2008). So far, only orobanchol-like SLs have been identified in rice: (-)-orobanchol (**1**), *ent*-2'-*epi*-5-deoxystrigol (**2**), orobanchyl acetate (**3**), 7-oxoorobanchyl acetate (**4**) (Umehara et al., 2008; Xie et al., 2013). In addition, three putative methoxy-5-deoxystrigol isomers (**5**) have been reported with unknown structure and stereochemistry (Jamil et al., 2011a).

Parasitic plant seeds and AM fungi have different sensitivities to different SL variants (Akiyama et al., 2010; Nomura et al., 2013). Interestingly, it was reported that orobanchol-like SLs (of the same type as found in rice exudates) are considerably less active at inducing *Striga hermonthica* seed germination (Nomura et al., 2013). Here, we extensively survey the chemical composition (SL content) and biological activity of rice root exudates to understand the relevance of the different SLs, and possible other signalling molecules, in the establishment of mycorrhizal symbiosis with the AM fungus, *Gigaspora rosea*, and infection by the parasitic plant, *Striga hermonthica*.

Materials and methods

Strigolactone standards

The synthetic SL GR24 (**9a-d**) and (\pm)-strigol (**8a,d**, R¹=CH₃; R²=OH) were kindly provided by Prof. Binne Zwanenburg (Radboud University Nijmegen, Netherlands); (-)-orobanchol (**1**) and (+)-*ent*-2'-*epi*-orobanchol (**6**), solanacol, orobanchyl acetate (**3**), 7-oxoorobanchyl acetate (**4**), 7-oxoorobanchol, and sorgomol (**7**) were

provided by Prof. Koichi Yoneyama (Utsunomiya University, Japan); (\pm)-2'-*epi*-strigol (**8b,c**, R¹=CH₃; R²=OH), (\pm)-2'-*epi*-5-deoxystrigol (**8b,c**, R¹=CH₃; R²=H) and (\pm)-5-deoxystrigol (**8a,d**, R¹=CH₃; R²=H) were a gift from Prof. Tadao Asami (University of Tokyo, Japan) (Structures **1** to **8a-d** represented in Figure 1).

Plant growth and root exudate collection

The exudates were collected from rice seedlings of the variety Nipponbare and the SL biosynthetic mutant line *d10-2* with Nipponbare background, kindly provided by Prof. Junko Kyojuka (University of Tokyo, Japan) (Umehara et al., 2008). The seeds were sown in pots of 14 cm diameter filled with quartz sand. The experiment was conducted with three pots per treatment. One pot containing 25 plants represents one replicate. Plants were watered every three days during the first week and every two days during the remaining weeks to full substrate saturation with half-strength modified Hoagland nutrient solution containing NH₄NO₃ (5.6 mM), K₂HPO₄ (0.4 mM), MgSO₄ (0.8 mM), FeSO₄ (0.18 mM), CaCl₂ (1.6 mM), K₂SO₄ (0.8 mM), MnCl₂ (0.0045 mM), CuSO₄ (0.0003 mM), ZnCl₂ (0.0015 mM), Na₂MoO₄ (0.0001 mM). After 3 weeks, phosphate starvation and phosphate starvation in combination with 0.01 μ M fluridone – an inhibitor of carotenoid and therefore SL biosynthesis – were applied. Control plants were watered with the half-strength modified Hoagland nutrient solution described above. For the phosphate starvation treatment, KNO₃ (0.8 mM) was substituted for K₂HPO₄ to maintain the same the K⁺ concentration. Residual phosphate was removed from the pots by applying 1 L of the P-less nutrient solution and draining the pots. Six days after the start of the treatments the latter treatment was repeated. Root exudates were collected 24 hours later by applying 1 L of the corresponding nutrient solution and collecting the flow through.

Sample preparation

The root exudates were concentrated using an SPE cartridge (GracePure™ SPE C18 – Max 500 mg) and eluted in 4 mL of 100% acetone. For HPLC, 250 μ L of water was added to 1 mL of this acetone eluent after which the acetone was evaporated under a flow of N₂. The remaining 250 μ L sample was injected into the HPLC and 1 min fractions (corresponding to 1 mL) were collected. The fractions were evaporated to dryness and dissolved in 200 μ L water for further analysis. For MRM-LC-MS

analysis, 50 μL of the C18 acetone eluent was diluted 3-fold in water and HPLC fractions were diluted 2-fold in water. For seed germination bioassays, C18 acetone eluents (crude exudates) were diluted 32-fold in water and HPLC fractions 5-fold. For the AM fungal hyphal branching bioassay the HPLC fractions of each replicate were pooled and tested in the same concentration as in the seed germination bioassay.

Fractionation of root exudates

Root exudates were fractionated by HPLC. The samples were injected into a XBridgeTM C18 column (4,6*150 mm from 5 μm , Waters) using a U6K injector (Waters). For the gradient model 510 pumps (Waters) were used. The mobile phase was water and the following gradient to acetonitrile used: 1 min 100% water, 2 min 27% acetonitrile, 15 min 45% acetonitrile, 24 min 80% acetonitrile and 24.2 min 100% acetonitrile which was maintained for 4 minutes to clean the column. The flow rate was 1 mL min^{-1} and the column temperature 25°C. Fractions of one minute were collected using a Biofrac fraction collector (Biorad).

MRM-LC-MS analysis

For LC-MS analysis, samples were filtered through mini syringe filters (Minisart SRP4). The retention times, mass transitions and MS/MS spectra of available SL standards such as (+)-*ent*-2'-*epi*-orobanchol (**6**), (-)-orobanchol (**1**), (\pm)-5-deoxystrigol (**8a,d**, $\text{R}^1=\text{CH}_3$; $\text{R}^2=\text{H}$), (\pm)-2'-*epi*-5-deoxystrigol (**8b,c**, $\text{R}^1=\text{CH}_3$; $\text{R}^2=\text{H}$), (\pm)-sorgolactone, (\pm)-strigol (**8a,d**, $\text{R}^1=\text{CH}_3$; $\text{R}^2=\text{OH}$), solanacol, orobanchyl acetate (**3**), (\pm)-7-oxoorobanchol and (\pm)-7-oxoorobanchyl acetate (**4**) were compared with each sample to quantify SLs using ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). Analyses were performed using a Waters Xevo tandem quadrupole (TQ) mass spectrometer equipped with an ESI source. Chromatographic separation was achieved on an Acquity UPLC BEH C18 column (150 x 2.1 mm, 1.7 μm) (Waters) by applying a water/ acetonitrile gradient to the column, starting from 5% (v/v) acetonitrile for 2.0 min and rising to 50% (v/v) acetonitrile at 8.0 min, followed by a 1.0 min gradient to 90% (v/v) acetonitrile, which was maintained for 0.1 min before going back to 5% (v/v) acetonitrile using a 0.2 min gradient, prior to the next run. Finally, the column was equilibrated for 2.8 min, using this solvent composition. Operation temperature and

flow-rate of the column were 50°C and 0.4 mL min⁻¹, respectively. Sample injection volume was 15 µL. The mass spectrometer was operated in positive electrospray ionization (ESI) mode. Cone and desolvation gas flows were set to 50 and 1000 L h⁻¹, respectively. The capillary voltage was set at 3.0 kV, the source temperature at 150°C and the desolvation temperature at 650°C. The cone voltage was optimized for each SL standard using the IntelliStart MS Console. Argon was used for fragmentation by MS/MS spectra in the collision cell.

The identification of SLs in rice root exudates and extracts was done using Multiple Reaction Monitoring (MRM) and by comparing retention times and MRM mass transitions with those of the available SL standards mentioned above. MRM transitions were optimized for each standard using the IntelliStart MS Console. The MRM transitions for putative 4-methoxy-5-deoxystrigol isomers were initially set based on the theoretically predicted fragmentation (see Results and Discussion section). MRM-transitions for the predicted putative SLs were incorporated in the MRM-method. The structures of all detected SLs were confirmed by MS/MS fragmentation spectra. Data acquisition and analysis were performed using MassLynx 4.1 software (Waters). Full mass scan and precursor ion scan for $m/z=97$ were performed to search for unknown SLs in biologically active HPLC-fractions 15 to 19. The LC-MS results of the measurements of (-)-orobanchol (**1**) and *ent*-2'-*epi*-5-deoxystrigol (**2**) (of 3 biological replicates) were compared using ANOVA followed by pair wise comparisons with t-test (LSD values).

S. hermonthica seed germination bioassay

Seeds of *Striga hermonthica* used for the bioassay were kindly provided by Bob Vasey and originating from a sorghum field in Sudan, collected in 1995. The bioassays were performed as described (Matusova et al., 2005). The samples were tested in three technical replicates (three discs) and 3 biological replicates were tested, one independent bioassay per biological replicate. Given the binomial distribution nature of the measurements the mean values of the seed germination scores (3 replicates per treatment per fraction) were compared using a Chi-square test.

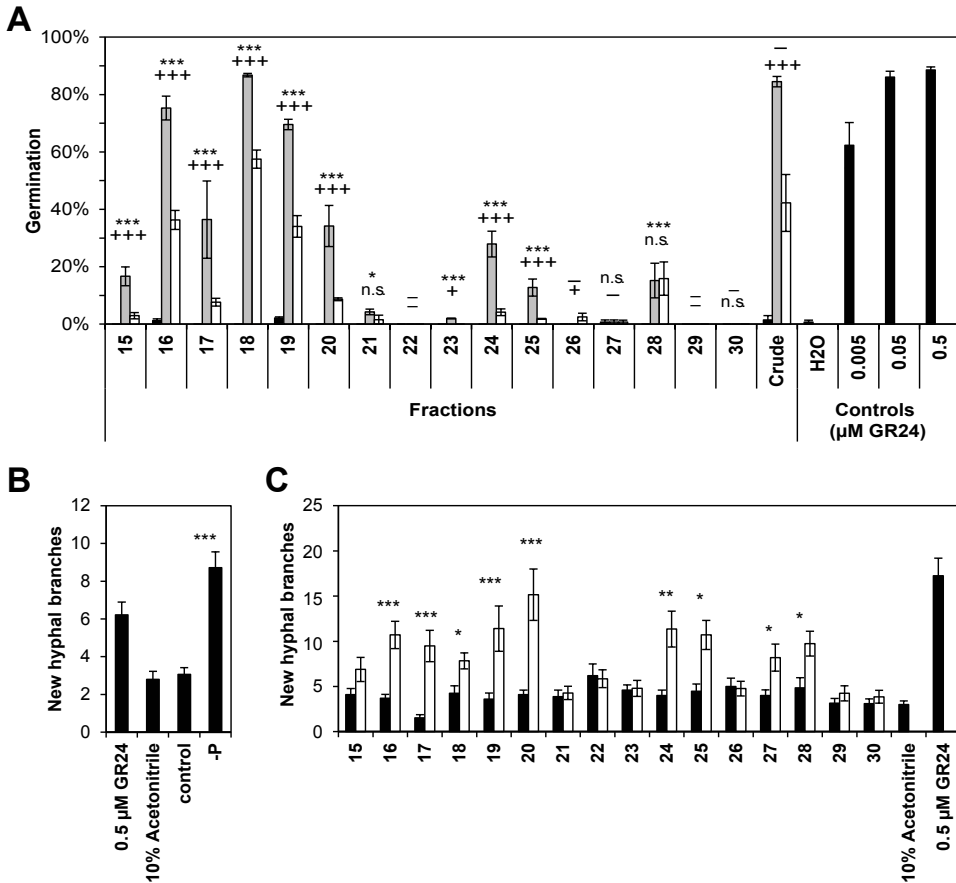


Figure 2. Activity profiles of rice root exudates. Germination of *S. hermonthica* (1 biological replicate; the other two are shown in Figure S1) obtained with crude exudates and exudate fractions from rice plants (**A**) treated with full nutrition (black bars); phosphate starvation (grey bars) and phosphate starvation plus 0.01 μM fluridone (white bars). Water and SL analogue GR24 (0.005, 0.05 and 0.5 μM) were used as controls. The error bars represent the standard error of 3 technical replicates. Significance levels between treatments as determined using a χ^2 test are indicated: */+ = $P < 0.05$; **/+ = $P < 0.01$; ***/+++ = $P < 0.001$; n.s. = $P > 0.05$; * = control vs. phosphate starvation treatment; + = phosphate starvation vs. phosphate starvation plus fluridone treatment. When germination values are close to zero the statistical test cannot be performed, which is indicated with “–”. AM hyphal branching induced by crude exudates (**B**) and exudate fractions (**C**) of rice treated with full nutrition (black bars) and phosphate starvation (white bars) in germinating *Gi. rosea* spores. The assay was performed with pooled samples of three biological replicates. GR24 (0.005, 0.05 and 0.5 μM) and 10% acetonitrile in water were used as controls. The bars represent the mean of the total number of new branches, the error bar the standard error of the mean ($n=20$). Significance values comparing means between control treatments and phosphate starvation treatment or between negative control and GR24 are indicated above the bars. (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

AM Fungal Hyphal Branching Bioassay

For the AM branching bioassay spores of *Gigaspora rosea* (DAOM 194757) were used. The spores were routinely produced in pots containing leek and collected by wet sieving. They were washed in water/0.05% Tween 20 (v/v), soaked with 2% (w/v) Chloramine T (Sigma) for 10 min, washed again three times in sterile water for 30 s per wash, and stored in an antibiotic solution containing 100 mg l⁻¹ gentamycin and 200 mg l⁻¹ streptomycin. After 2 days at 4 °C, a second treatment with Chloramine T was carried out under the same conditions. They were then stored in the antibiotic solution at 4°C before use. Branching bioassays were carried out according to Buee *et al.* (Buee *et al.*, 2000). Four spores of *Gi. rosea* were germinated (in 2% CO₂ at 30°C in dark) on M medium (Becard & Fortin, 1988) supplemented with 10 µM quercetin (Sigma) and solidified with 0.6 % Phytigel (Sigma). Seven days after inoculation, each spore produced a single germ tube growing upwards. Two small wells on each half of the Petri dish, near the hyphal tip, were made in the gel with a Pasteur pipette tip and 5µl of the test solution (SL analogue GR24 or purified fraction) or 10% acetonitrile (control) was injected in each well. After 24 h, hyphal branching was recorded by counting newly formed hyphal tips. Twenty to thirty spores were used for each treatment. Values of each tested fraction were compared with the corresponding control using the Student's t-test.

Results

Rice root exudates were profiled to find compounds responsible for *S. hermonthica* seed germination and AM fungi hyphal branching. Plants were submitted to phosphate starvation with and without the application of 0.01 µM fluridone, an inhibitor of carotenoid and hence SL biosynthesis (Matusova *et al.*, 2005). A reduction in seed germination stimulatory activity of root exudate fractions by fluridone treatment would suggest that the compound(s) responsible for the biological activity in that fraction is a (are) SL(s). The root exudates were fractioned by HPLC and the biological activity of the resulting fractions as well as the crude exudates (not fractioned) was assessed. Each of the three biological replicates was tested in independent seed germination bioassays of which one is shown in Figure 2 A, and the remaining are shown in Figure S1. Preliminary results showed that fractions eluting before 14 min

(fractions 1 to 14) do not exhibit seed germination stimulatory activity (data not shown). Fractioned and crude exudates of control plants with sufficient phosphate showed almost no activity across all fractions. The seed germination stimulatory activity was significantly increased by phosphate starvation in crude exudates and in fractions 15-21, 23-25 and 28 ($P < 0.05$ using χ^2 test). This was observed consistently in all replicates (Figure 2 A and Figure S1). Fluridone treatment significantly reduced the activity of crude exudates and of fractions 15 to 19 and 24) with 99.9% confidence ($P < 0.001$ using χ^2 test) in all biological replicates. In fractions 20, 21, and 25 fluridone treatment also reduced the activity but the effect was not consistently significant; in two replicates these fractions showed significance at $P < 0.001$ using the χ^2 test, but not in the third replicate, likely because the germination stimulatory activity in

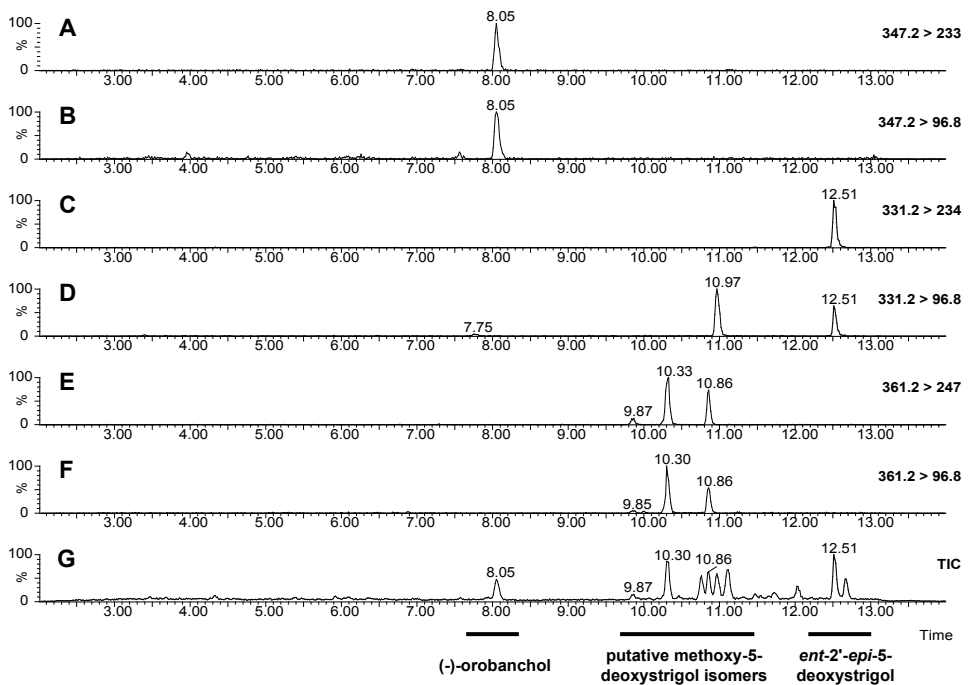


Figure 3. SL analysis of rice root exudates. Root exudates from rice plants grown under phosphate starvation were analyzed with liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) using multiple reaction monitoring (MRM). Chromatograms of (A) transitions 347.2 > 233 and (B) 347.2 > 96.8 for orobanchol; (C) transitions 331.2 > 234 and (D) 331.2 > 96.8 for 2'-*epi*-5-deoxystrigol; (E) transitions 361.2 > 247 and (F) 361.2 > 96.8 for three putative methoxy-5-deoxystrigol isomers; (G) the total ion count (TIC) showing of all measured transitions and where orobanchol (8.05 min), *ent*-2'-*epi*-5-deoxystrigol (12.51 min) and the three putative methoxy-5-deoxystrigol isomers (9.87; 10.33; 10.86 min) are visible.

these fractions was sometimes low. Also in fraction 28 that induces low germination activity the fluridone treatment did not significantly reduce seed germination. The effect of crude and fractioned root exudates on AM fungal hyphal branching was also assessed using *Gi. rosea* spores (Figure 2 B and C). Just as for *S. hermonthica* seed germination, phosphate starvation significantly increased branching stimulatory activity of crude exudates and most fractions (16-20, 24-25 and 27-28, at $P < 0.005$, using Student t-test). Crude and fractioned exudates of control, non-phosphate starved plants did not show a significant difference in branching activity compared with the negative control, 10% acetonitrile. All the active fractions (of phosphate starvation treated rice) induced hyphal branching to a similar level. Interestingly, however, fraction 18 - the most active in the *S. hermonthica* seed germination bioassay - induced less AM fungal hyphal branching than the other active fractions (Figure 2 C).

To gain insight into the identity of the compounds responsible for the biological activity, MRM-LC-MS analysis was performed. The MRM chromatograms of crude exudates revealed an intense peak in the channels m/z 347>233, 347>205 and 347>97 at retention time 8.05 min, which matches with an authentic standard of (-)-orobanchol (**1**) (Figure 3 A and B). In the channels m/z 331>234 and 331>97 there was a peak at 12.51 min, which matches with *ent*-2'-*epi*-5-deoxystrigol (**2**) (Figure 3 C and B). MS/MS fragmentation spectra, and the addition of authentic orobanchol and (\pm)-2'-*epi*-5-deoxystrigol (**8b,c**, $R^1 = \text{CH}_3$; $R^2 = \text{H}$) to the samples, confirmed that the compounds detected were indeed orobanchol and *ent*-2'-*epi*-5-deoxystrigol. The same two SLs were barely detectable in exudates of control plants supplied with full nutrient solution, were most abundant in the phosphate starvation treatment and were both significantly reduced by fluridone treatment ($P < 0.001$) (Figure 4 A). In addition to (-)-orobanchol (**1**) and *ent*-2'-*epi*-5-deoxystrigol (**2**) three unknown peaks were detected using the channels for 7-oxoorobanchol (m/z 361>247 and 361>97) at the retention times 9.9, 10.3 and 10.9 min (Figure 3 E and F), which is substantially later than 7-oxoorobanchol, which elutes at 3.7 min. The three unknown compounds were most abundant in the phosphate starvation treatment and were reduced by fluridone treatment (Figure 4 B - D) suggesting they are SL-like compounds.

MRM-LC-MS analysis was also performed on the HPLC fractions to try to correlate the presence of SLs with the seed germination and hyphal branching

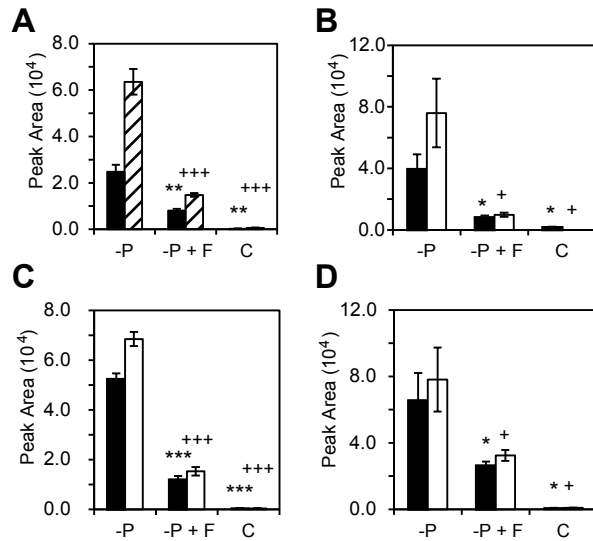


Figure 4. Abundance of (-)-orobanchol, *ent-2'-epi-5-deoxystrigol* and putative SL-like compounds in phosphate starvation and fluridone treatments. Peak areas obtained with liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis using multiple reaction monitoring (MRM) of root exudates of rice. **(A)** (-)-orobanchol (MRM transition 347.2>96.8; black bars) and *ent-2'-epi-5-deoxystrigol* (MRM transition 331.2>234; hatched bars); **(B-D)** three putative SL-like compounds measured in crude exudates with the retention times: *rt*= 9.87 **(B)**; *rt*=10.3 and **(C)** *rt*=10.9 **(D)** and the MRM transitions 361>96.8 (black bars) and 361>247 (white bars). All measurements taken from crude exudates of plants grown in different treatments: phosphate starvation (-P); phosphate starvation combined with fluridone (-P+F) and control treatment with full nutrient supply (C). The error bars represent the standard error of 3 biological replicates. Significance values are indicated with * (for *ent-2'-epi-orobanchol* and for 361>96.8 transition) and + (for *2'-epi-5-deoxystrigol* and for 331.2>234 transition) and compare phosphate starvation (-P) treatment vs. phosphate starvation with fluridone (-P+F) and -P vs. full nutrition (C) (*/+ = $P < 0.05$, **/+ = $P < 0.01$, ***/+++ = $P < 0.001$).

activity. (-)-orobanchol (**1**) was detected in fractions 19 to 21 with highest abundance in fraction 20 and *ent-2'-epi-5-deoxystrigol* (**2**) in fractions 27 and 28 with highest signal in fraction 28 (data not shown). The three methoxy-5-deoxystrigol isomers detected in the channel for 7-oxoorobanchol (*m/z* 361> 247 and 361>97) at the retention times 9.95, 10.3 and 10.9 min eluted in fractions 23-25 with highest abundance in fraction 24 and are likely responsible for the seed germination stimulant/hyphal branching activity peak in fractions 24-25. The activity of the three compounds could not be evaluated individually as they did not separate on HPLC due to their highly similar retention time.

