Elucidation of strigolactone biosynthesis in the host plant rice and the signal perception in the parasitic plant *Striga hermonthica*

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To my beloved parents and husband

献给我的父母和爱人

Genius is one percent inspiration, ninety-nine percent perspiration.

-by Thomas Edison

Chapter 1

General Introduction

During the evolution of angiosperms, the ability of plants to parasitize other plant species emerged multiple times. Parasitic plants are able to invade and attach to the shoots or roots of other plant species through organs called haustoria through which the parasite takes up water and nutrients from its host (Westwood et al., 2010). *Striga* spp. (for example *Striga hermonthica*, *Striga aspera* and *Striga asiatica*), that belong to the Orobanchaceae, are obligate root parasites. Their seeds will remain ungerminated in the soil until they sense specific compounds that are secreted by the roots of their hosts. Only then the seeds will germinate and their radicles grow out and penetrate the host root from which they will take water and nutrients to sustain their own development (Westwood et al., 2010) (**Figure 1**). *Striga* species mainly infect monocot plants, such as maize (*Zea mays*), sorghum (*Sorghum bicolor*), rice (*Oryza sativa*) and millet, but *Striga gesnerioides* parasitizes legumes, particularly cowpea (Mohamed et al., 2001; Westwood et al., 2010). The combination of *Striga*'s unique life cycle and the increasing use of agricultural monoculture has caused severe crop yield losses of up to 90%, especially in Africa, India and Southeast Asia (van Ast et al., 2005; Scholes and Press, 2008).

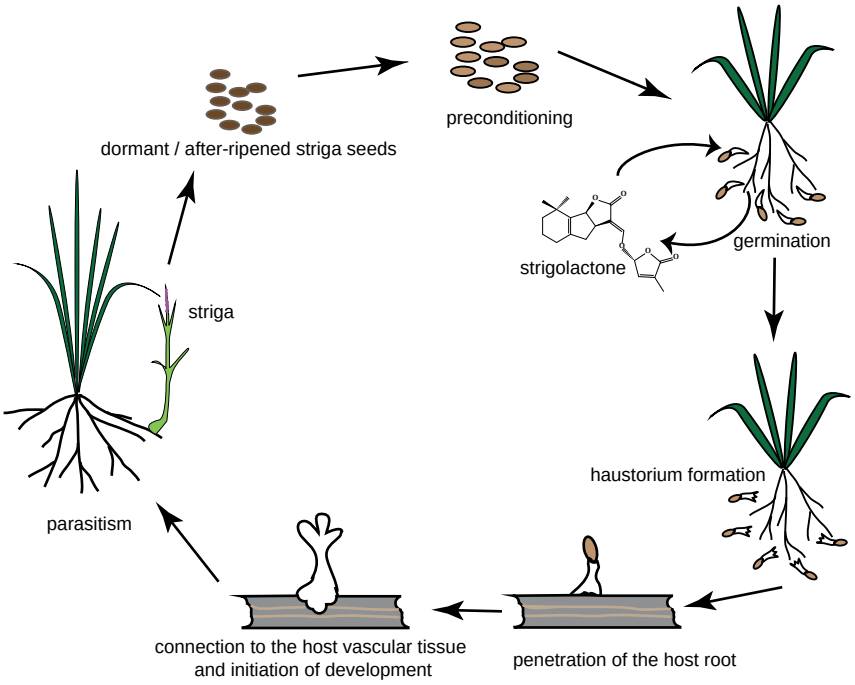

Figure 1. The life cycle of *Striga* spp.

One group of well-studied host derived signals that trigger *Striga* seed germination consists of host root secreted signalling molecules called strigolactones (SLs). In the

1960s, the first SL was isolated from root exudate of the false host plant cotton (*Gossypium hirsutum*) triggering *Striga lutea Lour.* seed germination, and was named strigol (Cook et al., 1966). Its absolute chemical structure was characterized afterwards (Cook et al., 1966; Cook et al., 1972; Brooks et al., 1985) (**Figure 2**, compound 1). Later on, strigol was also isolated from maize and proso millet, both true hosts of *Striga* (Siame et al., 1993). During this time, more strigol-like naturally occurring SLs, such as sorgolactone and alectrol (later renamed to orobanchyl acetate), were identified from several different host plant species (Hauck et al., 1992; Muller et al., 1992) (**Figure 2**). In 1995, Butler coined the name "strigolactone" for this class of strigol-like chemical cues.

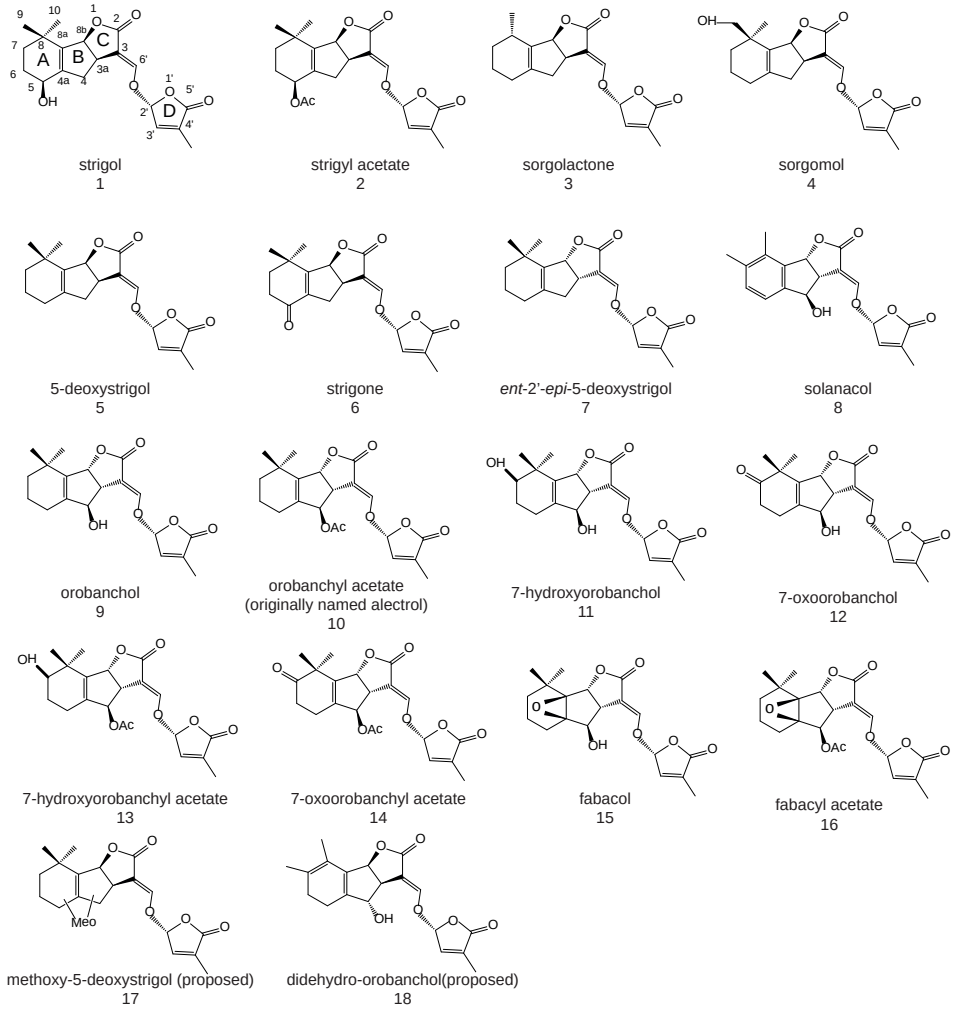

Figure 2. Chemical structures of some of the naturally occurring SLs.

The interaction of the parasitic plant with their hosts (mainly crops) makes it economically and biologically important to study both SL biosynthesis in the host plant and SL signal perception in the root parasite. This thesis is addressing both aspects in *Striga hermonthica* and its host *Oryza sativa*.

More biological activities of strigolactones

In addition to the "negative" role that SLs have in stimulating root parasitic plant seed germination, more recently SLs were also found to induce hyphal branching of arbuscular mycorrhizal (AM) fungi, which is beneficial for the host (Akiyama et al., 2005) (**Figure 3**). AM fungi establish obligate symbiotic interactions with the vast majority of land plants (Parniske, 2008). This symbiosis is able to improve the plants' nutrient (e.g. phosphorus) and water acquisition from a larger soil volume and hence protects plants against abiotic stress but has also been suggested to protect the plant from biotic stresses (Smith et al., 2010). To assure a symbiotic association with plants, hyphal branching during fungal spore germination is a crucial step prior to host plant colonization (Harris, 2008). Although it is still poorly understood how SLs stimulate AM fungal hyphal branching, Besserer *et al.* (2006) reported that the synthetic SL analogue GR24 rapidly induced a fungal mitochondrial response (Besserer et al., 2006).

AM fungi are able to form tripartite symbiosis with *Rhizobium* spp. and leguminous plants (de Varennes and Goss, 2007). Foo et al. showed that the SL deficient pea mutant (*rms1*/*ccd8*) but not the signalling mutant (*rms4*/*max2*) formed less nodules than wild-type pea after inoculation with *Rhizobium*, and suggested that SLs promote nodule formation independently of the auto-regulation of the nodulation pathway (Foo and Davies, 2011; Reid et al., 2011; Foo et al., 2013; Foo et al., 2014). Taken together, considering the role of SLs in triggering AM fungi hyphal branching, it may imply that SLs are playing a role in establishment of the AM-*Rhizobium*-legume tripartite symbiosis.

Another intriguing feature of SL bioactivity is their capacity to inhibit plant shoot branching/tillering as a phytohormone (Gomez-Roldan et al., 2008; Umehara et al., 2008). It was shown that the production of the SL *epi*-5-deoxystrigol was abolished in the high tillering/branching rice and pea *ccd7* and *ccd8* mutants, mutants of *CAROTENOID CLEAVAGE DIOXYGENASE* (*CCD*) 7 and 8 (*d17* and *d10* in rice; *rms1* in pea), while application of the synthetic SL GR24 restored the branching phenotype of biosynthetic mutants *max3-11* (*ccd7*) and *max4-7* (*ccd8*) but not the signalling mutant *max2* (mutant of *MORE AXILLARY GROWTH 2 (MAX2))* in *Arabidopsis* (Gomez-Roldan et al., 2008; Umehara et al., 2008). These studies provide the first evidence for the identity (SLs) of the many times reported elusive mobile signal involved in the inhibition of shoot branching in *Arabidopsis*, rice and pea (Beveridge et al., 2000; Foo et al., 2001; Stirnberg

et al., 2002; Sorefan et al., 2003; Booker et al., 2004; Zou et al., 2006; Arite et al., 2007). Later, it was demonstrated that SLs also affect other shoot architectural aspects. They stimulate cambium activity in the stem, causing the SL-deficient mutants to display reduced secondary growth compared with wild-type plants (Agusti et al., 2011). The identification of these molecules as phytohormones is a milestone for SL research, and brought broader significance to the study of its biosynthesis, perception, signalling and potential other physiological roles of this class of molecules in higher plants.

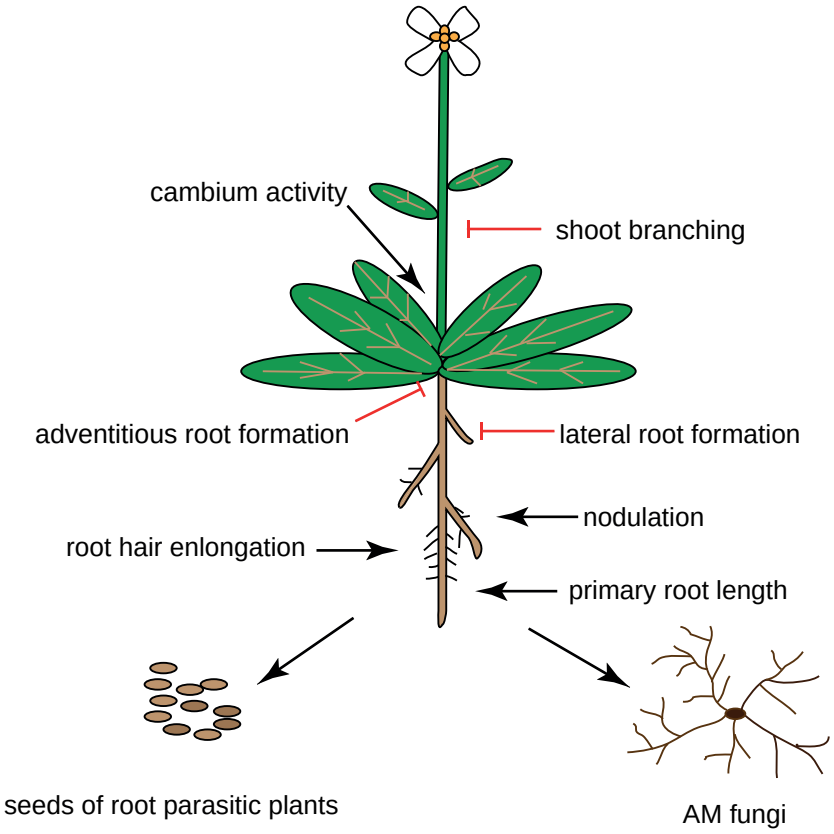

Figure 3. The multiple roles of SLs in the rhizosphere and as phytohormones. Black arrow represents a stimulatory role; red line represents repressive role.

SLs have also been demonstrated to play a role in the regulation of root architecture (Kapulnik et al., 2011b; Ruyter-Spira et al., 2011; Rasmussen et al., 2012). Under optimal nutrient conditions (sufficient phosphate, P), the SL-deficient and signalling mutants have more lateral and adventitious roots when compared with wild-type plants, and application of GR24 represses later root formation in a MAX2 dependent manner (Kapulnik et al., 2011b; Ruyter-Spira et al., 2011; Rasmussen et al., 2012). Under

these conditions GR24 also stimulates primary root growth, which is accompanied by an increase in size and cell number in the meristem and transition zone (Ruyter-Spira et al., 2011). Application of GR24 also increases root hair elongation in *Arabidopsis* wild-type and SL-deficient mutants (*max1*and *max4*), but not the signalling mutant *max2* (Kapulnik et al., 2011b).

Interestingly, the role of SLs in regulating root architecture is affected by nutrient availability. While SL deficient mutants have more lateral roots under control conditions, P starvation stimulates lateral root outgrowth to a higher extent in wild-type than in these mutants (Ruyter-Spira et al., 2011). Umehara et al. (2010) showed that in wild-type rice seedlings bud outgrowth was fully inhibited when P was omitted from the medium but not in SL mutants (Umehara et al., 2010). In line with this, it was shown that P-limiting conditions increase SL levels in roots and root exudates (Yoneyama et al., 2007b; Yoneyama et al., 2007a; Lopez-Raez et al., 2008; Umehara et al., 2010). Taken together, the adaptive architectural aspects that are beneficial during P starvation and the fact that SLs stimulate AM fungal symbiosis suggest that SLs are the mediators for plants to respond to low P conditions (Mayzlish-Gati et al., 2012).

Besides triggering parasitic plant seed germination as rhizosphere signalling compounds, exogenous GR24 application was also demonstrated to promote germination of *Arabidopsis* primary dormant and thermo-inhibited seeds in a MAX2 dependent manner (Nelson et al., 2011; Toh et al., 2012). Under controlled conditions, only the seeds of the signalling mutant *max2* show a more dormant germination phenotype (under continuous white light) and longer hypocotyl length (under continuous red light) but not the wild-type and SL-biosynthetic mutants (Nelson et al., 2011). Consistently, Shen et al. (2012) showed that SL-deficient mutants do not display any defects in germination and hypocotyl phenotypes under increasing fluence rates of red and far-red light (Shen et al., 2012). However, Tsuchiya et al. (2010) demonstrated that, upon far-red followed by red light pulses, both the SL-deficient mutant *max1* and signalling mutant *max2* exhibit reduced germination rates compared with wild-type seeds (Tsuchiya et al., 2010).

Although it is not conclusive whether or not the endogenous SLs are regulating *Arabidopsis* seed germination, it has been shown that GR24 application to thermo-inhibited *Arabidopsis* seeds affects the ratio of the seed germination hormones abscisic acid (ABA) and gibberellin (GA) by decreasing the levels of ABA and increasing the levels of GA (Toh et al., 2012).

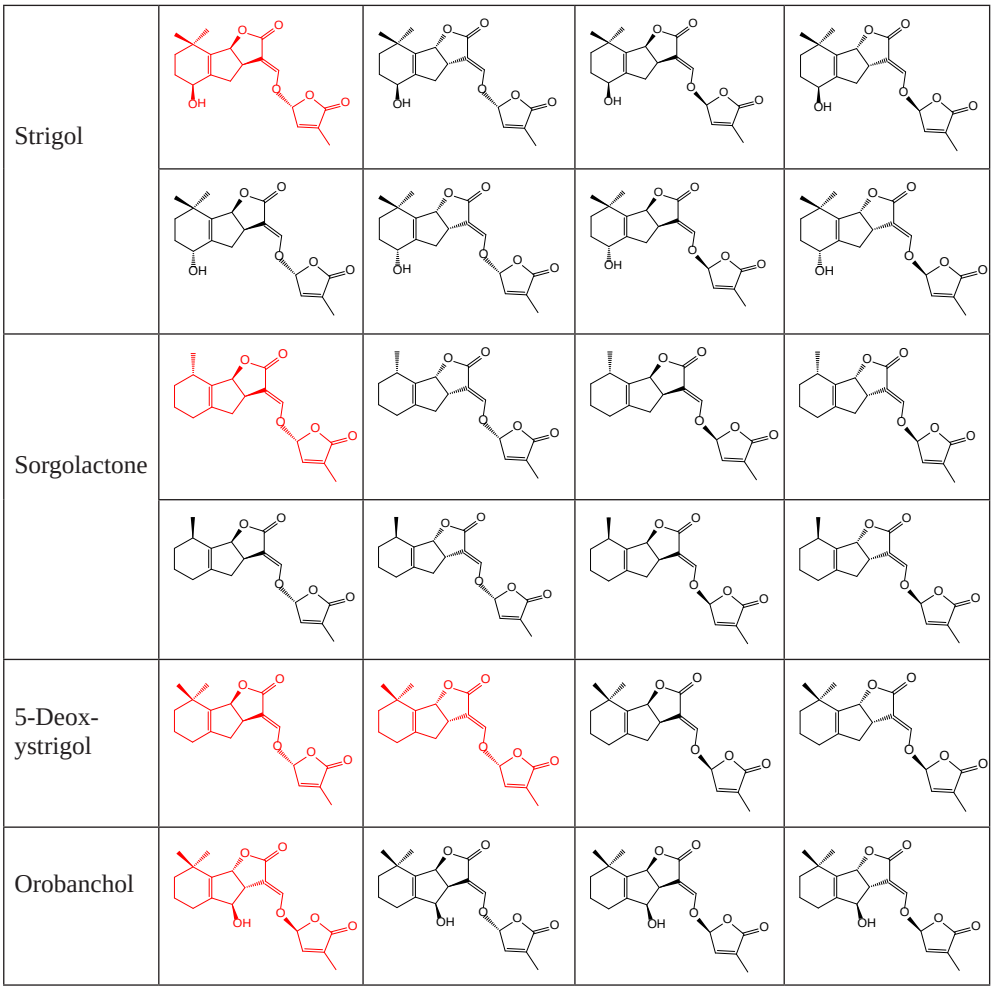

Figure 4. Some of the possible stereoisomers of the SLs strigol, sorgolactone, 5-deoxystrigol and orobanchol. The naturally occurring stereoisomers are indicated in red.

Similarly, inhibition of shoot branching and regulation of root architecture are also associated with the interaction and crosstalk of SLs with other plant hormones. For instance, it has been described that SLs regulate shoot branching by modulating polar auxin transport (Crawford et al., 2010; Shinohara et al., 2013). In the case of root development, SLs have been suggested to interact with auxin to regulate primary root length, root hair elongation and lateral root formation (Kapulnik et al., 2011a; Ruyter-Spira et al., 2011). Kapulnik *et al.* (2011) also suggested that SLs affect root hair elongation through ethylene synthesis (Kapulnik et al., 2011a). More aspects of hormonal cross-talk between SL and other phytohormones in regulating plant developmental processes are addressed in detail in **Chapter 2**.

Structural diversification of strigolactones and its biological relevance

SLs belong to a class of compounds with already quite extended structural diversity. In the past decades, more than 20 different SL structures have been discovered and (tentatively) structurally identified (Xie and Yoneyama, 2010; Liu et al., 2011; Jamil et al., 2012; Kisugi et al., 2013) (**Figure 2**). All characterized naturally occurring SLs consistently contain a four-ring skeleton: a tricyclic ring structure (ABC-part) and a D-ring connected to the C-ring via an enol ether bond. Different SLs may have different methyl-, hydroxy- and acetate substituents on their A and B rings. In addition, stereochemistry is another important feature for SLs that contain two stereo-centres, the C-3a/C-8b position and the C-2' position, that determine the orientation of the C-ring and the D-ring, respectively (**Figure 2**, compound 1). Accordingly, naturally occurring SLs have been classified into two groups that are called strigol- and orobanchol-type, with β -orientated and α -orientated C-ring, respectively (**Figure 2**) (Xie et al., 2013; Zwanenburg and Pospisil, 2013). Both groups share the same stereochemistry at the C-2' position (D-ring orientation).

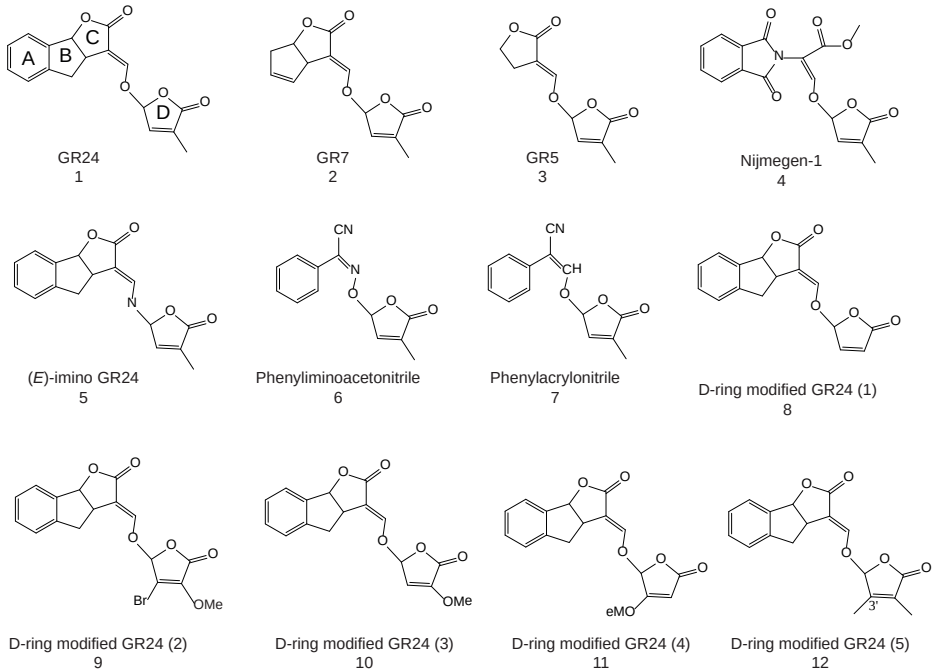

Figure 5. Chemical structures of some SL analogues.

The bioactivity of the SLs is significantly impacted by their stereochemistry. For instance, among all the possible stereoisomers of strigol and sorgolactone, which were

generated through chemical synthesis, the naturally occurring strigol and sorgolactone isomers had the highest activity in stimulating seed germination of Striga hermonthica (Sugimoto et al., 1998; Reizelman et al., 2000; Zwanenburg and Pospisil, 2013) (**Figure 4**). Similarly, the naturally occurring SLs 5-deoxystrigol (5DS), *ent*-2'-*epi*-5-deoxystrigol (*ent*-2'-*epi*-5DS) and orobanchol are more active than any of the other stereoisomers in inducing AM fungi hyphal branching (Akiyama et al., 2010) (**Figure 4**). This may suggest the C-2´stereochemistry of the natural SLs is evolutionarily selected for their bioactivity in the rhizosphere.

Structure-Activity Relationships (SAR) have been extensively studied for synthetic SL analogues that are structurally similar to naturally occurring SLs, such as GR5, GR7, GR24 and Nijmegen-1 (**Figure 5**) (Mangnus et al., 1992; Mangnus and Zwanenburg, 1992b; Nefkens et al., 1997). Among the tested synthetic SL analogues, GR24, having an aromatic A-ring, is the most widely used synthetic SL analogue with a relatively high parasitic plant seed germination stimulatory capacity (Mangnus and Zwanenburg, 1992b; Wigchert et al., 1999). It has been shown that the A- and-B rings are less important for germination stimulatory activity since GR7 (without the A-ring) and GR5 (without the A-B rings) still had high activity in inducing both *S. hermonthica* and *Orobanche crenata* seed germination in the concentration range of 10^{-7} to 10^{-6} M. When the ABC part without D-ring, or the D-ring without ABC part were tested, neither of them showed biological activity, suggesting that the C-D moiety is required for parasitic plant seed germination stimulatory activity (Zwanenburg et al., 2009). However, imino and phenylacrylonitrile analogues of GR24 also showed moderate germination stimulatory activity toward the seeds of *S. hermonthica* (Kondo et al., 2007) (**Figure 5**, compounds 6, 7), leaving the requirement of the enol ether bridge of the C-D moiety for SL activity in stimulating seed germination still undetermined. It is worth mentioning that Mangnus et al. (1992) showed that the methyl group at the C-4´ position of the D-ring is also important for the bioactivity in parasitic plant seed germination (Mangnus and Zwanenburg, 1992a).

Besides studies on the structural requirements of SLs with respect to root parasitic plant seed germination, SAR studies have also been performed for AM fungal hyphal branching stimulatory activity. Compared with GR24, the A-ring or AB-ring truncated SL analogues GR7 or GR5 displayed a dramatic reduction (GR7) or loss of function (GR5) in hyphal branching activity, suggesting that the C-D part connecting with at least one ring (A-ring or AB-ring) system is necessary for AM fungal hyphal branching activity (Akiyama et al., 2010). The GR24 imino analogue (in which the enol ether is replaced by a carbon-nitrogen double bond) showed only a10-fold reduction in hyphal branching stimulatory activity compared with GR24 (**Figure 5**, compound 5) (Akiyama et al., 2010). Taken together, the AM fungus hyphal branching stimulatory activity of

SLs also behaves in a structure-dependent manner, but the enol ether bridge in the C-D ring moiety is not essential for this activity (Akiyama et al., 2010).

To gain more insight in the SAR of SLs regarding their role as phytohormones, Boyer *et al.* (2012) used pea in a shoot branching inhibition assay. Results from this study revealed that acetate-SLs (strigyl acetate, orobanchyl acetate and solanacyl acetate) are more active in inhibiting shoot branching than their corresponding hydroxy-SLs (strigol, orobanchol and solanacol) (Boyer et al., 2012). It has been suggested that the oxygen on the A or B rings may compromise the branching inhibitory activity as 5DS and sorgolactone had a stronger effect than other natural SLs such as orobanchol, strigol and solanacol (Boyer et al., 2012) (**Figure 2**). Moreover, the stereochemistry at the C-2´ position had little effect on hormonal bioactivity (Boyer et al., 2012). Similar as for germination stimulatory activity, GR5, lacking the A-B rings, was as active as a branching inhibitor as the most active natural SL in the same concentration, suggesting that the A-B part of the SLs is also not essential for the branching inhibitory activity (Boyer et al., 2012). Notably, it has been reported that the stereochemistry at the D-ring (C-2´) and the C-ring (C3a/C8b) of solanacol and its acetate are not affecting bud outgrowth inhibitory activity (Boyer et al., 2012). Similarly, Chen *et al.* (2013) also reported that in contrast to the effect on stimulation of seed germination and AM fungal hyphal branching, the bud outgrowth inhibitory activity of SL analogues is not constrained by their absolute stereochemistry (Chen et al., 2013). In contrast, modifications to the D-ring resulted in strong effects on the branching inhibiting activity (Boyer et al., 2012). For instance D-ring modified GR24 analogues showed loss of activity (**Figure 5**, compounds 8-11), whereas a methyl group at the C-3' position is boosting up this activity (**Figure 5,** compound12).

Biosynthesis of strigolactones

SLs are carotenoid derived phytohormones (Matusova et al., 2005). As explained above, SLs consist of a four-ring skeleton; a tricyclic ring structure (ABC-part) and a D-ring connected via an enol ether bond (see **Figure 2**). Phenotypically, in many plant species, the mutants of *CAROTENOID CLEAVAGE DIOXYGENASE* 7 and 8 (*CCD7* and *CCD8*) show increased branching/tillering and reduced SL levels (Sorefan et al., 2003; Booker et al., 2004; Arite et al., 2007; Gomez-Roldan et al., 2008; Umehara et al., 2008). Plants carrying a mutation in the gene encoding an iron-containing protein *DWARF27* (*D27*) also display a branching/tillering phenotype (Lin et al., 2009; Waters et al., 2012a). Biochemically, CCD7 was demonstrated to cleave *all-trans*-β-carotene at the C9-10 position to obtain 10'-*apo*-β-carotenal *in vitro*. This product was subsequently cleave by CCD8 at the C13-14 position to form 13-*apo*-β-carotenone (Schwartz et al., 2004) (**Figure 6A**). However, this final product has never been detected *in planta*. Intriguingly, the characterization of the biochemical function of D27 changed the fate of CCD7 and

CCD8. Alder *et al.* (2012) showed that D27 is a carotenoid isomerase converting *all-trans*-β-carotene to 9-*cis*-β-carotene, which turned out to be the substrate for CCD7 that converts it to 9-*cis*-β-*apo*-10´-carotenal, which is in turn converted by CCD8 to a SL-like compound called carlactone (CL) (Alder et al., 2012) (**Figure 6B**). Although the D-ring is already present in CL, the B-C rings are not. Surprisingly, CL already has parasitic plant seed germination stimulatory activity, and is also able to inhibit branching/tillering and *Arabidopsis* hypocotyl growth (although with low activity). However, unlike GR24, CL did not stimulate primary dormant Arabidopsis seed germination (Alder et al., 2012; Scaffidi et al., 2013). Finally, CL was detected in both rice and Arabidopsis root extracts and demonstrated to be a SL precursor *in vivo* (Seto et al., 2014).

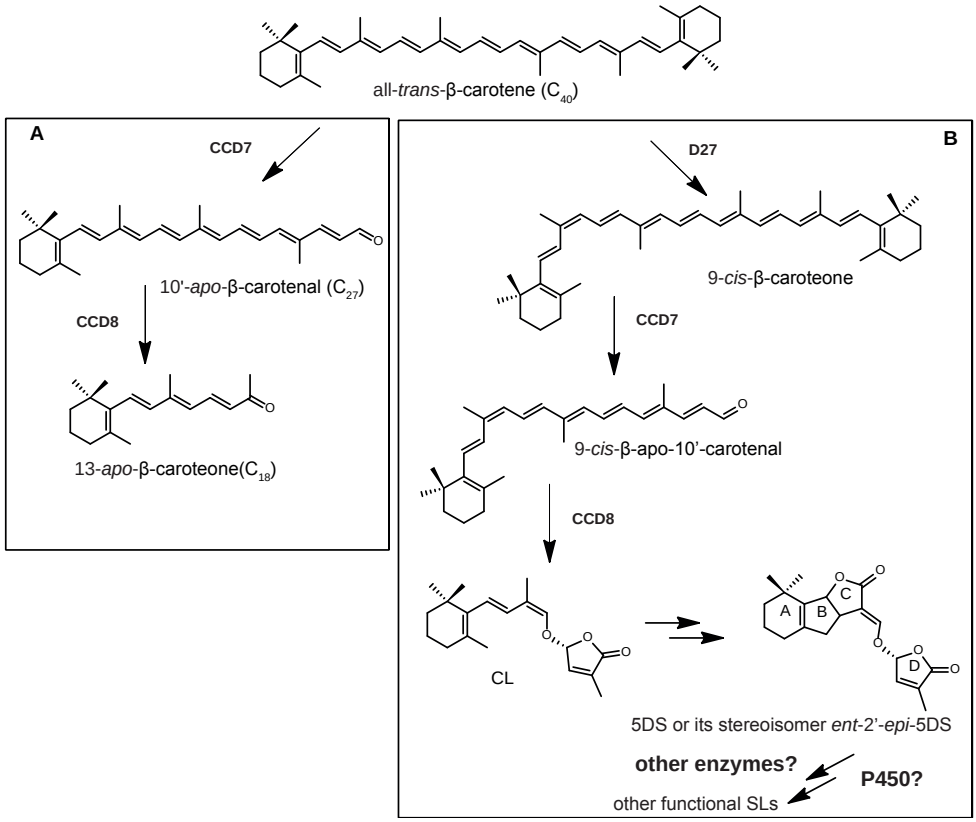

Figure 6. The proposed biosynthetic pathway of SLs. Solid arrows represent consolidated steps; dashed arrows represent hypothetical steps.

Arabidopsis plants mutated in *MORE AXILLARY GROWTH 1* (*MAX1*), encoding a cytochrome P450 family member, also display a bushy phenotype, suggesting that *MAX1* is also involved in the SL biosynthetic pathway (Stirnberg et al., 2002; Booker et al.,

2005). This was further confirmed by the fact that reduced SL levels were detected in the *Arabidopsis max1* mutant (Kohlen et al., 2011). Challis *et al.* (2013) proved that *MAX1* orthologs from different plant species were able to restore the branching phenotype of the *Arabidopsis max1* mutant (Challis et al., 2013). However, the enzymatic role of MAX1 in the SL biosynthetic pathway has not been characterized yet. It has been postulated that 5DS and its stereoisomer, *ent*-2'-*epi*-5DS , are the common precursors for all other natural SLs (Rani et al., 2008; Xie and Yoneyama, 2010; Ruyter-Spira et al., 2013). Therefore, the formation of various SLs requires B-C ring cyclisation of the precursor CL to form 5DS/*ent*-2'-*epi*-5DS. Decoration of these parent strigolactones on the A and B rings will subsequently yield all the currently known strigolactones (**Figure 2**). It was postulated that CL cyclisation is mediated by (a) cytochrome P450(s), possibly *MAX1* (Alder et al., 2012; Ruyter-Spira et al., 2013). Further conversion of 5DS/*ent*-2'-*epi*-5DS to other SLs may be mediated by enzymes such as (other) cytochrome P450s and acetyl transferases (**Figure 6B**).

SL downstream signalling

SLs are phytohormones and rhizosphere signalling compounds. The molecular mechanisms of SL perception and signalling in plant development have been partially unravelled. MORE AXILLARY GROWTH 2 (MAX2)/DWARF 3 (D3), an F-box protein that participates in an SCF^{MAX2} complex (including the core subunits ubiquitin-conjugating enzyme E2, Skp1, Cullin 1 and Rbx) has been shown to regulate the SL downstream signalling pathway(s) (**Figure 7**) (Stirnberg et al., 2002; Ishikawa et al., 2005; Stirnberg et al., 2007). The interaction of MAX2 with an α/β-hydrolase family member DWARF 14 (D14)/DECREASED APICAL DOMINANCE 2 (DAD2) was reported to be required for the SL response in inhibiting shoot branching (Arite et al., 2009; Hamiaux et al., 2012; Waters et al., 2012b). DWARF 53 (D53), a newly identified component involved in the SL mediated inhibition of rice tillering, is a repressor in the SL signalling cascade (Jiang et al., 2013; Zhou et al., 2013). It has been shown that in the presence of GR24, the interaction of D14 with D3 and D53 leads to D53 ubiquitination and subsequent degradation, which triggers expression of downstream targets, such as *TOPLESS* (*TPL*) and hence activates the SL signalling pathway (Jiang et al., 2013; Zhou et al., 2013) (**Figure 7A**). The rice DELLA protein SLENDER RICE1 (SLR1) has been shown to interact with D14 in the presence of GR24 and this interaction requires the binding of D-OH - the SL D-ring product resulting from the D14 hydrolysis activity- into the D14 cavity, supporting that the D-ring is important for SL signalling (Nakamura et al., 2013) (**Figure 7B**). Although this study did not show any involvement of MAX2/D3 in this SLR1-D14 interaction, the authors suggest that SLR1 might be a potential target for the D14-D3 complex for further unbiquitination and proteasomal

degradation (Nakamura et al., 2013). *Arabidopsis* BRI1-EMS-SUPPRESSOR1 (BES1) is also described to interact with MAX2 *in vitro* and *in vivo*, and GR24 can promote the MAX2-mediated BES1 degradation (Wang et al., 2013) (**Figure 7C**). SLR1 and BES1 are key regulators of the GA and brassinosteroid pathways, respectively, suggesting that SLs may interact with these pathways to regulate shoot branching (Yin et al., 2002; Sun, 2010; Hirano et al., 2012).

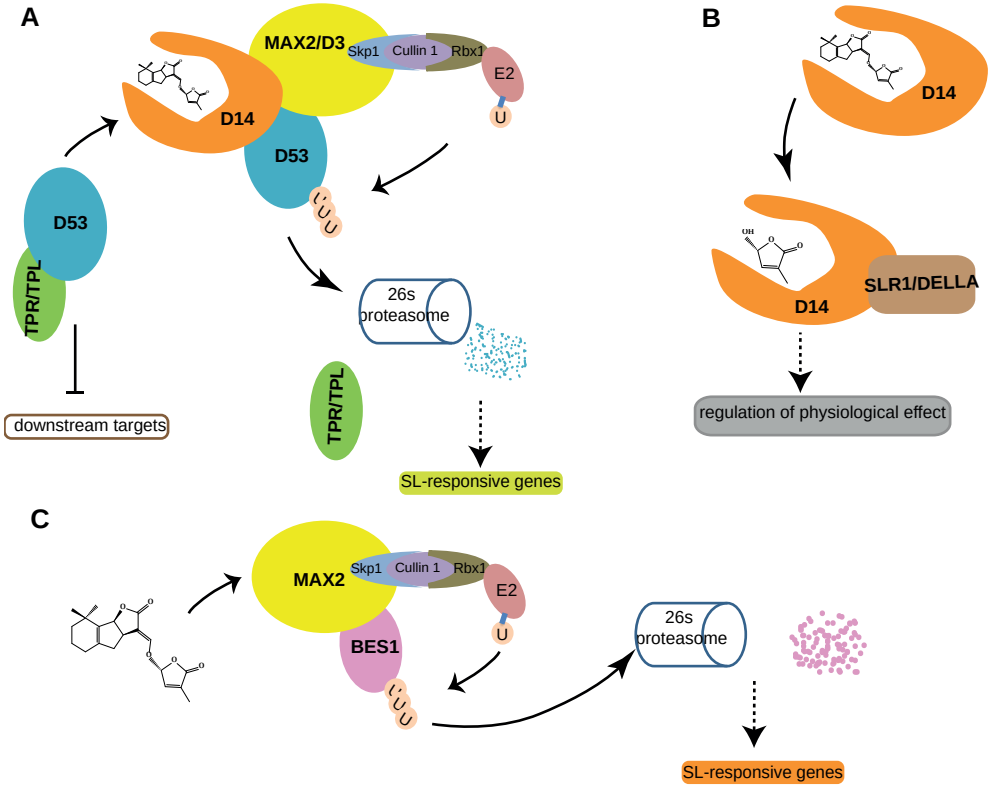

Figure 7. Putative mechanism of SL downstream signalling pathways.

However, the mechanism of SLs as rhizosphere signalling molecules involved in root parasitic plant seed germination and AM fungal colonization is still poorly understood. The SAR studies discussed above may shed light on which part(s) of the SL structure is playing a crucial role in the interaction/binding with the receptor(s) in these two biological systems (Yoneyama et al., 2009; Zwanenburg et al., 2009). It is known that parasitic plant seeds need a period of preconditioning before germination can be triggered by germination stimulants, such as SLs (Matusova et al., 2004; Joel et al., 2007). Plakhine *et al.* suggest that the seeds of root parasitic plants *Orobanche cumana*

and *Orobanche aegyptiaca* already become receptive before pre-conditioning (Plakhine et al., 2009). Joel *et al.* (2012) reported that in the root-parasitic plant *Phelipanche aegyptiaca* the putative SL receptors may be located in the perisperm cells underneath the micropylar opening (Joel et al., 2012). Application of GR24 to trigger *Striga hermonthica* seed germination led to an increase in cytokinin (CK) and GA levels, while it decreased the level of ABA (Toh et al., 2012). Consistent with this, Lechat *et al.* (2012) showed that the ABA level of *P. aegyptiaca* seeds decreased during preconditioning and GR24 application. In addition, expression of the ABA catabolic gene homologue in *P. aegyptiaca PrCYP707A1* strongly increased in cells close to the micropyle during GR24 induced germination (Lechat et al., 2012). Collectively, these results may suggest that similar to the observations in *Arabidopsis*, parasitic plant seed germination is a multi-hormonal regulated process, and accordingly SL perception may also be involved in the regulation of this hormonal cross-talk. Additional experimental evidence still needs to be provided to understand the unique feature that parasitic plant seeds exclusively germinate upon the perception of SLs, which discriminates them from other plant species.

Scope of this thesis

The aim of the research described in this thesis is to further elucidate the SL biosynthetic pathway in the host plant rice and the signal perception pathway in the root parasitic plant Striga hermonthica that parasitizes rice. After the general introduction in **Chapter 1**, **Chapter 2** reviews the current literature on strigolactone biosynthesis, perception and biology. As rhizosphere signalling molecules, the interactions mediated by SLs in the plant rhizosphere are introduced. Moreover, SLs are also participating in different plant developmental processes as plant hormones often in interaction with other plant hormones. **Chapter 3** describes a QTL mapping study for SL production in rice by using an Azucena×Bala F6 population for which the parents produce different levels of SLs. The objective of this chapter is to discover new genes that are involved in SL biosynthesis or its regulation. The genes underlying the identified QTL are orthologs of *Arabidopsis MAX1* that has been reported to be involved in the inhibition of shoot branching. **Chapter 4** subsequently describes the reconstitution of the SL biosynthetic pathway in Nicotinana benthamiana, an exciting and valuable tool to further biochemically verify the role of the OsMAX1s identified in the SL QTL mapping study in **Chapter 3** in SL biosynthesis. **Chapter 5** describes the characterization of an F-box protein MAX2 ortholog in the root parasitic plant *Striga hermonthica*, *ShMAX2*, which may shed light on SL signal perception during root parasitic plant seed germination. **Chapter 6** discusses several aspects of SLs biosynthesis and signalling and presents an in-depth discussion related to the findings of this thesis.

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Chapter 2

Strigolactone Biosynthesis and Biology

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Abstract

Strigolactones belong to a newly identified class of plant hormones that are involved in the inhibition of shoot branching. Prior to this finding, strigolactones were proven to be root rhizosphere signaling molecules that mediate plant-parasitic plant, and the symbiotic plant-AM fungi interactions. More recently, strigolactones were shown to have other biological functions as endogenous plant hormones in shoot development, root architecture, seed germination (also in non-parasitic plants) and to regulate plant developmental processes in interaction with other signaling pathways (i.e. light and senescence signaling) or hormones. Gene discovery in the strigolactone biosynthesis and signal perception pathways is a key step in elucidating the mechanism and mode of action of the existing roles and discovering potential additional roles of strigolactones. Furthermore, the insights in the strigolactone and strigolactone-associated pathways will provide more knowledge for the control of parasitic weeds and improvement of crop yield. In this chapter, we will outline different aspects of the roles that strigolactones play both in the rhizosphere and during plant development. Gene characterization in strigolactone pathways and strigolactone-related hormone cross-talk will also be addressed.

Introduction

The rhizosphere is the complex environment surrounding the roots of plants in which a diverse range of organisms interact with each other. Examples are plant-microbe (bacteria, fungi, oomycetes, and viruses), plant-insect, plant-nematode and plant-plant interactions. The fundamental insight in this interplay between plants and the rhizosphere have been extensively reviewed (Estabrook and Yoder, 1998; Buee et al., 2009; Lugtenberg and Kamilova, 2009; Kawaguchi and Minamisawa, 2010). Plants can benefit from some interactions, such as the symbiosis between plants and rhizobia or arbuscular mycorrhizal (AM) fungi, while other interactions, such as with parasitic plants or rhizosphere pathogens, are a source of biotic stress.

Plants or plant roots produce and release into the rhizosphere a multitude of metabolites including sugars, polysaccharides, amino acids, aliphatic acids, aromatic acids, fatty acids, sterols, enzymes, vitamins, phenolics and other secondary metabolites (Bertin et al., 2003; Steinkellner et al., 2007). The volatile plant hormones, ethylene, methyl jasmonate and methyl salicylate, play key roles in mediating plant communication as airborne signals aboveground (Arimura et al., 2002; Engelberth et al., 2004; Heil and Karban, 2010). Similarly, in the rhizosphere, it is likely that many root-derived compounds play roles in plant-plant, plant-microbe, and plant-insect chemical communication. For example, flavonoids have been shown to stimulate or inhibit rhizobial nod gene expression, cause chemo-attraction of rhizobia towards the root, inhibit root pathogens, stimulate mycorrhizal spore germination and hyphal branching, and mediate allelopathic interactions between plants (Cooper, 2004; Hassan and Mathesius, 2012).

Still, there are likely many more plant-derived molecules that await identification as signaling molecules and more biological functions of known signaling compounds to be discovered. The newly identified phytohormone strigolactone is one of the best examples of the discovery of new biological functions of known signaling compounds. Strigolactones are present in the root exudates of many plant species and were identified as seed germination stimulants for root parasitic plants of the Orobanchaceae such as *Striga, Orobanche, Alectra and Phelipanche* spp. decades ago (Cook et al., 1966). Only much later they were also shown to stimulate the symbiosis of plants with arbuscular mycorrhizal (AM) fungi by acting as hyphal branching factors (Akiyama et al., 2005). So far, a variety of different strigolactones have been isolated from a range of plant species and in most cases, one plant species produces more than one strigolactone (Xie et al., 2010).

In this chapter we will review the various roles that strigolactones play both in the rhizosphere and as endogenous plant hormone. In addition, we will present current

knowledge on the strigolactone biosynthetic and downstream signaling pathways and the interactions of strigolactones with other phytohormones.

Strigolactone Performance in the Rhizosphere

Parasitic Seed Germination Stimulants

Root parasitic plants are integral participants of the strigolactone story. Excellent reviews have focused on the biology, economic importance, and plant resistance approaches of the main root parasitic plant genera *Striga*, *Orobanche* and *Phelipanche* (Musselman, 1980; Rispail et al., 2007; Parker, 2009). Several species from these genera belong to the most damaging parasitic weed species worldwide. *Striga* spp. are a major pest in crops such as maize, sorghum, upland rice and millet throughout semi-arid Africa and parts of Asia, while *Orobanche* and *Phelipanche* spp. are problematic in legumes, tomato, tobacco, rapeseed, sunflower, etc. in southern and eastern Europe, the Middle East and North Africa (Rubiales, 2003). The nature of these parasites is the main reason why they cause significant economic losses in agriculture. They form a connection organ with the host plants' root system, called haustorium, through which they consume water, carbon and nutrients. This inevitably leads to a reduction in the host plant's growth, affects its assimilate partitioning and reproduction, and even disrupts the competitive balance between host and non-host species, leading to changes in community structure (Press and Phoenix, 2005).

The persistence of the parasites lies in their tiny seeds which can remain dormant and viable in the soil for over 10 years. They will only germinate upon the perception of specific seed germination stimulants that are released by the host roots (Bouwmeester et al., 2003; Hearne, 2009). This ensures that the parasite only germinates when a host plant is within reach. Extensive research work has been performed on characterization of these seed germination stimulants, which is of economical and scientific importance for the biology and management of parasitic weeds. In the 1960s and 1970s, strigol and strigyl acetate were discovered to be present in the root exudates of cotton, a non-host of *Striga* (Cook et al., 1966; Cook et al., 1972). More recent publications show that strigol also occurs in root exudates of several host species of *Striga* like maize, sorghum and proso millet, next to a closely related but even more active seed germination stimulating compound called sorgolactone (Hauck et al., 1992; Siame et al., 1993). Almost thirty years after the initial discovery of strigol, Butler (Butler, 1995) coined the name "strigolactones" for these strigol-related compounds. Besides strigol, strigyl acetate and sorgolactone, many other strigolactones have since then been isolated from the root exudates of a large variety of plants, all functioning – with varying activity - as *Striga* or *Orobanche*/*Phelipanche* seed germination stimulants. Alectrol, which recently

was proven to be (+)-orobanchyl acetate (Xie et al., 2008), was originally discovered in cowpea root exudates (Müller et al., 1992). Orobanchol, the first strigolactone isolated from a host of Orobanche (red clover) induces *Orobanche minor* seed germination (Yokota et al., 1998; Mori et al., 1999). Entering the 21st century, with the development of highly advanced quantitative and qualitative analytical methods, more novel natural strigolactones were identified from the root exudates of many different plant species (Akiyama et al., 2005; Awad et al., 2006; Xie et al., 2007; Matsuura et al., 2008; Xie et al., 2008; Xie et al., 2009b; Xie et al., 2009a; Yoneyama et al., 2010; Kohlen et al., 2011; Ueno et al., 2011a; Jamil et al., 2012) **(Table 1)**. Although in one plant species one or more major known strigolactones can be detected, evidence suggests that usually plant root exudates contain strigolactone mixtures including so far unidentified ones (Cardoso et al., 2011).

In addition to natural strigolactones, several strigolactone analogs have been synthesized and used in seed germination studies (Zwanenburg and Thuring, 1997), such as GR24, desmethyl sorgolactone, GR7 and Nijmegen1. Among them, GR24 is the most widely used in strigolactone studies.

Through bioassays with natural strigolactones and the synthesis of strigolactone analogs it has been confirmed that the D ring of the strigolactones is essential for parasitic plant seed germination stimulatory activity (Mangnus et al., 1992; Mangnus and Zwanenburg, 1992; Yoneyama et al., 2009a; Ueno et al., 2011b). Interestingly, natural strigolactones exhibit differential activity on different parasitic plant species (Yoneyama et al., 2009b; Kim et al., 2010). Usually, acetates are less active than the corresponding hydroxy-strigolactones (Yoneyama et al., 2009a). Orobanchyl acetate and strigyl acetate were 10 to 100-fold less active as germination stimulant in *O. minor* and *Orobanche ramosa* than orobanchol and strigol (Sato et al., 2005; Xie et al., 2008). However, 7-oxoorobanchyl acetate was more active than 7-oxoorobanchol in *O. minor* as well as *O. ramosa* seed germination (Xie et al., 2009a).

As parasitic weed seed germination stimulants, strigolactones seem to play a negative role in the rhizosphere. The fact that strigolactones are still being produced by plants suggests there must be a positive function for strigolactone as well, resulting in selection pressure leading to their persistence in nature.

Table1. Strigolactones present in different plant species (after year 2000).

strigolactones	Plant species	publications
5'-deoxystrigol and its isomers	*Lotus japonicus*	Akiyama et al., 2005
	Arabidopsis thaliana	Kohlen et al., 2011
	Oryza sativa	Jamil et al., 2011a
ent-2′-*epi*-orobanchol and its acetate	*Vigna unguiculata*	Ueno et al., 2011
	Trifolium pratense L	Xie et al., 2007
	Nicotiana tabacum L	
(+)-4-O-acetylorobanchol	*Vigna unguiculata*	Matsuura et al., 2008
(+)-orobanchyl acetate	*Trifolium pratense L*	Xie et al., 2008
solanacol	*Nicotiana tabacum L*	Xie et al., 2007
sorgomol	*Sorghum bicolor*	Awad et al., 2006;Yoneyama et al., 2009a
fabacyl acetate	*Pisum sativum*	Xie et al., 2009a
7-oxoorobanchol and its acetate	*Linum usitatissimum L*	Xie et al., 2009b
7-hydroxyorobanchol and its acetate	*Linum usitatissimum L*	Xie et al., 2009b

Branching Factors in AM Symbiosis- A Positive Role in the Rhizosphere

Arbuscular mycorrhizal (AM) fungi are symbiotic organisms in the plant rhizosphere and are playing an important role in agriculture. They can form a beneficial symbiosis with the roots of around 80% of vascular plants and have multiple positive functions in the rhizosphere. They can enhance plant uptake of inorganic phosphate (Pi) and other mineral nutrients from the soil, stimulate plant growth, increase tolerance to drought and protect plant roots against pathogens (Smith and Gianinazzi-pearson, 1988; Ruizlozano et al., 1995; Alan, 2000; Veresoglou and Rillig, 2011). AM fungi can not complete their life cycle without a host, and they have to colonize plant roots after spore germination to obtain carbon for their survival (Smith and Gianinazzi-pearson, 1988). This bidirectional benificial relationship between the two organisms consists of two phases, the pre-symbiotic and the symbiotic phase. Exchange of carbon and nutrients occurs through the extensively branched haustoria, known as arbuscules. However, the signaling events for the two phases are not yet fully understood. In the pre-symbotic phase AM fungi exist

in the soil as spores which are capable of spontaneous germination without host root contact. The germ tube grows for one to three weeks but will cease if a host root is not present in the soil. Over the years, experimental evidence has accumulated showing that host roots can release chemical compounds that serve as signals for directional growth and hyphal branching of AM fungi (Koske, 1982; Becard and Piche, 1989; Giovannetti et al., 1993; Harrison, 2005). This is a prerequisite for further root colonization by the fungus. The signal molecules released by the host are called "branching factors" (Buee et al., 2000). Signal molecules produced by AM fungi, called "Myc factors", are also required for successful colonization (Kosuta et al., 2003; Maillet et al., 2011).

Many groups have tried to characterize these branching factors. Preliminary evidence has suggested that this factor is a compound of <500D (Giovannetti et al., 1996). Buee et al. isolated a semi-purified fraction from exudates of eight mycotrophic plant species containing active AM fungi branching factors (Buee et al., 2000). Nevertheless, successful isolation and structural characterization were not achieved until 2005, with the discovery of 5-deoxystrigol as the AM fungal hyphal branching factor from *Lotus japonicus* (Akiyama et al., 2005). This breakthrough for the first time provided evidence that strigolactones, which were only believed to have negative effects, can also be of benefit plants, making them janus-faced molecules in the plant rhizosphere.

Later Besserer *et al.* (Besserer et al., 2006) proved that one of the branching factors from the monocot sorghum is sorgolactone. The same study also showed that GR24 stimulated hyphal branching of the AM fungus *Gigantea rosea* by provoking a rapid and strong cellular response which is associated with mitochondrial biogenesis. Further study showed that GR24 stimulates the mitosis and growth of the AM fungi by boosting its energy metabolism. Treatment of *G. rosea* with GR24 caused a rapid increase in the NADH concentration, the NADH dehydrogenase activity, and the ATP content of the fungal cell. This powerful action of GR24 on *G. rosea* cells suggests that strigolactones are important plant signals involved in switching AM fungi towards full germination and a presymbiotic state (Besserer et al., 2008). Based on this study, it was also proven that hyphal branching was completely inhibited with the suppression of mitochondrial biogenesis, confirming that AM fungi mitochondria can amplify the strigolactone response (Besserer et al., 2009).

