Biosynthesis of germination stimulants of parasitic weeds *Striga* and *Orobanche*

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Contents

Chapter 1

General introduction

Introduction adapted from Sun, Z., R. Matusova and H. Bouwmeester, 2007. Germination of *Striga* and chemical signaling involved: A target for control methods. In: Integrating new technologies for *Striga* control: Towards ending the witch-hunt, J. Gressel and G. Ejeta, Eds., World Scientific Publishing Co., Singapore, 47-60 and Bouwmeester, H.J., R. Matusova, Sun Zhongkui and M.H. Beale, 2003. Secondary metabolite signalling in host-parasitic plant interactions. Current Opinion in Plant Biology 6: 358-364

Parasitic plants

Parasitic plants are plants that obtain their resources (assimilates, water, nutrients) partly to even completely from another (host) plant. Parasitism in plants has evolved many times and hence parasitic plants show great phenotypic plasticity. Parasitic plants have a wide environmental tolerance and are represented by about 4100 species in approximately 19 families of flowering plants (Nickrent and Musselman 2004; Press and Phoenix 2005). They can be classified into two main types depending on the presence or absence of chlorophyll. Holoparasites do not contain chlorophyll and depend completely on their host for the supply of assimilates. Hemiparasites do contain (some) chlorophyll and can perform photosynthesis to some extent. Some of these hemiparasites can live either as a parasite or on their own roots, and these are called facultative parasites (Joel et al. 1995). Parasitic plants can attach to their host on several different organs. Mistletoes, for example, attach to branches of trees. Root parasites are parasitic plants that attach to the roots of their host. They belong to a number of families and can be non-photosynthetic or photosynthetic. The broomrapes (*Orobanchaceae*), for example the genera *Orobanche*, *Conopholis*, and *Boschniakia* have non-photosynthetic fleshy stems and therefore belong to the holoparasites. Other root holoparasites are found in the families *Lennoaceae, Hydnoraceae*, *Rafflesiaceae* (for example, Mitrostemon matudae and Bdallophyton americanum) and *Balanophoraceae*. Parasitic *Striga* spp. (also belonging to the *Orobanchaceae*), *Santalaceae* (the sandalwood family) and *Krameriaceae* (the *ratony* family) have photosynthetic leaves and thus belong to the hemiparasites. Nevertheless, a substantial portion of these parasites' carbon is derived from the host plant that is parasitized via its roots. The most economically important families are the *Orobanchaceae* (Broomrapes). This family includes the largest number of genera (85) and species (ca. 1650) of all the families of parasitic flowering plants (Nickrent and Musselman 2004). The genera *Striga* (witchweeds) (Fig. 1, Appendix Chapter 1 Fig. 1 in colour) and *Orobanche* (broomrapes) (Fig. 1, Appendix Chapter 1 Fig. 1 in colour), which cause the most crop damage, are described below:

Striga

The genus *Striga* (Orobanchaceae) is one of the most agriculturally important pests. Of the 28 *Striga* spp., *S. asiatica*, *S. aspera*, *S. forbesii* and particularly *S. hermonthica* (Fig. 1) parasitise cereal crops such as sorghum, maize, millet and rice (Oswald 2005; Parker and Riches 1993). Another species *Striga gesnerioides* can infect dicotyledonous crops such as cowpea (Dube and Olivier 2001). Although these parasites are able to develop green leaves and have intact chloroplast genomes (Thalouarn et al. 1991),

they exhibit only low rates of photosynthesis (Shah et al. 1987) and therefore they need to rob most of organic matter from their hosts (Press and Graves 1995). Moreover these parasitic plants exhibit high rates of transpiration and consequently they obtain a large amount of water and minerals from their hosts (Musselman 1980). These facts result in the parasites causing yield losses from a few percent to complete crop failure depending on crop species, crop variety and severity of infection (Parker and Riches 1993). It has been estimated that about two-thirds of the 70 million hectares used for cereal production in Africa are infested by *Striga*. Hence, *Striga* negatively affects the lives of some 300 million people (Cherfas 2002; Kim et al. 2002; Press et al. 2001) and is considered by many experts to be the greatest obstacle to food production in Africa, particularly in the Sahel region. *Striga* spp. are also distributed throughout other tropical and subtropical regions, such as south Asia, but there do not pose a serious threat to agriculture (Joel et al. 1995).

Striga hermonthica on rice *Orobanche ramosa on tomato*

Fig. 1 Pictures of *Striga* (photograph by Zhongkui Sun) and *Orobanche* (photograph by Radoslava)

Orobanche

Other notorious parasitic plants are the holoparasitic angiosperms of the genus *Orobanche* (Orobanchaceae). Of the more than 100 *Orobanche* spp., only *Orobanche crenata*, *Orobanche ramosa* (Fig. 1), *Orobanche cumana* and *Orobanche aegyptiaca* parasitise agricultural crops (Bouwmeester et al. 2003). They have a broad host range, for example, they affect major crops such as legumes, solanaceous crops (eggplant, tomato, tobacco, potato but not Capsicum peppers), umbelliferous crops (carrot, parsley, celery), Brassicaceae (cabbage, lettuce and cauliflower) and sunflower (Joel 2000; Nickrent and Musselman 2004; Press et al. 2001). *O. ramosa* can parasitize plants from eleven different dicot families, which is more than any other broomrape, and these include hosts of great economic importance such as cabbage, rapeseed, tomato, cauliflower, hemp, carrots, lettuce and some legumes (Nickrent and Musselman 2004). Unlike the *Striga* spp., the *Orobanche* spp. obtain all the assimilates they need from the host because the leaves lack the ability of photosynthesis due to deletions and rearrangements in the chloroplast genome (dePamphilis and Palmer 1990). In addition, *Orobanche* spp. do not develop normal functioning roots (Musselman 1980). Damage to crops by *Orobanche* spp. is common in warm and dry areas, such as the Middle East, India and large parts of Europe and North America (Joel et al. 1995; Press et al. 2001; Verkleij and Kuiper 2000), with yield losses ranging up to complete crop failure depending on the level of infestation (Foy et al. 1989). It has been estimated that the *Orobanche* spp. affect over a million hectare of agricultural land (Verkleij and Kuiper 2000).

Life cycle and chemical signalling between parasite and host

Although *Striga* and *Orobanche* species parasitise different hosts in different parts of the world, their lifecycles are broadly similar. Hence we discuss the two genera together. During the lifecycle of the parasite, several steps have been shown or suggested to be regulated or affected by signalling molecules that are exchanged between the parasite and its host (Fig. 2). The chemical stimuli that are initiating the lifecycle are called germination stimulants, are secreted by the host root and trigger the germination of the seeds of the parasites (Bouwmeester et al. 2003; Press and Graves 1995). However, before the parasite seeds can respond to these stimuli and germinate they require a pre-treatment at a suitable temperature under moist conditions, a treatment known as preconditioning (Joel et al. 1995) or warm stratification (Matusova et al. 2004). During preconditioning, metabolic pathways in the seed are activated, for example, respiration and synthesis of DNA, protein and hormones (Ejeta 2005; Joel et al. 1995). After the conditioning period, the parasite seeds will germinate only if exposed to sufficiently high concentrations of germination stimulants hence assuring that germination only occurs in close vicinity of the host root. The adaptation of parasites to respond to these germination stimulants is of evolutionary significance: their tiny seeds contain limited reserves and the seedlings will die within a number of days after germination unless a host root is invaded (Butler 1995). When the seeds have germinated, the radicle must grow towards the host root and this process is possibly directed by the concentration gradients of germination stimulants (Dube and Olivier 2001). The host root has also been reported to produce other

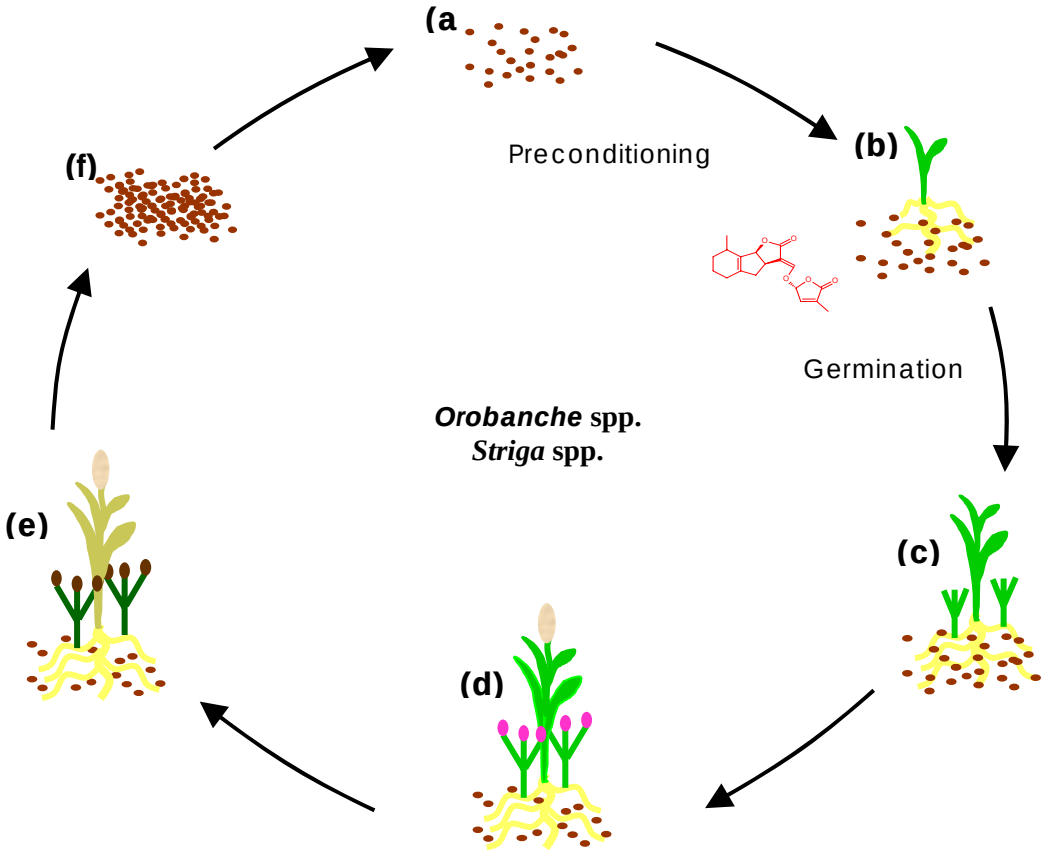

Fig. 2 Life cycle of *Striga spp*. (a) the seeds are buried in the soil; (b) they become sensitive to the germination stimulants exuded by the roots of the host plant and may germinate; (c) the germinated seeds form a haustorium by which they attach themselves to the host root, establish a xylem connection and emerge; (d) parasitic plants flower; (e) they produce mature seeds and end up in a new generation of seeds in soil; (f) in the next season the cycle starts again (a). From Matusova and Bouwmeester, 2006.

secondary metabolites, for example, as yet unidentified compounds that inhibit germination or the elongation of the radicle (Joel et al. 1995; Khan et al. 2002). When the radicle reaches the host root, the attachment and penetration of the host root are achieved by the formation of a special organ called haustorium. The haustorium penetrates the host and connects the vascular system of the host with that of the parasite. The formation of this organ requires other host-derived chemical signals to initiate and guide this developmental transition. The nature and role of these haustorium-inducing factors have been reviewed (Estabrook and Yoder 1998; Tomilov et al. 2006; Tomilov et al. 2005; Yoder 2001). Following the establishment of the connection with the host, the parasite will develop a so-called tubercle that helps to accumulate nutrients. At a certain stage it forms a shoot, emerges above the soil, flowers and produces seeds after which the lifecycle can start again (Fig. 2).