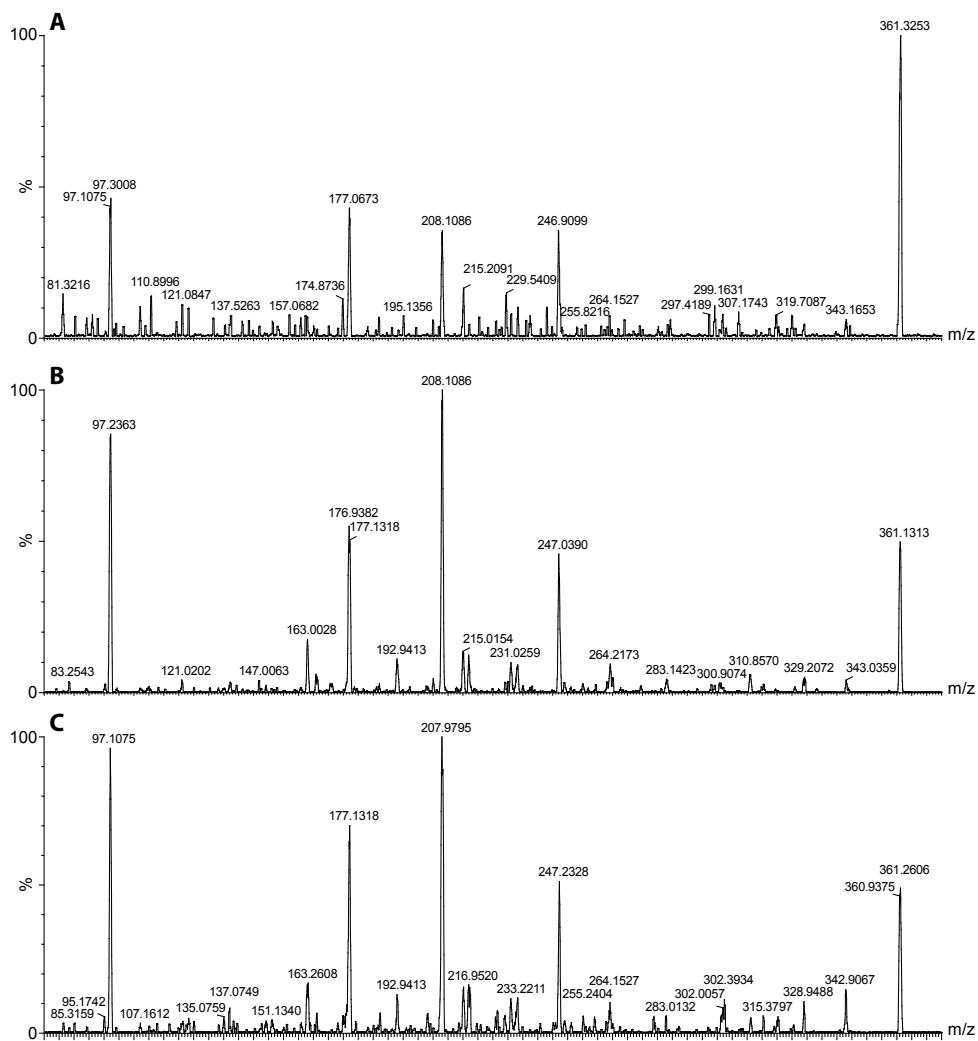


Figure 5. MS/MS spectra of putative SL-like compounds. The spectra were measured at the retention time of each isomer: 9.87min (**A**), 10.35min (**B**) and 10.95min (**C**).

The fractions with highest seed germination inducing activity (16 and 18) were also analyzed using known MRM transitions typical for SLs as well as full mass scan and precursor ion scan for $m/z=97$. However, we could not detect any masses that could be indicative for SLs and displayed an expected abundance pattern across the treatments similar as the known SLs: low in control, high in P starvation, low upon fluridone treatment.

To further investigate the nature of the active compounds in fractions 16, 18, 24 and 25, exudates of the SL biosynthetic mutant *d10-2* were studied (Umehara et al., 2008). HPLC fractions 16 – 25 collected from *d10-2* and its background Nipponbare were tested using the seed germination bioassay. All active fractions in the wild type had reduced activity in the mutant (Figure 6 A) supporting the SL (CCD8-dependent) nature of the compounds responsible for the biological activity of these fractions.

The MRM-LC-MS spectra of *d10-2* mutant root exudates confirmed that (-)-orobanchol (**1**) and *ent-2'-epi-5-deoxystrigol* (**2**) as well as the three methoxy-5-deoxystrigol isomers detected at 9.9, 10.35 and 10.95 were strongly decreased in *d10-2* mutant root exudates (Figure 6 B,C), further indicating that the latter three are SLs/require CCD8. Fractions 16 and 18 of *d10-2* exudates were also analyzed by LC-MS and compared with those from wild type plants using full-scan mass spectrometry, but no differential masses were found that could explain the seed germination activity in the wild type and give a hint on the identity of the seed germination stimulant(s) in these fractions.

The activity profiles obtained with the exudate fractions, when tested with the seed germination and hyphal branching are different. Some fractions that stimulate high seed germination percentages induce low fungal response and the contrary is also observed. To further investigate the differences in activity observed in our bioassays, we performed a seed germination bioassay using pure or racemic mixtures of SLs (Table 1). We observed that sorgomol (**7**) is the most active of the tested SLs inducing 36 % seed germination at 200 nM and 26 % seed germination at 20 nM followed by (+)-*ent-2'-epi-orobanchol* (**6**, 34 % seed germination at 200 nM and 8.7 % at 20 nM). The racemates of (\pm)-strigol (**8a,d**, R¹=CH₃; R²=OH); (\pm)-5-deoxystrigol (**8a,d**, R¹=CH₃; R²=H) and the racemic mixture of all 4 stereoisomers of GR24 GR24 (**9a-d**) have intermediate activity inducing 9.3 %; 6.0 % and 2.0 % seed germination at 20 nM and inducing 19 %; 26 % and 25 % seed germination at 200 nM, respectively. The racemate of (\pm)-2'-*epi-5-deoxystrigol* (**8b,c**, R¹= CH₃; R²=H) induced less seed germination (11 % at 200 nM) and was not active at 20 nM. The least active SLs were (-)-orobanchol (**1**) and the racemate of (\pm)-2'-*epi-strigol* (**8b,c**, R¹=CH₃; R²=OH) that induced less than 1% seed germination in both concentrations. Table 1 also summarises data from a study by Akiyama et al. that analysed the *Gigaspora margarita* hyphal branching activity of a range of different SLs (Akiyama et al.,

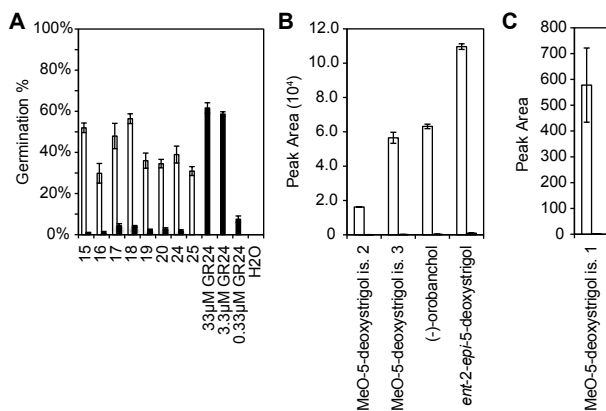


Figure 6. Characterization of rice mutant *d10* exudate. Germination assay with *S. hermonthica* seeds on exudate fractions of full nutrition (black bars) and phosphate starvation (white bars) treated plants (A). Water and GR24 (0.33, 3.3 and 33 μM) were used as controls. Peak areas of *ent*-2'-*epi*-5-deoxystrigol, (-)-orobanchol and putative methoxy-5-deoxystrigol isomers 2 and 3 (B) and putative methoxy-5-deoxystrigol isomer 1 (C) obtained with liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis using multiple reaction monitoring (MRM) of root exudates of *d10-2* (black bars) and WT (white bars) under phosphate starvation treatment.

2010). In contrast to what is observed with *S. hermonthica*, both orobanchol (1) and *ent*-2'-*epi*-5-deoxystrigol (2) are highly active at inducing hyphal branching and their activity is similar to their natural stereoisomers. Strigol (8a, $\text{R}^1=\text{CH}_3$; $\text{R}^2=\text{OH}$), sorgomol (7), GR24 (9a) and (\pm)-2'-*epi*-strigol (8b,c, $\text{R}^1=\text{CH}_3$; $\text{R}^2=\text{OH}$) were considerable less active (100 fold) than orobanchol (1), and the remaining GR24 stereoisomers (9b-d) were 10000- to 1000-fold less active than GR24 (9a).

Discussion

Rice root exudates were fractionated to evaluate the contribution of SLs and potentially other signalling molecules to the *S. hermonthica* seed germination stimulant and AMF hyphal branching activity of rice root exudate. MRMLCMS analysis of these HPLC fractionated rice root exudates showed the presence of (-)-orobanchol (1) in fractions 19, 20 and 21 and *ent*-2'-*epi*-5-deoxystrigol (2) in fraction 28 suggesting that these SLs are responsible for the seed germination and hyphal branching stimulatory activities of these fractions. These results confirm the presence of SLs found previously in root exudates of the rice variety Nipponbare except for orobanchyl acetate (3) that was not detected in the present study but is reported

by others (Xie et al., 2013). A fourth SL –7-oxoorobanchyl acetate (**4**) – was also reported to be produced in Nipponbare between days 10 to 17 after germination (Xie et al., 2013). In the present study the exudates were collected at a later stage and this SL was not detected.

The relative abundance of (-)-orobanchol (**1**) and *ent*-2'-*epi*-5-deoxystrigol (**2**), measured by MRM-LC-MS across the different treatments matches the seed germination stimulatory activity of the fractions where these SLs elute (19-20 and 28 respectively). Phosphate starvation induced the highest production of (-)-orobanchol (**1**) and *ent*-2'-*epi*-5-deoxystrigol (**2**) which resulted in the highest germination of *Striga* seeds. Fluridone application inhibited the biosynthesis of these SLs which resulted in a lower biological activity of the fractions and crude exudates, confirming the inhibitory effect of fluridone on SL production that was previously described (Matusova et al., 2005). MRM-LC-MS analysis of fractions 24-25 revealed the presence of three compounds with the same mass m/z 361 showing up in the 361.2>247 and 361.2>96.8 MRM channels. These metabolites were most abundant in exudates of phosphate starved plants and were reduced by fluridone application. The seed germination activity obtained with fractions 24 and 25 correlates with the abundance of the detected masses. MS/MS analysis of the compounds eluting in fractions 24 and 25 shows fragmentation patterns typical for SLs (Sato et al., 2003): loss of the D-ring and H₂O yields fragment ions $[M+H - D\text{-ring} - H_2O]^+$ with $m/z = 247$ and the fragment ion of the D-ring itself C₅H₅O₂ with $m/z = 97$ (Figure 5). The loss of methanol $[M+H - MeOH]^+$, yielding the fragment ion $m/z = 329$, is not typical for the fragmentation of known SLs and could indicate the presence of a methoxy-group in the molecule (Figure 5). This feature could explain the late retention time of these putative SLs compared with orobanchol (Figure 3 G) and other known SLs given that methyl ethers are less polar than alcohols (Figure 3 E and G). The MS/MS fragmentation spectra of all three compounds are very similar (Figure 5). Based on these data we suggest that the compounds eluting at 9.5, 10.3 and 10.9 are methoxy-5-deoxystrigol isomers (**5**). Isolation followed by NMR or chemical synthesis should give the final proof of the structure of these three isomers. As we do not have this proof as yet, we will refer to these new compounds under the combined name methoxy-5-deoxystrigol isomers (Jamil et al., 2011a). The absence of the putative methoxy-5-

Table 1. *Striga hermonthica* germination and *Gigaspora margarita* hyphal branching in the presence of SL standards

	<i>S. hermonthica</i> germination (%)		<i>Gi. margarita</i> hyphal branching ¹
	200 nM	20 nM	MEC ² in pg per disc
(±)-2'- <i>epi</i> -strigol	0.00 ± 0.00	0.00 ± 0.00	100
(-)-orobanchol ³	0.67 ± 0.67	0.00 ± 0.00	1
<i>ent</i> -2'- <i>epi</i> -5-deoxystrigol	-	-	3
2'- <i>epi</i> -5-deoxystrigol	-	-	30
(±)-2'- <i>epi</i> -5-deoxystrigol	11.33 ± 1.76	0.00 ± 0.00	-
GR24	-	-	100
<i>ent</i> -GR24	-	-	10000
2'- <i>epi</i> -GR24	-	-	1000
<i>ent</i> -2'- <i>epi</i> -GR24	-	-	1000
GR24 (4 stereoisomers)	25.33 ± 1.76	2.00 ± 1.15	
<i>ent</i> -5-deoxystrigol	-	-	30
5-deoxystrigol	-	-	3
(±)-5-deoxystrigol	26.00 ± 9.87	6.00 ± 3.05	-
(±)-strigol	19.33 ± 8.82	9.33 ± 4.67	100
<i>ent</i> -2'- <i>epi</i> -orobanchol ⁴	34.00 ± 1.15	8.67 ± 2.40	1
sorgomol	36.00 ± 3.05	26.00 ± 5.03	100

¹Results extracted from Akiyama et al. (Akiyama et al., 2010); ² MEC = minimum effective concentration; ³In Akiyama et al. (Akiyama et al., 2010) these compounds are named (+)-orobanchol and (+)-2'-*epi*-orobanchol respectively, before revision of stereochemical structure whereas the present table indicates the revised stereochemistry (Xie et al., 2013).

deoxystrigol isomers in *d10-2* exudate further supports that these compounds are produced from the SL pathway (Figure 6 B and C).

Fractions 16 and 18 induced the highest level of *S. hermonthica* germination (Figure 2 and Figure S1) and do not contain any of the SLs discussed above. As mentioned above, two other SLs, orobanchyl acetate (**3**) and 7-oxoorobanchyl acetate (**4**), were recently reported in rice (Xie et al., 2013). Orobanchyl acetate (**3**) elutes after (-)-orobanchol (**1**) and is unlikely to be responsible for the activity in fractions 16 and 18. 7-Oxoorobanchyl acetate (**4**) elutes before (-)-orobanchol (**1**) and could be present in fraction 16 to 18. This SL was previously detected in

exudates collected 10 to 17 days after germination (Xie et al., 2013). In the present study, exudates were collected in later stages and this SL was not detected in crude exudates nor in any of the fractions. We could also not detect any other known SLs in fractions 16 to 18; however the seed germination bioassays showed that the activity of fractions 16 and 18 followed the same trend across the treatments as the activity of SL containing fractions. They were increased by phosphate starvation and reduced by fluridone application (Figure 2 A). The seed germination stimulatory activity of these fractions in *d10-2* root exudate was also clearly reduced (Figure 6 A). All this strongly suggests that the activity in these fractions is caused by compounds derived from the SL pathway after carlactone (as CCD8 is required for their production). Considering their high activity in the induction of *S. hermonthica* germination it is of great interest to identify these compounds.

Overall, the activity profiles for *S. hermonthica* seed germination and AM fungal hyphal branching are similar but not the same (Figure 2). All active fractions in the seed germination bioassay exhibited hyphal branching stimulatory activity albeit to a different extent. Fraction 20 [(-)-orobanchol (**1**)] induced high AM fungal hyphal branching but stimulated little germination of *S. hermonthica* seeds compared with other fractions. Also, *ent-2'-epi-5-deoxystrigol* (**2**, fraction 28; low seed germination), the methoxy-5-deoxystrigol isomers (fractions 24-25; low seed germination) and fraction 16 and 18 (high seed germination) have very different activity with regard to the induction of seed germination whereas being quite similar in the induction of hyphal branching. Fraction 27 did not display seed germination stimulatory activity, but it did induce hyphal branching. This is probably due to the presence of *ent-2'-epi-5-deoxystrigol* (**2**) that is still detected in this fraction but at lower concentration than in fraction 28. Hence the concentration of *ent-2'-epi-5-deoxystrigol* (**2**) in fraction 27 may not be sufficient to induce *S. hermonthica* seed germination but is apparently high enough to induce AM fungal hyphal branching.

The separation on HPLC is not good enough to separate all the active compounds. This results in tailing peaks for example for (-)-orobanchol (**1**), present in fractions 19 to 21 and with highest abundance in fraction 20. Fraction 19 induces response of both *S. hermonthica* seeds and AM fungi to an extent that is intermediate to fractions 18 and 20. The activity in fraction 19 is probably a result from the cumulative effect of (-)-orobanchol (**1**) and the tail of the unknown active compound eluting mostly

in fraction 18. Similarly, the activity observed in fraction 17 might also be due to fronting of fraction 18 and tailing of fraction 16. However, we cannot exclude the presence of other active compounds in fractions 17 and 19.

The two activity profiles show that some of the most active mycorrhizal hyphal branching stimulants present in rice root exudates play only a minor role in the induction of *S. hermonthica* germination. The seed germination stimulatory activity of known concentrations of SLs was assessed and compared with results of a study relating structural differences in SLs to AM fungal hyphal branching stimulatory activity (Akiyama et al., 2010) (Table 1). The structure of (-)-orobanchol (**1**) and (+)-*ent*-2'-*epi*-orobanchol (**6**) have been revised after the study by Akiyama et al. (Akiyama et al., 2010) hence, these compounds were originally labeled (+)-orobanchol and (+)-2'-*epi*-orobanchol respectively (Akiyama et al., 2010; Xie et al., 2013; Ueno et al., 2011). As previously shown by Nomura et al. (Nomura et al., 2013) sensitivity of *S. hermonthica* seeds is highly dependent on the orientation of the C-ring, and is more sensitive to the strigol-type configuration. In our bioassays this preference is confirmed, the highest seed germination was obtained with sorgomol (**7**) and (+)-*ent*-2'-*epi*-orobanchol (**6**) while (-)-orobanchol (**1**) hardly induced any seed germination (Table 1).

AM fungi also have different sensitivity to different SL structures (Akiyama et al., 2010). The strigol-type configuration is sometimes more active, as was observed for 5deoxystrigol (**8a**, R¹=CH₃; R²=H) and GR24 (**8a**, R¹=H; R²=H) but not always (Akiyama et al., 2010). For instance, all strigol stereoisomers (**8a-d**, R¹=CH₃; R²=OH) have equal activity just as (-)-orobanchol (**1**) and (+)-*ent*-2'-*epi*-orobanchol (**6**) (Table 1) (Akiyama et al., 2010). Also the two natural stereoisomers of 5deoxystrigol (**8a** and **8c**, R¹=CH₃; R²=H) have each the same activity at inducing hyphal branching (Table 1) (Akiyama et al., 2010). Indeed, the activity of SLs to stimulate hyphal branching seems to be more influenced by modifications in rings A and B than by stereochemical variation (Akiyama et al., 2010; Zwanenburg and Pospíšil, 2013). In our hyphal branching assay with a different AM species, *Gi. margarita*, we obtained a similar response to the different SLs as reported for *Gi. rosea* (Akiyama et al., 2010). Fraction 20, where (-)-orobanchol (**1**) elutes, displays high activity in the branching bioassay whereas there is no clear activity peak in the seed germination bioassay (Figure 2). Similarly, *ent*-2'-*epi*-5-deoxystrigol (**2**)

detected in fractions 27 and 28 induced hyphal branching and only fraction 28 with highest amounts of this SL induced low seed germination (Figure 2). SL activity is also affected by different chemical and structural properties that influence diffusion and stability (Zwanenburg et al., 2009; Akiyama et al., 2010). However compared to other SLs, (-)-orobanchol (**1**) and *ent*-2'-*epi*-5-deoxystrigol (**2**) are highly active at stimulating hyphal branching. Therefore, the low activity of (-)-orobanchol (**1**) and *ent*-2'-*epi*-5-deoxystrigol (**2**) at inducing seed germination does not seem to be a result of instability or poor diffusion of these two SLs but rather a result of lower sensitivity of the seeds to these compounds.

Our bioassays suggest that strong hyphal branching stimulators make little contribution to the overall stimulation of parasitic seed germination. Moreover, the fractions showing the largest effect on seed germination (fractions 16 and 18) contain stimulants of unknown structure. The reduction of activity in these fractions by fluridone and by mutation in *D10* (*CCD8*) suggests that they are SL-like. The strong differences in activity across the exudate fractions suggest that *S. hermonthica* infection and potentially also the infection by other parasitic plant species could be reduced by altering the qualitative composition of SLs rather than just quantitatively reducing their production. New varieties with such altered SL composition could maintain their ability to establish symbiosis with AM fungi while at the same time they induce less *Striga* seed germination. In a recent study, 20 rice cultivars were screened for the abundance of SLs in their root exudates (Jamil et al., 2012). The authors observed that the relative amounts of (-)-orobanchol (**1**) and *ent*-2'-*epi*-5-deoxystrigol (**2**) differ across cultivars, suggesting that selection for different SL composition is possible.

ent-2'-*epi*-5-Deoxystrigol (**2**) and 5deoxystrigol (**8a**, R¹=CH₃; R²=H) have the most simple structure of all SLs so far identified in plants. They have been suggested to be produced from carlactone through the action of MAX1 [and possibly additional enzyme(s)] (Alder et al., 2012; Seto et al., 2014) which would imply that the orientation of the C-ring, a structural feature of major importance for the induction of seed germination as well as hyphal branching, is determined by MAX1. We propose that *ent*-2'-*epi*-5-deoxystrigol (**2**) is the precursor for the remaining rice SLs. Further supporting this hypothesis, it has been recently shown that a sorghum enzyme(s) - likely a cytochrome P450 - converts *ent*-2'-*epi*-5-deoxystrigol (**1**) and 5-deoxystrigol

(**8a**, R¹=CH₃; R²=H) into *ent-2'-epi-sorgomol* and *sorgomol* respectively (Motonami et al., 2013). Breeding for a different SL composition would be greatly aided by the characterization of these later steps in SL biosynthesis, that is the decoration of the SLs' core-structure.

As a word of caution, the sensitivity to specific SLs may vary between *Striga* species and/or races (Matusova and Bouwmeester, 2006). Therefore, assessment of the seed germination and hyphal branching requirements for the host/parasite and host/AM combination present in a certain region would be necessary in order to direct the development of new, locally adapted, cultivars that are less affected by *Striga* parasitism but still efficient in AM symbiosis establishment. A study performed in sorghum has shown that host plants are especially vulnerable to plant parasitism in the early stages of their life cycle (Gurney et al., 1999). Since SL composition in rice exudates changes according to the age of the plants (Xie et al., 2013) efforts to produce new rice varieties should take into consideration SL variation throughout the life cycle.

Finally, we can only speculate about the driving forces for the diversification in SL structures that we see in rice. Unlike for the gibberellins, it seems that there are not just one or two active molecules accompanied by inactive precursors and degradation products. In their role as rhizosphere signaling molecules the different SLs all display activity, albeit admittedly with different efficiency. With regard to their endogenous function, as plant hormones regulating a suit of developmental processes, we only just begin to understand the structure-activity relationships (Boyer et al., 2012). As a result of all these different functions the consequences of evolutionary and human (breeding) selection pressures are complex – which is reflected in the large structural diversification - and the resulting structural diversification so far difficult to explain.