The major advantage of the AM symbiosis for plants is that AM fungi provide a very effective Pi-uptake pathway by which Pi is scavenged from the soil and directly delivered to the cortical cells in the roots, reducing Pi depletion in the rhizosphere and improving the plants Pi nutrition (Smith et al., 2011). Pi avaibility can alter phytohormone production in plants. Cytokinin production and the receptor gene *CRE1* expression are repressed by Pi-limiting conditions in sunflower and *Arabidopsis* (Salama and Wareing,

1979; Franco-Zorrilla et al., 2002), but also symbotic AM fungi were able to induce changes in endogenous plant hormones like cytokinin and auxin (Danneberg et al., 1993; Kaldorf and Ludwig-Muller, 2000). Similarly, strigolactone production is also responsive to Pi deficiency. Reduced supply of phosphorus but not of other elements (N, K, Ca, Mg) to red clover significantly promoted the secretion of the strigolactone orobanchol (Yoneyama et al., 2007). Moreover, it has been proposed that increased AM fungal hyphal branching induced by exudates from Pi starved tomato plants is mediated by increased strigolactone production (Lopez-Raez et al., 2008). A more recent study demonstrated that the correlation between AM fungal colonization and strigolactone production is linked to shoot Pi rather than to the external Pi availability or local Pi concentrations present in the root (Balzergue et al., 2011). High Pi conditions, under which strigolactone are hardly produced, did not result in AM symbiosis. Curiously, exogenous GR24 application failed to restore AM symbiosis in these high phosphate-grown plants.Consistently, strigolactone deficient mutants showed slightly reduced AM fungi colonization (Gomez-Roldan et al., 2008).Taken together, although it is clear that strigolactones play an important role in AM symbiosis, it is likely there are also other factors involved in the regulation of this process (Nagahashi and Douds, 2007; Balzergue et al., 2011).

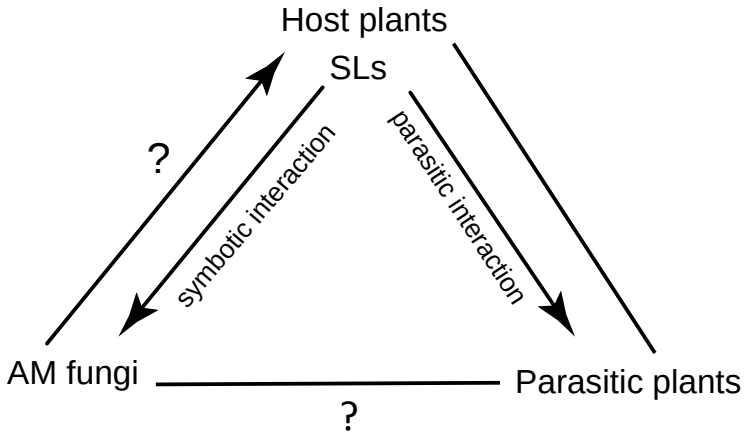

Figure 1. The trilateral relationship of host plant, arbuscular mycorrhizal (AM) fungus and parasitic plant. The host plant facilitates the symbiotic and parasitic interactions with AM fungi and parasitic plants through the exudation of strigolactones. "?" indicates that the mechanism is still unclear.

Interestingly, AM fungal colonisation leads to a decrease in seed germination of the parasitic plant *Striga* and subsequent attachment and emergence (Lendzemo et al., 2007; Sun et al., 2008), and strigolactone production was shown to significantly decrease upon AM symbiosis in tomato (Lopez-Raez et al., 2011). This suggests that the reduced

parasitic seed germination upon colonisation by AM fungi is, at least partially, due to decreased strigolactone levels. Possibly, besides the well-documented strigolactone action in plant- parasitic plant and plant-AM fungi symbiotic interactions (Bouwmeester et al., 2007), more complex trilateral relationships exist among host plants, parasites and symbionts mediated by strigolactones or other factors (**Figure 1**).

With respect to the structural requirements of strigolactones to induce hyphal branching in AM fungi, few studies were performed to date. Truncation of A-and AB-rings in the strigolactone structure caused a dramatic decrease in hyphal branching. The D-ring was shown to be essential for the branching, while the enol ether bridge in the C-D part was not necessary, concluding that the structural requirements for AM fungi hyphal branching are very simillar but not identical to those observed in root parasitic weeds seed germination (Yoneyama et al., 2009a; Akiyama et al., 2010).

Strigolactone Biosynthetic Pathway

In a study aimed at the elucidation of the biosynthetic origin of the strigolactones, Matusova *et. al*. treated maize, sorghum and cowpea plants with inhibitors of various pathways. Screening the plant root exudates for germination stimulatory activity of *Striga* seeds revealed that treatment of the plants with fluridone, an inhibitor of the carotenoid pathway, caused a significant decrease in the germination stimulatory activity of their root exudates (Matusova et al., 2005). Based on this finding, a hypothetical strigolactone biosynthetic pathway was constructed, starting with a carotenoid substrate such as β-carotene that would be cleaved by a carotenoid cleavage dioxygenase. Further hydroxylation, decarboxylation and oxidation would then lead to the formation of the A, B and C rings. The D ring (with unknown origin) was hypothesised to be enzymatically coupled to the ABC part as a last step (**Figure 2a**), leading to the formation of 5-deoxystrigol, which is believed to be the common precursor of other natural strigolactones (Rani et al., 2008; Xie et al., 2010).

As hypothesised in the above described study, in 2008 it was indeed discovered that two carotenoid cleavage dioxygeneases (CCDs) are required for strigolactone biosynthesis (Gomez-Roldan et al., 2008; Umehara et al., 2008). Prior to these findings, these two carotenoid cleavage dioxygeneaes (called *CCD7/MAX3* and *CCD8/MAX4* in *Arabidopsis*), were reported to be required for the synthesis of a novel plant signaling molecule controling shoot branching or tillering in *Arabidopsis*, pea (*RMS5* and *RMS1*), petunia (*DAD3* and *DAD1*) and rice (*HTD1*/*D17*/*D10*) (Morris et al., 2001; Sorefan et al., 2003; Booker et al., 2004; Snowden et al., 2005; Zou et al., 2006; Arite et al., 2007; Simons et al., 2007). Plants carrying mutations in these genes show an excessive bushy or branched phenotype. The conclusion from these studies is that strigolactones, or close

derivatives, are involved in the regulation of shoot branching. Both *CCD7* and *CCD8* enzymes were shown to be localized in plastids (Auldridge et al., 2006), where also the carotenoid substrates are produced.

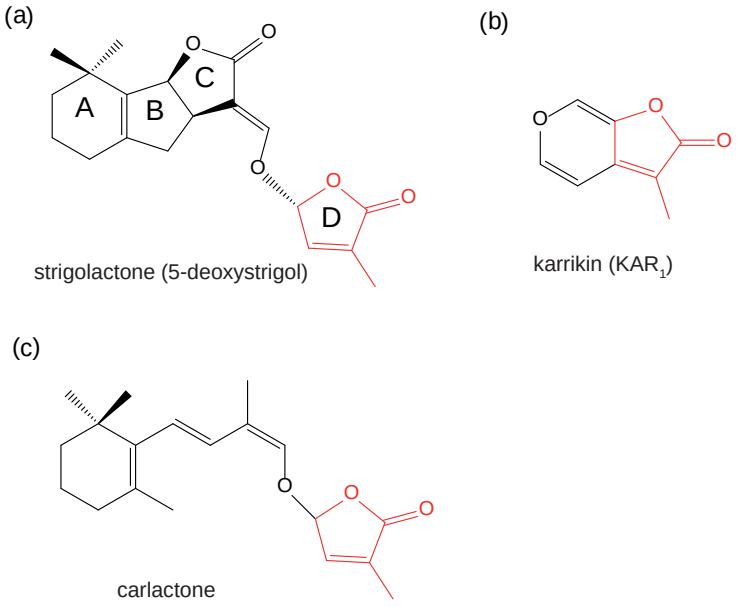

Figure 2. Chemical structures of strigolactones, karrikin and carlactone. a, structure of the strigolactone 5-deoxystrigol; b, structure of karrikin; c, structure of carlactone. Note the the butenolide moiety shared by these three compounds.

D27, an iron-containing protein also suggested to be involved in the strigolactone biosynthetic pathway in rice, shares the same plastidial localisation with the CCDs (Lin et al., 2009). Recently, it was shown that *D27* is an all-*trans* to 9-*cis*-β-carotene isomerase converting all-*trans*-β-carotene into the CCD7 substrate 9-*cis*-β-carotene (**Figure 3**). Subsequent incubation of the CCD7 product 9-*cis*-β-apo-10′-carotenal with CCD8 led to a compound called carlactone (**Figure 2c**). Carlactone restored the tillering phenotype of the strigolactone-deficient rice mutants *d27*, *htd1* (*ccd7*) and *d10* (*ccd8*) but not the signalling mutant *d3* (Alder et al., 2012). The presence of the D-ring in carlactone is highly surprising, makes this compound already strigolactone-like and greatly simplifies the strigolactone biosynthetic pathway. Carlactone clearly is an intermediate in strigolactone biosynthesis already quite close to 5-deoxystrigol (**Figure 3**).

MAX1 in *Arabidopsis*, encoding a cytochrome P450 *CYP711A1* gene, is involved in the production of the carotenoid-derived branching hormone, and has been suggested to act on a mobile substrate downstream of *CCD7* and *CCD8* (Stirnberg et al., 2002;

Booker et al., 2005). Additional experiments have shown that *MAX1* is expressed in all vascular-associated tissues throughout the plant, while the highest *CCD7/CCD8* activity is found mainly in the root (Sorefan et al., 2003; Booker et al., 2004; Booker et al., 2005). Because strigolactone levels are relatively low in *Arabidopsis*, it took some time before analytical evidence for *MAX1* activity in strigolactone production was provided. Kohlen et al. proved that less orobanchol was detected in Arabidopsis *max1* mutants root extracts and root exudates compared with the Col-0 wild-type under Pi-limiting conditions (Kohlen et al., 2011). It is likely that in other plant species, gene redundancy masks the mutational effect of a single CYP450 family member. In rice, there are five putative orthologs of *MAX1* (Booker et al., 2005; Umehara et al., 2010). Interestingly, using a genetic approach in rice, it has been shown that two of these rice P450s are co-localized with a major QTL for strigolactone production on chromosome 1. Both orthologs were capable to rescue the branching phenotype of the *Arabidopsis max1* mutant (Cardoso et al., 2013). Still, the exact enzymatic activity of these *MAX1* orthologs (nor of *MAX1* itself) has not been characterized. Since the strigolactone composition in the parents and offsprings of the mapping population was not affected, just the total level, it is likely that these cytochrome P450s are not acting in strigolactone modification but upstream in the common pathway. *MAX1* is a good candidate for the unidentified conversion of carlactone to 5-deoxystrigol (Alder et al., 2012) (**Figure 3**). Curiously, the moss *Physcomitrella patens* can produce several kinds of strigolactones, but in the moss genome so far no *MAX1* homolog was reported. This may imply that *MAX1* functions only in the very late steps of strigolactone biosynthesis that lead to shoot branching inhibitors in seed plants or that its function in moss is taken over by other P450s (Proust et al., 2011).

Two *GRAS*-type transcription factors, *NODULATION SIGNALING PATHWAY1* (*NSP1*) and *NSP2*, were postulated to affect the strigolactone biosynthetic pathway. Liu *et.al* showed that strigolactone production was disturbed in the *NSP1* and *NSP2* mutants both in the legume *Medicago truncatula* and in rice, suggesting that these two transcription factors are required for strigolactone biosynthesis. Both in rice and in *Medicago*, *D27* was down-regulated in the *nsp1* and *nsp2* mutants. In addition, in *M. truncatula NSP2* was essential for conversion of orobanchol into didehydro-orobanchol (Liu et al., 2011).

Strigolactone Signaling

Strigolactone Signal Perception and Downstream Signaling Transduction Pathway

Strigolactones are essential signaling molecules in establishing plant- parastic plant and plant-AM fungi symbiotic interactions. However, how parasitic plant seeds and

AM fungi sense strigolactones secreted from the host roots is still a mystery, awaiting identification of receptor genes to be solved. The sequencing projects of parasitic plants and symbiotic AM fungi are crucial to achieve this and will assist in the elucidation of these mechanisms (http://ppgp.huck.psu.edu/; http://striga.psc.riken.jp/) (Bonfante and Genre, 2008; Martin et al., 2008). For the parasitic plant interaction, studies of the seeds before and during germination could be one way to get a better understanding of putative recepter localisation. Joel et al. suggested that the small perisperm cells likely are the location of the germination stimulant receptors in *Phelipanche aegyptiaca* seeds (Joel et al., 2012). Localisation of putrative receptors is one thing but understanding the molecular mechanisms of strigolactone signal perception is still far way.

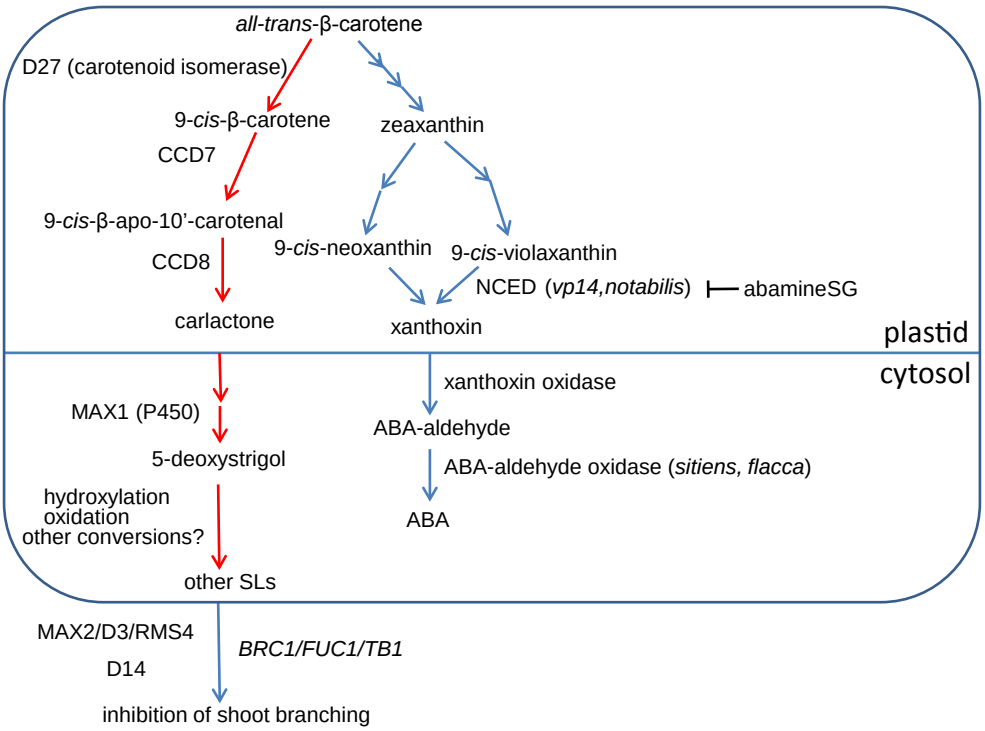

Figure 3. Schematic overview of strigolactone and abscisic acid (ABA) biosynthesis and downstream signalling pathway.

Identification of genetic mutants in non-parasitic plants has resulted in a lot of progress in the search for putative strigolactone receptors. *Arabidopsis* mutants *max2-1* and *max2-2* showing a bushy phenotype were isolated from independent M_2 bulks in a screening of M_2 plants for altered shoot branching, indicating that *MORE AXILLARY BRANCHES 2* (*MAX2*) is involved in the regulation of shoot branching (Stirnberg et al.,

2002). Later on, grafting experiments showed that wild-type roots did not restore the *max2* bushy phenotype confirming that MAX2 acts in the shoot branching inhibition, while *max2* roots could restore the bushy *max3* phenotype, indicating that MAX2 is not required for the synthesis of the branching signal molecule (Booker et al., 2005). GR24 can rescue the branching phenotype of the strigolactone biosynthetic mutants *max1, max3* and *max4* but not of *max2* (Umehara et al., 2008; Crawford et al., 2010), while in rice the *MAX2* orthologous gene *D3* mutant line (*d3*) is producing even more strigolactone than the wild-type (Yan et al., 2007; Umehara et al., 2008). *MAX2*, encoding an F-box protein with a leucine-rich repeat domain, has been shown to be expressed throughout the plant and is required in the green tissue at each individual node for repression of its associated axillary bud, acting either in the bud itself or close to it. Through a loss of-function test by deleting the F-box domain, it has been proven that this domain is required for *MAX2* function in shoot branching, indicating that *MAX2* is a component of the puative SCF^{MAX2} complex (Stirnberg et al., 2007). Further evidence for this is provided by Stirnberg *et al* who proved that in the SCF^{MAX2} complex MAX2 interacts with the SCF subunits ASK1 and AtCUL1 *in vivo* (Stirnberg et al., 2007). Similarly, the auxin receptor TIR1 and jasmonic acid receptor COI1 are also active in SCF complexes (Tan et al., 2007; Thines et al., 2007; Katsir et al., 2008).

In addition to its role in the regulation of shoot branching, *MAX2* was also described to be involved in light signalling. *MAX2*, then named *PLEIOTROPIC PHOTOSIGNALING* (*PPS*), was shown to be essential in both red (R) and far-red (FR) light-induced seed germination and the *pps* mutant seedling was hyposensitive to red and far-red light in a *PHY*-dependent manner (Shen et al., 2007). In paralle, Tsuchiya *et al* demonstrated that *max2* seeds had reduced germination compared with wild-type seeds under far-red followed by red light pulses and this germination phenotype could not be rescued by GR24 application. The same study indicated that in a short term (1 hour) situation, strigolactone signals, acting through *LONG HYPOCOTYl 5* (*HY5*) which is a light signaling trancription factor in *Arabidopsis*, can positively regulate *Arabidopsis* seedling growth in a *MAX2* dependent manner. In a long term condition (over 24 hours), strigolactone accumulation may inhibit *CONSTITUTIVE PHOTOMORPHOGENIC1*(*COP1*) function (Tsuchiya et al., 2010). In the dark, COP1 localizes to the nucleus as a repressor of light signaling by degrading the transcription factor *HY5*, while *COP1* will be inactivated by responding to light to move into the cytoplasm (Osterlund et al., 2000). Strigolactone inhibition of *COP1* function is similar to that of light, which may explain why strigolactones and light can enhance each others function (Tsuchiya et al., 2010).

Interestingly, *MAX2* was also found to be required for the signal transduction of another group of germination stimulating molecules - the karrikins which are smoke

derived butenolide compounds (Flematti et al., 2004; Nelson et al., 2011) (**Figure 2b**). Karrikins as well as synthetic strigolactone GR24 can enhance the germination of primary dormant *Arabidopsis* seed in a MAX2 dependent manner (Kipreos and Pagano, 2000; Nelson et al., 2011). In addition to their germination stimulatory activity, both karrikins and strigolactones were shown to be involved in the regulation of hypocotyl elongation mediated by *MAX2* (Nelson et al., 2011). Curiously, in contrast to strigolactones, karrikins can not suppress shoot branching (Nelson et al., 2011). In conclusion, *MAX2* is both required for germination and photomorphogenic responses to karrikins and strigolactone, and for strigolactone mediated shoot branching inhibition, indicating that the F-box protein *MAX2* probably has dual roles in both strigolactone and karrikin signaling pathways and that the plants can make a distinction between these two compounds (Nelson et al., 2011). Therefore, it is likely that *MAX2* targets different repressors involved in different signaling pathway during different physiological processes (shoot branching, seed germination and photomorphogenesis) when mediating karrikin and strigolactone signaling.

In addition to *MAX2*, in rice the *HIGH TILLERING 14* (*D14*) was also shown to act in the strigolactone downstream signaling transduction pathway. *D14* was proven to encode an α/β-hydrolase family protein (Arite et al., 2009). Two other rice genes, *HTD2* and *D88*, turned out to be allelic with *D14* (Gao et al., 2009). In *Arabidopsis*, the *D14* orthologous gene *AtD14* and another *D14-like* α/β-hydrolase family protein (*KAR2*) were also characterized (Waters et al., 2012). Interestingly, the gibberellin (GA) receptor GID1 and a salicylic acid-binding protein *SABP2* also belong to this α/β-hydrolase superfamily (Forouhar et al., 2005; Ueguchi-Tanaka et al., 2005). Based on the GA signaling pathway, a working model has been hypothesised in which *MAX2* could target two types of repressors for proteasomal degradation upon its specific association with At*D14* or *KAR2* in strigolactone and karrikin signaling pathways, respectively (Nelson et al., 2011; Waters et al., 2012). Moreover, it has also been proven that *KAR2* is only responsible for strigolactone and karrikin signaling in the early developmental stages (germination and photomorphogenesis), while *AtD14* is the modulator of *MAX2* required for the regulation of shoot branching, since *kar2* did not display the branching phenotype typical for the strigolactone mutants (Waters et al., 2012). Moreover, additional experimental data showed that *D14* was highly expressed in the axillary buds where *MAX2::GUS* activity was also detected (Stirnberg et al., 2007; Arite et al., 2009). This spatio-temporal consistency is in line with the proposed model. Although it is still not clear how *MAX2* is interacting with *D14* and *KAR2* in the strigolactone and karrikin signaling pathway, it is clear that *D14* and *KAR2* are the mediators that allow plants to discriminate between strigolactones and karrikins in different developmental stages (Waters et al., 2012).

In addition to putative receptor genes in the strigolactone perception pathway, several studies have focused on the strigolactone downstream transcription factors. More than two decades ago, *TEOSINTE BRANCHED 1* (*TB1*) in maize was already proven to be a major contributor to the change in maize apical dominance during domestication, and the *tb1* mutant showed a branched phenotype (Doebley et al., 1995; Doebley et al., 1997). Later on, this gene was characterized to belong to the *TCP* gene family of putative basic helix-loop-helix transcription factors that are involved in the regulation of organ growth (Cubas et al., 1999). Also in rice, the mutant *fine culm1* (*fc1*), with a loss-of-function mutation in *OsTB1*, displayed a hight-tillering phenotype (Takeda et al., 2003; Choi et al., 2012). Further more, the high-tillering phenotype of *fc1* was shown not to be rescued by GR24 application, while overexpression of *FC1* was able to rescue the tillered phenotype of the strigolactone-insensitive mutant *d3-2*, suggesting that *FC1* and its orthologs act downstream of the strigolactone pathway. Interestingly, *FC1* transcription levels were not affected by GR24 application. However, they are downregulated by cytokinin. This may imply that *FC1* is the common converging point through which both strigolactone and cytokinins interact (Minakuchi et al., 2010; Dun et al., 2012).

Strigolactone Transport

As a plant hormone and rhizosphere signaling molecule, strigolactone transport is expected to play a key role both in plant development and the rhizosphere, but just few studies exist on this important aspect. The early grafting experiments proved that the long-distance signals, later known as strigolactones, are produced in the roots and transported to the shoot in *Arabidopsis* (Turnbull et al., 2002). Consistently, the strigolactone biosynthetic genes *CCD7* and *CCD8* are expressed highest in the plant root (Sorefan et al., 2003; Booker et al., 2004). Recently, analytical evidence has shown that strigolactones are indeed transported through the xylem from root to shoot. MRM-LC-MS/MS analysis confirmed that the strigolactone orobanchol was present in xylem sap of *Arabidopsis Col-0* and tomato (Kohlen et al., 2011). However, transport is not a prerequisite for shoot branching inhibition, since wild-type shoots grafted to the strigolactone-deficient mutant *max1* and *max3* roots did not show excessive branching (Turnbull et al., 2002). This may imply that local production of strigolactones in the shoot is able to suppress bud activation (Domagalska and Leyser, 2011).

ATP binding cassette (ABC) transporters are well known to be involved in the translocation of the phytohormones absicic acid (ABA) and auxin (Petrasek and Friml, 2009; Kuromori et al., 2010). Recently, a strigolactone transporter, *PDR1*, belonging to the same protein family was identified in *Petunia hybrida* (Kretzschmar et al., 2012). *PDR1* was demonstrated to be expressed in the lateral roots in individual subepidermal

cells, which are probably the hypodermal passage cells which serve as entry points for AM hyphae. *Pdr* mutants showed reduced AM fungal colonization compared with the wilde-type. Consistently, *pdr* mutant root exudates contained reduced strigolactone levels. However, the strigolactone levels in the root extracts were not affected. *PDR1* overexpression lines showed a higher tolerance to the root growth inhibitory effect of high GR24 concentrations (10 and 25µM) also indicating that *PDR1* is indeed functioning as a strigolactone export carrier (Kretzschmar et al., 2012).

Strigolactones as Endogenous Plant Hormone

Strigolactone Regulated Shoot Branching Responses to Environmental Cues

Phytohormones coordinate plant development by modulating growth in response to intrinsic and environmental cues. Light is one of the important environmental signals regulating shoot architecture. A low ratio of red light to far-red light (R/FR) perceived by phytochrome *B* (*PHYB*) reduces shoot branching which is part of the shade avoidance response (Franklin and Whitelam, 2005; Franklin, 2008). It was proven that *BRC1*, *MAX2* and *MAX4* are required for the phytochrome mediated regulation of shoot branching in *Arabidopsis* (Finlayson et al., 2010). In sorghum, it was also shown that *SbTB1*, an ortholog of *BRC1*, is expressed higher in the buds of *phyb-1* than in the wild-type, suggesting that *PHYB* mediates the regulation of axillary bud outgrowth in response to light signals by suppressing the expression of *SbTB1* (Kebrom et al., 2006). Taken together, these data suggest that strigolactones play a role in the response of shoot branching to light.

Prior to the identification of the role of strigolactones in shoot branching, it was already observed that rice plants growing under Pi deficiency show reduced tillering (Luquet et al., 2005). Low Pi and nitrogen conditions increased strigolactone levels in the root exudates and extracts of various plant species (Yoneyama et al., 2007; Lopez-Raez et al., 2008). In rice it was also found that the expression levels of the strigolactone biosynthetic genes *D10*, *D17*, *D27* and two putative *MAX1* orthologs decreased after transferring rice plants from Pi-limiting to Pi-sufficient medium. Consistently, both in *Arabidopsis* and rice it was demonstrated that Pi deficiency led to an inhibition of bud outgrowth in the wild-type but not in strigolactone biosynthetic mutants (Umehara et al., 2010). Hence, enhanced strigolactone production during Pi limiting conditions offers the plant an evolutionary advantage by stimulating nutrient allocation not only within the plant (allocation of nutrients from shoot to root) but also from outside the plant by stimulating its mycorrizhal symbiosis (Umehara, 2011).

Root Development

Besides regulating shoot architecture as a phytohormone, strigolactones have also been proven to affect plant root development and architecture. In *Arabidopsis*, primary root length of strigolactone-deficient and -insensitive mutants were shorter than those of wild-type plants, and showed a reduction in meristem cell number, which could be rescued by application of GR24 in all mutants except in the strigolactone-insensitive mutant *max2* (Ruyter-Spira et al., 2011). In rice, strigolactones positively regulate the length of crown roots. GR24 application complemented the crown root defect in strigolactone-deficient mutants but not in the insensitive mutant. Also here, the meristematic zone was shorter in strigolactone mutants than in wild-type plants, suggesting that strigolactones may exert their effect on roots via the control of cell division. Under phosphate starvation, the crown roots of wild type, but not of strigolactone mutants, were longer, implying that the increase in strigolactone biosynthesis under low Pi conditions can promote crown root elongation (Arite et al., 2012).

In *Arabidopsis*, strigolactone-biosynthetic and signaling mutants showed a higher lateral root density than wild-type *Col-0* (Koltai, 2011; Ruyter-Spira et al., 2011). GR24 application negatively affected the formation and subsequent development of lateral roots in wild-type and strigolactone biosynthetic mutants, but not in the *max2* mutant (Ruyter-Spira et al., 2011).

Furthermore, strigolactones positively affect root hair (RH) elongation in a *MAX2*-dependent manner. These authors showed that GR24 (10^{-6}M) increases root-hair (RH) length in WT, *max3-11* and *max4-1* of *Arabidopsis*, but not in the strigolactone-insensitive mutant *max2* (Kapulnik et al., 2011b). However, higher concentrations (27 µM) of GR24 led to asymmetric root growth and inhibition of root-hair elongation in tomato (Koltai et al., 2010).

Strigolactones were also proven to regulate adventitious root formation in *Arabidopsis* and pea (Rasmussen et al., 2012). The *Arabidopsis* strigolactone-mutants *max1, max2, max3* and *max4* all had an increased number of adventitious roots, which could be restored by GR24 treatment in all genotypes except *max2*. These authors showed that strigolactones suppressed the adventitious root formation at or even before the first divisions of the founder cells. Similarly, strigolactones reduced the size of the adventitious rooting zone in pea and GR24 inhibited the numbers of adventitious in a dose-dependent manner.

Seed Germination

Besides investigating the germination of parasitic plant seeds triggered by exogenous strigolactone application, strigolactones are now also being studied as endogenous seed

germination stimulating hormones. To understand the roles of strigolactones in seed germination, recent studies used *Arabidopsis* as a model system. GR24 stimulated germination of primary dormant (PD) *Arabidopsis Ler* seeds but an approximately 100-fold higher concentration was required than for the least active karrikin. Curiously, the most active karrikin (KAR_1) was unable to trigger germination of *O. minor*. These findings suggest that strigolactones and karrikins are acting in a distinct species-specific manner (Nelson et al., 2009). It is likely that the discovery of karrikins, which are structurally related to the strigolactones by sharing a common butenolide moiety (**Figure 2**), drew the attention of scientists to study the roles of strigolactones in seed dormancy and germination, which are likely mediated by the F-box protein *MAX2* (Waters et al., 2011). Moreover, Toh et al. (Toh et al., 2012) demonstrated that GR24 rescued the observed reduced germination percentage of thermo-inhibited strigolactone biosynthetic mutant seeds (*max1* and *max3*) but not the signaling mutant *max2*.

Other Functions

Besides the roles of strigolactone in seed germination, AM fungal symbiosis, shoot and root development, even more roles for strigolactones are being discovered at quick pace. Similarly to inhibiting hypocotyl growth in *Arabidopsis* (Tsuchiya et al., 2010), strigolactones negatively regulate mesocotyl elongation in rice in darkness (Hu et al., 2010). In addition, strigolactones were shown to stimulate cambial activity in *Arabidopsis* secondary growth in the stem (Agusti et al., 2011). Moreover, strigolactones were found to regulate the nodule number in pea (Foo and Davies, 2011), and can regulate colony growth in moss (Proust et al., 2011). The latter could suggest that strigolactones in the rhizosphere may also act as a quorum sensing-like molecule.

It has also been reported that petunia *CCD8* is involved in the regulation of leaf senescence and flower development (Snowden et al., 2005). Similarly, in kiwi the reduction of *CCD8* expression in RNAi lines resulted in a delay in leaf senecence (Ledger et al., 2010). Consistently, in *Arabidopsis*, MAX2 (in this study named ORE9) was shown to be a positive regulator acting upstream in the regulatory cascade of the senescence pathway (Woo et al., 2001; Kim et al., 2011). Taken together, MAX2 may be the coordinator or mediator in other, yet to be discovered, strigolactone signaling pathways during plant development. Studying its exact localization throughout the plant may shed more light on these novel aspects.

Mechanisms of Strigolactones Interaction with Other Plant Hormones

Plants are sessile organisms and must adjust their growth and development to continuously changing environmental conditions (Wolters and Jürgens, 2009). Plant hormones such as auxin, abscisic acid (ABA), cytokinin (CK), gibberellin (GA),

ethylene, brassinosteroids (BRs), jasmonic acid (JA), salicylic acid (SA), nitric acid (NO) and strigolactones (Santner and Estelle, 2009) play a central role in regulating plant growth and development in response to these environmental changes. However, these plant hormones do not act alone but are interconnected and modulate each other at the level of biosynthesis, degradation and signalling by cross-talk.

In this part, we summarize the recent findings describing the cross-talk of strigolactones with other plant hormones. Special emphasis will be on the interaction of strigolactones with ABA, auxin, CK and ethylene.

Strigolactones and Abscisic acid

In their study aimed at the elucidation of the origin of strigolactones, Matusova et al. showed that the ABA-deficient mutant *viviparous14* (*vp14*) of maize induced less germination of *S. hermonthica* seeds (Matusova et al., 2005). Later, a similar result was obtained with the tomato mutant *notabilis (not)*, carrying a mutation in a homolog of *vp14* (Lopez-Raez et al., 2008) (**Figure 3**). Indeed just as in maize *vp14*, in tomato *notabilis* strigolactone levels were decreased by about 40%. *Vp14* and *notabilis* encode a 9-*cis*-epoxycarotenoid dioxygenase (NCED) catalyzing the cleavage of 9-*cis*-epoxycartenoids to xanthoxin, the first step in ABA biosynthesis (Bouwmeester et al., 2007) (**Figure 3**). The fact that the low ABA levels in these mutants correlate with reduced strigolactone production suggests a role for ABA in the regulation of strigolactone biosynthesis and/or vice versa. Because the possibility that *NCED* itself is involved in the strigolactone biosynthetic pathway, could initially not be excluded, two additional ABA-deficient mutants in tomato - *flacca* and *sitiens* - involved in the last step of ABA biosynthesis were also analysed (Lopez-Raez et al., 2010) (**Figure 3**). Since also these mutants showed a strong reduction in strigolactone levels, it is more likely that ABA affects strigolactone production rather than that these enzymes are involved in both pathways. However, the mechanism of how ABA affects strigolactone biosynthesis is still unknown.

ABA plays a vital role in regulating seed development, dormancy and stress tolerance (Davies et al., 2005; Jiang and Hartung, 2008). In *Arabidopsis*, Toh et al. have shown that strigolactones positively regulate seed germination, by counteracting the inhibitory effect of ABA during thermo-inhibition. Under high temperatures (32ºC), application of GR24 decreased ABA levels in *max1-1* but not in *max2-1* seeds. In accordance with this, the expression of the ABA biosynthetic gene *NCED9* was suppressed by GR24. These results suggest that strigolactones decrease the ABA response via the MAX2 F-box protein. Also in the parasitic plant *Striga hermonthica*, application of GR24 during germination reduced ABA compared to the control treatment (Toh et al., 2012) (**Figure 4d**). Hence the evidence is accumulating that strigolactones regulate seed dormancy/germination by interacting with other plant hormones.

Strigolactones and Auxin

At the physiological level, several studies have provided evidence for the cross-talk between auxin and strigolactones. For instance, in the regulation of shoot branching, it is suggested that strigolactones act synergistically with auxin. Hayward et al. showed that auxin up-regulates strigolactone biosynthetic genes *MAX3* and *MAX4*, whereas in *N*-1-naphthylphthalamic acid (NPA)-treated or decapitated plants the expression of these genes is down-regulated. The regulation by auxin of strigolactone biosynthetic genes is *AXR1* dependent and acts via the AXR1-TIR1 signalling pathway (Hayward et al., 2009; Leyser, 2009). Still, the exact mechanism of how strigolactones inhibit the outgrowth of axillary buds has not been resolved. It is hypothesised that strigolactones act as a secondary messenger of auxin inside the buds to repress its outgrowth (Brewer et al., 2009; Dun et al., 2009; Ferguson and Beveridge, 2009) (**Figure 4b**). Other studies suggested that strigolactones act primarily to reduce the capacity of the polar auxin transport (PAT) from the shoot apical meristem. This would inhibit or limit auxin export from the bud into the main PAT stream resulting in inhibition of bud outgrowth (Bennett et al., 2006; Mouchel and Leyser, 2007; Ongaro and Leyser, 2008; Leyser, 2009; Domagalska and Leyser, 2011) (**Figure 4a**).

Auxin and strigolactones affect each other's levels by feedback mechanisms (Hayward et al., 2009). GR24 application to *Arabidopsis* seedlings resulted in reduced levels of free auxin in rosette leaves (Ruyter-Spira et al., 2011). The strigolactone biosynthetic mutant *max1-1* displayed a higher auxin transport capacity and increase in signal intensity of the auxin reporter *DR5::GUS* in the lower stem and *MAX3* and *MAX4* expression were higher in all max mutants (Hayward et al., 2009). The latter is likely mediated by increased auxin levels caused by the higher auxin transport capacity.

Like in shoot branching, strigolactones also act synergistically with auxin to stimulate secondary growth in plants. It was previously shown that vascular cambium-mediated secondary growth depends on auxin transport (Snow, 1935). Interestingly, Agusti et al. showed that although there is an auxin dependent (through *AXR1*) component involved in the strigolactone mediated induction of cambial growth, strigolactones can also directly influence cambium activity independently or downstream of auxin (Agusti et al., 2011). With this new knowledge, it is not unexpected that strigolactone deficient mutants displayed a reduction in cambial activity, and that exogenous application of GR24 could complement this phenotype.

Several studies show that strigolactones interact with auxin to regulate primary root length (Koltai et al., 2010; Ruyter-Spira et al., 2011) (**Figure 4c**). In *Arabidopsis*, auxin treatment resulted in decreased primary root length accompanied by reduced cell

elongation (Rahman et al., 2007). However, higher concentrations of GR24 were able to eliminate the suppressive effect of exogenous auxin application on primary root length in tomato (*Solanum lycopersicon*), accompanied by increased cell length and reduced cell division (Koltai et al., 2010). In addition, exogenous application of GR24 to *Arabidopsis* roots, led to a decrease in PIN1/3/7::GFP intensities in the provascular tissue of primary root tip (Ruyter-Spira et al., 2011). However, since after simultaneous application of NAA and GR24 no reduction in PIN protein levels could be observed, the effect of GR24 is likely not direct but is mediated through reduced local IAA levels instead. Indeed, as previously mentioned, GR24 treated plants were shown to have decreased levels of free IAA in their rosette leafs, implying that strigolactones negatively feedback on auxin levels throughout the entire plant. Consistent with this, *Arabidopsis* strigolactone mutants had shorter primary roots, which were accompanied by higher DR5::GUS signals in the primary root tips.

As described above, lateral root initiation and their subsequent outgrowth are both suppressed by GR24 application (Ruyter-Spira et al., 2011). Interestingly, application of GR24 in the presence of NAA counteracted the inhibitory effect of NAA and stimulated a more rapid outgrowth of lateral root primordia instead. It was postulated that the effect of strigolactones on lateral root formation was mediated through the modulation of local auxin levels (Ruyter-Spira et al., 2011) (**Figure 4c**). Analogous to this finding, lateral root development during sufficient Pi conditions was faster in strigolactone deficient plants than in the wild-type, while endogenous strigolactones in wild-type plants were found to stimulate lateral root outgrowth during limiting Pi conditions instead (Ruyter-Spira et al., 2011). It has been hypothesized that the low Pi conditions increase the auxin sensitivity in the roots to such an extent that auxin becomes inhibitory to lateral root outgrowth. Strigolactones will reduce auxin concentration and hence stimulate lateral root outgrowth.

Strigolactones and Ethylene

Ethylene is a gaseous plant hormone that acts synergistically with auxin to regulate root hair elongation (Pitts et al., 1998). In addition to auxin, strigolactones also show a cross-talk with ethylene to regulate root hair elongation (Kapulnik et al., 2011a). Using root hair elongation as an assay, it was found that the effect of GR24 was reduced in the ethylene signalling mutants *ein2* and *etr1* and eliminated when ethylene biosynthesis was blocked. Moreover, root treatment with GR24, resulted in up-regulation of the expression of the ethylene biosynthesis gene *AtACS2*, suggesting that strigolactones and ethylene positively regulate root hair elongation via the same pathway (Kapulnik et al., 2011a). Therefore, the effect of strigolactones on root hair elongation might either be directly via modulation of the auxin flux or indirectly through modulation of ethylene

synthesis (Koltai, 2011) (**Figure 4c**).

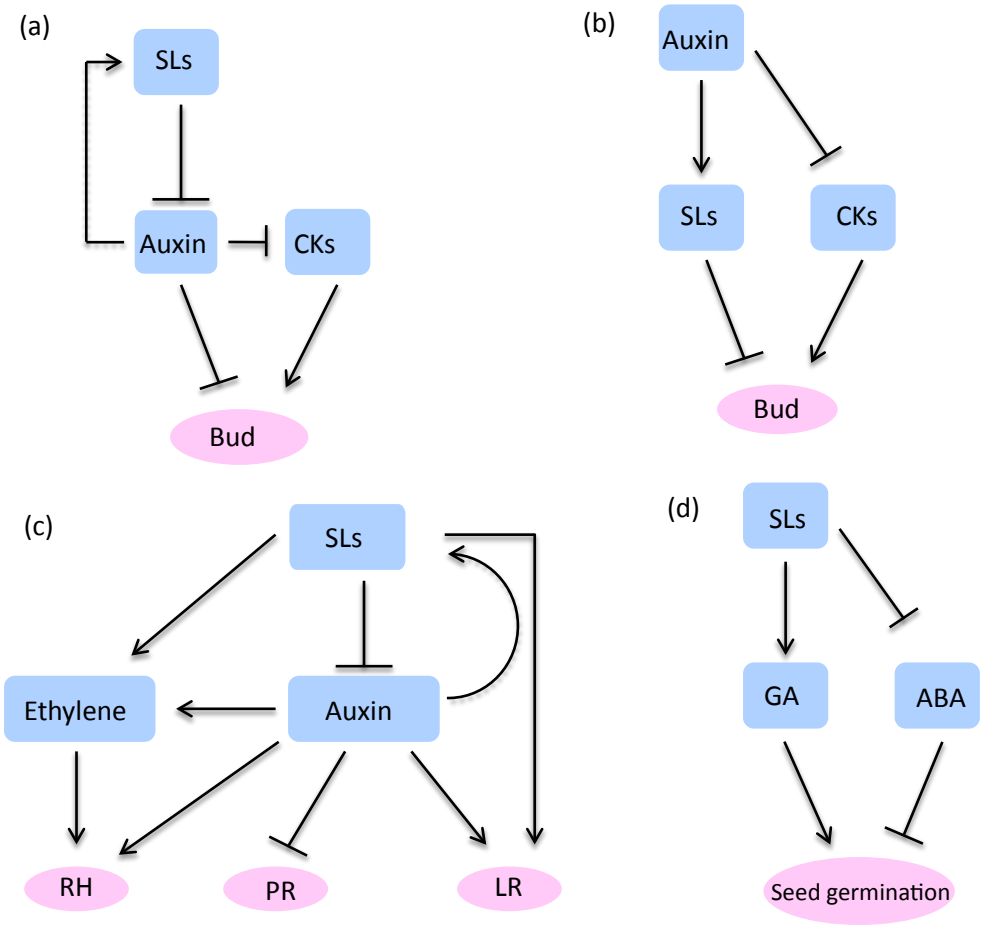

Figure 4. Schematic representation of strigolactones interacting with other plant hormones. (a), (b) For shoot branching; (a) canalization hypothesis for bud activation; (b) secondary messenger hypothesis for bud activation; (c) For root architecture; (d) For seed germination. Arrows represent positive regulation, flat ended lines represent negative regulation, and broken arrows represent feedback. Abbreviations: SLs, strigolactones; CKs, cytokinins; GA, gibberellin; ABA, abscisic acid; RH, root hair elongation; PR, primary root length and LR, lateral root formation.

Strigolactones and Cytokinin

Cytokinins (CKs) are known to act as a promoter of bud outgrowth (Dun et al., 2009; Leyser, 2009; Beveridge and Kyozuka, 2010). Physiological studies suggest that strigolactones act antagonistically with CK to regulate bud outgrowth (Brewer et al., 2009;

Crawford et al., 2010; Liang et al., 2010). Recent work in pea sheds more light on the underlying mechanism (Dun et al., 2012). It was found that axillary buds in strigolactone deficient mutant plants (*rms1*) showed an increased response to 6-benzylaminopurine (BA, synthetic CK), when compared with WT buds. In addition, exogenous application of GR24 in combination with BA, led to a reduction in bud outgrowth in *rms1* but not in the *rms4* strigolactone insensitive mutant. This suggests that strigolactones decrease the CK response via MAX2. Furthermore, molecular data show that strigolactones and CK act antagonistically via the common target of *PsBRC1* expression in the bud. Exogenous application of GR24 led to an increase in expression of *PsBRC1*, whereas in decapitated or BA treated pea plants, decreased *PsBRC1* expression was observed (Dun et al., 2012).

Strigolactones and other hormones

There are a few studies indicating the cross-talk between strigolactones and other hormones. Recent work by Toh et al. has shown that strigolactones act synergistically with GA to regulate seed germination in *Arabidopsis* (Toh et al., 2012) (**Figure 4d**). Particularly when exposing *Arabidopsis* seeds to high temperature (32°C), GR24 application increased endogenous GA_4 levels in *max1-1* but not in *max2-1* seeds. This suggests that strigolactones increase the GA response via MAX2. However, GR24 application did not increase the transcription of gibberellin-3-oxidase 2, a key enzyme in GA biosynthesis. Therefore, this suggests that the effect of strigolactones on GA is via the regulation of other steps in the GA biosynthetic pathway or through its catabolism or both.

Future Perspectives

As an important signal compound in the rhizosphere and a novel phytohormone, strigolactone may have other biological functions besides the above described functions. According to the description above, it is likely that the function and/or activity of the natural strigolactones differs due to structural diversity. To date, over twenty natural strigolactons have been identified, some of which show different capacity in inducing parasitic seed germination and AM fungi branching. Characterization of strigolactone functional diversification and specifity is highly relevant for agricultural practices. For instance, screening of crop cultivars producing strigolactones which are less potent to trigger parasitic plant seed germination but stimulate more AM fungi colonization, can be a breeding approach to control parasitic weeds (Cardoso et al., 2011). Hence further unravelling of the strigolactone biosynthesic pathway, including the strigolactone diversification steps, is an important research question. Cytochrome P450s, and O-methyl and acetyl transferases are good candidates for the decoration of strigolactone structures starting from 5-deoxystrigol. In order to discover these genes, a genetic approach, using

parental lines that produce different strigolactone profiles, would provide an excellent tool to resolve these questions and to discover enzymes that create the natural varation of strigolactones among plant species.

Moreover, from the view of parasitic weed control, elucidation of the strigolactone biosynthesic pathway will also offer posibilities for the development of chemical inhibitors of strigolactone production. With respect to the carotenoid pathway origin, inhibitors of this pathway have been examined in several studies. Fluridone specifically inhibits the second dedicated enzyme in the carotenoid pathway, phytoene desaturase (Li et al., 1996). Treating maize, sorghum and cowpea with fluridone, Matusova et al. showed that the root exudates from the treated plants were less active in triggering parasitic seed germination (Matusova et al., 2005). Similarly, the *NCED* specific competative inhibitor abamineSG (**Figure 3**) and the *CCD* specific inhibitor D2 also reduced strigolactone-production (Lopez-Raez et al., 2010). In rice, irrigation application of the carotenoid inhibitors fluridone, norflurazon, clomazone and amitrole significantly decreased strigolactone production, *Striga hermonthica* germination and *Striga* infection (Jamil et al., 2010). A screen of a chemical library of triazole derivatives for strigolactone biosynthesis inhibitors revealed that TIS13 [2,2-dimethyl-7-phenoxy-4-(1 H -1,2,4-triazol-1-yl)heptan-3-ol], which induced outgrowth of secondary tiller buds of wild-type seedlings, could reduce the strigolactone level in rice in a dose-dependent manner (Ito et al., 2010). In a subsequent study, TIS108, a new TIS13 derivative, was found to be a more specific strigolactone biosynthesis inhibitor than TIS13. Treatment of rice seedlings with TIS108 reduced strigolactone levels in a concentration-dependent manner and did not reduce plant height (Ito et al., 2011).

Besides the above mentioned breeding approach and the use of chemical inhibitors of the strigolactone biosynthetic pathway, synthetic strigolactone analogues may also be used to induce parasitic weeds suicidal germination. Therefore, synthesis of cheap strigolactone analogs having high germination stimulatory activity remains a hot topic (Zwanenburg and Mwakaboko, 2011). Furthermore, the control of soil fertility (Pi and N) and AM fungal colonization are also useful methods to reduce the parasitic weed problem in agricultural practices (Lopez-Raez et al., 2009).

With regard to strigolactones as a phytohormone, it will be of interest to further explore the hormonal cross-talk with other plant hormones. Currently, most progress has been made at the physiological level, while at the molecular and biochemical level, our knowledge is still fragmented. It is not unlikely that strigolactones are involved in even more biological processes than we already know in different development stages, environmental conditions and different tissues. For instance, ABA is a well-known stress hormone known to be involved in drought stress tolerance. The interaction between

ABA and strigolactones may imply that strigolactones also play a role during drought stress. However, the mechanisms underlying the ABA-strigolactone cross-talk are still unknown. The fact that they share a common biosynthetic origin (**Figure 3**) makes it even more intriguing to find out the mechanism by which they influence each other's levels.

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Chapter 3

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

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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 QTL - 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 51-59 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.

Significance Statement

Strigolactones are a new class of plant hormones regulating plant shoot and root architecture in response to the environment. Also present in root exudates, strigolactones stimulate the germination of parasitic plant seeds. This report describes a genomic polymorphism - associated with the *Indica*/*Japonica* ssp divide in rice that has a major impact on the biosynthesis of strigolactones, plant tillering and germination of the parasitic plant *Striga hermonthica* - consisting of the deletion of two strigolactone biosynthetic genes orthologous to *Arabidopsis MAX1*. Both these genes rescued the *Arabidopsis max1-1* highly branched mutant phenotype and increased the strigolactone level when overexpressed in the *Indica* rice variety Bala. This finding is of great interest for plant physiologists, plant evolutionary biologists and breeders.

Keywords: Strigolactones, Rice, *Striga hermonthica*, QTL, *MAX1*

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 (1). 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 (2). SLs are also signalling molecules for the establishment of the symbiosis with arbuscular mycorrhizal (AM) fungi (3) that help the plant to improve nutrient uptake. Under low phosphate availability, SL exudation into the rhizosphere is strongly enhanced, hence promoting AM symbiosis (4). As a negative consequence, however, agricultural areas with poor soils and low fertilizer input are strongly affected by *Striga* (1, 4). In addition to their rhizosphere role, SLs also function as plant hormones inhibiting shoot branching and modulating root architecture (5–7), also in response to phosphate deficiency (8, 9). SL biosynthesis or signalling mutants have increased axillary bud outgrowth, resulting in a bushy and dwarf phenotype (10). 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 DIOXIGENASE 8 (CCD8), which results in the formation of carlactone (11–16). The gene(s) responsible for the conversion of carlactone to a strigolactone has/have not been identified, although *MORE AXILLARY GROWTH 1* (*MAX1*), encoding a cytochrome (CYP) P450 in *Arabidopsis*, has been suggested to be a candidate (8, 11, 17, 18). SL signalling is mediated by an F-Box protein (MAX2 in *Arabidopsis*; D3 in rice) and an α/β-hydrolase protein (D14) (5, 6, 19, 20).

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 x Azucena F_6 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 (21). Here we show that the QTL is due to a rearrangement of a 51-59 Kbp stretch between 28.9 and 29.0 Mbp of chromosome 1 in the Bala genome. This rearrangement results in the deletion of two cytochrome P450 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 two weeks after first emergence, there was on average 1 *Striga* shoot in Azucena and 2 in Bala (Fig. 1*A*). After 2 weeks, however, the number of *Striga* shoots increased more rapidly in Azucena and after 5 weeks Azucena had on average 19 *Striga* shoots while Bala had only 10.

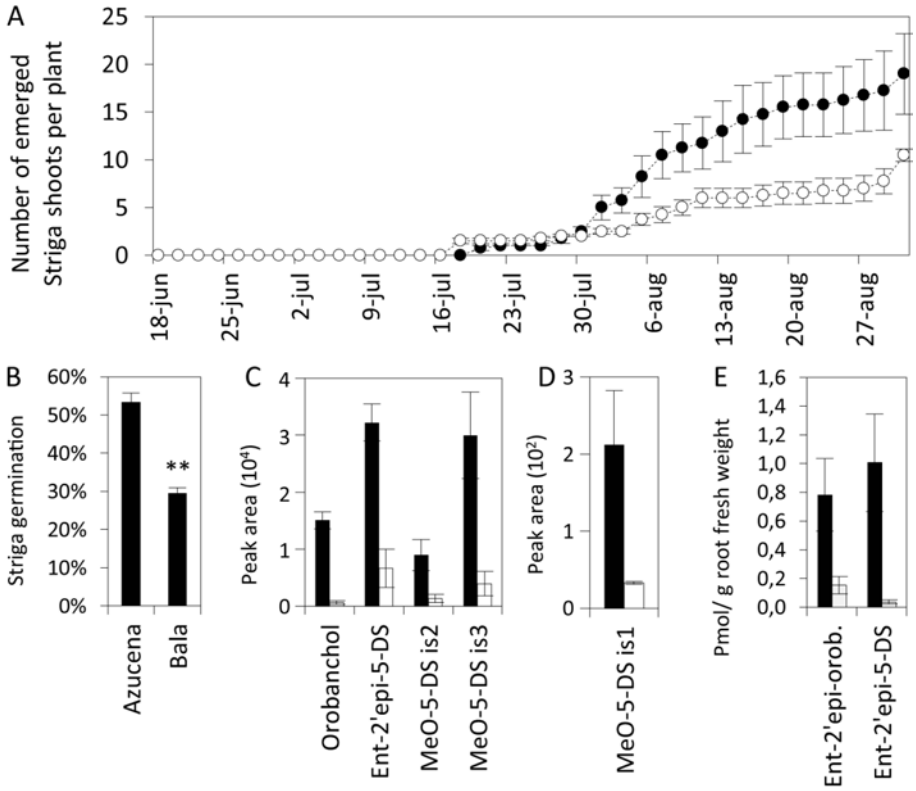

Fig. 1 Analysis of the parental lines of the RIL population Bala x Azucena. Emergence of *Striga* shoots per pot during a period of 78 days. (● – Azucena; ○ – Bala) (A), germination percentage of *Striga* seeds exposed to crude exudates of the parental lines (B), LC-MS peak areas of orobanchol, *ent*-2'-*epi*-5-deoxystrigol (*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.

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 (Fig. 1*B*). 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 (22, 23) (Fig. 1*C*, *D* and *E*).

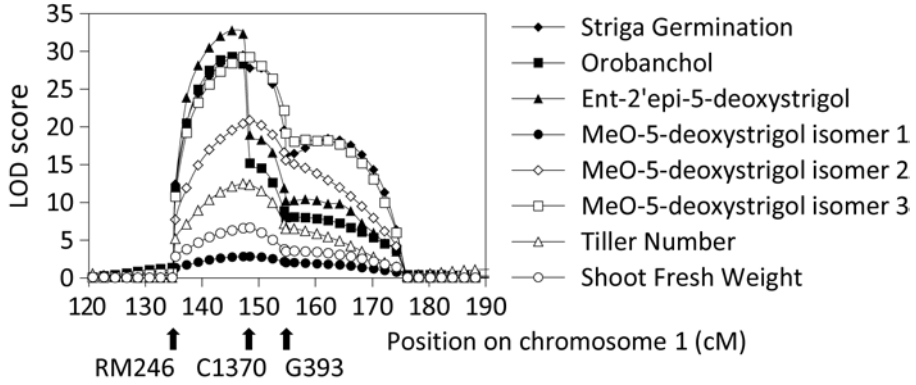

Fig.2 LOD score distribution of the QTLs for SL production (MeO-5-DS- methoxy-5-deoxystrigol), *Striga* germination, tiller number and plant shoot fresh weight that co-locate on rice chromosome 1. The 5% genome wide threshold for QTL detection was determined after 1000 permutations and ranged from LOD 3.3 to 4.2. Arrows mark the position of the three closest markers.