Germination stimulants

Origin and distribution

A number of different classes of secondary metabolites have been described to have germination stimulant activity: dihydrosorgoleone, the strigolactones and the sesquiterpene lactones. Lynn and coworkers have suggested that dihydrosorgoleone is the active stimulant in the root exudates of sorghum and other monocotyledonous hosts [Keyes et al., 2001]. However, it has been suggested that dihydrosorgoleone is less likely to be the germination stimulant in vivo (Butler 1995; Zwanenburg and Thuring 1997) and we have shown that in our bioassay germination stimulant formation of sorghum is almost completely dependent on carotenoid biosynthesis (which excludes that dihydrosorgoleone is the stimulant) (Matusova et al. 2005). Also the capacity of sesquiterpene lactones - that share some structural features with the strigolactones - to induce germination of *Striga asiatica* has been reported (Fischer et al. 1989). Macías and coworkers (Galindo et al. 2002; Pérez de Luque et al. 2000) demonstrated that several sesquiterpene lactones induced the germination of the sunflower parasite *O. cumana*, but not of *O. crenata* and *O. ramosa*, at concentrations as low as 10^{-7} M. Interestingly, *O. cumana* is a specialist parasite of sunflower, and sunflower is known to contain large amounts of sesquiterpene lactones (Bouwmeester et al., 2003).

The best explored class of germination stimulants so far are the strigolactones. Up to now seven natural strigolactone germination stimulants were isolated and characterized (Fig. 3). The first germination stimulant strigol was isolated from the non-host cotton (Cook et al. 1972). Later on, strigol was also detected in the exudates of genuine hosts such as maize, and proso millet and in lower amounts in sorghum (Awad et al. 2006; Siame et al. 1993). Alectrol (now renamed to orobanchyl acetate) together with orobanchol were isolated from cowpea and the *Orobanche* host : red clover and soybean (Muller et al. 1992; Xie et al. 2007; Yokota et al. 1998) Sorgolactone was isolated from sorghum (Hauck et al. 1992) and recently an isomer of strigol, named sorghumol, was also detected in sorghum cultivars (Awad et al. 2006) but the structure of this compound has not yet been elucidated. 5-Deoxystrigol is the major strigolactone present in the root exudates of maize, proso millet and sorghum (Awad et al., 2006). Surprisingly, sorgolactone was not present in the sorghum cultivars examined by these authors. Recently, several existing but also new strigolactones were detected in the exudates of tomato, tobacco, spinach and white lupin by Yoneyama and coworkers (Xie et al. 2006; Yoneyama et al. 2006; Yoneyama et al. 2004) suggesting that the strigolactones are a class of secondary metabolizes that are produced by many different plant species. Of one of these, solanacol, recently the structure was elucidated, showing that it

had an aromatic A-ring (Fig. 3) (Xie et al., 2007). A valuable tool in the research on germination of parasitic plant seeds is GR24, a synthetic germination stimulant, which is used worldwide in parasitic weed research to stimulate parasitic weed seed germination (Fig. 3) (Wigchert et al. 1999).

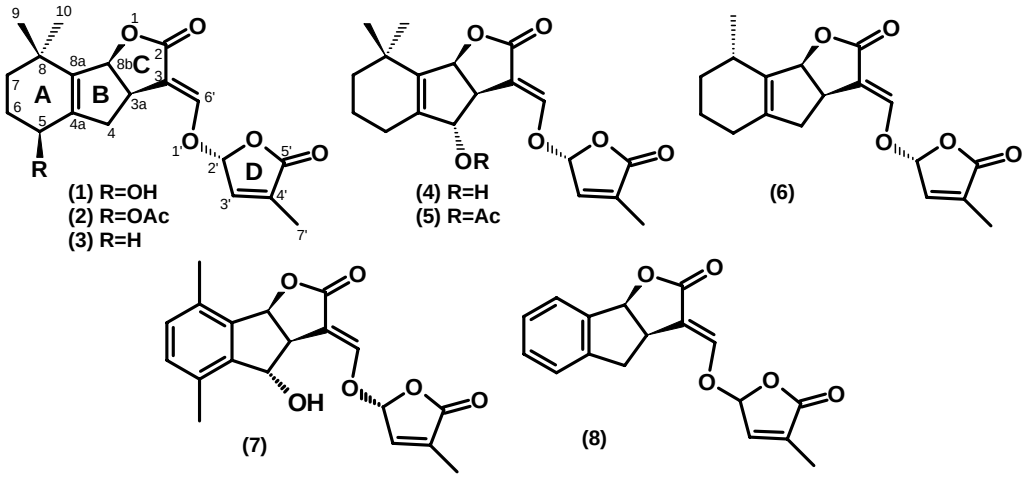

Fig. 3 Structures of strigolactones: strigol (1), strigyl acetate (2), 5-deoxystrigol (3), orobanchol (4), orobanchyl acetate (5), sorgolactone (6), solanacol (7), the synthetic germination stimulant GR24 (8).

Perception of germination stimulants

The availability of the synthetic germination stimulant GR24, of which in principle relatively large concentrations can be used, seems to have obscured the relevance of dormancy in parasitic plant seeds. In many cases, a standard preconditioning treatment is used to break dormancy and to study the response to GR24. However, if lower concentrations of GR24 or natural germination stimulants are used, it becomes suddenly clear that the conditions during preconditioning strongly affect the responsiveness of the seeds to the stimulants (Matusova et al. 2004; Van Hezewijk et al. 1993). Preconditioning at an optimal temperature (e.g., about 30°C for *S. hermonthica* and 21°C for *O. ramosa*) releases dormancy within 2-3 weeks and increases the sensitivity to GR24 by several orders of magnitude. After reaching maximum sensitivity, prolonged preconditioning induces secondary dormancy, i.e., decreases sensitivity to GR24 (Matusova et al. 2004). It is important to note that the rapid changes in sensitivity during prolonged preconditioning are only (or particularly) visible at low concentrations of GR24. At higher concentrations, GR24 almost always induces high germination, regardless of the preconditioning period.

Parasitic plant seeds are highly sensitive to the germination stimulant for a short period of time only, and then enter into secondary dormancy relatively quickly. These large changes in sensitivity to germination stimulants are suggestive of a safety mechanism that ensures that seeds can respond to the germination stimulants produced by their host only during a restricted period of the year. This is of ecological importance for the parasite as it requires a long enough period of time to grow and reproduce, and germination during the later stages of host development would not allow this.

Implication in host specifity

The survival of the parasite fully depends on its ability to detect the presence of a host plant. Therefore the parasitic plants have developed a mechanism to recognize host exuded chemical signals to ensure that the roots of the host are in close vicinity. However, even though the recognition mechanism at the

germination stage is most crucial (point of no return), it seems it is not fully specific. The induction of germination of parasitic plant seeds by non-host plants is the most obvious example of this non-specific recognition. In addition, orobanchyl acetate (originally called alectrol) was identified in cowpea as a germination stimulant for *S. gesneroides* (Muller et al. 1992), but has also been identified in red clover and soybean as a germination stimulant for *O. minor* and *O. crenata* (Xie et al. 2007; Yokota et al. 1998). Also the fact that germination of *Striga* and *Orobanche* seeds can be induced by the synthetic germination stimulant GR24, regardless of the parasitic plant species or its host history, is not suggestive of a strong specificity during the germination phase. On the other hand, there are several indications that the composition of root exudates indeed plays a role in determining host specificity during the germination phase. For example, *Striga* seeds collected from maize and sorghum fields respond differentially to the germination stimulants present in the root exudates of maize (host), cowpea (nonhost) and GR24 (Matusova and Bouwmeester 2006). *Striga* seeds collected from maize responded well to the maize root exudate (36% germination), the cowpea root exudate (51% germination) and 0.001 $mg.l^{-1}$ GR24 (44% germination). In contrast, *Striga* seeds collected from sorghum germinated to 37% in response to the maize root exudate, to only 22% in response to the cowpea exudate and to 49% in response to 0.001 $mg.^{-1}$ of GR24. *Striga* seeds collected from sorghum field responded to maize and cowpea root exudates by very low germination (15 and 14%, respectively), even though germination in 0.001 $mg.l^{-1}$ GR24 was as high as 28%. The slightly different response of the two sorghum-collected *Striga* batches could be due to the fact that different sorghum varieties can have different root exudate compositions (Awad et al. 2006). The novel evidence for the presence of several different strigolactones in root exudates of e.g. sorghum and tomato (Yoneyama et al. 2004), and the presence of the same strigolactones (e.g. strigol, 5-deoxystrigol, orobanchol) in the exudates of several other different species may help (but also makes it more difficult) to unravel the recognition of the germination stimulants by parasitic plants and mechanism of host plant selectivity at the germination stage.

Ecological significance of strigolactones

A puzzling question that was asked from the first discovery of the strigolactones being germination stimulants of parasitic plants onward until recently was why plants produce these signalling molecules when they induce germination of one of their worst enemies. In 2005, Akiyama and co-workers showed that the strigolactones are required by AM fungi for their host root colonisation process (Akiyama et al. 2005). Most agricultural plants (including the majority of plants that are hosts of *Orobanche* and *Striga spp*) engage in symbiosis with arbuscular mycorrhizal fungi (AM fungi). The benefits of AM fungi and the possibilities to exploit them to improve (sustainable) agriculture have been discussed by several authors (see (Johansson et al. 2004) for review) and particularly on poor soils the benefits of AM fungi and their possible role to improve agriculture have been suggested (Bagayoko et al. 2000; Gworgwor and Weber 2003). Interestingly, several groups have reported that AM fungi can also reduce the infection of crops such as sorghum and maize by *Striga* (Gworgwor and Weber 2003; Lendzemo and Kuyper 2001; Lendzemo et al. 2005). The extent of the effect seems to depend on mycorrhizal species (Gworgwor and Weber 2003) and host variety (Lendzemo and Kuyper 2001; Lendzemo et al. 2006). Possible explanations for this are that host plants with improved nutritional status are more resistant to parasitic plants or that AM fungi induce defense gene expression in their host, which makes the host more resistant to parasitic plants (Sun et al. 2007). However, the exact mechanism is still unknown.

Biosynthetic origin of germination stimulants

The germination stimulants are exuded from the roots of host plants in extremely low concentrations and are often instable which makes the isolation and characterization of these compounds quite difficult (Sato et al. 2005). For the same reasons, the study of the biosynthesis of these compounds is also difficult. However, by using the germination of *Striga* or *Orobanche* seeds as the most sensitive method available for the detection of strigolactones, we could analyse the production of germination stimulants by single plants (Matusova et al. 2005; Sun et al. 2007). Using this system we analysed germination stimulant production from single maize plants treated with specific inhibitors of the isoprenoid biosynthetic