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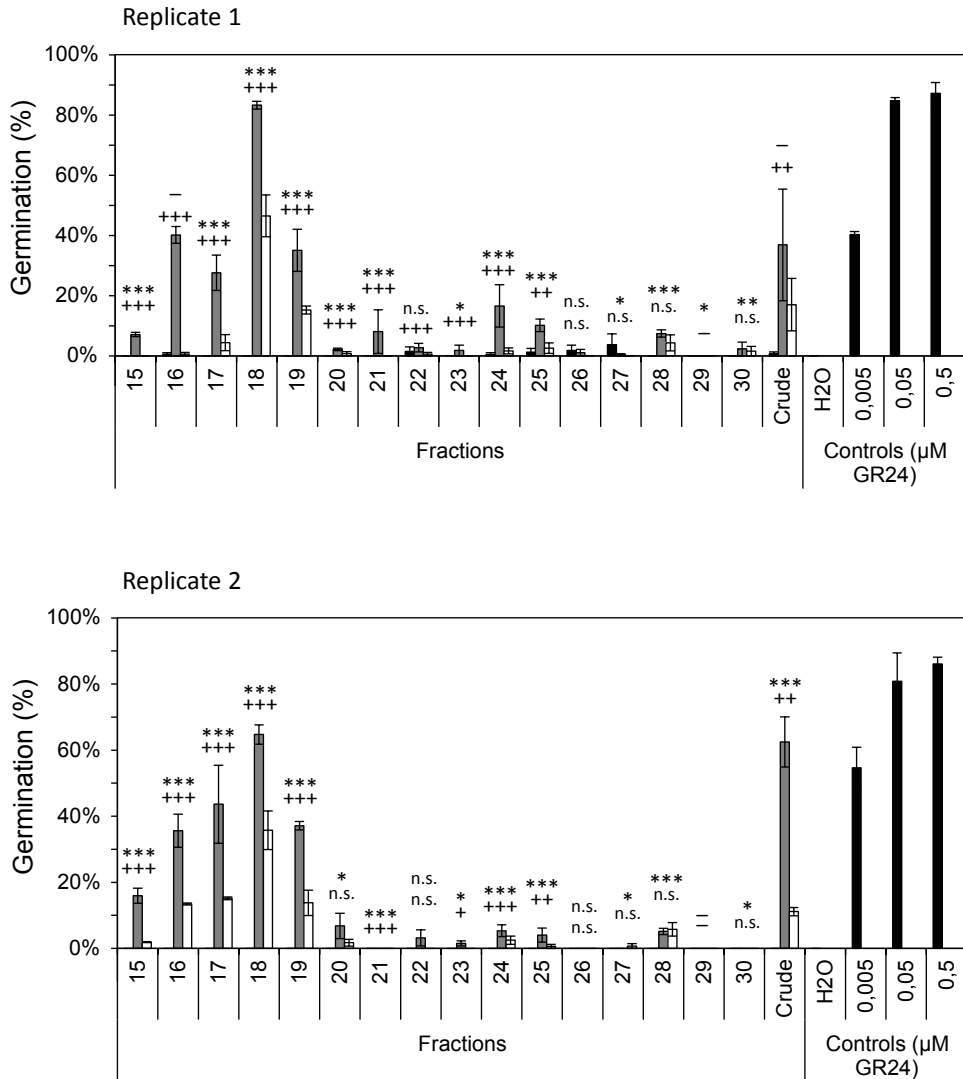


Figure S1. Activity profiles of rice root exudates tested with *S. hermonthica* seed germination assay. Two biological replicates are shown here and the third replicate is shown in Figure 2A. Crude exudates and exudate fractions from rice plants treated with full nutrition (black bars); phosphate starvation (grey bars) and phosphate starvation plus 0.01 μM fluridone (white bars). Water and SL analogue GR24 (0.005, 0.05 and 0.5 μM) were used as controls. The error bars represent the standard error of 3 technical replicates. Significance levels between treatments as determined using a χ^2 test are indicated: */+ = $P < 0.05$; **/++ = $P < 0.01$; ***/+++ = $P < 0.001$; n.s. = $P > 0.05$; * = control vs. phosphate starvation treatment; + = phosphate starvation vs. phosphate starvation plus fluridone treatment. When germination values are close to zero the statistical test cannot be performed, which is indicated with “–”.



Natural variation of rice strigolactone biosynthesis is associated with the deletion of two MAX1 orthologs

Catarina Cardoso^{a,‡}, Yanxia Zhang^{a,‡}, Muhammad Jamil^{a,‡}, Jo Hepworth^{b,‡}, Tatsiana Charnikhova^a, Stanley O. N. Dimkpa^c, Caroline Meharg^d, Mark H. Wright^e, Junwei Liu^a, Xiangbing Meng^f, Yonghong Wang^f, Jiayang Li^f, Susan R. McCouch^e, Ottoline Leyser^{b,g}, Adam H. Price^c, Harro J. Bouwmeester^{a,h}, and Carolien Ruyter-Spira^{a,i}

[‡] C.C., Y.Z., M.J., and J.H. contributed equally to this work.

^a Laboratory of Plant Physiology, Wageningen University, The Netherlands

^b Department of Biology, University of York, United Kingdom

^c Institute of Biological and Environmental Sciences, University of Aberdeen, UK

^d Institute of Global Food Security, Queen's University Belfast, Northern Ireland

^e Department of Plant Breeding and Genetics, Cornell University, Ithaca, NY

^f State Key Laboratory of Plant Genomics and National Center for Plant Gene Research, Chinese Academy of Sciences, Beijing, China

^g Sainsbury Laboratory, University of Cambridge, United Kingdom

^h Centre for Biosystems Genomics, Wageningen, The Netherlands

ⁱ Bioscience, Plant Research International, Wageningen, The Netherlands

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Abstract

Rice (*Oryza sativa*) cultivar Azucena—belonging to the *Japonica* subspecies—exudes high strigolactone (SL) levels and induces high germination of the root parasitic plant *Striga hermonthica*. Consistent with the fact that SLs also inhibit shoot branching, Azucena is a low-tillering variety. In contrast, Bala, an *Indica* cultivar, is a low-SL producer, stimulates less *Striga* germination, and is highly tillered. Using a Bala × Azucena F₆ population, a major quantitative trait locus—qSLB1.1—for the exudation of SL, tillering, and induction of *Striga* germination was detected on chromosome 1. Sequence analysis of the corresponding locus revealed a rearrangement of a 51- to 59-kbp stretch between 28.9 and 29 Mbp in the Bala genome, resulting in the deletion of two cytochrome P450 genes—*SLB1* and *SLB2*—with high homology to the Arabidopsis SL biosynthesis gene, *MAX1*. Both rice genes rescue the Arabidopsis *max1-1* highly branched mutant phenotype and increase the production of the SL, *ent-2'-epi-5-deoxystrigol*, when overexpressed in Bala. Furthermore, analysis of this region in 367 cultivars of the publicly available Rice Diversity Panel population shows that the rearrangement at this locus is a recurrent natural trait associated with the *Indica/Japonica* divide in rice.

Introduction

The root parasitic *Striga* spp. parasitize on roots of crops in tropical and subtropical areas. The species typically parasitize cereals, including economically important crops such as maize, sorghum, millet and rice (Parker, 2009). The parasitic relationship is dependent on the ability of the parasite to detect the host, which is mediated by the perception of strigolactones (SLs), molecules exuded by the host into the rhizosphere, by the seeds of the parasite (Cardoso et al., 2011). SLs are also signalling molecules for the establishment of the symbiosis with arbuscular mycorrhizal (AM) fungi (Akiyama et al., 2005) that help the plant to improve nutrient uptake. Under low phosphate availability, SL exudation into the rhizosphere is strongly enhanced, hence promoting AM symbiosis (Bouwmeester et al., 2007). As a negative consequence, however, agricultural areas with poor soils and low fertilizer input are strongly affected by *Striga* (Bouwmeester et al., 2007; Parker, 2009). In addition to their rhizosphere role, SLs also function as plant hormones inhibiting shoot branching and modulating root architecture (Gomez-Roldan et al., 2008; Umehara et al., 2008; Ruyter-Spira et al., 2011), also in response to phosphate deficiency (Kohlen et al., 2011; Umehara et al., 2010). SL biosynthesis or signalling mutants have increased axillary bud outgrowth, resulting in a bushy and dwarf phenotype (Domagalska and Leyser, 2011). Biosynthesis of SLs proceeds through isomerization of β -carotene by β -CAROTENE ISOMERASE (D27), followed by cleavage by CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7) and CAROTENOID CLEAVAGE DIOXYGENASE 8 (CCD8), which results in the formation of carlactone (Sorefan et al., 2003; Booker et al., 2004; Zou et al., 2006; Arite et al., 2007; Lin et al., 2009; Alder et al., 2012). The gene(s) responsible for the conversion of carlactone to a SL has/have not been identified, although *MORE AXILLARY GROWTH 1* (*MAX1*), encoding a cytochrome P450 (CYP) in Arabidopsis, has been suggested to be a candidate (Kohlen et al., 2011; Booker et al., 2005; Alder et al., 2012; Ruyter-Spira and Bouwmeester, 2012). SL signalling is mediated by an F-Box protein (*MAX2* in Arabidopsis; *D3* in rice) and an α/β -hydrolase protein (*D14*) (Gomez-Roldan et al., 2008; Umehara et al., 2008; Arite et al., 2009; Hamiaux et al., 2012).

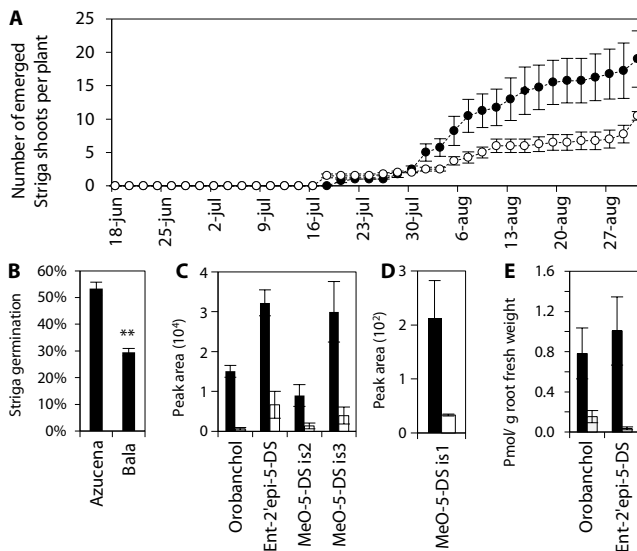
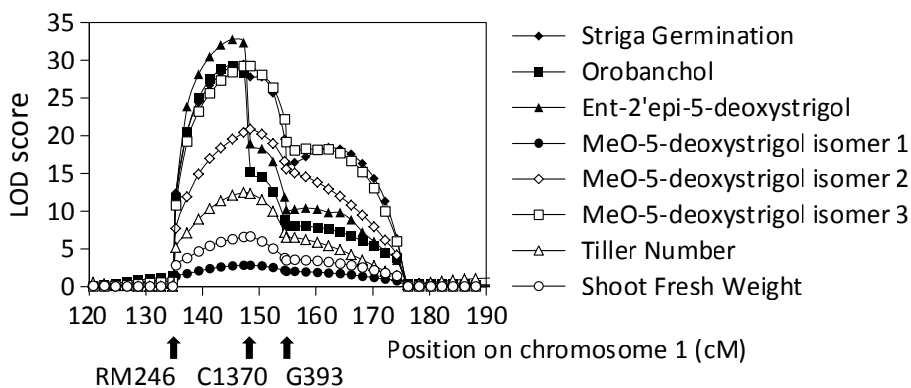


Figure 1 Analysis of the parental lines of the RIL population Bala x Azucena. Emergence of *Striga* shoots per pot during a period of 78 days. (● – Azucena; ○ – Bala) **(A)** germination percentage of *Striga* seeds exposed to crude exudates of the parental lines. **(B)** LC-MS peak areas of orobanchol, *ent-2'-epi-5-deoxystrigol* (*ent-2'-epi-5-DS*) and methoxy-5-deoxystrigol isomers 1-3 (MeO-5-DS is) in root exudates **(C,D)** and orobanchol and *ent-2'-epi-5-deoxystrigol* in root extracts **(E)**. In A-E error bars represent standard error of the mean, n=4 (A), n=3 (B-E). In B-E plants were grown under low P nutrition for one week, prior to exudate collection. In C-E black bars correspond to Azucena, white bars to Bala.



In the present study molecular genetics was used to further elucidate the SL biosynthetic pathway. We had observed that the rice cultivars Bala and Azucena differ greatly in SL biosynthesis and susceptibility to *Striga* infection. The Bala × Azucena F₆ recombinant inbred line (RIL) population was used to map quantitative trait loci (QTL) related to SLs. A major QTL was detected explaining most of the variation in the concentrations of all five SLs detected in rice exudates. This locus was also detected as a QTL for rice–*Striga* interaction in a previous study that used the same population (Kaewchumnong and Price, 2008). Here we show that the QTL is due to a rearrangement of a 51- to 59-kbp stretch between 28.9 and 29.0 Mbp of chromosome 1 in the Bala genome. This rearrangement results in the deletion of two CYP genes, which we show are orthologs of the Arabidopsis *MAX1*. The rearrangement of this locus is a recurrent natural trait, observed in several rice cultivars.

Results

Rice Varieties Bala and Azucena Show Differential Susceptibility to Striga hermonthica Infection.

When the two parental lines were grown in soil infected with *Striga* seeds, emergence of *Striga* occurred fastest on Bala, and during the 2 wk after first emergence, there was on average one *Striga* shoot in Azucena and two in Bala (Figure 1A). After 2 wk, however, the number of *Striga* shoots increased more rapidly in Azucena, and after 5 wk Azucena had on average 19 *Striga* shoots, whereas Bala had only 10.

Low Striga Infection Rate in Bala Correlates with Reduced SL Exudation.

To investigate whether the difference in *Striga* emergence and infection between Bala and Azucena is the result of differences in SL exudation, root exudates and extracts of the parental lines were analyzed. Azucena root exudates induced a higher percentage of *Striga* seed germination than Bala exudates (Figure 1B). Liquid chromatography (LC)-MS analysis of root exudates and extracts of Bala and Azucena showed that the higher germination in Azucena root exudate correlates with higher amounts of the SLs orobanchol, *ent*-2'-*epi*-5-deoxystrigol, and three methoxy-5-deoxystrigol isomers in Azucena (Jamil et al., 2011; Xie et al., 2013) (Figure 1C, D and E).

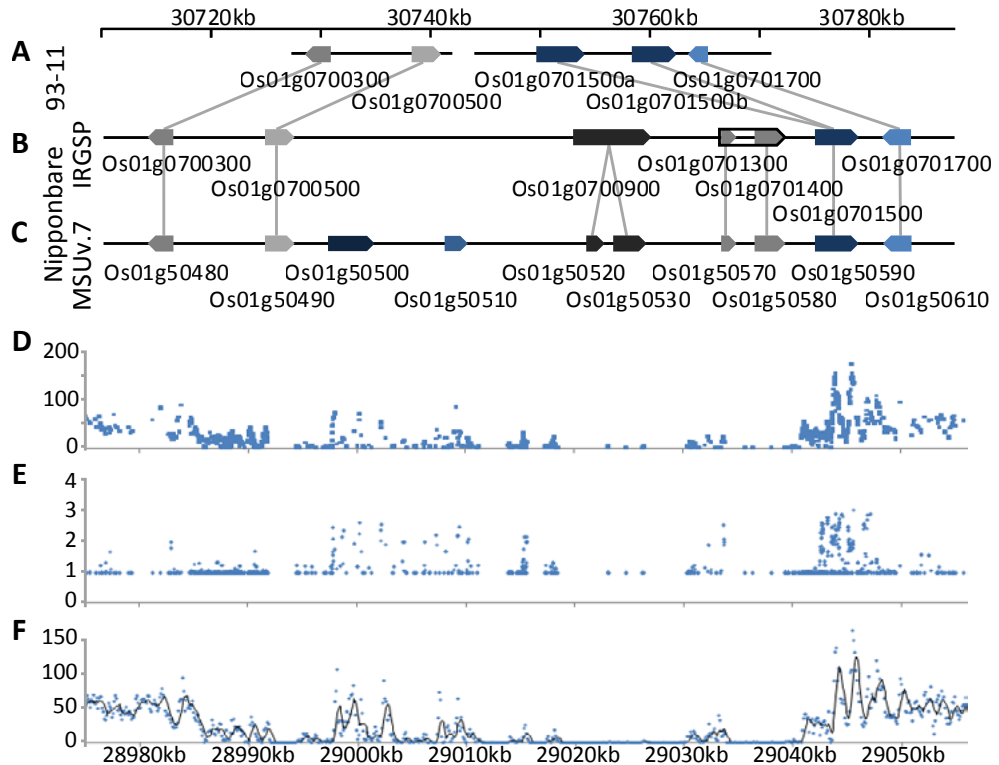


Figure 3 Alignment of genomic sequences of 93-11, Nipponbare and Bala in the region where the major QTL for SL production was identified. **(A)** Gene models for cultivar 93-11 were adapted from the genome browser of the Beijing Institute of Genomics (<http://rice.genomics.org.cn/rice/index2.jsp>) **(B)** Gene models for cultivar Nipponbare as predicted in the genome browser of the Genome Sequencing Project (IRGSP-1.0: <http://rapdb.dna.affrc.go.jp/viewer/gbrowse/irgsp1/>). *Os01g0700900* was confirmed by RACE PCR, *Os01g0701300* and *Os01g0701400* turned out to be one single gene indicated with black outline. **(C)** Annotation by the genome browser of the Rice Genome Annotation Project (RGAP) (<http://rice.plantbiology.msu.edu/>). Genes that are homologous between the different annotations are linked by a line. **(D-F)** show the alignment of the 55x coverage of Bala Illumina sequence reads to the MSU Nipponbare reference. SNP frequency **(D)**, Ratio of Bala sequence read depth to SNP frequency **(E)**, Bala sequence read depth **(F)**. Note the evidence of a deletion between 28985 kb and 29040 kb since read depth and SNP frequency are close to zero (except around the transposon *Os01g50500* and the expressed protein gene *Os01g50510*) and the evidence of a duplication for *Os01g50590* since both read depth and SNP frequency are high and a ratio of read depth to SNP frequency of 2 indicates that two alleles align to the same place. Note the coordinates at the bottom of the image that refer to the RGAP (MSUv.7) annotation to which Bala sequences were aligned and do not match the IRGSP build5 coordinates that are indicated at the top of the image.

QTL Mapping of SL Levels and Related Phenotypes.

Given the different amounts of SLs found in the parental lines, we used the Bala × Azucena mapping population (Kaewchumnong and Price, 2008) to map *Striga* germination, the level of SLs in root exudates, shoot and root fresh weight (fwt), and tillering. The broad sense heritabilities of all traits were high (78–97%) with the exception of methoxy-5-deoxystrigol isomer 1 (44%). A major QTL for *Striga* germination, qSTRIGOLACTONE BIOSYNTHESIS 1.1 or qSLB1.1 [logarithm of the odds ratio (LOD) = 29.42, R^2 = 66%], was identified on chromosome 1 at 143,8 cM near marker C1370 (Figure 2, Table S1, and Figure S1). At the same position, QTLs were detected for the levels of orobanchol (LOD = 29.25, R^2 = 70.2%), *ent-2'-epi-5-deoxystrigol* (LOD = 32.7, R^2 = 71.2%), and methoxy-5-deoxystrigol isomers 1, 2, and 3 (LOD = 2.83, R^2 = 6.7%; LOD = 20.83, R^2 = 49.2%; and LOD = 29.17, R^2 = 52.8%, respectively). For all these traits, the positive allele was from Azucena. In addition, tiller number (LOD = 12.43, R^2 = 28.8%) and shoot fwt (LOD = 6.64, R^2 = 14.6%) mapped to this region, however, with the positive effect from Bala. Minor QTLs for SL levels and tillering mapped to chromosome 6 and 10 (Table S1 and Figure S1). Although these QTLs have lower LOD scores (2.5–4.6), the colocalization of QTLs for SL level and a SL-related phenotypic trait makes them interesting candidate loci for additional SL regulatory, biosynthetic, and/or signaling genes.

Molecular Analysis of the Major QTL on Chromosome 1 Reveals a Genome Rearrangement in Bala.

The genomic region for the major QTL contained a genomic rearrangement (Figure 3). Alignment of the genomic sequence and predicted genes of *Indica* cultivar 93-11 from the Beijing Institute of Genomics (<http://rise2.genomics.org.cn/page/rice/download.jsp>) with the International Rice Genome Sequencing Project (IRGSP-1.0: <http://rapdb.dna.affrc.go.jp/viewer/gbrowse/irgsp1/>; Kawahara et al., 2013; Sakai et al., 2013) Nipponbare reference sequence showed the following: (i) three predicted Nipponbare genes between 30,750 and 30,771 kbp are missing in the *Indica* sequence; (ii) there is a gap in the *Indica* sequence within that region; and (iii) the *Indica* cultivar appears to have two genes with homology to a single gene, *Oso1g0701500*, in Nipponbare (Figure 3A, B and C). Alignment of Bala

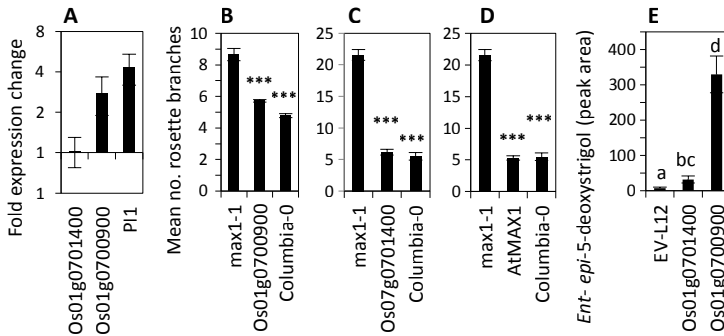


Figure 4 Expression analysis (A) and functionality of rice CYP genes *Os01g0700900* and *Os01g0701400* in Arabidopsis (B–D) and rice (E). Regulation of gene expression of *Os01g0700900*, *Os01g0701400* and the low-P marker gene *PI* by low P nutrition measured by qRT-PCR, n=4-5 (A). Complementation of axillary bud outgrowth of *max1-1* by overexpression of *Os01g0700900* (B), *Os01g0701400* (C) and *AtMAX1* (D) under control of the 35S promoter (n = 2-9). Significance values (Students t-test) are shown as * ($P < 0,05$); ** ($P < 0,01$); *** ($P < 0,001$). Levels of *ent-2'epi-5-deoxystrigol* measured in root exudates of Bala cultivar transformed with *Os01g0701400*, *Os01g0700900* under control of the 35S promoter and empty vector control pHm43GW (n=2-4) (E). Different letters indicate significantly different means at $P < 0.05$, using ANOVA followed by Student t-test on log transformed data. Values shown in graph are back transformed. In A–E bars represent mean values \pm SE.

genomic sequence reads (88- to 120-bp lengths at 55 \times genome coverage) to the IRGSP Nipponbare reference sequence revealed a stretch of between 51 and 59 kbp with a Bala read depth close to zero (Figure 3F). This alignment also confirms that *Oso1g0701500* is duplicated (Figure 3D, E and F) and that the Bala genes in this region share 100% homology with the 93-11 *Indica* sequence. Two of the genes in the rearranged region that appear to be missing in 93-11 and Bala, have conserved domains that classify them as CYP (*Oso1g0700900* and *Oso1g0701400*). At the border of the deleted region, *Oso1g0701500*, which is present in Nipponbare and is duplicated in 93-11 and Bala, also contains this conserved domain. When annotation of the recent Build 5 of the IRGSP-1.0 was compared with the MSU v.7 annotation [Rice Genome Annotation Project (RGAP)], it was found that *Oso1g0700900* is split into two genes, *LOC_Oso1g50520* and *LOC_Oso1g50530* (Figure 3B and C). *Oso1g0701300* and *Oso1g0701400* correspond to *LOC_Oso1g50570* and *LOC_Oso1g50580*, respectively, whereas *Oso1g0701500* is the same as *LOC_Oso1g50590* (Figure 3B and C).

The Rearranged Region on Bala Chromosome 1 Contains MAX1 Homologs.

A BLASTX search, using the predicted ORFs of the deleted genes *Oso10700900* and *Oso1g0701400*, revealed high similarity to the SL biosynthetic gene *MORE AXILLARY BRANCHES 1* (*At2g26170*) from *Arabidopsis*, which belongs to the CYP711A1 family. We could confirm experimentally by RACE PCR that the IRGSP annotation for *Oso10700900* is correct, although with different splicing form and start codon (Genbank accession no.: JX235697, Figure S2A), whereas the RGAP annotation for *Oso1g50520/30* is incorrect. We could also confirm that *Oso1g0701300* and *Oso1g0701400* and the corresponding RGAP-annotated *Oso1g50570/80* are one single gene, which from here on will be referred to as *Oso1g0701400* (Genbank accession no.: JX235696; Fig. S2B). The protein sequences of *Oso10700900* and *Oso1g0701400* show, respectively, 57.6% and 60.3% identity to *AtMAX1* (Figure S3). Quantitative RT-PCR (qRT-PCR) showed that expression of *Oso10700900* in Nipponbare was induced by low-P treatment—just as expression of the P starvation marker *OsPI1* (Wasaki et al., 2003) - but not of *Oso1g0701400* (Figure 4A).