QTL mapping of SL levels and related phenotypes

Given the different amounts of SLs found in the parental lines, we used the Bala x Azucena mapping population (21) 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 (Fig. 2, Table S1 and Fig. 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%; LOD= 29.17, R^2= 52.8%, respectively). For all these traits the positive allele was from Azucena. Also 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 Fig. S1). Although these QTLs have lower LOD scores (2.5 - 4.6) the co-localization 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.

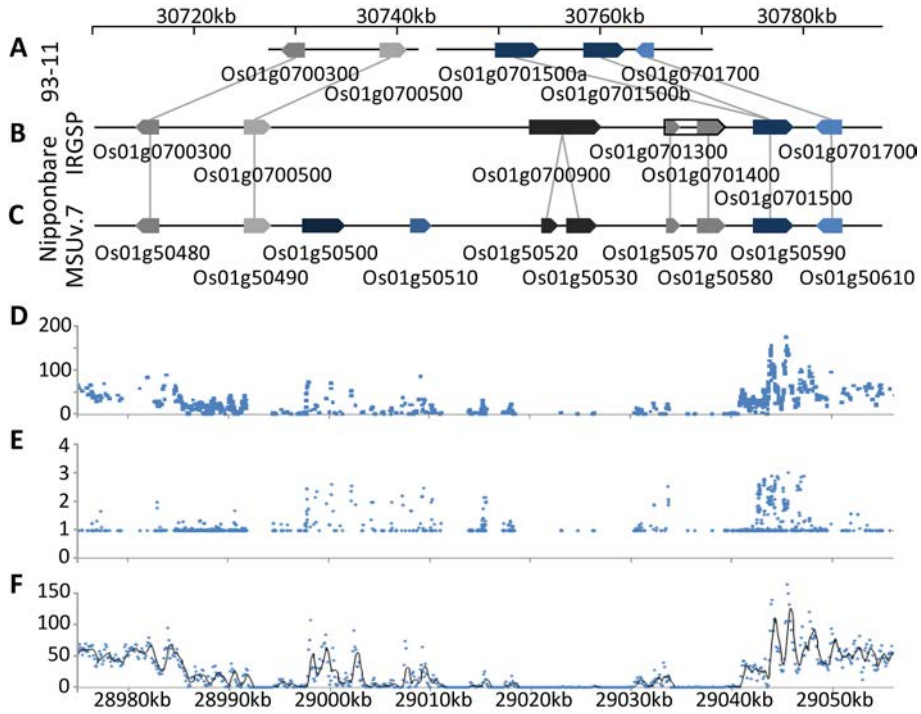

Fig. 3 Alignment of genomic sequences of 93-11, Nipponbare and Bala in the region where the major QTL for SL production was identified. A - Gene models for cultivar 93-11 were adapted from the genome browser of the Beijing Institute of Genomics (http://rice.genomics.org.cn/rice/index2.jsp) (A); B - Gene models for cultivar Nipponbare as predicted in the genome browser of the International Rice 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 *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.

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 (Fig. 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/) (24, 25) Nipponbare reference sequence showed that (i) three predicted Nipponbare genes between 30,750 Kbp 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, *Os01g0701500*, in Nipponbare (Fig. 3*A*, *B* and *C*). Alignment of Bala genomic sequence reads (88-120 bp lengths at 55x 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 (Fig. 3*F*). This alignment also confirms that *Os01g0701500* is duplicated (Fig. 3*D*, *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 cytochrome P450s (CYP) (*Os01g0700900* and *Os01g0701400*). At the border of the deleted region, *Os01g0701500*, 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 International Rice Genome Sequencing Project (IRGSP-1.0) was compared to the MSU v.7 annotation (Rice Genome Annotation Project, RGAP), it turned out that *Os01g0700900* is split into two genes, *LOC_Os01g50520* and *LOC_Os01g50530* (Fig. 3*B* and *C*). *Os01g0701300* and *Os01g0701400* correspond to *LOC_Os01g50570* and *LOC_Os01g50580* respectively, while *Os01g0701500* is the same as *LOC_Os01g50590* (Fig. 3*B* and *C*).

The rearranged region on Bala chromosome 1 contains MAX1 homologs

A BLASTX search, using the predicted open reading frames (ORFs) of the deleted genes *Os010700900* and *Os01g0701400* 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 *Os01g0700900* is correct although with different splicing form and start codon (Genebank accession no: JX235697, Fig. S2*A*) while the RGAP annotation for *Os01g50520/30* is wrong. We could also confirm that *Os01g0701300* and *Os01g0701400* and the corresponding RGAP annotated *Os01g50570/80* are one single gene from here on referred to as *Os01g0701400* (Genebank accession no: JX235696, Fig. S2*B*).The protein sequences of *Os01g0700900* and *Os01g0701400* show respectively 57.6% and

60.3% identity to *AtMAX1* (Fig. S3). Quantitative RT-PCR showed that expression of *Os01g0700900* in Nipponbare was induced by low P treatment - just as expression of the P starvation marker *OsPI1* (26) - but not of *Os01g0701400* (Fig. 4*A*).

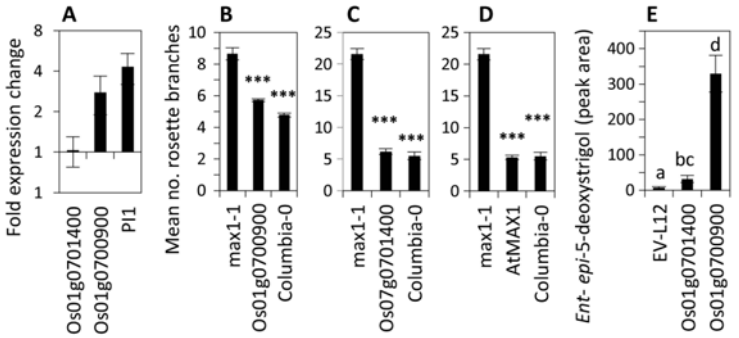

Fig. 4 Expression analysis (A) and functionality of rice CYP genes Os01g0700900 and Os01g0701400 in Arabidopsis (B-D) and rice (E). Regulation of gene expression of Os01g0700900, Os01g0701400 and the low-P marker gene PI by low P nutrition measured by qRT-PCR, n=4-5 (A). Complementation of axillary bud outgrowth of max1-1 by overexpression of Os01g0700900 (B), Os01g07001400 (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 ± SE.

Os01g0700900 and Os01g0701400 rescue the branched phenotype of Arabidopsis max1

Arabidopsis max1-1 was transformed with the *Os01g0700900* and *Os01g07001400* cDNA under the control of the CaMV35S promoter (p35S). p35S:*AtMAX1* was used as a positive control. *Os01g0700900*, *Os01g07001400* and *AtMAX1* all fully restored the branching phenotype of *max1-1 (P*<0.001) (Fig. 4 *B-D*, and Fig. S4) showing that these two rice CYP450 genes are *AtMAX1* orthologs.

Os01g0700900 and Os01g0701400 increase SL levels in root exudates of Bala

Bala was independently transformed with *Os01g0700900* and *Os01g0701400*, driven by the p35S promoter. The levels of *ent*-2'-*epi*-5-deoxystrigol in root exudates of P starved p35S:*Os01g0700900* and p35S:*Os01g0701400* transformed T1 Bala plants (for which transgene expression was confirmed) both significantly increased compared with the empty vector control, but the effect of p35S:*Os01g0700900* was much stronger than that of p35S:*Os01g0701400* (Fig. 4*E*).

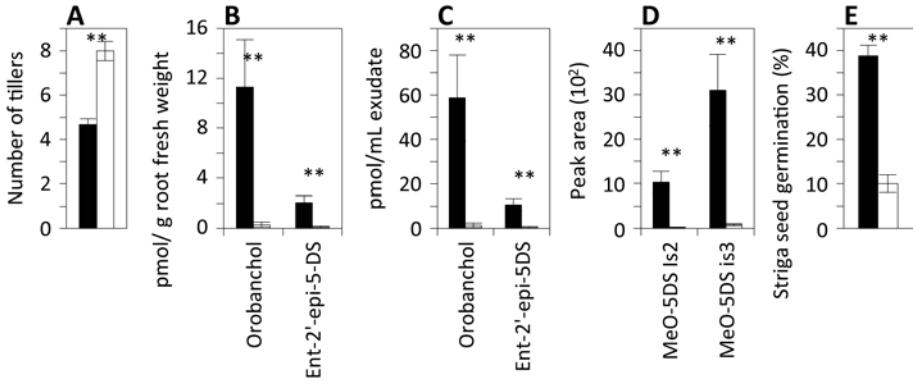

Fig. 5 Impact of the rearrangement on the (A) number of tillers, (B) the strigolactone content in root tissue and (C, D) root exudates and (E) on the stimulation of *Striga* germination by root exudates. Orobanchol and e*nt*-2'-*epi*-5-deoxystrigol (*ent*-2'-*epi*-5-DS) are determined in pmol/ g fresh 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 ±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).

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 (*Os01g0700900*, *Os01g0701400* and *Os01g0701500*) which all give a product in Azucena but for which only *Os01g0701500* 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 (27) the Nipponbare/Azucena allele frequency was 3/74 (4.1%) and 4/59 (6.8%), respectively, while in the *temperate* and *tropical japonicas* within the *Japonica* subspecies (27) 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 which differed in the allele under study but were otherwise from the same subspecies and share the same country of origin (Table1). 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 (Fig. 5, Table S2) than the genotypes containing the Azucena alleles in both the *Indica* and the *Japonica* genetic backgrounds.

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

[a]According to Zhao et al. (27)

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 51-59 Kbp rearrangement compared with Nipponbare. The rearrangement spans two CYP genes (*Os01g0700900, SLB1*; *Os01g0701400, 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 (8, 17). Overexpression of either of the two genes in *Arabidopsis max1-1* rescued the branched mutant phenotype and overexpression in Bala - particularly of *Os01g0700900* - increased the level of *ent*-2'-*epi*-5-deoxystrigol in the root exudate. While 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 *Os01g0700900* and *Os01g0701400* 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 (Fig. 1*C*). This 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: *Os01g0701500*, *Os06g0565100* and *Os02g0221900*. Two of these, *Os02g0221900* and *Os06g0565100*, also rescued the branched phenotype of *Arabidopsis max1-1* (28) suggesting that they also have MAX1-like activity. In fact, several other monocotyledonous species such as maize and sorghum have 2 to 5 *MAX1* orthologs, while in dicotyledonous species such as petunia and *Medicago* generally only one, sometimes two are present (28, 29).

Gene duplication allows for diversification in metabolic regulation. This is also observed for *Os01g0700900* and *Os01g0701400* in the present study. The expression of *Os01g0700900* was increased by P starvation but not that of *Os01g0701400* (Fig. 4*A*). In line with this, the expression of *Os01g0700900* and *Os02g0221900*, as well as the SL biosynthetic genes *D10*, *D17* and *D27* was repressed by P replenishment whereas expression of *Os01g07001400*, *Os01g07001500* and *Os06g0565100* were not (9). Finally, the levels of *ent*-2'-*epi*-5-deoxystrigol obtained in root exudates of Bala overexpressing *Os01g0701400* were considerably lower than those of Bala overexpressing *Os01g0700900* (Fig. 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 while in *Arabidopsis* this approach was successful (10). It also demonstrates the power of QTL mapping to address more fundamental questions such as plant hormone biosynthesis, as 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-strigolactone 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 also supports the hypothesis that *Indica* and *Japonica* were domesticated from divergent populations of their wild ancestor, *O. rufipogon* (30). The fact that the *Indica* low-strigolactone allele is present in approximately 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 *O. sativa* has been previously documented in other studies (31–34).

SLs regulate overall plant architecture. In shoots, SLs inhibit tillering, in roots SLs influence root and root hair elongation and lateral root development (5, 7, 9, 35, 36). Indeed, tillering mapped to the same strigolactone 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 (37, 38). These findings emphasize the potential of qSLB1.1 in the improvement of important agronomic traits in rice. Our study demonstrates once more the positive correlation between SL production and *Striga* germination (22, 39). However, although decreased SL biosynthesis results in less *Striga* germination, it is unclear if 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 (21), even though in the present study we showed there were more *Striga* emerging on Azucena in controlled environments (Fig. 1*A*). 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 (40). Intriguingly, *Striga* post attachment resistance mapped to the same position on chromosome 1 as the QTL discussed in the current study (near marker C1370) in a cross between Nipponbare and Kasalath (21) with the Nipponbare allele conferring greater resistance (41). 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 recombinant inbred lines (RILs) derived from Bala x Azucena described in Price *el al* (42) was used. The experiments were conducted under controlled conditions (28°C/25°C, 450μM 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 fresh weight determined. Allelic frequencies of the rearrangement in the different subpopulations were assayed using 367 diverse accessions from the publicly available Rice Diversity Panel (27, 34). Eleven of these lines (Table 1) were multiplied in Aberdeen and sent to Wageningen for physiological characterisation. *Arabidopsis* growing conditions are described in SI Material and Methods.

Complementation of Bala

Transformation of Bala with constructs p35S:*Os01g0700900*, p35S:*Os01g0701400* and empty vector pHm43GW is described in Text S1-*B*. 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 (3 plants per pot) for root exudate collection (see below). Statistical tests were performed using Genstat (Genstat for Windows 15th Edition, VSN International, Hemel Hempstead, UK).

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 5 plants as described (39). For the transgenic rice, root exudates were collected from pots containing three 4-wk old plants. The exudates were collected on 3, 6 and 9 days after the start of P starvation and the three samples pooled for SL analysis. The root exudates were passed through an SPE C18-Fast column (500 mg/3mL) and the SL eluted with 6 mL 100% acetone. For root extracts 1 g fresh weight of ground root tissue was extracted following the method described previously (39) but the resulting extracts were evaporated to dryness, taken up in hexane, loaded on pre-equilibrated Silica gel Grace Pure SPE (200 mg/3ml) columns and eluted with 2 mL hexane:ethyle acetate (1:9) for further purification. The solvent was evaporated and the residue redissolved in 200 µl of 25% acetonitrile in water and filtered through Minisart SRP4 0.45 µm filters (Sartorius, Germany) before LC-MS/MS analysis.

SL analysis using liquid chromatography – tandem mass spectrometry (LC-MS/MS)

SLs were analysed 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 UPLC system using the settings previously described (39) with some modifications specified in *SI Material and Methods*. The analyses were performed in three biological replicates.

Striga germination bioassay

Root exudate germination stimulatory activity was assessed using a germination bioassay with *S. hermonthica* as described previously (39, 43). Approximately 50 to100 preconditioned *Striga* seeds on a 9-mm diameter glass fibre filter paper disc (Sartorius, Germany) were exposed to the column purified root exudates (50 µL per disc) after

acetone evaporation. Germination was scored after 48 h incubation in darkness at 30ºC. The synthetic SL GR24 (3.3 µM) 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 previously described (39). About 25 mg *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. Pre-germinated 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 70% and 400 µM m^{-2} 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 five days prior to tissue collection of roots. 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% ethanol (v/v), 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 synthetized using the iScript cDNA Synthesis Kit (BioRad) using 1µg of total RNA per sample following the manufacturer's instructions. The qRT-PCR reactions were prepared using iQ SYBR Green Supermix (BioRad). Per reaction 0,3 µM of each primer and 1µl 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 5 (control) or 4 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 (Rapid Amplification of cDNA Ends) cDNA Amplification Kit (Clontech, Palo Alto, CA). The primers and nested primers of RACE PCR were designed based on the predicted mRNA sequences in 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 Material and Methods*. 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 (seven to five plants per line) for p35S:*Os01g0701400*. Rosette branching was measured using the decapitation method (44).

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 http://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.* (45). The molecular map (46) contains 164 markers covering 1833 cM on 12 linkage groups. The identification of QTLs was performed by composite interval mapping using QTLCartographer version 1.15 (47). 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 1000 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 Analyser 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 using Panati (48) and ref 49. Fastq data has been deposited in the Short Read Archive at NCBI as Acc_ID SRA050654.1.

Assessing allelic diversity

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

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Supporting information

SI Material and Methods

Complementation of Arabidopsis max1-1 mutants

Open reading frames were amplified from cDNA using primers containing restriction sites or the Gateway™ 5'-CACC sequence in the forward cloning primer for directional cloning, as described in Table S4. The products were cloned either by restriction digest directly into the pART7 binary vector (1) containing the 35S promoter (*Os01g0700900*), or into Gateway™ entry vector pENTR/D-TOPO (Invitrogen), (*Os01g0701400* and *AtMAX1*). The pENTR vectors carrying *Os1g0701400* and *AtMAX1* were each combined with pENTR-p4p1r containing a CaMV 35S promoter and pENTR-p2rp3 containing a NOS terminator (kindly offered by the Department of Molecular Biology of Wageningen UR) into the destination expression vector pHm43GW (2) by a multisite gateway LR reaction (Invitrogen), resulting in 35S::*Os01g0701400* and 35S::*AtMAX1* in pHm43GW.

Constructs were transformed into max1-1 plants by floral dip (3). Single insertion lines were selected by antibiotic resistance, and brought to homozygosity. For assessment of branching phenotype, 35S::*Os01g0700900* plants were sown on F2 compost treated with Intercept 70WG (both Levington Horticultures, UK), and were stratified for 2 days at 4°C prior to moving to plant growth rooms or greenhouses with temperatures between 18-24°C and light period defined by the decapitation method experiment (4). 35S::*Os01g0701400* and 35S::*AtMAX1*, were grown in Arabidopsis soil pots in the climate room of Wageningen UR greenhouse with temperatures between 18-22°C and 60% humidity. Branching was scored using decapitation method (4).

Rice Bala variety complementation

Mature calli were prepared from mature seeds of Bala surface sterilized with 30 sec treatment of 75% ethanol followed by 30 min 2.5% sodium hypochlorite. Calli were induced on N6B5 basal medium (N6 medium basal salt mixture and B5 vitamins) (5, 6), supplemented with 30 g/L sucrose, 500 mg/L L-proline, 2 mg/L glycine, 500 mg/L L-glutamine, 300 mg/L casein acid hydrolysate, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 4 g/L phytagel, pH=5.8, in the dark at 28°C; transferring to fresh medium every week. Embryogenic calli were selected after 5-6 weeks to sub-culture on the same medium for 4 days before infection with agrobacterium. Calli were transformed as describe in Ozawa et al with modifications (7). The constructs p35S:*Os01g0700900*, p35S:*Os01g0701400* and empty pHm43GW with the CaMV35 promoter and nos-terminator elements were introduced separatedly by electroporation into *Agrobacterium* strains EHA105. After 3-day co-cultivation, calli were cultured for 3 weeks on N6B5

medium containing 400 mg/L carbenicillin and 50 mg/L hygromycin; transferring to fresh medium every week. For regeneration, the actively growing calli were transferred on MS basal medium supplemented with 500 mg/L L-glutamine, 500 mg/L L-proline, 30 g/L sucrose, 30 g/L sorbitol, 0.5 mg/L kinetin, 2 mg/L 6-BA, 0.2 mg/L NAA, 30 mg/L hygromycin B, and 4 g/L phytagel, pH=5.8, for 3 weeks at 28°C, 16-hour photoperiod. Shoots grown from the calli were transferred to rooting medium (1/2 MS medium, 30g/L sucrose, 50mg/L hygromycin, pH=5.8) to obtain T0 seedlings.

Detection and quantification of strigolactones by liquid chromatography – tandem mass spectrometry (*LC-MS/MS*)

Chromatographic separation was obtained on an Acquity UPLC BEH C18 column (100 mm, 2.1 mm, 1.7 mm; Waters, USA) by applying a water/acetonitrile gradient to the column starting from 5% of acetonitrile for 0.33 min and rising to 27% (v/v) of acetonitrile in 0.67 min, followed by a 4.33 min gradient to 40% (v/v) acetonitrile, followed by a 4.0 min gradient to 65% (v/v) acetonitrile, which was maintained for 0.67 min, followed by a 0.2 min gradient to 90% (v/v) acetonitrile, which was maintained for 0.46 min before going back to 5% acetonitrile using a 0.2 min gradient, prior to the next run. Finally, the column was equilibrated for 2.47 min using this solvent composition. Total run was 12 min. The column was operated at 50 ºC with a flow-rate of 0.5 ml min-1. Sample injection volume was 20 µl. The mass spectrometer was operated in positive 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 150ºC and the desolvation temperature at 650ºC. The cone voltage was optimized for each standard compound using the IntelliStart MS Console (Waters). Argon was used for fragmentation by collision induced dissociation (CID). Multiple reaction monitoring (MRM) was used for strigolactone identification and quantification. Parent-daughter transitions for the standards, strigolactones and $[^2H]_6$-2'-*epi*-5-deoxystrigol, deuterium labelled strigolactones (used as internal standards), were set using the IntelliStart MS Console. MRM-transitions selected for identification of strigolactones in rice were: for *ent*-2'-*epi*-orobanchol mass-to-charge ratio (*m/z*) 347.20 > 97.00 at a collision energy of 22 eV, 347.20 > 205.20 at 18 eV and 347.20 > 233.15 at 12 eV; for *ent*-2'-*epi*-5-deoxystrigol *m/z* 331.20 > 97.00 at 22eV, 331.20 > 216.15 at 15 eV and 331.20 > 234.15 at 10 eV, for methoxy-5-deoxystrigol isomers *m/z* 361.20 > 96.96 at 20 eV and 361.20 > 247.00 at 10 eV; for $[^2H]_6$-2'-*epi*-5-deoxystrigol *m/z* 337.16 > 97.02 at 22eV and 337.16 > 240.19 at 10eV. Cone voltage was set to 18, 15, 20 and 16 eV, correspondingly.

Strigolactones were quantified using a calibration curve with known amount of standards and based on the ratio of the peak areas of the MRM-transition for strigolactone standards (*m/z* 347.20 > 97.00; 331.20 > 97.00) to the MRM transition for $[^2H]_6$-2'-*epi*-

5-deoxystrigol (*m/z* 337.16 > 97.02). Methoxy-5-deoxystrigol isomers were quantified using only peak areas for the transition *m/z* 361.20 > 247.00. Data acquisition and analysis were performed using MassLynx 4.1 (TargetLynx) software (Waters).

References for the Supplemental materials and methods

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SI Figures

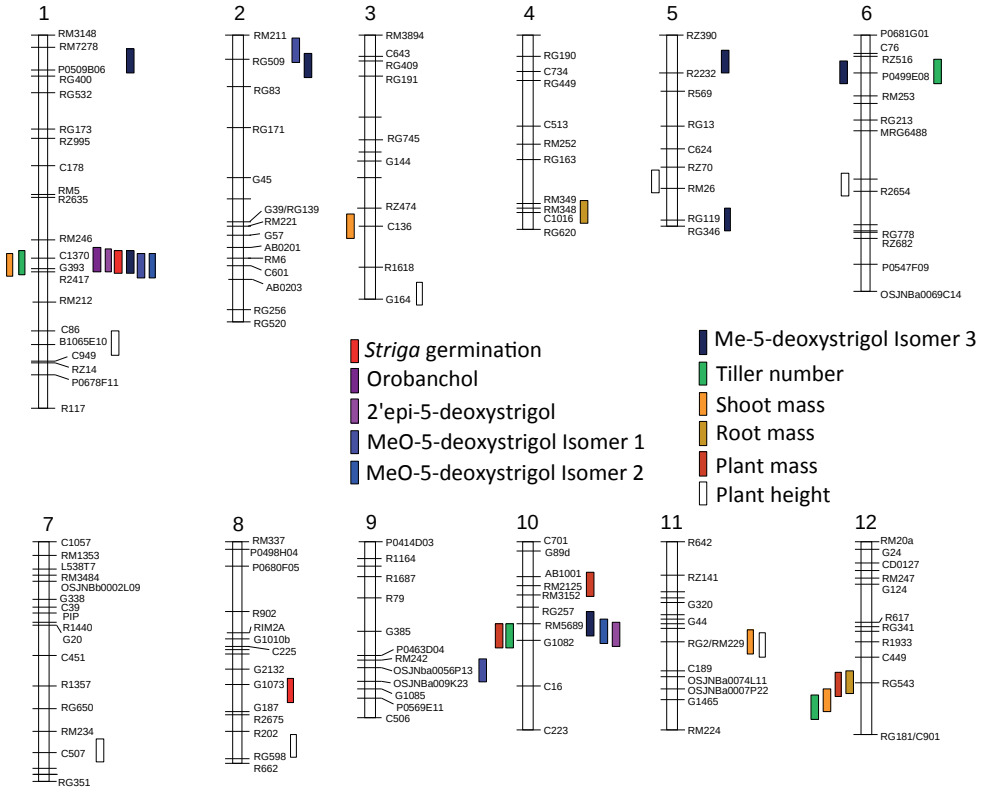

Fig. S1 Molecular map of QTLs for traits analysed in a Bala x Azucena mapping population. The indicated markers are RFLP and microsatellite markers (AFLP marker names omitted unless mentioned in the text). All indicated QTLs have a LOD score above 2.5. QTLs indicated on the right of a chromosome indicate a positive contribution by the Azucena allele, QTLs on the left indicate a positive contribution of the Bala allele. See Table S1 for details.

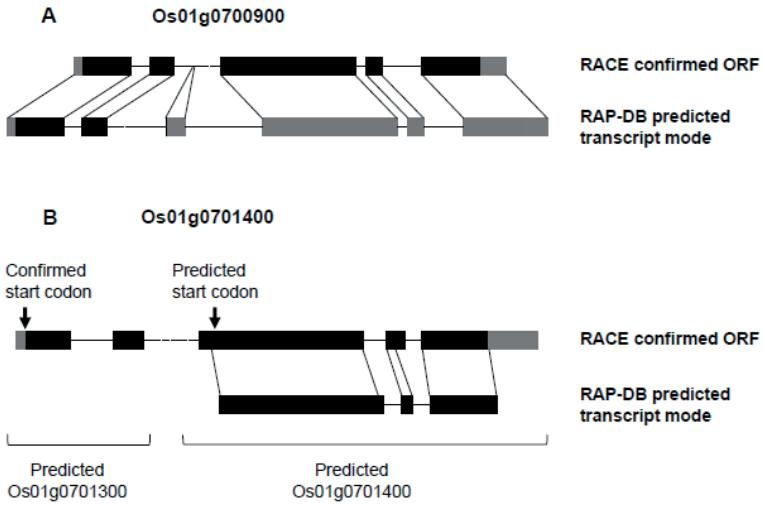

Fig. S2 *Os01g0700900* (A) and *Os01g0701400* (B) mRNA sequence analyses. Comparison between the gene model predicted by IRGSP and the open reading frame identified with RACE-PCR. Grey shaded bars represent untranslated regions and black shaded bars the coding region between START and STOP codons. The IRGSP predicts *Os01g0701300* and *Os01g0701400* genes that were confirmed to be one single ORF.

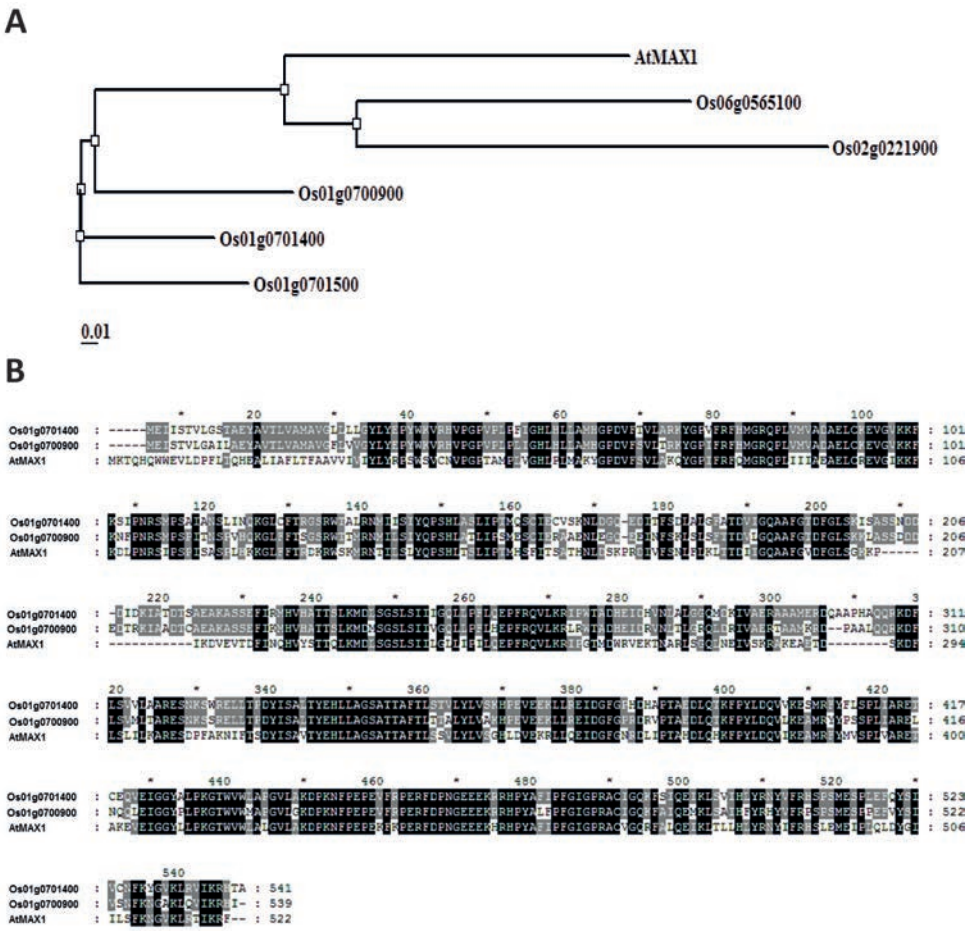

Fig. S3 *MAX1* orthologs sequence analyses. (A) Similarity tree of the CYP711A1 found in the rice genome and *AtMAX1* (NP_565617) protein sequences using the sequences confirmed by RACE-PCR of *Os01g0700900* and *Os01g0701400* and the predicted sequences of *Os01g0701500*, *Os02g0221900* and *Os06g0565100*. The tree was built using clustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). (B) Sequence alignment of the amino acid sequences of *AtMAX1* (NP_565617), and the confirmed sequences of *Os01g0700900* and *Os01g0701400*.

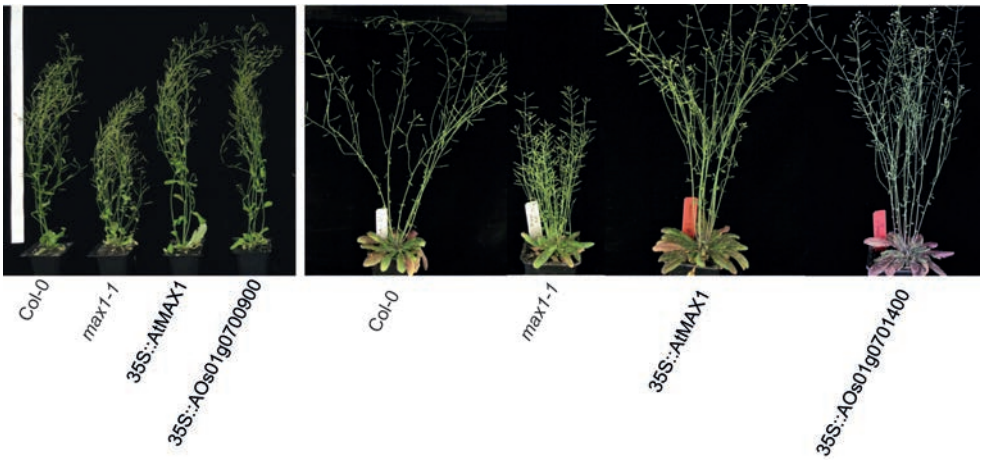

Fig. S4 Effect of complementation of *Arabidopsis max1-1* with p35S:*AtMAX1*, p35S:*Os01g0700900* and p35S:*Os01g0901400* on branching phenotype and comparison with wild-type Col-0 and *max1-1*.

SI tables

Table S1 Main-effect quantitative trait loci for traits measured on the Bala x Azucena recombinant inbred line population.[1]MeO-5-DS = methoxy-5-deoxystrigol

Trait	Chromo-some	QTL position		LOD	R^2 (%)	Donor of positive allele
		in cM	in Mbp (approx.)			
Striga 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
ent-2'*epi*-5-deoxystrigol	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[1]	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[1]	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[1]	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	2.9	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

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. [a]A= Azucena allele. B= Bala allele; [b]According to Zhao et al. (17); [c] Phi – Philippines, Ban – Bangladesh, Chi – China, Ind – India; [4]*ent*-2'-epi-5-DS – *ent*-2'-epi-5-deoxystrigol; [5]MeO-5DS – methoxy-5-deoxystrigol.

			Impact of the presence of the rearrangement on strigolactone levels and number of tillers in different rice lines								
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
				orobanchol	*Ent*-2'-epi-5-DS[4]	orobanchol	*Ent*-2'-epi-5-DS	MeO-5DS isomer 2[5]	MeO-5DS isomer 3[5]		
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
Sinaguing	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

Table S3 Primers used for quantitative RT-PCR

Primers for quantitative RT-PCR		
Primer pair		**Locus / Gene**
GGCTTCTCTGCTTGCTGCTTC	ACCAACGGTTGCCTTCCC	*Os01g0700900*
ACGACGGCGTTCACTCTCTC	TCCGAACCCGTCAATCTCC	*Os01g0701400*
AGCGAAGGTATCCACTAGGC	CCGGGGATATAGACACTC	OsPI1 - *Os01g0838350*
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**
Os01g0700900 5RACE primer	GTGGATCGCTGACAATTTCATCTC
Os01g0700900 5RACE Nested primer	GCAGCTCCCTCGAGGATTTGTTCG
Os01g0700900 3RACE primer	GCAAGATCGCGGCCGATACCTG
Os01g0700900 3RACE Nested primer	CGGACGGCTGCAATGAAGCGAG
Os01g0700900 Forward Cloning primer (with EcoRI site)	gggggaattcATGGAGATCAGCACAGTG
Os01g0700900 Reverse Cloning primer (with ClaI site)	ggggatcgatTTATATATGCCTCTTGATGACCTG
Os01g0701400 5RACE primer	CATATCCTCCAATTTCAACCTGTTCGCAC
Os01g0701400 5RACE nested primer	GCGTAAGAAGCTCCCGCCAGG
Os01g0701400 3RACE primer	CGGGAGCTTCTTACGCCGGAC
Os01g0701400 3RACE nested primer	CGTGCGAACAGGTTGAAATTGGAGGATATG
Os01g0701400 cloning forward primer(with pENTR/D-TOPO cloning site)	caccATGGAGATCATCAGCACAGTGCTG
Os01g0701400 cloning reverse primer	CTATGCAGTGTGCCTCTTGATGACCCG
AtMAX1 cloning forward primer(with pENTR/D-TOPO cloning site)	caccATGAAGACGCAACATCAAT
AtMAX1 cloning reverse primer	TCAGAATCTTTTGATGGTTCTGAGC

Table S5 Primer used for assessing allelic diversity

Primer for assessing allelic diversity			
Primer pair		**Locus / Gene**	**Product size**
GGACGTGTTCTCAGTGCTCA	TCCTTGCACAACGCATC	*Os01g0700900*	200 bp
ACAGTGCTGGGCTCAACG	GGCTCGTACAAGTAGCCCAAC	*Os01g0701400*	100 bp
TCAAAGCTGCCAGTCACACC	ATCCGGACAGGTCCATCTTG	*Os01g0701500*	150 bp

Chapter 4

Rice cytochrome P450 *MAX1* homologs catalyse distinct steps in strigolactone biosynthesis

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A revised version has been accepted by Nature Chemical Biology (2014) as a research article

Abstract

Strigolactones are a class of phytohormones and rhizosphere signaling compounds. By reconstituting the strigolactone biosynthetic pathway in *Nicotiana benthamiana*, we show that a rice homolog of *Arabidopsis MORE AXILLARY GROWTH 1* (*MAX1*), a cytochrome P450 CYP711 subfamily member, mediates the stereo-selective conversion of carlactone into *ent*-2'-*epi*-5-deoxystrigol. A second rice *MAX1* homolog then catalyzes the conversion of *ent*-2'-*epi*-5-deoxystrigol to orobanchol. We therefore report the first enzymes involved in B-C ring closure and a subsequent structural diversification step of strigolactones.

Strigolactones (SLs) are plant hormones that regulate various aspects of plant architecture and development, such as shoot branching/tillering and root architecture[1-3]. SLs are also secreted by plants as rhizosphere signaling molecules that induce hyphal branching in arbuscular mycorrhizal (AM) fungi and seed germination in root parasitic plants[4,5]. All SLs contain a tricyclic-lactone (ABC-rings) connected to a butenolide (D-ring) via an enol ether bridge[6] (**Scheme 1**). The naturally occurring SLs are classified into two groups of diastereoisomers with opposite C-ring orientation but identical stereochemistry at C-2´, coined strigol-type and orobanchol-type SLs[7,8] (**Supplementary Results, Supplementary Fig. 1a**). It has been proposed that 5-deoxystrigol (5DS) and *ent*-2´-*epi*-5-deoxystrigol (*ent*-2'-*epi*-5DS) are the key precursors for these two groups of SLs[6,9,10]. The presence of substituents in the A- and B-rings of these two precursors defines a series of different SLs, of which several may occur within one plant species[6,11].

SLs are biosynthetically derived from the carotenoids[12]. Mutants in *DWARF27* (*D27*), *CAROTENOID CLEAVAGE DIOXYGENASE 7* (*CCD7*) and *CAROTENOID CLEAVAGE DIOXYGENASE 8* (*CCD8*) have reduced SL levels suggesting their involvement in the SL biosynthetic pathway[1,2,13]. It was recently demonstrated that D27 catalyses the isomerisation of all-*trans*-β-carotene to 9-*cis*-β-carotene, which is sequentially cleaved by CCD7, to form 9-*cis*-β-apo-10′-carotenal, and then by CCD8 to yield C9-C10 *cis*-configured carlactone (CL), (*Z*)-CL[14] (**Scheme 1a**). Theoretically, the C-11 stereochemistry of (*Z*)-CL could be either (*R*) or (*S*) (**Supplementary Fig. 1b**). However, Seto *et al.* (2014) detected only the (*11R*)-configured (*Z*)-CL in rice (*Oryza sativa*) and *Arabidopsis* and demonstrated that this isomer is a precursor of SLs *in vivo*[15]. The enzyme(s) responsible for the conversion of CL to SLs - during which the BC lactone moiety is formed - is unknown, but it is likely that it is mediated through oxidation followed by a ring closure, possibly involving the cytochrome P450 *MORE AXILLARY GROWTH 1*, *MAX1*[14-17]. In contrast to *Arabidopsis* that only has one *MAX1* gene, rice has five *MAX1* homologs (*OsMAX1*s): *Os01g0700900* (*Os900*), *Os01g0701400* (*Os1400*), *Os01g0701500* (*Os1500*), *Os02g0221900* (*Os1900*) and *Os06g0565100* (*Os5100*).

To investigate the role of these *OsMAX1s*, we transiently expressed the SL biosynthetic genes *OsD27*, *OsCCD7* and *OsCCD8* in combination with each of the *OsMAX1s* in *Nicotiana benthamiana* leaves using *Agrobacterium tumefaciens.* Subsequent analysis of leaf extracts from these plants by MRM-LC-MS/MS showed that simultaneous expression of *OsD27*, *OsCCD7* and *OsCCD8* resulted in the production of CL (**Fig. 1a**). To verify the stereochemistry of the CL produced, a racemic mixture of (*Z*)-CL was synthesised as previously described[18], and separated into two enantiomers by semi-preparative chiral HPLC (**Supplementary Fig. 2a**). Circular dichroism (CD)-spectra were used to confirm the stereochemistry of the CL isomers[15] (**Supplementary**

Fig. 2b). To assign which of the two synthetic isomers occurs *in planta*, we compared CL produced *in vitro* by OsCCD8 and CL produced using transient expression of *OsD27*, *OsCCD7* and *OsCCD8* (the CL biosynthetic genes) in *N. benthamiana* with the two synthetic (*Z*)-CL enantiomers using chiral LC-TOF-MS analysis. This demonstrated that CL products are exclusively (*Z*)-(*R*)-CL with (*11R*)-configuration in both cases (**Supplementary Fig. 3a, b**).

We then carried out co-expression of each of the *OsMAX1s* with the CL biosynthetic genes in *N. benthamiana*. Among the five *OsMAX1s*, *Os900* exhibited 800- to 10,000-fold higher activity than the other *OsMAX1s* as indicated by the increase in *epi*-5DS with a corresponding decrease in (*Z*)-(*R*)-CL (**Fig. 1a, b**). However, *Os1500* did not show any activity (**Fig. 1b**), which is consistent with its premature stop codon and its inability to rescue the *Arabidopsis max1* bushy phenotype[19]. To assign which 5-deoxystrigol stereoisomer was produced we used chiral LC-MS/MS and confirmed the formation of predominantly *ent*-2'-*epi*-5DS which has been shown to be the 5DS stereo-isomer present in rice root exudates (**Supplementary Fig. 4a**)[7]. A trace of 5DS was detected with individual co-expression of *Os900*, *Os1400*, *Os1900* and *Os5100*, but at around 1000- to 10,000-fold lower than the production of *ent*-2'-*epi*-5DS (**Supplementary Fig. 4a-c**).

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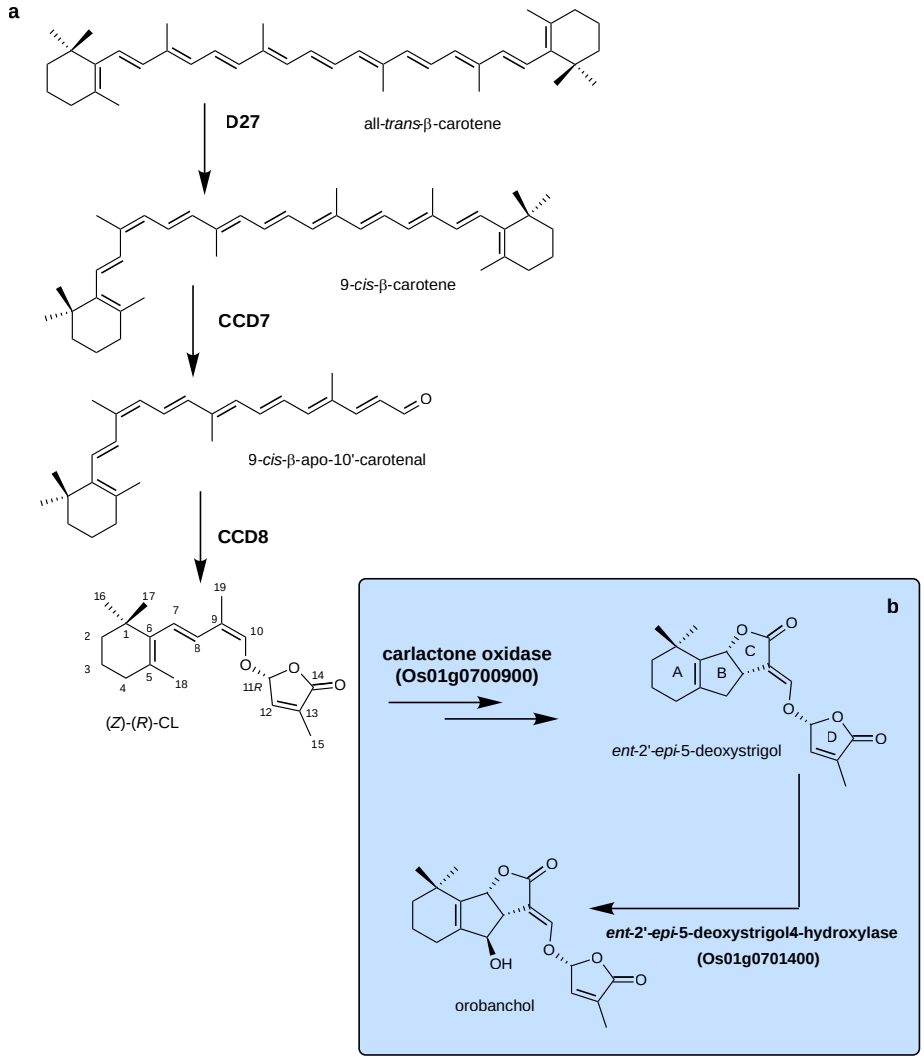
Interestingly, simultaneous expression of all four active *OsMAX1s* with the CL biosynthetic genes resulted in a pronounced decline in *ent*-2'-*epi*-5DS compared with co-expression of *Os900* alone (**Fig. 1b**). By targeted screening of the major known SLs, we observed an almost 800-fold increase in orobanchol level (**Fig. 1c**). Further chiral LC-TOF-MS analysis revealed that the orobanchol produced consists exclusively of the rice orobanchol isomer (**Supplementary Fig. 4d, e**). This suggests that one of the other three OsMAX1s (encoded by *Os1400*, *Os1900* or *Os5100*) catalyses the hydroxylation at C-4 of *ent*-2'-*epi*-5DS to form orobanchol. Different combinations of *OsMAX1* homologs with the CL biosynthetic genes were assayed and this revealed that orobanchol production was the result of *Os1400* expression (**Supplementary Fig. 5a,b**). We hence conclude that Os1400 is an *ent*-2'-*epi*-5-deoxystrigol-4-hydroxylase and that in rice, *ent*-2'-*epi*-5DS is the parent SL from which orobanchol is derived.

To further confirm the function of the OsMAX1s, we expressed *Os900*, *Os1400*, *Os1900* and *Os5100* in the yeast WAT11 strain, which also expresses the *Arabidopsis NADPH-P450 REDUCTASE 1* (*ATR1*) gene, and incubated the resulting microsomal preparations with natural (*Z*)-(*R*)-CL produced *in vitro* using OsCCD8[14]. Subsequent MRM-LC-MS/MS analysis showed *ent*-2'-*epi*-5DS formation only with the microsomes isolated from yeast expressing *Os900*, but not with microsomes that contain other *OsMAX1s* (**Supplementary Fig. 6**). This is in agreement with the results obtained

with *N. benthamiana* showing that only Os900 can convert CL to *ent*-2'-*epi*-5DS. We therefore conclude that Os900 has carlactone oxidase activity both *in vitro* and *in vivo*.



Scheme 1. Strigolactone biosynthesis. (**a**) The path from all-*trans*-β-carotene to the strigolactone precursor carlactone. The enzymes D27, CCD7 and CCD8 catalyse the formation of a single carlactone isomer with C-11 *R*-configuration from all-*trans*-β-carotene. (**b**) The formation of the strigolactone B-C rings by *Os01g0700900* and orobanchol by *Os01g0701400* in rice. *Os01g0700900* encodes a carlactone oxidase, which catalyses formation of the B- and C-rings of *ent*-2´-*epi*-5DS, while the D-ring is maintained from (*Z*)-(*11R*)-CL. Hydroxylation of *ent*-2´-*epi*-5DS at C-4 to orobanchol is catalysed by Os01g0701400, an *ent*-2´-*epi*-5-deoxystrigol-4-hydroxylase. The blue box indicates the bioconversion steps demonstrated in the current study.

To explore the stereospecificity of the Os900 carlactone oxidase activity, we then separately incubated (*Z*)-(*R*)-CL and (*Z*)-(*S*)-CL with yeast microsomes expressing Os900, and found that only (*Z*)-(*R*)-CL was converted to *ent*-2'-*epi*-5DS, indicating that Os900 has high substrate specificity and is enantio-specific (**Fig. 2a)**. The conversion of CL to *ent*-2'-*epi*-5DS most likely involves oxidation at C-18 and C-19 resulting in a ring closure and B-C lactone formation (**Supplementary Fig. 7**). Kinetic analysis of Os900 activity as a carlactone oxidase in yeast microsomes revealed a very low estimated *Km* (0.69 µM) *in vitro*, demonstrating a high affinity for (*Z*)-(*R*)-CL as a substrate, albeit with low maximum enzyme velocity (V_{max}) and end product yield (**Supplementary Fig. 8a; Supplementary Table 1**). Multistep oxidation reactions catalysed by one cytochrome P450 are not uncommon in plants[20,21]. The catalysis of such reactions in yeast may however result in reduced efficiency if the intermediate reaction products leave the active site. It could also be that Os900 requires a cofactor[22], or has a particular codon distribution that yeast does not adequately recognize[23]. Hence, we conclude that Os900 is a carlactone oxidase involved in the formation of the SL B-C moiety, and that the D-ring orientation is maintained from (*Z*)-(*R*)-CL.

To further characterise the activity of Os1400, we performed yeast microsome assays using the four possible 5-deoxystrigol stereoisomers (5DS, *ent*-5DS, 2´-*epi*-5DS and *ent*-2´-*epi*-5DS) as substrates. This demonstrated that Os1400 exclusively converts *ent*-2´-*epi*-5DS to orobanchol (**Fig. 2b**) with a K_m and V_{max} of 0.74 µM and 50.69 pmol·min^{-1}·µg protein^{-1}, respectively (**Supplementary Fig. 8b**; **Supplementary Table 1**), demonstrating a high affinity for *ent*-2´-*epi*-5DS. Similar assays with microsomes containing the other OsMAX1 homologs did not show any conversion.

To underpin our experimental results, we also performed protein modelling and substrate docking studies which show that the OsMAX1 proteins exhibit differences in their ability to dock (*Z*)-(*R*)-CL. Particularly Os900 and Os1400 were predicted to dock (*Z*)-(*R*)-CL in orientations consistent with its conversion to *ent*-2'-*epi*-5DS, that is with a small enough distance (< 5Å) between both C-18 and C-19 and the heme, where oxidation should take place to result in B-C-ring closure[14] (**Supplementary Table 2; Supplementary Fig. 7, 9a, b**). Docking studies using all four 5-deoxystrigol stereoisomers as substrate predict that of the five OsMAX1s, Os1400 is best able to dock them in an orientation consistent with conversion to orobanchol (distance between C-4 and heme < 5Å) (**Supplementary Fig. 9c, d**), but with no clear difference between the four stereoisomers (**Supplementary Table 3**). Given the fact that Os900 and Os1400 belong to the same MAX1 clade in monocots but have different catalytic activities[19], it is interesting that there are only a few amino acid residues (Phe124/Lys228/Ser408 in Os900 and Cys124/Arg227/Leu409 in Os1400) in the immediate neighbourhood of the

docked substrate which distinguish Os900 from Os1400 (**Supplementary Fig. 9b, d, 10**).

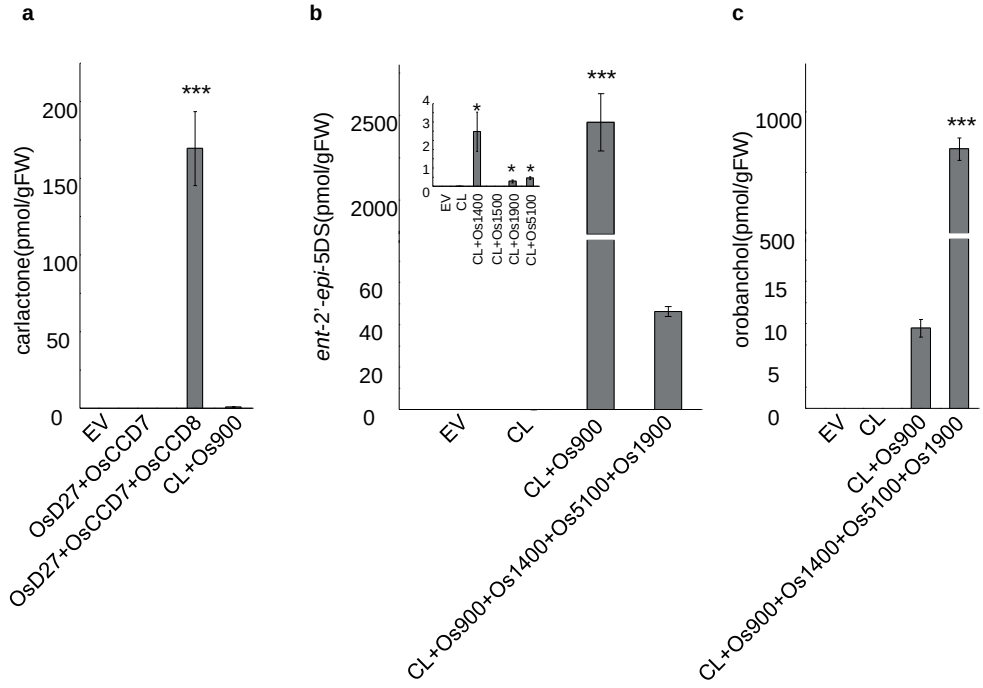


Figure 1. Strigolactone biosynthesis in Nicotiana benthamiana by MRM-LC/MS analysis. (a) Transient expression of *OsD27*, *OsCCD7* and *OsCCD8* in *N. benthamiana* results in the formation of carlactone (transition [M+H]+ m/z 303>207) (compared with *OsD27*+*OsCCD7* treatment), which is reduced upon *Os900* co-expression (compared with *OsD27*+*OsCCD7*+*OsCCD8* treatment). (b) Transient expression of carlactone biosynthesis genes (*OsD27*, *OsCCD7* and *OsCCD8*) together with the *OsMAX1s* results in the formation of *ent*-2´-*epi*- 5DS (transition [M+H]+ m/z 331.2>234.15) (compared with CL treatment). (c) Orobanchol formation in *N. benthamiana* transient expression assay (transition [M+H]+ m/z 347.2>233.15) (compared with CL+900 treatment). Bars represent mean ± SE (n=5). * and *** indicate significant differences at P<0.05 and P<0.001, respectively, as determined by a pairwise Tukey's HSD test performed using R 3.0.2. CL represents gene combination *OsD27*, *OsCCD7* plus *OsCCD8*. EV represents control samples infiltrated with empty vector.

In this study we show that rice cytochrome P450 Os900 is involved in catalysing the conversion of (*Z*)-(*R*)-CL to the parent SL, *ent*-2'-*epi*-5DS **(Scheme 1b**). A second rice MAX1 homolog, Os1400, efficiently hydroxylates *ent*-2´-*epi*-5DS at C-4 to form orobanchol, which represents the ideotype of one of the two SL subfamilies in plants (**Scheme 1b**). Intriguingly, *Arabidopsis* contains only one *MAX1* homologue, *AtMAX1*. We questioned whether or not this one *MAX1* gene is able to convert (*Z*)-(*R*)-CL to orobanchol, one of the SLs reported to be produced by *Arabidopsis*[16]. Therefore, we

reconstructed the *Arabidopsis* SL biosynthetic pathway in *N. benthamiana* by co-expressing *AtD27*, *AtMAX3*, *AtMAX4* and *AtMAX1*. Similar as what was observed for the rice *MAX1s Os1900* and *Os5100*, we detected a small amount of 5DS and *epi*-5DS (although the amount is not enough to resolve their stereochemistry), but no orobanchol (**Supplementary Fig. 11**). The low amounts of 5DS and *epi*-5DS produced by the expression of the *Arabidopsis* pathway in *N. benthamiana* may indicate that another (co-) factor(s) and/or enzyme present in *Arabidopsis* is needed to produce more substantial amounts of 5DS/*epi*-5DS. Another possibility is that in *Arabidopsis* AtMAX1 produces mostly other compounds from carlactone, such as SL-LIKE1 reported by Seto et al. (2014)[15]. But in our assay we did not detect this compound nor any putative SL-LIKE1 precursor, or any similar compounds.