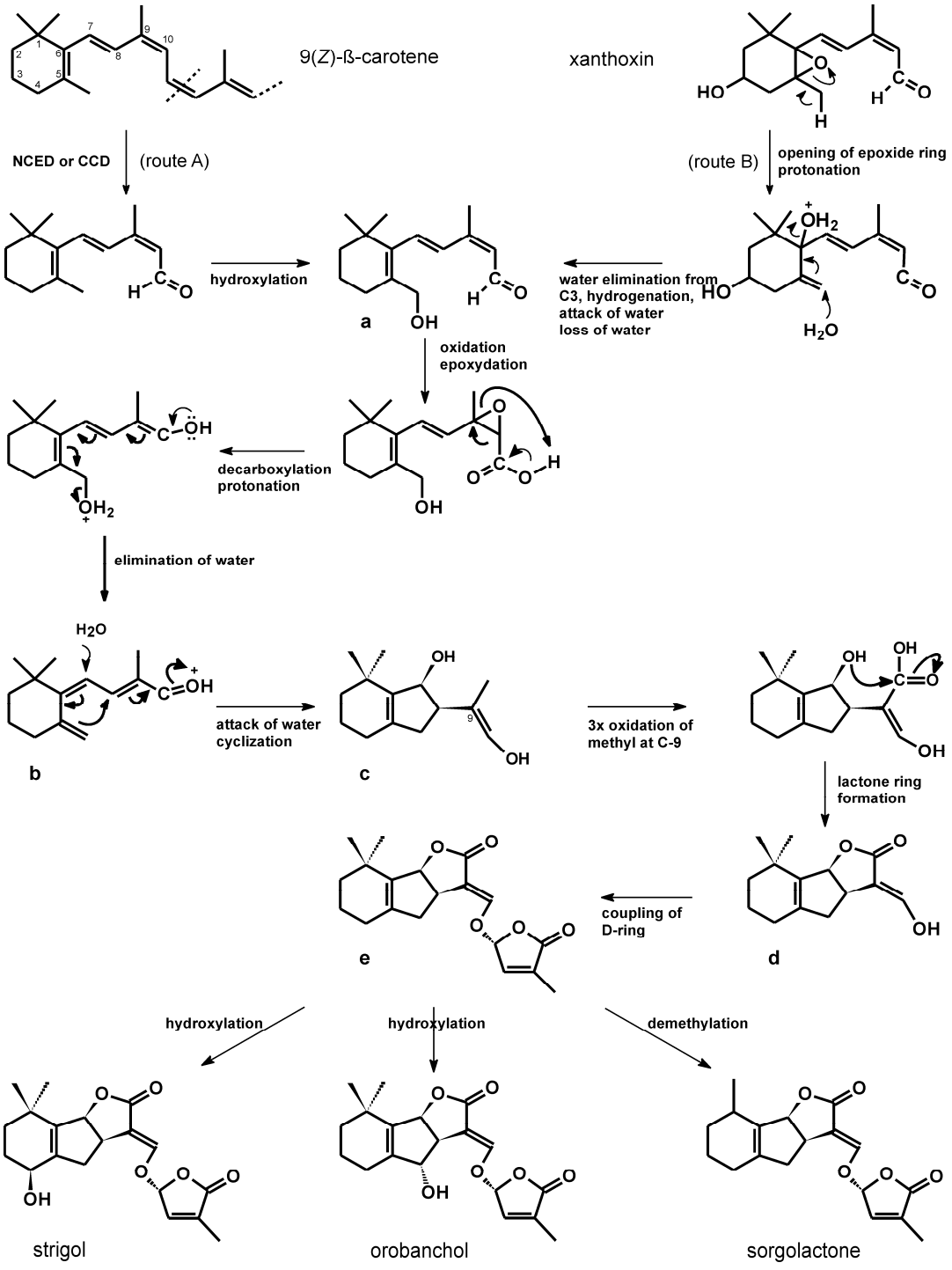

Fig. 4 Postulated biosynthetic pathway of germination stimulants (from Matusova et al 2005)

pathway. The carotenoid-pathway inhibitors fluridone and amitrole reduced maize root-exudate-induced germination by about 80% compared with control seedlings, showing that the germination stimulants produced by maize are derived from the carotenoid pathway (Fig. 4) (Matusova et al. 2005). This conclusion was confirmed by the use of mutants. A series of carotenoid mutants of maize (obtained from the Maize Genetics COOP Stock Center, Urbana, Illinois) induced lower germination of *Striga* seeds than their corresponding wild-type phenotype siblings (Matusova et al. 2005). Also the root exudates of fluridone-treated cowpea induced about 80% less germination of *Striga* than those of non-treated cowpea and fluridone treatment of sorghum seedlings almost completely blocked subsequent exudate-induced germination of *Striga* seeds (Matusova et al. 2005). These results show that the germination stimulant(s) of *Striga* exuded from the roots of cowpea and sorghum is (are) also derived from the carotenoid pathway which fits with the identification of the strigolactones: sorgolactone, 5-deoxystrigol, sorghumol and alectrol (now renamed to orobanchyl acetate) in the exudates of these two plant species (Awad et al. 2006; Hauck et al. 1992; Kamioka et al. 2006; Muller et al. 1992).

The exact position in the carotenoid pathway where strigolactone biosynthesis branches off from the main pathway has not yet been identified. However, it is clear that carotenoid cleavage is involved in germination-stimulant biosynthesis. Carotenoids are C40 molecules whereas the strigolactones are C14 compounds (excluding the D-ring) (Matusova et al. 2005). Carotenoid cleavage is a common biosynthetic reaction that occurs in a number of biosynthetic pathways, for example leading to the production of volatile flavour/fragrance molecules such as ß-ionone (Simkin et al. 2004). However, also the production of important plant signalling molecules proceeds through the action of carotenoid cleavage enzymes, such as the formation of the plant hormone abscisic acid through the action of 9-cis-epoxycarotenoid cleavage dioxygenases (Seo and Koshiba 2002) and the formation of a new, as yet unidentified, plant hormone involved in the control of plant architecture, through the action of two carotenoid cleavage dioxygenases (Booker et al. 2004; Schwartz et al. 2004). We have also postulated how, after carotenoid cleavage, further enzymatic conversions likely leads to the production of all strigolactones known to date (Fig. 4) (Matusova et al. 2005).

Control of parasitic plants

The tremendous impact of parasitic plants on world agriculture has prompted much research aimed at preventing infestation. Many potential control methods were developed against the parasite problem including physical, cultural, chemical and biological (Joel 2000). So far these methods however have only had a limited impact on controlling the menace of the parasites and today there is no single control method that can effectively solve this problem (Ejeta 2005; Joel 2000; Kleifeld and Goldwasser 2004; Oswald 2005).

Potentially more effective methods can be developed if these are based on detailed knowledge of the host plant - parasite interaction. Control methods that affect parasite seed germination are expected to be more effective than those affecting later stage of development because they prevent parasitism prior to crop damage and could also reduce the seed bank in the case of overexpression of the germination stimulants, for example with trap crops (Joel et al. 1995). A number of currently existing strategies to limit *Striga* and *Orobanche* damage are already based on the germination stimulants (Fig. 5) (Sun et al. 2007).

Suicidal germination using chemicals. There has been a great interest in using the germination stimulants as a method for control of parasitic plants in a cropping situation and the introduction of a germinating

agent before a crop is planted can potentially reduce parasite populations via suicidal germination (Eplee 1992; Joel et al. 1990; Zwanenburg and Thuring 1997). Work on synthetic germination stimulants in the group of B. Zwanenburg has led to the development of molecules that have potential as parasitic weed control agents through the induction of suicidal germination (Mwakaboko 2003; Wigchert and Zwanenburg 1999). Limitations of this approach are that the synthetic stimulants should be very cheap for farmers in the Third World being able to buy them and that application of the chemicals to sufficient depth in the soil requires suitable equipment and possibly large amounts of water.

Trap and catch crops. Another control strategy, based on suicidal germination is the use of trap and catch crops in monoculture or in intercropping. Usually, these non-host species produce germination stimulants, sometimes in high amounts, and hence induce massive germination of the parasite, but they are resistant in a later stage of the parasite's lifecycle (trap crops) or harvested before the seeds of the parasite are shed (catch crops) (Chittapur et al. 2001; Khan et al. 2002; Ross et al. 2004). The efficacy of catch and trap crops could possibly be increased if overproduction of germination stimulants can be achieved through selection or molecular breeding. The latter can be achieved by overexpression of one or more (rate-limiting) genes from the strigolactone biosynthetic pathway (see below under Breeding). This can be done in catch and trap crop species but possibly also in host crops, if it is proven that very high germination stimulant production induces germination (only) at sufficient distance from the root, so that the radicle of the germinating seed can not reach the host root. In addition, overexpression of strigolactone formation could possibly improve mycorrhizal colonisation efficiency and hence benefit the crop even more.

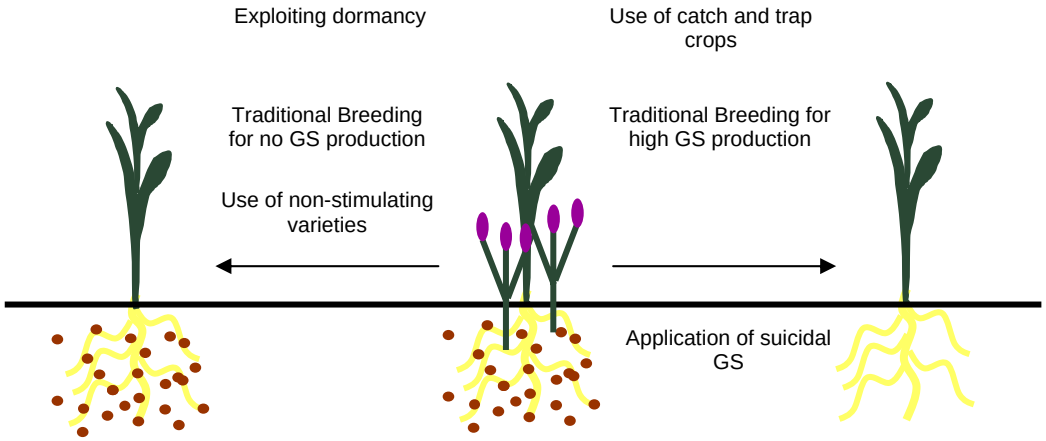

Fig. 5 Diagram showing existing control methods based on the knowledge about germination stimulants.

Dormancy. As described above dormancy of parasitic plant seeds is released during preconditioning and induced again upon prolonged preconditioning. For the parasites this is a safety mechanism restricting germination to a suitable period of the year (and/or suitable phase of crop development), but possibly this phenomenon can also be used to control parasitic weeds. Indeed, there are several publications showing that a later crop sowing date strongly reduces infection by parasitic plants, for example of sunflower by *O. cumana* (Eizenberg et al. 2003) and a 10-day delay in sowing of sorghum considerably reduced emergence of *Striga* on sorghum in Mali (A. van Ast, personal communication). Although there is no direct proof that this is due to the induction of dormancy (= a decrease in sensitivity to germination stimulants), it seems worthwhile to investigate whether this plays a role in the positive effect of delayed

sowing and whether it could be developed into a control strategy, if the availability of water does not exclude the possibility to delay sowing.

Through selection. In sorghum a selection program for low-germination stimulant formation has resulted in low-stimulant sorghum varieties with improved resistance (or decreased sensitivity) to *Striga* (Mohamed et al. 2001). Also variation in resistance to *O. crenata* due to low induction of parasite seed germination has been found in wild species of *Cicer* (chickpea) (Rubiales et al. 2003). The authors postulate this may provide a new source of resistance for cultivated chickpea.

Host specifity. As described above there is ample evidence that the composition of the mixture of germination stimulants that is exuded may vary between different crop species as well as between varieties of one crop species. Detailed knowledge about the germination stimulant composition in the exudates of a crop variety to be sown and the effect of this on germination of a certain field population of parasite may help to choose or design (through breeding) a crop (variety) or combination of crops (varieties), for example in rotation, with the aim to reduce germination of parasite seeds from the local seed bank or to exhaust the seed bank as quickly as possible. Choosing the right varieties of one crop based on this knowledge may be useful in areas where a broad rotation of different crops is not possible.

Objective and approaches of this thesis

The objective of this thesis is to gain further insight into the regulation of the formation of the germination stimulants of the parasitic plants *Striga* and *Orobanche*. To achieve this goal, several host plant – parasite models were used. We started with the interaction between maize and *Striga* and tried to understand the regulation of germination stimulant formation. Based on the earlier work in our group described above, potential biosynthetic genes were cloned from maize and characterized by in vitro expression in *E. coli* or in transgenic maize plants. Because maize proved to be not the most suitable model to study *Striga* germination stimulant biosynthesis, the interaction of S*triga* with rice was studied to see whether rice could be used as a model in the elucidation of strigolactone biosynthesis. In addition, the germination stimulant and its biosynthetic origin in *Arabidopsis* were uncovered. Based on the knowledge collected in this thesis work, we anticipate that new and more efficient control methods will become available.