Oso1g0700900 and *Oso1g0701400* Rescue the Branched Phenotype of *Arabidopsis max1*.

Table 1 Lines from the diversity panel selected to test the impact of the genomic rearrangement on strigolactone levels, tillering and *Striga* germination.

Azucena Allele			Bala Allele		
Line	Sub-group ^a	Country of origin	Line	Sub-group ^a	Country of origin
Azucena	TRJ	Philippines	Bala	IND	India
DZ-78	AUS	Bangladesh	Dhala-S	AUS	Bangladesh
Aswina-330	AUS	Bangladesh	Kachilon	AUS	Bangladesh
Kun-Min-TH	IND	China	Guan-Yin-T	IND	China
Ta-Mao-T	TEJ	China	Sung-Liao-2	TEJ	China
Sinaguing	TRJ	Philippines	Asse Y Pung	TRJ	Philippines
			Kinastano	TRJ	Philippines

^aAccording to Zhao et al. (2010)

Arabidopsis max1-1 was transformed with the *Oso1g0700900* and *Oso1g07001400* cDNA under the control of the CaMV35S promoter (p35S). p35S:*AtMAX1* was used as a positive control. *Oso1g0700900*, *Oso1g07001400*, and *AtMAX1* all fully restored the branching phenotype of *max1-1* ($P < 0.001$) (Figure 4 B, C, D, and Figure S4) showing that these two rice CYP450 genes are *AtMAX1* orthologs.

Oso1g0700900 and *Oso1g0701400* Increase SL Levels in Root Exudates of Bala.

Bala was independently transformed with *Oso1g0700900* and *Oso1g0701400*, driven by the p35S promoter. The levels of *ent-2'-epi-5-deoxystrigol* in root exudates of P-starved *p35S:Oso1g0700900*- and *p35S:Oso1g0701400*-transformed T1 Bala plants (for which transgene expression was confirmed) both significantly increased compared with the empty vector control, but the effect of *p35S:Oso1g0700900* was much stronger than that of *p35S:Oso1g0701400* (Figure 4E).

Rearrangement in Rice Chromosome 1 Is Associated with Low SL Levels in a Collection of Accessions.

To establish the presence of the Bala rearrangement on chromosome 1 across rice germplasm, a PCR assay was developed using multiplexed primers for three genes (*Oso1g0700900*, *Oso1g0701400*, and *Oso1g0701500*), which all give a product in Azucena, but for which only *Oso1g0701500* gives a product in Bala. The multiplex test was applied to 367 cultivars of the publicly available Rice Diversity Panel. In the *indica* and *aus* subpopulations within the *Indica* subspecies (Zhao et al., 2010) the Nipponbare/Azucena allele frequency was 3/74 (4.1%) and 4/59 (6.8%), respectively, whereas in the *temperate* and *tropical japonicas* within the *Japonica* subspecies (Zhao et al., 2010) it was 93/96 (96.9%) and 63/94 (67%), respectively, showing that the genome rearrangement is associated with the *Indica/Japonica* divide in rice. To evaluate how the deletion affects SL biosynthesis in different genetic backgrounds, the SL content of root exudates and root extracts was analyzed in pairs of cultivars that differed in the allele under study but were otherwise from the same subspecies and the same country of origin (Table 1). The lines containing the Bala alleles (carrying the rearrangement) had more tillers, exuded lower amounts of SL, had lower SL root content, and induced lower *Striga* germination (Figure 5, Table S3) than the

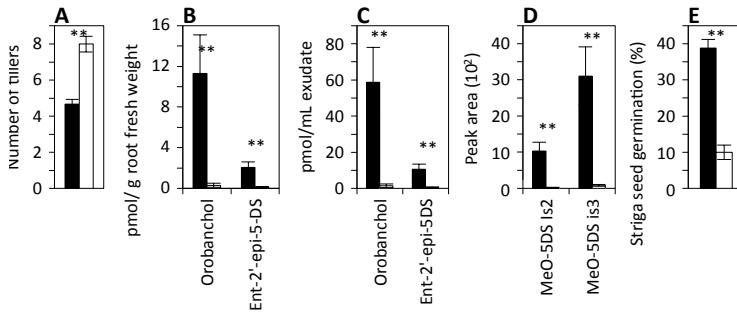


Figure 5 Impact of the rearrangement on the **(A)** number of tillers, **(B)** the strigolactone content in root tissue and **(C, D)** root exudates and **(E)** on the stimulation of *Striga* germination by root exudates. Orobanchol and *ent-2'-epi-5*-deoxystrigol (*ent-2'-epi-5-DS*) are determined in pmol/ g fresh weight in B or pmol/ ml exudate in C. In D, levels of methoxy-5-deoxystrigol isomers 1-2 (MeO-5-DS is) in root exudates are determined in peak area. The lines used for this comparison are listed in Table 1. Bars represent the mean values \pm SE obtained from lines containing the Azucena allele (black bars) or the Bala allele (white bars) with the rearrangement. Significance values (Wilcoxon rank sum test) are shown as * ($P < 0,05$); ** ($P < 0,01$); *** ($P < 0,001$).

genotypes containing the Azucena alleles in both the *Indica* and the *Japonica* genetic backgrounds.

Discussion

A strong QTL for SL production in rice—qSTRIGOLACTONE BIOSYNTHESIS (qSLB1.1)—was mapped to chromosome 1. In this locus the Bala genome contains a 51- to 59-kbp rearrangement compared with Nipponbare. The rearrangement spans two CYP genes (*Oso1g0700900*, *SLB1*; and *Oso1g0701400*, *SLB2*) that are present in Azucena but absent in Bala. Both genes share high similarity with *AtMAX1*, the CYP that is required for the biosynthesis of SLs (Kohlen et al., 2011; Booker et al., 2005). Overexpression of either of the two genes in *Arabidopsis max1-1* rescued the branched mutant phenotype, and overexpression in Bala—particularly of *Oso1g0700900*—increased the level of *ent-2'-epi-5*-deoxystrigol in the root exudate. Although it is possible that there are other elements or genomic features present in the rearranged region that may also contribute to the differential expression of SLs, the results show that *Oso1g0700900* and *Oso1g0701400* contribute to SL biosynthesis in rice and that the deletion of these genes in the Bala genome causes the low SL levels that are observed.

Our SL analyses show an overall reduction of all SL in Bala exudates compared with Azucena but no differences in the composition (Figure 1C). This result suggests that the *MAX1* orthologs in rice discovered in the present work contribute to the synthesis of all SLs present in rice, possibly at an earlier step of the biosynthetic pathway, rather than to the biosynthesis of specific structural SL variants. The big difference in SL levels driven by the Azucena allele also suggest that the SL biosynthetic CYP genes characterized in this study make an important contribution to SL biosynthesis across different genetic backgrounds. Nevertheless, SLs are still produced in Bala, showing that there must be redundancy for this biosynthetic step. In Arabidopsis, only a single *MAX1* ortholog is present, but indeed in rice, besides the two CYP genes described in the present study, three other CYP genes homologous to *MAX1* are present in the rice genome: *Oso1g0701500*, *Oso6g0565100*, and *Oso2g0221900*. Two of these, *Oso2g0221900* and *Oso6g0565100*, also rescued the branched phenotype of Arabidopsis *max1-1* (Challis et al., 2013) suggesting that they also have *MAX1*-like activity as well. In fact, several other monocotyledonous species such as maize and sorghum have two to five *MAX1* orthologs, whereas in dicotyledonous species such as petunia and *Medicago*, generally only one and sometimes two are present (Challis et al., 2013; Drummond et al., 2012).

Gene duplication allows for diversification in metabolic regulation. This diversification was also observed for *Oso1g0700900* and *Oso1g0701400* in the present study. The expression of *Oso1g0700900* was increased by P starvation but not that of *Oso1g0701400* (Figure 4A). In line with this finding, the expression of *Oso1g0700900* and *Oso2g0221900*, as well as the SL biosynthetic genes *D10*, *D17*, and *D27*, was repressed by P replenishment, whereas expression of *Oso1g07001400*, *Oso1g07001500*, and *Oso6g0565100* were not (Umehara et al., 2010). Finally, the levels of *ent-2'-epi-5-deoxystrigol* obtained in root exudates of Bala overexpressing *Oso1g0701400* were considerably lower than those of Bala overexpressing *Oso1g0700900* (Figure 4E). This difference may be caused by diversification in gene function after duplication, resulting in differences in enzymatic efficiency or specificity. Combined, these observations suggest that *MAX1* duplication has led to diversification in the regulation of SL biosynthesis in rice and other grass species, but not in dicots, for as-yet-unknown reasons. The existence of multiple *MAX1* orthologs in the rice genome does explain why mutant screens have been unsuccessful

in detecting these genes, whereas in *Arabidopsis* this approach was successful (Domagalska and Leyser, 2011). It also demonstrates the power of QTL mapping to address more fundamental questions, such as plant hormone biosynthesis, because it allowed us to obtain more insight into the SL biosynthetic pathway in rice. Two rice *MAX1* orthologs were revealed, and additional QTLs with minor influence on SL levels and tillering were also detected on chromosomes 6 and 10.

Population genetic analysis provides insights into the evolutionary history of genomes and traits, and our analysis of a Rice Diversity Panel revealed a striking difference in the frequency of the rearrangement found in this study between the two major rice varietal groups. The prevalence of the high-SL Azucena allele in the *Japonica* group and its virtual absence in the *Indica* group suggests that it is likely to have originated in a *Japonica* ancestor. By extension, this finding also supports the hypothesis that *Indica* and *Japonica* were domesticated from divergent populations of their wild ancestor, *Oryza rufipogon* (Kovach et al., 2007). The fact that the *Indica* low-SL allele is present in 33% of the tropical *japonica* varieties in the Diversity Panel is consistent with introgression from *indica* and *aus* varieties into tropical *japonica* varieties growing sympatrically in tropical environments. This pattern of allele divergence and introgression in *Oryza sativa* has been documented in other studies (Famoso et al., 2011; Sweeney et al., 2007; Takano-Kai et al., 2009; Zhao et al., 2011).

SLs regulate overall plant architecture. In shoots, SLs inhibit tillering; in roots, SLs influence root and root hair elongation and lateral root development (Gomez-Roldan et al., 2008; Koltai et al., 2010; Umehara et al., 2010; Kapulnik et al., 2011; Ruyter-Spira et al., 2011). Indeed, tillering mapped to the same SL locus on chromosome 1. Interestingly, in previous QTL studies using an IR64–Azucena and the Bala–Azucena mapping populations, several root architectural traits were mapped in the *same region*, suggesting that they may also be controlled by this locus (Champoux et al., 1995; Topp et al., 2013). These findings emphasize the potential of qSLB1.1 for the improvement of important agronomic traits in rice. Our study demonstrates once more the positive correlation between SL production and *Striga* germination (Jamil et al., 2011, 2012). However, although decreased SL biosynthesis results in less *Striga* germination, it is unclear whether SL levels also affect *Striga* attachment. In earlier work, *Striga* tolerance mapped to the same region

as qSLB1.1, with the Azucena allele increasing *Striga* tolerance (Kaewchumng and Price, 2008), even though in the present study we showed there were more *Striga* emerging on Azucena in controlled environments (Figure 1A). The later establishment of infection in Azucena likely explains the higher tolerance to *Striga*, similar to what was observed in sorghum, where tolerant varieties generally exhibit later *Striga* emergence (Gurney et al., 1999). Intriguingly, *Striga* postattachment resistance mapped to the same position on chromosome 1 as the QTL discussed in the present work (near marker C1370) in a cross between Nipponbare and Kasalath (Kaewchumng and Price, 2008) with the Nipponbare allele conferring greater resistance (Gurney et al., 2006). Nipponbare contains the same allele as Azucena, and we confirmed that Kasalath carries the same allele as Bala for the locus under study. This observation offers the intriguing hypothesis that higher SL levels increase *Striga* germination but reduce the subsequent efficiency of parasitization.

Materials and Methods

Mapping Population Plant Material.

A mapping population of 115 F6 RILs derived from Bala × Azucena described in Price et al (Price et al., 2000) was used. The experiments were conducted under controlled conditions (28 °C/25 °C; 450 μM·m⁻²·s⁻¹; 10-h light/14-h dark; and 70% relative humidity) in randomized design with three replicates, each consisting of one pot with five plants. After root exudate collection, the number of tillers per pot was counted, plants were removed from the pots, and root and shoot fwt were determined. Allelic frequencies of the rearrangement in the different subpopulations were assayed by using 367 diverse accessions from the publicly available Rice Diversity Panel (Zhao et al., 2010, 2011). Eleven of these lines (Table 1) were multiplied at the University of Aberdeen and sent to Wageningen University for physiological characterization. Arabidopsis growing conditions are described in SI Materials and Methods, Complementation of Arabidopsis *max1-1* Mutants.

Complementation of Bala.

Transformation of Bala with constructs *p35S:Oso1g0700900*, *p35S:Oso1g0701400*, and empty vector pHm43GW is described in SI Materials and Methods, Rice Bala Variety Complementation. Transgenic T1 plants were selected for exudate collection after confirmation on selection medium with hygromycin and verification of transgene expression by qRT-PCR. Equivalent average expression levels of the transgenes were ensured when plants were pooled and transferred to pots (three plants per pot) for root exudate collection (see below). Statistical tests were performed by using Genstat (Genstat for Windows 15th Edition; VSN International).

SL Collection from Root Exudates and Root Extracts.

For the mapping population, SLs were collected from 5-wk-old rice plants in three replicates with each replicate consisting of one pot with five plants as described (Jamil et al., 2012). For the transgenic rice, root exudates were collected from pots containing three 4-wk-old plants. The exudates were collected at 3, 6, and 9 d after the start of P starvation, and the three samples were pooled for SL analysis. The root exudates were passed through an SPE C18-Fast column (500 mg per 3 mL), and the SL was eluted with 6 mL of 100% acetone. For root extracts, 1 g fwt of ground root tissue was extracted following the method described (Jamil et al., 2012) but the resulting extracts were evaporated to dryness, taken up in hexane, loaded on preequilibrated Silica gel Grace Pure SPE (200 mg/3 mL) columns, and eluted with 2 mL of hexane:ethyl acetate (1:9) for further purification. The solvent was evaporated, and the residue was redissolved in 200 μ L of 25% (vol/vol) acetonitrile in water and filtered through Minisart SRP4 0.45- μ m filters (Sartorius) before LC-tandem MS (LC-MS/MS) analysis.

SL Analysis Using LC-MS/MS.

SLs were analyzed by comparing retention times and mass transitions with those of SL standards using a Waters Xevo TQ mass spectrometer equipped with an electrospray-ionization source and coupled to a Waters Acquity ultraperformance LC system using the settings described (Jamil et al., 2012) with some modifications specified in SI Materials and Methods, Detection and Quantification of Strigolactones

by Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS). The analyses were performed in three biological replicates.

Striga Germination Bioassay.

Root exudate germination stimulatory activity was assessed by using a germination bioassay with *S. hermonthica* as described previously (Jamil et al., 2012; Matusova et al., 2005). Approximately 50–100 preconditioned *Striga* seeds on a 9-mm diameter glass fiber filter paper disk (Sartorius) were exposed to the column-purified root exudates (50 μ L per disk) after acetone evaporation. Germination was scored after 48-h incubation in darkness at 30 °C. The synthetic SL GR24 (3.3 μ M) was used as positive and water as negative control. Three biological replicates and three discs per replicate were used.

Striga Emergence.

Striga emergence was studied as described (Jamil et al., 2012). Approximately 25 mg of *Striga* seeds were mixed thoroughly with 1 L of the 50:50 sand and soil mixture, which was then used to fill 1.5-L pots. Pregerminated rice seeds were transferred to the pots (one seed per pot) and grown at 28 °C day (14 h) and 25 °C night (10 h) with relative humidity at 70% and 400 μ M \cdot m⁻² \cdot s⁻¹ of light. *Striga* emergence was assessed at 2-d intervals in four replicates.

RNA Extraction.

RNA was extracted from roots of Nipponbare rice grown with full nutrition or P deprived for 5 d before tissue collection of roots. The RNA was purified from 70 mg of homogenized ground roots using 500 μ l Trizol (Invitrogen) and further purified with chloroform. After precipitation with 70% (vol/vol) ethanol, the RNA was recovered with an RNAeasy Mini Kit column (Qiagen) and DNA was removed using the DNAase I Kit (Qiagen), according to manufacturer's instructions.

Gene Expression Analysis.

cDNA was synthesized by using the iScript cDNA Synthesis Kit (BioRad) using 1 μ g of total RNA per sample following the manufacturer's instructions. The qRT-PCR reactions were prepared by using iQ SYBR Green Supermix (BioRad). Per reaction,

each primer at a concentration of 0.3 μ M and 1 μ L of 10-fold diluted template cDNA was used. The amplification was detected with a BioRad qRT-PCR detection system and thermocycler. The primers are listed in Table S3. The expression data are the average of five (control) or four biological replicates (P deprived).

Characterization of Os01g0700900 and Os01g0701400 Transcripts.

RNA from Nipponbare rice roots was used for cDNA synthesis according to instructions for the SMART RACE cDNA Amplification Kit (Clontech). The primers and nested primers of RACE PCR were designed based on the predicted mRNA sequences in the National Center for Biotechnology Information (NCBI) (GI: 115439412; accession no. NM_001050521) and are listed in Table S4. The 5' and 3' RACE PCR products were cloned into pJET1.2 (Fermentas) and sequenced.

Complementation of Arabidopsis max1-1.

Constructs *p35S:Os01g0700900* and *p35S:Os01g0701400* were transformed into Arabidopsis *max1-1* plants (Columbia-0 background) as described in SI Materials and Methods, Complementation of Arabidopsis *max1-1* Mutants. Rosette branching was measured on independent single-insertion homozygous lines: nine lines (20 plants per line) for *p35S:Os01g0700900*, five lines (three to five plants per line) for *p35S:AtMAX1*, and two lines (five to seven plants per line) for *p35S:Os01g0701400*. Rosette branching was measured by using the decapitation method (Greb et al., 2003).

Phylogenetic Studies of Rice MAX1 Homologs.

The confirmed amino acid sequences of Os01g0700900 and Os01g0701400 and the predicted sequences of Os01g0701500, Os06g0565100, and Os02g0221900 in IRGSP were aligned with Arabidopsis MAX1, and a phylogenetic tree made with ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2).

QTL Mapping.

Before QTL analysis, germination percentage and SL production were log-transformed. QTL analysis was conducted as described in Price *et al.* (Price et al., 2002). The molecular map (Norton and Price, 2008) contains 164 markers covering

1,833 cM on 12 linkage groups. The identification of QTLs was performed by composite interval mapping using QTLCartographer version 1.15 (by C.J. Basten, B.S. Weir, and Z.B. Zeng, Department of Statistics, North Carolina State University). Background markers (maximum of 10) for composite interval mapping were selected by forward stepwise regression with backward elimination using the default setting. The 5% genome-wide threshold for QTL detection was determined after 1,000 permutations.

Bala and Azucena Genome Sequencing.

The Bala and Azucena genomic DNA was extracted, made into pair-end libraries, and sequenced on an Illumina Genome Analyzer II at Cornell University providing reads of 88-, 100-, and 120-bp lengths for Bala and 100 bp lengths for Azucena. To report SNP calls, reads were aligned to the Nipponbare reference by using “Panati” (Wright, MH. 2009-2012 <http://panati.sourceforge.net/>) and (Li and Durbin, 2010). Fastq data have been deposited in the NCBI Short Read Archive (Acc_ID SRA050654.1).

Assessing Allelic Diversity.

Allelic diversity was accessed by multiplex PCR in a single reaction (25- μ L mix) of 5-min denaturation at 95 °C, 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, and 5 min of final extension at 72 °C. Primers are listed in Table S5.

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Table S1 Main-effect quantitative trait loci for traits measured on the Bala x Azucena recombinant inbred line population

Trait	Chromosome	QTL position		LOD	R ² (%)	Donor of positive allele
		in cM	in Mbp (approx.)			
<i>Striga</i> Germination	1	147.3	29.3	29.4	66.0	Azucena
	8	98.4	21.1	3.3	6.1	Azucena
Orobanchol	1	145.3	29.0	29.2	70.2	Azucena
<i>Ent-2'epi-5-deoxystrigol</i>	1	145.3	29.0	32.7	71.2	Azucena
	10	61.7	13.7	2.5	3.3	Azucena
MeO-5-DS isomer 1 ^a	1	148.5	29.4	2.8	6.7	Azucena
	2	10.0	3.0	2.7	10.3	Azucena
	9	84.4	18.8	3.1	7.8	Azucena
MeO-5-DS isomer 2 ^a	1	148.5	29.4	20.8	49.2	Azucena
	10	59.7	13.6	3.0	5.8	Azucena
MeO-5-DS isomer 3 ^a	1	18.1	3.3	3.0	4.6	Azucena
	1	147.3	29.3	29.1	52.8	Azucena
	2	20.4	4.0	2.7	4.2	Azucena
	5	18.0	2.7	3.5	5.7	Azucena
	5	121.7	28.8	2.6	2.7	Azucena
	6	24.3	3.9	3.0	3.8	Bala
	10	53.7	13.2	4.6	5.1	Azucena
Tiller number	1	147.3	29.3	12.4	28.8	Bala
	6	24.3	3.9	3.4	7.2	Azucena
	10	61.7	13.7	3.0	6.1	Bala
	12	117.5	25.9	V	8.8	Bala
Root fresh weight	4	111.8	32.5	3.3	8.3	Azucena
	12	100.7	23.4	4.6	14.6	Bala
Shoot fresh weight	1	148.5	29.4	6.6	14.6	Bala
	3	126.0	26.7	2.6	5.6	Bala
	11	65.2	17.5	3.8	8.8	Azucena
	12	115.5	25.6	2.7	9.8	Bala
Total Plant fresh weight	10	29.5	4.7	3.8	9.2	Azucena
	10	61.7	13.7	3.6	10.9	Bala
	12	103.5	23.8	3.7	9.8	Bala
Plant Height	1	205.0	38.4	8.7	19.4	Azucena
	3	170.3	33.6	10.6	27.1	Azucena
	5	97.9	26.6	4.8	13.0	Bala
	6	97.7	16.2	5.5	12.0	Bala
	7	138.9	26.6	3.0	9.4	Azucena
	8	133.1	25.7	2.6	8.0	Azucena

^aMeO-5-DS = methoxy-5-deoxystrigol

Table S2 SL levels in root exudates and root tissue, number of tillers and stimulation of *Striga* germination by root exudates of rice lines varying for the presence and absence of the rearrangement. *Ent-2'-epi-orobanchol*, 5-deoxystrigol and methoxy-5-deoxystrigol isomers 2 and 3 were measured in plants grown after one week/10 days of Pi starvation, n=3. *Striga* germination induced by root exudates (n=3) was measured in a germination bioassay. A maximum of 58.41%±2.68 germination was obtained with the synthetic strigolactone control GR24 at 3.3 µM concentration. The table shows the mean values ± SE.