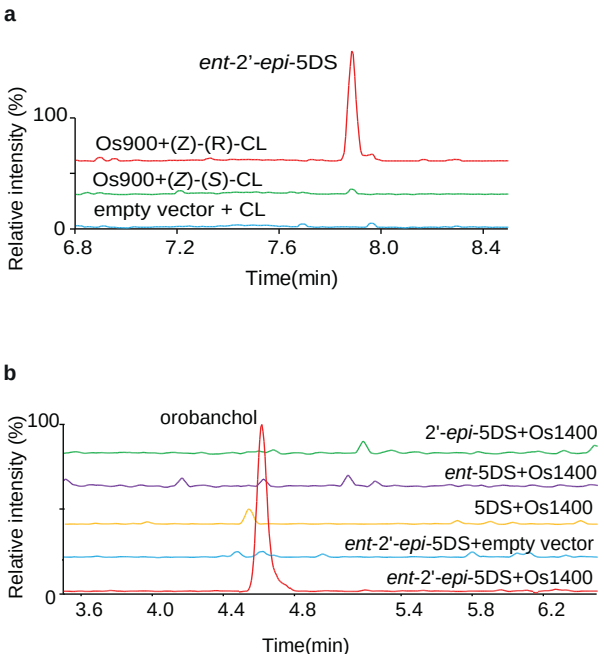


Figure 2. MRM-LC-MS/MS analysis of product formation by microsomes of yeast expressing OsMAX1s. (**a**) Representative MRM-LC-MS/MS chromatograms of *ent*-2´-*epi*-5DS (transition $[M+H]^+$ *m/z* 331.2 > 216.15) produced by microsomes isolated from *Os900* expressing yeast with either (*Z*)-(*11R*)-CL or (*Z*)-(*11S*)-CL as substrate. (**b**) MRM-LC-MS/MS analysis showing the formation of orobanchol (transition $[M+H]^+$ *m/z* 347.2 > 205.2) by microsomes isolated from *Os1400* expressing yeast from *ent*-2´-*epi*- 5DS. Other 5-deoxystrigol isomers were not converted. The chromatograms show representative results obtained in more than three independent experiments with multiple technical replicates (with empty vector pYeDp60 as control).

Intriguingly, a recent study showed that a deletion of *Os900* and *Os1400* in rice

cultivar Bala caused a strong reduction (but not complete disappearance) of SLs in this cultivar[22]. This implies that other enzymes are sufficient for (low level) SL production in this rice cultivar[24]. Our current data show that Os900 is the primary enzyme responsible for carlactone oxidation to *ent*-2'-*epi*-5DS, the precursor of the other known rice SLs such as orobanchol. However, Os1400, Os1900 and Os5100 also produce small amounts of *ent*-2'-*epi*-5DS/5DS upon expression in *N. benthaminana* (**Fig. 1b, Supplementary Fig. 4**). This is consistent with the observation that all four rice homologs of *MAX1* are able to repress shoot branching in the *Arabidopsis max1* mutant[19]. Of all the rice *OsMAX1s* expressed in yeast, only *Os1400* was able to convert *ent*-2'-*epi*-5DS to orobanchol (**Fig. 2b**). Nevertheless, Bala, in which *Os1400* is deleted, still produces orobanchol. This and the fact that AtMAX1 cannot produce orobanchol in *N. benthamiana* (even though *Arabidopsis* has been reported to produce it[16]) suggest that, in rice as well as *Arabidopsis*, an additional gene, probably not a *MAX1* homolog, is responsible for the biosynthesis of orobanchol.

To date, all characterized natural SLs have C-2´ *R*-configuration[7,8], while the orientation of the C-ring can be α or β. We have shown that rice *MAX1 Os900* is involved in catalysing the B-C ring closure to generate *ent*-2'-*epi*-5DS, the precursor of the orobanchol-type SLs with α oriented C-ring. We postulate that carlactone oxidase homologs in plant species predominantly producing strigol-type SLs convert (*Z*)-(*R*)-CL to the other parent SL, 5DS (**Supplementary Fig. 7**).

According to Challis et al. (2013), the five *OsMAX1s* belong to three distinct CYP711 clades: a chromosome 1 (*Os900*, *Os1400* and *Os1500*), a chromosome 2 (*Os1900*) and a chromosome 6 clade (*Os5100*). Among them, the chromosome 1 clade has diverged only recently, while the chromosome 2 and chromosome 6 clades have diverged earlier (soon after the origin of *MAX1s* in dicots)[19]. It will be of great interest to study what (other) enzymatic reactions are catalysed by the *MAX1* homologs in the different CYP711 clades in monocot and dicot plants[19], whether they can create different stereochemistry and/or whether they are involved in other structural diversification reactions in SL biosynthesis. Just as intriguing is the fact that there must be other enzymes involved in SL formation and it will be interesting to see to which classes they belong to.

The selection pressure(s) that has resulted in MAX1 functional diversification also provides an intriguing research question, considering that there is large variation in the biological activity of the different SLs, including of different enantiomers, in different biological processes[11,25,26]. One intriguing fact is that orobanchol is the most potent natural SL in inducing AM fungi hyphal branching among those tested by Akiyama et al. (2010)[27], whereas it is much less active in stimulating *Striga hermonthica* seed germination than *ent*-2'-*epi*-5DS[26]. Possibly, the orobanchol producing activity of

Os1400 has resulted from an evolutionary benefit, i.e. making SLs with high activity towards AM fungi but not towards *Striga*, which has resulted in *MAX1* diversification in rice. Identification of the other enzymes involved in SL structural diversification will open up possibilities to modify SL composition in plants at will, allowing us to study the biological relevance of SL structural diversity in plants and apply this knowledge in crop breeding.

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Author contributions

Y.Z, C.R and H.J.B designed the research. Y.Z, A.S, G.R.F, M.H, T.C, F.V, and J.H performed the experiments. A.S and B.Z synthesised standards. A.D.J.D conducted the protein modelling and docking. Y.Z, A.D.J.D, A.S, G.R.F, S.K, O.L, S.M.S, B.Z, S.A, C.R, and H.J.B wrote the manuscript. C.R and H.J.B supervised the project.

Competing financial interests

The authors declare no competing financial interests.

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Supplementary Information

Supplementary Methods

1. Plasmids construction

Full-length ORFs of rice MAX1s (*Os01g0700900*, *Os01g0701400*, *Os01g0701500*, *Os06g0565100* and *Os02g0221900*) were obtained from the rice annotation project database (http://rapdb.dna.affrc.go.jp/) and confirmed by RACE PCR[1,2]. Full-length ORFs of *Arabidopsis* genes (*AtD27*, *AtMAX3*, *AtMAX4* and *AtMAX1*) were obtained from TAIR (http://www.arabidopsis.org/). The ORF fragments were amplified by Phusion polymerase (New England Biolabs) from rice Nipponbare wild-type cDNA and subsequently transformed into the cloning vector pJET1.2 (Fermentas) for sequencing. The positive clones were sub-cloned by digestion and ligation to linearized impact vector PIV1A_2.1 containing a CaMV35S promoter[3], yeast expression vector pYeDP60 or a modified pYeDP60 vector with Not I and Pac I restriction sites [4,5].

For *Nicotiana benthamiana* agro-infiltration, *OsCCD7* (*Os04g550600*) and *OsCCD8* (*Os01g0706400*) were amplified from *p35S:OsCCD7:PC1390* and *p35S:OsCCD8b:PC1390*, pCAMBIA1390 derivatives expressing *OsCCD7* or *OsCCD8* under the control of CaMV35S promoter, using the primers with the desired restriction sites. *OsD27* was amplified from plasmid *p35s:OsD27:PJTK13* kindly provided by Prof. Yonghong Wang (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences), using the primers with the desired restriction sites. The *Arabidopsis* genes (*AtD27*, *AtMAX3*, *AtMAX4* and *AtMAX1*) were isolated from *Arabidopsis* leaf cDNA. Amplified fragments were then inserted into pIV1A_2.1. The pIV1A_2.1 entry clones were confirmed by sequencing and LR reactions were performed to transfer the gene fragments into the pBin-Plus binary vector[6], using Gateway LR clonase II enzyme mix (Invitrogen) according to the manufacturer's instructions. *Os06g0565100* was cloned into binary vector PART7-PART27 system[1]. The binary vectors were introduced into *Agrobacterium tumefaciens* AGL0 by electroporation.

For yeast expression, *Os06g0565100* was amplified from p*35s:Os5100:PART27* using the Advantage GC2 polymerase mix (Clontech, Canada) and the corresponding primers and cloned into pGEMT-easy for sequence confirmation. Digestion and ligation were performed to clone *Os06g0565100* into pYeDP60. The other four *OsMAX1s* were cloned from Nipponbare wild-type cDNA into pYeDP60 as above. The resulting pYeDP60-OsMAX1s plasmids were transformed into *Saccharomyces cerevisiae* strain WAT11[4].

2. Primers used in this study

Primers used for constructing of agro-infiltration and yeast expression. Restriction sites are included in the primer sequences.

Primer	Restriction site	Sequence (5´-3´)
OsD27-Aro-F	NcoI	catgCCATGGCAATGGAGACCACCACGCTTG
OsD27-Agro-R	NotI	atttGCGGCCGCTCAGATGGAGCAATTCACACC
OsCCD7-Agro-F	Xbal	CTAGtctagaATGGCAACACAAGCGATTG
OsCCD7-Agro-R	BamHI	cgGGATCCTCATTCATCTCCCCAGAAACC
OsCCD8-Agro-F	Xbal	CTAGtctagaATGTCTCCCGCTATGCTGC
OsCCD8-Agro-R	NcoI	catgCCATGGTTACTTGCTGTTCCTTTTCCTGG
Os900-Agro-F	NotI	atttGCGGCCGCTATGGAGATCAGCACAGTGC
Os900-Agro-R	SacI	CgagctcTTATATATGCCTCTTGATGACCTG
Os900-Pyed-F	NotI	ATTTGCGGCCGCATGGAGATCAGCACAG
Os900-Pyed-R	PacI	GGTTAATTAATTATATATGCCTCTTGATGACC
Os1400-Agro-F	NotI	atttGCGGCCGCTATGGAGATCATCAGCACAGTG
Os1400-Agro-R	SacI	CgagctcCTATGCAGTGTGCCTCTTGATG
Os1400-Pyed-F	NotI	ATTTGCGGCCGCATGGAGATCATCAGCAC
Os1400-Pyed-R	PacI	GGTTAATTAACTATGCAGTGTGCCTC
Os1500-Agro-F	NotI	atttGCGGCCGCTATGGACATCAGCGAGGTGC
Os1500-Agro-R	SacI	CgagctcCTAGAACTCGAGAGGGGACTCCAT
AtD27-Agro-F	NcoI	catgCCATGGCAATGAACACTAAGCTATCACTTTCTCA
AtD27-Agro-R	NotI	atttGCGGCCGCCTAATGCTTCACACCGTAGCT
AtMAX3-Agro-F	NotI	atttGCGGCCGCtATGTCTCTCCCTATCCCGCC
AtMAX3-Agro-R	SacI	CgagctcTTATTTGTGTGAAAGGTGAAAAGCAATA
AtMAX4-Agro-F	Xhol	CCGctcgagATGGCTTCTTTGATCACAACCA
AtMAX4-Agro-R	NotI	atttgcggccgcTTAATCTTTGGGGATCCAGCAA
AtMAX1-Agro-F	NcoI	catgCCATGGCAATGAAGACGCAACATCAATGG
AtMAX1-Agro-R	NotI	atttGCGGCCGCTCAGAATCTTTTGATGGTTCTGAG
Os1500-Pyed-F	NotI	ATTTGCGGCCGCATGGACATCAGCGAGGTGC
Os1500-Pyed-R	PacI	GGTTAATTAACTAGAACTCGAGAGGGGACTCCAT
Os1900-Agro-F	XhoI	CCGctcgagATGCAAGCATCTTCCATG
Os1900-Agro-R	BamHI	cgGGATCCTCAGGTGTTGGTCCTCTTG
Os1900-Pyed-F	BamHI	cgcGGATCCATGCAAGCATCTTCCATGGA
Os1900-Pyed-R	EcoR1	ccgGAATTCTCAGGTGTTGGTCCTCTTGATG
Os5100-Pyed-F	BamHI	ATTTGCGGCCGCATGGAGGCTCTAGTGGCGGC
Os5100-Pyed-R	EcoRI	GGTTAATTAACTAGGCGGCGGCGGC

3. Yeast culture media, transformation, and gene induction

Wild-type yeast strain WAT11 was maintained in YPGA medium[4] containing 200 mg·L^{-1} adenine, 10 g·L^{-1}yeast extract, 10 g·L^{-1} bacto-peptone, and 20 g·L^{-1} glucose. For solid medium, 20 g L^{-1} agar was supplemented. Transformed yeast was maintained in SGI medium (1 g·L^{-1} bactocasamino acids, 7 g·L^{-1} yeast nitrogen base without amino acids, 20 mg·L^{-1} tryptophan, 20 g·L^{-1} glucose and 20 g·L^{-1} agar if necessary). All yeast expression plasmids prepared were independently transformed into yeast strain WAT11 using the polyethylene glycol (PEG)-LiOAc method[7].

For induction of gene expression, a starter 5 mL positive yeast colony culture was grown in 50 mL SGI medium with initial OD_{600} of 0.1~0.25 for 36 h at 30°C with constant shaking at 300 rpm. The cultures were subsequently transferred to 250 mL YPL medium (10 g·L^{-1} yeast extract, 10 g·L^{-1} Bactopeptone, 20 g·L^{-1} galactose) and cultured at 30°C for another 24 h.

4. Microsomal protein preparation

Yeast microsomes were prepared as described[4]. The yeast cells were collected by centrifugation at 5500 rpm, and resuspended in 100 mL microsome extraction buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.6 M sorbitol and 10 mM β-mercaptoethanol) for 10 min at room temperature. Subsequently, the cells were collected by centrifugation, and resuspended in 5 mL microsome extraction buffer and transferred to a 50 mL plastic tube. After adding 20 mL of glass beads (450-500 μm), the cells were intensively shaken at 4°C. The resulting suspension was collected and centrifuged at 9400 rpm for 10 min at 4°C and the supernatant was recentrifuged at 45.000 rpm (4°C) in a Beckman-L7 ultracentrifuge, model L7-80, for at least 2 h (Beckman Instruments, Fullerton, CA). The pellets were re-dissolved in ice chilled 50 mM Tris-HCl buffer (pH=7.5) containing 1mM EDTA and 20% (v/v) glycerol and stored at -80°C for further study. The protein concentration was determined using Bradford assay[8].

5. Enzyme Assays and Kinetics study

Carlactone or individual 5DS isomers (1mM or 0.02 mM stock in DMSO) were incubated with 70 μL (190 μg protein·mL^{-1}) of microsomal protein preparation in a 500 μL reaction containing 1 mM NADPH and 40 mM phosphate buffer (PH=7.5). The assays were carefully agitated at a moderate speed (200 rpm) for 3 h at 30°C. The reaction was stopped by adding 1.5 mL ethyl acetate and extracted.

To determine enzyme kinetics, serial dilutions of (*Z*)-(*R*)-CL or *ent*-2'-epi-5DS were used for enzyme assays. Reactions were initiated by the addition of carlactone (at

0.04 µM, 0.16 µM, 0.8 µM, 2 µM, 4 µM, 16 µM and 20 µM) or *ent*-2'-*epi*-5DS (at 0.02 µM, 0.04 µM, 0.12 µM, 0.2 µM, 0.8 µM, 1.6 µM, 4 µM, 8 µM, 12 µM, 40 µM and 100 µM). The assays were incubated for 3.5 h at 30°C under constant shaking at 250 rpm. The products of the reactions were analyzed by MRM- LC-MS/MS and values of V_{max} and K_m were estimated using nonlinear fitting by Graph Pad Prism6.

6. Transient expression in leaves of *Nicotiana benthamiana*

Liquid Agrobacterium strain cultures (AGL0) harboring different genes were grown at 28°C at 220 rpm for 2 days in LB media with antibiotics corresponding to the binary vectors and rifampicillin (35 mg/L). Cells were harvested by centrifugation for 15 min at 4000 rpm at room temperature and then re-suspended in 10 mM MES-KOH buffer (PH=5.7) containing 10 mM $MgCl_2$ and 100 mM acetosyringone (49-hydroxy-39,59-dimethoxyacetophenone, Sigma) to a final OD600 of 0.5, followed by rolling at room temperature for 2-4 h. For the different gene combinations, equal concentration of the agrobacterium strains harboring different constructs were mixed, and strains carrying empty vectors were used for compensate the dosage of each gene in each combination. In all experiments, an agrobacterium strain harboring a gene encoding the TBSV P19 protein was added to maximize protein production by suppression of gene silencing[9]. *Nicotiana benthamiana* plants were grown in soil pots in greenhouse with 16 h light at 25°C /8 h dark at 22°C. Construct combinations in agrobacterium were infiltrated into leaves of five-week-old *N. benthamiana* plants by using a 1mL syringe. Leaves of the same stage were selected to minimize the variability. For each gene combination, two or three leaves from each plant were infiltrated and in total 4-5 plants were used for individual biological replicates. The bacterial suspension in buffer was slowly injected into the abaxial side of the leaf to spread to the whole leaf area. Six days after infiltration, the infiltrated leaves were harvested for further analysis.

7. Carlactone and strigolactone extraction from *Nicotiana benthamiana* leaves

For carlactone and strigolactone analysis in the *N. benthamiana* leaves, 510 mg fine-ground agro-infiltrated leaf tissue was used. Ethyl acetate was used as the extraction solvent. The extractions steps followed previous methods[10], with the exception that d_6-*epi*-5-deoxystrigol was used as internal standard. Grace Pure Silica SPE (500 mg/3 ml) columns were used for further purification. A crude extract of strigolactone or carlactone in a mixture of ethyl acetate (50 µL) and 3.95 mL n-hexane was applied to the silica column. After the samples were eluted through the pre-conditioned (with 3 ml n-hexane) silica column, the column was washed by absolute n-hexane. Subsequently, three fractions were eluted and collected with the following solvents mixture (A through C): (A) n-hexane (80 mL): ethyl acetate (35 mL); (B) n-hexane (20 mL): ethyl acetate

(90 mL); (C) 2% methanol in ethyl acetate. Of these three fractions, carlactone and *ent*-5DS/5DS are enriched in fraction A, while *ent*-2'-*epi*-5DS/2'-*epi*-5DS and orobanchol are more abundant in fraction B. Fractions were evaporated under vacuum and dissolved in 250 µL acetonitrile: water (1: 3). All the fractions were filtered with a minisart SRP4 0.45 µm filter (Sartorius Stedim Biotech, Germany) for further analysis.

8. Strigolactone extractions from yeast expression reactions

For extractions of strigolactones from the yeast assay, the total 500 µL enzyme assay mixture was transferred to a 10 mL glass tube, followed by adding 1.5 mL ethyl acetate, vortexing and centrifugation. The upper phase was then transferred to 4 mL glass vial. The extractions steps were repeated and the upper organic phases were combined for drying. The residues were re-dissolved in 200 µL 25% acetonitrile/water followed by filtration for MRM-LC-MS/MS analysis.

9. Carlactone and strigolactone detection and quantification by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The LC-MS/MS analysis of carlactone and strigolactone in *N. benthamiana* leaf extracts and yeast microsomal incubations were performed by comparing the retention time and mass transitions with those of carlactone and 12 major authentic strigolactones standards according to the method described by Kohlen *et al.*[10] with some modifications. A Waters Xevo tandem quadrupole mass spectrometer equipped with an electrospray ionization source and coupled to an Acquity UPLC system (Waters) was used. Chromatographic separation was achieved on a non-chiral Acquity UPLC BEH C18 column (100 x 2.1 mm, 1.7 µm; Waters) by applying a water/acetonitrile gradient (with 0.1% formic acid) to the column. For carlactone detections, the gradient started from 5% acetonitrile for 0.15 min and raised to 60% (v/v) acetonitrile in 2.0 min, followed by a 5.25-min gradient to 90% (v/v) acetonitrile, which was maintained for 0.75 min before going back to 5% acetonitrile using a 0.25-min gradient, and maintained for 1.85 min to equilibrate the column prior to the next run. For strigolactones, gradients started from 5% acetonitrile for 0.33 min and raised to 27% (v/v) acetonitrile in 0.34 min, followed by a 4.33-min gradient to 40% (v/v) acetonitrile, then rising to 65% acetonitrile in 3 min and maintained for 0.67 min, then raised to 90% acetonitrile in 0.2 min, which was maintained for 0.46 min before going back to 5% acetonitrile using a 0.2-min gradient, and maintained for 2.47 min to equilibrate the column prior to the next run. Operation temperature and flow rate of the column were 50ºC and 0.5 mL min^{-1}, respectively. Sample injection volume was 10 to 20 µL. The mass spectrometer was operated in positive electrospray ionization 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 standard compound using the Waters IntelliStart MS Console. MRM was used for identification of carlactones and strigolactones by comparing retention times and MRM mass transitions with racemic standards (carlactone and 12 major strigolactones). Data acquisition and analysis were performed using MassLynx 4.1 (combined with TargetLynx) software (Waters).

10. Preparation of carlactone produced by rice CCD8

9-*cis*-β-apo-10'-carotenal was produced *in vitro* by the *Arabidopsis* CCD7[11] from 9-*cis*-β-carotene and incubated with the supernatant of *Escherichia. Coli* BL21 cells overexpressing thioredoxin-OsCCD8 fusion[12]. Crude assays were extracted according to Alder *et al*., 2012. For HPLC purification of carlactone from *E. coli* assays, a Waters system equipped with a photodiode array detector (model 996) was used. Carlactone was preparatively purified using a YMC-Pack C30-reversed phase column (250 × 4.6 mm i.d., 5 µm) with the solvent system developed at a flow-rate of 1.4 ml/min with isocratic condition of 95.5 % B for 5 min, followed by a gradient from 95.5% B [MeOH:water:tert-butylmethyl ether (30: 10: 1, v/v/v)] to 92% B/8%A within 8 min, then finally to 100% A [methanol:tert-butylmethyl ether (1: 1, v/v)] with a flow-rate of 2 ml/min within 1 min, maintaining the final conditions for another 6 min. The collected fractions were dried *in vacuo*, dissolved in DMSO for enzyme assays.

11. Preparation of carlactone and strigolactone standards

Racemic (*Z*)-CL[13], racemic 5DS[14], racemic 2'-*epi*-5DS[14], racemic orobanchol and racemic 2'-*epi*-orobanchol[15,16] were prepared as previously described. The racemic mixtures were separated into their individual enantiomers using semi-preparative high-pressure liquid chromatography performed on a Hewlett-Packard 1050 HPLC with a multi-wavelength UV detector (MWD). Separation was achieved with an Astec® Cellulose DMP chiral HPLC column (250 x 10 mm id, 5 µm particle size, Supelco, Bellafonte, PA, USA). Separation of the (Z)-CL enantiomers was achieved using a flow rate of 1.5 mL/min of 10% isopropanol/hexane and monitored at a UV wavelength 260 nm. Separation of the 5DS isomers was achieved using a flow rate of 3 mL/min of a 8:1:1 mixture of hexane/methanol/methyl-tert-butyl ether and monitored at a UV wavelength of 240 nm. Separation of the orobanchol isomers was archived using a flow rate of 2 mL/min of a 8:1:1 mixture of hexane/methanol/methyl-tert-butyl ether and monitored at a UV wavelength of 240 nm. CD-spectra were recorded on a Jasco J-810 spectropolarimeter in acetonitrile to assign the relevant stereochemistry.

12. Chiral LC-MS

Chiral LC-TOF-MS for carlactone and orobanchol was conducted with a Waters Alliance e2695 HPLC connected to a Waters LCT Premier XE time-of-flight (TOF) mass spectrometer with an atmospheric chemical ionization source (APCI). Separation was achieved with an Astec® Cellulose DMP chiral HPLC column (250 x 4.6 mm id, 5 µm, Supelco, Bellafonte, PA, USA) using a flow rate of 0.5 mL/min. For carlactones, the column was eluted with 5% isopropanol/hexane. For 5DS and orobanchol stereoisomers, separation was achieved with a 6:1:1 hexane/methanol/methyl-tert-butyl ether . The column was held at 25ºC and sample injection volume was 5 to 20 µL. The mass spectrometer was operated in positive APCI mode. Cone and desolvation gas flows were set to 250 and 650 $L \cdot h^{-1}$, respectively. The corona voltage was set at 3.0 kV, the source temperature at 100°C, and the desolvation temperature at 350°C. For chiral LC-MS/MS of 5DS stereoisomers, an Agilent 6460 LC-QQQ with a Jetstream Electrospray source, coupled with 1290 UPLC was used. The same chiral column, solvents and conditions were employed as above. For MRM of the 5DS stereoisomers, a fragmenter voltage of 135V and collision energy of 15V was used.

13. SL-LIKE1 analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS)

LC-MS/MS analysis of *N. benthamiana* leaf samples co-expressing *AtD27, AtMAX3, AtMAX4* and *AtMAX1* was conducted using an Agilent 6460 LC-QQQ with a Jetstream Electrospray source, coupled with 1290 UPLC. Separation was achieved with an Alltima C18 column (150 x 2.1 mm id, 5 µm, Grace Discovery Sciences) using a flow rate of 0.3 mL/min. The column was initially eluted with 20% acetonitrile/water (containing 0.1% formic acid) which increased over 40 mins to 100% acetonitrile which was held for 10 mins. The column temperature was maintained at 25ºC and sample injection volume was 10 µL. MRM transitions of *m/z* 347.2 ->97.1 and *m/z* 347.2 ->315.1 were monitored using a fragmenter voltage of 135V and collision energy of 15V.

14. MAX1 Modeling and docking

Modeler 9v6 was used for protein structure modeling[17]. Based on sequence similarity and the requirement that a substrate-bound structure is used as template, PDB structure 1w0f (human CYP3A4) was used as template[18,19]. Sequence identity between the MAX1 sequences and this template is 22-24% (see sequence alignment in **Supplementary Fig. 12**). Because this sequence identity value indicates that template based structure modelling may be challenging, we checked the structural similarity of the template with plant P450 structures available in the PDB using jFATCAT[20-22]. The resulting RMSD was ~3.0Å, indicating a quite reasonable structural similarity. The rice MAX1 sequences

modelled here have a higher sequence similarity with the template than the available plant P450 protein structures, suggesting that the structural similarity between the rice sequences and the template is even better.

Missing residues in the crystal structure were modeled using the Modeler automodel class. Subsequently, for each of the OsMAX1 proteins 1,000 structures were generated using the automodel class. Out of these, the best 10 according to objective score were selected. Protein structure figures were generated using MolScript and Raster3D[23,24]. Carlactone and 5-deoxystrigol coordinates were obtained from Pubchem[25]. PRODRG was used to generate additional stereoisomer structures[26]. Ligand and protein structures were converted to pdbqt format using AutoDockTools[27]. Docking was performed using Vina[28] in two steps: first the heme/Fe was docked into the active site, and subsequently the ligand (carlactone or 5-deoxystrigol isomers). Default selection of active torsions by AutoDockTools was used.

Docking as described above was performed using non-oxygenous heme because of the known CYP450 reaction cycle, in which the substrate binds before the oxygen[29,30]. However, we also investigated the influence of oxygen binding by repeating the docking using oxygenous heme. This showed that the results reported in the main text for non-oxygenous heme (Os900 and Os1400 predicted to dock carlactone in orientations consistent with conversion to *ent*-2′-*epi*-5DS, and Os1400 predicted to be best able to convert 5-deoxystrigol to orobanchol) did not change when using oxygenous heme.

Supplementary Results

Supplementary Table 1. Enzyme kinetic parameters of OsMAX1s[a]

OsMAX1	Substrate	*Km* (μM)	*Vmax* (pmol·min^{-1}·μg^{-1})
Os01g0700900	(*Z*)-(*R*)-CL	0.69±0.30	7.0x10^{-6}±0.6x10^{-6}
Os01g0701400	*ent*-2'-*epi*-5DS	0.74±0.10	50.7±1.2

[a]The enzyme activity was evaluated in 3 independent replicates (n=3). Data are means ± SEM.

Supplementary Table 2. Overview of carlactone docking results[a]

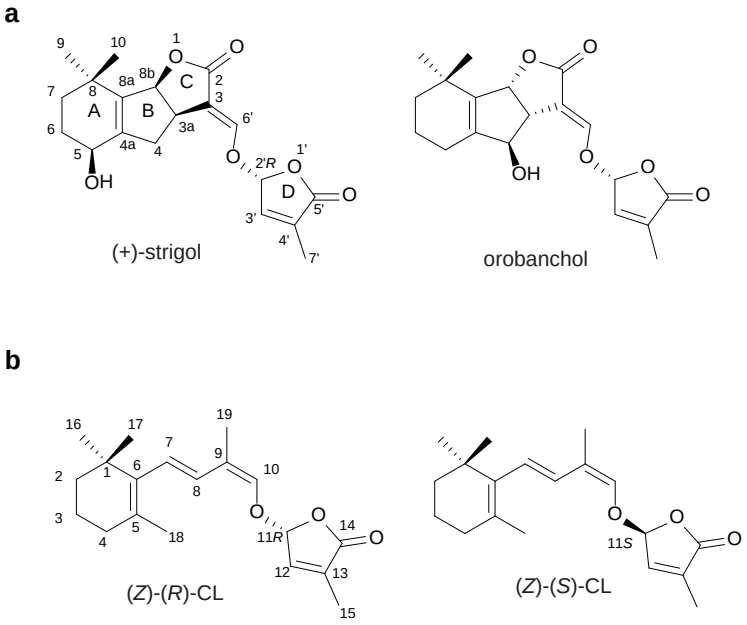
Os-MAX1	Affinity (kcal/mol)	Distance C-18-heme iron (Å)	Distance of C-19-heme iron (Å)
Os1400	-7.6	4.37	9.06
	-5.5	4.99	8.25
	-4.6	4.70	6.16
Os900	-7.3	4.24	8.32
Os1900	-5.5	4.22	9.13
Os1500	NA	NA	NA
Os5100	NA	NA	NA

[a]Docking of (Z)-(R)-CL isomer to the five OsMAX1 proteins was performed as described in the supplementary materials. All productive docking results are shown; docking modes were considered productive if carlactone C-18 was within 5 Å of the heme iron (note that the distance to the ferryl oxygen would be smaller). Using this criterion, Os900 and Os1400 gave the largest number of productive docking modes with most favorable affinities (as calculated by Vina). Although for Os1900 one productive docking mode was obtained, this had less favorable affinity compared with the Os1400 and Os900 docking results. An alternative definition of productive docking modes using C-19 instead of C-18 did not discriminate Os1400 and Os900 from the other OsMAX1 homologs. No docking solutions were obtained with both C-18 and C-19 simultaneously within 5 Å of the heme iron.

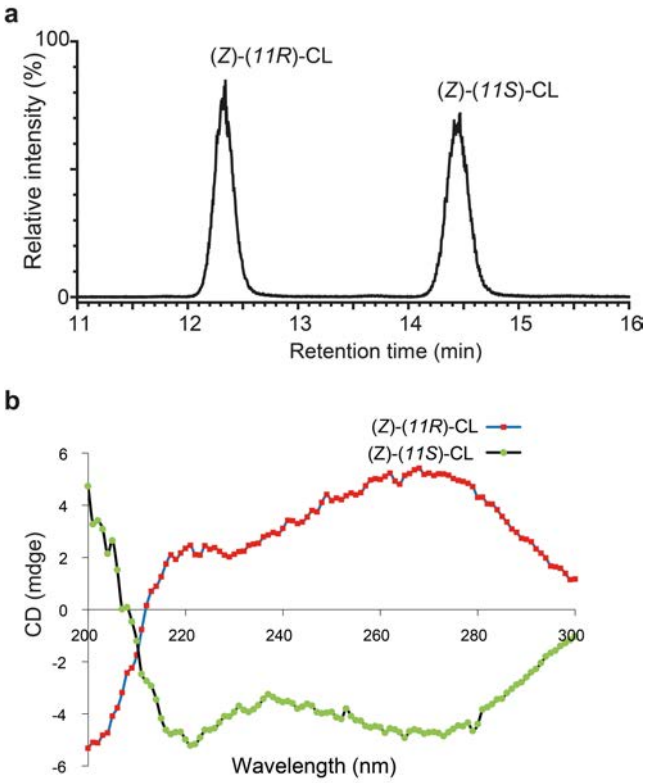
Supplementary Table 3. Overview of 5-deoxystrigol isomers docking results[a]

5DS isomers	Affinity (kcal/mol)	Distance C4-heme iron (Å)
	Os1400	
2'-*epi*-5DS	-6.6	4.61
2'-epi-5DS	-4.9	4.55
2'-*epi*-5DS	-3.9	4.85
5DS	-7.6	4.77
5DS	-4.6	4.78
ent-2'-*epi*-5DS	-7.0	4.89
ent-5DS	-8.6	4.67
	Os900	
5DS	-6.0	4.81
ent-2'-*epi*-5DS	-6.2	4.45
ent-5DS	-6.2	4.95
ent-5DS	-3.8	4.76
	Os1500	
2'-*epi*-5DS	-5.6	4.34
ent-5DS	-7.4	4.84
	Os5100	
ent-5DS	-2.4	5.02
5DS	-6.0	5.86
ent-2'-*epi*-5DS	-5.8	5.82
ent-5DS	-1.3	5.81
	Os1900	
2´-*epi*-5DS	-1.0	5.76
5DS	-2.5	5.62
ent-5DS	-4.8	5.95

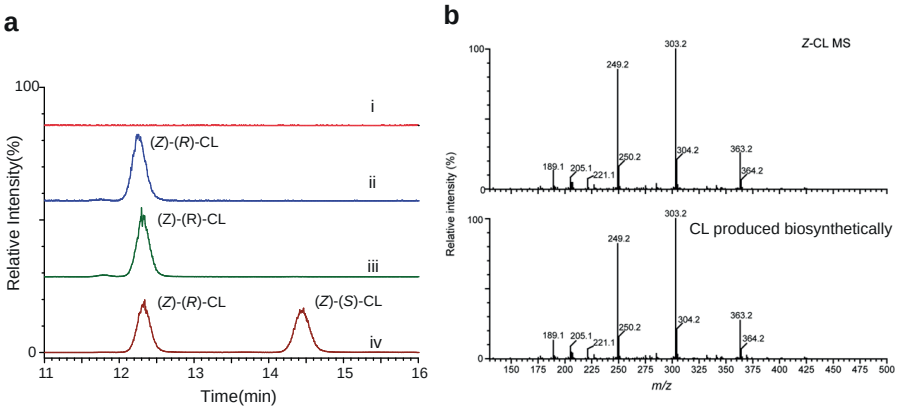
[a]Docking of the 5DS isomers to the five OsMAX1 proteins was performed as described in supplementary materials. All productive docking results are shown. Docking modes were considered productive if C-4 was within 5 Å of the heme iron. Although for Os900 and Os1500 a few productive docking results were obtained, these have less favorably affinities (as calculated by Vina) compared with the Os1400 docking results. For Os5100 and Os1900, no docking results were obtained with C-4 within 5Å of the heme iron. For these proteins, therefore results with C-4 within 6Å of the heme iron are shown for comparison.



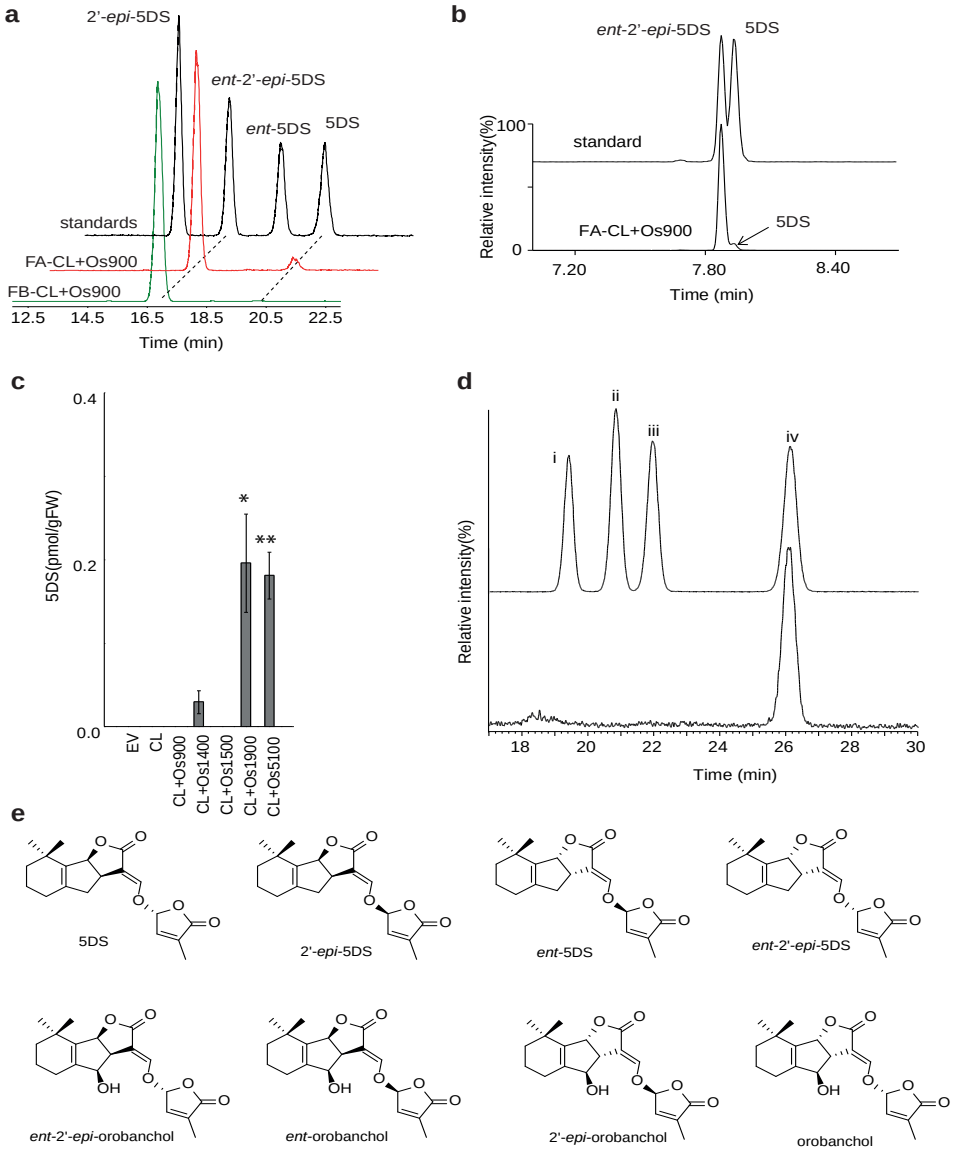
Supplementary Figure 1. The structure of the naturally occurring strigolactone families and carlactone enantiomers. (**a**) Two naturally occurring strigolactone families: strigol-type and orobanchol-type. Both types show identical stereochemistry at C-2' with (*R*) configuration, but have opposite C-ring orientation. (**b**) The two possible (Z)-CL enantiomers, with C-11 (*R*) and (*S*) configuration.



Supplementary Figure 2. Separation of the two (Z)-CL enantiomers by chiral LC- MS and their circular dichroism (CD) spectra. (**a**) Extracted ion chromatogram ($[M+H]^+$ *m/z* 303.1) showing the chiral separation of the two (Z)-CL enantiomers, (*Z*)-(*11R*)-CL and (*Z*)-(*11S*)-CL. (**b**) (*Z*)-(*11R*)-CL and (*Z*)-(*11S*)-CL show opposite CD patterns from 220nm to 300nm.

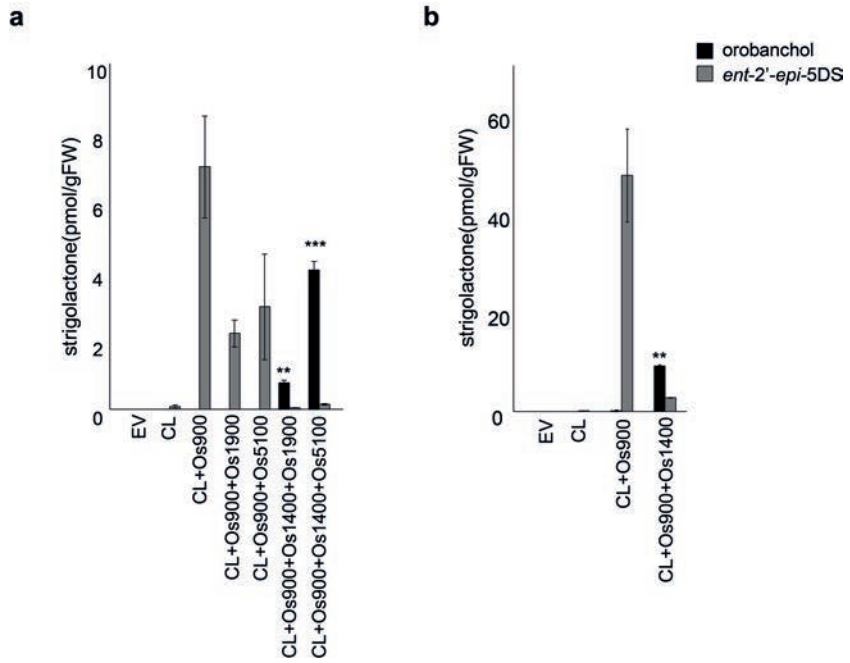


Supplementary Figure 3. Chirality of carlactone produced *in vitro* (by OsCCD8) and in *N. benthamiana* compared with two synthetic CL isomers. (**a**) Chiral LC-MS extracted ion chromatogram ($[M+H]^+$ *m/z* 303.1) overlay of carlactone isomers: (i) chromatogram of the empty vector control of *N. benthamiana* assays; (ii) CL produced *in vitro* using AtCCD7 and OsCCD8; (iii) the product of transient expression of *OsD27*, *OsCCD7* plus *OsCCD8* in *N. benthamiana*; (iv) two synthetic (*Z*)-CL isomers. (**b**) MS spectra of CL produced biosynthetically (using AtCCD7 and OsCCD8 through *in vitro* assay) and the standard (*Z*)-CL.

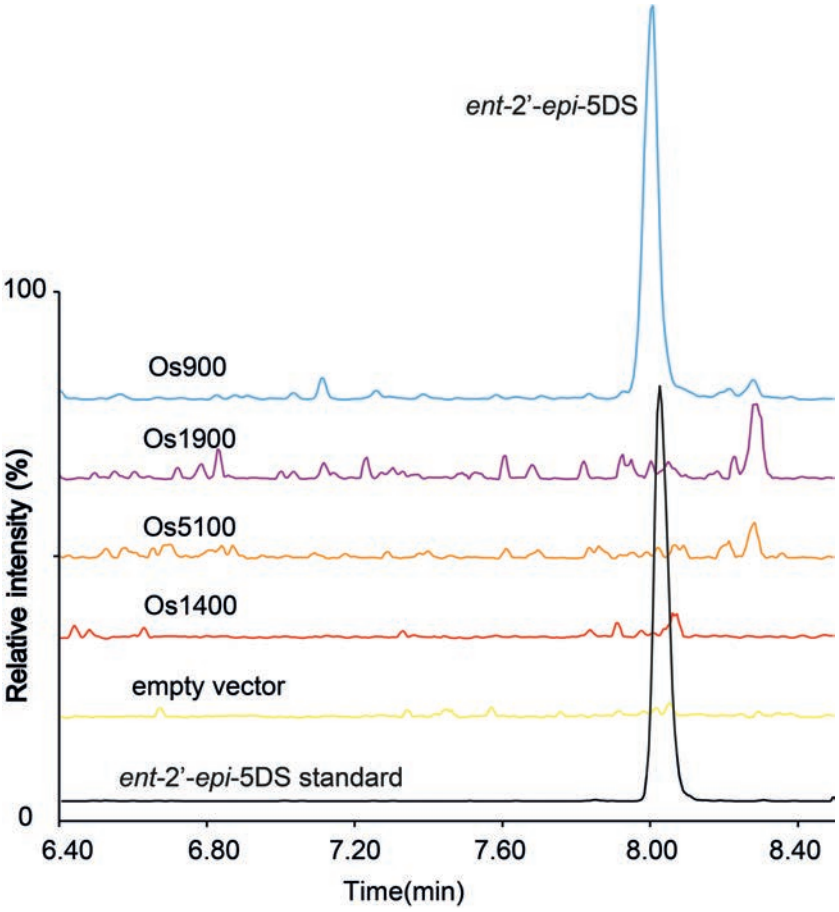


Supplementary Figure 4. LC-MS analysis of 5-deoxystrigol (5DS) produced in *N. benthamiana* and chiral-LC/MS identification of 5DS and orobanchol stereoisomers. (**a**) Chiral LC-MS/MS chromatogram overlay of 5-deoxystrigol stereoisomer standards with SPE fraction A (FA, red color) and B (FB, green color, only contains *ent*-2'-*epi*-5DS) (transition $[M+Na]^+$ *m/z* 353>256) produced from co-expression of *Os900* with carlactone biosynthetic genes in *N. benthamiana*. In *Os900* co-expression samples, only *ent*-2'-*epi*-5DS (fraction B) and a trace of 5DS (fraction A only) are detected. (**b**) Representative chromatograms of fraction A enriched in 5DS (transition $[M+H]^+$ *m/z* 331.2>216.15)

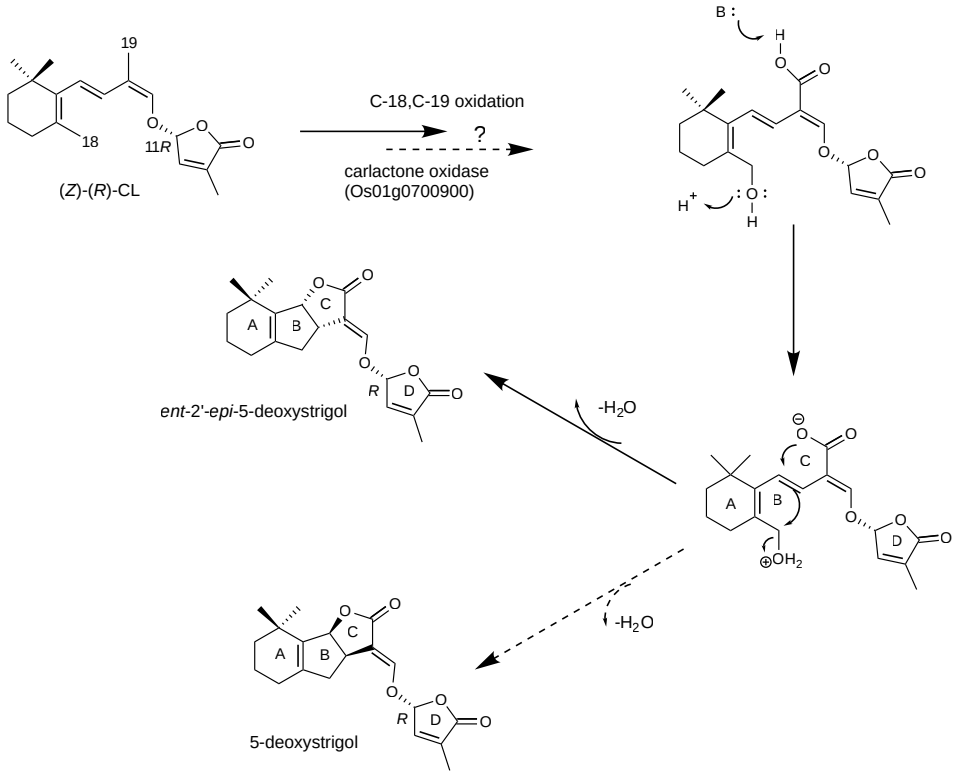
produced by *Os900* in *N. benthamiana* assay (indicated by arrow) and the standards. (**c**) Quantitative analysis of 5DS (transition $[M+H]^+$ *m/z* 331.2>216.15) production through transient expression of carlactone biosynthetic genes (CL) in combination with *OsMAX1s* in *N. benthamiana*. Bars represent means ± SE (n=3 to 5). ∗ and ∗∗ indicate significant differences from the CL treatment at $P<0.05$ and $P<0.01$, respectively, as determined by Student's T-test. EV represents control samples infiltrated with the empty vector. (**d**) Orobanchol stereoisomer identification by comparison with four synthetic stereoisomers standards on LC-TOF-MS. i, ii, iii and iv, represents the orobanchol isomer standards. i, *ent*-orobanchol; ii, 2'-*epi*-orobanchol; iii, *ent*-2'-*epi*-orobanchol; iv, orobanchol (naturally occurring orobanchol isomer). (**e**) Chemical structures of 5DS and orobanchol stereoisomers mentioned in current figure.



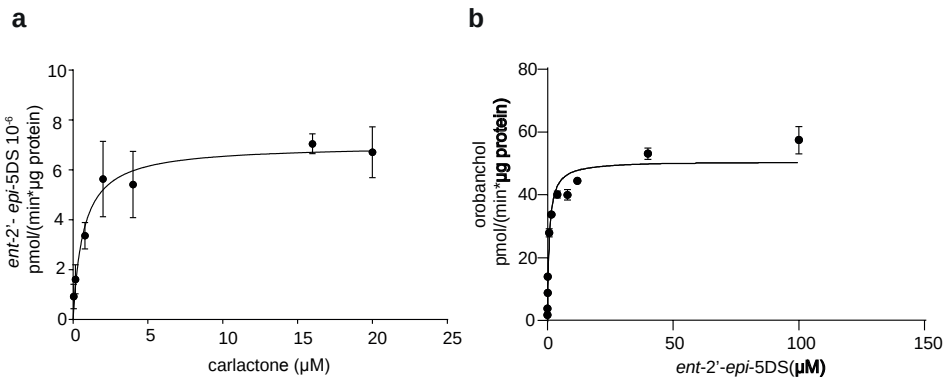
Supplementary Figure 5. The effect of transient co-expression of carlactone biosynthetic genes with different combinations of *OsMAX1s* in *N. benthamiana* on the production of strigolactones. (**a**) and (**b**) show that transient expression of *Os1400* in combination with the other three *OsMAX1s* (*Os900*, *Os1900* and *Os5100*) increases orobanchol (transition $[M+H]^+$ *m/z* 347.2>233.15) (compare with treatment without *Os1400*) and decreases *ent*-2´-*epi*-5-deoxystrigol (transition $[M+H]^+$ *m/z* 331.2>234.15). Bars represent means ± SE (n=3 to 5). *** αnd ** indicate significant differences at P<0.001 and P<0.01, respectively, as determined by a pairwise Tukey's HSD test performed using R 3.0.2. CL represents gene combination *OsD27*, *OsCCD7* plus *OsCCD8*. EV represents control samples infiltrated with the empty vector. (**a**) and (**b**) are representing data from two individual experiments.



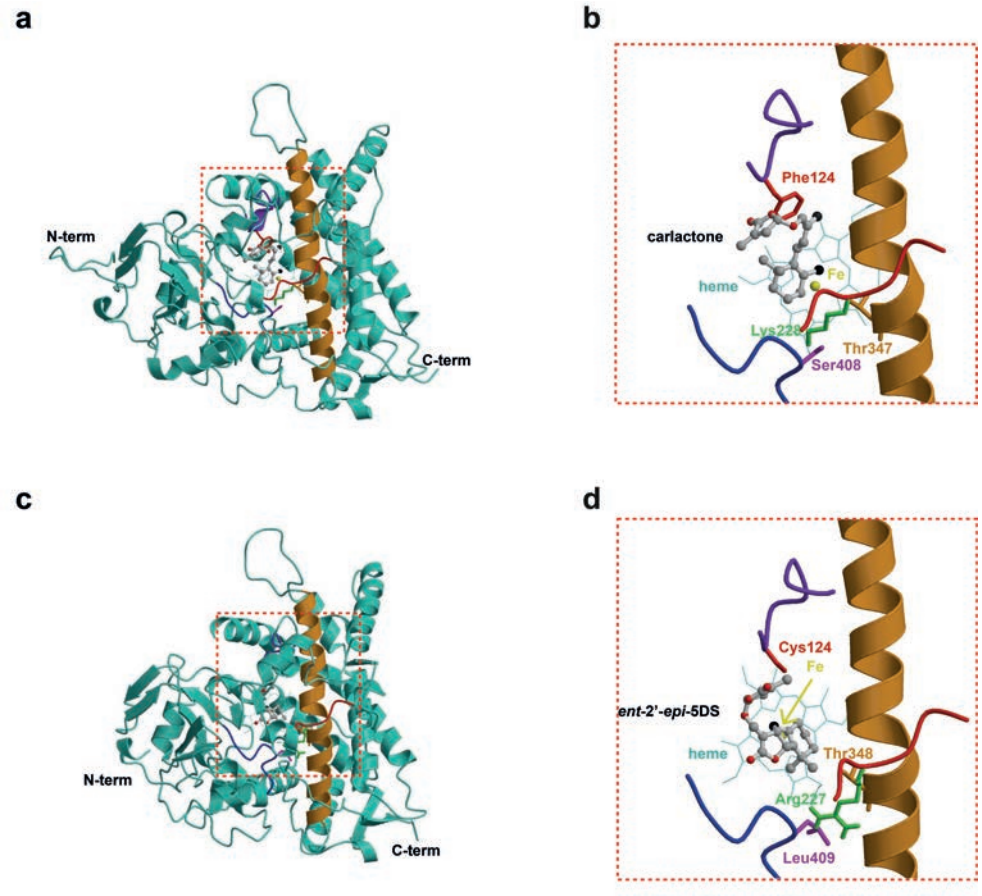
Supplementary Figure 6. MRM-LC-MS/MS analysis of the products formed from carlactone using yeast microsomes expressing OsMAX1s. Representative chromatograms of *ent*-2´-*epi*-5-deoxystrigol (*ent*-2´-*epi*-5DS) (transition $[M+H]^+$ *m/z* 331.2 > 216.15) from *in vitro* assays with yeast microsomes expressing OsMAX1s (*Os900*, *Os1400*, *Os190*0 and *Os510*0) using natural rice carlactone (obtained from *in vitro* assays with OsCCD8). The *ent*-2´-*epi*-5DS peak only results from the incubation of yeast microsomes expressing *Os900*. The chromatograms show representative results observed in more than three independent experiments with multiple technical replicates (with empty vector pYeDp60 as control).