Outline of the thesis

Following the above introduction, Chapter 2 describes the cloning and characterization of a maize carotenoid cleavage dioxygenase gene (*ZmCCD1*). Higher expression of this gene was observed in maize roots colonised by AM fungi which at the same time produced less germination stimulants. In Chapter 3, a cluster of carotenoid cleavage dioxygenase enzymes (belonging to the 9-*cis*-epoxycarotenoid dioxygenases or NCED cluster) was silenced in maize using RNAi constructs that were transformed using particle bombardment. Two generations of transgenic maize plants were characterized at the molecular level and by using a germination bioassay. To further study the germination stimulants and elucidate their biosynthetic origin also the model plant *Arabidopsis* was used. Chapter 4 focuses on the germination stimulants from *Arabidopsis*. In Chapter 5, the germination stimulants from the monocot model plant rice and its biosynthetic origin are discussed. In Chapter 6, all the results are discussed in a broader context including the possible implication of the study for *Striga* and *Orobanche* management.

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Chapter 2

Cloning and characterisation of a maize carotenoid cleavage dioxygenase (*ZmCCD1*) and its involvement in the biosynthesis of apocarotenoids with various roles in mutualistic and parasitic interactions

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Abstract

Colonisation of maize roots by arbuscular mycorrhizal (AM) fungi leads to accumulation of apocarotenoids (cyclohexenone and mycorradicin derivatives). Other root apocarotenoids (strigolactones) are involved in signalling during early steps of the AM symbiosis but also in stimulation of germination of parasitic plant seeds. Both apocarotenoid classes are predicted to originate from cleavage of a carotenoid substrate by a carotenoid cleavage dioxgenase (CCD), but the precursors and cleavage enzymes are unknown. A *Zea mays CCD (ZmCCD1)* was cloned by RT-PCR and characterised by expression in carotenoid accumulating *E. coli* strains and analysis of cleavage products using GC-MS. ZmCCD1 efficiently cleaves carotenoids at the 9,10 position and displays 78% amino acid identity to *Arabidopsis thaliana* CCD1 having similar properties. *ZmCCD1* transcript levels were shown to be elevated upon root colonisation by AM fungi using northern blot analysis. Mycorrhization led to a decrease in seed germination of the parasitic plant *Striga hermonthica* as examined in a bioassay. *ZmCCD1* is proposed to be involved in cyclohexenone and mycorradicin formation in mycorrhizal maize roots but not in strigolactone formation. Possibly, cyclohexenone and mycorradicin formation competes with other carotenoid cleavage enzymes for common carotenoid precursors, hence reducing the formation of strigolactones and consequently parasitic plant seed germination.

Key words

Apocarotenoids, arbuscular mycorrhizal fungi, carotenoid cleavage dioxygenase, germination stimulants, maize, *Striga* spp.

Abbreviations

ABA	abscisic acid
AM fungi	arbuscular mycorrhizal fungi
DXR	deoxyxylulose-5-phosphate reductoisomerase
NCED	9-cis-epoxycarotenoid dioxygenase
ZmCCD1	*Zea mays* carotenoid cleavage dioxygenase 1

Introduction

Carotenoid cleavage dioxygenases (CCDs) and 9-cis-epoxycarotenoid dioxygenases (NCEDs) constitute a family of enzymes that catalyse the cleavage of carotenoids at specific double bonds. The cleavage products are collectively called apocarotenoids (Auldridge et al. 2006b; Schwartz et al. 2001). The first carotenoid cleaving enzyme (*Vp14)* was isolated from the abscisic acid (ABA) deficient viviparous maize mutant. Vp14 is an NCED and catalyses the rate-limiting step in ABA (also an apocarotenoid) biosynthesis, the cleavage of the 9-*cis*-isomer of neoxanthin or violaxanthin at the 11,12 position (Schwartz et al. 2001; Tan et al. 1997). Based on the sequence homology to *Vp14*, nine *CCDs* have been identified in the *Arabidopsis thaliana* genome. Among them, five are *Vp14*-like (*NCED2*, *NCED3*, *NCED5*, *NCED6* and *NCED9*) and are supposed to be involved in ABA biosynthesis (Tan et al. 2003). The other four have been given the generic designation carotenoid cleavage dioxygenase (*CCD1*, *CCD4*, *CCD7* and *CCD8*) (Auldridge et al. 2006a). *Arabidopsis* CCD1 (AtCCD1) symmetrically cleaves multiple *trans*-carotenoid substrates (ß-carotene, lutein, zeaxanthin, trans-violaxanthin) at the 9,10 and 9',10' double bonds producing a C_{14} dialdehyde and two C_{13} products that vary depending on the carotenoid substrate (Schwartz et al. 2001). Although AtCCD7 cleaves at the same position as AtCCD1, AtCCD7 was shown to cleave lycopene, ß-carotene, ζ-carotene and zeaxanthin asymmetrically and therefore produces a C_{13} and a C_{27} product (Schwartz et al. 2004). This C_{27} apocarotenoid can be further catabolised by AtCCD8 yielding a C_{18} apocarotenoid (Schwartz et al. 2004). The function and enzymatic activities of AtCCD4 so far remain unknown but the ortholog in chrysanthemum (*Chrysanthemum morifolium* Ramat.), *CmCCD4a*, showed specific expression only in white petals which led to the conclusion that CmCCD4a cleaves carotenoids into, as yet unidentified, colourless compounds (Ohmiya et al. 2006).

Orthologous enzymes of AtCCDs have been reported in many other plant species and usually have the same cleavage activity as their *Arabidopsis* counterpart. For example, orthologous enzymes of AtCCD1 have been found in *Crocus sativus*, *Lycopersicon esculentum*, *Petunia hybrida*, *Vitis vinifera* and *Cucumis melo* and all cleave several carotenoid substrates at the 9,10 and 9',10' double bonds (Bouvier et al. 2003b; Ibdah et al. 2006; Mathieu et al. 2005; Simkin et al. 2004a; Simkin et al. 2004b). However, from some plant species CCDs were cloned that cleave carotenoid substrates at positions different from the *Arabidopsis* CCDs. For example, Bouvier and colleagues cloned *BoLCD* from *Bixa orellana* and demonstrated that the enzyme catalyses cleavage of lycopene at the 5,6 position (Bouvier et al. 2003a) and *CsZCD* from *Crocus sativus* that catalyses cleavage of zeaxanthin at the 7,8 position (Bouvier et al. 2003b).

Apocarotenoids resulting from the oxidative cleavage of carotenoids serve as important signalling molecules in a variety of biological processes. The plant hormone ABA has long been known to be involved in regulating plant responses to various environmental stresses, especially drought and salinity

and also in long-distance signalling within the plant (Davies et al. 2005). Furthermore, a novel, unidentified, apocarotenoid phytohormone that regulates plant lateral shoot branching was recently postulated to be produced from an as yet unidentified carotenoid substrate by sequential cleavage by CCD7 and CCD8 (Beveridge 2006; Booker et al. 2004; Schwartz et al. 2004; Zou et al. 2006). In addition to being plant hormones, some of the apocarotenoids (such as ß-ionone, ß-cyclocitral, geranial, geranial acetone, theaspirone, α-damascenone and ß-damascenone) contribute to the flavour and/or aroma of flowers or fruits of a variety of agricultural products (Auldridge et al. 2006b; Winterhalter and Rouseff 2001). For example, AtCCD1 can cleave ß-carotene to produce the C_{13} derivative ß-ionone, an important fragrance compound in the flowers of many plant species (Schwartz et al. 2001).

The derivatives of the apocarotenoids mycorradicin (an acyclic C_{14} polyene) and a C_{13} cyclohexenone accumulate during colonisation of roots by arbuscular mycorrhizal (AM) fungi, causing the yellow colour of maize roots that are colonised by AM fungi (Fester et al. 2002; Klingner et al. 1995). These apocarotenoids are predicted to originate from an unknown C_{40} carotenoid precursor by cleavage at positions 9,10 and 9',10' by an unknown carotenoid cleavage enzyme (Fester et al. 2002; Strack and Fester 2006; Walter et al. 2000) (Fig. 1). During AM colonisation, carotenoid biosynthesis is upregulated in the roots of several plant species suggesting that the apocarotenoids play some important role in the symbiosis (Fester et al. 2002; Fester et al. 2005; Strack and Fester 2006; Walter et al. 2007).

Another class of interesting apocarotenoids are the strigolactones (Fig.1), which form a separate group of structurally closely related molecules (Bouwmeester et al. 2003). Strigolactones are germination stimulants of the root parasitic *Striga* spp. and *Orobanche* spp., obligate parasitic plants that can only survive and reproduce when attached to the root of a host plant from which they obtain water, nutrients and assimilates. The seeds of these parasitic plants will only germinate in the presence of these germination stimulants (Bouwmeester et al. 2003; Musselman 1980; Parker and Riches 1993; Press et al. 2001). Interestingly, strigolactones also induce branching in germinating AM fungal spores, a process required for host root colonisation (Akiyama et al. 2005; Besserer et al. 2006). Recently we have demonstrated that the strigolactones are derived from the carotenoid pathway probably with the involvement of a carotenoid cleaving enzyme (Matusova et al. 2005). In addition to this common signalling molecule, AM fungi and parasitic plants have another relationship. In pot and field trials it was demonstrated that enhanced colonisation with AM fungi can reduce *Striga* infection in maize and sorghum (Gworgwor and Weber 2003; Lendzemo and Kuyper 2001; Lendzemo et al. 2007).

In recent years, large progress was made with the characterisation of plant CCD enzymes and their apocarotenoid products (Auldridge et al. 2006a; Auldridge et al. 2006b; Bouvier et al. 2003a; Bouvier et al. 2005; Bouvier et al. 2003b; Ibdah et al. 2006; Mathieu et al. 2005; Schwartz et al. 2004; Schwartz et al. 2001; Schwartz et al. 1997; Simkin et al. 2004a). However, the biosynthetic origin of some biologically important apocarotenoids is still unknown. For example, although it is very likely that the "yellow pigment" formed in AM colonised roots is derived from carotenoids by oxidative cleavage, neither the carotenoid precursor nor the cleavage enzyme are known. The same holds for the strigolactones. In this study, we have cloned and characterized the maize CCD1 that is responsible for the 9,10 cleavage of carotenoids. We provide arguments for its involvement in the formation of the "yellow pigment" apocarotenoids and discuss its possible relation to the formation of strigolactones.