Line	Allele ^a	Sub-group ^b	Country of origin ^c	pmol/mL exudate		pmol/g root fresh weight	LC-MS peak areas exudate		Striga Germination (%)	Number of tillers	
				<i>Ent-2'-epi-orobanchol</i>	<i>Ent-2'-epi-5-DS^d</i>		<i>Ent-2'-epi-orobanchol</i>	MeO-5DS isomer 2 ^e			MeO-5DS isomer 3 ^e
Azucena	A	TRJ	Phi	152.08±19.20	22.27±4.16	30.08±4.33	4.41±0.88	1656.97±319.75	3942.09±775.65	47.42±2.32	4±0
DZ-78	A	AUS	Ban	102.45±16.49	9.71±4.68	19.09±3.22	1.81±0.88	2063.75±283.89	7592.69±1196.57	42.67±3.06	4.67±0.067
Aswina-330	A	AUS	Ban	26.39±6.56	4.80±2.26	5.35±1.29	0.97±0.46	479.12±48.17	1835.51±347.77	33.94±2.59	5±0.2
Kun-Min-TH	A	IND	Chi	29.52±2.97	5.16±0.69	5.48±0.74	0.96±0.16	547.84±111.01	1767.85±255.44	30.84±3.69	4.27±1.05
Ta-Mao-T	A	TEJ	Chi	32.98±10.84	18.86±2.64	6.20±1.66	3.62±0.31	577.09±197.51	2456.21±597.66	33.13±2.52	4.2±0.11
Sinaguang	A	TRJ	Phi	8.35±2.63	3.29±1.53	1.56±0.46	0.60±0.28	187.78±66.00	874.90±236.34	44.5±4.4	5.93±0.13
Bala	B	IND	Ind	0±0	0.34±0.27	0±0	0.06±0.05	12.95±8.04	89.87±4.64	13.61±1.45	10±0.23
Dhala-S	B	AUS	Ban	6.024±1.16	0.05±0.04	1.15±0.24	0.01±0.01	61.28±59.5	198.00±159.13	2.67±.42	7.93±0.35
Kachilon	B	AUS	Ban	0±0	0.02±0.01	0±0	0.00±0.00	12.65±3.48	34.80±1.18	10.88±1.28	8.07±0.48
Guan-Yin-T	B	IND	Chi	4.78±0.73	0.015±0.01	0.96±0.14	0.00±0.00	13.42±5.85	63.051±5.27	3.63±0.52	6.8±0.2
Sung-Liao-2	B	TEJ	Chi	0±0	2.58±0.01	0±0	0.51±0.00	15.46±6.95	76.83±6.62	17.03±2.01	6.8±0.11
Asse Y Pung	B	TRJ	Phi	0±0	0.30±0.08	0±0	0.06±0.01	12.07±4.95	34.86±13.11	12.9±2.65	8.73±0.18
Kinastano	B	TRJ	Phi	0±0	0.30±0.03	0±0	0.05±0	2.75±2.43	88.24±8.11	8.98±2.14	7.6±0.23

^aA= Azucena allele. B= Bala allele

^bAccording to Zhao et al. (17)

^cPhi – Philippines, Ban – Bangladesh, Chi – China, Ind – India

^d*Ent-2'-epi-5-DS - Ent-2'-epi-5-deoxystrigol*

^eMeO-5DS – methoxy-5-deoxystrigol

Table S3 Primers used for PCR

Primers for quantitative RT-PCR		
Primer pair		Locus / Gene
GGCTTCTCTGCTTGCTGCTTC	ACCAACGGTTGCCTTCCC	Oso1g0700900
ACGACGGCGTTCACTCTCTC	TCCGAACCCGTCAATCTCC	Oso1g0701400
AGCGAAGGTATCCACTAGGC	CCGGGGATATAGACACTC	OsPI1 - Oso1g0838350
CTTGTCGCCTGGTACGAC	GTCGATGACACGGTTGCTGTA	GADPH used as internal control

Table S4. Primers used for RACE-PCR and cloning PCR

Primer for RACE-PCR and cloning PCR	
Primers	Sequence
Oso1g0700900 5RACE primer	GTGGATCGCTGACAATTCATCTC
Oso1g0700900 5RACE Nested primer	GCAGCTCCCTCGAGGATTTGTTCG
Oso1g0700900 3RACE primer	GCAAGATCGCGGCCGATACCTG
Oso1g0700900 3RACE Nested primer	CGGACGGCTGCAATGAAGCGAG
Oso1g0700900 Forward Cloning primer (with EcoRI site)	gggggaattcATGGAGATCAGCACAGTG
Oso1g0700900 Reverse Cloning primer (with ClaI site)	ggggatcgatTTATATATGCCTCTTGATGACCTG
Oso1g0701400 5RACE primer	CATATCCTCCAATTTCAACCTGTTCGCAC
Oso1g0701400 5RACE nested primer	GCGTAAGAAGCTCCCGCCAGG
Oso1g0701400 3RACE primer	CGGGAGCTTCTTACGCCGGAC
Oso1g0701400 3RACE nested primer	CGTGCGAACAGGTTGAAATTGGAGGATATG
Oso1g0701400 cloning forward primer (with pENTR/D-TOPO cloning site)	caccATGGAGATCATCAGCACAGTGCTG
Oso1g0701400 cloning reverse primer	CTATGCAGTGTGCCTCTTGATGACCCG
AtMAX1 cloning forward primer (with pENTR/D-TOPO cloning site)	caccATGAAGACGCAACATCAAT
AtMAX1 cloning reverse primer	TCAGAATCTTTTGATGGTTCTGAGC

Table S5. Primers used for assessing allelic diversity

Primers for assessing allelic diversity			
Primer pair		Locus / Gene	Product size
GGACGTGTTCTCAGTGCTCA	TCCTTGACAACGCATC	Oso1g0700900	200 bp
ACAGTGCTGGGCTCAACG	GGCTCGTACAAGTAGCCCAAC	Oso1g0701400	100 bp
TCAAAGCTGCCAGTCACACC	ATCCGGACAGGTCCATCTTG	Oso1g0701500	150 bp



Do strigolactones and d'orenone have independent signalling pathways?

Catarina Cardoso¹, Tatsiana Charnikhova¹,
Francel Verstappen^{1,2}, Harro Bouwmeester^{1,2} &
Carolien Ruyter-Spira^{1,3}

¹*Laboratory of Plant Physiology, Wageningen University, the Netherlands*

²*Centre for Biosystems Genomics, Wageningen, the Netherlands*

³*Plant Research International, Bioscience, Wageningen, the Netherlands*

Abstract

CAROTENOID CLEAVAGE DIOXIGENASES 7 and 8 convert 9-*cis*- β -carotene into carlactone, an intermediate in strigolactone biosynthesis. *In vitro* the same enzymes can also cleave all-*trans*- β -carotene into β -apo-13'-carotenone, or d'orenone. The presence of d'orenone in plants has not been confirmed but previous reports have shown that it does induce a physiological response, blocking root hair elongation by altering PIN2 protein abundance. Because d'orenone and strigolactones share common biosynthetic enzymes and both act in root developmental processes, the objective of the present study was to investigate possible interactions between the two pathways. In contrast to strigolactones, in *Arabidopsis* d'orenone application stimulates lateral root development while inhibiting primary root elongation. Consistent with this, d'orenone application results in a minor decrease in the expression of genes encoding strigolactone biosynthetic enzymes. However, the effect of d'orenone on root architecture is independent from MORE AXILLARY GROWTH 2 (MAX2), the F-box protein involved in strigolactone signalling, suggesting that the main effect of d'orenone on root architecture is not mediated by strigolactones. Our results therefore suggest that d'orenone is an independent endogenous plant signalling compound. A model for the effect of d'orenone and strigolactones in auxin homeostasis and development of the root tip is presented.

Introduction

D'orenone and strigolactones are both carotenoid derived compounds. Strigolactones are well studied, as plant hormones that regulate the development of plant architecture, and as signalling molecules that promote colonization of the plant host secreting them into the rhizosphere by arbuscular mycorrhizal fungi as well as infection by parasitic plants (Umehara et al., 2008; Gomez-Roldan et al., 2008; Ruyter-Spira et al., 2011; Kapulnik et al., 2011; Koltai et al., 2010; Akiyama et al., 2005; Agusti et al., 2011). The first committed step in strigolactone biosynthesis is the isomerization of all-*trans*- β -carotene to 9-*cis*- β -carotene by the carotenoid isomerase DWARF27 (D27) that was first identified in rice (Lin et al., 2009, 27) and later also in Arabidopsis (Waters et al., 2012) (Fig. 1). 9-*cis*- β -Carotene is subsequently cleaved into 9-*cis*- β -carotenal by CAROTENOID CLEAVAGE DIOXIGENASE 7 (CCD7; D17 or HTD1 in rice and MAX3 in Arabidopsis), which is then further cleaved by CAROTENOID CLEAVAGE DIOXIGENASE 8 (CCD8; D10 in rice and MAX4 in Arabidopsis) into carlactone (Alder et al., 2012) (Fig. 1). The subsequent enzymatic step(s) that result in strigolactone formation have not yet been elucidated. One more enzyme known to be involved in strigolactone biosynthesis is a CYTOCHROME P450 initially identified in Arabidopsis (MAX1; Booker et al., 2005; Kohlen et al., 2011). In rice, five homologs of MAX1 have been identified (Challis et al., 2013), two of which (*SLB1* and *SLB2*) play an important role in strigolactone biosynthesis in rice varieties of the *Japonica* subspecies (Chapter 4, this thesis).

Just as the strigolactones, d'orenone (β -apo-13'-carotenone) can also be produced from all-*trans*- β -carotene, but requiring just the strigolactone biosynthetic enzymes CCD7 and CCD8 (and not D27) (Alder et al., 2008; Schwartz et al., 2004; Schlicht et al., 2008). In this case, the all-*trans* isomer of β -carotene is the substrate for cleavage by CCD7 producing all-*trans*- β -apo-10'-carotenal, which is then further cleaved by CCD8 into d'orenone (Fig. 1). These reactions seem to be conserved across species. CCD7 and CCD8 homologs from rice, Arabidopsis and pea all cleaved all-*trans*- β -carotene and all-*trans*- β -apo-10'-carotenal, respectively, all producing d'orenone (Alder et al., 2008; Schwartz et al., 2004). Besides the cleavage of 9-*cis*- β -apo-10'-carotenal into carlactone *en route* to the strigolactones, all CCD8 homologs also cleaved all-*trans*- β -apo-10'-carotenal, but not other all-*trans*-apo-carotenoids

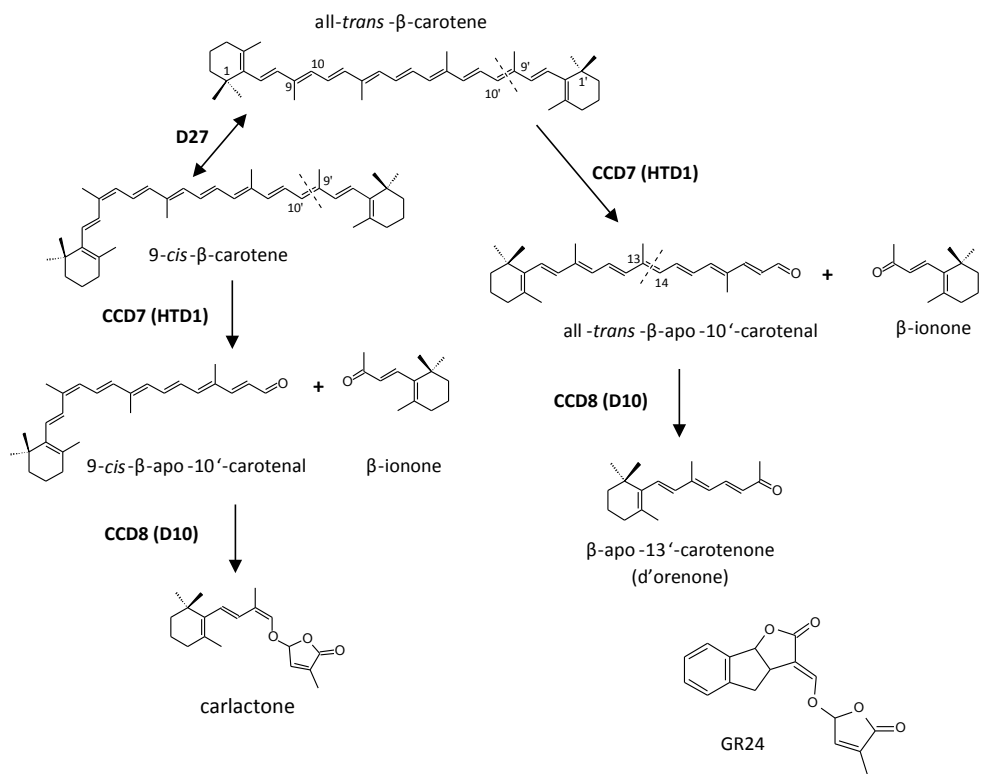


Figure 1 - The Pathway from β-carotene to carlactone and d'orenone and structure of the strigolactone synthetic analogue GR24

(Alder et al., 2008, 2012). D'orenone is known as a natural precursor of trisporic acids, pheromone compounds produced by zygomycete fungi (Schlicht et al., 2008). All the work on the involvement of plant CCD7s and CCD8s in the biosynthesis of d'orenone described above was done *in vitro* with enzymes produced in *E. coli*. Neither the conversion of all-trans-β-carotene into d'orenone nor the presence of d'orenone have ever been demonstrated *in planta*.

Physiological effects of d'orenone and strigolactones

Even though the occurrence of d'orenone in plants is unknown, several physiological effects have been described upon application of the compound to plants. Schlicht et al. (2008) have shown that d'orenone in *Arabidopsis* inhibits root gravitropism and root hair elongation. This is accompanied by a reduction of PIN2 protein abundance in the cells of the meristem and lateral root cap and a concomitant increase of PIN2 in the transition zone, extending further into the elongation zone. D'orenone changes

PIN2 abundance probably by affecting protein degradation rate (Schlicht et al., 2008). PIN proteins are auxin efflux carriers responsible for polar auxin transport (PAT). PIN2, for example, is involved in basipetal auxin transport through the root epidermal layer and acropetal transport through the cortex of the meristem (Blilou et al., 2005; Abas et al., 2006). PIN2 is required for root gravitropism, induction of root hair elongation and together with other auxin transporters also contributes to the auxin reflux in the root tip (Blilou et al., 2005; Grieneisen et al., 2007; Abas et al., 2006; Rahman et al., 2010). In d'orenone treated plants, root hair elongation is inhibited and can be rescued by application of auxin (Schlicht et al., 2008) suggesting that d'orenone interferes with auxin transport through changes in PIN2 abundance, possibly decreasing auxin supply to the root hair cells (Jones et al., 2008; Lee and Cho, 2006). Strigolactones inhibit bud outgrowth and modulate root development. Just as d'orenone, strigolactones also affect auxin transport. Strigolactones promote clathrin mediated PIN1 depletion from the plasma membrane, thereby reducing the PAT stream that channels auxin from the shoots to the roots (Bennett et al., 2006; Prusinkiewicz et al., 2009; Shinohara et al., 2013). It has been postulated that this reduction of the PAT stream prevents auxin export out of the axillary buds thereby inhibiting their outgrowth (Domagalska and Leyser, 2011), but there may be other mechanisms involved in strigolactone mediated inhibition of bud outgrowth (Dun et al., 2012). In contrast to d'orenone, strigolactones induce root hair elongation. This effect likely also involves changes in PIN mediated auxin transport but the PIN proteins involved have not yet been identified (Kapulnik et al., 2011; Koltai et al., 2010).

As was described above, d'orenone and strigolactones share two enzymes, CCD7 and CCD8 that are required for their biosynthesis but use different substrates. If d'orenone is naturally produced in plants, there could be crosstalk between the two pathways by means of competition for the same substrate (all-*trans*- β -carotene) or for the activity of the same enzymes. Likewise, application of d'orenone may interfere with the strigolactone biosynthetic pathway through feedback regulation of common enzymes. The biosynthesis of d'orenone in plants has not been confirmed but since d'orenone is an *in vitro* product of CCD8, application of d'orenone to plant tissues may block the catalytic centre of CCD8. The fact that both d'orenone and strigolactones affect auxin transport with opposite effects on root hair elongation further suggests

a possible interaction. In this study we therefore compared the effects of d’orenone and strigolactones on a number of plant architectural aspects such as axillary bud outgrowth, primary root elongation and lateral root development. Potential feedback mechanisms were studied by assessing the effect of d’orenone application on the expression of strigolactone biosynthetic genes. Finally, we propose a model to explain how d’orenone and strigolactones differentially affect auxin transport in the root meristem and how this results in different effects on primary root growth and root-hair elongation.

Results

D’orenone does not affect axillary bud outgrowth of rice and Arabidopsis

The effects of GR24 and d’orenone application on rice tillering and Arabidopsis branching were compared. GR24 reduces axillary bud outgrowth in Arabidopsis and rice strigolactone biosynthetic mutant lines impaired in CCD7 (*d17-1,max3-11, max3-12*) and CCD8 (*d10-1/max4-1*) but fails to rescue the strigolactone signaling mutants impaired in the F-BOX protein (*d3-1/max2-1*) (Umehara et al., 2008;

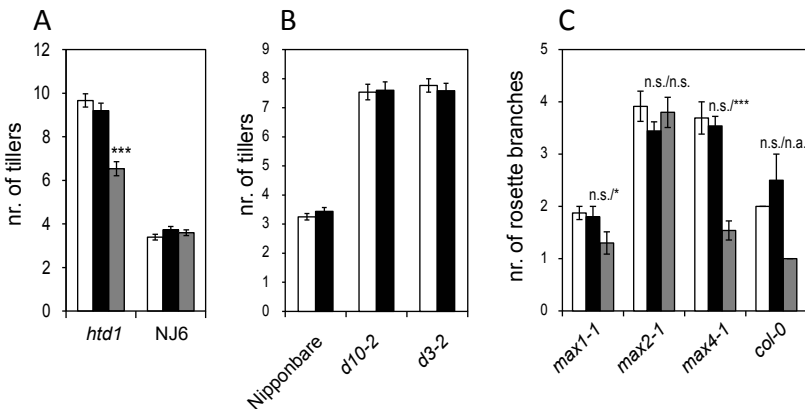


Figure 2 Bud outgrowth inhibition in rice (**A**) *htd1* and wild type NJ6, treated with 2 μ M d’orenone or 1 μ M GR24; (**B**) rice *d10-2*, *d3-2* and wild-type Nipponbare treated with 40 μ M d’orenone and (**C**) Arabidopsis *max1-1*, *max2-1*, *max4-1* and wild-type Col-0 treated with 2,5 μ M d’orenone or GR24. T-test significance tests indicated as: control vs. GR24 / control vs. d’orenone; ***($P < 0.001$), **($P < 0.01$), *($P < 0.05$), n.s. ($P \geq 0.05$), n.a. (not possible to calculate significance values because standard deviation = 0 in both samples).

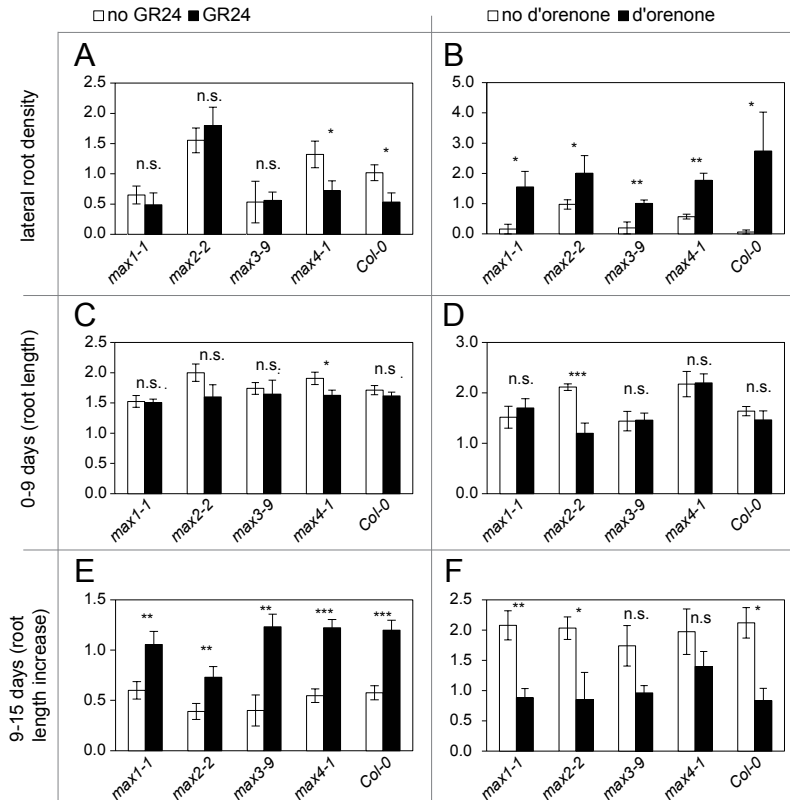


Figure 3 Effect of d'orenone and GR24 on root developmental traits of *max1-1*, *max2-1*, *max3-9*, *max4-1* and Col-0. **(A, B)** lateral root density; **(C,D)** primary root elongation in the first nine days after germination, **(E-F)** primary root elongation from nine to 15 days after germination. Plants treated with 2,5 μ M d'orenone or 2,5 μ M GR24. T-test significance tests indicated as: treated vs. non-treated; ***($P < 0.001$), **($P < 0.01$), *($P < 0.05$), n.s. ($P \geq 0.05$)

Gomez-Roldan et al., 2008). In this study, rice *htd1* impaired in OsCCD7 and its wild-type background NJ6 (Zou et al., 2006) were treated with nutrient solution containing 2 μ M d'orenone or 1 μ M GR24 from day seven after germination (Fig 2A). After two weeks of treatment, *htd1* responded to GR24 with a significant reduction in tillering. No response to d'orenone treatment was observed. The wild-type NJ6 already had a low number of tillers and did not respond to GR24 nor to the d'orenone treatment. To exclude the possibility of a lack of activity due to degradation, a higher concentration of 40 μ M d'orenone was tested using the rice mutant lines *d10-2* and *d3-2* (Umehara et al., 2008; Yoshida et al., 2012) and their wildtype Nipponbare (Fig. 2B). Again, tillering was not affected by d'orenone three weeks after the start of the treatment. The Arabidopsis strigolactone biosynthetic mutants *max1-1*, *max3-9*,

max4-1 and wild-type Col-o showed reduced rosette branching in response to 2.5 μM GR24 but not to 10 μM d'orenone (Fig. 2C). The strigolactone signaling mutant *max2-1* did not respond to any of the treatments.

D'orenone effect on Arabidopsis root development:

The effects of GR24 and d'orenone treatment on root elongation and lateral root formation were compared, using the same Arabidopsis strigolactone biosynthetic and insensitive mutants as in the branching/tillering experiment described above. Seedlings were grown in vertical agar plates supplemented with 2.5 μM d'orenone or 2.5 μM GR24. Primary root elongation was measured at day 9 and 15, and lateral root density at day 15 after germination (Fig. 3A, B). D'orenone significantly increased lateral root density in both Col-o and strigolactone biosynthetic and signalling mutant lines. In contrast, 2.5 μM GR24 either reduced lateral root density (*max4-1*, WT) or did not have an effect (*max1-1*, *max3-9*, *max2-1*), but lateral root density was already low in the untreated *max1-1* and *max3-9* control plants.

The effects of GR24 and d'orenone on primary root elongation were assessed nine and 15 days after germination (Fig 3C-F). During the first nine days, GR24 did not affect primary root elongation in any of the lines except for a small but significant decrease in *max4-1* (Fig. 3C). In the following period from nine to 15 days after germination, GR24 increased primary root elongation in all lines including *max2-1*, albeit at a lower rate in the latter (Fig. 2E). Also *max4-1* plants, which showed a slightly reduced root elongation during the first nine days of GR24 treatment, showed a significant increase in root elongation in the next period, from nine to 15 days after germination.

During the first nine days, d'orenone treatment reduced the primary root length of *max2-1* but no effect was observed in the remaining lines (Fig. 3D). From nine to 15 days after germination, a significant reduction of primary root length by d'orenone was observed in *max2-1*, *max1-1* and Col-o (Fig. 3F). Although the trend in *max4-1* and *max3-9* was similar as in the other lines, the effect was not significant.

Effect of d'orenone on gene expression

To study a possible interaction between d'orenone application and strigolactone biosynthesis, we analysed gene expression of strigolactone biosynthetic genes

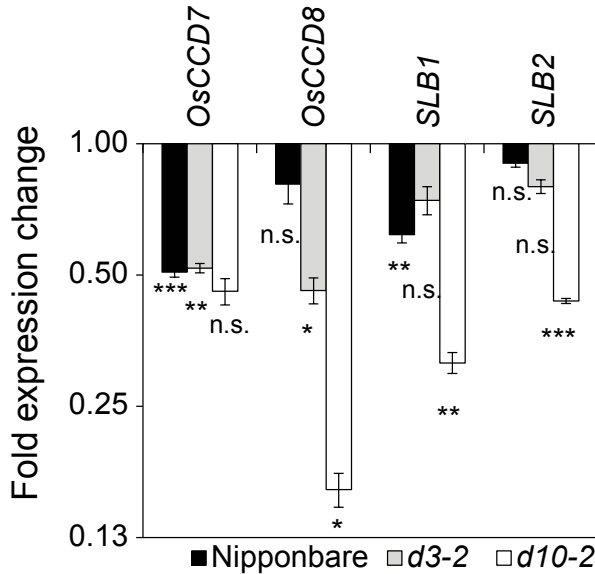


Figure 4 Gene expression response of *OsCCD7*, *OsCCD8*, *SLB1* and *SLB2* to 40 μ M d'orenone treatment in homogenized roots of *d10-2*, *d3-2* and wild-type Nipponbare. Bars indicate the fold expression change compared to non treated roots of the same genotype. T-test significance tests indicated as: treated vs. non-treated; ***($P < 0.001$), **($P < 0.01$), *($P < 0.05$), n.s. ($P \geq 0.05$)

in d'orenone treated plants. The lines *d3-2*, *d10-2* and the wildtype Nipponbare background were treated from day seven after germination with nutrient solution containing 40 μ M d'orenone. Gene expression was measured in roots, collected after three weeks of treatment. Expression changes were most pronounced in *d10-2* where all tested genes were significantly down-regulated except *OsCCD7*, with *OsCCD8* being about 4-fold and *SLB1* and *SLB2* about 2-fold down-regulated. In *d3-2*, *OsCCD7* and *OsCCD8* were both about 2-fold down-regulated while *SLB1* and *SLB2* were not significantly affected. In the wild-type Nipponbare, only expression of *OsCCD7* and *SLB1* was significantly repressed although not by more than 2-fold.