Supplementary Figure 7. Proposed mechanism of carlactone [(*Z*)-(*R*)-CL] conversion to *ent*-2'-*epi*-5-deoxystrigol (*ent*-2'-*epi*-5DS) and 5-deoxystrigol (5DS), the two parent representatives of naturally occurring strigolactones. We postulate that Os900 initiates carlactone oxidation by catalysing carboxylation of C-19 and hydroxylation of C-18. Upon proton abstraction of the carboxyl group and proton addition to the alcohol, stereo-controlled closure of the B and C rings occurs, with water as leaving group, yielding the parent SL, *ent*-2'-*epi*-5DS (solid arrow). Homologous enzymes from species making strigol-type strigolactones will catalyze the same reactions but now resulting in stereo-controlled closure of the B and C rings yielding 5DS (dashed arrow). In both cases the C-2' *R*-configuration of CL is retained.



Supplementary Figure 8. Michaelis-Menten kinetics of carlactone oxidase (Os900) and *ent*-2´-*epi*-5-deoxystrigol-4-hydroxylase (Os1400) incubated with their substrates. (**a**) Michaelis-Menten kinetics of carlactone conversion to *ent*-2'-*epi*-5-deoxystrigol (*ent*-2'-*epi*-5DS) catalysed by microsomes isolated from yeast WAT11 expressing Os900 and *ATR1*. (**b**) Michaelis-Menten kinetics of *ent*-2'-*epi*-5DS conversion to orobanchol catalysed by microsomes isolated from yeast WAT11 expressing Os1400 and *ATR1*. Error bars represent the SEM from three independent biological replicates (n=3). Curves were obtained using Graph pad Prism6 with nonlinear Michaelis-Menten curve-fitting.



Supplementary Figure 9. Predicted docking of carlactone and *ent*-2'-*epi*-5DS in OsMAX1s. (**a**) and (**b**) Docking of carlactone in carlactone oxidase (Os900) and its active site. (**c**) and (**d**) Docking of *ent*-2'-*epi*-5-deoxystrigol (*ent*-2'-*epi*-5DS) in *ent*-2´-*epi*-5-deoxystrigol-4-hydroxylase (Os1400) and its active site. Protein N-terminus and C-terminus are indicated. Amino acid residues in the active pockets that differ between Os900 and Os1400 are indicated. (**b**) and (**d**) show a phenylalanine (Phe) present in the active site of Os900 at the equivalent position to a Cys in Os1400. Lys228/Ser408 in Os900 and Arg227/Leu409 in Os1400, could also be involved in discriminating between Os900 and Os1400 activity. The conserved catalytic threonine (Thr347 in Os900, Thr348 in Os1400) is shown in orange. Carlactone [(Z)-(R)-CL] and *ent*-2'-*epi*-5DS are shown in balls-and-sticks with oxygen atoms in red and carbon atoms in grey. Carbon atoms C-18 and C-19 in carlactone and C-4 in *ent*-2'-*epi*-5DS are in black. Heme is shown in cyan sticks; the heme iron is shown in yellow. The amino acid numbers are referring to their positions in the protein sequences of Os900 (Genebank accession NO. AGI60164.1) and Os1400 (Genebank accession NO. AGI60163.1), respectively.

```
S.moellendorffii|gi|300160069          ------------------------------------------MALIIAVFFVIVVTIL
Z.mays|gi|413926093                    ----------------MEITASCDDGAVTAGAVS-----------GLLLASVLSLFGAFL
B.distachyon|Bradi3g08360.1            --------------------MAAITNCSIALVTSTNGHSAAASPTTAALLLLSLIIAFL
H.vulgare|gi|326530386                 NAVTRPNAGKQTKLLAPSSSSMEASNCSIALETTGHAAAHGTSAPALLLLSSVAAIGAFL
Os02g0221900                           --------------------MQASSMEASNCSIALEISHVATPGLPVLLLGSSLALLAVFL
Z.mays|gi|226491964                    ----------------MEITASCDDGAVTAGAVS-----------GLLLASVLSLFGAFL
S.bicolor|Sb04g007880.1                --------------------MEIALTVSAVSHQSV----------PVLVLISFLSLFSAFL
S.italic|Si016823m                     --------------------METTTICNGALGPVA------HQSVPVLVLISFVSLFSAFL
B.distachyon|Bradi4g09040.1            ----------------------MMGGVGVLLSSWI---------EGSPSFSAVFFTLAALV
S.bicolor|Sb03g032210.2                ----------------------MGWGEIISSQLL---IESSSSSLPAVLFTAAALAAGAF
S.italic|Si015313m                     ------------------------------------------------------------
B.distachyon|Bradi1g75310.1            ----------------------------MESPL-----------AAILFTVAALAAGAF
H.vulgare|gi|326492025                 --------------------MEGAGAAPVWGQPP---------SFPAMLFTVAAMAAGAL
B.distachyon|Bradi1g37730.1            ----------------------------MAPVGE----------WLPCISTLACCLLGLV
Os06g0565100                           ----------------------MEALVAAAAAAAR-------DQPWLLLPWSWLAGVVVVV
S.bicolor|Sb10g022310.1                ----------------------MEMAGAAGTAE---------TWLPYVTTAASCAVAVF
Z.mays|gi|224033291                    ----------------------MEMAGAAGTEA----------WLPYVTTVASCAVGVF
Z.mays|gi|226493876                    ------------------------------------------------------------
A.thaliana|AtMAX1                      --------------------MKTQHQWWEVLDPFL----------TQHEALIAFLTFAAVV
V.viniferagi|296081643                 ----------------MLEFFLRDWEWSLQGLIQ-IGVSFIRTPMAPAFFTVLAMLGGLL
C.papaya|supercontig_7.164             ---------------MGLVEMLMGVRWFNTTLPP----------AVSTFFTILAVAAGIL
S.tuberosum|PGSC0003DMP400014456       --------------------------MMFLSSAIQ----------ESPIVSTIFTILAGVL
P.hybrida|gi|329739341                 ----------------------MEFLSTNIQHSIVDTVEVLTRPPMTSTICTILALLATVL
C.sativus|Cucsa.299480.1               --------------------MDNFFQSMVASVPM---------GVGTILFTTFCLVGGGL
P.trichocarpa|POPTR_0018s07540.1       ----------------------MSTDLQVLFTPM----------VTPLCTVLAMLLGLL
P.trichocarpa|POPTR_0006s24320.1       ----------------------MDLQVLFTDVPV----------VTAIICTVFAMLLGLL
R.communis|gi|255550067                ---------------MGFGLIQEAFFSTCSNSTS----------VTIVLALVFTALAIVL
M.esculenta|cassava4.1_005510m         ----------------------MAEAFFNSVPM----------LTVIFTALALVLG--
M.truncatula|gi|357437385              ----------------------MLFISVILNVPL----------ASTIFILVTLMGGLV
G.max|Glyma17g34530.1                  ------------------------------------------------------------
G.max|Glyma14g11040.1                  ------------------------------------------------------------
M.truncatula|gi|357465755              --------------------MVFMDLEWLFPIPIS----------VSFASTILALAGGWL
G.max|Glyma04g05510.1                  -----------------MVVFMDYLEWLFAIPSV---------PSASAMFTLLALIGGLL
G.max|Glyma06g05520.1                  -------------------------------------------------MFTLLALIGGLL
B.distachyon|Bradi4g08970.1            --------------------------MGMLPMLL----------GEYAVTVVAMAVGFL
Os01g0700900                           ----------------------MEISTVLGAIL----------AEYAVTLVAMAVGFL
Os01g0701400                           ----------------------MEIISTVLGST----------AEYAVTLVAMAVGLL
Os01g0701500                           ----------------------MDISEVLGA-----------TAEWAVTLVAMAVGLL
S.italic|Si004997m                     -------------------------MGTVLGAME----------EYTFTFVAMVVGFL
Z.mays|gi|237908823                    ------------------------------------------MEECTFTSAAMAVGFL
S.bicolor|Sb03g032220.1                ----------------------MEMGTVLGAME----------EYTFTFLAMAVGFL
S.italic|Si000975m                     -------------------------MEMVLGAMV----------EEYTFTFLAMAVGFL
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S.moellendorffii|gi|300160069          --IYLQWPAWKLSKIPAAPYISGLGHLPLMAKYQAGVFIKLAKQLGPIYR----------
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B.distachyon|Bradi3g08360.1            --AYFHLPFWAVRKVPGPPTRFPLGHLHLLAQHGPDILRAMAQEHGPIFR----------
H.vulgare|gi|326530386                 --VYFYAPFWAVRRVPGPPTRFPLGHLHLLANDGPDVFRAIAKEHGPIFR----------
Os02g0221900                           --VYFYAPFWSLRTVPGPPTRFPIGHLHLLAKNGPDVFRAITKEYGPIFR----------
Z.mays|gi|226491964                    --VYFYAPFWSVRRVPGPPARFPIGHLHLLARNGPDVFRAIAKEYGPIFR----------
S.bicolor|Sb04g007880.1                --IYFYAPLWSVRRVPGPPTRFPIGHLHLLAKNGPDVFRAIAKEYGPIFR----------
S.italic|Si016823m                     --IYFYAPFWSVRKVPGPPTRFPLGHLHLLAKNGPDVFRAIAKEYGPIFR----------
B.distachyon|Bradi4g09040.1            FAVYFYEPSWRVRRVPGPLAFPLIGHLPLLAKHGPEVFGVLAERYGPIYS----------
S.bicolor|Sb03g032210.2                A-VYFYIPSWRVRRVPGPVALPLVGHLPLFAKHGPGLFRMLAKEYGPIYR----------
S.italic|Si015313m                     ------------------------------------------------------------
B.distachyon|Bradi1g75310.1            A-VYFYAPSWRLRRVPGPLAYGLIGHLPLFTKHGPEVFGVLARRYGPIYR----------
H.vulgare|gi|326492025                 AVLYFYAPSWRLRRVPGPLAYGLVGHLPLLDKHGSQAFGVLAKKYGPIYR----------
B.distachyon|Bradi1g37730.1            --LYFYAPYWGVRRVPGPPALPLVGHLPLLARHGPDVFGLLAQKYGPIFR----------
Os06g0565100                           --VYFYAPWWGVRRVPGPAALPVVGHLPLLAAHGPDVFAVLAKKYGPIFR----------
S.bicolor|Sb10g022310.1                FLLYFYAPQWAVRGVPGPPALPVVGHLPLLARHGPDIFGLLAKKYGPIFR----------
Z.mays|gi|224033291                    FLLYFYAPHWRIRDVPGPPALPVVGHLPLLARHGPDVFGLLAKKYGPIFR----------
Z.mays|gi|226493876                    ------------------------------------------------------------
A.thaliana|AtMAX1                      IVIYLYRPSWSVCNVPGPTAMPLVGHLPLMAKYGPDVFSVLAKQYGPIFR----------
V.vinifera|gi|296081643                --GYLYEPYWRVRRVPGPPVFPLVGHLPLMAKYGHDVFSVLAKKYGPIFR----------
C.papaya|supercontig_7.164             --VYLYGPYWGVRRVPGPPIIPLVGHLPLMAKYGPDVFSVLAKRHGPIFR----------
S.tuberosum|PGSC0003DMP400014456       --VYLYGPYWRVRKVPGPPAFPLVGHLPLMAKYGPDVFSVLAKQYGPIYR----------
P.hybrida|gi|329739341                 --VYFYGPYWRVRKVPGPPGFPLVGHLPLMARYGPDVFSVLAKQYGPIFR----------
C.sativus|Cucsa.299480.1               --VYFYGPYWGVRRVPGPPAIPLVGHLPLLAKYGPDVFSVFASQYGPIFR----------
P.trichocarpa|POPTR_0018s07540.1       --GYLYGPYWGVRKVPGPPVIPLLGHLPLMAKHGPDVFTLLAKLYGPIFR----------
P.trichocarpa|POPTR_0006s24320.1       --GYLYGPYWGVRKVPGPPVIPLLGHLPLMAKHGPDVFSVLAKRYGPIFR----------
R.communis|gi|255550067                --GYLYKPFWGVRRVPGPPSVPLLGHLPLLAKYGPDVFSVLAKQYGPIFR----------
M.esculenta|cassava4.1_005510m         ---YLYRPYWGVRKVPGPPAIPLLGHLPLLAKYGPDVFSVLAKQYGPIFR----------
M.truncatula|gi|357437385              --GYLYWPFWKLRKVPGPPSLPLVGHLPLLAKYGPDVFSVLAKQYGPIYRIKKEAVIVGI
G.max|Glyma17g34530.1                  ------------------------------------------------------------
G.max|Glyma14g11040.1                  ------MRNNNIFRNGRPSYLELISFIKTFSWKC---------------R----------
M.truncatula|gi|357465755              --IYLYEPYWRVRKVPGPPSLPLVGHLHLLAKHGPDVFSVLAKQYGPIYR----------
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G.max|Glyma06g05520.1                  --VYLYAPYWGVRKVPGPPSLPLVGHLPLLAKYGPDVFSVLAKQYGPIYRCRRGIQVVED
B.distachyon|Bradi4g08970.1            VATYLYEPYWKMRHVPGPVPLPLIGNLHLLAWHGPDVFSVLARKHGPVFR----------
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Os01g0701500                           VVAYLYEPYRKVWHVPGPVPLPLIGHLHLLAMHGPDVFSVLARKHGPVFR----------
S.italic|Si004997m                     LIVYMYEPYWKVRHVPGPVPLPLIGHLHLLAKHGPDVFSVLAKKHGPIFR----------
Z.mays|gi|237908823                    LVVYLYEPYWKVRHVPGPVPLPFVGHLHLLARHGPDVFLVLAKKYGPIFR----------
S.bicolor|Sb03g032220.1                VLVYLYEPYWKVRHVPGPVPLPLIGHLHLLAKHGPDVFPVLAKKHGPIFR----------
S.italic|Si000975m                     VVVYLYEPYWKVRHVPGPVPLPLIGHLHLLAKHGPDVFPVLAKKHGPVFR----------
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Z.mays|gi|413926093                     ------------------------------------------------------------F
B.distachyon|Bradi3g08360.1             ------------------------------------------------------------F
H.vulgare|gi|326530386                  ------------------------------------------------------------F
Os02g0221900                            ------------------------------------------------------------F
Z.mays|gi|226491964                     ------------------------------------------------------------F
S.bicolor|Sb04g007880.1                 ------------------------------------------------------------F
S.italic|Si016823m                      ------------------------------------------------------------F
B.distachyon|Bradi4g09040.1             ----------------------------------------------------------PVSRF
S.bicolor|Sb03g032210.2                 ------------------------------------------------------------F
S.italic|Si015313m                      ------------------------------------------------------------
B.distachyon|Bradi1g75310.1             ------------------------------------------------------------F
H.vulgare|gi|326492025                  ------------------------------------------------------------F
B.distachyon|Bradi1g37730.1             ------------------------------------------------------------F
Os06g0565100                            ------------------------------------------------------------F
S.bicolor|Sb10g022310.1                 ------------------------------------------------------------F
Z.mays|gi|224033291                     ------------------------------------------------------------F
Z.mays|gi|226493876                     ------------------------------------------------------------
A.thaliana|AtMAX1                       ------------------------------------------------------------F
V.vinifera|gi|296081643                 ------------------------------------------------------------F
C.papaya|supercontig_7.164              ------------------------------------------------------------F
S.tuberosum|PGSC0003DMP400014456        ------------------------------------------------------------F
P.hybrida|gi|329739341                  ------------------------------------------------------------F
C.sativus|Cucsa.299480.1                ------------------------------------------------------------F
P.trichocarpa|POPTR_0018s07540.1        ------------------------------------------------------------F
P.trichocarpa|POPTR_0006s24320.1        ------------------------------------------------------------F
R.communis|gi|255550067                 ------------------------------------------------------------F
M.esculenta|cassava4.1_005510m          ------------------------------------------------------------F
M.truncatula|gi|357437385               KIKLNTCHDMQNLQTLFDEDELQ-----------------------------------F
G.max|Glyma17g34530.1                   ------------------------------------------------------------
G.max|Glyma14g11040.1                   ------------------------------------------------------------F
M.truncatula|gi|357465755               ------------------------------------------------------------F
G.max|Glyma04g05510.1                   ------------------------------------------------------------F
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B.distachyon|Bradi4g08970.1             ------------------------------------------------------------F
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Os01g0701400                            ------------------------------------------------------------F
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S.italic|Si004997m                      ------------------------------------------------------------F
Z.mays|gi|237908823                     ------------------------------------------------------------F
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S.italic|Si000975m                      ------------------------------------------------------------F
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Z.mays|gi|413926093                    HMGRQPLVIVANAELCKEVGIKKFKDIPNRSTPPPSIGS-LHQDALFLT-RDSTWSAMRS
B.distachyon|Bradi3g08360.1            HMGRQPLVMAASAELCKEVGIKRFRDIRNRSAPPPTAGSPLHRDALFLA-RDSAWASMRS
H.vulgare|gi|326530386                 HMGRQPLVIVANAELCKEVGIKKFKDIRNRSTPPPTVGS-LHQDALFLT-RDSTWSSMRN
Os02g0221900                           HMGRQPLVIVANAELCKEVGIKKFKDIRNRSTPPPNVGT-LHQDALFLT-RDSTWSSMRN
Z.mays|gi|226491964                    HMGRQPLVIVANAELCKEVGIKKFKDIPNRSTPPPSIGS-LHQDALFLT-RDSTWSAMRS
S.bicolor|Sb04g007880.1                HMGRQPLVIVANAELCKEVGIKKFKDIRNRSTPPPSIGS-LHQDALFLT-RDSTWSAMRS
S.italic|Si016823m                     HMGRQPLVIVANAELCKEVGIKKFKDIRNRSTPPPTVGS-LHQDALFLT-RDSTWSAMRN
B.distachyon|Bradi4g09040.1            HMGRQPLVMVASPELCREVGIKKFKSIPNRSMPSPIRCSPIHHKGLFFT-RDTRWQTMRN
S.bicolor|Sb03g032210.2                HMGRQPLVMVADAELCKEVGIKKFKSIPNRSIPTPIRGSPIHNKGLFFT-RDSRWQSMRN
S.italic|Si015313m                     ----------------------------------------------------DSRWQSMRN
B.distachyon|Bradi1g75310.1            YLGRQPVVVIADAELCREAGIKKFKSVVDRSVPSTIRSSPIHFKSLLFT-KGSRWQSMRN
H.vulgare|gi|326492025                 YMGRQPLVVLADPELCREAGIKKFKSITDRSVPVTIASSPIHYKSLLFT-KGSTWQAMRN
B.distachyon|Bradi1g37730.1            HLGRQPLVIVADPELCKEVGIRQFKSIPNRSTPSPIAGSALHQKGLFFT-RDARWSAMRN
Os06g0565100                           HLGRQPLVIVAEAELCKEVGIRQFKSIANRSLPAPIAGSPLHQKGLFFT-RDARWSAMRN
S.bicolor|Sb10g022310.1                HLGRQPLVIVADPELCREVGVRQFKLIPNRSLPAPIAGSPLHQKGLFFTSRDERWSAMRN
Z.mays|gi|224033291                    HLGRQPLVIVADPELCREVGVRQFKLIPNRSLPAPIAGSPLHQKGLFFT-RDERWSAMRN
Z.mays|gi|226493876                    ----------------------------------------------------------MRN
A.thaliana|AtMAX1                      QMGRQPLIIIAEAELCREVGIKKFKDLPNRSIPSPISASPLHKKGLFFT-RDKRWSKMRN
V.vinifera|gi|296081643                HVGRQPLVIVADAELCREVGIKKFKDIPNRSIPSAISASPLHQKGLFFT-RDARWSTMRN
C.papaya|supercontig_7.164             HMGRQPLIIVADPELCREVGIKKFKDIPNRSIPSPISASPLHQKGLFFT-RDARWSTMRN
S.tuberosum|PGSC0003DMP400014456       HMGRQPLVIVADAELCREVGIKKFKDIPNRSIPSPIAASPLHQKGLFFT-RDSRWSTMRN
P.hybrida|gi|329739341                 HMGRQPLVIVADAELCREVGIRKFKDIPNRSIPSPIAASPLHQKGLFFT-RDSRWSTMRN
C.sativus|Cucsa.299480.1               HMGRQPLIIIADPELCKEVGIKKFKDIPNRSVPSPISASPLHQKGLFFT-RDARWSTMRN
P.trichocarpa|POPTR_0018s07540.1       HMGRQPLIIVADPELCREIGIKKFKDIPNRSIPSPISASPLHQKGLFFT-RDAIWSTMRN
P.trichocarpa|POPTR_0006s24320.1       HMGRQPLIIVADPELCKEVAIKKFKDIPNRSVPSPISASPLHQKGLFFT-RDARWSTMRN
R.communis|gi|255550067                HMGRQPLIIVADPELCKEVGIKKFKDISNRSIPSPIAASPLHKKGLFFT-RDSRWSTMRN
M.esculenta|cassava4.1_005510m         HMGRQPLIIVADAELCKEVGIKKFKDIPNRSIPSPISASPLHQKGLFFT-RDTRWSTMRN
M.truncatula|gi|357437385              HMGRQPLIIIADAELCKEVGIKKFKEIPNRSIPSPISASPLHQKGLFFT-RNSQWSTMRN
G.max|Glyma17g34530.1                  -MGRQPLILVADPELCKEVGIKKFKDIPNRSIPSPISASPLHQKGLFFT-RDSRWSTMRN
G.max|Glyma14g11040.1                  HMGRQPLILVADPELCKKVGIKQFKDIPNRSIPSPISASPLHQKGLFFT-RDSRWSAMRN
M.truncatula|gi|357465755              HMGRQPLIIVADAELCKEVGIKKFKDIPNRSTPSPIKASPLHQKGLFFS-RDSQWSTMRN
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G.max|Glyma06g05520.1                  HMGRQPLIIIADAELCKEAGIKKFKDISNRSIPSPISASPLHQKGLFFS-----------
B.distachyon|Bradi4g08970.1            HMGRQALIMVADAELCRQVGIRKFKSFRNRSLPSPIAKSPILEKGLFVT-RDSRWSAMRN
Os01g0700900                           HMGRQPLVMVADAELCKEVGVKKFKNFPNRSMPSPITNSPVHQKGLFFT-SGSRWTTMRN
Os01g0701400                           HMGRQPLVMVADAELCKEVGVKKFKSIPNRSMPSAIANSLINQKGLCFT-RGSRWTALRN
Os01g0701500                           HMGRQPLIIVADAELCKEVGVKKFKSIPNRSMPSPIANSPIHKKGLFFI-RGPRWTSMRN
S.italic|Si004997m                     HMGRQPMIMVANAEICKDVGIKKFKSIPNRSMPSPIANSTIHQKGLFFT-RDSRWSSMRN
Z.mays|gi|237908823                    HMGRQPLVIVANAELCKEVGIKKFKSMPNRSLPSAIANSPIHLKGLFST-RDSRWSALRN
S.bicolor|Sb03g032220.1                HVGRQPLIIVADAELCKEVGIKKFKSMPNRSLPSPIANSPIHRKGLFAT-RDSRWSAMRN
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 S.moellendorffii|gi|300160069        ILLPTFHTEKLSAYVPLMERVMGQVVEIL-------DKHANAGED-VNMTQLLQRMALDV
Z.mays|gi|413926093                   TVVPLYQPARLAGLIPVMQSYVDTLAANI-------AA--CPDQDCVPFCQLSLRMAIDI
B.distachyon|Bradi3g08360.1           TVVPLYQPARLAQLVPTMRASVDALVDAV-------DQ--DQGSY-VPFSQLSLRLAIDI
H.vulgare|gi|326530386                TVIPLYQPARLACLVPTMQPYVDALVDSV-------AA--CPDQDCVPFCQLSLRMAIDI
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S.italic|Si016823m                    TVVPLYQPARLAGLIPVMQSYVDALVANI-------AG--CPDQDCIPFCQLSLRMAIDI
B.distachyon|Bradi4g09040.1           VIISIYQPSHLASLIPAIQPYVERAGRLL-----------RHGEE-ITFSDLSLKLFSDT
S.bicolor|Sb03g032210.2               VILTIYQPSHVASLIPAIQPYVERAGRLL-----------HPGEE-ITFSDLSLKLFNDT
S.italic|Si015313m                    VIISIYQPSHVASLIPAIQPYVERAGRLL-----------HPGEE-ITFSDLSLKLFSDT
B.distachyon|Bradi1g75310.1           VIIAIYQPSHLASLIPAVHPYIRRAARLL-----------HPGQE-VAFSDLAVKLFSDT
H.vulgare|gi|326492025                VIISIYQPSHLASLIPAIQPYIERAGSLF-----------CPGEE-ITFSDVSIRLFTDV
B.distachyon|Bradi1g37730.1           AILSLYQPSHLAGLIPTMQRCVERAADTI-------ST--VNDGD-FDFSDLALKLATDV
Os06g0565100                          TIISLYQPSHLAGLIPTMHSCVARAADAI-------AA--AEQRD-VDFSDLSLKLATDV
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Z.mays|gi|224033291                   TIISLYQPSHLAGLVPTMQHCIERAADAI-------PAMVVQENGLVDFSDLSLKLATDI
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A.thaliana|AtMAX1                     TILSLYQPSHLTSLIPTMHSFITSATHNL--------D--SKPRD-IVFSNLFLKLTTDI
V.vinifera|gi|296081643               TIISVYQQSHLANLVPTMQAFIEPAFRNL-------PS--SEEED-ITFSNLSLKLATDV
C.papaya|supercontig_7.164            TIVSVYQPSHLASLVPTMQEFIESATQNL-----------ESQQD-VNFSNMSLKLATDV
S.tuberosum|PGSC0003DMP400014456      TILSVYQPSYLAKLVPIMQSYIESATKNL-----------ESEGD-ITFSDLSLKLATDV
P.hybrida|gi|329739341                TILSVYQPSYLAKLVPIMQSFIESATKNL-----------DSEGD-LTFSDLSLKLATDV
C.sativus|Cucsa.299480.1              TILSVYQPSHLMRLIPTMQSIIETATQNL-------HS--SVEED-IPFSNLSLKLTTDV
P.trichocarpa|POPTR_0018s07540.1      SILSVYQPSHLASLVPTMQSFIESATENF-------QS--LKEEE-ITFSNLSLKLATDV
P.trichocarpa|POPTR_0006s24320.1      TILSVYQPSHLASLVPTMQSFIESATDNF-------Q---SSNEE-ITFSNFSLKLATDV
R.communis|gi|255550067               TILSVYQPSHLASLVPTMQSFIESATANF-------QS--SKDED-ITFSNLSLKLATDV
M.esculenta|cassava4.1_005510m        TILSVYQPSHLASLVPTMQSFIESATANF-------QSFEEQEED-ITFSNLSLKLATDV
M.truncatula|gi|357437385             TILSVYQPSHLANLVPKMQSFIESATQNL-------DD--TSKED-IIFSNLSLRLATDV
G.max|Glyma17g34530.1                 TILSVYQPSHLASLVPTMQSFIESATQNL-------D---TPNED-IIFSNLSLRLATDV
G.max|Glyma14g11040.1                 TILSVYQPSHLASLVPMMQSFIESATQNL-------D---TPNED-IIFSNLSLRLATDV
M.truncatula|gi|357465755             TILSVYQPSHLSRLVPTMQSFIESATQNL-------D---SQKED-IFFSNLSLKLATDV
G.max|Glyma04g05510.1                 TILSMYQPSYLSRLVPTMQSFIESATQNL-------D---SQKED-IIFSNLSLRLATDV
G.max|Glyma06g05520.1                 ------------RLVPTMQSFIESATQNL-------D---SQKED-IIFSNLSLRLATDV
B.distachyon|Bradi4g08970.1           TVASIYQPSHLASLVPTMHSYIQRAARNI-------GGV-GGGQD-VDFSTLAVSLFTDV
Os01g0700900                          MILSIYQPSHLATLIPSMESCIERAAENL-----------EGQEE-INFSKLSLSFTTDV
Os01g0701400                          MIISIYQPSHLASLIPTMQSCIECVSKNL-----------DGQED-ITFSDLALGFATDV
Os01g0701500                          MIISIYQPSHLASLIPTMESCIQRASKNL-----------DGQKE-ITFSDLSLSLATDV
S.italic|Si004997m                    IIVSIYQPSHLAGLIPTMESYIERAATNL-----------QHGEE-VDFSKLALSLFTDV
Z.mays|gi|237908823                   IIVSIYQPSHLAGLIPSMQSHIERAATNL-------DD--GGEAE-VAFSKLALSLATDV
S.bicolor|Sb03g032220.1               VIVSIYQPSHLAGLMPTMESCIERAATTN-------LG--DGEE--VVFSKLALSLATDI
S.italic|Si000975m                    IIVSIYQPSHLAGLIPAMESCIQRAATNL-----------DDGEE-VVFSDLAVRLATDV
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S.moellendorffii|gi|300160069          IGESAFGTGFKLVKPSWADG----------------------RSEDKDMVNAVLNS-LDT
Z.mays|gi|413926093                    IGRTAFGIEFGLSKNAAGTGSSSSE----------SPGGGEGEGDVREFLREYKRS-MEF
B.distachyon|Bradi3g08360.1            IGKTAFGIEFGLLSKQGTNG----------------------DDEARELLGEYERS-MEF
H.vulgare|gi|326530386                 IGKTAFGIDFGLCKKVAAADG---------------------GEDVRGFLEEYKRS-MEF
Os02g0221900                           IGKTAFGIEFGLSRKAADTAAG-------------DDGDGDDDDDVKEFLREYKKS-MEF
Z.mays|gi|226491964                    IGRTAFGIEFGLSKNAAGTGSSSSE----------SPGGGEGEGDVREFLREYKRS-MEF
S.bicolor|Sb04g007880.1                IGKTAFGIEFGLSKNAAGGGG--------------ETEGGEGDDNVREFLKEYKRS-MEF
S.italic|Si016823m                     IGKTAFGVEFGLSKDSAGSG----------------CSGGEVDDDIREFLKEYKRS-MEF
B.distachyon|Bradi4g09040.1            IGQVAFGVDFGLTKGKGAEAE---------------------ESIPDGFIRKHFYA-TTE
S.bicolor|Sb03g032210.2                IGQVAFGVDFGLTKDDTTAATSPAAQQQPAH--GGANANQSVDDPATDFIRKHFRA-TTS
S.italic|Si015313m                     IGQVAFGVHFGLTKDMATSPPPLQ-----------EPAAAKSVDPATDFIRKHFHATTTS
B.distachyon|Bradi1g75310.1            IGQAAFGVDFGLTKPDDANNV--------------DSTINNEKTATDDFIEKHLYA-LTS
H.vulgare|gi|326492025                 IGQAAFGVDFGLTKDADDAEKII-------------------HDAPRDFIQKHLYA-TTS
B.distachyon|Bradi1g37730.1            IGQAAFGVDFALSAPPAGDGTK--------------------DASAAEFIAEHVQS-TTS
Os06g0565100                           IGQAAFGVDFGLTAAAAAAPR--------------SDDADADGGEAAEFIREHVHS-TTS
S.bicolor|Sb10g022310.1                IGQAAFGVDFGLTASGDP-----------------------GGEAAEFIREHVHS-TTS
Z.mays|gi|224033291                    IGEAAFGVDFGLTASGP------------------------GCEAAEFIREHVHS-TTS
Z.mays|gi|226493876                    IGEAAFGVDFGLTASGP------------------------GCEAAEFIREHVHS-TTS
A.thaliana|AtMAX1                      IGQAAFGVDFGLSGKKPIK----------------------DVEVTDFINQHVYS-TTQ
V.vinifera|gi|296081643                IGQAAFGVHFGLSKPPSSNEVKN-------------------SDEVSEFINQHIYS-TTN
C.papaya|supercontig_7.164             IGRAAFGVNFGLSKPQSIDESINKKT---------NQDDNVDDHEVSSFINQHIYS-TTQ
S.tuberosum|PGSC0003DMP400014456       IGQASFGVDFGLSKPISD-----------------KMSHHQDDSEVQEFIKQHIYS-TTQ
P.hybrida|gi|329739341                 IGQAAFGVDFGLSKPITDK----------------MNHQEK-DSEVQEFINQHNYS-TTQ
C.sativus|Cucsa.299480.1               IGTAAFGVNFGLSNPHATKT---------------TNDQDSKNDEVSDFINQHIYS-TTQ
P.trichocarpa|POPTR_0018s07540.1       IGQAAFGVDFGLSKPQSTSDSFNS----------FHSQGKDNTDVSEFIKQHIYS-TTQ
P.trichocarpa|POPTR_0006s24320.1       IGQAAFGVDFGLSKPQSASDSINS----------FHNQGKDNCDVSEFINQHIYS-TTQ
R.communis|gi|255550067                IGQAAFGVDFGLSKPQSTTDSAN-----------TFRNQENGKEVSDFINQHVYS-TTQ
M.esculenta|cassava4.1_005510m         IGQAAFGVDFGLSKPQSAKN---------------ISHNQENGNEVSDFINQHVYS-TTQ
M.truncatula|gi|357437385              IGDAAFGVNFGLSKPHSICESMNN----------VEQSSANSDEVSIFINQHIYS-TTQ
G.max|Glyma17g34530.1                  IGEAAFGVNFGLSKPHS----------------------------VSDFINQHIYS-TTQ
G.max|Glyma14g11040.1                  IGEAAFGVNFGLSKPISV---------------------------LSDFINQHIYS-TAQ
M.truncatula|gi|357465755              IGQAAFGVNFGLSQSHSVHNESKNVATDNK-----DLMNASGSNEVTDFINQHIYS-TTQ
G.max|Glyma04g05510.1                  IGHAAFGVNFGLSRPHSVCD----------------------SIKISDFIDQHIYS-TTQ
G.max|Glyma06g05520.1                  IGHAAFGVNFGLSSPHSVCDSIKNVNVNNNNNNASASSSNSNDNEVSDFINQHIYS-TTQ
B.distachyon|Bradi4g08970.1            MGQAAFGLDFGLTAADKNPGGDSSS-----------------NKQAQEFVKMHAHV-TTS
Os01g0700900                           LGQAAFGTDFGLSKKLASSDDDEDTRKI-------AADTCAEAKASSEFIKMHVHA-TTS
Os01g0701400                           IGQAAFGTDFGLSKISASSNDDDIDKI--------ATDTSAEAKASSEFIRMHVHA-TTS
Os01g0701500                           IGLAAFGTDFGLSKLPVTPDDSNIDKI--------AADTSVEAKASSEFIKMHMHA-TTS
S.italic|Si004997m                     IGQAAFGADFGLSRKPTPPGEDGKSHAR-------DDAGSTQANASSEFIKMHLQA-TTS
Z.mays|gi|237908823                    IGQAAFGADFGLTTKPAAPPPHHGPPRQHGE----EDGDGSHSTRSSEFIKMHIHS-TTS
S.bicolor|Sb03g032220.1                IGQAAFGTDFGLSGKPVVPDDDMKGVDVV------VGDAAKAKASSSEFINMHIHS-TTS
S.italic|Si000975m                     IGQAAFGADFGLSGKPPVPGDEDSKG---------ADDGGAAAKASSEFINMHIHS-TTS
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S.moellendorffii|gi|300160069          LTMSEKAPVSTFAGLFFPFLQHPIREIMKRIPGTGDWNQYTGNLLLEAQMRALLERREA-
Z.mays|gi|413926093                    VKMDLTSSLSTILGLFLPCVQTPCKRLLRRVPGTADYKMDQNERRLCSRIDAIIAGRRRD
B.distachyon|Bradi3g08360.1            MKMDLSSSLSTILGLFLPCLQTPCKRLLRRVPGTADHKMEQNERRLCRRIDAIIAARRR-
H.vulgare|gi|326530386                 IKMDLSSSLSTILGLFLPCAQTPCKRLLRRVPGTADYKMEENERLLCRRIDAIIAGRRR-
Os02g0221900                           IKMDLSSSLSTILGLFLPCVQTPCKRLLRRVPGTADYKMDQNERRLCRRIDAIIAGRRRD
Z.mays|gi|226491964                    VKMDLTSSLSTILGLFLPCVQTPCKRLLRRVPGTADYKMDQNERRLCSRIDAIIAGRRRD
S.bicolor|Sb04g007880.1                VKMDLSSSLSTILGLFLPCVQTPCKRLLRRVPGTADYKMNENERRLCSRIDAIIAGRRRD
S.italic|Si016823m                     IKMDLSSSLSTILGLFLPCVQTPCRRLLRRLPGTADYKMDENERRLCRRIDAIIAGRRRD
B.distachyon|Bradi4g09040.1            LKMDLSGSLSMLLGMVAPLMQDPVRQLLLRVPGSADRRMEDTNLALSGLLDGIVAERAAL
S.bicolor|Sb03g032210.2                LKMDLSGPLSIVLGQFVPFLQEPVRQLMLRVPGSADRRLEEANSDMSGLLDEIVAERAA-
S.italic|Si015313m                     LKMCLSGSLSIVLGQFVPFLQEPLRQLLLRVPGSADRRLEETNSAMSGLLDEIVAERAA-
B.distachyon|Bradi1g75310.1            LKADLNGSLSMVLGTVAPLLQEPARQLLLRVPGSADRLMDETNRALSGLVDAIVAERAAM
H.vulgare|gi|326492025                 LKMDTSGSLSMLVGTFLPALQKPLRKLMLSVPGSMDRRMDDTNSALSGELDVIVAERAA-
B.distachyon|Bradi1g37730.1            LKMDLSASLSIVLGLVAPALQEPARRLLSRVPGTADRRTARANERLQARVEEIVASREQ-
Os06g0565100                           LKMDLSGSLSIVLGLVAPALQGPARRLLSRVPATADWRTARANERLRARVGAVVARRERA
S.bicolor|Sb10g022310.1                LKMDLSAPLSVALGLVAPALQGPVRRLLSRVPGTADWKVARTNARLRARVDEVVAARAR-
Z.mays|gi|224033291                    LKMDLSAPLSVVLGLVAPALQGPVRHLLSRVPGTADWRVARTNARLRARVDEIVVSRARG
Z.mays|gi|226493876                    LKMDLSAPLSVVLGLVAPALQGPVRHLLSRVPGTADWRVARTNARLRARVDEIVVSRARG
A.thaliana|AtMAX1                      LKMDLSGSLSIILGLLIPILQEPFRQVLKRIPGTMDWRVEKTNARLSGQLNEIVSKRAK-
V.vinifera|gi|296081643                LKMDLSGSFSIILGLLVPILQKPVQHILKRIPGTMDWKIYQTNKKLSSRLDEIVAKRMK-
C.papaya|supercontig_7.164             LKMDLSGSFSIILGLLIPILQEPFRQILKRIPGAMDRKVDQTNRKISRKLDEIVTKRMK-
S.tuberosum|PGSC0003DMP400014456       LKMDLSGSVSIILGLLVPILQEPFRQVLKRIPGTMDWKVERTNKNLSSRLDEIVAKRME-
P.hybrida|gi|329739341                 LKMDLSGSVSIILGLLVPILQEPFRQILKRIPGTIDWKVERTNKNLSRRLDEIVAKRME-
C.sativus|Cucsa.299480.1               LKMDLSGSFSIILGLLVPILQEPFRQVLKRIPFTMDWKVDRTNQKLSGRLNEIVDKRMK-
P.trichocarpa|POPTR_0018s07540.1       LKMDLSGSFSIILGLLVPILQEPFRQILKRIPGTMDWKVDRTNKNISGRLEEIVRKKME-
P.trichocarpa|POPTR_0006s24320.1       LKMDLSGSFSIIIGLLVPILQEPFRQILKRIPGTMDWKVDRTNRNISGRLDEIVRKKME-
R.communis|gi|255550067                LKMDLSGSFSIIIGLLIPILQEPFRQILKRIPGTMDWKVDRTNKNISGKLDEIVTKRMK-
M.esculenta|cassava4.1_005510m         LKMDLSGSFSIIIGLLIPILQEPFRQILKRIPGTMDWKVDRTNKNLSGKLDEIVTKRMN-
M.truncatula|gi|357437385              LKMDLSGSFSIIIGLIAPILQEPIRQILKRIPGTMDWKMECTNKNLTGRLDDIVKKRME-
G.max|Glyma17g34530.1                  LKMDLSGSFSIILGLLAPILQEPFRQILKRIPGTMDSKIESTNEKLSGPLDEIVKRRME-
G.max|Glyma14g11040.1                  LKMDLSGSFSIILGLLAPILQEPFRQILKRIPGTMDRKIESTNEKLSGRLDEIVKRRME-
M.truncatula|gi|357465755              LKMDLSGSFSIILGLLVPILQEPFRQILKRIPGTMDWKIERTNEKLGGRLDEIVEKRTK-
G.max|Glyma04g05510.1                  LKMDLSGSLSIILGLLLPILQEPFRQILKRIPGTMDWKIERTNQKLSGRLDEIVEKRMK-
G.max|Glyma06g05520.1                  LKMDLSGSLSIILGLLLPILQEPFRQILKRIPGTMDWKIEHTNQKLSGRLDEIVEKRMK-
B.distachyon|Bradi4g08970.1            LKMDMTGSLSSIVGQLVPSLHRPFQEVLRRVPGTADRETDRVNRELRRQMDAIVAD--A-
Os01g0700900                           LKMDMSGSLSIIVGQLLPFLHEPFRQVLKRLRWTADHEIDRVNLTLGRQLDRIVAERTA-
Os01g0701400                           LKMDLSGSLSIIIGQLLPFLQEPFRQVLKRIPWTADHEIDHVNLALGGQMDKIVAERAA-
Os01g0701500                           LKMDLSGSLSILVGMLLPFLQEPFRQVLKRIPGMGDYKIDRVNRALKTHMDSIVAEREA-
S.italic|Si004997m                     LKMDLSGSLSIIVGQLMPFLHQPFRQVLTRIPGSADREIDRVNNELSRQMDGMVADRIA-
Z.mays|gi|237908823                    LKMDLSGSLSTIVGTLLPVLQWPLRQLLLRVPGAADREIQRVNGALCRMMDGIVADRVA-
S.bicolor|Sb03g032220.1                LKMDLSGSLSTIVGALVPFLQNPLRQVLLRVPGSADREINRVNGELRRMVDGIVAARAA-
S.italic|Si000975m                     LKMDLSGSLSTIVGMFLPFLQKPLRQVLLRVPGSADREITRVNGELRRMMDGIVAARERA
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S.moellendorffii|gi|300160069          -EMRDGVVRS------------DALSLLLDARAK-SQEMRE--------LLTDERVLALA
Z.mays|gi|413926093                    RATRRRCGPGAAPAPA----PLDFIAALLDAMESGRRRRRR--------CRCQQGLRAGG
B.distachyon|Bradi3g08360.1            ---RSSSPATAL----------DFIAALLED----SRGRVA--------ALEDRHVRALA
H.vulgare|gi|326530386                 -DRENSASEPGAAL--------DFIAALLDARES---GARDL-------ALEDKHVRALA
Os02g0221900                           RDAGDGAAL-------------DFIAALLDARES-GGGGHGG------FALEDRHVRALA
Z.mays|gi|226491964                    RATRRRCGPGAAPAPA----PLDFIAALLDAMES-GGGGGGGAGANKDFALADRHVRALA
S.bicolor|Sb04g007880.1                RATRRRGGDGVSEDDAA---PLDFIAALLDAMEN-GGGAKD-------FALADRHVRALA
S.italic|Si016823m                     RASRRRDGDGDGDGDAARSAPLDFIAALLDAMES-GGGKEL--------ALEDRHVRALA
B.distachyon|Bradi4g09040.1            PE-LERGQK-------------NFLSVLLNARES-TEALRN--------VFTPDYVSALT
S.bicolor|Sb03g032210.2                -Q-ADRGQQK------------NFLSVLLNARES-TEAMKK--------LLTPDYVSALT
S.italic|Si015313m                     -Q-ADGDQK-------------NFLSVLLNASES-TEVTKK--------LLTPDYLGIP-
B.distachyon|Bradi1g75310.1            EAQSEGEKK-------------NFLSVLLKARES-SHAMRE--------LFTADYVSALT
H.vulgare|gi|326492025                 -Q-ADRGQK-------------NFLSVLLNGIDT-SDAMRK--------LFTPDYVSALT
B.distachyon|Bradi1g37730.1            -QSLQRRRQKSQISKR------DFLSALLDARDGGDGKMRE--------LLTPVYVGALT
Os06g0565100                           GGEARRARR-------------DFLSAVLNARDGGSDRMRA--------LLTPDYVGALT
S.bicolor|Sb10g022310.1                -A-RERRRHGEARTK-------DFLSAVLDARDR-SAALRE--------LLTPDHVSALT
Z.mays|gi|224033291                    RGQHGERRK-------------DFLSAVLDARDR-SAALRE--------LLTPDHVSALT
Z.mays|gi|226493876                    RGQHGERRK-------------DFLSAVLDARDR-SAALRE--------LLTPDHVSALT
A.thaliana|AtMAX1                      -E-AETDSK-------------DFLSLILKARES-DPFAKN--------IFTSDYISAVT
V.vinifera|gi|296081643                -D-KDRGSK-------------DFLSLILNARES-EKAMKN--------IFTSDYLNAVT
C.papaya|supercontig_7.164             -DIDKRSNV-------------DFLSLILRARES-GTAAKN--------VFSPDYISAVT
S.tuberosum|PGSC0003DMP400014456       -E-KDRSSK-------------DFLSLIMQARES-EKLAKN--------VFTSDYISAVT
P.hybrida|gi|329739341                 -E-KYGSSK-------------DFLSLILQARES-EKLAKN--------VFTSDYISAVT
C.sativus|Cucsa.299480.1               -C-NDRGSK-------------DFLSLILRARES-ETVSRN--------VFTPDYISAVT
P.trichocarpa|POPTR_0018s07540.1       -E-KNKGSK-------------DFLSLILRARES-ETLSKN--------AFTPDYISAVT
P.trichocarpa|POPTR_0006s24320.1       -E-KNRGSK-------------DFLSLILRARES-ETLSKK--------VFTPDYISAVT
R.communis|gi|255550067                -D-KNRGSK-------------DFLSLILSARES-ETLSKN--------VFSPDYISAVT
M.esculenta|cassava4.1_005510m         -D-KNRGSK-------------DFLSLILSARES-ETLSRN--------VFTPDYISAVT
M.truncatula|gi|357437385              -D-KSRTSK-------------NFLSLILNTRES-KSVSEN--------VFSFDYISAVT
G.max|Glyma17g34530.1                  -D-KNRTSK-------------NFLSLILNARES-KKVSEN--------VFSPDYISAVT
G.max|Glyma14g11040.1                  -N-KNRTSK-------------NFLSLILNARES-KKVSEN--------VFSPDYVSAVT
M.truncatula|gi|357465755              -D-RTRSSK-------------DFLSLILNARES-KAVSEN--------VFTPEYISAVT
G.max|Glyma04g05510.1                  -D-KARSSK-------------DFLSLILNARET-KAVSEN--------VFTPDYISAVT
G.max|Glyma06g05520.1                  -D-KTRSSK-------------DFLSLILNARET-KSVSEN--------VFTPEYISAVT
B.distachyon|Bradi4g08970.1            -A-RERDLHYSRQQQK----KNDFLSVVL------GGAAEK--------LLTPDYIGALA
Os01g0700900                           -A-MKRDPAA--LQQR----K-DFLSVMLTARES-NKSSRE--------LLTPDYISALT
Os01g0701400                           -A-MERDQAAPHAQQR----K-DFLSVVLAARES-NKSWRE--------LLTPDYISALT
Os01g0701500                           -A-MEHDLAA--SQQR----K-DFLSVVLTARES-NKSSRE--------LLTPDYISALT
S.italic|Si004997m                     -A-REHSPAS---EQH----K-DFLSVVLAAKER-GASAQE--------LLTPDYLSGLT
Z.mays|gi|237908823                    -A-RERAPQA---QRQ----K-DFLSVVLAARDS-DAAARK--------LLTPDYLSALT
S.bicolor|Sb03g032220.1                -E-RERAPAATAAQQH----K-DFLSVVLAARES-DASTRE--------LLSPDYLSALT
S.italic|Si000975m                     PA-ASRQRK-------------DFLSVVLAARES-DASTRE--------LLSPDYLSALI
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S.moellendorffii|gi|300160069          YELMMAGSEST-----GTNLCYTLYFIAA--------HPEVASKMVKEIDEL--APL-GS
Z.mays|gi|413926093                    QARARAGVRAPHRRHQDHGVHAVVGGVPGLVPPARGGQAAAGVGRLRAAPRA--RARAGR
B.distachyon|Bradi3g08360.1            YEHLIAGTKTT-----AFTLSSLVYLVSC--------HRPVEEKLLAELDAF--GPQ-SQ
H.vulgare|gi|326530386                 YEHLIAGTKTT-----AFTVSSVLYLVSC--------HPRVEEKLLAEVDAF--D---GA
Os02g0221900                           YEHLIAGTKTT-----AFTVSSVVYLVSC--------HPRVEERLLREIDGF--APR-GR
Z.mays|gi|226491964                    YEHLIAGTKTT-----AFTLSSVVYLVSC--------HPLVEAKLLRELDGF--APRRGR
S.bicolor|Sb04g007880.1                YEHLIAGTKTT-----AFTLSSVVYLVSC--------HPRVEEKLLREVDGF--APRHGR
S.italic|Si016823m                     YEHLIAGTKTT-----AFTLSSVVYLVSC--------HPRVEEKLLRELDGS--APPGGR
B.distachyon|Bradi4g09040.1            YEHLLAGAVTM-----SFTLSSLVYLVAA--------HPEVEEKLLREIDAF--GPK-DV
S.bicolor|Sb03g032210.2                YEHLLAGSVTM-----SFTLSSLVYLVAM--------HPEVEEKLLREIDAF--GPK-DV
S.italic|Si015313m                     ----------------------VAM--------HPEVEEKLLREIDAF--GPK-DV
B.distachyon|Bradi1g75310.1            YEHLLAGSGSM-----SFTLSGLAYRVAM--------HPEVEEKMLSEIDAF--GPK-DL
H.vulgare|gi|326492025                 YEHLLAGSGTM-----SFTLSSLVYLVST--------HPEVEEKLLQEIDAF--GPK-DV
B.distachyon|Bradi1g37730.1            YEHLLAGSATT-----SFTLASAVYLVAG--------HPEVEAKLLAEIDRY---PP-AA
Os06g0565100                           YEHLLAGSATT-----AFTLSSAVYLVAG--------HPGVEAKLLDEVDRF--GPP-DA
S.bicolor|Sb10g022310.1                YEHLLAGSATT-----AFTLSSAVYLVAG--------HPEVEAKLLAEVDGF--GPR-GA
Z.mays|gi|224033291                    YEHLLAGSATT-----AFTLSSAVYLVAG--------HPEVEAKLLAEVDAF--GPR-GA
Z.mays|gi|226493876                    YEHLLAGSATT-----AFTLSSAVYLVAG--------HPEVEAKLLAEVDAF--GPR-GA
A.thaliana|AtMAX1                      YEHLLAGSATT-----AFTLSSVLYLVSG--------HLDVEKRLLQEIDGF--GNR-DL
V.vinifera|gi|296081643                YEHLLAGSATT-----SFTLSSTIYLIAE--------HPEVEKKLLAEIDGF--GPP-DQ
C.papaya|supercontig_7.164             YEHLLAGSATT-----SFTLSSVLYLVAG--------HPEVEKKLLAEIDSF--GPH-KK
S.tuberosum|PGSC0003DMP400014456       YEHLLAGSATT-----SFTLSSIIYLVAC--------HPEVEQKLLAEIDAF--GPD-DH
P.hybrida|gi|329739341                 YEHLLAGSATT-----SFTLSSIIYLVAG--------HPKVEQKLIAEIDAF--GPD-DH
C.sativus|Cucsa.299480.1               YEHLLAGSATT-----AFTLSSVVYLVAG--------HPEVEKKLLEEIDNF--GPS-DQ
P.trichocarpa|POPTR_0018s07540.1       YEHLLAGSATT-----AFTLSSVVYLIAQ--------HPEVEKKLLAEIDGF--GPH-EQ
P.trichocarpa|POPTR_0006s24320.1       YEHLLAGSITT-----SFTLSSVVYLVAQ--------HPETEKKLLAEIDGF--GPH-EQ
R.communis|gi|255550067                YEHLLAGSATT-----AFTLSSIVYLVAG--------HPEVEKKLLAEIDVF--GPP-DQ
M.esculenta|cassava4.1_005510m         YEHLLAGSATT-----SFTLSSIVYLVAG--------HPEVEKKLLAEIDGF--GPP-DQ
M.truncatula|gi|357437385              YEHLLAGSATT-----SFTLSSIVYLVAG--------HPNVEEKLLQEIDGF--GPH-DK
G.max|Glyma17g34530.1                  YEHLLAGSATT-----AFTLSSIVYLVAG--------HREVEKKLLQEIDGF--GPP-DR
G.max|Glyma14g11040.1                  YEHLLAGSATT-----AFTLSSIVYLVAG--------HIEVEKKLLQEIDGF--GTP-DR
M.truncatula|gi|357465755              YEHLLAGSATT-----SFTLSSVVYLVAA--------HPEVEKKMLEEIDGY--GSL-DQ
G.max|Glyma04g05510.1                  YEHLLAGSATT-----SFTLSSVVYLVAG--------HPEVEKKLLHEIDGF--GPV-DQ
G.max|Glyma06g05520.1                  YEHLLAGSATT-----SFTLSSVVYLVAG--------HPEVEKKLLHEIDGF--GPV-DQ
B.distachyon|Bradi4g08970.1            YEHILAGSASP-----AFTLSTVVYLVSK--------HPEVEDRLLKEVDAFFLDHD-DR
Os01g0700900                           YEHLLAGSATT-----AFTLTTALYLVAK--------HPEVEEKLLREIDGF--GPR-DR
Os01g0701400                           YEHLLAGSATT-----AFTLSTVLYLVSK--------HPEVEEKLLREIDGF--GPH-DH
Os01g0701500                           YEHLLAGSTTT-----AFTLSTVLYLVAK--------HPEVEEKLLKEIDAF--GPR-YC
S.italic|Si004997m                     YEHLLAGSATT-----SFTLSCTVYLIAK--------HPEVEEKLLREIDAF--GAH-DR
Z.mays|gi|237908823                    YEHLLAGSATT-----AFTLSSVLYLVAQ--------HPRVEEKLLREVDAF--GPP-DR
S.bicolor|Sb03g032220.1                YEHLIAGPATA-----AFTLSSVVYLVAK--------HPEVEEKLLREMDAF--GPR-GS
S.italic|Si000975m                     YEHLLAGSATT-----AFTLSSVVYLVAK--------HPEVEEKLLREIDAF--GPR-DS
```