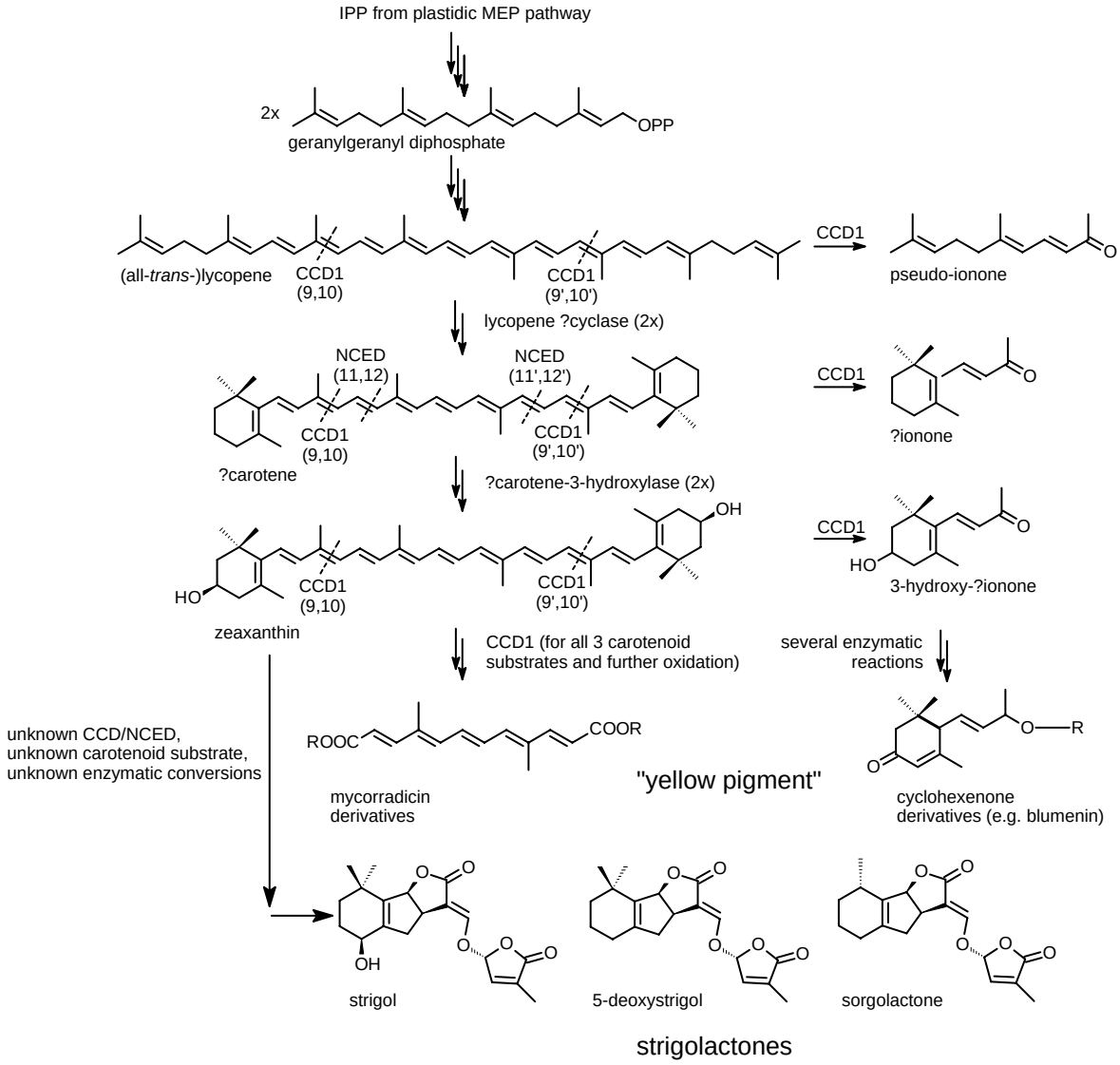


Fig. 1 Schematic overview of reactions catalysed by ZmCCD1. The three carotenoid substrates tested (lycopene, ß-carotene, and zeaxanthin) were cleaved by ZmCCD1 yielding: pseudo-ionone (6,10-dimethyl-3,5,9-undecatrien-2-one), β-ionone (9-apo-β-caroten-9-one) and 3-hydroxy-β-ionone (3-hydroxy-9-apo-β-caroten-9-one), respectively. In mycorrhizal roots ZmCCD1 is involved in the formation of the "yellow pigment", consisting of cyclohexenone and mycorradicin derivatives, from an as yet unknown carotenoid precursor (Strack and Fester 2006). Also strigolactones, of which three examples are shown, are derived from carotenoids by an unknown route (Matusova et al. 2005).

Materials and methods

Plant materials and chemicals

Maize (*Zea mays* L.) seeds of cultivar MBS 847 (Dent type) were washed with 70% ethanol for 10 minutes. Then the seeds were washed with 25 ml of 2% sodium hypochlorite with 0.02% (v/v) Tween-20 for 30 minutes. Subsequently the seeds were washed four times using sterilized tap water. Finally the seeds were pre-germinated for two days in a dark climate room at 25°C in a Petri dish with moist filter paper. Maize plants were then grown in 1-L plastic containers containing perlite under greenhouse conditions with a day/night temperature of 25ºC (16 h) / 18ºC (8h). The plants were only given tap water.

After two weeks, roots and leaves were harvested separately and ground under liquid nitrogen for total RNA extraction.

To assess the effect of mycorrhizal colonisation of maize on the germination of *Striga hermonthica* (Del.) Benth., maize plants of cv MBS 847 (Dent type) were grown under the same conditions as described above but in expanded clay (Lecaton, 2–5 μm particle size; Fibo Exclay, Pinneberg, Germany). The expanded clay consisted of 1 part expanded clay on which leek plants (*Allium porrum* L.) inoculated with the AM fungus *Glomus intraradices* had been growing, and two parts of clean expanded clay. Plants were watered with half-strength Hoagland's solution containing 1/10 of the normal phosphate concentration. Four plants for each treatment were carefully removed from the expanded clay at 14, 21, 28 and 34 days after inoculation and root exudates were collected from each single plant separately for 24 hours in demineralised water. Rates of colonisation by AM fungi were estimated by staining roots with trypan blue (Maier et al. 1995). The exudates were diluted to the same concentration of g root fresh weight per ml root exudate and induction of *S. hermonthica* germination was assessed. Before the germination bioassay, the *S. hermonthica* seeds were preconditioned. Hereto, seeds were surface-sterilized in 2% sodium hypochlorite containing 0.1 % Tween 20 for 5 minutes. Then seeds were rinsed three times with sterile demineralised water, and excess water was removed by filtration through a Büchner funnel. The sterile seeds were allowed to air dry for 2 hours and subsequently approximately 50-100 seeds were placed on 9-mm diameter glass filter paper (Sartorius, Germany) discs. Twelve discs were placed in 9-cm diameter Petri-dishes with a filter paper (Whatman, UK) moistened with 3 ml demineralised water. The Petri dishes were sealed with parafilm and wrapped in aluminium foil and placed in a growth chamber at 30ºC for 10 days. Before applying root exudates, the discs of the seeds were dried on sterile filter paper for 3 minutes and transferred to a new Petri dish with a 1-cm wide filter paper ring (outer diameter of 9 cm), moistened with 1 ml sterile demi water to keep a moist environment inside the Petri dish. Fifty μl of the root exudates to be tested were applied to triplicate discs. The synthetic strigolactone analogue GR24 (0.1 $mg.l^{-1}$) was used as a positive control and sterile demineralised water as a negative control in each germination assay. Seeds were then incubated at 30ºC in darkness for 2 days. After 2 days, germination was assessed using a binocular microscope. Seeds were considered to be germinated if the radical protruded through the seed coat.

For Northern blot analysis, maize (cv dwarf-1) was grown in expanded clay in 250-ml plastic pots under a 16-h light/8-h dark regime in a growth chamber at 25 ºC. Plants were fertilised once a week using Long Ashton nutrient solution with 1/10 of the original phosphate content. Inoculation with AM fungi was done as described before (Hans et al. 2004).

The *Striga* seeds used in the experiments were collected from a *S. hermonthica* population growing on maize in Kibos, Kenya in 1994. They were kindly provided by Vicky Child of Long Ashton Research Station, Bristol, UK. Seeds of maize inbred line Dent MBS 847 were obtained from J.C.Robinson Seeds, Ottersum, The Netherlands. The synthetic germination stimulant GR24 was provided by Prof. B. Zwanenburg, Radboud University, Nijmegen, The Netherlands. Ampicillin and chloramphenicol were from Duchefa, Haarlem, The Netherlands. Pentane and diethylether were from Biosolve, Valkenswaard, The Netherlands. All other chemicals were from Sigma-Aldrich, Zwijndrecht, The Netherlands unless otherwise specified.

Statistical analysis

Germination data were transformed by taking the arcsine of the square root of the proportion of germinated seeds prior to analysis of variance (ANOVA).

Cloning and characterisation of ZmCCD1

Total RNA was extracted from maize roots and leaves using Tri Reagent and quantified using the NanoDrop ND-1000 spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands). RT-PCR was performed in a 20 µl volume, with 10 µl (1 µg) of total RNA as the template, 1 µl of Primer Oligo dT21 (25ngµl^{-1}), 2 µl of 0.1M DTT (0.1M), 2 µl dNTP (10 mM), 4 µl of 5X RT buffer and 1 µl Superscript II Reverse Transcriptase (200 units.µl^{-1}) (all from Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. A full-length fragment was amplified using 35 cycles and the following nested specific primers: forward primer 5'-CTTCGCTACAAGTCATCTCG-3', reverse primer 5'-AGTGAAGATACGGCACCTGC-3'; and nested forward primer 5'-CAAGTCATCTCGCCGCAACC-3', nested reverse primer 5'-GCAGGACGTGTATTCGAACC-3'. Primers were designed according to a TC sequence from maize (TC220599 TIGR), which is highly similar to the *Arabidopsis* CCD1 and obtained from Biolegio, Nijmegen, The Netherlands. The PCR fragments were cloned into the pGEM®-T Easy vector using the TA-cloning kit (Promega, Leiden, The Netherlands) and sequenced on a DNA sequencer model 3730X DNA Analyzer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

The obtained *ZmCCD1* sequence showed two possible start codons in the same reading frame. Therefore we amplified *ZmCCD1A* (long) by PCR using the forward primer 5'-CGCAGGATCCATGGGGACGGAGG-3', and the reverse primer 5'ATATGAATTCGCAGGTGCCGTATCTTCAC-3', and *ZmCCD1B* (short) using the forward primer 5'-GGATCCATGGACAGCCACCG-3' and the reverse primer 5'-GCCACCGCTGAGCAATAACTA-3'. The resulting PCR products were cloned into the *BamHI* and *EcoRI* sites in pRSETA (Invitrogen Breda, The Netherlands). Plasmid pRSETA-*ZmCCD1A*, plasmid pRSETA-*ZmCCD1B,* plasmid pRSETA-*AtCCD1* as positive control and pRSETA (empty vector) as a negative control were transformed to *E. coli* BL21 (DE3) pLysS carrying expression plasmids for lycopene, ß–carotene, and zeaxanthin biosynthesis (Cunningham Jr et al. 1996). The transformed *E. coli* were grown overnight at 37ºC on LB solid medium containing 50µg.ml^{-1} of ampicillin, 35µg.ml^{-1} chloramphenicol and 1% glucose. Selected colonies were streaked on the same LB solid medium and incubated at 21ºC for 4-7 days in darkness for expression. In this system carotenoid cleavage activity is visualised by the absence of accumulating carotenoids, hence the absence of the yellow to orange colour. Enzyme activity was further analysed using GC-MS. Briefly, a 1 ml aliquot of a culture of each construct grown overnight at 30°C was used to inoculate 25 ml of LB medium containing 50µg.ml^{-1} ampicillin and 35µg.ml^{-1} chloramphenicol in a 250 ml conical flask. Cultures were grown overnight in darkness at 22°C with shaking at 250 rpm. Then the 25 ml liquid cultures were mixed with 5 ml of pentane:diethylether (1:4) and the phases separated in a separation funnel after thorough mixing. The organic phase was transferred into a glass centrifugation tube and centrifuged at 1200*x*g for 5 min to further separate the organic phase from the water. The organic phase was passed over a short column containing anhydrous Na_2SO_4 into a new vial and concentrated under a flow of N_2 until about 1ml. Of this 1 ml, 2 µl were injected into the injection port of a gas chromatograph coupled to a mass spectrometer (5890 series II, Hewlett-Packard GMI, USA) with a Zebron ZB-5ms column (30 m × 0.25 mm I.D. × 0.25 µm film thickness)

(Phenomenex, USA). The oven was programmed at an initial temperature of 45°C for 1 min, with a ramp of 10°C per min to 280°C, and final time of 5.5 min. The injection temperature was 250°C, and the detection temperature was 290°C. Products were identified by comparison to reference standards.