Discussion

Although heterologously produced CCD7 and CCD8 can produce d'orenone *in vitro* from the ubiquitous plant carotenoid all-*trans*- β -carotene, the biosynthesis of d'orenone in plants has not yet been confirmed. Still, d'orenone triggers physiological responses in plants (Schlicht et al., 2008) (Fig. 2 and 3). The existence

of common enzymes required for the biosynthesis of d'orenone and strigolactones and the opposite response of root hair growth to these two compounds suggest that d'orenone may not induce a direct plant response but instead reduce strigolactone biosynthesis. This reduction might be the result of a feedback response that reduces the expression of the enzymes common in the biosynthesis of both compounds, or be caused by inhibition of their enzymatic activity. In this study we compared the effects of d'orenone and GR24 application to strigolactone biosynthetic and signaling mutants to investigate possible interactions between d'orenone treatment and strigolactone signaling in plants. We observed opposite responses to the two compounds in root development, but in contrast to the response to GR24, the response to d'orenone does not require the MAX2 signaling pathway. No effect of d'orenone on bud outgrowth was observed in rice and Arabidopsis (Fig. 2). However, d'orenone did change the expression of some of the strigolactone biosynthetic genes in rice roots and this effect was strongest in the strigolactone biosynthetic mutant *d10-2* compared to the strigolactone insensitive mutant *d3-1* and the wild-type Nipponbare (Fig. 3).

D'orenone effect is independent of the strigolactone MAX2 signaling pathway

D'orenone and GR24 had opposite effects on primary root elongation and lateral root density. We confirmed that GR24 reduces lateral root density and stimulates primary root elongation. Also the strigolactone signaling mutant *max2-1* responded to GR24, with an increase in primary root elongation although to a lesser extent than that observed in the other lines. Indeed a small response of *max2* to strigolactone induction of primary root elongation has been reported before, however, the response of this line is always smaller than in the biosynthetic mutant lines and the wildtype (Ruyter-Spira et al., 2011; Kapulnik et al., 2011; Shinohara et al., 2013). D'orenone treated *max2-1*, *max1-1* and Col-0 had an equally strong, but opposite, response, with reduced primary root elongation and increased lateral root density. The fact that this is also observed in *max2* indicates that the response to d'orenone is not a result of reduced strigolactone activity in the d'orenone treated plants but is mediated by an alternative regulatory mechanism, independent of the MAX2 signaling pathway.

Interestingly, the decrease in primary root elongation by d'orenone was not significant in *max3-9* and *max4-1*. Possibly the effect of d'orenone is masked in *max3-9* because untreated plants already have a shorter primary root length compared with the other lines, that cannot be further reduced by d'orenone. However, *max4-1* is clearly not as responsive to d'orenone as the Col-0, *max2-1* and *max1-1*. Also, the lateral root density was significantly increased in *max3-9* and *max4-1* but to a lesser extent than in the other lines, particularly in *max3-9*. MAX3 and MAX4 catalyze the formation of d'orenone and maybe their presence is required for the efficient conversion by putative downstream enzymes of d'orenone into a bioactive compound. If the d'orenone response indeed involves further conversion, this may also explain why we cannot detect d'orenone in plant tissues. This could also explain why previous reports observed that d'orenone was highly active in blocking root hair elongation, while small changes in structure such as a longer side-chain, altered polarity or stereochemistry greatly reduced the activity (Schlicht et al., 2008).

D'orenone application may interact with the regulation of the strigolactone biosynthetic pathway

To further investigate a possible effect of d'orenone on the expression of strigolactone biosynthetic genes, we quantified the level of gene expression of *OsCCD7*, *OsCCD8* and the CYP450 genes *SLB1* and *SLB2* in rice *d3-1*, *d10-2* and Nipponbare in response to d'orenone. In general, the expression of strigolactone biosynthetic genes was down-regulated by d'orenone and the effect was most pronounced in *d10-2*. If d'orenone is naturally present in plants, *d10-2* should be deficient for it. *OsCCD8* (encoding the putative d'orenone producing enzyme in plants) showed the strongest down-regulation by d'orenone and also most pronounced in the putative d'orenone deficient *d10-2* and hence behaves as could be expected from a d'orenone biosynthetic gene. In the strigolactone insensitive mutant *d3-2*, *OsCCD8* was also down-regulated but to a lesser extent than in *d10-2* and no changes in *OsCCD8* expression were detected in the wild-type Nipponbare.

The CYP450 genes *SLB1* and *SLB2* (*Os01g0700900* and *Os01g0701400* respectively) acting downstream of *OsCCD8* were both more than twofold downregulated in *d10-2* but not in *d3-2* nor in Nipponbare, suggesting that the effect of d'orenone application on the regulation of strigolactone biosynthetic genes

is only strong enough in *d10-2*. Since *d10-2* cannot produce the substrate for *SLB1* and *SLB2* the regulation of these two genes by d'orenone could be indirect instead of a result of lower levels of substrate.

Since d'orenone is a product of *OsCCD8* *in vitro*, *in planta* d'orenone could act as a competitive inhibitor of *OsCCD8*. This would result in increased levels of upstream substrates and likely in an up-regulation of *OsCCD8* expression. However, our results show the opposite effect on *OsCCD8* expression, suggesting that d'orenone is not acting as a competitive inhibitor of *CCD8*. Rather, it seems that d'orenone reduces strigolactone biosynthesis through down regulation of gene expression. This effect is strongest in *d10-2* (that produces only residual levels of strigolactones) and less pronounced in *Nipponbare* and *d3-2* hence, further studies are necessary to confirm the effect of d'orenone on strigolactone levels and the extent of such a reduction. The strong reduction in *OsCCD8* expression in *d10-2* upon d'orenone treatment suggests that d'orenone could be a natural product of *CCD8* in addition to carlactone. In addition, the root developmental responses are equally strong in *max2-2* and *Col-0* suggesting that d'orenone acts independently of the strigolactone signaling pathway in the modulation of root development. If this can be confirmed, it would have implications for the current interpretation of the phenotype of *CCD7* and *CCD8* mutant lines. A detailed study would then be required that compares their phenotypes with other strigolactone mutant lines not impaired in d'orenone biosynthesis. The existence of at least two more carotenoid derived signaling molecules involved in plant development has been reported. One of these molecules – *BYPASS* – is involved in shoot development (Van Norman et al., 2004). Genetic analyses of *max4* and *max3* lines crossed with the *bps1* mutant showed no relation between *BYPASS* and the strigolactone biosynthetic pathway (Van Norman and Sieburth, 2007). The other is a shoot derived signal necessary to determine the positioning of lateral roots that is thought to be an apocarotenoid, probably derived from β -carotene (Norman et al., 2014). However, *CCD8* and *CCD7* are also not required for the synthesis of this unknown signaling molecule. Hence, if d'orenone is a plant signaling compound it is not related to *BYPASS* nor to the unknown signalling compound involved in lateral root positioning. Nevertheless, these reports point out the existence of alternative, apocarotenoid-mediated, signaling pathways in plant development, and d'orenone could be involved in yet another one.

A model for the effect of d'orenone on auxin transport in the root meristem

Changes in PIN protein distribution that cause changes in auxin homeostasis in the root tip could trigger the developmental effects observed upon d'orenone and strigolactone application. Understanding these mechanisms can help us dissect the different effects of d'orenone and strigolactones on root development (Figure 5). Several PIN proteins with specific localization in the cell membrane (basal, apical or lateral) direct auxin flow in the root tip (Blilou et al., 2005; Grieneisen et al., 2007). Shoot derived auxin arrives to the quiescent centre (QC) via the main PAT formed by PIN1, 3 and 4 that are localized in the provascular tissue. From the QC, auxin is redistributed through the columella and lateral root cap to the epidermis by PIN2, 3, 4 and 7. Auxin is then transported basipetally along the epidermis and at the transition zone it is redirected from the epidermis, back to the provascular cells where it re-enters the main PAT. PIN2 that is localized basipetally in the epidermal cells and acropetally in the cortex cells of the meristem contributes to this auxin reflux at the root meristem (Blilou et al., 2005). The auxin reflux in the root tip maintains an auxin gradient with a maximum at the QC (Blilou et al., 2005; Grieneisen et al., 2007). A reduction of auxin reflux leads to a less steep auxin gradient, a longer meristem and a less well defined zonation between the division and elongation zones (Grieneisen et al., 2007). This effect resembles the phenotypic response of root tips treated with GR24 and it was proposed that strigolactones reduce the lateral auxin reflux, by directly controlling PIN protein activity at the transition zone, where MAX2 is expressed (Ruyter-Spira et al., 2011, 2013; Brady et al., 2007) (Figure 5, strigolactone treatment). A fraction of the meristem derived auxin is also transported along the epidermal and cortical cells up to the differentiation zone to promote root-hair elongation (Jones et al., 2008). The effect of strigolactones on root hair elongation is probably a result of reduced auxin reflux at the transition zone leaving more auxin available to be transported along the epidermis towards the trichoblasts (Kapulnik et al., 2011; Pandya-Kumar et al., 2014). This hypothesis could be investigated using modeling studies of auxin fluxes in the root meristem. Further suggesting that strigolactones act indirectly on root hair elongation, it has been reported that MAX2 expression in endodermal cells of the *max2* mutant is

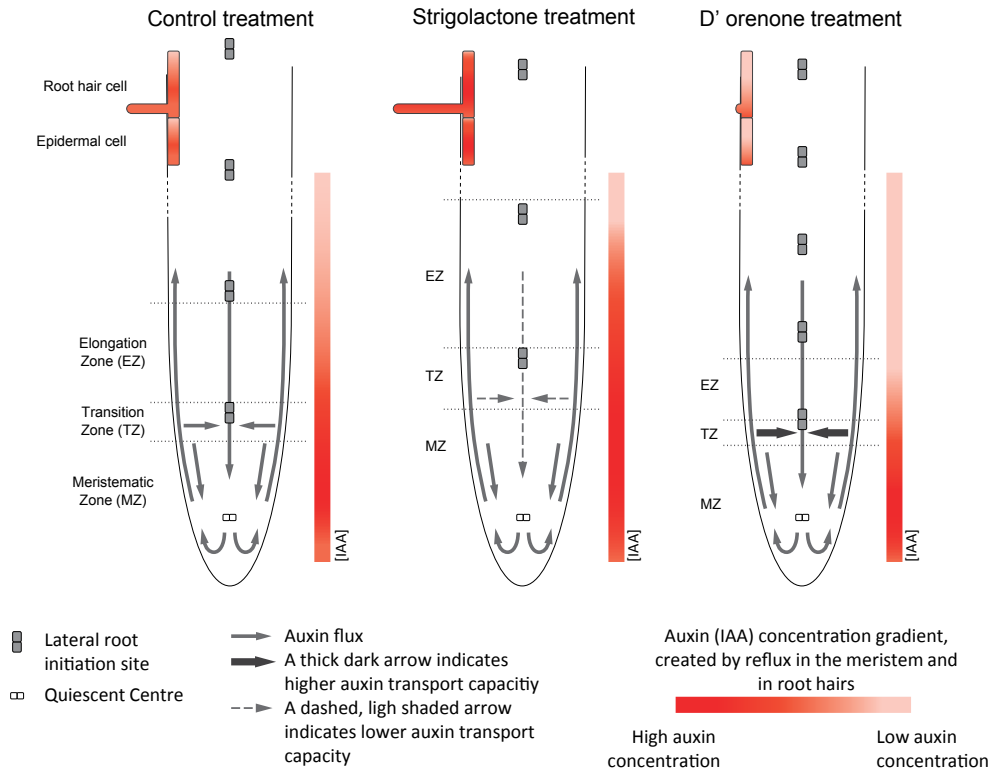


Figure 5 – Model for the putative effect of d'orenone and strigolactones on auxin homeostasis and root development. Arrows represent auxin (IAA) transport capacity. Thick dark arrows correspond to higher auxin transport capacity and dashed arrows represent reduced auxin transport in comparison to control treatment. In strigolactone-treated roots, auxin loading from the shoots to the quiescent centre (QC) and auxin reflux at the transition zone (TZ) are reduced creating an auxin gradient that is less steep (represented by the red bar at the right hand side of the root) and longer meristematic (MZ), transition (TZ) and elongation (EZ) zones. In the strigolactone treatment, more auxin is available to be transported basipetally along the epidermis and loaded to trichoblasts, promoting root-hair elongation. Epidermal and root hair cells at the top-left side of each root are colored in darker or lighter red corresponding to higher or lower auxin concentration respectively. In d'orenone-treated roots the auxin reflux is increased creating a steeper auxin concentration gradient, the zones of the meristem are shorter and root elongation is slower (not represented in the model). Due to slow root tip elongation and increased auxin concentration in the pericycle, the density of lateral root initiation sites is higher compared to control and strigolactone treatments. The increased auxin reflux at the transition zone reduces the amount of auxin available to be transported along the epidermis which inhibits root-hair elongation. In contrast to the d'orenone effect on root hair elongation, strigolactones reduce the auxin reflux at the transition zone letting a higher amount of auxin to be transported along the epidermis, promoting root hair elongation.

sufficient to stimulate root hair elongation in a non-cell autonomous manner (Koren et al., 2013).

In d'orenone treated roots, measurements of auxin influx from the medium into the roots suggest that the auxin reflux is increased (Schlicht et al., 2008). Auxin influx measurements show a typical maximum of auxin influx at the transition zone which is thought to be a consequence of the internal auxin reflux, that also occurs at that same region of the root (Mancuso et al., 2005). Upon d'orenone treatment, this maximum is further increased suggesting that d'orenone treatment increases the auxin reflux at the transition zone, probably as a consequence of higher PIN2 protein abundance at that region (Schlicht et al., 2008). This could explain the reduction in primary root elongation by d'orenone because enhanced auxin reflux in the transition zone results in a steeper auxin gradient along the root, and a shorter meristematic and elongation zone which slows down root tip elongation (Grieneisen et al., 2007) (Figure 5, d'orenone treatment). It is however not clear if the increase in PIN2 abundance in the transition zone by d'orenone application is sufficient to increase auxin reflux or if d'orenone also affects the activity of other auxin transporters.

The increased auxin reflux as a consequence of d'orenone treatment results in depletion of auxin in the epidermal layer and this might explain why root hair elongation is blocked (Schlicht et al., 2008). This is consistent with the observation that direct application of auxin to the root hairs of d'orenone treated roots restores their elongation (Schlicht et al., 2008). PIN2 together with AUX1 maintain the supply of auxin to the trichoblasts to promote root-hair elongation (Jones et al., 2008; Ikeda et al., 2009). Root hair elongation is dependent on a dynamic balance between AUX1 mediated auxin influx and PIN2 mediated auxin efflux from the root-hair cells (Jones et al., 2008; Ikeda et al., 2009). Possible changes in PIN2 abundance by d'orenone would also disrupt this balance however the effect of d'orenone on PIN2 expression in trichoblasts has not been described.

We have also observed that d'orenone increases lateral root density whereas strigolactones reduce it (Figure 3 A, B). Two factors might contribute to increased lateral root density in d'orenone treated roots. D'orenone treatment slows down elongation rate which might be sufficient to increase frequency of pericycle cell priming sites – the first step that determines the lateral root initiation sites and positioning of lateral roots. Indeed, Arabidopsis roots growing under conditions that

reduce primary root elongation have the same number and thus a higher density of lateral root initiation sites (Moreno-Risueno et al., 2010). In addition, the possible increase of auxin reflux by d'orenone may raise the auxin concentration in the pericycle cells and increase the frequency of lateral root initiation (Benková et al., 2003). In this study we have only measured the number of emerged lateral roots. To confirm the two suggested mechanisms it is necessary to measure the density of root initiation sites, lateral root primordia and subsequent developmental phases separately.

Once the development of lateral root primordia has been initiated, cell division continues, forming a new root meristem that will emerge from the primary root (Lavenus et al., 2013). The emergence of lateral root primordia is dependent on auxin levels, derived from the shoot via the main PAT stream (Wu et al., 2007; Bhalerao et al., 2002). Strigolactones reduce lateral root density by arresting their development before emergence, probably as a result of reduced auxin loading from the shoot via the PAT stream (Ruyter-Spira et al., 2011). There is however no evidence that d'orenone is changing auxin loading to lateral root primordia since it did not have an effect on axillary bud outgrowth, suggesting that d'orenone (when applied to roots) does not change the PAT in the shoot.

Conclusion

Carotenoid derived hormones appear to become of increasing importance. Next to strigolactones and abscisic acid, two important carotenoid derived plant hormones, studies have reported the presence of other yet unknown carotenoid derived signals: *BYPASS*, that inhibits shoot development and a shoot derived signal necessary for priming cells for the establishment of root primordia (Van Norman et al., 2004; Norman et al., 2014). Our results suggest that d'orenone potentially represents an endogenous plant signal, pointing out at a possible new branch of apocarotenoid metabolism in plants and an expanding family of carotenoid derived signaling compounds involved in the regulation of plant development.

Material and Methods

Plant Material and growth conditions

Seeds from *d10-2* (Umehara et al., 2008; Arite et al., 2007) and *d3-2* (Yoshida et al., 2012) rice mutants of cv. Nipponbare were kindly provided from Prof. Junko Kyojuka (University of Tokyo). Seeds of *htd1* and its wildtype NJ6 (Zou et al., 2006) were kindly provided by Prof. Lihuang Zhu (Chinese Academy of Sciences). Rice seeds were planted in pots (one seed/pot) filled with quartz sand and watered with distilled water. After leaf emergence at 7th day the plants were watered with 0.5x Hoagland solution with 1 μ M MES buffer adjusted to pH=5.8. The treatments were prepared in stock solution dissolved in acetone and diluted to final concentration of 1 μ M GR24, and 2 or 40 μ M d'orenone or respective volume in acetone for control treatment in the nutrient solution before application to the plants. All pots and individual pot trays were light protected to prevent degradation of d'orenone. Plants were grown in a climate chamber at 28°C/25°C 10 h light/ 14 h dark cycle (450 μ M m⁻² s⁻¹) and 70% relative humidity. Tillering was counted at the end of the second or third week of treatment for *d3-2/d10-2*/Nipponbare or *htd1*/NJ6 plants respectively. Seeds from *max1-1* and *max2-1* (Stirnberg et al., 2002), *max4-1* (Sorefan et al., 2003) and *max3-9* (Booker et al., 2005) and Columbia-0 wild-type Arabidopsis seeds were kindly provided by Prof. Ottoline Leyser (University of York). The Arabidopsis seeds were surface sterilized in 10% (w/v) chlorine bleach, followed by 70% ethanol and at last sterile distilled water. The seeds were spread in wet filter paper and pre-conditioned for two to four days at 4 °C in dark. To test branching, Arabidopsis seeds were transferred to sterile plastic cups (5 seeds per cup) containing 0.5x MS salts supplemented with 1x Gamborg's B5 vitamin mix and 0.8% adjusted to pH5.8 (w/v) agar (Daichin) without sucrose and the treatment of 2.5 μ M GR24, 10 μ M d'orenone or respective volume of acetone for the control treatment. The agar was covered with a thin layer of sterilized vermiculite and the cups wrapped in aluminium foil at the sides and bottom to protect the d'orenone from light degradation. For root trait analyses, the seeds were sown in plates with the same nutrient medium and treatments as described before. The plates were placed vertically and the roots covered with aluminium foil to protect from light. In both

experiments, the plants were grown at 22°C /18 °C and 16 h - light /8 h - dark cycle (80µmol m⁻² s⁻¹). Rosette branching was counted 2.5 weeks after bolting.

Root trait phenotyping

Root images were taken with a digital camera (Canon EOS d) and root elongation was measured with a curvimeter on enlarged printed images. Emerged lateral roots were scored visually.

RNA extraction

RNA was extracted from roots of d3-2, d10-2 and Nipponbare roots treated with d'orenone harvested, after the tillering was scored. The RNA was purified from 70 mg of homogenized ground roots using 500 ml Trizol (Invitrogen) and further purified with chloroform. After precipitation with 70% (vol/vol) ethanol, the RNA was recovered with an RNAeasy Mini Kit column (Qiagen) and DNA was removed using the DNAase I Kit (Qiagen), according to manufacturer's instructions.

Gene expression analysis

cDNA was synthesized from 1µg of total RNA per sample, using the iScript cDNA Synthesis Kit (BioRad) and following the manufacturer's instructions. The qRT-PCR reactions were prepared using iQ SYBR Green Supermix (BioRad). Gene expression was measured in pooled cDNA from 5 to 6 biological replicates. For the reactions 0,3 µM of each primer and 1µl of 10-fold diluted template cDNA were used. The amplification was detected with a BioRad qRT-PCR detection system and thermocycler. The following primers were used: reverse primer 5'CTTGTCGCCTGGTACGACAA-3' and forward primer 5'-GTCGATGACACGGTTGCTGTA-3' for *OsGAPDH* (used as reference); reverse primer 5'AATGCACTTGTGGCAAACTAGAG-3' and forward primer 5'-CATTGGAAAAGTGAGGTTCTTTGG-3' for *OsCCD7*; reverse primer 5'CGTTGCGGGCATTGGGTTAC-3' and forward primer 5'-GATGGCTGCGCGAGTCTTT-3' for *OsCCD8*; reverse primer 5'GCAAGGGAATAACCAACAGC-3' and forward primer 5'-GGCTCTGGGAAATTTTTGGG-3' for *SLB1*; reverse primer 5'ACGACGGCGTTCCTCTCTC-3' and forward primer 5'-TCCGAACCCGTC AATCTCC-3' for *SLB2*.

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Chapter 6

General discussion

Catarina Cardoso

The research of strigolactone biosynthesis and its regulation is an interesting case study that addresses both evolutionary and ecological aspects in plant physiology. Strigolactones were first described as the signaling compounds that trigger the germination of parasitic plants (Cook et al., 1972). Hence, they were considered to be detrimental and the evolutionary advantage for the conservation of their biosynthetic pathway was not understood. This changed when new roles for the strigolactones were discovered, as signaling molecules for the symbiotic arbuscular mycorrhizal fungi (AMF) and as plant hormones (Umehara et al., 2008; Akiyama et al., 2005; Gomez-Roldan et al., 2008). The findings in the past five years, to which this thesis contributes, have given a new perspective on the conservation of strigolactone biosynthesis and the evolution of their ecophysiological roles, which will be discussed in this chapter.

Conservation of the strigolactone pathway

The symbiosis with AMF is considered to have been important to enable plants to colonize land (Wang et al., 2010). The discovery of the involvement of strigolactones in the establishment of the symbiotic interaction with AMF led scientists to hypothesise that strigolactone biosynthesis dates back to this event. However, recent data suggest that strigolactone biosynthesis is probably even older than that. Strigolactones (or their biosynthetic genes) have been found in Lycohytes (*Marchantia* spp. and *Lunularia cruciata*) Bryophytes (*Physcomitrella patens*) and in algae of the order Charales, but probably not in the Chlorophytes (Delaux et al., 2012). This suggests the emergence of strigolactone biosynthesis in the green algae of the *Charophyta* division, prior to land colonization. Figure 1 shows the results of a recent phylogenetic study relating major algae and plant divisions, in which I summarized in which clades strigolactones/strigolactone biosynthetic genes have been detected (Delaux et al., 2012; Finet et al., 2012). The early role of strigolactones does not seem to be restricted to the communication with symbionts. The strigolactone analogue GR24 stimulates rhizoid elongation in mosses, liverworts and in species from the Charales (Delaux et al., 2012). In *P. patens*, strigolactones seem to be involved in conspecific neighbour detection, restricting protonema branching, in a *quorum sensing* manner (Proust et al., 2011). This suggests that the

primary role of strigolactones was to regulate rhizoid and protonema development and that they were recruited afterwards to act as signaling molecule in the symbiosis with AMF (Delaux et al., 2012).