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S.moellendorffii|gi|300160069          --TVAFEDV-DKFKYVDQVIKESMRMITFSPVVAREAMEDIKVVGYHIPK--------GT
Z.mays|gi|413926093                    RRAPERVPLPRPGHQGGHAVLRRLAAHRQADLRARGDR------GLRSPQ--------GR
B.distachyon|Bradi3g08360.1            --SPDADELHTKFPYLDQIIKESMRFHLVSPLIARETSEAVEIGGYLLPK--------GT
H.vulgare|gi|326530386                 --APDADDLQGRFPYLDLVVKEAMRFHLVSPLIARETSEEVEIAGYQLPK--------GT
Os02g0221900                           --VPGADELHAGLPYLNQVIKEAMRFHLVSPLIARETSEPVEIAGHLLPK--------GT
Z.mays|gi|226491964                    GRAPDADELQSGFPYLDQVIKEAMRFYVVSPLIARQTSERVEIGGYVLPK--------GA
S.bicolor|Sb04g007880.1                --APDADELQSRFPYLDQVIKEAMRFHLVSPLIARQTSERVEIGGYVLPK--------GA
S.italic|Si016823m                     --APNAEELQSRFPYLDQVIKEAMRFHLVSPLIARQTSERVEIGGHVLPK--------GA
B.distachyon|Bradi4g09040.1            --VPSAEELHNNFPYLEQVLKETMRFFTVSPLIAREASEDVEIGGYLLPK--------GT
S.bicolor|Sb03g032210.2                --VPSSDDLETKFPYVEQVVKETMRFYTASPLVARQASEDVEVGGYLLPK--------GT
S.italic|Si015313m                     --VPSSDDLQTKFPYVERFLKETMRFCTASSLVTREASEDVEVGAYLLPKRDIICATVGT
B.distachyon|Bradi1g75310.1            --VPDAEELNTKFTYLEQVLKETMRFYSSSPLVSRETTEDVEIGGYLLPK--------GT
H.vulgare|gi|326492025                 --VPDADDLRTKFTYLEQVLKETMRYFPGSPVVSREATADVEIGGYLLPK--------GT
B.distachyon|Bradi1g37730.1            --VPTAEDLQQKFPYLDQVIKEAMRFYTVSPLIARETSREVEIGGYALPK--------GT
Os06g0565100                           --VPTADDLEHKFPYLDQVIKEAMRFYTVSPLIARETSEQVEVGGYTLPK--------GT
S.bicolor|Sb10g022310.1                --VPTADDLHHRFPYLDQVIMEAMRFYTVSPLIARVTSRRTELGGHELPK--------GT
Z.mays|gi|224033291                    --VPTADDLQHRFPYLDQVIKEAMRFYTVSPLIARVTSRQTELGGHTLPK--------GT
Z.mays|gi|226493876                    --VPTADDLQHRFPYLDQVIKEAMRFYTVSPLIARVTSRQTELGGHTLPK--------GT
A.thaliana|AtMAX1                      --IPTAHDLQHKFPYLDQVIKEAMRFYMVSPLVARETAKEVEIGGYLLPK--------GT
V.vinifera|gi|296081643                --MPTAHDLQHKFPYLDQAKSLAMRFYTVSPLVARETSAEVEIGGYVLPK--------GT
C.papaya|supercontig_7.164             --LPTFHHLQYNFPYLDQVIKESMRFYLVSPLIARETSKDVEIGGYFLPK--------GT
S.tuberosum|PGSC0003DMP400014456        --IPTANDLQQKFPYLDQVIKEAMRCYTVSPLVARETSAEVEIGGYKLPK--------GT
P.hybrida|gi|329739341                 --MPTANDLQQKFSYLDQVIKEAMRCYTVSPLVARETSAEVEIGGYKLPK--------GT
C.sativus|Cucsa.299480.1               --IPTANDLQQKFPYLDQVIKESMRFYTVSPLVARETSKDVEIGGYLLPK--------GT
P.trichocarpa|POPTR_0018s07540.1       --MPTAQDLQNEFPYLDQVVKEAMRFYVVSPLIARETSKEVEIGGYLLPK--------GT
P.trichocarpa|POPTR_0006s24320.1       --IPTALDLQNKFPYLDQVVKEAMRFYVVSPLVARETSKEVEIGGYVLPK--------GT
R.communis|gi|255550067                --TPTSQDLQTRFPYLDQVIKEAMRFYVVSPLVARETSKEVEIGGYLLPK--------GT
M.esculenta|cassava4.1_005510m         --MPTAHDLQTKFPYLDKVIKESMRFYVVSPLVARETSKDVEIGGYLLPK--------GT
M.truncatula|gi|357437385              --IPNAKDLNESFPYLDQVIKEAMRIYTVSPLVARETSNEVEIGGYLLPK--------GT
G.max|Glyma17g34530.1                  --IPTAQDLHDSFPYLDQVIKEAMRFYTVSPLVARETSNEVEIGGYLLPK--------GT
G.max|Glyma14g11040.1                  --IPIAQDLHDSFPYLDQVIKEAMRFYTVSPLVAREASNEVEIGGYLLPK--------GT
M.truncatula|gi|357465755              --IPTSQDLHDKFPYLDQVIKEAMRFYIVSPLVARETSNEVEIGGYLLPK--------GT
G.max|Glyma04g05510.1                  --IPTSQDLHNKFPYLDQVIKEAMRFYTVSPLVARETSNEVEIGGYLLPK--------GT
G.max|Glyma06g05520.1                  --IPTSQDLHDKFPYLDQVIKEAMRFYTVSPLVARETSNEVEIGGYLLPK--------GT
B.distachyon|Bradi4g08970.1            --LPTADDLHTNFPYLDQVVKESMRFYMSSPLVARESSDKVDIGGYVLPK--------GT
Os01g0700900                           --VPTAEDLQTKFPYLDQVLKEAMRYYPSPLIARELNQQLEIGGYPLPK--------GT
Os01g0701400                           --APTAEDLQTKFPYLDQVVKESMRFYPSPLIARETCEQVEIGGYALPK--------GT
Os01g0701500                           --VPMADDLQTKFPYLDQVVKESMRFYIMSPLLARETLEQVEIGGYVLPK--------GT
S.italic|Si004997m                     --VPTADDLQTKFPYLDQVLKESMRFYMVSPLVARETSERVEIGAYVLPK--------GT
Z.mays|gi|237908823                    --VPTAEDLQSRFPYTDQVLKESMRFFMVSPLVARETSEQVDIAGYVLPK--------ST
S.bicolor|Sb03g032220.1                --VPTADDLQTKFPYLDQVVKESMRLFMVSPLVARETSERVEIGGYVLPK--------GA
S.italic|Si000975m                     --VPTADDLQTKFPYLDQVVKESMRFFMVSPLVARETSERVEIGGYVLPK--------GT
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S.moellendorffii|gi|300160069          WVWLVINALAQDEED-F-PEPHLFRPERFDPDCAE-AKKRHPYAHSPFGIGPRMCIGYKL
Z.mays|gi|413926093                    VRLAGAGCAGPGRGA-V-PGPGGVPAGAVRAGGGG-GAGAPPVRAHPLRRRAQGV-----
B.distachyon|Bradi3g08360.1            CVWLAPGVLARDAAQ-F-PDPDEFRPERFAADGEE-ERARHPYAHIPFGIGPRACVGHRF
H.vulgare|gi|326530386                 YVWLAPGVLARDARQ-F-PEPEEFRPERFAPEGEE-ERGRHPYAHIPFGIGPRACVGHRF
Os02g0221900                           YVWLAPGVLARDAAQ-F-PEPEEFRPERFAAGAAE-ERARHPYAHIPFGIGPRACVGHRF
Z.mays|gi|226491964                    YVWLAPGVLARDAAQ-F-PDPEEFRPERFAPEAEE-ERARHPYAHIPFGVGPRACIGHKF
S.bicolor|Sb04g007880.1                YVWLAPGVLARDAAQ-F-PDPEEFRPERFAPEAEE-ERTRHPYAHIPFGVGPRACIGHKF
S.italic|Si016823m                     YVWLAPGVLARDAAQ-F-PEPEEFRPERFAAGAEE-ERARHPYAHIPFGVGPRACIGHKF
B.distachyon|Bradi4g09040.1            WIWLAPGVLAKDPKQ-F-PDPYVFRPERFDPESEE-CKQRHPYAFIPFGIGPRACIGQKF
S.bicolor|Sb03g032210.2                WVWLAPGVLAKDPKD-F-PDPDVFRPERFDPESEE-CKRRHPYAFIPFGIGPRACIGQKF
S.italic|Si015313m                     WVWLTPGVLAKDPKDDF-LDPDMFRPERFVPESEEYVQAKAPLRVHPLRHRPRACIGQKF
B.distachyon|Bradi1g75310.1            WVWLATGQLSKDPKH-F-PDPYTFRPERFDPEDEE-CKRRHPYAFLPFGIGPRGCPGQKF
H.vulgare|gi|326492025                 WVWLATAVLGRDPEQ-F-PEPEAFRPERFDPESEE-CKRRHPYAYIPFGIGPRACPGQKF
B.distachyon|Bradi1g37730.1            WLWLAPGVLARDPAQ-FAPDPGAFRPERFEAGSEE-EKARHPYAQIPFGLGPRACVGQRF
Os06g0565100                           WVWLAPGVLSRDEAQ-F-RDAGEFRPERFDAGGEE-ERRRHAYAHVPFGLGPRACPGRRF
S.bicolor|Sb10g022310.1                WLWMAPGVLSRDAAS-FFPDPGAFRPERFDPASEE-QRGRHPCAHIPFGIGPRACVGQRF
Z.mays|gi|224033291                    WLWMAPGVLSRDAAN-F-EDPGAFRPERFDPASEE-QRRRHPCAHIPFGIGPRACVGQRF
Z.mays|gi|226493876                    WLWMAPGVLSRDAAN-F-EDPGAFRPERFDPASEE-QRRRHPCAHIPFGIGPRACVGQRF
A.thaliana|AtMAX1                      WVWLALGVLAKDPKN-F-PEPEKFKPERFDPNGEE-EKHRHPYAFIPFGIGPRACVGQRF
V.vinifera|gi|296081643                WIWLAPGVLAKDPKN-F-PEPDKFKPERFDPNCEE-EKQRHPYALIPFGIGPRACLGQKF
C.papaya|supercontig_7.164             WVWLAPGVLAKDPKN-F-PEPDKFKPERFDPNCEE-EKQRHPYAFIPFGIGPRACIGQKF
S.tuberosum|PGSC0003DMP400014456       WVWLALGVLAKDPKN-F-PEPEKFKPERFDPNCAE-EKQRHPYANIPFGIGPRACIGQKF
P.hybrida|gi|329739341                 WVWLALGVLAKDPKN-F-PEPEKFRPERFDPNCKE-EKQRHPYANIPFGIGPRACIGQKF
C.sativus|Cucsa.299480.1               WVWLALGVLAKDPKN-F-PEPDKFKPERFDPNEEE-EKRRHPYALIPFGIGPRACIGQKF
P.trichocarpa|POPTR_0018s07540.1       WIWLAPGVLAKDPKN-F-PEPDKFKPERFDPNCEE-EKRRHPYALIPFGLGPRACIGQKF
P.trichocarpa|POPTR_0006s24320.1       WIWLAPGVLAKDPKN-F-PEPDRFKPERFDPNCEE-EKRRHPCALIPFGIGPRACIGKKF
R.communis|gi|255550067                WVWLALGVLAKDPKN-F-PEPDKFKPERFDPKCEE-EKQRHPYALIPFGIGPRACIGQKF
M.esculenta|cassava4.1_005510m         WVWLALGVLAKDPKN-F-PEPDKFKPERFDPNCEE-EKQRHPYALIPFGIGPRACIGQKF
M.truncatula|gi|357437385              WVWLALGVLAKDSRN-Y-AEPEKFKPERFDPKCGE-MKRRHPYAFIPFGIGPRACIGQKF
G.max|Glyma17g34530.1                  WVWLALGVLAKDPRN-F-PEPEKFKPERFDPKCEE-MKRRHPYAFIPFGIGPRACIGQKF
G.max|Glyma14g11040.1                  WVWLALGVLAKDPRN-F-PEPEKFKPERFDPKCEE-MKRRHPYAFIPFGIGPRACIGQKF
M.truncatula|gi|357465755              WVWLALGVLAKDHKN-F-PEPEKFKPERFDPNCEE-MKQRHPYAFIPFGIGPRACIGQKF
G.max|Glyma04g05510.1                  WVWLALGVPAKDPKN-F-PEPEKFKPDRFDPNCEE-MKRRHPYAFIPFGIGPRACIGKQF
G.max|Glyma06g05520.1                  WVWLALGVPAKDPRN-F-PEPDKFKPERFDPNFEE-MKRRHPYAFIPFGIGPRACIGRQF
B.distachyon|Bradi4g08970.1            WVWLAPGVLAKDPIN-F-PDPELFRPERFDPTGDE-DKRRHPYAFIPFGIGPRICIGYKF
Os01g0700900                           WVWMAPGVLGKDPKN-F-PEPEVFRPERFDPNGEE-EKRRHPYALFPFGIGPRACIGQKF
Os01g0701400                           WVWLAPGVLAKDPKN-F-PEPEVFRPERFDPNGEE-EKRRHPYAFIPFGIGPRACIGQKF
Os01g0701500                           WVWLAPGVLAKDPKN-F-PEPEIFRPERFDPNGEE-ERRRHPYAFIPFGIGPRVCIGQKF
S.italic|Si004997m                     WVWLALGVLAKDPNN-F-PDPELFRPERFEPTCDE-ERRRHPYAFIPFGIGPRACIGQKF
Z.mays|gi|237908823                    WVWMAPGVLAKDPVN-F-PEPELFRPERFDPAGDE-QKRRHPYAFIPFGIGPRICIGQRF
S.bicolor|Sb03g032220.1                WVWMAPGVLAKDAHN-F-PDPELFRPERFDPAGDE-QKKRHPYAFIPFGIGPRVCIGQKF
S.italic|Si000975m                     WVWMAPGVLAKDPIN-F-PDPELFRPERFDPAGDE-QKKRHPYAFIPFGIGPRVCIGQKF
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S.moellendorffii|gi|300160069          AYLEMKLALIHFYQRYTFEHSPAMENPLAVRLSIVVRPIHGVKLRVRKRGIC----
Z.mays|gi|413926093                     HRAQVRAATGEACRGGALP-------PVRVPTLAVHGVAHPVRL------------
B.distachyon|Bradi3g08360.1             ALQQVKLAVVGLYRHFVFRHSPDMESPVEFDFDLVLGFRHGVKLRAIRRTND----
H.vulgare|gi|326530386                  ALQQVKLAVVHLYRRYVFRHSPAMESPIQFDFDLVLGFRHGVKLRAIRRTTRD---
Os02g0221900                            ALQQVKLAAVGLYRRYVFRHSPAMESPLQFDFDLVLAFRHGVKLRAIKRTNT----
Z.mays|gi|226491964                     ALQQVKLAVVELYRRYVFRHSPSMESPIQFDFDLVLAFRHGVKLRAIRRG------
S.bicolor|Sb04g007880.1                 ALQQVKLAVVELYRRYTFRHSPAMESPLQFDFDLVLAFRHGVKLRAIRRS------
S.italic|Si016823m                      ALQQVKLAVVGLYRRYVFRHSPAMESPIQFDFDLVLAFRHGVKLRAIRRE------
B.distachyon|Bradi4g09040.1             SMQQLKLVVVHLYRQYVFRHSPNMEAPLQFQFSIVVNFKHGVKLHVIERNA-----
S.bicolor|Sb03g032210.2                 AMQQLKLVVIHLYRNYIFRHSPRMEFPLQFQYSILVNFKYGVKVQVIERKN-----
S.italic|Si015313m                      AMQQLKLVVIHLYRNYIFRHSPRMEFPLQFQYFILVNFKYGVKVQVIERKN-----
B.distachyon|Bradi1g75310.1             AMQQLKLVVIHLYRRYVFRHSPGMEFPLQLEFSIVNNFKHGVKLQVIDREEH----
H.vulgare|gi|326492025                  AFQQLKLTLIHLYRRYVFRHSPRMESPLKLQFSIVTNFKNGVKLQVLERNN-----
B.distachyon|Bradi1g37730.1             ALQEVKLAMVHMYRRFVFRRSPRMESPPEFQFGMVLSFRHGVKLRAIKRLTRNEAV
Os06g0565100                            ALQEVKLAMAHLYRRFVFRRSPRMESPPELQFGMVLSFRRGVKLTAVERRHAAAA-
S.bicolor|Sb10g022310.1                 ALQELKLSMVHLYQRFLFRRSPQMESPPELQFGIVLNFKNGVKLVAVERCAAMS--
Z.mays|gi|224033291                     ALQEVKLSMLHLYRRFLFRRSPRMESPPELQFGIVLNFKKGVKLVAVERCAAMPL-
Z.mays|gi|226493876                     ALQEVKLSMLHLYRRFLFRRSPRMESPPELQFGIVLNFKKGVKLVAVERCAAMPL-
A.thaliana|AtMAX1                       ALQEIKLTLLHLYRNYIFRHSLEMEIPLQLDYGIILSFKNGVKLRTIKRF------
V.vinifera|gi|296081643                 SLQEVKLSLIHLYQRYVFRHSPNMEKPLELEYGIILNFKHAVKLRAIKRHP-----
C.papaya|supercontig_7.164              SIQEIKLLLIHLYRNYVFRHSPNMENPIELEYGIVLNFKYGVKLRVIKRT------
S.tuberosum|PGSC0003DMP400014456        SIQEIKLSLIHLYRKYIFQHSPLMESPLELEYGIVLNYKHGVKVQAIKRK------
P.hybrida|gi|329739341                  SIQEIKLSLIHLYRKYIFRHSPLMEKPLELEYGIVLNYKHGVKVCAIKRK------
C.sativus|Cucsa.299480.1                ALQELKLSLIHLYRKFVFRHSLSMEQPLELEYGVILNFKSGVKLRVINRK------
P.trichocarpa|POPTR_0018s07540.1        SIQEIKLSLIHLYRKYLFRHSPHMEKPLELDFGIVLNFRHGVKLRIVKRT------
P.trichocarpa|POPTR_0006s24320.1        SIQEIKLSLIHLYRKYLFRHSPTMEKPLEFEFGIVLNFKRGVKLRIIKRT------
R.communis|gi|255550067                 SLQELKLSLIHLYRKYIFRHSPNMEKPLELEYGIVLNFKHGVKLRVIKRN------
M.esculenta|cassava4.1_005510m          SLQELKLSLIHLYRKFIFRHSPNMEKPLELEYGIVLNFKHGVKLRVIKRR------
M.truncatula|gi|357437385               SLQEIKLTLIHLYRKYIFRHSLNMEKPVELEYGLVLNFKHGIKLRVIKRT------
G.max|Glyma17g34530.1                   SLQEIKLTLIHLYQKYVFRHSVDMEKPVEMEYGMVLNFKHGIKLRVIRRT------
G.max|Glyma14g11040.1                   SLQEIKLSLIHLYRKYVFRHSLDMENPVEMEYGMVLNFKHGLKLRVIRRT------
M.truncatula|gi|357465755               SMQEIKLSLIHLYKKYLFRHSADMESPLELEYGIVLNFKHGVKFSVIKRTEMSC--
G.max|Glyma04g05510.1                   SLQEIKISLIHLYRKYLFRHSPNMENPLELQYGIVLNFKHGVKLRVIKRTETC---
G.max|Glyma06g05520.1                   SLQEIKLSLIHLYRKYLFRHSPNMENPLELQYGIVLNFKHGVKLRAIKRKEAC---
B.distachyon|Bradi4g08970.1             SIQEIKLAIIHLYRQYIFRHSPSMESPLQFQYGVIVNFKHGVKLQVIHRHKE----
Os01g0700900                            AIQEMKLSAIHFYRHYVFRPSPSMESPPEFVYSIVSNFKNGAKLQVIKRHI-----
Os01g0701400                            SIQEIKLSVIHLYRNYVFRHSPSMESPLEFQYSIVCNFKYGVKLRVIKRHTA----
Os01g0701500                            SIQEIKLSMIHLYRHYVFRHSPSMESPLEF--------------------------
S.italic|Si004997m                      SIQEIKLSLIHLYRHFVFRHSPSMESPLEFQYGIVVNFKYGVKLQVIRRQRD----
Z.mays|gi|237908823                     SIQEIKLALIHLYRQYVFRHSPSMESPLEFQFGVVLNFKHGVKLQSIKRHKC----
S.bicolor|Sb03g032220.1                 AIQEIKLAIIHLYQHYVFRHSPSMESPLEFQFGIVVNFKHGVKLHVIKRHVENN--
S.italic|Si000975m                      AIQEIKLAVIHLHQRYVFRHSPSMESPLEFQFGIVVNFKHGVKLQAIKRYKNY---
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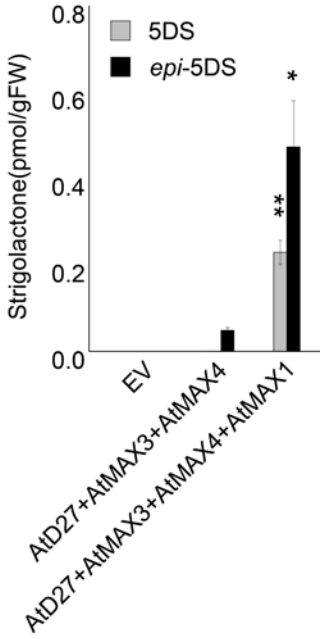
Supplementary Figure 10. Alignment of *MAX1* homologs from different plant species with OsMAX1s. The amino acid pairs that may distinguish the function of Os900 and Os1400 are shaded: Phe124 (Os900)/Cys124 (Os1400) is shaded in red; Lys228 (Os900)/Arg227 (Os1400) is shaded in green; Ser408 (Os900)/Leu409 (Os1400) in pink. Amino acids that may be involved in the catalytic function (Thr347 in Os900, Thr348 in Os1400) and coordinating the heme iron (Cys483 in Os900) are shaded in orange and grey, respectively. Alignment was performed using Muscle[31]. All the MAX1 homologous protein sequences were extracted from NCBI or PlantGDB (http://www.plantgdb.org/) by using AtMAX1 as query sequence and using blast-p.



Supplementary Figure 11. The effect of transient co-expression of *Arabidopsis* carlactone biosynthetic genes with *AtMAX1* in *N. benthamiana* on the production of strigolactones. Transient expression of *AtMAX1* in combination with carlactone biosynthetic genes of *Arabidopsis* (*AtD27*, *AtMAX3* and *AtMAX4*) increases 5-deoxystrigol (5DS) and *epi*-5-deoxystrigol (*epi*-5DS) production (transition $[M+H]^+$ *m/z* 331.2>234.15). Bars represent means ± SE (n=4). ∗ and ∗∗ indicate significant differences from the *AtD27+AtMAX3+AtMAX4* treatment at P<0.05 and P<0.01, respectively, as determined by a Student's T-test .

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1W0F             -------------------------MAYGTHSHGLFKKLGIPGPTPLPFLGN--ILSYH
Os01g0700900     ME-ISTVLGAILAEYAVTLVAMAVGFLVVGYLYEPYWKVRHVPGPVPLPLIGHLHLLAMH
Os01g0701400     MEIISTVLGST-AEYAVTLVAMAVGLLLLGYLYEPYWKVRHVPGPVPLPFIGHLHLLAMH
                                         :  *      :*   :***.***::*:  :*: *

1W0F             KGFCMFDMECHKKYGKVWGFYDGQQPVLAITDPDMIKTVLVKECYSVFTNRRPFGPVG--
Os01g0700900     -GPDVFSVLT-RKYGPIFRFHMGRQPLVMVADAELCKEVGVKK-FKNFPNRSMPSPITNS
Os01g0701400     -GPDVFTVLA-RKYGPVFRFHMGRQPLVMVADAELCKEVGVKK-FKSIPNRSMPSAIANS
                  *  :* :   .*** :: *: *.**:: ::*.:: * * **: :. :.**   ..:

1W0F             -FMKSAISIAEDEEWKRLRSLLSPTFTSGKLKEMVPIIAQYGDVLVRNLRREAETGKPVT
Os01g0700900     PVHQKGLFFTSGSRWTTMRNMILSIYQPSHLATLIPSMESCIERAAENLEGQEE----IN
Os01g0701400     LINQKGLCFTRGSRWTALRNMIISIYQPSHLASLIPTMQSCIECVSKNLDGQED----IT
                  . :..: :: .. *. :*.:: . : ..:*  ::* : .  :    **  : :    :.

1W0F             LKDVFGAYSMDVITSTSFGVNID---SLNNPQDPFVENTKKL------------------
Os01g0700900     FSKLSLSFTTDVLGQAAFGTDFGLSKKLASSDDD--EDTRKIAADTCAEAKASSEFIKMH
Os01g0701400     FSDLALGFATDVIGQAAFGTDFGLSKISASSNDD---DIDKIATDTSAEAKASSEFIRMH
                 :..:  .:: **: .::**.::.      ..:*    :  *:

1W0F             ------LRFDFLDPFFLSI-TVFPFLI-PILEVLNIC-------------VFPREVTNFL
Os01g0700900     VHATTSLKMDMSGSLSIIVGQLLPFLHEPFRQVLKRLRWTADHEIDRVNLTLGRQLDRIV
Os01g0701400     VHATTSLKMDLSGSLSIIIGQLLPFLQEPFRQVLKRIPWTADHEIDHVNLALGGQMDKIV
                       *.:*: ..: : :  ::***  *: :**:               .:  :: .::

1W0F             RKSVKRMKESRLEDTQKHRVDFLQLMIDSQNSKETESHKALSDLELVAQSIIFIFAGYET
Os01g0700900     AERTAAMKRDPAA--LQQRKDFLSVMLTARESNKS-SRELLTPDYISALTYEHLLAGSAT
Os01g0701400     AERAAAMERDQAAPHAQQRKDFLSVVLAARESNKS-WRELLTPDYISALTYEHLLAGSAT
                  : .  *: .      ::* ***.::: :.:*:::  .: *:   : * :  .::**  *

1W0F             TSSVLSFIMYELATHPDVQQKLQEEIDAVLP-NKAPPTYDTVLQMEYLDMVVNETLRLFP
Os01g0700900     TAFTLTTALYLVAKHPEVEEKLLREIDGFGPRDRVPTAEDLQTKFPYLDQVLKEAMRYYP
Os01g0701400     TAFTLSTVLYLVSKHPEVEEKLLREIDGFGPHDHAPTAEDLQTKFPYLDQVVKESMRFYF
                 *: .*:  :* ::.**:*::**  ***.. * :..*.: *   :: *** *::*::* :

1W0F             IAMRLERVCKKDVEINGMFIPKGVVVMIPSYALHRDPKYWTEPEKFLPERFSKKNKDNI-
Os01g0700900     SSPLIARELNQQLEIGGYPLPKGTWVWMAPGVLGKDPKNFPEPEVFRPERFDPNGEEEKR
Os01g0701400     LSPLIARETCEQVEIGGYALPKGTWVWLAPGVLAKDPKNFPEPEVFRPERFDPNGEEEKR
                  :  : *   :::**.*  :***. * :.. .* .*** :.*** * ****. :.:::

1W0F             -DPYIYTPFGSGPRNCIGMRFALMNMKLALIRVLQNFSFKPCKETQIPLKLSLGGLLQPE
Os01g0700900     RHPYALFPFGIGPRACIGQKFAIQEMKLSAIHFYRHYVFRPSPSMESPPEFVYSIVSNFK
Os01g0701400     RHPYAFIPFGIGPRACIGQKFSIQEIKLSVIHLYRNYVFRHSPSMESPLEFQYSIVCNFK
                   **   *** *** *** .*:: ::**: *.. .:: *. . . : * ::  . : : :