Expression analysis

Total RNA was extracted from maize roots with and without AM fungi and used for Northern analysis as described before (Hans et al. 2004). Hybridisations were done in 7% SDS (w/v), 250 mM NaPi, pH 7.0, 250 mM NaCl and 1 mM EDTA at 60°C overnight using an [α-32P] dATP-labeled *ZmCCD1* cDNA fragment as probe. Final washes were in 0.5× SSC, 0.1 % (w/v) SDS at 65°C.

Results

Cloning of ZmCCD1

To find the *Zea mays* orthologue of *AtCCD1*, we blasted *AtCCD1* against the TIGR maize EST database (http://tigrblast.tigr.org/tgi/). This yielded one contig, TC220599, with 64% identity to *AtCCD1*. According to the TC220599 sequence, specific nested primers were designed and used to amplify the full-length cDNA by PCR using maize cv. MBS 847 root cDNA as a template. This yielded one clear band of the expected size (~1600 bp) after agarose gel electrophoresis. This band was purified, subcloned in pGEM-T Easy and sequenced. The maize *CCD1* had two open-reading frames starting with an ATG, one of 1650 bp (*CCD1A*) and another of 1623 bp (*CCD1B*), encoding proteins of 550 and 541 amino acids, respectively, with calculated molecular weight of about 61 kD (Fig. 2). Based on the alignment with other plant CCD1s (Fig. 2) and expression in *E. coli* (see below) we assume that the shorter version (CCD1B) represents the natural protein. The predicted maize CCD1 protein has a high similarity (77-78% identity) to CCD proteins of crocus (CsCCD1), petunia (PhCCD1), tomato (LeCCD1A), grape (VvCCD1) and *Arabidopsis* (AtCCD1) that all catalyse the symmetrical 9,10 (9',10') cleavage of several linear and bicyclic carotenoids (Bouvier et al. 2003b; Mathieu et al. 2005; Schwartz et al. 2001; Simkin et al. 2004a; Simkin et al. 2004b) (Fig. 2). Because of its high identity to these proteins the maize CCD1 was designated as *ZmCCD1* (Genbank accession number DQ539625). A second variant of *ZmCCD1* was isolated from a maize cv. dwarf-1 cDNA library. The nucleotide sequence obtained was 99% identical to the *ZmCCD1* amplified by PCR, resulting in only one conserved amino acid change (K160E) (Genbank accession number AY773278).

Characterisation of ZmCCD1 catalytic activity

To investigate the catalytical function of ZmCCD1, the cDNA cloned from maize cv. MBS 847 was cloned into the *E. coli* expression vector pRSETA, which was transformed to *E. coli* strains that were engineered to accumulate lycopene, ß-carotene and zeaxanthin (Cunningham Jr et al. 1996). Colonies of these three carotenoid accumulating *E. coli* strains develop an orange-like, yellowish colour. Loss of colour upon introduction of a putative carotenoid cleaving enzyme encoding cDNA indicates that the carotenoids are metabolized to colourless compounds. When the short version of *ZmCCD1 (ZmCCD1B)* was expressed in cells producing lycopene, ß-carotene, and zeaxanthin, colonies indeed failed to develop the yellow to orange colour that did show up in the empty vector control, showing that ZmCCD1B cleaves the carotenoids produced by the *E. coli* strains (Fig. 3). This decolouration did not occur with the

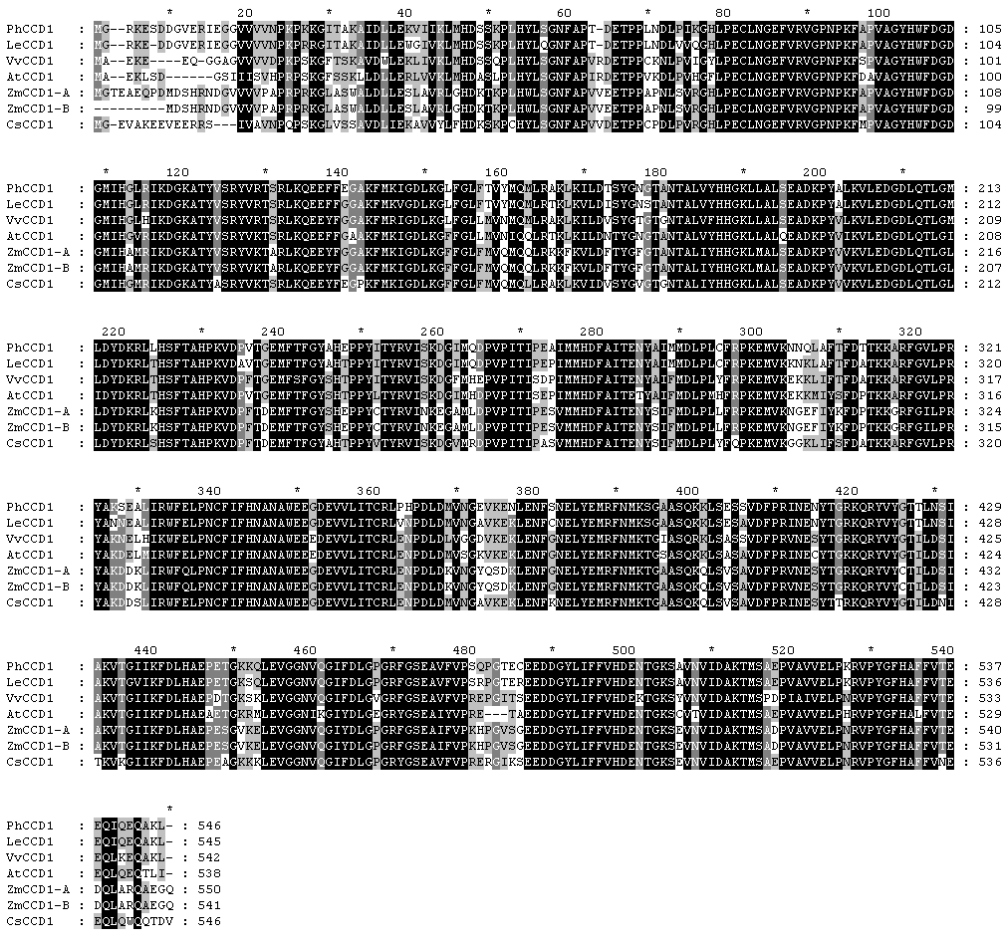


Fig. 2 Alignment of the deduced amino acid sequences of maize CCD1A (ZmCCD1-A) and CCD1B (ZmCCD1-B; DQ539625) with CCD1 from petunia (PhCCD1; AY576003) (Simkin et al. 2004b), tomato (LeCCD1; AY576001) (Simkin et al. 2004a), grape (VvCCD1 ; AY856353) (Mathieu et al. 2005), crocus (CsCCD1 ; AJ132927) (Bouvier et al. 2003b) and *Arabidopsis* (AtCCD1 ; AJ005813) (Neill et al. 1998).

long version of *ZmCCD1 (ZmCCD1A)* which indicates that the extended open reading frame version is not active. The colonies lost colour more effectively when ß-carotene and zeaxanthin were the substrate than with lycopene (Fig. 3). ZmCCD1B more efficiently prevented carotenoid accumulation than AtCCD1, particularly for lycopene and ß-carotene (Fig. 3, Appendix Chapter 2 Fig. 3 in colour). The two enzymes seemed equally efficient for zeaxanthin cleavage.

To characterise the cleavage products, the *E. coli* strains were grown overnight in liquid medium which was subsequently extracted with pentane-diethylether. GC-MS analysis revealed the presence of pseudo-ionone in the culture of lycopene accumulating *E. coli* (Fig. 4A), pseudo-ionone and ß-ionone in the culture of ß-carotene accumulating *E. coli* (Fig. 4B) and pseudo-ionone, ß-ionone and 3-hydroxy-ß-ionone in the culture of zeaxanthin accumulating *E. coli* (Fig. 4C). All cleavage products were absent in the empty vector controls (Fig. 4). The positive control, *AtCCD1*, was slightly less active than *ZmCCD1*, but the product patterns were similar (data not shown). We did not detect any other cleavage products but assume that ZmCCD1, analogous to other CCD1s characterised, is also cleaving symmetrically by 9,10 and 9',10' cleavage (Fig. 1) and hence also yields the C_{14} dialdehyde. This product is not volatile and prone to further modifications and will thus not show up in GC-MS analysis.

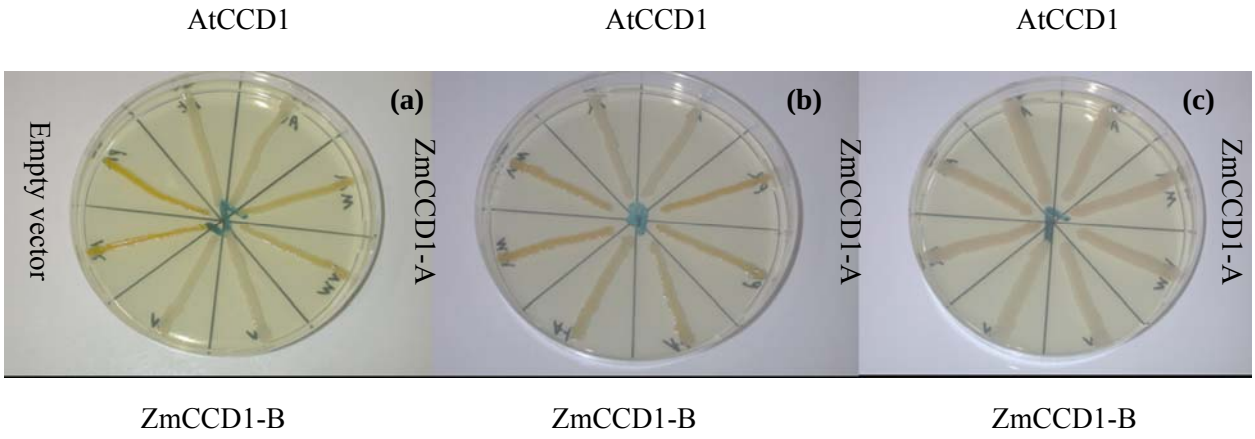


Fig. 3 Expression of *ZmCCD1* in *E. coli* strains accumulating different carotenoid substrates: (A) lycopene (pACLYC); (B) ß-carotene (pACBETA); (C) zeaxanthin (pACZEAX) (Cunningham Jr et al. 1996). *Arabidopsis thaliana CCD1* (AtCCD1) was used as positive, an empty vector as negative control; *ZmCCD1-A* is a variant of *ZmCCD1-B* having a 27-bp 5' extension starting with an ATG in the same reading frame as *ZmCCD1-B.*