In vascular plants, strigolactones are derived from all-*trans*- β -carotene that is first isomerized by DWARF27 (D27), and then cleaved in two consecutive steps by CAROTENOID CLEAVAGE DIOXIGENASES7 AND 8 (CCD7 and CCD8) (Alder et al., 2008). In the latter cleavage step, CCD8 also catalyses a molecular rearrangement producing carlactone. After this step, carlactone is probably further converted by MORE AXILLARY GROWTH 1 (MAX1), a cytochrome P450 (CYP450) enzyme described in Arabidopsis (Booker et al., 2005; Kohlen et al., 2011; Seto et al., 2014). The enzymatic step performed by MAX1 is not yet fully elucidated, and the conservation of this enzyme will be discussed below in more detail. Two genes involved in strigolactone perception and signaling have been described. DWARF14 (D14), encoding an α/β -hydrolase that hydrolyses the butenolide D-ring of the strigolactones (Figure 2), and in the presence of the substrate interacts with MORE

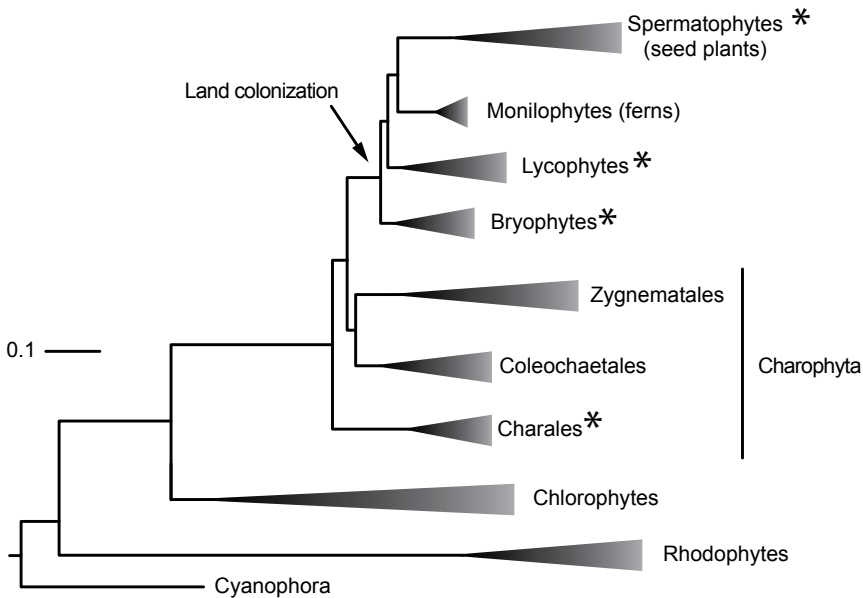


Figure 1. Phylogenetic relationship of major algae and plant clades, adapted from Finet et al. 2012. The star (*) indicates that strigolactones or strigolactone biosynthetic genes have been detected in species belonging to those clades.

AXILLARY GROWTH 2 (MAX2)/DWARF3 (D3) an F-BOX subunit of the SCF complex that triggers strigolactone mediated signaling responses.

Interestingly, despite all the evidence that strigolactones are ancient signaling molecules, phylogenetic studies reveal that several genes encoding strigolactone biosynthetic and perception components are not conserved across the taxa (Delaux et al., 2012; Challis et al., 2013). For example, no *CCD8* orthologs were found in strigolactone producing Charales or liverworts suggesting that there is a strigolactone biosynthesis pathway in these organisms that is independent from *CCD8* (Delaux et al., 2012; Challis et al., 2013). In contrast, *P. patens* does have a *CCD8* and the *P. patens ccd8* mutant has reduced levels of several strigolactones, although strigol and 7-orobanchyl acetate are still detected suggesting that in bryophytes there are two pathways acting in parallel (Proust et al., 2011). In addition, the Arabidopsis *more axillary growth 4 (max4)* and rice *dwarf 10 (d10)* mutants (both impaired in *CCD8* activity) contain low but still detectable levels of strigolactones (Kohlen et al., 2011; Umehara et al., 2008). It is possible that the mutations in these lines do not cause a complete knockout of *CCD8* activity but it may also indicate the presence of an alternative pathway – just as suggested for the Charales and *P. patens* - that is also active in higher plants.

The data reported in Chapter 5 suggest that *CCD8* and *CCD7* participate in the biosynthesis of yet another compound, besides strigolactones. *In vitro*, these two enzymes catalyse the cleavage of all-*trans*- β -carotene to produce an apocarotenoid called d'orenone (Alder et al., 2008; Schwartz et al., 2004), but it is still elusive if d'orenone is also produced in plants. In Chapter 5 I investigated the interaction between the biosynthetic pathway of strigolactones and d'orenone and compared the physiological response to the two compounds. I found that d'orenone inhibits primary root elongation and increase of lateral root density, while the synthetic strigolactone analogue GR24 increases primary root elongation and decreases lateral root density. Though opposite to the effect of GR24, the two physiological responses triggered by d'orenone are independent of the strigolactone signaling pathway (Chapter 5, this thesis). All in all this suggests that d'orenone could be a new signaling molecule in vascular plants. The orthologs of *CCD7* and *CCD8* in rice, pea as well as Arabidopsis are conserved for the ability to produce both strigolactones and d'orenone (Alder et al., 2008). Hence, the two enzymes seem to be under selective pressure to maintain

both functions. If d'orenone biosynthesis occurs and is exclusive to higher plants, the absence of such selective pressure in algae could result in divergence of carotenoid cleavage enzymes dedicated to strigolactone biosynthesis among the taxa. It would therefore be interesting to study if more divergent homologs of *CCD8*, in Bryophytes, Lycophytes and Charales can produce both carlactone and d'orenone.

It is unclear if *D27* is conserved between vascular plants, bryophytes and strigolactone producing algae. Delaux et al. identified *D27* sequences in all strigolactone producing taxa and considered them true *D27* orthologs (Delaux et al., 2012). In another study, Challis et al. concluded in their phylogenetic study that the algal and early embryophyte sequences are unlikely to be *D27* orthologs and are instead closely related to *D27-like* that is not involved in strigolactone biosynthesis in higher plants (Challis et al., 2013). This could imply that there is a *D27* independent strigolactone biosynthetic pathway. Also *MAX1* is not conserved in algae and embryophytes (Challis et al., 2013).

The existence of alternative metabolic pathways has been described in higher plants for several metabolites such as isopentenyl diphosphate, β -carotene and abscisic acid (Schwartz et al., 2003; Lichtenthaler, 1999; Ronen et al., 2000). The emergence of a new pathway might be advantageous if it provides higher efficiency or different possibilities of regulation. These aspects are of interest to consider when studying strigolactone biosynthesis in early embryophytes and algae. Phosphate starvation is a stimulus well-known for strongly promoting strigolactone biosynthesis in higher plants (López-Ráez et al., 2008a; Umehara et al., 2010). The evolutionary advantage of this regulation is thought to be two-fold. First, strigolactones alter

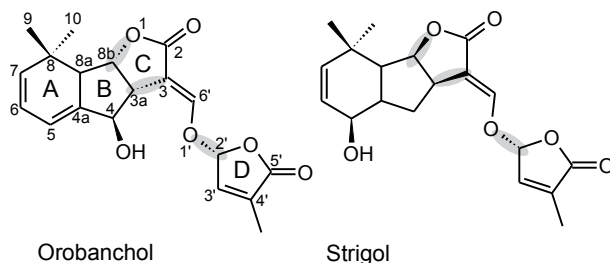


Figure 2. Structures of orobanchol and strigol showing the two natural stereochemical variants in the strigolactones, which define the orobanchol and strigol-like strigolactone families. The stereocenters are shaded in light gray.

plant architecture by inhibiting axillary branching which reduces the development of shoot biomass and by increasing root expansion and branching which promotes higher exploration of the soil under unfavourable nutrient conditions (Kohlen et al., 2011; Umehara et al., 2010). Secondly, strigolactones promote AMF colonization that improves phosphate supply (Akiyama et al., 2005). Primitive plants, however, do not interact with mycorrhiza and the postulated primary role of strigolactones in these organisms is in rhizoid development. It would be interesting to test if in these organisms the phosphate levels - as well as other factors such as the availability of other nutrients or drought stress - also affect strigolactone biosynthesis.

Another open question is how the physiological function of strigolactones has evolved in the plant kingdom. Strigolactones regulate plant development in vascular plants by reducing abundance of PIN FORMED (PIN) - auxin efflux carriers - and polar auxin transport capacity changing auxin homeostasis (Ruyter-Spira et al., 2013; Shinohara et al., 2013). In Charales and embryophytes strigolactones were found to be involved in rhizoid development by stimulating cell elongation, two processes in which auxin is also involved (Cooke et al., 2002; Delaux et al., 2012; Jones and Dolan, 2012). However, there is so far no evidence of strigolactones and auxin interacting in algae to modulate development even though Polar Auxin Transport (PAT) was found to occur in Charales (Boot et al., 2012). In vascular plants PAT is mostly governed by PIN proteins – which are strigolactone targets as described above - but so far no *PIN* homologs were found in the Charales genome (Boot et al., 2012). Finally, neither *MAX2* nor *D14* homologs were found in algae, while in bryophytes only putative *MAX2* homologs were found (Challis et al., 2013). This suggests that the mechanisms influenced by strigolactones and their perception in algae and bryophytes are different from what is currently described in higher plants.

Structural diversification of strigolactones

Plants produce a range of different strigolactone molecules. More than 16 structures have already been identified and this is still expanding (Xie et al., 2010; Kisugi et al., 2013). Strigolactones have a common core structure formed by a tricyclic lactone connected via an enol-ether bridge to a butenolide group (Figure 2). The different strigolactones so far identified maintain this general structure with some variations.

The tricyclic lactone is formed by three rings, (A, B and C) and can have different substituents at the A- and B- rings. Strigolactones can also vary according to their stereochemistry. The stereochemical configuration of some strigolactones was recently revised, and it is currently proposed that strigolactones are divided into two families depending on the α or β orientation of the C-ring (Xie et al., 2013) (Figure 2). Strigol-like strigolactones have the C-ring in β -orientation whereas orobanchol-like strigolactones have the C-ring in α -orientation (Figure 2).

One plant species can produce several different strigolactones. For example, tomato produces orobanchol, solanacol, orobanchyl acetate, 7-oxoorobanchol, two isomers of 7-hydroxyorobanchol and four isomers of didehydro-orobanchol (Kohlen et al., 2013; López-Ráez et al., 2008a, 2008b). In the rice variety Nipponbare, four different strigolactones have been confirmed: *ent*-2'-*epi*-5-deoxystrigol, orobanchol, orobanchyl acetate and 7-oxoorobanchyl acetate (Xie et al., 2013). Additionally, three putative methoxy-5-deoxystrigol isomers have been reported (Jamil et al., 2011) and further characterized in Chapter 3. Also in Chapter 3, the exudates of rice Nipponbare were profiled for the presence of additional parasitic seed germination and hyphal branching stimulants, revealing the presence of two strong germination stimulants that are possibly related to the strigolactone biosynthetic pathway but are not known strigolactones. Rice has been an exception so far because the strigolactones identified in most grasses are strigol-like whereas in rice only orobanchol-like strigolactones have been confirmed (Awad et al., 2006; Xie et al., 2013). Tobacco produces strigolactones from both families suggesting that in this, and perhaps other, dicotyledonous species both the orobanchol and the strigol-like strigolactone biosynthetic pathway operate (Xie et al., 2013). Since the stereochemical structure of some strigolactones have been elucidated and amended only very recently, it would be helpful if more structures were confirmed so we better understand the variation in the α and β orientation of the C-ring among plant species. It is intriguing how and why plants produce such a high number of different strigolactones. In this section I will focus on how this variation may have evolved and in the following section I will discuss possible evolutionary reasons for the variation.

It has been postulated that 5-deoxystrigol is the first dedicated strigolactone and that all other strigolactones are derived from this molecule by hydroxylation, methylation and/or acetylation in rings A and B (Rani et al., 2008; Xie et al.,

2010). The C-ring orientation is most likely determined upon biosynthesis of this first strigolactone, so resulting in 5-deoxystrigol, the precursor of the strigol-type strigolactones or *ent-2'-epi-5-deoxystrigol*, the precursor for the orobanchol-type strigolactones.

A study by Motonami et al. (2013) showed that sorghum plants can bio-convert externally provided 5-deoxystrigol (strigol-like, with the C-ring in β -orientation) into sorgomol (a strigolactone present in sorghum) but also *ent-2'-epi-5-deoxystrigol* (orobanchol-like, with the C-ring in α -orientation) into *ent-2'-epi-sorgomol* (which is not naturally present in sorghum) suggesting that 5-deoxystrigol and its stereoisomer are the precursors for all strigolactones in the two families. The authors also showed that conversion from one stereochemical family to another did not occur. So far, only strigol-like strigolactones (with the C-ring in β -orientation) have been reported in sorghum (Awad et al., 2006) suggesting that this species lacks the enzyme(s) that produce the precursor for orobanchol-like strigolactones, even though it does contain enzymes that can convert the orobanchol-like *ent-2'-epi-5-deoxystrigol*. Interestingly, in the same study by Motonami et al., the conversion of both 5-deoxystrigol enantiomers was inhibited by a CYP450 inhibitor but to different extents, suggesting that either two CYP450 enzymes are involved in the bioconversion steps of each enantiomer or that the stereochemistry of the reaction influences the effect of the inhibitor (Motonami et al., 2013).

An important candidate enzyme for the formation of the different strigolactone variants is the CYP450 enzyme MAX1. This enzyme is necessary for strigolactone biosynthesis, but the performed enzymatic step is so far unknown (Booker et al., 2005, 1; Kohlen et al., 2011) (and Chapter 4 in this thesis). It is tempting to speculate that MAX1 converts carlactone into the parent strigolactone, 5-deoxystrigol, and hence determines the stereochemistry of downstream strigolactone structures. The accumulation of carlactone in the Arabidopsis *max1* mutant supports this hypothesis (Seto et al., 2014). However, Arabidopsis contains only one MAX1 homolog in its genome, while this is not the case in many other species. A detailed study on MAX1 homologs from vascular plants by Challis et al. reveals that, many species contain more than one MAX1 homolog (Challis et al., 2013). This is particularly the case in grasses with at least 3 homologs, whereas dicots contain only one with the exception of some species that contain two (Challis et al., 2013). The different MAX1

homologs could be involved in the synthesis of different strigolactone structures thus contributing already in this step of the biosynthesis to structural diversification. The same phylogenetic study shows that the *MAX1* sequences from dicots are all clustered in one clade, independent from the monocot sequence clade suggesting that the divergence in *MAX1* sequences occurred after the monocot/dicot division. Moreover, the duplication in dicots (when present) seems to have occurred only very recently since the resulting homolog sequences cluster in a species or family specific clade. In grasses the *MAX1* homologs are clustered in three distinct clades and all grass species investigated so far contained at least one gene in each of these clades (Challis et al., 2013).

If gene duplication in *MAX1* has led to specialization in enzymatic function resulting in production of different strigolactones, the phylogenetic structure of *MAX1* orthologous genes suggests that this would yield strigolactone variants specific to monocots and dicots. However, many strigolactones produced by monocots are also found in dicots. Regarding stereochemistry, both strigol-type and orobanchol-type strigolactones are found in tobacco, showing that at least some dicot species can produce both enantiomers. In contrast, in grasses that contain many *MAX1* homologs, distributed over three conserved clades, most species only produce strigol-like strigolactones while in rice only orobanchol-like strigolactones were found so far (Xie et al., 2013; Awad et al., 2006). Some variations in A- and B-rings that could also be a consequence of different *MAX1* homolog activities appear in both monocots and dicots such as orobanchol and orobanchyl acetate whereas others have only been found in grasses (strigol, sorgomol and sorgolactone) or only in dicots (solanacol and didehydro-orobanchol). Apparently, although only one or maximally two *MAX1* homologs are found in dicots, this does not constrain the variability of strigolactones structures found in these species compared to the variability found in grasses. In conclusion, the phylogeny of *MAX1* homologs does not reflect the diversity of strigolactone structures across species. This could imply that the *MAX1* homologs only have a (redundant) enzymatic function in the early steps of the strigolactone pathway, upstream of the diversification of strigolactone structures and that other enzymes must be involved in the downstream structural diversification.

Indeed, in Chapter 4 of this thesis I show that deletion of two *MAX1* homologs in the rice variety Bala [*STRIGOLACTONE BIOSYNTHESIS1 (SLB1)* and *STRIGOLACTONE BIOSYNTHESIS2 (SLB2)*] results in an overall reduction in the production of all rice strigolactones compared with the variety Azucena where these genes are present. This suggests that the two deleted genes contribute to the biosynthesis of all strigolactones detected in rice. However, the identification of strigolactones in rice and other species is far from complete. In Chapter 3, by testing fractionated root exudates of the rice variety Nipponbare, I provide evidence for the presence of three putative methoxy-5-deoxystrigol isomers and two strong additional germination stimulants yet unidentified but very likely to be strigolactones or strigolactone-like. The germination activity studies in Chapter 3 also showed that the two unknown germination stimulants have a major contribution to the overall germination of *S. hermonthica* seeds. The presence of those strong germination stimulants in Bala and Azucena has not yet been tested however, the deletion of *SLB1* and *SLB2* characterized in Chapter 4 resulted in overall reduction of *S. hermonthica* germination stimulatory activity. It would be interesting to test if these stimulants are also produced by Bala and Azucena and if their biosynthesis is dependent on the activity of *SLB1* and *SLB2* homologs like all detected strigolactones. This test could provide more evidence to the strigolactone nature of the unknown compounds as well as give more insight into the role of the *MAX1* homologs in strigolactone biosynthesis. Bala complementation with *SLB1* or *SLB2* increased *ent-2'-epi-5-deoxystrigol* levels in root exudates showing that both genes contribute to the synthesis of this strigolactone. The functional diversity of the *MAX1* homologs may also reside in differential regulation of strigolactone biosynthesis in relation to stress responses or other stimuli. For example, in Chapter 4 I observed that only the rice *MAX1* homolog *SLB1* responded to phosphate starvation treatment with increased expression levels but not *SLB2*. Finally, testing if carlactone can be converted by *MAX1* and its homologs is ultimately necessary to understand if this enzyme contributes to strigolactone structural diversification. Structural elucidation of all rice strigolactones will also give us more information on strigolactone diversity and on the possible roles of *MAX1* homologs in rice and grasses in general.

It will be a challenge to identify the enzymes responsible for strigolactone diversification. Simple plant architecture screens or germination and hyphal

branching assays using whole root exudates are not suitable to screen for different strigolactone composition in a mutant screen because changes in strigolactone composition probably result merely in small changes in overall activity. The structural variation so far found in natural strigolactones has little effect on their shoot branching regulatory activity (Boyer et al., 2012). Parasitic plants and AMF have different sensitivities to strigolactones as will be discussed below. The differences are however not dramatic, and in species that produce a large variety of strigolactones, the loss of one very active compound may not have a big impact on the overall activity of the total exudate, in assays with parasitic plant seeds or AMF as well (Akiyama et al., 2010; Kim et al., 2010; Nomura et al., 2013). The use of fractionated exudates as I used in Chapter 3 can however give meaningful results, also because it allows us to find stimulants of unknown structure, but is also not optimal for extensive mutant screens due to its low throughput. The recent advances in metabolic profiling methods to detect strigolactones and the increasing knowledge of strigolactone variants will probably enable high throughput screens based on an analytical rather than biological approach in the near future. Besides mutant screens, another alternative to find genes that confer structural diversity in strigolactones is to look for genetic variation between varieties or ecotypes of the same species such as corn, sorghum and rice and dicots like tomato or tobacco, the latter a producer of both orobanchol- and strigol-like strigolactones. If genetic variation is present, GWAS or mapping studies using RIL populations could help us to find the genes involved in strigolactone structural diversification. In fact, sorghum varieties have been reported to produce different mixtures of strigolactones and could be a useful germplasm for such studies (Awad et al., 2006; Satish et al., 2012). This approach also enables us to detect genes with redundant functions that cannot be found in mutant screens. Chapter 4 in this thesis shows how QTL mapping, using metabolic profiling of root exudates in a RIL population, was a viable strategy to successfully identify MAX1 homologs in rice.

Structure-activity relationship of strigolactones in different organisms

The evolutionary causes and advantages for the diversity in strigolactones found in nature and in one plant species are intriguing. In Chapter 3 I observed that rice produces a large number of germination and hyphal branching stimulants, of which most are strigolactones or strigolactone-related compounds. The compounds that are responsible for the activity of two very active fractions were not yet elucidated although there is strong evidence that they are strigolactones or at least biosynthetically related to strigolactones. I showed that the structural features required for biological activity are different between the parasitic plant (*S. hermonthica*) and symbiotic fungi (*Gigaspora margarita* and *Gigaspora rosea*). This can possibly be extended to other species of parasitic plants and AMF. Other in depth studies, using a wide range of strigolactone structures and analogues have also concluded that high activity to induce parasitic plant germination, arbuscular mycorrhizal hyphal branching or to inhibit bud outgrowth requires different structural features (Akiyama et al., 2010; Kim et al., 2010; Nomura et al., 2013; Boyer et al., 2012). Nevertheless, there is one structural feature that is essential for the activity in inhibiting bud outgrowth, parasitic plant seed germination and AMF hyphal branching and that is the D-ring (Akiyama et al., 2010; Zwanenburg and Pospíšil, 2013). However, the different biological systems have different requirements for the connection between the tricyclic lactone and the D-ring. For example, the inhibition of bud outgrowth and the induction of parasitic plant seed germination require the presence of the unsaturated bond in the enol-ether bridge (Zwanenburg and Pospíšil, 2013; Boyer et al., 2012). In contrast, the stimulation of hyphal branching still occurs if the connection is replaced by a saturated bond (Akiyama et al., 2010). The stereochemistry also affects the activity differently in these systems. Variations in the 2'C (enol-ether bond) and 3aC/8bC (C-ring, Figure 2) stereo centres do not affect the activity of inhibition of bud outgrowth (Boyer et al., 2012). But *Striga hermonthica* and *Orobancha minor* are most sensitive to strigol-like stereochemistry and of all possible stereochemical combinations, orobanchol-like strigolactones are generally much less active, especially in *Striga hermonthica* (Nomura et al., 2013; Thuring et al., 1997; Sugimoto et al., 1998). AMF

hyphal branching is equally stimulated by orobanchol- and strigol-like strigolactones whereas non-natural stereochemical configurations are less active (Akiyama et al., 2010). Finally, the impact of substitutions in the A- and B-rings as well as the presence/absence of the A- and B-rings is most pronounced in hyphal branching. Removal of the A- or AB- rings strongly affects hyphal branching activity, but not the induction of seed germination nor the inhibition of bud outgrowth (Zwanenburg and Pospíšil, 2013; Akiyama et al., 2010; Boyer et al., 2012). In fact, strigolactone analogues in which the A- and B-rings are lacking maintain or even show increased activity at inducing parasitic plant seed germination and inhibiting bud outgrowth (Boyer et al., 2012; Zwanenburg and Pospíšil, 2013). Hydroxylation of C4 in the B-ring, leading to the formation of orobanchol isomers, increases hyphal branching activity (Akiyama et al., 2010). These orobanchol isomers are the most active in inducing hyphal branching, even in less favourable stereochemical configurations, but this is not observed in the inhibition of bud outgrowth nor parasitic plant seed germination. Given its proximity to the D-ring it was proposed by Akiyama et al. that this substitution could increase specificity to the elusive receptor in AMF (Akiyama et al., 2010). Other types of modifications in the AB-rings affect molecule stability and polarity and therefore the changes in activity could also be related to different half-lives in the soil and/or diffusion across membranes, rather than to receptor-substrate specificity in AMF or parasitic plants (Akiyama et al., 2010).