1W0F             KPVVLKVESRDGTVSGAHHHH
Os01g0700900     NGAKLQVIKR--------HI-
Os01g0701400     YGVKLRVIKR--------HTA
                   . *.* .*        *
```

Supplementary Fig. 12. Alignment of human CYP3A4 (1W0F) template with OsMAX1 Os900 and Os1400. The alignment was done by Muscle.

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Chapter 5

Striga hermonthica MAX2 restores branching but not the Very Low Fluence Response in the *Arabidopsis thaliana max2* mutant

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Summary

- Seed germination of *Striga* spp. (witchweeds), one of the world's most destructive parasitic weeds, cannot be induced by light but is specifically induced by strigolactones. It is not known whether *Striga* uses the same components for strigolactone signaling as host plants, whether it has endogenous strigolactone biosynthesis and whether there is post-germination strigolactone signaling in *Striga*.
- Strigolactones could not be detected in *in vitro* grown *Striga*, while for host-grown *Striga*, the strigolactone profile is dominated by a subset of the strigolactones present in the host.
- Branching of *in vitro* grown *Striga* is affected by strigolactone biosynthesis inhibitors. *ShMAX2*, the *Striga* ortholog of *Arabidopsis MORE AXILLARY BRANCHING 2 (AtMAX2)* - which mediates strigolactone signaling - complements several of the *Arabidopsis max2-1* phenotypes, including the root and shoot phenotype, the High Irradiance Response and the response to strigolactones. Seed germination of *max2-1* complemented with *ShMAX2* showed no complementation of the Very Low Fluence Response phenotype of *max2-1*.
- Results provide indirect evidence for *ShMAX2* functions in *Striga*. A putative role of *ShMAX2* in strigolactone dependent seed germination of *Striga* is discussed.

Key words: *Striga hermonthica*, strigolactone, biosynthesis, signaling, *MAX2*

Introduction

Striga species (witchweeds) are among the world's most destructive weeds as they parasitize important crop species and reduce yields by 30-90% (Scholes & Press, 2008, and references there in). More than 50 million hectares of the arable land used for cereal and legume production in sub-Saharan Africa are infested with at least one *Striga* species, resulting in annual losses of more than US$10 billion (Westwood *et al.*, 2010, and refenences there in).

Striga species have evolved a life strategy in which germination is delayed until a potential host is sensed through the presence of strigolactones in the host root exudate (Bouwmeester *et al.*, 2003; Matusova *et al.*, 2004). After germination, the *Striga* radicle will grow towards and then invade the host root through a parasitic structure called a haustorium (Kuijt, 1969).

Strigolactones also have an endogenous signaling role in plants acting as a plant hormone controlling shoot branching/tillering by inhibiting the outgrowth of axillary lateral buds (Stirnberg *et al.*, 2002; Booker *et al.*, 2004; Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). Strigolactones also affect different developmental processes like primary root, lateral root and root hair growth (Kapulnik *et al.*, 2011a; Kapulnik *et al.*, 2011b; Ruyter-Spira *et al.*, 2011; Mayzlish-Gati *et al.*, 2012).

The effect of strigolactones on branching requires the F-box protein MAX2 (Stirnberg *et al.*, 2002; Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). MAX2 also has a role in seed germination and seedling de-etiolation (Shen *et al.*, 2012; Toh *et al.*, 2012). However, the role of strigolactones in these MAX2-related phenotypes is disputed, mainly because strigolactone biosynthesis mutants often lack these phenotypes (Shen *et al.*, 2012). At least some of the strigolactone induced phenotypes in non-parasitic plants require the presence of an LRR F-box protein (MAX2 in *Arabidopsis*) and a member of the α/β-hydrolase superfamily (e.g., OsD14 in rice, AtD14 in *Arabidopsis*, DAD2 in petunia) which has been identified as a second strigolactone signaling component (Ishikawa *et al.*, 2005; Stirnberg *et al.*, 2007; Arite *et al.*, 2009; Gao *et al.*, 2009; Liu *et al.*, 2009; Gaiji *et al.*, 2012; Hamiaux *et al.*, 2012; Waters *et al.*, 2012). Some α/β-hydrolase family members are also involved in signaling by karrikins, which are smoke-derived compounds with a structure that resembles the active part of strigolactones and are potent seed germination stimulants (Nelson *et al.*, 2009; Nelson *et al.*, 2012; Waters *et al.*, 2012). Recently, DAD2, the D14 homolog from petunia was shown to bind to and cleave the synthetic strigolactone GR24 and the presence of GR24 increases DAD2 binding to *Ph*MAX2A (Hamiaux *et al.*, 2012).

Strigolactones are derived from all-*trans*-β-carotene through isomerization, cleavage and several (putative) modification reactions (Matusova *et al.*, 2005; Alder *et al.*, 2012) (Fig. 1). The presence of strigolactones is conserved in the plant kingdom (Delaux *et al.*, 2012; Waters *et al.*, 2012; Brewer *et al.*, 2013), but endogenous strigolactones, strigolactone biosynthesis genes or strigolactone signaling components have not been reported in parasitic plants. Only a putative CCD7 was reported from *Phelipanche ramosa* recently (Péron *et al.*, 2012). The dependence of parasitic plant seed germination on exogenous strigolactones suggests absence of endogenous strigolactones or a malfunctioning of strigolactone related signaling in their seeds. Here we present evidence for endogenous strigolactone biosynthesis and signaling during *Striga* plant development and we focus on characterization of a homolog of MAX2 in *Striga* (*Sh*MAX2). Because of the lack of mutants and problems with transformation of *Striga*, functional characterization of the *Sh*MAX2 was achieved by overexpression in an *Arabidopsis max2-1* mutant background and characterization of the complementation of several different *max2-1* phenotypes. Results suggest that *Sh*MAX2 can function in *Arabidopsis* as most *max2-1* related phenotypes are fully complemented by ShMAX2, except for the VLFR seed germination phenotype.

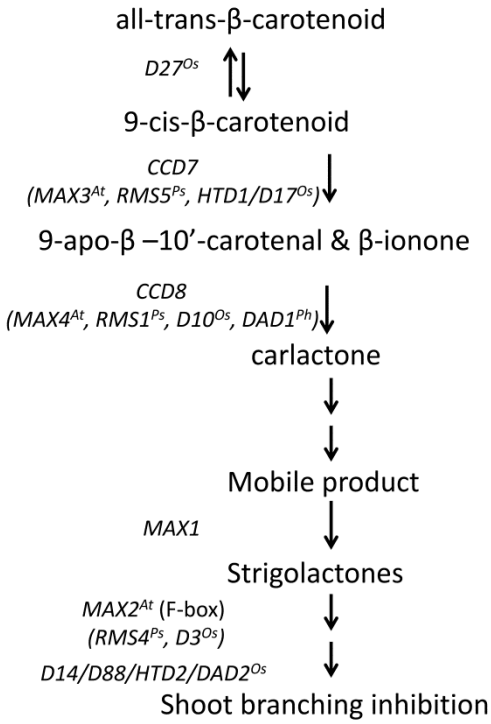

Fig.1. Biosynthetic pathway of strigolactones.Gene superscripts identify species; *At*, *Arabidopsis thaliana*; *Ps*, *Pisum sativum* (pea); *Os*, *Oryza sativa* (rice); and *Ph*, *Petunia hybrida*.

Methods

Plants and growing conditions

Seeds of *max2-1* (Stirnberg *et al.*, 2002) and the parental Columbia-0 wild-type were kindly provided by Prof. Ottoline Leyser (University of Cambridge, UK). *Striga* (*Striga hermonthica* Del. Benth) seeds were collected from a maize field in Kibos, Kenya, as described by Matusova *et al.* (2005).

Arabidopsis plants were grown on sterile plates and then on soil in 16-h photoperiods as described by Bennett *et al.* (2006). Other conditions used are outlined in the paragraphs below.

Inhibitor treatment of in vitro grown Striga hermonthica

Three *in vitro* grown *S. hermonthica* explants were grown in a plastic jar with half strength MS medium (containing 20 g L^{-1} sucrose) as a pool. For carotenoid biosynthesis inhibitor treatments, fluridone and D2 (dissolved in acetone) were added to the medium to reach the desired concentration. An equal amount of acetone was added to all treatments and the controls. For each treatment, four replicates were used. After three weeks in a climate chamber (25 °C, 16 hours day length), the branch numbers were scored.

Strigolactone extraction from Striga hermonthica

Strigolactones were extracted from *in vitro* grown *Striga* (see above) and from *in vivo* grown *Striga*, grown on rice (*Oryza sativa*), maize (*Zea mays*) and sorghum (*Sorghum bicolor*). *Striga* underground shoot and root were carefully removed from the host with all host material excluded. For strigolactone analysis, 500 mg of *Striga* material was extracted with ethyl acetate and purified with Silica gel Grase Pure SPE (200 mg 3 mL^{-1}) columns as described by Kohlen *et al. (2011)* or extracts were fractionated by HPLC for *Phelipanche ramosa* seed germination bioassays (see Methods S1 and S2).

Gene expression analysis by Real Time PCR

Gene expression analysis for *ShCCD7*, *ShCCD8* and *ShMAX2* was done by real time PCR with total RNA isolated from *Striga* tissues (see Methods S3).

Isolation of ShMAX2

Genomic DNA was isolated from *Striga* shoot material using the Plant Genomic DNA miniprep Kit (Sigma-Aldrich, USA) according to manufacturers' protocol. Degenerate primers were designed based on the protein sequences of *Arabidopsis* MAX2, pea RMS4, and *Vitis vinifera* and used to amplify fragments of the *ShMAX2* gene from DNA. Then Genome-walking (Kilstrup & Kristiansen, 2000) was used to

obtain additional sequences information. DNA sequence analysis showed that the *ShMAX2* does not contain any introns and the full length *ShMAX2* coding sequence was amplified for cloning into an expression vector. For primer sequences and details of cloning into expression vector and plant transformation see Methods S4. Alignment of predicted amino acid sequences of *ShMAX2* compared with other reported MAX2 was performed by CLC workbench (USA).

Decapitation assay

The *Arabidopsis* decapitation assay was performed according to the description of Stirnberg *et al.* (2007) with modifications. Following imbibition in water for 3 days at 4 °C, seeds were sown onto soil in 49 cm^2 pots (four seeds per pot later thinned to one). They were first grown at 20 °C/18 °C in an 8h light/16h dark photoperiod at a light intensity of 120 µmol cm^{-2} sec^{-1}. After 30 days, the plants were moved to a 12 h light photoperiod (same light intensity) for one week and then to a 16 h light photoperiods to induce flowering. To encourage branching from the rosette, the primary inflorescence of each plant was removed when it was 2-4 cm long. Rosette branches with a length of at least 2 cm were counted 10 days later. Sample size varied between 7 and 30 plants per treatment between the different experiments.

Seed germination assays

Plates with germinating seeds were imaged by a digital camera (Nikon D80 with Nikkor AF-S 60 mm f/2.8 G Micro ED; Nikon, http://www.nikon.com). The experiment was carried out in an air-conditioned room (20 °C). Image processing and data analysis were performed with the GERMINATOR package (Joosen *et al.*, 2010). Germination was defined by emergence of the radicle from seeds. All germination assays were tested with two independent transgenic lines.

Seed germination rate under continuous white light**:** Six-well (12.7 x 8.5 cm) plates (Greiner bio-one, Germany) were used for germination experiments according to the description of Joosen *et al.* (2010) with modifications. One layer of white filter paper and one layer of blue filter paper (Anchor Paper Company, http://www.seedpaper.com) were placed in each well and 1 mL of water added. Approximately 50-80 mature *Arabidopsis* seeds were dispersed on the filter paper using a mask to ensure an accurate and reproducible spacing. Clustering of seeds was prevented as much as possible. Then the plates were sealed by parafilm (Pechiney Plastic Packaging Company, USA). A maximum of 20 plates were stacked together. On top of the stack, three empty plates were wrapped in aluminum foil to ensure equal distribution of light to other plates. The whole stack was wrapped in a closed transparent plastic bag and placed in a 20 °C incubator (type 5042; Seed processing Holland, http://www.seedprocessing.nl).

Seed germination percentage after FR or R light pulse treatment**:** After stratified at 4 °C for 4 days, seeds were surface-sterilized and plated on ½MS growth medium (0.5 x MS slats supplemented with 1 x Gamborg's B5 vitamin mix, 0.8% [w/v] agar [Daishin], without sucrose at pH5.8) within 15 minutes' imbibition (50-100 seeds/genotype). Then they were placed under dark conditions, or exposed to 5 min of far-red light or red light before returning to the dark for 2-3 days at room temperature.

Seed dark germination percentage in response to GR24*:* For GR24 treatment, seeds were treated as described above and then plated on ½ MS growth medium with minimal or various concentrations of GR24 as indicated.

Hypocotyl elongation assay

Before sowing on MS plates, seeds were surface sterilized in a container containing 50 mL bleach and 1.5 mL concentrated HCl for 4 hours and then transferred to a flow cabinet for 1 hour. Seeds were imbibed on wet filter paper at 4 °C for 3 days and plated on ½MS agar plates. Plants were grown on near vertical plates in a climate chamber under a 22 °C/ 18°C 16h-light/8h-dark regime (80 µmol m^{-2} s^{-1}). Image capturing was done by a digital camera (Nikon D80 with Nikkor AF-S 60 mm f/2.8 G Micro ED; Nikon, http://www.nikon.com). Hypocotyl length was measured with ImageJ from digitally computed images of 5-day-old seedlings.

Accession number

Sequence data from this article can be found in the GenBank data libraries under the following accession numbers: JX565467 for *ShMAX2*, NM_129823 for *AtMAX2*, DQ403159 for *PsRMS4*, Q5VMP0 for *OsD3*, XP_003607592 for putative *MtORE9*, and XP_002320412 for putative *PtMAX2*.

Results

Evidence for endogenous strigolactone production in Striga

Strigolactones are not detected but germination stimulants are present in in vitro grown Striga

To determine if *Striga* produces strigolactones, seeds were stimulated to germinate with GR24 *in vitro* and the resulting shoots were sub-cultured *in vitro* at least three times on medium without GR24. Subsequently, shoot tissue was extracted for analysis of strigolactones. No signal was detected for any of the known strigolactones using LC-MRM-MS/MS. The extracts of *in vitro* grown *Striga* were assessed for germination stimulant bioactivity in a *Phelipanche ramosa* seed germination bioassay (Kohlen *et al.*, 2011). Extracts were fractionated by HPLC into thirty fractions and several fractions were able to stimulate *P. ramosa* seed germination (Fig. 2). Many of these fractions coincide with the elution position of known strigolactones or active fractions of non-parasitic plant root exudates.

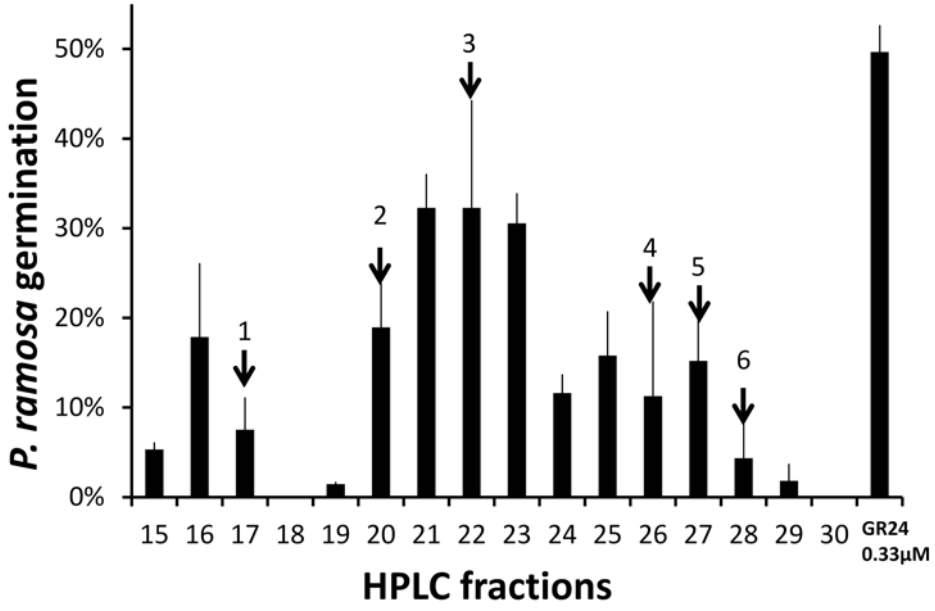

Fig.2. Germination assay of HPLC fractions of *in vitro* grown *Striga hermonthica*. *Phelipanche ramosa* seeds were used for germination bioassay. GR24 (0.33 µM) was used as positive control. Each bar represents mean of three biological replicates +/-SE. Arrows point to fractions in which strigolactone standards elute: (1) solanacol and 7-hydroxyorobanchyl acetate (2) 2´-*epi*-orobanchol, orobanchol, strigol and sorgomol (3) GR24 (4) orobanchyl acetate (5) sorgolactone (6) 5-deoxystrigol.

Host derived strigolactones are detectable in extracts of host attached Striga plants

To determine if strigolactones are present in *Striga*, *Striga* plants attached to a host, rice (*Oryza sativa*), maize (*Zea mays*) or sorghum (*Sorghum bicolor*), were extracted for strigolactone analysis using LC-MRM-MS/MS. For comparison, the roots of the host plants were also extracted and analyzed for the presence of strigolactones. In the extract of *Striga* attached to rice, only the strigolactone 2′-*epi*-5-deoxystrigol was identified, while in rice root extracts the strigolactones 2′-*epi*-5-deoxystrigol, orobanchol and two putative methoxy-5-deoxystrigol isomers were identified (Table 1, Fig. S1a ,d). In *Striga* grown on sorghum, 5-deoxystrigol and sorgomol were identified, while 5-deoxystrigol, sorgomol, and orobanchol were detected in the corresponding sorghum root extracts (Table 1, Fig. S1b, d). A number of unknown strigolactones, which previously have been reported from maize (Jamil *et al.*, 2012), were detected in *Striga* grown on maize. Again, these unknown strigolactones were a subset of the strigolactones present in the maize root extract (Table 1, Fig. S1c, d). Overall the results show that different types of strigolactones are detected in host-attached *Striga* and that, in all cases, these strigolactones represent a subset of the strigolactones present in the host root, suggesting that strigolactones are transported from the host to *Striga*.

Strigolactone biosynthesis in Striga

Nevertheless, the bioassays suggested that *Striga* can also produce strigolactones itself. In the *Striga* EST sequences (http://ppgp.huck.psu.edu/) (Das *et al.*, Submitted) homologs of the strigolactone biosynthesis genes *AtMAX3* (*CCD7*) and *AtMAX4* (*CCD8*) were identified that were named *ShCCD7* and *ShCCD8*, respectively. The expression of both *ShCCD7* and *ShCCD8* genes in different tissues and stages of *Striga* was quantified by real time RT-PCR (Fig. 3). In general, *ShCCD8* expression was higher than that of ShCCD7 but their expression patterns were otherwise similar in that most stages were statistically indistinguishable (Fig. 3a, b). Preconditioning of *Striga* seeds is required to make them responsive to strigolactones (Matusova *et al.*, 2004). Surprisingly, expression of *ShCCD7*was transiently up-regulated during seed conditioning, suggesting endogenous strigolactone biosynthesis in *Striga* seeds at this stage, while at the same time seeds become strictly responsive to exogenous strigolactones for induction of germination.

Table1. Strigolactones identified from in vivo grown *Striga* plants and its host by LC- MRM-MS/MS.

	2'-*epi*-5-de-oxystrigol (pmol g^{-1})	orobanchol (pmol g^{-1})	5-deoxystrigol (pmol g^{-1})	sorgomol (pmol g^{-1})	methoxy-5-de-oxystrigol isomers (peak area)	SL1-4* (peak area)	SL-5* (peak area)
Striga on rice	-	-	0.5	-	-	-	-
Rice root	1.7	0.7	0.3	-	2.1 ~ 132.2	-	-
Striga on sor-ghum	-	-	4.5	0.18	-	-	-
Sorghum root	-	0.4	79.4	207.5	-	-	-
Striga on maize	-	-	-	-	-	-	3321.8
Maize root	-	-	-	-	-	22.2 ~ 446.0	4263.6

Note:

SL, strigolactone.

* putative identification.

- not detected.

Evidence for strigolactone signaling in Striga

Strigolactones have been shown to suppress branching in plants. As strigolactones are derived from carotenoids, branching can be induced by inhibition of strigolactone biosynthesis with the carotenoid biosynthesis inhibitor fluridone or a more specific inhibitor D2 (Sergeant *et al.*, 2009). In order to get further confirmation of the presence of strigolactones in parasitic plants and to test whether they have a similar function in parasitic plant development, *in vitro* grown *Striga* plants were treated with different concentrations of fluridone and D2. Treatment with both fluridone (Fig. 4a, b) and D2 (Fig. 4c) resulted in enhanced branching in *Striga*, showing that also in *Striga* endogenous strigolactone biosynthesis and strigolactone signaling are involved in the regulation of branching.

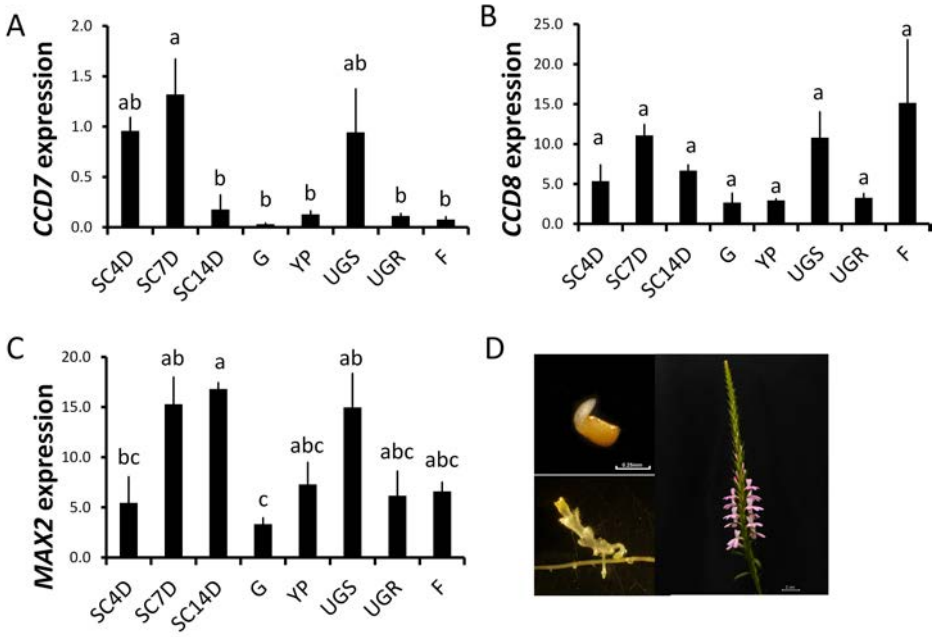

Fig.3. Expression of strigolactone signaling and putative biosynthetic genes from *Striga*. Expression of putative CCD7 (a), putative CCD8 (b) and MAX2 (c) in *Striga hermonthica* were measured by real time RT-PCR. Y axis for (a) (b) (c) is copy number / ShUBQ1 copy number X 1000. (d) Picture of germinated *Striga* seed (upper left), underground root and shoot (lower left), and floral buds (right). Each bar represents mean of three biological replicates +/-SE. Bars with different letters are significantly different according to Tukey test (P=0.05). Abbreviations: SC4d-seeds conditioned at 30 °C for 4 days, SC7d-seeds conditioned for 7 days, SC14d-seeds conditioned for 14 days, G-germinating seeds, YP-young plants, UGS-underground shoots, UGR-underground roots, F-floral buds.

MAX2 homolog expression in Striga

One of the proteins required for strigolactone signaling is MAX2 (Stirnberg *et al.*, 2002; Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008; Hamiaux *et al.*, 2012). *AtMAX2* encodes an F-box leucine-rich repeat protein, which probably functions as the substrate-recognizing subunit of SCF-type ubiquitin E3 ligase in protein ubiquitination (Stirnberg *et al.*, 2007). We isolated the *Striga* homolog of *AtMAX2*, *ShMAX2*. Comparison of the *Sh*MAX2 amino acid sequence with *MAX2* orthologs from *Arabidopsis*, pea and rice shows an N-terminal extension of 25 amino acids in *Sh*MAX2 compared with the longest MAX2 from non-parasitic plants (Fig. S2). The expression of *ShMAX2* was analyzed by real time RT-PCR in *Striga* seeds. Because *Striga* seeds need to perceive exogenous strigolactones to trigger germination (Matusova *et al.*, 2004), the *ShMAX2* could be involved in this specific signaling event. Indeed, *ShMAX2* expression increased upon preconditioning at 30 °C (Fig. 3C), which correlates with an increase in strigolactone sensitivity during preconditioning (Matusova *et al.*, 2004). This pattern of expression is different from *AtMAX2* in *Arabidopsis* seeds, which declines in seeds upon imbibition (Fig.S3). *ShMAX2* expression was detected in all tissues analyzed, including young plants, underground shoots and roots and floral buds (Fig.3c).

Functional analysis of ShMAX2 in Arabidopsis

In order to test the functionality of *ShMAX2* in *Striga*, it would be best to obtain mutants or to interfere with *ShMAX2* gene expression by use of specific RNAi or amiRNA constructs. However, currently there is no transformation protocol for this species. Therefore, the function of *ShMAX2* was tested by complementation analysis in the *Arabidopsis max2-1* mutant. The *Arabidopsis max2-1* mutant displays increased branching (Stirnberg *et al.*, 2002), delayed seed germination (Shen *et al.*, 2007) and has an increased hypocotyl length in light grown seedlings (Tsuchiya *et al.*, 2010). For expression in *Arabidopsis*, the *ShMAX2* full length coding sequence was cloned into a binary expression vector (pBIN:*35S:ShMAX2*) under the control of the Cauliflower Mosaic Virus 35S promoter (see Methods S5). To test whether the N-terminal extension has an effect on *ShMAX2* functionality, we also cloned a truncated version of *ShMAX2* without the first ATG and with the transcription start site at a similar position as *AtMAX2* (35S:*ShMAX2*^{S}) (Fig. S2). As a positive control, *35S:AtMAX2*, which was provided by Professor *Otoline Leyser* was used for complementation of the *max2-1* mutant. 35S:*ShMAX2*, 35S:*ShMAX2*^{S} and 35S:*AtMAX2* were introduced into the *Arabidopsis max2-1* mutant background by floral dip transformation.

For each construct, 9-23 independent transgenic lines (T0) were selected on kanamycin. Preliminary analysis of T0 plants indicated that all three constructs were

able to suppress the branching phenotype of *max2-1* (Fig. S4). For each construct two lines displaying the highest reduction in branching were selected for further analysis. The branching phenotype was quantified in kanamycin resistant T1 progeny plants using the decapitation assay developed by Leyser and coworkers (Stirnberg *et al.*, 2007). Figure 5A shows the branching phenotype for each of the constructs. Under the conditions used, wild-type (WT) plants had on average 5.1 branches while the *max2-1* mutant had 28.1. In *max2-1* mutant plants with ectopic expression of *AtMAX2* the number of side branches was strongly reduced and not significantly different from the WT, indicating that ectopic expression of *AtMAX2* can fully complement the branching phenotype (Fig. 5b). The four selected *max2-1* lines with ectopic expression of *ShMAX2* or *ShMAX2^S* also showed significant reduction in the number of branches (between 16.9 and 8.1) compared with untransformed *max2-1*, but still had significantly higher numbers of branches than WT.

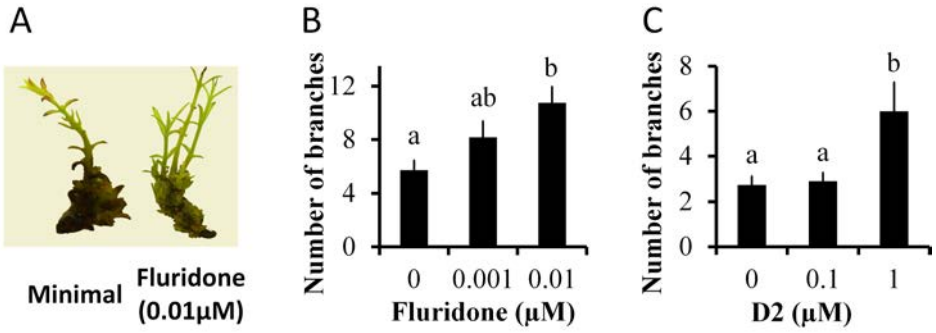

Fig. 4. Carotenoid biosynthesis inhibitor induces branching in *in vitro* grown *Striga hermonthica*. (**a**) *In vitro Striga hermonthica* grown on 0.01µM fluridone (carotenoid biosynthesis inhibitor) or on minimal medium. Arrows indicate the emerged shoots. (**b**) Effect of fluridone on branching numbers of *in vitro* grown *Striga hermonthica*. (**c**) Effect of D2 (strigolactone biosynthesis inhibitor) on branching numbers of *in vitro* grown *Striga hermonthica*. Means ± SE were obtained from 12 independent plants.

ShMAX2 complements the Arabidopsis max2-1 root growth phenotype

Strigolactones affect root phenotypes in a MAX2 dependent way (Kapulnik *et al.*, 2011a; Ruyter-Spira *et al.*, 2011). To determine whether *ShMAX2* can complement the root growth phenotype of the *max2-1* mutant, the primary root length of 5-day-old seedlings of *35S:ShMAX2*, *35S:ShMAX2^S* and *35S:AtMAX2* transgenic plants was determined. Both *ShMAX2* and *AtMAX2* showed suppression of root elongation of the *max2-1* mutant, demonstrating that they can rescue *MAX2* signaling in roots (Fig. 5c). Constitutive expression of *ShMAX2^S* reduced the mean length of the root from 1.104 cm in *max2* to 0.631-0.517 cm, resulting in plants indistinguishable from WT, with a root length of 0.594 cm. Constitutive expression of *ShMAX2* was slightly less effective,

reducing the primary root length to 0.694-0.755 cm. Combining these results suggested that *ShMAX2* gene functions similar to the *AtMAX2* gene in complementing the root growth phenotype of *max2-1*.

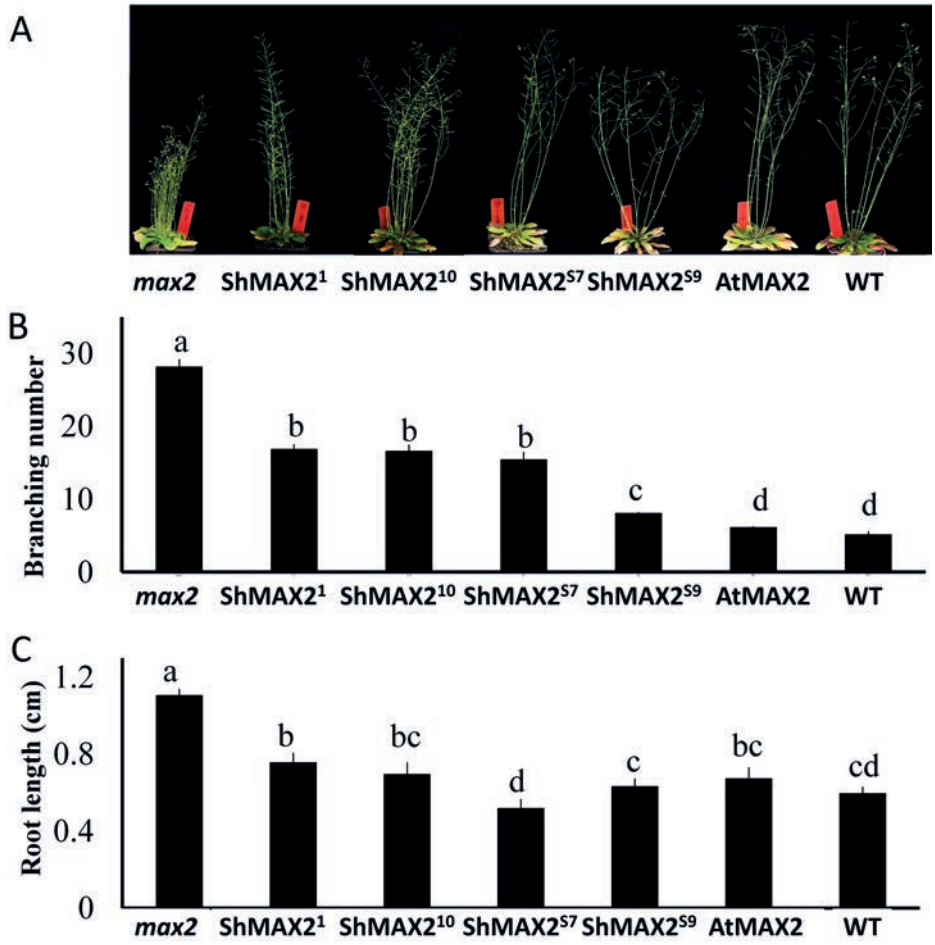

Fig. 5. Complementation of *Arabidopsis max2-1* branching and root phenotype with *ShMAX2*. (**a**) Comparison of branching phenotypes of *max2-1*, *max2-1* transformed with *ShMAX2* line1 and line10, *max2-1* transformed with *ShMAX2*^{S} line7 and line9, *max2-1* transformed with *AtMAX2*, and WT. (**b**) Branch numbers of *max2-1*, *max2-1* transformed with *ShMAX2* line1 and line10, *max2-1* transformed with *ShMAX2*^{S} line7 and line9, *max2-1* transformed with *AtMAX2*, and WT. Each bar represents the mean of three biological replicates +/-SE. Bars with different letters are significantly different according to Tukey test (P=0.05). (**c**) Primary root length of 5-day-old seedlings of *max2-1*, *max2-1* transformed with *ShMAX2* line1 and line10, *max2-1* transformed with *ShMAX2*^{S} line7 and line9, *max2-1* transformed with *AtMAX2*, and WT grown on vertical MS plates containing no sucrose were measured. Each bar represents the mean of three biological replicates +/-SE. Bars with different letters are significantly different according to Tukey test (P=0.05).

ShMAX2 complements the Arabidopsis max2-1 High Irradiance Response phenotypes

MAX2 is involved in light-regulated changes in gene expression and the resulting photomorphogenesis such as light-repressed hypocotyl elongation and seed germination under continuous white light (Tsuchiya *et al.*, 2010). Both of these processes represent a response to radiation with relatively high energy for a relatively long period of time and therefore are representative of the High Irradiance Response (HIR) of plants (Briggs *et al.*, 1985). To determine whether *ShMAX2* can complement the HIR hypocotyl phenotype of the *max2-1* mutant, the hypocotyl length of representative transformants with *35S:ShMAX2*, *35S:ShMAX2*^{S} or *35S:AtMAX2* was quantified. *Arabidopsis max2-1* has a similar hypocotyl length as WT when grown in the dark (Shen *et al.*, 2012), but when grown in continuous light or grown in the dark after germination was triggered with a red light pulse, it has a longer hypocotyl than WT (Tsuchiya *et al.*, 2010). Both *ShMAX2* and *AtMAX2* complemented the mutant hypocotyl phenotype resulting in a hypocotyl length that was not significantly different from WT (Fig. 6a). Also, there was no difference in complementation between the full length *ShMAX2* and its truncated version *ShMAX2*^{S}, suggesting that the N-terminal extension does not influence the function of *ShMAX2* in this process.

To determine whether *ShMAX2* can complement the HIR seed germination phenotype of the *max2-1* mutant, the seed germination rate under continuous white light and seed germination percentage after a red light pulse were quantified for *max2-1* transformants with *35S:ShMAX2*, *35S:ShMAX2*^{S} or *35S:AtMAX2*. For all our seed germination assays we made sure that seeds were harvested at the same time from plants grown under the same conditions and seeds were stored under similar conditions to ensure equal after-ripening for the different genotypes. Under continuous white light *max2-1* seeds have a lower germination rate than WT seeds (Shen *et al.*, 2007; Tsuchiya *et al.*, 2010). Also in our experiments the germination rate of *max2-1* was significantly lower than that of WT seeds (Fig. 6b). Although overexpression of *AtMAX2* in this mutant background resulted in a significant increase in the seed germination rate, it was still significantly lower than that of the WT. *ShMAX2* generally complemented the reduced seed germination rate of *max2* to a similar extent as *AtMAX2*, with the strongest complementation (the same rate as WT) achieved in line *ShMAX2*^{S7}. Previously it was shown that the branching phenotype of the Arabidopsis max2 mutant can also almost fully be complemented by one of the MAX2 homologs from petunia (PhMAX2A) (Drummond *et al.*, 2011). However in these studies other Arabidopsis *max2* related phenotypes, such as germination, HIR, VLFR, were not characterized, so it is not known whether *Ph*MAX2A homologs can complement these function in the Arabidopsis *max2*-background as well.

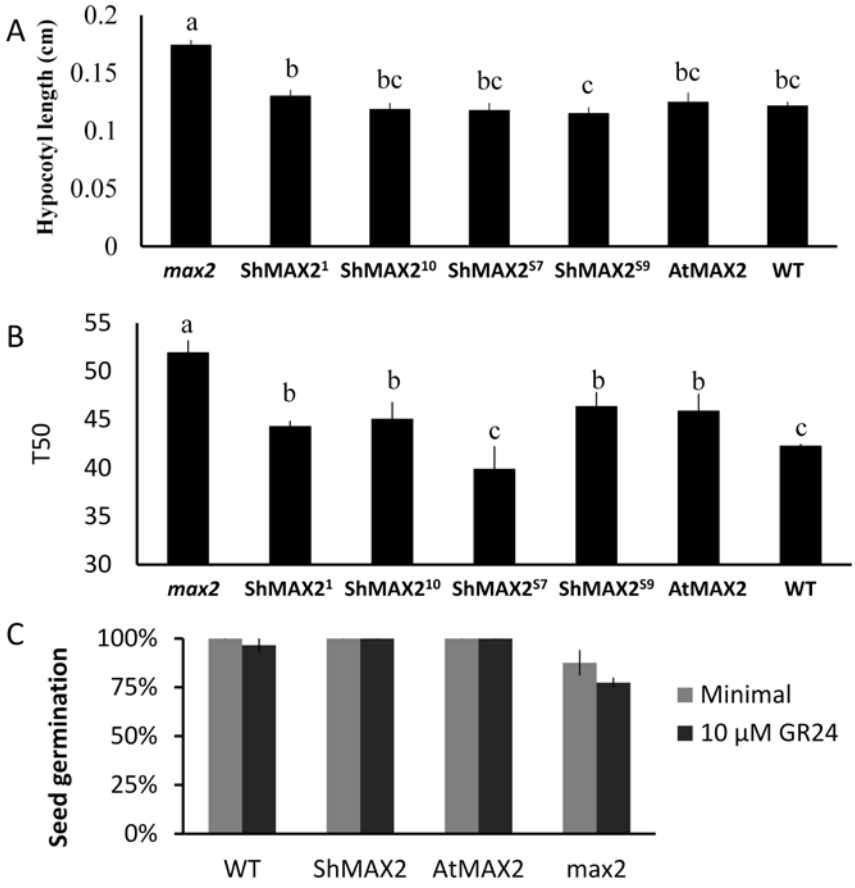

Fig. 6. Complementation of *Arabidopsis max2-1* hypocotyl, seed germination under continuous light condition and after red-light pulse. (**a**) Hypocotyl length of 5-day-old seedlings of *max2-1*, *max2-1* transformed with *ShMAX2* line1 and line10, *max2-1* transformed with *ShMAX2*^{S} line7 and line9, *max2-1* transformed with *AtMAX2*, and WT grown on vertical MS plates containing no sucrose under a 16h-light/8h-dark regime were measured. Each bar represents the mean of three biological replicates +/-SE. Bars with different letters are significantly different according to Tukey test (*P*=0.05). (**b**) Seed germination rate of *max2-1*, *max2-1* transformed with *ShMAX2* line1 and line10, *max2-1* transformed with *ShMAX2*^{S} line7 and line9, *max2-1* transformed with *AtMAX2*, and WT after three days' imbibition at 4 °C in dark followed by continuous light condition was scored. Y axis: Time (hour) of 50% germination. Each bar represents the mean of three biological replicates +/-SE. Bars with different letters are significantly different according to Tukey test (*P*=0.05). (**c**) Seed germination rate of *max2-1*, WT, *max2-1* transformed with *ShMAX2*, and *max2-1* transformed with *AtMAX2* or without GR24 (10µM) after three days' imbibition at 4 °C in dark followed by 5 minutes' red light treatment. Seeds were put into dark condition afterwards at room temperature. Seed germination was scored 2 days later. Each bar represents mean of three biological replicates +/-SE. Grey bars, minimal; black bars,10µM GR24.

Seed germination was also quantified in response to a red light pulse. Seeds were stratified at 4°C for 4 days after which they received a 5 min pulse of red light in the absence or presence of GR24 to test if strigolactones affect the response at this stage. Two days after the red light pulse, seeds of all genotypes, except *max2-1*, showed almost 100% germination (Fig. 6c), regardless of the presence of GR24.

ShMAX2 complements the max2-1 seed germination response to GR24

Max2-1 mutant seeds show reduced germination in the dark after a R or FR light pulse (Shen *et al.*, 2007), which cannot be complemented by addition of the synthetic strigolactone GR24 (Tsuchiya *et al.*, 2010). To determine whether this GR24 response can be restored by *ShMAX2*, the seed germination phenotype of WT, *max2-1* and *max2-1* complemented with *ShMAX2* or *AtMAX2* was assessed at four different concentrations of GR24 (Fig. 7). After 96 hours, seed germination of WT and *max2-1* complemented with *AtMAX2* was close to 100% with or without GR24, while no germination was observed in *max2-1* seeds. In *ShMAX2* complemented *max2-1* germination percentage increased to 81.9% with increasing GR24 concentrations, but at the highest GR24 concentration declined again to 45.5%. The whole experiment was repeated with a different line from each genotype, and showed similar results. These results show that *ShMAX2* can only partially restore the seed germination response to GR24 in *max2-1* and that endogenous strigolactone levels are already sufficient for the full germination response in WT and *max2-1* complemented with *AtMAX2*.

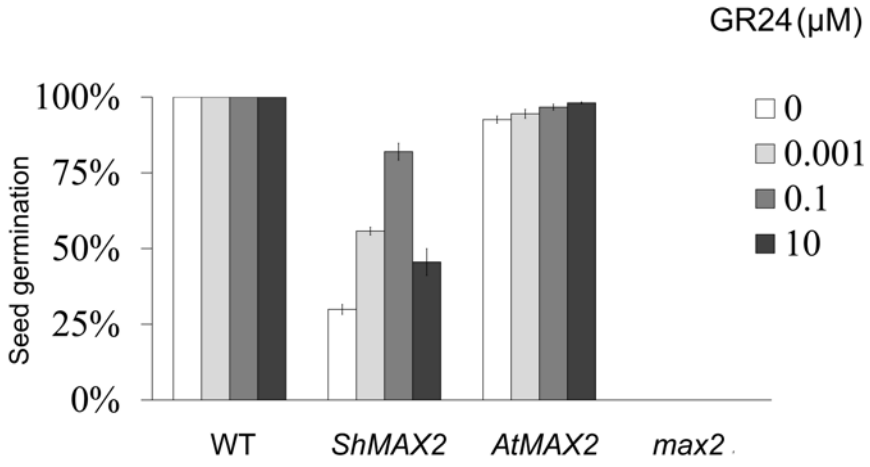

Fig. 7. Complementation of *Arabidopsis max2*-1 seed germination phenotypes under dark condition. Seed germination of *max2-1*, WT, *max2-1* transformed with *ShMAX2* line1 and *max2-1* transformed with *AtMAX2* with or without GR24 (0.001, 0.1, and 10 µM) after three days' imbibition at 4 °C in dark was scored after in dark condition for 4 days. Each bar represents the mean of three biological replicates +/-SE.

ShMAX2 does not complement the VLFR of max2-1 seed germination

MAX2 is required for seed germination under very low light intensities or after a Far Red (FR) light pulse (Botto *et al.*, 1996; Shen *et al.*, 2007), a response that can be classified as a Very Low Fluence Response (VLFR; Botto *et al.*, 1996). To determine whether the hyposensitivity to far-red of *max2-1* seed germination can be restored by *ShMAX2*, the far-red light induced seed germination phenotype of WT, *max2*, and *max2* complemented with *ShMAX2* or *AtMAX2* was tested. Seeds were stratified at 4°C for 4 days in dark after which they received a 5 min pulse of far-red light in the absence or presence of GR24. Three days after the far red light pulse, *max2-1* showed no seed germination, while germination of WT and *max2-1* complemented with *AtMAX2* were as high as 50% and 80%, respectively (Figure 8). Under these conditions, there was only 2% germination of *max2-1* complemented with *ShMAX2*. In these assays the addition of GR24 had either no effect or resulted in a slight decrease in germination percentage. The results suggest that, contrary to AtMAX2, *ShMAX2* cannot complement for the Very Low Fluence Response (VLFR) of seed germination.

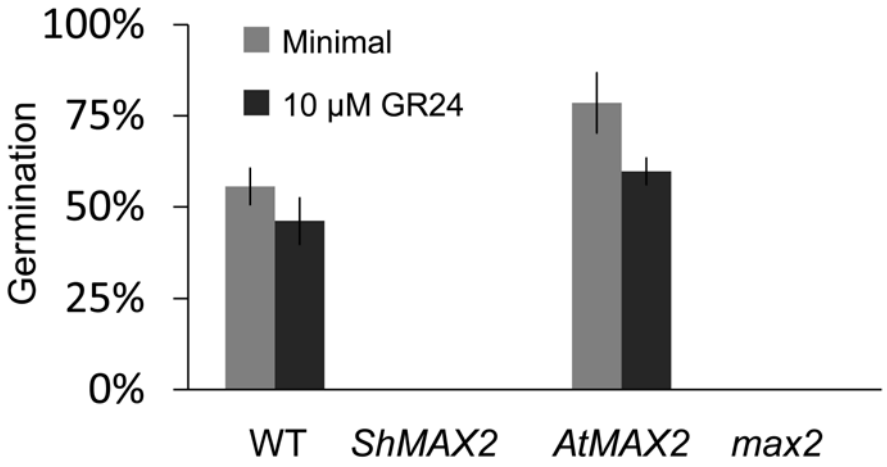

Fig. 8. Complementation of *Arabidopsis max2-1* far-red light sensitivity with *ShMAX2*. Seed germination rate of *max2-1*, WT, *max2-1* transformed with *ShMAX2*, and *max2-1* transformed with *AtMAX2* with or without GR24 (10 µM) after first three days' imbibition at 4 °C in dark followed by 5 minutes' far-red light treatment. Seeds were put into dark condition afterwards at room temperature. Seed germination was scored 3 days later. Each bar represents mean of three biological replicates +/- SE. Grey bars, minimal; black bars,10µM GR24.

Discussion

Strigolactones are required for germination of the parasitic plant *Striga*, but before this study it was unknown whether *Striga* itself produces strigolactones, whether strigolactone signaling plays a role during *Striga* development after germination and whether *Striga* has similar signaling components as non-parasitic plants, e.g. such as *AtMAX2* in *Arabidopsis* (Stirnberg *et al.*, 2007). The results presented in this paper provide evidence for strigolactones and strigolactone signaling in *Striga* beyond germination and we demonstrate that *ShMAX2*, the *AtMAX2* homolog in *Striga*, can replace most AtMAX2 functions except for its role in the Very Low Fluence Response (VLFR) of seed germination.

Evidence for strigolactones and strigolactone signaling in Striga

The germination of most parasitic plants seeds, e.g. *Striga, Orobanche* and *Phelipanche* spp., does not respond to light, but requires the presence of exogenous stimulants, for instance strigolactones (Matusova *et al.*, 2004). The strict dependence of germination of the parasitic plant *Striga* on host derived strigolactones raised the question whether these parasitic plants have lost the capacity for endogenous strigolactone biosynthesis, thus making them strictly dependent on exogenous strigolactones. Although direct measurement of strigolactones in different *Striga* tissues was not successful (no known strigolactones could be detected by LC-MRM-MS/MS), the HPLC fractioned extracts of sub-cultured *in vitro* grown *Striga* did contain fractions that were able to trigger germination of *P. ramosa* seeds (Fig. 2). Some active fractions coincided with the elution time of known strigolactones, suggestive of strigolactones presence. It is worthy to note that a similar experiment conducted independently by Das *et al*. (Submitted) reached the same conclusion. Even though the concentration of these putatively present strigolactones was extremely low (below the detection limit of our LC-MRM-MS machine), they were still functional in suppression of branching as inhibition of endogenous strigolactone biosynthesis by fluridone resulted in an increase in branching (Fig. 4). For *Striga* plants attached to host plants, the strigolactone profile differed depending on the host to which the *Striga* plant was attached, and the detected strigolactones always matched a subset of the strigolactones detected in the host. In non-parasitic plants, strigolactone biosynthesis is most active in the roots, but *Striga* does not have an extensive root system of its own. Strigolactones are transported through the xylem to the shoot (Kohlen *et al.*, 2011) and presumably the vascular connection between *Striga* and its host allows for the transfer of host produced strigolactones to the parasite. This explains why the strigolactone profile in *Striga* is dominated by host-derived strigolactones. Putative strigolactone biosynthesis genes are present and expressed in *Striga* (*ShCCD7*, *ShCCD8*; Fig. 3). The overall patterns of expression of

these genes in different plant tissues are only slightly different from those of *AtCCD7* (*MAX3*) and *AtCCD8* (*MAX4*) in *Arabidopsis* (compare Fig.3 with Fig. S1).

The increased branching in *in vitro* grown *Striga* as a result of the application of strigolactone biosynthesis inhibitors strongly suggests that strigolactones play a similar role in the control of shoot branching of *Striga* as in non-parasitic plants. Although none of the experiments on their own are conclusive, combined these results give sufficient evidence that *Striga* can produce its own strigolactones, and that endogenous strigolactone signaling in relation to shoot branching is conserved in *Striga*.

ShMAX2 complements Arabidopsis max2-1 shoot, root and HIR phenotypes

Germination of *Striga* seeds cannot be induced by light. This suggests that in *Striga* a component required for light-induced germination was lost or altered. In *Arabidopsis*, *MAX2* is such a component as it is involved in both the high irradiance response (HIR) and very low fluence response (VLFR) of seed germination (Shen *et al.*, 2007).

Because no fast transformation procedure exists for *Striga* plants and no relevant *Striga* mutants are available at present, the functionality of the *ShMAX2* was tested by complementing the *Arabidopsis max2-1* mutant. This mutant is characterized by its increased shoot branching (Stirnberg *et al.*, 2002), a HIR related seedling hypocotyl (Nelson *et al.*, 2011), a HIR and VLFR seed germination phenotype (Shen *et al.*, 2007), and a root elongation phenotype (Ruyter-Spira *et al.*, 2011). Our results show that, when transformed into *max2*, *ShMAX2* complements the branching, HIR and root phenotypes (Fig. 5, 6, and 7). This indicates that *ShMAX2* has not lost any of these known strigolactone related signaling functions, suggesting that in *Striga ShMAX2* may have similar functions as that of *AtMAX2*.

ShMAX2 does not complement the Arabidopsis max2-1 VLFR seed germination phenotype

The *max2-1* mutant also has reduced sensitivity to Far Red (FR) induced germination (Shen *et al.*, 2007), which is a typical VLFR phenotype. Our germination assay in response to a short FR light pulse indicates full complementation by the *AtMAX2* but no complementation by the *ShMAX2* (Fig. 8). Results also show that strigolactones affect the VLFR for *max2-1/ShMAX2* seeds (Fig. 8). Because VLFR is supposedly mediated by *PHYA*, this suggests that ShMAX2 has little interaction with the PHYA signaling components of *Arabidopsis*. The higher germination percentage of the *max2-1*/AtMAX2 seeds than WT suggests that this MAX2 mediated VLFR may normally be limited by endogenous MAX2 expression levels: overexpression of *AtMAX2* in the *max2-1* background results in a higher sensitivity to FR than in WT *Arabidopsis* (Fig. 8).

It is not clear whether the *max2* seed germination phenotypes relate to strigolactone signaling as there are conflicting reports about the seed germination phenotype of the different strigolactone biosynthesis mutants (Shen *et al.*, 2007; Nelson *et al.*, 2009; Tsuchiya *et al.*, 2010; Shen *et al.*, 2012; Toh *et al.*, 2012). Also, MAX2 promotes seedling de-etiolation in response to light (Shen *et al.*, 2007), but again the strigolactone biosynthetic mutants (*max1-1*, *max3-100*, and *max4-100*) do not display a seedling de-etiolation phenotype in response to different fluence rates of R or FR light (Shen *et al.*, 2012). Moreover, high levels of the synthetic strigolactone analog GR24 promote photomorphogenesis by inducing nuclear exclusion of COP1, which targets the photomorphogenesis related transcription factor HY5 for degradation and this effect seems to be at least partly independent of MAX2 (Tsuchiya *et al.*, 2010). It might be that seed germination and de-etiolation are extremely sensitive to strigolactones so that even very low levels in leaky strigolactone biosynthesis mutants are sufficient to promote photomorphogenic responses (for recent reviews see Foo and Reid (2012) and Brewer *et al*. (2013)).

The lack of a germination response after a FR light pulse (seeds were in darkness before and after FR light pulse) of *max2-1* or *max2-1* complemented with *ShMAX2* was not rescued by the addition of GR24 (Fig. 8). In strong contrast to this was the germination response in total darkness (Fig. 7). Under this condition, germination was dependent on a functional MAX2 gene, while the *ShMAX2* was less effective in complementing the *max2-1* phenotype. Moreover, in complete darkness the *max2-1/ShMAX2* seeds responded to added strigolactones (GR24) in a dosage dependent manner (Fig. 7). Combined, the results show that there is an interaction between strigolactones and MAX2, but that effects of strigolactones are very much condition dependent. In full darkness exogenous strigolactones stimulate germination in *max2-1*/*ShMAX2*, while for WT and *max2-1/AtMAX2* presumably the endogenous strigolactone levels are already sufficient. However, after a FR light pulse this stimulatory role of endogenous strigolactones is abolished in *max2-1/ShMAX2* seeds and results in reduced germination in WT and *max2-1/AtMAX2* seeds (Fig. 8). It has been shown that *At*MAX2 functions in the D14 and KAI2 signaling in combination with the signaling molecules strigolactones and karrikins (Waters *et al.*, 2012). While the interaction of KAI2 with MAX2 is mostly triggered by binding of karrikins, the interaction of D14 with MAX2 is triggered by binding of strigolactones (Waters *et al.*, 2012). Therefore, the difference in the complementation action of *Sh*MAX2 in the *max2-1* mutant background (compared to *At*MAX2) could be due to difference in interaction of *Sh*MAX2 with *At*D14 and the KAI2 protein of *Arabidopsis*. Such difference has been observed for the germination VLFR complementation of *max2-1* (Fig. 8). It has been shown that in *Arabidopsis* KAI2 signaling is most active during seed germination, while during control of shoot

branching the signaling of D14 is more prominent (Waters *et al.*, 2012). Therefore, the action of *Sh*MAX2 during germination is most likely due to interaction with KAI2, while the complementation of the branching phenotype is most likely due to interaction of *Sh*MAX2 with *At*D14. The lack of response to FR light in max2-1/*Sh*MAX2 indicates a reduced VLFR, which is mediated by PHYA. This result therefore suggests that the putative signaling complex *Sh*MAX2/KAI2 has reduced interaction with one of the PHYA signaling components. The lack of complementation of the VLFR seed germination response by *ShMAX2* in *Arabidopsis max2-1* could be that the F-box from Striga cannot recognize the protein(s) from Arabidopsis, hence resulting less efficient interaction of ShMAX2 with KAI2 than of AtMAX2 with KAI2.

Why is *Striga* seed germination dependent on exogenous strigolactones?

Strigolactone signaling through AtMAX2 has been shown to be involved in the control of *Arabidopsis* shoot branching. Here we have shown that *ShMAX2* can function in this strigolactone signaling pathway in *Arabidopsis* by demonstrating complementation of the *max2-1* branching phenotype by *ShMAX2* (Fig. 5, 6 and 7). As *in vitro* grown *Striga* also shows a branching phenotype in response to strigolactone biosynthesis inhibitors (Fig. 4) we assume that *ShMAX2* also functions in strigolactone controlled shoot branching in *Striga* itself. The inhibitor experiment suggests that *Striga* itself can produce strigolactones. Indeed, putative strigolactone biosynthesis genes, *ShCCD7* and *ShCCD8* were identified and are expressed in different tissues (Fig. 3). The expression profile of *ShCCD7* and *ShMAX2* in pre-conditioned *Striga* seeds is different from the expression profile of the *Arabidopsis CCD7* (*MAX3*) and *AtMAX2* as retrieved from publicly available microarray expression data at TAIR (expression data for *AtCCD8/MAX4/*at4g32810 are not available). Both *AtMAX3* and *AtMAX2* have relatively high expression in developing and dry seeds, especially in the chalazal seed coat, in *Arabidopsis* (Winter *et al.*, 2007; Bassel *et al.*, 2008). However, in *Arabidopsis*, expression of both genes rapidly declines upon imbibition. In contrast, in *Striga* expression of *ShCCD7* and *ShCCD8* show a transient maximum around seven days of seed conditioning, after which expression of these genes declines to low levels in germinating seeds (Fig. 3 and Fig. S1). Most interestingly, the expression of *ShMAX2* increases during *Striga* seed conditioning, and only declines in germinating seeds. This expression profile of *ShMAX2* during seed conditioning correlates well with the increased sensitivity of *Striga* for strigolactones in response to the preconditioning treatment (Fig. 3) (Matusova *et al.*, 2004). Germination of *Striga* seeds depends on exogenous strigolactones (also after conditioning). Therefore, it is not clear what the significance is of transient *ShCCD7* and *ShCCD8* expression during preconditioning. Possibly, *Striga* endogenous strigolactones do play a role in seed germination but are not sufficient to induce germination. Possibly, the lack of a VLFR in *Striga* seed germination is due to ShMAX2. However, the lack of a HIR in

Striga seed germination does not seem to be caused by ShMAX2, as it can complement this phenotype in the *Arabidopsis max2-1* mutant. Identifying the target proteins of the SCF^{ShMAX2} protein complex will be of interest to further address this enigma.

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Supporting Information

Methods S1 HPLC fractionation

Two hundred-fifty µl of filtered sample were fractioned with an XBridge C18 column (4.6×3×150 mm; Waters) using the following gradient: 1 min of 100% water, 2 min of 27% (v/v) acetonitrile in water, 15 min of 45% (v/v) acetonitrile, 24 min of 80% (v/v) acetonitrile, and 24.2 min of 100% acetonitrile, which was maintained for 4 min to clean the column. The flow rate was 1 ml min^{-1}, and the column temperature was set at 25ºC. One-minute fractions were collected using a Biofrac fraction collector (Bio-Rad). In total, thirty 1-ml fractions were collected. The HPLC fractions were dried by speed vacuum, re-dissolved in 175 µl MQ water and used for *Phelipanche ramosa* seed germination bioassay. For each treatment, three biological replicates were used.

Methods S2 Phelipanche ramosa seed germination bioassay

Germination assays with *Phelipanche ramosa* seeds (from rape seed host) were conducted as reported by Matusova *et al.* (2005). Seeds of *Phelipanche ramosa* were cleaned using a sucrose gradient and preconditioned for 14 d at 21ºC. Aliquots (70 µl) of re-dissolved HPLC fractions (without organic solvent) were added to triplicate 1cm discs containing approx. 40 preconditioned seeds each. The synthetic germination stimulant GR24 and MQ water were included as positive and negative controls in each bioassay. After 6 d dark incubation at 21°C, the germinated and non-germinated seeds were counted using a stereomicroscope. Seeds were considered germinated when the radicle had protruded through the seed coat.

Methods S3 Gene expression analysis by Real Time PCR

Total RNA was isolated from *Striga* tissues using Trizol reagent (Invitrogen, USA) and further purified with RNAeasy mini spin columns (Qiagen, USA). Quality and quantity of the DNase-treated RNA samples were assessed by Agilent Bioanalyzer 2100 analyses (Agilent Technologies, USA) and determined to contain high quality RNA with no DNA contamination. First strand cDNA was synthesized using high capacity cDNA reverse transcription kit (Applied Biosystems). Taqman chemistry was used to measure the expression level of the targeted strigolactone genes in *S. hermonthica*. Primers used for *ShCCD7* were a forward, 5'-ACGCGCCGAGACCTC-3', a reverse 5'-CCGGGAGGGCGGATG-3', and a probe, 5'-CTCCCCGTCATCAGCG-3'. Primers used for *ShCCD8* were a forward, 5'-GCAAAGAGCTGGATGGAAAACG-3', a reverse 5'-TTCGTGGCCCGTCCG-3', and a probe, 5'-ACCCTCCGAACCCTTC-3'. Primers used for *ShMAX2* were a forward, 5'-CCGACACGAACGCCTTGT-3', a reverse 5'-AGAGTAGCTGCGTTGATCTTAGC-3', and a probe, 5'-GACGAGCGCGAGGG-3'. For each developmental stage, three biological replicates and two technical replicates

were used. Real time RT-PCR was performed in 7300 real-time PCR system (Applied Biosystems) using a cycling regime of 95ºC for 30 s followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min. The default parameters of SDS 7300 system SDS software were used to determine the threshold cycle (Ct). The number of transcript copies were calculated based on an external standard curve and subsequently normalized based on input cDNA quantity. The reference gene used was *S. hermonthica* Ubiquitin 1, using primer sequences and amplification conditions exactly as described in Fernández-Aparicio *et al*. (2013). The normalized values of transcript copy number in each biological sample were expressed as: (gene transcript copy number/ *ShUBQ1* copy number) X 1000. Statistical analyses were performed in JMP 8 (SAS Institute Inc.).

Methods S4 Isolation of the ShMAX2 Gene

Genomic DNA was isolated from *Striga* shoot material using the GenElute Plant Genomic DNA miniprep Kit (Sigma-Aldrich, USA) according to manufacturers' protocol.

For *ShMAX2*, degenerate primers were designed based on the protein sequences of *Arabidopsis* MAX2, pea RMS4, and hypothetical protein from *Vitis vinifera* and used to amplify fragments of the *ShMAX2* gene from DNA. Then Genome-walking (Kilstrup & Kristiansen, 2000) was used to obtain additional sequences information. The full length of *ShMAX2* was amplified suing a forward primer 5'-TAAGTATGACCCCCAAAACG-3', and a reverse primer 5'-TTACGAATTCGTTTTA-3'. Then a truncated version of *ShMAX2* (*ShMAX2*^{S}) was cloned, in which the first ATG was deleted, resulting in a coding sequence with the first ATG at similar position as in the *AtMAX2* gene (NCBI accession: NM_129823).

Methods S5 Expression constructs and plant transformation

Polymerase chain reactions (PCR) for cloning were performed using Phusion DNA polymerase and PCR-derived clones were confirmed by colony PCR and sequencing. Full length *ShMAX2*^{L} and *ShMAX2*^{S} was amplified from *Striga* genomic DNA using primers with flanking BamHI and PacI sites (forward primer 5'-CCCGGATCCTAAGTATGACCCCCAAAACG-3', and reverse primer 5'-GGGGCGGCCGCTTACGAATTCGTTTTA-3'). The PCR product was digested with BamHI and PacI and ligated into the pIV1A entry vector which contains 35S promoter. Subsequently, the two DNA fragments were sub-cloned into destination vector pBIN (Vanengelen *et al.*, 1995), generating pBIN(35S:*ShMAX2*^{S}) and pBIN(35S:*ShMAX2*). Plasmid pBIN(35S:*AtMAX2*) was kindly provided by Dr P. Stirnberg (University of York, UK) and Prof. O. Leyser (University of Cambridge, UK). The binary vectors were purified from *Escherichia coli* and electro-incorporated into *Agrobacterium tumefaciens*

GV3101. Transformation was performed with a transformed clone by floral dipping (Clough & Bent, 1998). The independent transformants were screened on MS agar medium containing 50 mg l^{-1} kanamycin. Independent, homozygous T1 lines were used for the following analysis.

Supporting figures

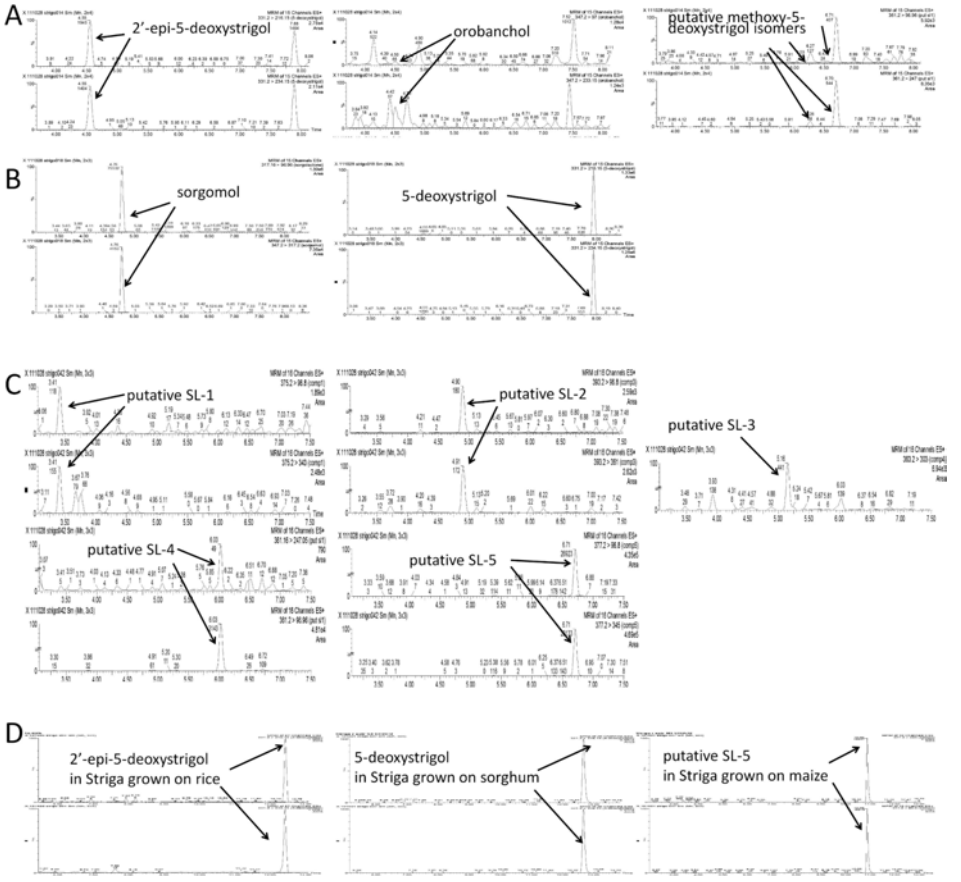

Fig. S1 Strigolactones identified from *in vivo* grown *Striga* plants and its host by MRM-LC-MS/MS. (A) 2'-*epi*-5-deoxystrigol (transitions 331.2>216.15, and 331.2>234.15), orobanchol (transitions 347.2>97, and 347.2>233.15) and two putative methoxy-5-deoxystrigol isomers (transitions 361.2>96.96, 361.2>247) were identified in root extracts of host plant rice (*Oryza sativa*). (B) 5-deoxystrigol (transitions 331.2>216.15, and 331.2>234.15) and sorgomol were identified in root extracts of host plant sorghum (*Sorghum bicolor*). (C) Five putative strigolactones, SL-1 (transitions 375.2>96.8, and 375.2>343), SL-2 (transitions 393.2>96.8, and 393.2>361), SL-3 (transition 363.2>303), SL-4 (transition 361.16>247.05, and 361.16>96.96), and SL-5 (transitions 377.2>96.8, and 377.2>345) were identified in root extracts of host plant maize (*Zea mays*). (D) 2'-*epi*-5-deoxystrigol (transitions 331.2>216.15, and 331.2>234.15), 5-deoxystrigol (transitions 331.2>216.15, and 331.2>234.15), and SL-5 (transitions 377.2>96.8, and 377.2>345) were identified in *Striga* plants grown on rice, sorghum, and maize, separately.

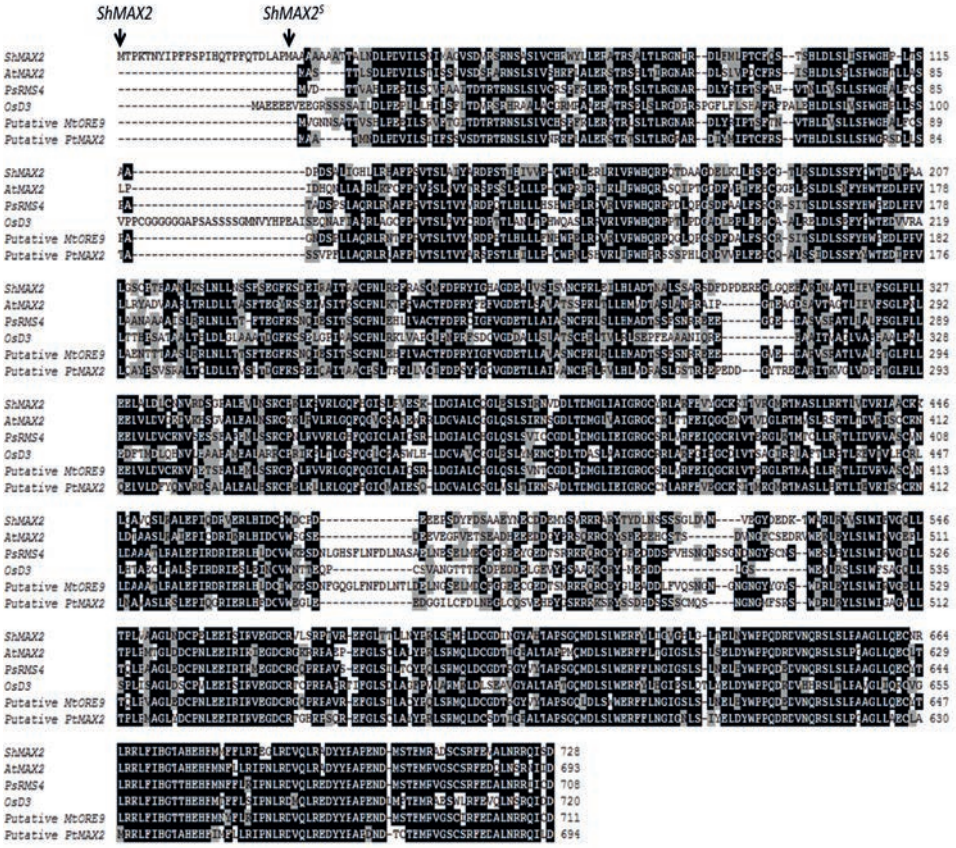

Fig. S2 Isolation of *ShMAX2*. Alignment of predicted amino acid sequences of *ShMAX2* compared with *Arabidopsis thaliana* (*AtMAX2*, NM_129823), *Pisum sativum* (*PsRMS4*, DQ403159), *Oryza sativa* (*OsD3*, Q5VMP0), *Medicago truncatula* (*putative MtORE9*, XP_003607592), *Vitis vinifera* (*putative VvMAX2*, CAN59822), and *Populus trichocarpa* (*putative PtMAX2*, XP_002320412).

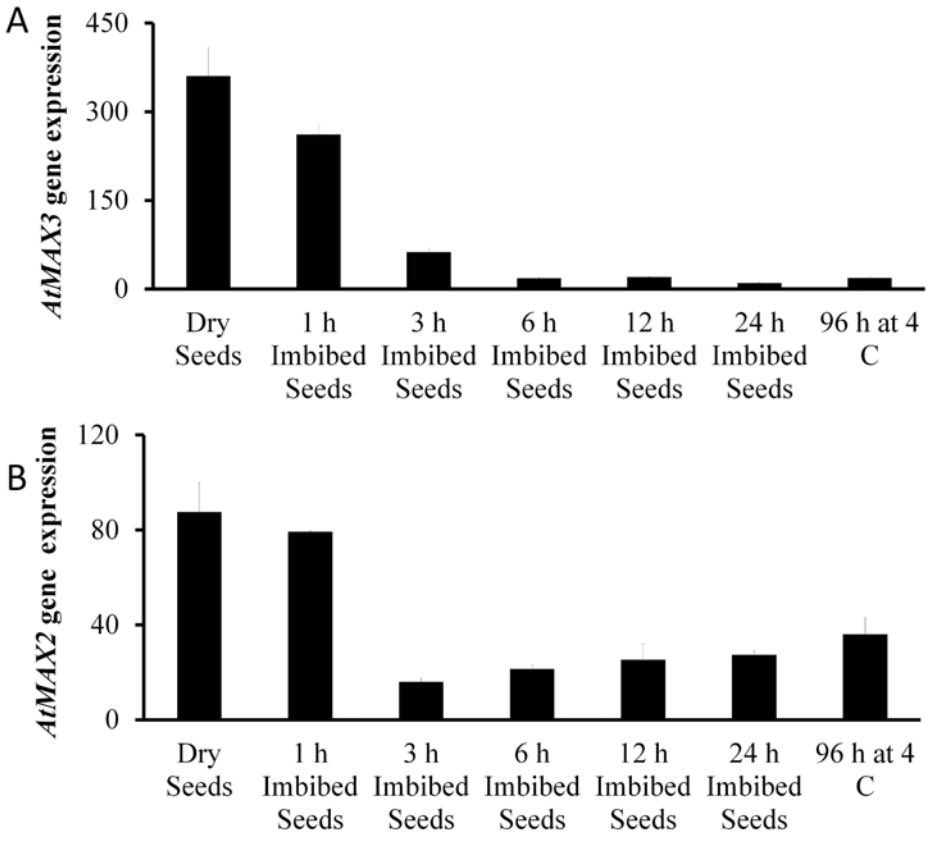

Fig. S3 Expression of *AtMAX3* (A) and *AtMAX2* (B) in *Arabidopsis* seeds. Source data are from TAIR (http://www.arabidopsis.org/). Y axis means relative gene expression level.

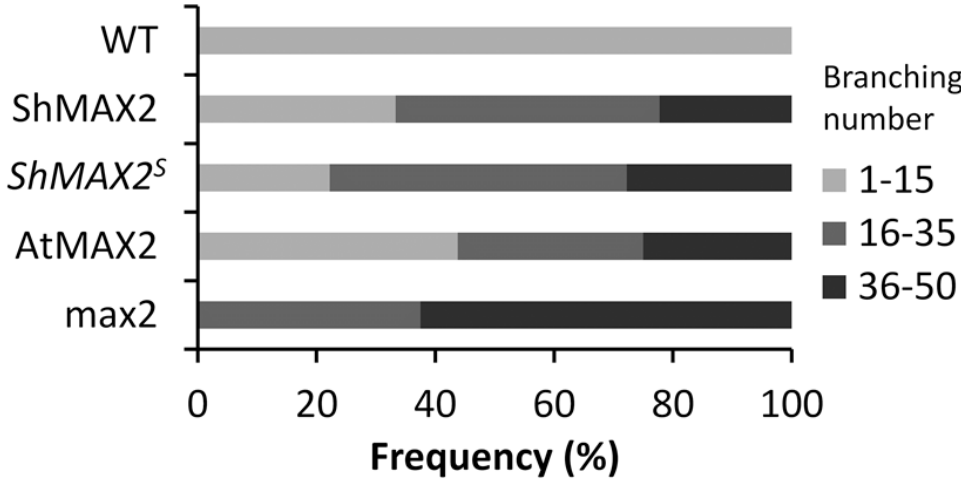

Fig. S4 Distribution of branching numbers of WT, *max2-1*, T0 *max2-1* plants transformed with *ShMAX2*, *ShMAX2*^{S} and *AtMAX2*. Frequency was obtained from 9~19 plants.

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Chapter 6

General Discussion

Strigolactones (SLs) are important rhizosphere signalling molecules and newly identified phytohormones. For a better understanding of their rhizosphere signalling functions, it is of importance to study both the biosynthesis of these molecules in the host plants as well as the signal perception in the parasitic plants. The biosynthetic pathway of SLs has been partially elucidated. In various plant species, several mutants were identified that were shown to be associated with the biosynthesis and signal transduction pathways of these molecules. However, how the formation of their ABC-D structure and their structural diversification are catalysed was unclear when I started the work on my thesis. Moreover, nothing was known about the perception and signalling of the SL in the seeds of parasitic plants in which these rhizosphere signalling molecules trigger germination. In this thesis, the SLs were therefore studied from different angles: the biosynthesis and biology of SLs including the cross-talk with other phytohormones (**Chapter 2**); the discovery of genes associated with SL production in a QTL mapping study using an F_6 population of rice cultivars Azucena × Bala (**Chapter 3**); the enzymatic characterization of the discovered candidate genes from the QTL mapping study (**Chapter 4**); and finally, the functional analysis of a candidate gene for SL signal perception in the root parasitic plant *Striga hermonthica* (**Chapter 5**). Here, in **Chapter 6**, I discuss the results in my thesis in a broader perspective. I discuss whether the origin of SLs can be identified from the presence of homologs of its biosynthetic genes in the predecessors of vascular plants; how one of these biosynthetic genes, *MAX1*, is involved in the creation of structural as well as stereochemical variation in the SLs; what may be the evolutionary selection pressures that have caused this structural variation and finally how the multifunctional MAX2 functions in SL signalling.

Is the origin of strigolactones correlated with the evolution of *MAX1* or other strigolactone biosynthetic genes?