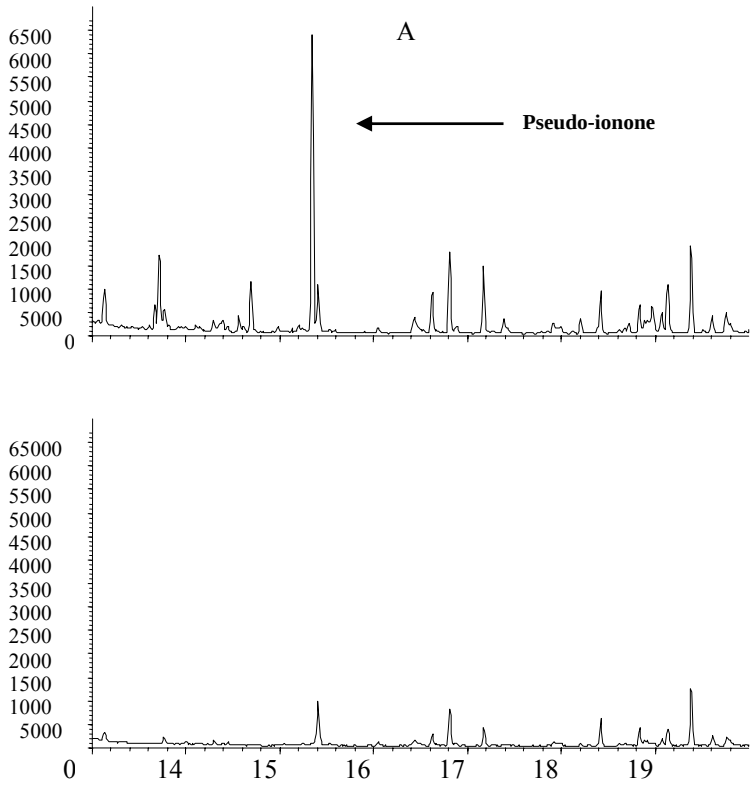
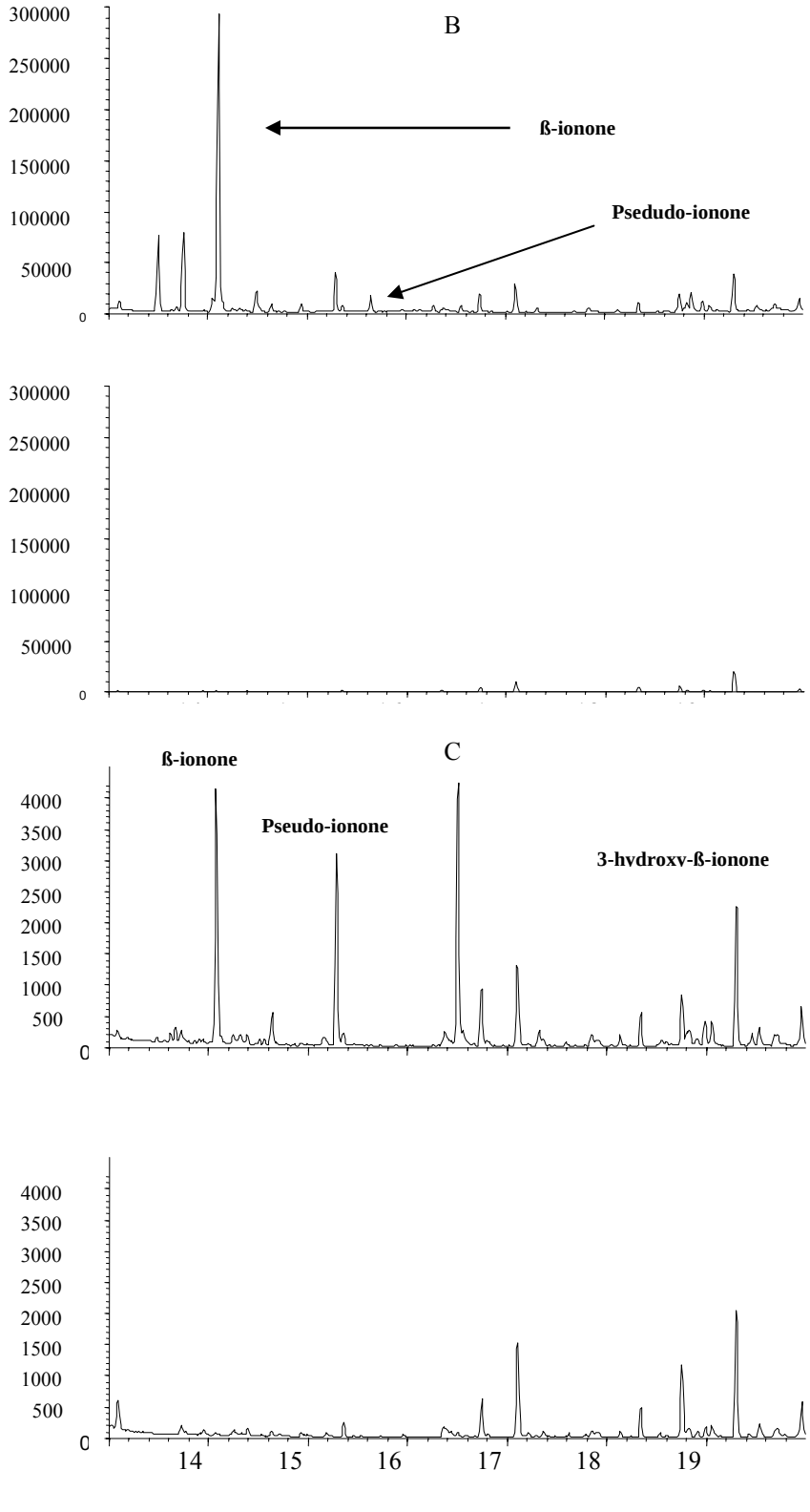


Fig. 4 GC-MS analysis of ZmCCD1 cleavage products. ZmCCD1 was expressed in *E. coli* strains accumulating different carotenoid substrates: (A) lycopene (pACLYC); (B; next page) ß-carotene (pACBETA); (C; next page) zeaxanthin (pACZEAX) (Cunningham Jr et al. 1996). Cultures of these *E. coli* were grown overnight in liquid medium. The medium was extracted with pentane-diethylether. Cleavage products were analysed using GC-MS. The identity of the peaks was confirmed using authentic standards.


B
ß-ionone
Psedudo-ionone
C
ß-ionone
Pseudo-ionone
3-hydroxy-ß-ionone
300000
250000
200000
150000
100000
50000
0
4000
3500
3000
2500
2000
1500
1000
500
14
15
16
17
18
19

ZmCCD1 is up-regulated in AM maize roots

Because ZmCCD1 cleaves carotenoids at 9,10 and 9',10'positions, it could be involved in cyclohexenone/mycorradicin formation in maize (Fig. 1). We therefore analysed *ZmCCD1* expression in AM fungi colonised maize roots. The full-length sequence of *ZmCCD1* was used as a probe in a Northern blot analysis with total RNA isolated from both AM and non-AM maize (cv. dwarf-1) roots. The probe recognized a single band of about 1600 bp. *ZmCCD1* transcript accumulation was increased by 1.5 to 2-fold upon mycorrhizal colonisation by *G. mossae* and *G. intraradices*, respectively (Fig. 5).

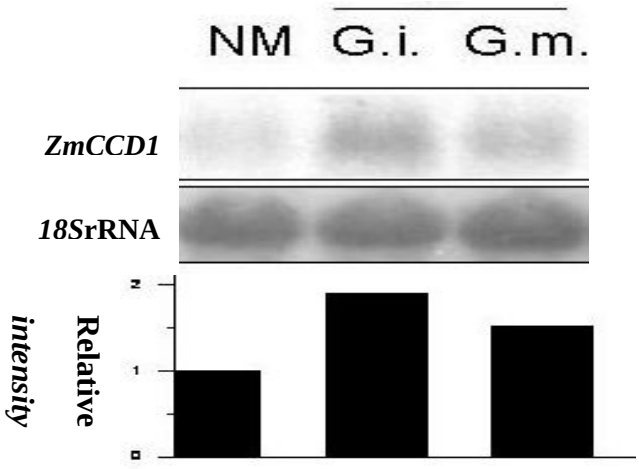


Fig. 5 *ZmCCD1* expression in maize roots with or without mycorrhizal colonisation. Maize plants (cv dwarf-1) were grown in expanded clay with and without inoculum of two arbuscular mycorrhizal fungi, *Glomus intraradices* and *Glomus mosseae.* Six weeks after inoculation roots were collected from the expanded clay and total RNA isolated. RNA was separated on a formamide gel (5 µg per lane), blotted onto HyBond N membrane and hybridized with the full-length sequence of *ZmCCD1*. Quantification was done by phosphor imager (Molecular Dynamics, Sunnyvale CA) and results were normalised to *18S* rRNA used as loading control. Expression in non-mycorrhizal roots was set at 1 and expression in mycorrhizal roots is shown relative to the control. NM, non-mycorrhizal; G.i., *G. intraradices* colonised roots; G.m., *G. mosseae* colonised roots.

Mycorrhizal colonisation of maize roots results in decreased Striga *germination*

Since it has been shown that maize and sorghum colonised by AM fungi are infected to a lesser extent by *Striga* than non-colonised control plants, we decided to study whether this reduction of infection is caused by the decreased production of strigolactone germination stimulants by AM maize roots. Maize roots were inoculated with *G. intraradices* and progress of fungal colonisation of roots was inspected at regular intervals. A colonisation rate of about 11% of total root length was observed after 14 days. This percentage increased to 47% after 34 days, when hyphae, vesicles and arbuscules were clearly visible. Exudates of mycorrhizal and control roots were collected and applied to preconditioned seeds of *S. hermontica*. Throughout the whole experiment, the root exudates of maize plants that were colonised by *G. intraradices* induced significantly ($P<0.01$) lower germination than the root exudates of control plants (Fig. 6).

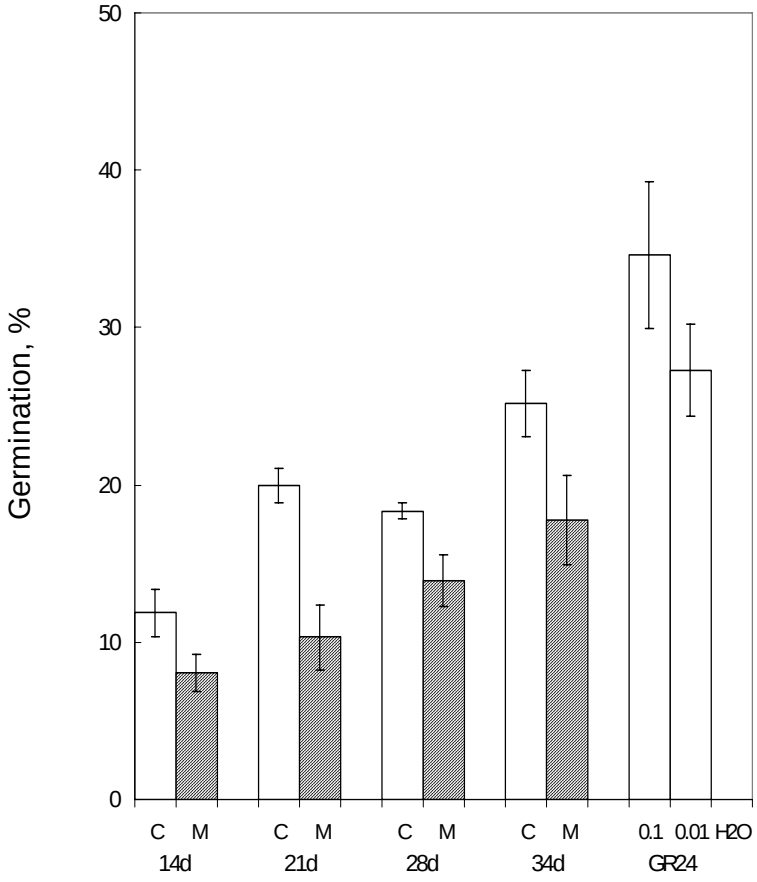


Fig. 6 Effect of colonisation by *Glomus intraradices* of maize roots on the induction of *Striga hermonthica* seed germination. Root exudates from four plants per treatment (different plants for each time point) were collected for 24 hours in demineralised water at 14, 21, 28 and 34 days after inoculation. The exudates were diluted to the same concentration of grams of root fresh weight per millilitre root exudate and induction of *S. hermonthica* germination was assessed. GR24 (in $mg.L^{-1}$) was used as positive control and demineralised water as negative control. C: non-mycorrhizal, control plants; M: mycorrhizal plants. Error bars represent the standard error of the mean of 4 individual plants.