In Chapter 2 I reviewed and discussed the ecological role of strigolactones and its significance for host specificity. In that review chapter I have postulated that the structural diversification of strigolactones could result from an arms race in a three party co-evolution involving the plant host, the parasite and the symbionts. The plant host has to communicate efficiently with its symbiotic partner while remaining unrecognized by the parasite. For that, it is advantageous to produce a strigolactone structure that is highly active at stimulating hyphal branching but has reduced (or preferably no) activity in inducing parasitic plant germination. After the formulation of this hypothesis, described in Chapter 2, the knowledge on structural variation of strigolactones and its effect on activity have increased. The recent studies on structure-activity relationships reported in Chapter 3 and in other studies suggest that stereochemical variation is more likely to affect the stimulation of seed germination than hyphal branching (Akiyama et al., 2010; Nomura et al., 2013 and

Chapter 3). For example, there is a dramatic difference in the sensitivity of *Striga hermonthica* to the two stereoisomers of 5-deoxystrigol, with highest preference to the strigol-type while AMF have a similar response to these two strigolactones (Akiyama et al., 2010; Nomura et al., 2013). Hence, stereochemical variation could have emerged and be preserved to avoid recognition by parasites, especially in plants that are naturally exposed to *Striga* and *Orobanche/Phelipanche* spp. In Chapter 3, I observed that production of orobanchol and *ent-2'-epi-5-deoxystrigol* in rice (both orobanchol-type) might be advantageous since both have low activity at stimulating the germination of *Striga hermonthica* - compared with the other rice germination stimulants (possibly strigolactones) - but are strong stimulators of AMF *Gigaspora margarita* and *Gigaspora rosea* hyphal branching.

On the other hand, certain AB-ring modifications such as those found in orobanchol isomers seem to have a stronger impact on the stimulation of hyphal branching activity of *Gigaspora margarita* than on seed germination of the parasites. This type of structural variation could be advantageous to the host plant to produce efficient strigolactone variants to promote the symbiotic interaction since it has little effect on parasitism. Only one AMF species has been tested so far in an extensive structure-activity study (Akiyama et al., 2010). It would be interesting to study if other mycorrhizal species have similar or different preferences. If variation is found, exudation of different strigolactone structures might contribute to variation in colonization by different AMF species as well.

The structural requirements for parasitic plant seed germination discussed here refer to data obtained with *S. hermonthica* and *Orobanche crenata*. However, the level of specificity of parasitic plants to strigolactone structures varies across species. *Striga gesnerioides*, for example, has strong specificity to a narrow range of strigolactone variants and some strigolactones can even have an inhibitory effect (Nomura et al., 2013). *S. hermonthica* and *O. crenata* seem to share similar requirements for increased sensitivity to differences in strigolactone structures and no strigolactone was reported so far to inhibit seed germination in these species. This suggests that the perception mechanism diverged further in *S. gesnerioides* and it is more conserved between *S. hermonthica* and *O. crenata*. Structure-activity relationship studies, such as the one reported in Chapter 3, are important to understand the perception mechanisms of strigolactones by different organisms.

Not less important is their contribution to the development of new crop varieties with improved mixtures of strigolactones that can be effective at communicating with AMF, regulate plant development and induce low or no parasitism. Common agricultural systems promote the spread of the parasitic seeds that are produced in high numbers whereas the host germplasm is replanted every year with no selection for the synthesis of optimal strigolactone structures. Therefore, it is important to study host plant varieties in natural ecosystems to understand the evolutionary pressures that drive structural diversification of strigolactones. Having said that, the study using a RIL population from a cross between two rice cultivars (Bala and Azucena) in Chapter 4, has opened up many questions regarding the role of strigolactones in tolerance to *Striga* parasitism in rice. In Chapter 4 I show that a major QTL common to most varieties in the Japonica group contributes to higher production of strigolactones. Interestingly, the same allele also contributes to increased post-attachment tolerance in *Striga hermonthica* parasitism (Kaewchumnong and Price, 2008). This suggests that even though higher levels of strigolactone biosynthesis increase parasitic plant seed germination and subsequent infection, strigolactones may also have a role in post-attachment tolerance. Further studies are necessary to determine whether this effect is due to increased levels of strigolactones or to other elements present in the QTL. In Chapter 4 transgenic rice lines of Bala were generated overexpressing the genes that were deleted in Bala and caused the described QTL. These lines have increased strigolactone production and are a great tool to further test this hypothesis. Also other biosynthetic and strigolactone perception mutants may help us to better understand this putative tolerance mechanism.

Concluding remarks

In this chapter, I discussed some of the recent advances made in the field of strigolactone research and how they relate to my research presented in this thesis as well as some of the perspectives for the strigolactone field in the coming years. The study of strigolactones is interesting to a broad range of research areas, from plant symbiotic fungi to plant development, adaptation to biotic and abiotic stresses and evolution of land colonization by plants. All these lines of research are also of great importance for the development of better adapted cultivars and to improve

agricultural techniques, especially on fields with poor nutrient and water availability and infested by parasitic plants. In many cases, these problems cause serious economic losses and put food security at risk. This justifies intense research in the biology of strigolactones and I expect that in the near future we will again see significant advancements in strigolactone research just as we have witnessed in the recent years.

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Summary

Strigolactones are plant derived molecules that play important eco-physiological roles. Strigolactones are released into the rhizosphere to function as signaling compounds for the establishment of beneficial arbuscular mycorrhizal (AM) symbiosis. Seeds of the parasitic plants belonging to the genera *Striga*, *Orobancha* and *Phelipanche*, take advantage of this communication and recognize the presence of a host by perceiving strigolactones upon which they germinate and infect the host's roots.

Crop infestation by these parasitic plants causes considerable economic losses especially in areas of the Mediterranean region and Sub-Saharan Africa. Farmers are often forced to abandon contaminated fields or have to switch to less economically viable crops that are not affected by the parasites and in some cases food security is put at risk. Recently, novel roles of strigolactones were discovered. These compounds are also plant hormones that modulate shoot and root architecture and mediate developmental responses to low phosphate availability. This finding reinforced the interest in strigolactone research not only to fight plant parasitism but also to increase the understanding of plant development and as a potential tool to improve crop yields.

In **Chapter 2** I have reviewed the current knowledge on the life cycle of the parasitic plants, the ecological significance of strigolactones as signaling compounds for beneficial symbiotic AM fungi and parasitic plants and I have discussed current and new possible strategies to fight plant parasitism, with particular emphasis on the role of strigolactones. In **Chapters 3 and 4** I have used rice and the parasitic plant *Striga hermonthica* as research models, to get a better understanding of the biosynthesis of strigolactones, its regulation and the impact of strigolactone structural variation on plant parasitism and AM symbiosis. Strigolactones form a large group of compounds that share a common core structure of four rings (A-D), of which two lactone moieties, but otherwise vary in their stereochemical configuration and

Summary

functional groups on the A-B rings. Plants produce and exude a mixture of several strigolactones and this mixture varies according to the plant species or even cultivar. It has been reported that rice produces orobanchol, *ent-2'-epi-5-deoxystrigol*, 7-oxoorobanchol and orobanchyl acetate.

In this thesis I have profiled rice exudates to detect novel germination stimulants and to test the contribution of each stimulant to *Striga hermonthica* germination and AM fungi hyphal branching (**Chapter 3**). I detected the presence of strong germination stimulants in rice exudates that are likely strigolactones (but not known ones) or other, but biosynthetically closely related, molecules. Furthermore I have shown that some of the strong germination stimulants present in rice exudates give little contribution to AM fungi hyphal branching. These results suggest that selection of cultivars producing different mixtures of strigolactones could be a strategy to reduce parasitic plant infestation while maintaining the ability to attract mycorrhiza, as I suggested in Chapter 2.

In **Chapter 4**, I have assessed genetic variation in strigolactone biosynthesis in rice using a RIL population derived from a cross between the rice cultivars Bala and Azucena, known to have different susceptibilities to *Striga hermonthica* infestation. In this study I have identified a QTL containing *SLB1* and *SLB2* – two strigolactone biosynthetic genes, orthologs of Arabidopsis *MAX1*, that are deleted in the allele associated to low strigolactone production. This polymorphism is highly associated to the *indica/japonica* divide. Moreover, this polymorphism has a strong impact on strigolactone biosynthesis and related traits in all tested varieties belonging to the Rice Diversity Panel and is of great interest for the development of new rice varieties for increased tillering or tolerance to parasitism.

Finally, I have investigated the interaction between the strigolactone biosynthetic pathway and plant responses to d'orenone (**Chapter 5**). *In vitro*, d'orenone formation from β -carotene is catalysed by enzymes (Carotenoid Cleavage Dioxygenases 7 and 8) that are also involved in strigolactone biosynthesis. Earlier reports have shown that d'orenone triggers physiological responses such as changes in the distribution and abundance of the auxin transporter PIN2, blocks root hair elongation and reduces gravitropic response in root meristems, although d'orenone has so far never been detected *in planta*. In this thesis, I show that d'orenone also affects primary root elongation and lateral root density. Strigolactones and d'orenone

trigger opposite responses regarding root hair and primary root elongation and lateral root density. Also, the effect of d'orenone on gene expression suggest that d'orenone may down-regulate strigolactone biosynthesis. However, I also observed that the physiological response to d'orenone is independent from the strigolactone signalling pathway. The data in this thesis suggest that d'orenone or a downstream derivative could be another carotenoid derived signalling compound in plant development. A model is proposed on how d'orenone application to roots might affect auxin homeostasis and trigger developmental responses in the root meristem.

With this thesis, I hope I made a contribution to the understanding of the biology of strigolactones, its biosynthetic pathway and the possible involvement of strigolactone-related compounds in plant development. I have also identified valuable genetic resources for the development of low strigolactone producing rice cultivars and pointed out new solutions to fight plant parasitism.



Samenvatting

Strigolactonen zijn door planten geproduceerde moleculen met een belangrijke eco-fysiologische rol. Strigolactonen worden in de rhizosfeer uitgescheiden waar ze dienen als communicatiestoffen om symbiotische arbusculaire mycorrhiza (AM) schimmels aan te trekken. Zaden van de parasitaire planten uit de genera *Striga*, *Orobanche* en *Phelipanche* maken misbruik van dit signaal en gebruiken de strigolactonen ook om de aanwezigheid van een gastheer te detecteren, waarna ze kiemen en de wortels van de gastheer infecteren.

Deze plantparasieten veroorzaken aanzienlijke economische schade aan gewassen, voornamelijk in gebieden rond de Middellandse Zee en in Sub-Sahara Afrika. Boeren zijn vaak genoodzaakt besmette akkers te verlaten of moeten overgaan op minder waardevolle gewassen die niet door de parasiet worden aangetast, met als gevolg een risico voor de voedselzekerheid. Recentelijk is ook een nieuwe rol voor de strigolactonen ontdekt: het zijn ook plantenhormonen die de architectuur van de wortel en de scheut beïnvloeden en vooral functioneren als signaalstof voor de plantontwikkeling bij lage fosfaat beschikbaarheid. Deze nieuwe kennis heeft het belang van het onderzoek aan strigolactonen verder versterkt, omdat het niet meer alleen van belang is in de strijd tegen parasitaire onkruiden, maar ook voor een beter begrip van de plantontwikkeling wat mogelijk kan leiden tot verbetering van landbouwgewassen voor verhoogde opbrengst.

In **Hoofdstuk 2** bediscussieer ik de huidige kennis van de levenscyclus van de parasitaire planten en van de ecologische betekenis van strigolactonen als signaalstoffen voor de communicatie met symbiotische AM schimmels en parasitaire planten. Vervolgens bespreek ik de huidige strategieën om parasitaire planten te bestrijden en stel ik nieuwe strategieën voor die gebaseerd zijn op de kennis over strigolactonen.

In de **Hoofdstukken 3 en 4** doe ik verslag van onderzoek naar de biosynthese van strigolactonen en naar de regulatie van de vorming van strigolacton structuur varianten en de impact op plant-parasitisme en AM symbiose. Voor deze onderzoeken gebruikte ik rijst en de parasitaire plant *Striga hermonthica* als onderzoeksmodellen. Strigolactonen vormen een grote groep verbindingen met een gemeenschappelijke kernstructuur van twee (A-B) ringen, een lacton ring (C) en een butenolide D-ring, maar die verder verschillen in hun stereochemische configuratie en in hun functionele groepen aan de A-B ringen. Planten produceren een mix van verschillende strigolactonen en die mix verschilt tussen plantensoorten en soms zelfs tussen cultivars. Rijst, bijvoorbeeld, produceert de strigolactonen, orobanchol, *ent-2'-epi-5-deoxystrigol*, *7-oxoorobanchol* en orobanchyl acetaat.

In dit proefschrift heb ik rijstexudaat gefractioneerd om nieuwe kiemingstimulerende stoffen op te sporen en nauwkeurig het effect van dit exudaat te bepalen op kieming van *Striga hermonthica* en de hyfevertakking van AM schimmels (**Hoofdstuk 3**). Ik heb sterke kiemingstimulerende fracties gevonden die waarschijnlijk nog onbekende strigolactonen bevatten, of biosynthetisch verwante moleculen. Verder heb ik aangetoond dat sommige van de rijst exudaat fracties die sterk de kieming stimuleren, weinig bijdragen aan de hyfevertakking van AM schimmels. Deze resultaten suggereren dat het mogelijk is cultivars te selecteren met verschillende mengsels van strigolactonen als strategie om parasitaire planten besmetting te verminderen zonder het aantrekken van mycorrhiza te beïnvloeden, zoals ik ook heb geopperd in Hoofdstuk 2.

In **Hoofdstuk 4** analyseer ik de genetische variatie in strigolacton-biosynthese in rijst met behulp van een RIL populatie, afkomstig uit een kruising tussen de rijst cultivars Bala en Azucena, die verschillen in *Striga hermonthica* gevoeligheid. In deze studie heb ik een QTL geïdentificeerd met de genen *SLB1* en *SLB2* - strigolacton biosynthese genen, ortholoog met Arabidopsis *MAX1* – die niet aanwezig zijn in het allel geassocieerd met lage strigolacton productie. Dit polymorfisme is sterk verbonden met de *indica/japonica* scheiding. Daarnaast is deze variatie geassocieerd met strigolacton biosynthese en gerelateerde kenmerken in alle geteste rassen die behoren tot de '*Rice Diversity Panel*'. Deze kennis is van groot belang voor de ontwikkeling van nieuwe rijstsoorten met meer halmen en/of verhoogde tolerantie voor parasitaire planten.

Tot slot onderzoek ik of de aan strigolactonen verwante verbinding d'orenon een effect heeft op de strigolactonbiosynthese (**Hoofdstuk 5**). D'orenon wordt *in vitro* geproduceerd door splitsing van β -caroteen door enzymen die ook betrokken zijn bij strigolacton biosynthese (Carotenoid Cleavage Dioxygenases 7 en 8). Eerder onderzoek heeft aangetoond dat d'orenon bij toediening aan planten fysiologische reacties initieert: het verandert bijvoorbeeld de lokalisatie en de dichtheid van de auxine transporter PIN2, blokkeert haar wortel strekking, en vermindert de gravitropische reactie van de wortelmeristemen. D'orenon is tot nu toe echter nog nooit *in planta* gedetecteerd. In dit proefschrift laat ik zien dat d'orenon ook van invloed is op de primaire wortel strekking en laterale worteldichtheid. Strigolactonen en d'orenon hebben een tegengesteld effect op de strekking van wortelharen en de primaire wortel en op de laterale worteldichtheid. Ook het effect van d'orenon op de expressie van strigolacton biosynthese genen suggereert dat d'orenon de strigolactonbiosynthese kan *down*-reguleren. Ik heb echter ook waargenomen dat de fysiologische reactie op d'orenon onafhankelijk is van de strigolacton signaalroute. De gegevens in dit hoofdstuk suggereren dat d'orenon, of een *downstream* afgeleide van d'orenon, een van de carotenoïden afgeleide signaalstof voor plantontwikkeling zou kunnen zijn. Ik beschrijf een model over hoe d'orenon toediening aan wortels van invloed kan zijn op de auxinehuishouding en de ontwikkeling van het wortelmeristeem.

Met dit proefschrift hoop ik een bijdrage te hebben geleverd aan het begrip van de biologie en de biosynthese van strigolactonen, en van de mogelijke betrokkenheid van strigolacton verwante verbindingen in de ontwikkeling van planten. Verder heb ik waardevolle genetische informatie gevonden die gebruikt kan worden voor de ontwikkeling van rijstcultivars met lagere strigolacton productie en nieuwe oplossingen gesuggereerd in de strijd tegen parasitaire onkruiden.



Curriculum Vitae

Catarina Sofia de Moura Luis Cardoso was born on 9 September 1979 in Lisbon, Portugal. She graduated as an Agronomic Engineer at the *Instituto Superior de Agronomia, Universidade Técnica de Lisboa* in Lisbon, with specialization in plant breeding, molecular biology and plant physiology. The final research project for her licentiate was carried out at the Laboratory of Molecular Biology of Wageningen University, supported by an Erasmus mobility grant. In this project Catarina studied heterochromatin formation during cell differentiation in the *Arabidopsis* primary root meristem, and the role of the then newly discovered RNA Polymerase IV in directing RNA mediated DNA methylation and heterochromatin formation. After completing her degree in 2006, Catarina joined the Laboratory of Plant Physiology, as a research assistant at the group of Prof. Harro Bouwmeester. In the same year, she was accepted as a Ph.D student in the same group to study the biosynthetic pathway and the regulation of strigolactone biosynthesis under the supervision of Prof. Harro Bouwmeester and Dr. Carolien Ruyter-Spira of which the results are described in this thesis.

In 2010, Catarina started working at Cropdesign, a BASF Plant Science Company in Zwijnaarde, Belgium as Senior Scientist. Here she has worked in a large project aimed at selecting genes to be transferred to crop species for yield improvement. Catarina provided scientific expertise and coordinated teams to set-up phenotypic screens for gene selection. In parallel Catarina used data from several high-throughput phenotypic screens to select genes for further testing in development pipelines.

During her Ph.D and work in industry, Catarina has developed interest in studying plant development and nutrient recycling as means to improve crop yields and sustainability. From November 1st of 2014, she will start working as a post-doctoral researcher at the Laboratory of Dr. Peter Bozhkov of the Swedish University of Agricultural Sciences in Uppsala (Sweden) to study the regulation of plant cell autophagy.

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Bookchapter

- C Ruyter-Spira, J A López-Ráez, **C Cardoso**, T Charnikhova, R Matusova, W Kohlen, M Jamil, R Bours, F Verstappen and H Bouwmeester. 2013 Strigolactones: a cry for help results in fatal attraction. Is Escape Possible? In: *Isoprenoid Synthesis in Plants and Microorganisms: New Concepts and Experimental Approaches*, Springer, pp 199-211.

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During my Ph.D many people helped me in many different ways with supervision, scientific discussions, advices, collaborations and no less important with friendship, support, motivation or simply creating great fun moments to get my mind away from work. So, this section is to you all that in a way or another contributed to make my Ph.D a better and happier journey. Although many people (including myself) have seen this part of the thesis to some extent as a *cliché*, as I'm writing it, I feel very happy for this opportunity to express my gratitude.

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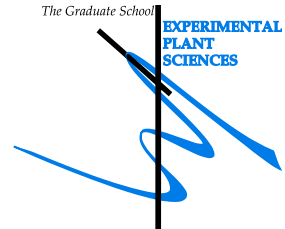
Acknowledgements

“*de beste Amsterdamse roti*” especially for us. **Ingrid** and **Tim**, it was great visting you and it’s a shame that we can’t meet as often as we would like. On the other hand, thanks for giving us a sweet nephew with a Californian accent with whom I can play!

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Education Statement of the Graduate School Experimental Plant Sciences



Issued to: Catarina Sofia de Moura Luis Cardoso

Date: 10 October 2014

Group: Plant Physiology, Wageningen University & Research Centre

1) Start-up phase	date
<ul style="list-style-type: none"> First presentation of your project PRI-Bioscience, Wageningen University 	Apr 04, 2007
<ul style="list-style-type: none"> Writing or rewriting a project proposal The biosynthesis of strigolactones, its role and regulation in the symbiosis with the arbuscular mycorrhizal fungi. 	Nov 2007
<ul style="list-style-type: none"> Writing a review or book chapter Strigolactones and root infestation by plant-parasitic Striga, Orobanche and Phelipanche spp., Plant Sci 2011 Mar; 180 (3): pp 414-420. 	Nov 2010
<ul style="list-style-type: none"> MSc courses Laboratory use of isotopes 	
<i>Subtotal Start-up Phase</i>	13.5 credits*

2) Scientific Exposure	date
<ul style="list-style-type: none"> EPS PhD Student Days EPS PhD student day, Wageningen University EPS PhD student day, Wageningen University EPS PhD student day, Leiden University 	Sep 19, 2006 Sep 13, 2007 Feb 26, 2009
<ul style="list-style-type: none"> EPS Theme Symposia EPS theme 3 symposium 'Metabolism and Adaptation', Wageningen University EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', Utrecht University EPS theme 1 symposium 'Developmental Biology of Plants', Leiden University EPS theme 3 symposium 'Metabolism and Adaptation', University of Amsterdam 	Nov 06, 2007 Jan 22, 2009 Jan 30, 2009 Feb 18, 2009
<ul style="list-style-type: none"> NWO Lunteren days and other National Platforms NWO-ALW meeting 'Experimental Plant Sciences', Lunteren, NL NWO-ALW meeting 'Experimental Plant Sciences', Lunteren, NL NWO-ALW meeting 'Experimental Plant Sciences', Lunteren, NL 	Apr 2-3, 2007 Apr 7-8, 2008 Apr 6-7, 2009
<ul style="list-style-type: none"> Seminars (series), workshops and symposia Flying seminar Rob Martienssen 16th Symposium ALW-Discussion Group - "Secondary Metabolism in Plant and Plant Cell", Leiden University Invited seminar Ineke Braakman Invited seminar Guillaume Bécard Flying seminar Hiroo Fukuda Invited seminar Jaakko Kangasjärvi Flying seminar Zhenbiao Yang Invited seminar Jian Kang Zhu Invited seminar Sjef Smeekens 	Oct 23, 2006 Oct 6, 2006 Feb 19, 2007 Sep 17, 2007 Nov 26, 2007 Mar 13, 2008 Jun 23, 2008 Nov 3, 2008 Nov 27, 2008

Education statement

Invited seminar Jan de Ruijter	Mar 09, 2009
Invited seminar Ottoline Leyser	Sep 18, 2009
WEES seminar Toby Kiers	Jun 17, 2010
Invited seminar Adam Prize	Sep 17, 2010
• Seminar plus	
Discussion with Adam Prize	Sep 17, 2010
Discussion with Ottoline Leyser	Sep 18, 2009
• International symposia and congresses	
5th International Conference in Mycorrhiza (ICOM 5), Spain	Jul 23-27, 2006
2nd International Plant Phenotyping Symposium, Forschungszentrum Jülich	Sep 05-07, 2011
• Presentations	
Oral: EPS theme 3 symposium 'Metabolism and Adaptation', Wageningen University	Nov 06, 2007
Oral: Laboratory of Genetics, Wageningen University	Aug 30, 2010
• IAB interview	
Meeting with a member of the International Advisory Board of EPS	Dec 05, 2008
• Excursions	

Subtotal Scientific Exposure 9.8 credits*

3) In-Depth Studies	date
• EPS courses or other PhD courses	
System Biology:statistical analysis of ~omics data'	Dec 11-14, 2006
EPS Summerschool on Arabidopsis Environmental Signaling	Aug 27-29, 2007
EPS Summerschool on Arabidopsis Environmental Signaling	Aug 24-26, 2009
• Journal club	
participant in a literature discussion group at Plant Physiology	2008-2009
• Individual research training	

Subtotal In-Depth Studies 4.5 credits*

4) Personal development	date
• Skill training courses	
WGS Workshop: Scientific Publishing	Nov 05, 2008
Introductory Biostatistics for researchers (UMC, Utrecht)	Jun-Jul 2010
Expectations Career Day	Nov 19, 2010
Course of Soft Skills	Feb 06-08 2011
Time management course	Sep 03, 2012
• Organisation of PhD students day, course or conference	
• Membership of Board, Committee or PhD council	

Subtotal Personal Development 4.8 credits*

TOTAL NUMBER OF CREDIT POINTS*	32.6
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits	
* A credit represents a normative study load of 28 hours of study.	



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