SLs are newly identified phytohormones that are regulating multiple biological processes in flowering plants (angiosperms). To date, many different SLs have been identified from different flowering plant species (see **Figure 2** in **Chapter 1**). Chapter 5 also provides evidence that SLs are produced by the root parasitic plant *Striga hermonthica*. Intriguingly, Delaux et al. (2012) reported that active SLs (two sorgolactone isomers) were detected in the exudate of *Charales* spp., the closest relative of green land plants, and in tissue extracts of liverworts *Marchantia spp.* (5-deoxystrigol, solanacol, two orobanchol isomers, fabacyl acetate and orobanchyl acetate), an early diverging lineage of the basal land plants (Karol et al., 2001; Delaux et al., 2012) (**Figure 1**). SLs strigol, orobanchol, fabacyl acetate, orobanchyl acetate, 7-oxobanchyl acetate and 7α-hydroxyorobanchyl acetate were detected in the moss *Physcomitrella patens*, another example of a basal land plant (Proust et al., 2011) (**Figure 1**). Surprisingly, GR24 application was able to

promote rhizoid growth of Charales, liverworts and moss, suggesting that SLs have a hormonal role in the development of these species (Delaux et al., 2012). Interestingly, Proust et al. (2011) showed that the *ccd8* mutant of *Physcomitrella patens* was not able anymore to respond to the proximity of neighbouring colonies which in the wild-type plants causes the arrest of the colony extension, suggesting that SLs are acting as quorum-sensing molecules for the communication between colonies in moss (Proust et al., 2011). Molecular evidence has shown that the divergence of green algae and fungi occurred much earlier (1000 million years ago) than that of land plants (700 million years ago), and AM fungi are able to establish symbiotic interaction with the very basal land plants, the liverworts, although we do not know whether this is SL related or not (Heckman et al., 2001; Humphreys et al., 2010). Nevertheless, it is clear that SLs are very ancient signalling compounds with hormonal activity originating from the algae or very early land plants (embryophytes).

The emergence of SLs is associated to some extent with the evolution of the SL biosynthetic genes. The putative homologs of the known SL biosynthetic genes *D27*, *CCD7* and *CCD8* can be retrieved the early lineage of green algae and land plants, including the very basal land plants (liverworts and mosses) although homologs of *CCD7* and *CCD8* are missing in some genera of the green algae (Delaux et al., 2012; Challis et al., 2013) (**Figure 1**). However, another SL biosynthetic gene *MAX1*, belonging to the cytochrome P450 CYP711clan, is an exception. A homology search with the AtMAX1 protein sequence using Blast-P against the NCBI (The National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/) and moss genome (http://www.plantgdb.org/PpGDB/) database, did not yield any homologs in Charales, liverwort and moss (data not shown). However, CYP711-like sequences were reported in the green algae genus *Chlamydomonas* which predates the Charales and liverworts, but there is no active SLs found in either the exudates or tissue extracts of *Chlamydomonas* (Nelson, 2006; Delaux et al., 2012) (**Figure 1**). This absence of CYP711 or CYP711-like sequence in Charales and liverworts may be due to the incompleteness of the sequence databases, or indeed CYP711 genes are lost in their genomes and this clade of CYP450s evolved only in vascular plants.

Gene localization studies have revealed that SL biosynthesis occurs in the plastids through the D27 catalysed isomerization of all-*trans*-β-carotene to 9-*cis*-β-carotene, which is subsequently cleaved by two CAROTENOID CLEAVAGE DIOXYGENASEs (CCD7 and CCD8) to the SL precursor carlactone (Auldridge et al., 2006; Lin et al., 2009). Although there is still no experimental evidence showing how carlactone is transported from the plastids to the target location where there is the cytochrome P450 *MAX1* (Schuler and Werck-Reichhart, 2003), in **Chapter 4** I showed that carlactone is

the direct substrate of the rice MAX1 Os900. In the case of Charales, liverworts and moss, where *MAX1* is absent, it is likely that there are either bypasses with different undiscovered genes leading to SL biosynthesis, or the MAX1 activity is replaced by other enzymes.

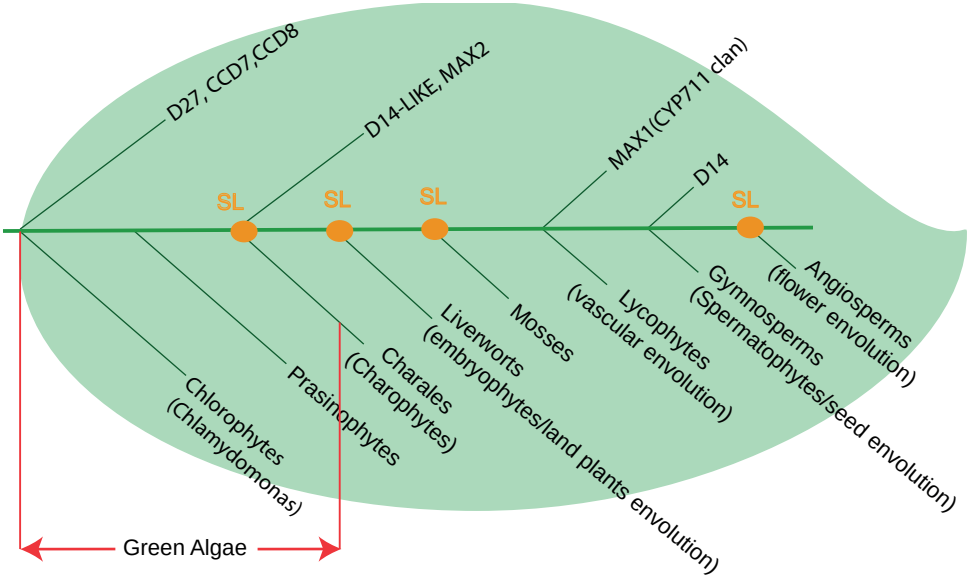

Figure 1. Plant evolution age and SL-related genes emergence. Orange dots are the detected SL signals.

In this thesis, QTL mapping of SL levels in rice using an F_6 population derived from cultivars Azucena × Bala, which produce different levels of SLs, identified a major QTL for the SLs on chromosome 1. Comparison of the genome sequence of the parental lines revealed that in the lower SL producer Bala there is a genome rearrangement in this QTL region, affecting two members of the rice *MAX1* family. Gene functional characterization showed that these two candidates (*Os01g0700900* and *Os01g0701400*) from rice cultivar Nipponbare are indeed regulating the SL level in rice. Previously, it has been genetically and analytically proven that *AtMAX1* is involved in SL production in *Arabidopsis* (Stirnberg et al., 2002; Booker et al., 2005; Kohlen et al., 2011). Taken together, this is indeed strongly suggesting that *MAX1* is tightly associated with SL presence in the flowering plants.

CYP450s have been extensively reported to be involved in many different primary and secondary metabolism pathways to catalyse different reactions, such as oxidation and hydroxylation in plants (Mizutani and Ohta, 2010). It is not uncommon that CYP450s are also involved in the phytohormone metabolic pathways. *Arabidopsis ent*-kaurene oxidase, a member of the CYP701A family, is involved in gibberellic

acid (GA) biosynthesis and catalyses a three-step oxidation to form *ent*-kaurenoic acid from *ent*-kaurene (Helliwell et al., 1999). A member of the CYP74A subfamily, allene oxide synthase (AOS), is oxidizing 13- hydroxyperoxyoctadecatrienoic acid *en route* to the defence hormone jasmonic acid (JA) (Song et al., 1993). CYP707A family P450s, especially CYP707A1 and CYP707A2, function as abscisic acid (ABA) 8'-hydroxylases, and are reported to play a major regulatory role in controlling abscisic acid (ABA) level in plants (Kushiro et al., 2004; Okamoto et al., 2006). *MAX1* is a cytochrome P450 CYP711A sub-family gene (Booker et al., 2005). Unlike other P450s involved in the hormonal pathways, the CYP711 clan CYPs are usually found as a single copy in dicotyledons but multiple (four to five) copies in monocotyledons, such as rice, maize and sorghum (Nelson and Werck-Reichhart, 2011; Challis et al., 2013). However, the dicot plant species *Populus trichocarpa*, *Glycine max*, *Medicago truncatula* and *Fragaria vesca* contain two copies of *MAX1* homologues. As already mentioned before, *MAX1* evolution can be traced back to where the vascular plants originated (after the mosses had emerged). Challis et al. (2013) tested the *Arabidopsis max1* phenotype complementation ability of *MAX1s* from phylogenetically diverse flowering plants (dicots or monocots), *Selaginella moellendorffii* (lycophytes) and the gymnosperm *Picea glauca*. They showed that all *MAX1* homologues could completely or partially restore the *Arabidopsis max1* branching and leaf phenotypes, except for one (out of three tested) rice *MAX1* ortholog, *Os01g0701500*, and one (out of two tested) *Medicago truncatula* ortholog, *Medtr1g015860* (Challis et al., 2013). These findings suggest that there is functional conservation of *MAX1* in different plant species in complementing the SL related shoot branching phenotype. However, in **Chapter 4** I show that the story of MAX1 is slightly more complicated than this and that not all MAX1 homologs dot the same thing. I analysed the enzymatic function of all five rice *MAX1* orthologs, which belong to three different MAX1 clades: the chromosome 1 clade (*Os01g0700900*, *Os01g701400* and *Os01g0701500*), the chromosome 2 clade (*Os02g0221900*) and the chromosome 6 clade (*Os06g0565100*) (Challis et al., 2013). The chromosome 1 clade is a more recent divergence in the monocotyledons, while the other two diverged rapidly after the dicotyledon *MAX1s* (Challis et al., 2013). Thus, this could explain that the chromosome 1 clade could encode new functions in SL biosynthesis and that different *MAX1s* from different clades have different activities. For instance, the enzyme encoded by *Os01g0700900* is catalysing SL B-C ring closure to form the ABC-D structure; the enzyme encoded by *Os01g0701400*, however, is catalysing SL structural diversification to orobanchol; the genes from the chromosome 2 and chromosome 6 clade show substantially weaker activity in catalysing 5DS production, even though they (except for *Os01g0701500*) were active in complementing the *Arabidopsis max1* phenotype (Challis et al., 2013). This suggests that these *MAX1* homologs have a function in SL biosynthesis but we do not exactly know what function.

Is *MAX1* determining the stereochemistry of strigolactones?

Many cytochrome P450s catalysed reactions are stereo-specific (Morant et al., 2003). In the context of stereo-structural diversification of SLs, the formation of certain SL stereoisomers is likely determined by specific enzymes. The naturally occurring SLs are classified into two groups- strigol-type and orobanchol-type containing the opposite C-ring orientation, but with the same D-ring stereochemistry which originates from the C-11 *R*-configured carlactone of which the formation is catalysed by CCD8. Chapter 4 shows that rice MAX1s (Os01g0700900 and Os01g0701400) are stereo-specifically producing the orobanchol-type SLs with α-orientated C-ring. This raises the question whether MAX1 (especially Os900-type MAX1) is controlling the C-ring conformation in the SLs. Prior to answering this question, another question has to be considered first. Can the conversion from the precursor carlactone to SLs be achieved in one step? In Chapter 4, a multiple-step proposition was made for this conversion because both the C-18 and C-19 positions of carlactone have to be oxidised followed by a ring-closure to form the B-C lactone ring. According to this proposal, there are two possibilities: (i) Os900 is the sole enzyme catalysing both C-18 and C-19 oxidations followed by a stereo-controlled B-C ring closure, forming the parent rice SL *ent*-2´-*epi*-5DS. This SL can be further converted to other rice SLs by adding functional groups on the A-B rings, resulting in SLs such as orobanchol that share the same C-ring stereochemistry; (ii) Considering the complexity of the B-C ring formation, *MAX1* (*Os01g0700900*) may only be involved in one step of this multiple-step reaction. However, this is very unlikely since in the *in vitro* yeast microsomal assays the precursor carlactone is exclusively converted to *ent*-2'-*epi*-5DS although with low efficiency. In contrast to the yeast assay, the parent SL *ent*-2'-*epi*-5DS was predominately and highly efficiently formed from the precursor carlactone by *MAX1 Os900* in the *Nicotiana benthamiana* in *planta* system. This may mean that in *N. benthamiana* another enzyme is present helping Os900 to complete the stereo-controlled ring-closure more efficiently.

The stereo-specificity of MAX1 Os900 in both *N. benthamiana* and yeast expression systems described in **Chapter 4** provides compelling evidence supporting the hypothesis that Os900-type MAX1 is evolved to be stereo-specific and determining the C-ring stereochemistry of SLs. Similarly, another *MAX1 Os1400*, which has very close homology to *Os900* in the same CYP711 sub-clade, is evolutionarily selected to stereo-selectively convert only *ent*-2´-*epi*-5DS to orobanchol. Collectively, it is likely that both *Os900* and *Os1400* have evolved to produce orobanchol-type SLs (*ent*-2'-*epi*-5DS and orobanchol), with an α-orientated C-ring, which are the main rice SLs.

As mentioned above, the five copies of the rice *MAX1* homologs belong to three sub-clades. Except for *Os01g0701500* (from cultivar Nipponbare) which lost its function, the

chromosome 1 clade homologues were, in Chapter 4, already shown to be stereo-specific and highly selective and to form orobanchol-type SLs although they are acting in distinct steps. Moreover, this clade of *MAX1* is mainly expressed in root tissue, while the *MAX1* homolog located in chromosome 2 is mainly expressed in leaves, and the *MAX1* from chromosome 6 mainly in stem tissue (**Figure 2**). Although only small amount of 5DS products (5DS and *epi*-5DS) were produced in *N. benthamiana* upon co-expression of these two *MAX1s* (*Os02g0221900* and *Os06g0565100*) with the carlactone biosynthetic genes (*D27*, *CCD7* and *CCD8*) (**Chapter 4**), there was a strong reduction in the level of carlactone upon addition of these *MAX1s* (data not shown), suggesting that an unknown compound is produced from carlactone by these two *MAX1s*, perhaps a compound similar to SL-LIKE1 that is produced from carlactone in *Arabidopsis* (Seto et al., 2014).

The SL 5-deoxystrigol is also a parent SL, from which the strigol-type SLs are likely derived, just as *ent*-2'-*epi*-5DS is the parent SL for the orobanchol-type SLs (Xie et al., 2010). This type of SLs have an opposite C-ring orientation (β-orientated) compared with the orobanchol-type SLs, which implies that the ring-closure step is controlled by (a) different enzyme (s). It is very likely that this β-orientated B-C ring closure is also catalysed by a CYP450 MAX1, just like Os900 but with different stereo-specificity, leading to a SL profile with opposite stereochemistry as in rice, as has indeed been observed in some other plant species. For instance, another monocot, sorghum, and the legume species *Lotus japonicus* are both producing strigol-type 5-deoxystrigol, and the former also contains relatively high level of sorgomol (Liu et al., 2013; Motonami et al., 2013), also a strigol-type SL, of which the formation might be catalysed by another *MAX1* just as *Os1400* that produces orobanchol from *ent*-2'-*epi*-5DS .

What are the multiple functions of MAX2? Are they (all) associated with strigolactones?

MAX2, which is conserved in green algae, mosses and vascular plants, is a leucine-rich repeat (LRR) F-box family protein, and participates in the SCF multi-subunit E3 ubiquitin ligase complex (Deshaies, 1999; Stirnberg et al., 2007; Delaux et al., 2012). These type of protein complexes play essential roles during plant growth and development, especially in phytohormone signalling pathways, such as auxin, JA and GA signalling pathways (Xie et al., 1998; Dill et al., 2004; Dharmasiri et al., 2005; Kepinski and Leyser, 2005). In line with this, more than a decade ago *MAX2* was already described to act downstream of a long-distance shoot branching inhibiting signal, which was recently identified to be SL (Stirnberg et al., 2002; Stirnberg et al., 2007; Gomez-Roldan et al., 2008; Umehara et al., 2008). **Chapter 5** shows that the parasitic plant *Striga MAX2* homologue *ShMAX2* can complement the shoot branching phenotype of *Arabidopsis max2*. More recently, the identification of another SL signaling pathway

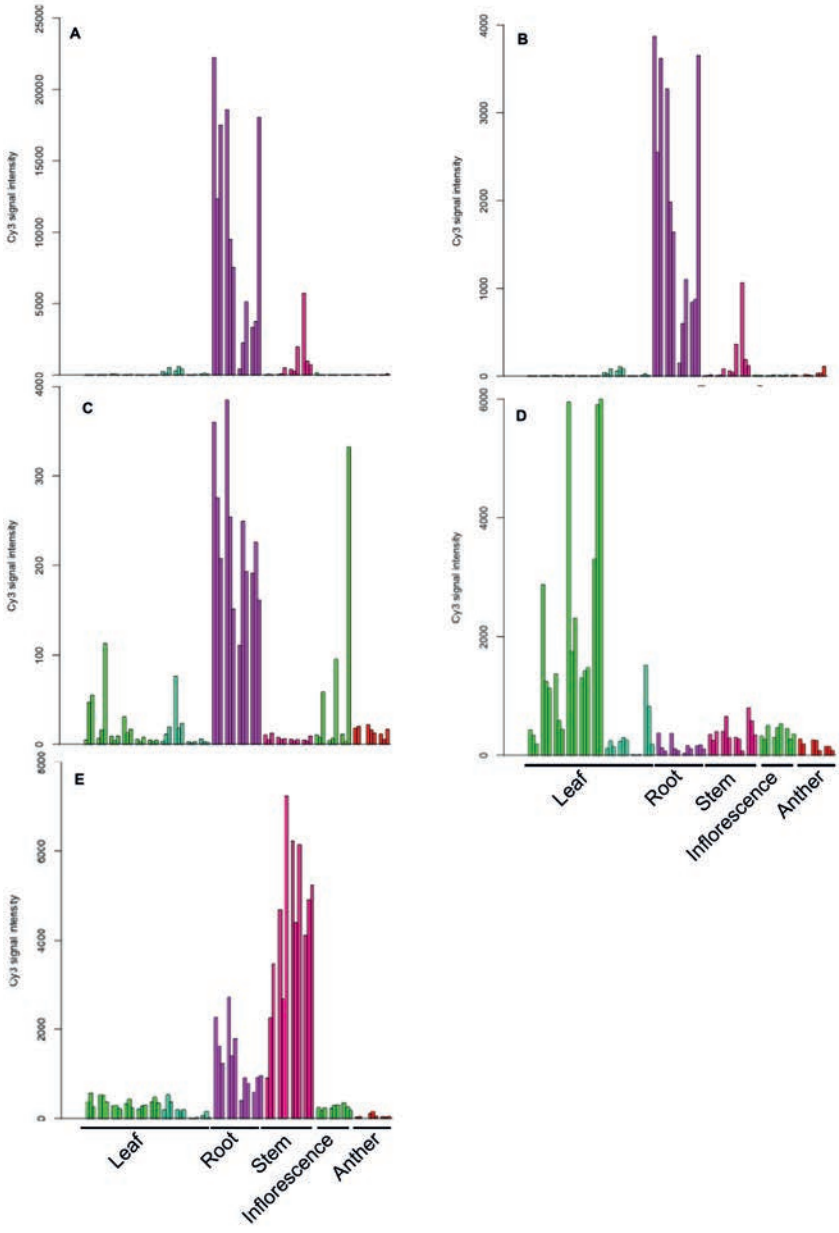

Figure 2. Expression profiles of members of the rice CYP711 sub-family (MAX1) (from the RiceXpro database, http://ricexpro.dna.affrc.go.jp/). **A**, the expression profile of *Os01g0700900*; **B**, the expression profile of *Os01g0701400*; **C**, the expression profile of *Os01g0701500*; **D**, the expression profile of *Os02g0221900*; E, the expression profile of *Os06g0565100*.

candidate *DWARF14* (*D14*), an α/β-hydrolase family member, provided more insight in the SL signal perception mechanism (Arite et al., 2009). Hamiaux et al. (2012) showed that petunia *DAD2*, a homolog of *D14* is likely to be involved in SL perception mediating the suppression of shoot branching by interacting with *PhMAX2A*, the *MAX2* ortholog in petunia (Hamiaux et al., 2012). The participation of D14-MAX2 in this mechanism was further elucidated by Zhou et al. (2013) and Jiang et al. (2014), demonstrating that the interaction of MAX2/D3 and D14 was promoted by the synthetic SL analogue GR24. Furthermore, a newly identified repressor of the SL signalling transduction pathway, DWARF53 (D53), was found to be degraded by the D14-SCF^{D3} complex leading to the activation of SL downstream signalling that results in the inhibition of shoot branching/tillering (Jiang et al., 2013; Zhou et al., 2013). The proposed model assumes that a SL binds the pocket of D14 forming a SL-D14 complex which can then be recognized by the SCF^{D3} complex to further interact with D53, leading to D53 ubiquitination and degradation by the 26s proteasome which results in the activation of the expression of SL target genes (Waters et al., 2012; Jiang et al., 2013; Zhou et al., 2013) (see **Figure 6** in **Chapter 1**).

Another striking feature of MAX2 is its participation in the signalling pathway of the smoke derived compound karrikin (**Figure 3**) (Nelson et al., 2011). Karrikin, sharing an essential butenolide moiety with the SLs, promotes *Arabidopsis* seed germination (Flematti et al., 2004). The study performed by Waters et al. (2012) implicates that MAX2 mediates *Arabidopsis* seed germination in response to karrikin through the interaction with KAI2, a paralogue of D14 also belonging to the α/β-hydrolase family. The *kai2* mutant shows a karrikin insensitive phenotype similar to that was observed for *max2* (Waters et al., 2012). Indeed, using crystallography and ligand-binding experiments, Guo et al. (2013) provided the direct biochemical evidence for KAI2 binding of karrikins (Guo et al., 2013). However, it is not yet clear how the KAI2-karrikin complex interacts with MAX2 to trigger the downstream signalling cascade of karrikin to regulate seed germination.

In addition, *Arabidopsis max2* mutants, in contrast to the SL biosynthetic mutants *max1*, *max3* and *max4*, has a dormant seed germination phenotype under continuous white light, implying that *MAX2* is involved in regulation of seed dormancy/seed germination (Nelson et al., 2011). However, the primary dormant (PD) seeds of *Arabidopsis* need 100-fold higher concentration of GR24 to trigger germination than of karrikins, although the response to both is mediated by *MAX2* (Nelson et al., 2009; Nelson et al., 2011). Thus, these *Arabidopsis* seed germination studies suggest that during PD seed germination, under optimal light conditions, MAX2 is involved more in karrikin than in SL signalling

MAX2 has been reported to regulate photomorphogenesis during *Arabidopsis*

seed germination and seedling development (**Figure 3**). The mutant of *MAX2/PPS* (*PLEIOTROPIC PHOTOSIGNALING*) was shown to have longer hypocotyls and smaller cotyledons under continues R (Red), FR (Far-Red) and B (Blue) light conditions compared with the wild-type, suggesting *MAX2* may play critical roles in R, FR, and B light-signalling pathways (Shen et al., 2007). In Chapter 5, describing the functional characterization of the *Striga MAX2* ortholog *ShMAX2*, we showed that *ShMAX2* only partially restores the high irradiance response (HIR) (continuous light after a red-light pulse) phenotype of *Arabidopsis max2* seedlings to the wild-type hypocotyl phenotype. Moreover, after a far-red light pulse followed by dark imbibition, *ShMAX2* was not able to significantly rescue the Very Low Fluence Response (VLFR) phenotype of *max2* seed germination compared with the *Arabidopsis* homologue *AtMAX2*. Interestingly, VLFR phenotype complementation of *max2* by *ShMAX2* could be enhanced by the supplementation of GR24 in a concentration dependent manner. These results suggest that *ShMAX2* is not able to respond to light properly, which would explain why *Striga* seed germination cannot be induced by light, but only by chemical signals, such as SLs. Both Nelson et al. (2011) and Shen et al. (2012) showed that except *max2*, all the SL biosynthetic mutants (*max1*, *max3* and *max4*) did not show any defects in seed germination and seedling phenotype under controlled light conditions, suggesting SLs are only weakly involved in photomorphogenesis (seed germination and seedling development) or the light-induced signalling pathway is can respond to the trace SL levels possibly remaining in the mutants (Nelson et al., 2011; Shen et al., 2012).

Although karrikins have been demonstrated to induce *Arabidopsis* seed germination under continuous light, karrikin treatment does not or hardly induce germination after a far-red (FR) pulse followed by dark condition, suggesting karrikin cannot mimic the light signalling pathway in seed germination (Nelson et al., 2009) . The light signalling pathway transcription factor *HY5* regulates photomorphogenesis in a MAX2 independent manner, but the *hy5* mutant displays sensitivity to both karrikin and SLs, suggesting that the HY5 dependent light pathway is distinct from both the karrikin and the SL pathway (Waters and Smith, 2013).

MAX2/ORE9, participating in the SCF^{MAX2} complex, was also suggested to regulate *Arabidopsis* leaf senescence by removing the target proteins that are required for delaying leaf senescence (Woo et al., 2001) (**Figure 3**). The SL biosynthetic genes *CCD7*, *DAD1/PhCCD8* were also demonstrated to modulate leaf senescence in *Lotus japonicus* and *Petunia hybrida*, respectively (Snowden et al., 2005; Liu et al., 2013). Therefore, it is likely that SLs are involved in the regulation of plant leaf senescence although the mechanism behind it is still undetermined.

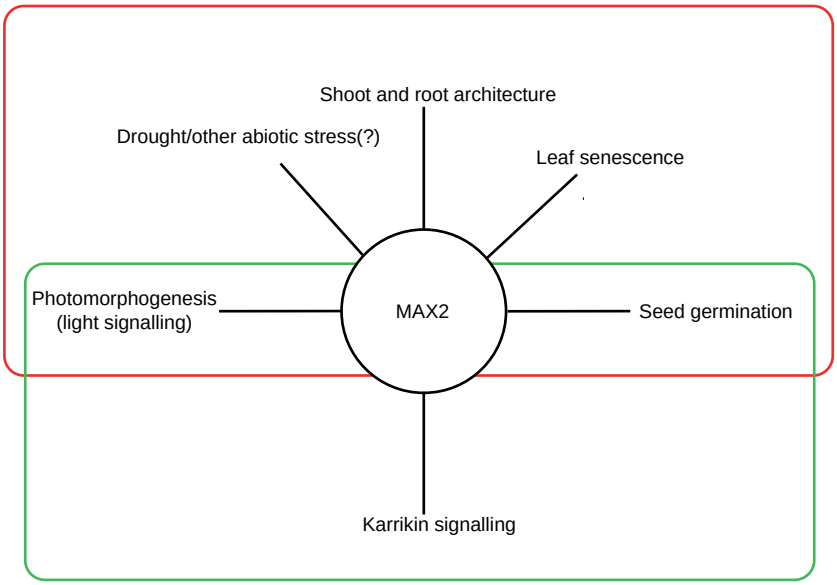

Figure 3. The multiple roles of MAX2. SL-related roles are in the red box; karrikin-related roles are in the green box.

More recently, *MAX2* was also demonstrated to play roles during the plants' response to drought and salt stress (Bu et al., 2014; Ha et al., 2014). The *Arabidopsis max2* mutant was reported to be hypersensitive to drought and salt stress compared with wild-type (Bu et al., 2014; Ha et al., 2014). Interestingly, in *Arabidopsis* adult plants, not only the *max2* mutant, but also the SL deficient mutants (*max3-11* and *max4-7*) showed hypersensitivity to drought and salt stresses, and exogenous application of GR24 rescued the drought sensitive phenotype of *max3-11* and *max4-7*, suggesting that SL is a positive regulator involved in these stress tolerance responses (Bu et al., 2014). Furthermore, the SL *max* mutants (both biosynthesis and signalling mutants) displayed slower stomatal closure than the wild-type plants in response to ABA treatment (Ha et al., 2014). However, during the seedling stage, only the SL signalling mutant *max2* plants showed hypersensitivity to exogenous ABA and osmotic stress (Bu et al., 2014). It is believed that the stress hormone ABA plays an important role in drought tolerance and also affects SL biosynthesis in plants (Iuchi et al., 2001; Lopez-Raez et al., 2010). The involvement of SLs in the drought stress response may act through the cross-talk with ABA but in a developmental stage specific manner. However, Haider et al. (2014) found perhaps a more direct connection between ABA and SLs. He reported that the rice *d27* mutant contains lower ABA levels and has much lower survival rates (upon resuming watering after drought) compared with other SL *d* mutants (biosynthetic as well as signalling mutants) and wild-type rice (Haider et al. 2014, PhD thesis), suggesting that *D27* is - in

addition to SL biosynthesis - also involved in ABA biosynthesis and hence plays a role in drought tolerance regulation.

In conclusion, F-box protein MAX2 within the SCF^{MAX2} complex has been proven to participate in a range of physiological processes in different plant developmental stages and under different environmental cues, suggesting that the SCF^{MAX2} has different targets to interact with to further initiate the various signalling pathways. Searching for the MAX2 targets (through for example yeast two hybrid screening, RNAseq or CoIP) could be an approach to understand more about this protein and its physiological effect. Whether or not all these MAX2 functions also involve SLs is an intriguing question.

What are the selection pressures for strigolactone biosynthesis?

Considering the negative consequence of the SLs mediated stimulation of the root parasitic plant seed germination, it seems not wise for plants to synthesize and secrete such signals into the rhizosphere. However, the presence of SLs in the rhizosphere also triggers hyphal branching of AM fungi which assist their host plants to improve nutrient uptake from a larger soil volume. Which came first, the evil root parasites or the positive AM fungi? AM fungi originated around 600 million years ago, which is millions of years before the colonization of land by plants some 400 million years ago (Corradi and Bonfante, 2012). AM fungi thus share about 400 million years of co-evolutionary history with land plants, forming a benefit for our crops when they are grown in infertile soils (Redecker et al., 2000; Bonfante and Genre, 2008). Although it is unclear whether the start of co-evolution of AM fungi and plants is associated with SLs or not, as mentioned above, SLs are also ancient signals produced by single-celled algae and basal land plants (liverworts and moss), to coordinate rhizoid growth and communications between cells (Proust et al., 2011; Delaux et al., 2012), which later evolved into the role of coordination of shoot and root growth in higher plants (Gomez-Roldan et al., 2008; Umehara et al., 2008; Ruyter-Spira et al., 2011). The major SLs in the legume plants *Lotus japonicus*, red clover and medicago are 5-deoxystrigol, orobanchol and didehydro-orobanchol, respectively, and both 5-deoxystrigol and orobanchol have high activity in AM fungal hyphal branching stimulation (Akiyama et al., 2010). This potentially stimulates AM-Rhizobium-plant tripartite symbiosis to help plants overcome deficiencies in both nitrogen and phosphorous. Although didehydro-orobanchol, only tentatively structurally elucidated, is not a well-characterised SL yet (see Figure 2 in Chapter 1), it would not be a surprise if this compound is also highly active in inducing AM fungal hyphal branching. The presence of these SLs could be the consequences of selection pressure, driving the synthesis of SLs that are positive for the establishment of AM symbiosis. During the evolution of root parasitism in plants, the root parasitic plants have evolved a mechanism to take advantage of the SL signals as a reliable host-presence signal that

stimulates their seed germination after which the parasite attacks the host plant to get water and nutrients. In Chapter 4, I proved that the rice *MAX1* homologue (*Os1400*) stereo-specifically converts *ent*-2'-*epi*-5DS to orobanchol, which is more active than other SLs in the stimulation of AM fungal hyphal branching tested by Akiyama et al. (2010) but much weaker (60% lower than the SL *ent*-2'-*epi*-5DS) in *S. hermonthica* seed germination induction (Akiyama et al., 2010; Nomura et al., 2013). It is intriguing to speculate that in order to escape from this biotic stress in the rhizosphere, higher plants could evolve new functions for *MAX1* to make different SLs with new (or altered) bio-functions.

From the side of the plant itself, although SLs were detected in moss to regulate protonema branching and filament growth of neighbouring colonies, the receptor of SLs *D14* is unique to seed plants (Gymnosperms and Angiosperms) (Proust et al., 2011; Delaux et al., 2012) (**Figure 1**). Zhou et al. (2013) and Jiang et al. (2014) both described the requirement of the interaction of MAX2 and D14 in suppressing the rice tillering phenotype (Jiang et al., 2013; Zhou et al., 2013). However, *D14-LIKE*, a paralogue of *D14*, required for the karrikin response during *Arabidopsis* seed germination, is already present in both algae and moss, which is earlier than the *D14* clade (Nelson et al., 2011; Delaux et al., 2012) (**Figure 1**). This may suggest that it is *D14-LIKE* that is playing an important role in the basal land plants' developmental process rather than *D14*. Interestingly, Waters et al. (2012) showed that *KAI2* also weakly mediated the GR24 response in *Arabidopsis* seedlings and proposed that *AtD14* and *KAI2* have functional redundancy in the *Arabidopsis* seeding stage, which may imply that they have similar ancestral activity but then diverged to obtain the specificity to the SL or karrikin response (Waters et al., 2012). Therefore, also the evolution of receptors might be a factor that affects the (co-)evolution of SL biosynthesis and structural diversification in plants.

Perspectives

SLs are very important phytohormones controlling plant architecture and rhizosphere signalling processes. The knowledge about how SL molecules are synthesised in plants and how SLs are perceived and transducing their signal, both in *planta* and in the rhizosphere (in parasitic plant seeds and AM fungi), is of great agronomic importance for crop breeding and root parasitic plant management. In this thesis I characterized the enzyme responsible for the B-C ring closure in SL biosynthesis and an enzyme involved in SL structural diversification. However, there are still many other SLs for which the biosynthetic enzymes have not been identified yet. Genetic mapping of SL related traits (such as shoot branching and parasitic plant seed germination) in different crops (with a different SL profile from rice) could be an approach to uncover new genes involved in SL biosynthesis and diversification processes. The knowledge gained from

these studies can be further applied in crop breeding to improve resistance against root parasitic plants, while at the same time not compromising other (positive) biological functions of the SLs. Furthermore, although the working model of SL regulating shoot branching has been partially unravelled by the interaction of MAX2/D3 with D14 and the repressor D53, the exact mechanism by which SLs bind to the receptor D14 and whether even more downstream targets are involved, still need to be determined. This will further help us understand more about how SL downstream signalling works and how SLs are interacting with other phytohormones in the regulation of plant architecture. In addition, comprehensive analysis of the genomic sequences and trancriptomics data of root parasitic plants and AM fungi will allow us to explore how the SLs are perceived by these two organisms. Also this knowledge could help in the improvement of crops and agricultural practices to avoid infection by parasitic plants but at the same time stimulate AM symbiosis.

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Summary

Strigolactones (SLs) are an important class of plant signalling molecules with in- and external functions, above- as well as belowground. In Chapter 1 I introduce the SLs, their structural diversity, biosynthesis and perception and their rhizosphere role as signalling molecules that stimulate hyphal branching in arbuscular mycorrhizal (AM) fungi, a process that is beneficial for the establishment of an efficient symbiosis between the fungus and its host. Unfortunately, the SLs also induce the germination of root parasitic plants, such as *Striga hermonthica*. As a survival strategy, seeds of these parasitic plants will only germinate when they perceive this germination signal from their host which is betraying its presence. After germination, the parasitic plants attach to the host root by forming a root invasive organ called haustorium, through which water and nutrients are taken up from the host. This causes tremendous yield losses in crops worldwide. Just quite recently it was discovered that the SLs are also newly identified phytohormones with multiple physiological roles in various plant developmental processes, such as the regulation of shoot branching and root architecture. This new role has tremendous implications for the evolution of parasitism and complicates control measures based on altered strigolactone production by crops.

Rice (*Oryza sativa*) is an important cereal crop supplying food to more than half of the world population. Rice also secretes SLs into the rhizosphere which is taken advantage of by *Striga*. Understanding the mechanism of how SLs are synthesised in the host plant rice and perceived by the parasitic plants is important for the management of root parasitic weeds in agriculture. The objective of my work was to gain more insight in the biosynthetic pathway of the SLs in the host plant rice and the molecular mechanism of signal perception in the parasite *Striga*. To achieve the first objective, I used a genetic approach to map quantitative trait loci (QTL) related to SL production using an F_6 recombinant inbred line (RIL) population of Bala x Azucena (Chapter 3). I showed that the susceptibility to *Striga* infection correlates with the SL levels in the host plant, with the lowest *Striga* emergence occurring with the low SL producing parent line Bala. A major QTL (qSLB1.1) for *Striga* germination, production of SLs *ent*-2'-*epi*-5-deoxystrigol (*ent*-2'-*epi*-5DS) and orobanchol and several other SL related traits was identified on rice chromosome 1. Subsequent molecular analysis of this QTL region revealed the presence of a rearrangement in the genome of Bala, causing a natural deletion of two rice cytochrome P450 (CYP) *MAX1* homologues, *Os01g0700900* (*Os900*) and *Os01g0701400* (*Os1400*). *Arabidopsis MAX1* has been reported to play a role in SL biosynthesis and in order to investigate whether this also holds for the rice *MAX1* homologs, we used a complementation approach and transformed *Arabidopsis max1* and Bala with these *MAX1* homologues (cloned from Nipponbare). Both genes rescued the branching phenotype of *Arabidopsis max1* and in Bala increased the level of

the SL, *ent*-2'-*epi*-5DS, confirming the strong association of SL production with these two rice *MAX1* homologues.

Subsequently, I characterized the biochemical function(s) of these two CYP proteins in SL biosynthesis by reconstitution of the SL biosynthetic pathway in *Nicotiana benthamiana* (Chapter 4). Previously, the biochemical evidence of the sequential involvement of *DWARF 27* (*D27*), *CAROTENOID CLEAVAGE DIOXYGENASE 7* and *-8* (*CCD7* and *CCD8*) in the biosynthesis of the SL precursor carlactone (CL) from all-*trans*-β-carotene had been provided. Transient overexpression of these genes in *N. benthamiana* leafs, resulted in the production of CL. Co-expression of the *MAX1* ortholog *Os900*, which was identified in the SL QTL mapping study, with this set of CL biosynthetic genes, resulted in the consumption of CL to form predominately the SL *ent*-2'-*epi*-5DS, suggesting that *Os900* is involved in the ring closure of CL to form SL. Intriguingly, the second rice *MAX1* homologue from the SL QTL region, *Os1400*, was found to be stereo-selectively converting *ent*-2'-*epi*-5DS to orobanchol. This is the first enzyme involved in SL structural diversification that is identified.

As SLs are secreted by host plants to the rhizosphere where they stimulate germination of root parasitic plant seeds, it is of great importance to study why parasitic plant seed germination largely depends on SL signalling and how these molecules act during this process. In Chapter 5, I show the conservation of the SL biosynthesis genes (*CCD7* and *CCD8*) in *Striga*. Although in our study we observed that the SL profile identified in *Striga* mirrors the SL profile of its hosts and is thus not conclusive in answering the question whether *Striga* produces SLs, fluridone treatment of *in vitro* grown *Striga* plants (without host) resulted in increased shoot branching, suggesting the inhibition of endogenous SL production. Subsequently, I characterized the homolog of the SL signalling component F-box protein MAX2 from *Striga*, ShMAX2. ShMAX2 showed the capacity to restore various characteristic *Arabidopsis max2* mutant phenotypes, including shoot branching, primary root length, high irradiance response (HIR) and seed germination upon GR24 application under optimal light conditions. However, ShMAX2 was not able to complement the Very Low Fluence Response (VLFR) of *max2* mutant seed germination. Together these results start to shed light on the question why *Striga* needs exogenous SLs for its seed germination.

Finally, I discussed several intriguing questions that are related to the main findings of this thesis, which are important for understanding the biosynthesis and signal transduction of SLs (Chapter 6). Taken together, as a breakthrough, this thesis provides the first scientific evidence of how the SL *ent*-2'-*epi*-5DS is formed and how this molecule is converted to orobanchol, representing the first identified SL diversification step in the host plant. Furthermore, this thesis identified the first SL signalling component,

ShMAX2, from a root parasitic plant species, which is paving the way for furthering our understanding of how SLs are perceived by the parasites. The knowledge gained can likely also be used to improve crop breeding or design for parasitic weed resistance.

Samenvatting

De strigolactonen vormen een belangrijke klasse van signaalmoleculen in planten met in- en uitwendige functies, boven- zowel als ondergronds. In Hoofdstuk 1 introduceer ik de strigolactonen, de verschillende structuren die ze kunnen hebben, hun biosynthese en detectie in planten. Ook beschrijf ik hun rol in de bodem als rhizosfeer signaalmoleculen, die het vertakken van zogenaamde arbusculaire mycorrhiza (AM) schimmels stimuleren, een proces dat belangrijk is om het ontstaan van symbiose tussen de plant en deze AM schimmels tot stand te brengen. Echter, de strigolactonen stimuleren naast deze goedaardige AM schimmels ook de kieming van wortel-parasitaire planten zoals *Striga hermonthica*. Zaden van deze parasitaire planten kiemen alleen als ze dit signaal waarnemen, dat de aanwezigheid van hun waardplant verraadt, wat een belangrijke overlevingsstrategie is voor deze obligate parasieten. Na kieming hechten ze zich aan de wortel van hun waardplant met behulp van een zogenaamd haustorium. Hiermee dringen ze binnen in het vaatweefsel van hun waardplant en ontnemen ze water, assimilaten en nutriënten van hun gastheer. Dit veroorzaakt wereldwijd enorme verliezen in vele verschillende gewassen. Nog maar kort geleden is ontdekt dat de strigolactonen, naast deze rol in de bodem, ook functioneren als (nieuw) plantenhormoon met een reeks van verschillende fysiologische effecten in planten, zoals de regulatie van scheutvertakking en wortelarchitectuur. Deze rol in de plant heeft enorme gevolgen voor ons denken over de evolutie van plant-parasitisme en zou ook grote implicaties kunnen hebben voor de mogelijke strategie om parasitaire planten te bestrijden door de productie van kiemstimulantia in gewassen te verminderen.

Rijst (*Oryza sativa*) is één van de belangrijkste graangewassen op aarde, dat voor meer dan de helft van de wereldbevolking de primaire bron van voedsel is. Rijst scheidt ook strigolactonen uit in de bodem, waar *Striga* gebruik van maakt om het gewas te kunnen parasiteren. Begrip van hoe strigolactonen worden gemaakt in rijst en hoe ze worden waargenomen in de parasiet is belangrijk voor een betere beheersing van deze wortel-parasitaire onkruiden in de landbouw. Het doel van mijn werk was dan ook om meer inzicht te verkrijgen in de biosynthese van strigolactonen in rijst en het moleculaire mechanisme waarmee *Striga* die strigolactonen waarneemt.

Om dat eerste doel te bereiken heb ik een genetische benadering gevolgd en heb ik in rijst zogenaamde quantitatieve trait loci (QTLs) bepaald voor de strigolactonproductie, en een aantal daaraan gerelateerde andere eigenschappen, met behulp van een F_6 recombinant inbred line (RIL) populatie ontwikkeld uit een kruising tussen Bala en Azucena (Hoofdstuk 3). In die studie laat ik zien dat de gevoeligheid voor *Striga* infectie correleert met de hoeveelheid strigolactonen, die wordt geproduceerd, met de laagste *Striga* opkomst in de laagst producerende ouderlijn Bala. De QTL mapping leverde een significante QTL op voor *Striga* kieming, productie van de strigolactonen,

ent-2'-*epi*-5-deoxystrigol (*ent*-2'-*epi*-5DS) en orobanchol, en een aantal andere aan strigolactonen gerelateerde eigenschappen, op één en dezelfde plek op chromosoom 1. Verdere analyse van deze QTL liet zien dat er op deze plek in het genoom van Bala een verandering is opgetreden waardoor twee cytochroom P450 genen zijn verdwenen, *Os01g0700900* (*Os900*) en *Os01g0701400* (*Os1400*). Dit zijn homologen van *MAX1* van Arabidopsis waarvan bekend is dat het een rol speelt bij de biosynthese van strigolactonen. Om de functionaliteit van de verdwenen rijst homologen te onderzoeken hebben we de Arabidopsis *max1* mutant en Bala getransformeerd met de verdwenen rijst homologen (die we gecloneerd hadden uit Nipponbare). Beide rijst *MAX1* homologen complementeerden het sterk vertakte fenotype van *max1* en verhoogden in Bala de productie van *ent*-2'-*epi*-5DS, wat bevestigt dat er een nauwe relatie is tussen deze twee rijst *MAX1* homologen en strigolacton productie.

Vervolgens heb ik de biochemische functie van deze twee cytochroom P450 enzymen in de strigolacton biosynthese bepaald door de hele strigolacton biosynthese route in *Nicotiana benthamiana* te reconstitueren (Hoofdstuk 4). Eerder was al bewezen dat *DWARF 27* (*D27*) en *CAROTENOID CLEAVAGE DIOXYGENASE 7* en *-8* (*CCD7* en *CCD8*) verantwoordelijk zijn voor de biosynthese van de strigolacton precursor carlacton (CL) uit *trans*-β-caroteen. Transiënte expressie van deze genen in *N. benthamiana* resulteerde inderdaad in de productie van CL. Als vervolgens het *MAX1* homoloog, *Os900*, dat we in de QTL mapping studie in Hoofdstuk 3 hadden gevonden, samen met de CL biosynthese genen tot expressie werd gebracht, verdween carlacton waarbij *ent*-2'-*epi*-5DS werd gevormd. Dit laat zien dat *Os900* betrokken is bij de ringsluiting van CL waarbij het eerste echte strigolacton wordt gevormd. De tweede *MAX1* homoloog die we in Hoofdstuk 3 hadden geïdentificeerd, *Os1400*, zette vervolgens *ent*-2'-*epi*-5DS stereo-specifiek om in orobanchol. Hiermee hebben we het eerste enzym geïdentificeerd dat is betrokken bij het creëren van de structurele variatie in de strigolactonen.

Omdat strigolactonen door de plant in de bodem worden uitgescheiden en daar de kieming van parasitaire-plantenzaden stimuleren is het belangrijk te weten hoe het kan dat de kieming van die parasitaire planten zo afhankelijk is van de strigolactonen en wat het mechanisme is van die stimulering. In Hoofdstuk 5 laat ik zien dat de strigolacton biosynthese genen *CCD7* en *CCD8* ook in *Striga* geconserveerd zijn. We vonden ook strigolactonen in volwassen *Striga* planten, maar deze bleken een afspiegeling te zijn van de strigolactonen aanwezig in de waardplant waarop de *Striga* groeide. Desalniettemin bleek behandeling met fluridon, een remmer van de strigolacton biosynthese, van *Striga* plantjes *in vitro* te resulteren in verhoogde scheutvertakking, wat suggereert dat die *Striga* plantjes strigolactonen maken. Vervolgens heb ik de perceptie van strigolactonen in *Striga* bestudeerd. Hiervoor heb ik het homologe gen van *Striga*,

ShMAX2, van het Arabidopsis F-box eiwit MAX2 – waarvan bekend is dat het een rol speelt bij de strigolacton signalering - gecloneerd. Dit *ShMAX2* heb ik gebruikt in een complementatie benadering door transformatie van *Arabidopsis max2*. *ShMAX2* kon diverse eigenschappen herstellen die verstoord waren in de Arabidopsis mutant, zoals vertakking, primaire wortellengte, de zogenaamde High Irradiance Response (HIR) en kieming onder invloed van het synthetische strigolacton, GR24, onder optimale lichtcondities. *ShMAX2* kon echter niet de Very Low Fluence Response (VLFR) van de kieming herstellen. Alles bij elkaar werpen deze resultaten licht op de vraag waarom *Striga* exogene strigolactonen nodig heeft om te kunnen kiemen.

Tenslotte bediscussieer ik in Hoofdstuk 6 een aantal intrigerende vraagstukken die samenhangen met de belangrijkste resultaten uit mijn proefschrift en die van belang zijn bij het begrijpen van de strigolacton biosynthese en signaaltransductie. Daarnaast bediscussieer ik de belangrijkste doorbraken van mijn werk en de bredere implicaties daarvan. Zo heb ik aangetoond hoe *ent*-2'-*epi*-5DS wordt gevormd en hoe het wordt omgezet in orobanchol, waarmee het eerste enzym betrokken bij de strigolacton diversificatie is geïdentificeerd. Daarnaast heb ik een belangrijke stap gezet om te begrijpen hoe parasitaire planten de strigolactonen van hun gastheer kunnen herkennen, door de karakterisering van *ShMAX2*. Ik bediscussieer hoe deze kennis kan worden gebruikt om de veredeling op resistentie tegen deze parasitaire onkruiden te verbeteren.

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I would like to express my sincere thanks to my friends, my present and former colleagues in the parasitic plant group (Xi, Beatriz, Yunmeng, Giovanni, Mahdere, Nasr, Andrea, Imran, Jamil and Catarina) and in Plant Physiology. All of you together build up such a nice atmosphere for doing research. I enjoyed a lot the fantastic Monday morning seminars, Thursday morning literature discussions and the " staff PhD meetings" with our staff (Harro, Henk, Sander, Dick, Wilco, Leónie and Richard). I would like to take the chance to show my appreciation to Imran. You were a very nice colleague. You were the first colleague I met when I arrived at the IBL in Leiden, and you showed me where the office was. However, the first time I was in Radix in Wageningen, I also met you! Such a coincidence! It was so nice to have you as a colleague both in Leiden and Wageningen. My nice neighbour and friend Rik, thanks for your help with my computer problems. Thanks Bea and Karen, for organizing great workshop for our PhD fellows to make a lot fun. I am very grateful to Karen for being my paranymph to support me during my defense. Phuong and Maria-Cecilia it is so nice to have you as friend here to share a lot of fun in the life. Jimmy, you and Ya-Fen are such nice friends and colleagues, and you guided me a lot from personal life in Wageningen to lab work when I just started my PhD study. Catarina and Qing, I so much appreciate to have collaborated with both of you on my research from which I benefit a lot. Many thanks to my students Esti, Marta and Mahdere (who is a colleague now) for your hard work during your MSc study with

me and for giving me the chance to learn how to be a supervisor.

To my nice and lovely friends from the Chinese community in Wageningen (Hui, Hanzi, Xi, Junwei, Bo, Yuanyuan, Yunmeng, Bing, Jun, Xiaohua, Chen Shuangchen, Jianhua, Yanli, Zhen Wei, Guiling, Tingting, Chunting, Wei Liu, Wei Song, Qing, Ting, Huchen, Defeng, Tian Zeng, Zhu Feng, Du Yu, Wang Yan, Guozhi, Xu Cheng, Fengjiao, Zhao Chao and all other Chinese friends that I forgot to mention here), I have spent a lot of pleasant time with all of you, and enjoyed a lot of delicious Chinese food, great parties and events. Hui, we have been friends since our master study in Beijing. I enjoyed a lot the many great activities (traveling, cook together and chatting about our future and ideals….) that we did together during our PhD life in Wageningen. Hanzi, Xi and Bo, it is my luck to meet all of you here in Wageningen, to have you as sweet friends in my life and also as my great colleagues….So many evenings we spent our "dinner" time together in the coffee corner and encouraged each other. And Bo also thanks for your support as my paranymph during my defense. All of these will be the memories for life.

To my family, I express my deepest gratitude. To my parents, you are the most wonderful parents in my heart. Thousands of words and sentences, but I cannot choose any of them to express my emotions to both of you. I truly and deeply appreciate your endless love, understanding and supporting, even sacrifice (我由衷地感谢我的父母。你们在我心里是最伟大的父母。我心里有千言万语，但是都不能表达我对你们的情感。我只想说一句感谢你们一直对我无尽的爱，理解和甚至牺牲自我的支持。). Many thanks to my dear brother, for these years, you have been supporting me and taking care of our parents when I was far away from home although I am the elder sister. To my parents-in-law and sister-in-law, i am very grateful your understanding, caring, support and love, and they have been encouraging me over these years (致我的公公婆婆及姐姐，我非常感谢你们这几年来对我的理解，关心，支持和爱护，这些爱和支持一直鼓励着我). My dear husband, Song Jun, you always told me your belief is our love, and I am so appreciated and touched. In my life, you are not only my husband, but also my soul mate and best friend. During these years, you shared all my feelings, pain and happiness. Your love, constant understanding and support encouraged me when I was down. I am so lucky and appreciated to have you in my life.

Finally, I would like to sincerely acknowledge my funding organization China Scholarship Council (CSC) for offering me the scholarship to be in Netherlands to pursue my PhD degree.

Yanxia
2nd August, 2014
Wageningen

About the author

Curriculum Vitae

Yanxia Zhang was born in Langfang city, People's Republic of China, on 17th April 1982. In 2002, she graduated from high school and started her 4-year bachelor study on biology in Jiamusi University, China. During her four years study on biology, including courses on animal science, microbiology and plant science, she started to show her interests towards plant science. In 2006, she obtained her bachelor degree and got the scholarship for studying her MSc in Beijing Forestry University, China. She studied at the major of Botany, on the project of "plant secondary metabolism under abiotic stress" under the supervision of Prof. dr. Yujun Liu. During her MSc study, due to her outstanding performance, she got the "Excellent MSc student" awards in 2007. In 2009, she obtained her Master's degree in Botany and got the scholarship from the China Scholarship Council (CSC) for supporting her PhD study in the Netherlands. At the beginning of March, 2010, Yanxia started her PhD study in the Laboratory of Plant Physiology under the supervision of Prof. dr. Harro Bouwmeester and Dr. Carolien Ruyter-Spira, focusing on the research topic "Strigolactone biosynthesis in the host plant rice and signal perception in the parasitic plant *Striga hermonthica*". During her PhD study, she got involved in several scientific collaborations and achieved a few publications (see the publication list). She handed in her PhD thesis in 2014. From March, 2014, she started as post-doc researcher in the same group in the field of strigolactone perception.

Publication list

Publications related to this thesis

Zhang Y*, Haider I*, Ruyter-Spira C, Bouwmeester HJ (2013) Strigolactone biosynthesis and Biology.In: Bruijn FJd (ed) ***Molecular Microbial Ecology of the Rhizosphere***. John Wiley& Sons, Inc., Honboken, NJ, USA, Vol.1, pp 355-371

Liu Q*, Zhang Y*, Matusova R, Charnikhova T, Amini M, Jamil M, Fernandez-Aparicio M, Huang K, Timko MP, Westwood JH, Ruyter-Spira C, van der Krol S. Bouwmeester HJ (2014) *Striga hermonthica MAX2* restores branching but not the Very Low Fluence Response in the *Arabidopsis thaliana max2* mutant. ***New Phytologist*** 202: 531-541

Cardoso C*, Zhang Y*, Jamil M*, Hepworth J*, Charnikhova T, Dimkpa SON, Meharg C, Wright MH, Liu JW, Meng XB, Wang YH, Li JY, McCouch SR, Leyser O, Price AH, Bouwmeester HJ, Ruyter-Spira C (2014) Natural variation of rice strigolactone biosynthesis is associated with the deletion of two *MAX1* orthologs. ***Proceedings of the National Academy of Sciences of the United States of America*** 111: 2379

Zhang Y, van Dijk ADJ, Scaffidi A, Flematti GR, Hofmann M, Charnikhova T, Verstappen F, Hepworth J, van der Krol S, Leyser O, Smith SM, Zwanenburg B, Al-Babili S, Ruyter-Spira C, Bouwmeester HJ (2014) Rice cytochrome P450 *MAX1* homologs catalyse distinct steps in strigolactone biosynthesis. ***Nature Chemical Biology*** (In press).

Other publications

Hu Y, Sun LW, Mokgolodi N.C, Zhang Y, Wen CX, Xie XL, Liu YJ (2010) Primary identifications and palynological observations of *Perilla* in China. ***Journal of Systematics and Evolution*** 48 (2): 133–145

Zhang HT*, Hedhili S*, Montiel G*, Zhang Y*, Chatel G, Pre M, Gantet P, Memelink J (2011) The basic helix-loop-helix transcription factor CrMYC2 controls the jasmonate-responsive expression of the ORCA genes that regulate alkaloid biosynthesis in *Catharanthus Roseus*. ***The Plant Journal*** 67: 61–71

Shi L, Wang C, Zhou X, Zhang Y, Liu YJ, Ma C (2013) Production of salidroside and tyrosol in cell suspension cultures of *Rhodiola crenulata*. ***Plant Cell, Tissue Organ Culture*** 114: 295–303

***Shared first authorship.**

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: Yanxia Zhang
Date: 15 September 2014
Group: Plant Physiology, Wageningen University & Research Centre

1) Start-up phase		*date*
►	**First presentation of your project**	
	Elucidation of strigolactone production and detection in the parasitic plants and their hosts	Mar 10, 2010
►	**Writing or rewriting a project proposal**	
	Elucidation of strigolactone production and detection in the parasitic plants and their hosts	Jun10, 2010
►	**Writing a review or book chapter**	
	Strigolactone biology and biosynthesis, IN: 'Molecular Microbial Ecology of the Rhizosphere', Vol. 1, (Ed. Frans J. de Bruijn), 2013, pp 355-371. John Wiley & Sons, Inc.	Mar 2012
►	**MSc courses**	
	MOB-30806 Regulation of plant development	Mar-Apr 2011
►	**Laboratory use of isotopes**	
	Subtotal Start-up Phase	***16.5 credits****

2) Scientific Exposure		date
►	**EPS PhD student days**	
	EPS PhD student day, Leiden University, NL	Feb 26, 2009
	EPS PhD student day, University of Amsterdam, NL	Nov 30, 2012
►	**EPS theme symposia**	
	EPS theme 3 symposium 'Metabolism and Adaptation', Utrecht University, NL	Feb 18, 2009
	EPS theme 1 symposium 'Developmental Biology of Plants', Leiden University, NL	Jan 20, 2011
	EPS theme 1 symposium 'Developmental Biology of Plants', Wageningen University, NL	Jan 19, 2012
	EPS theme 3 symposium 'Metabolism and Adaptation', Wageningen University, NL	Mar 11, 2014
►	**NWO Lunteren days and other National Platforms**	
	ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 06-07, 2009
	ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 19-20, 2010
	ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 04-05, 2011
	ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 02-03, 2012
	ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 14-15, 2014

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►	**Seminars (series), workshops and symposia**	
	Mini-symposium"how to write a world-class article"	Oct 26, 2010
	Invited seminar: Exploring roots-selective root placement in nutrient rich hotpots	Dec 08, 2010
	CBSG Technology symposium	Nov 25, 2010
	Symposia "parasitic plants and strigolactones more than rhizosphere communication"	Nov 07, 2011
	Invited seminars (Jennifer McEwain, Steffen Abel, Pierre Hilson, Jill Farrant, Ruth Finkelstein, Aaron Fait)	Jan-Dec 2012
	Invited seminar Wim van den Ende	Dec 13, 2013
	Invited seminars (Daniel van Damme, Dani Zamir, Theo Ruissen)	Jan-Jun 2014
	I-plant workshop	Mar 03-04, 2014
►	**Seminar plus**	
	Discussion with Steffen Abel after the seminar	Mar 16, 2012
	Discussion with Theo Ruissen after seminar	Jun 04, 2014
►	International symposia and congresses	
	European retreat for PhD students in Experimental Plant Sciences, Cologne, Germany	Apr 15-17, 2010
	Terpnet, Kalmar, Sweden	May 22-26, 2011
	ICAR Arabidopsis Conference, Vienna, Austria	Jul 03-07, 2012
	3rd international conference on "Plant metabolism", Xiamen, China	Jul 02-05, 2014
►	**Presentations**	
	EPS summer school 'Rhizosphere Signalling' (oral)	Aug 24, 2010
	Terpnet, Kalmar Sweden (poster)	May 22-26, 2011
	EPS theme 3 symposium (oral)	Mar 11, 2014
	3rd international conference on 'Plant metabolism' (poster)	Jul 02-05, 2014
►	**IAB interview**	
	Meeting with a member of the International Advisory Board	Jan 21, 2011
►	**Excursions**	

Subtotal Scientific Exposure ***17.2 credits****

3) In-Depth Studies		date
►	**EPS courses or other PhD courses**	
	EPS summer school 'Rhizosphere Signalling'	Aug 22-24, 2010
	PhD workshop 'Plant Metabolics'	Apr 2011
	EPS summer school 'Enviromental Signalling'	Aug 22-24, 2011
	Postgraduate course 'Microscopy and Spectroscopy in Food and Plant Sciences'	May 07-11, 2012
	EMBO-EBI Practical Course 'Plant Bioinformatics: Going -OMICs'	Jun 10-19, 2012

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►	**Journal club**	
	Plant physiology Journal club	2010-2013
►	**Individual research training**	

Subtotal In-Depth Studies ***9.9 credits****

4) Personal development		date
►	**Skill training courses**	
	Acdamic writing I	Sep 2010-Jan 2011
	Techniques for writing and presenting a scientific paper	Sep 06-09, 2011
	Acdamic writing II	Sep 2011-Feb 2012
	PhD scientific writing	Apr-Jun 2012
	PhD competence assessment	Sep 2011
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	

Subtotal Personal Development ***8.1 credits****

TOTAL NUMBER OF CREDIT POINTS*	**51.7**

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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Cover image: Strigolactone (5-deoxystrigol) structure; rice; flower of *Striga hermonthica*

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Liu Fahui (lowen695@hotmail.com)

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