Discussion

Maize CCD1

In this paper we have shown that a maize carotenoid cleavage dioxygenase, ZmCCD1, cleaves carotenoid substrates (lycopene, ß-carotene and zeaxanthin) at the 9,10 (and 9',10') positions leading to the formation of C_{13} apocarotenoids, that vary according to the substrate, and most likely a C_{14}-diadehyde (not detected in our GC-MS analysis) (Figs 1,4). These results are consistent with previously reported *in vitro* results for the orthologous enzyme AtCCD1 which cleaves carotenoids (ß-carotene, lutein, zeaxanthin, trans-violaxanthin) at the same position as ZmCCD1 (Schwartz et al. 2001) and other enzymes orthologous to AtCCD1 (Bouvier et al. 2003b; Mathieu et al. 2005; Simkin et al. 2004a; Simkin et al. 2004b). In addition, when using ß-carotene and zeaxanthin accumulating *E. coli* strains we also detected the cleavage products of the carotenoid intermediates (pseudo-ionone in ß-carotene accumulating *E. coli* resulting from lycopene cleavage; pseudo-ionone and ß-ionone in zeaxanthin accumulating *E. coli* resulting from lycopene and ß-carotene cleavage, respectively) (Figs 4B,C). All these data show that the

CCD1 enzymes have a broad substrate specificity but high regioselectivity for cleavage at the 9,10 and 9'10' position.

We did not detect the C_{14} dialdehyde cleavage product in the *E. coli* cell extracts, because it is not volatile enough for GC-MS analysis. Analogous to plant roots, the presence of the C_{14} dialdehyde should make the bacterial colonies yellow, which does not occur (Fig.3). Considering its reactive nature it is highly likely that further catabolism of the C_{14} dialdehyde occurs in *E. coli* by additional cleavage or oxidation of double bonds. Also in plants the primary cleavage products are further modified (oxidised, esterified, glycosylated) which makes them water soluble so they can be stored in the vacuole or vesicles, but these products remain yellow (Fester et al. 2002; Strack and Fester 2006; Walter et al. 2000).

Carotenoid cleavage is commonly assumed to occur in plastids, after which the cleavage products are exported to the cytosol (Cutler and Krochko 1999; Laule et al. 2003). However, other CCD1 studies have suggested that this family of CCD enzymes resides in the cytosol. Indeed, AtCCD1 is the only *Arabidopsis* carotenoid cleaving enzyme which is not localised in the plastids but in the cytosol (Auldridge et al. 2006a) and immunolocalisation indicated that the crocus CCD1 protein is also localised in the cytoplasm (Bouvier et al. 2003b). Prediction algorithms (SignaIP 3.0) also clearly suggest that all CCD1 enzymes are devoid of a plastid targeting signal. This also applies to ZmCCD1 indicating that its site of action is the cytosol. Nothing is known about the mechanism by which carotenoids produced in plastids come into contact with a cleaving enzyme in the cytosol. Transport of carotenoids across the plastidial membrane or degradation of the plastids could be possible explanations.

Possible biological functions of ZmCCD1

We have demonstrated that *ZmCCD1* is up-regulated in AM maize roots upon colonisation by AM fungi (Fig. 5). The 1.5 to 2-fold upregulation of *ZmCCD1* is similar to the upregulation upon mycorrhizal colonisation of *DXR* of which expression was localised in plastids in arbuscule containing plant cells (Hans et al. 2004). ZmCCD1 cleaves carotenoids at the position 9,10 (and 9',10'), which was also postulated for cyclohexenone and mycorradicin formation (Fester et al. 2005). Also considering the similarity of *ZmCCD1* to *MtCCD1*, of which expression is also up-regulated upon mycorrhizal colonisation (Lohse et al. 2005; Walter et al. 2007) it is likely that ZmCCD1 is indeed the enzyme that is responsible for cyclohexenone and mycorradicin formation in maize roots (Fig. 1). In addition to its role in yellow pigment formation in AM colonised maize roots, ZmCCD1 is also expected to be involved in the biosynthesis of apocarotenoid flavour and aroma volatiles in maize leaves or flowers. *CCD1s* have been found to be constitutively expressed in many plant tissues, especially in fruits, flower and leaves and the cleavage products contribute to the flavor and aroma of many agricultural products (Bouvier et al. 2003a; Ibdah et al. 2006; Mathieu et al. 2005; Schwartz et al. 2001; Simkin et al. 2004a; Simkin et al. 2004b). For example, tomato CCD1 can cleave several carotenoid substrates and some of the cleavage products are present in and/or emitted from tomato fruits, such as ß-ionone and geranylacetone, which play a key role in tomato flavour (Simkin et al. 2004a). Similarly, a petunia CCD1 is also leading to ß-ionone biosynthesis in the flowers when ß-carotene is cleaved and this volatile is possibly involved in the attraction of pollinating insects (Simkin et al. 2004b).

The actual carotenoid precursor of the cyclohexenone and mycorradicin derivatives in AM maize roots that is cleaved by ZmCCD1 is still unknown, since ZmCCD1 can cleave a number of carotenoid substrates at the 9,10/9',10' position (Figs 3, 4). It was postulated that cyclohexenone and mycorradicin

biosynthesis could involve zeaxanthin cleavage at the 9,10/9',10' position by a CCD-like enzyme (Fester et al. 2002). Indeed, we showed that ZmCCD1 can cleave zeaxanthin (Fig. 3). Two other candidates are lutein or lactucaxanthin (Siefermann-Harms et al. 1981). Especially the latter one shows ionone motives structurally closely related to the accumulating cyclohexenone derivatives in AM-colonised roots but this carotenoid has not been reported in plant roots so far (Strack and Fester 2006). Generally, the carotenoid composition of plant roots has hardly been studied but it is well-documented that roots do contain carotenoids, for example ß-carotene, α-carotene, lutein and violaxanthin (Baranska et al. 2006; Maudinas and Lematre 1979).

The cyclohexenone and mycorradicin derivatives are commonly produced in roots of many plant species when colonised by AM fungi, such as maize, *Medicago truncatula*, tomato, tobacco, and *Ornithogalum umbellatum* (Fester et al. 2002; Schliemann et al. 2006; Strack et al. 2003; Walter et al. 2000). In sorghum, mycorrhizal roots display a yellow colour as well (Lendzemo 2004). Although in sorghum the responsible compounds have not been identified, these are likely also carotenoid-derived (Fester et al. 2002). The function of these compounds in the mycorrhizal symbiosis is unknown.

AM fungi not only provide nutrients and water to their hosts, the symbiosis often also confers resistance to biotic stresses. In a number of studies with the parasitic plant *S. hermonthica* it was demonstrated that maize and sorghum have a 30-50% reduction in the number of *S. hermonthica* shoots after inoculation with AM fungi (Lendzemo et al. 2005). AM fungi may also confer resistance to other biotic stresses. For example, there are a number of reports showing that plants colonised by AM fungi are protected against subsequent infection with nematodes and plant pathogenic fungi (Borowicz 2001; Declerck et al. 2002; Elsen et al. 2003; Johansson et al. 2004). This protection has been suggested to be due to improved nutritional status of the host but there is ample evidence that this cannot be the (only) explanation (Harrison 2005; Johansson et al. 2004). Several studies have shown that during mycorrhizal symbiosis defence-related genes are induced (Kuster et al. 2004; Li et al. 2003; Pozo et al. 2002; Taylor and Harrier 2003). Increased defence gene expression could possibly also explain why sorghum and maize that are colonised by AM fungi are infected to a lesser extent by *Striga* (Lendzemo et al. 2005). There is ample evidence that defence gene expression could play a role in *Striga* resistance (Gowda et al. 1999). However, improved defence cannot be the only explanation for the lower infection of mycorrhizal sorghum and maize by *Striga* as we showed that the exudates of maize roots, colonized by AM fungi, induce less germination of *Striga* seeds than control root exudates (Fig. 6). Control experiments in which the synthetic strigolactone analog GR24 was mixed with exudates of AM colonised maize showed that this effect was not due to the presence of inhibitors. A similar and even more convincing result was obtained with sorghum where germination of *Striga* seeds induced by root exudates of plants colonized by AM fungi was dramatically reduced (Lendzemo et al. 2007). These all suggest that the reduction of *Striga* infection of sorghum and maize, when colonised by AM fungi, is caused at least partly by a decrease in the formation or secretion of strigolactone germination stimulants. In another study a positive effect of AM fungal colonisation on parasitic plants has been reported. Mycorrhizal colonisation of *Trifolium pratense* improved growth of the host as well as the attached parasitic plant *Rhinanthus serotinus* (Salonen et al. 2001) arguing against an effect of AM fungi on the defence capacity of plants against parasitic plants. However, the facultative parasite *R. serotinus* does not require a strigolactone apocarotenoid germination signal (Matthies 1995). Therefore, a reduction in strigolactone formation in *T. pratense* upon mycorrhizal colonisation is not expected to reduce *R. serotinus* germination and hence mycorrhizal colonisation will not reduce infection with this facultative parasite. Also in a study with

cucumber, it was shown that the exudate of AM-colonised cucumber is less stimulatory to AM fungi than the exudate of control plants (Pinior et al. 1999). In retrospect the authors now assume this is due to a lower secretion of strigolactones (Steinkellner et al. 2007). Nevertheless, we cannot completely exclude the presence of *Striga*-inhibitory compounds in or in the vicinity of mycorrhizal roots produced by the AM fungi, the plant itself in response to the AM fungi, or by microorganisms in an altered rhizosphere (Bais et al. 2004; Lendzemo et al. 2007; Singh et al. 2004; Werner et al. 2002).

ZmCCD1 may affect strigolactone precursor availability

Strigolactones are apocarotenoid host-signalling compounds for AM fungi in an ancient symbiotic relationship, which are apparently abused by parasitic plants to also detect the presence of a plant host (Bouwmeester et al. 2007; Matusova et al. 2005). While strigolactones are involved in early recognition processes of the AM symbiosis in very low concentrations (Akiyama et al. 2005), other apocarotenoids (cyclohexenone and mycorradicin derivatives) accumulate to high concentrations in later stages of the symbiosis (Maier et al., 1995; Walter et al., 2000). Any potential functional relationship between these different apocarotenoids or their carotenoid precursors is unknown at present. Different carotenoid cleavage enzymes may be involved in the formation of the different kinds of apocarotenoids but these enzymes could act on the same carotenoid precursor. Cyclohexenone and mycorradicin formation is preceded by the up-regulation of many genes of the carotenoid biosynthetic pathway such as deoxyxylulose-5-phosphate synthase 2 (*DXS2*), deoxyxylulose-5-phosphate reductoisomerase (*DXR*), and phytoene desaturase (*PDS*) (Walter et al. 2000; Walter et al. 2007) probably leading to a considerable increase in carotenoid precursor pools in the roots of plants colonised by AM fungi. Why then is there a reduction in strigolactone formation and hence *Striga* germination (Fig. 6) despite this increased pool of root carotenoids? A possible explanation could be the efficient depletion of the carotenoid precursor pools by ZmCCD1. This possibly depletes not only the AM-induced carotenoid precursors but also the basal levels of root carotenoids normally available for strigolactone formation in non-mycorrhizal plants.

Despite many attempts to identify the AM-induced carotenoid precursor of the mycorrhizal apocarotenoids only tiny amounts of potential parent carotenoids could be detected, indicating that the AM-induced carotenoids are immediately cleaved into apocarotenoids (Fester et al. 2002). Strigolactone formation does not benefit from a high activity of CCD1-type enzymes in maize roots but is rather reduced instead (Figs 5 and 6). It is therefore unlikely that ZmCCD1 contributes to strigolactone formation. This is in line with the previous proposal that strigolactone biosynthesis proceeds by carotenoid cleavage at the 11,12/11',12' position (Matusova et al. 2005). As a result of high ZmCCD1 activity carotenoid precursor availability may become limiting to strigolactone biosynthesis ultimately reducing its steady state levels. In addition, it is possible that - through a signalling mechanism mycorrhizal colonisation has a direct down-regulating effect on the strigolactone biosynthetic pathway. A direct effect of existing AM fungal colonisation on the further production/secretion of a recognition and branching factor for newcomer AM fungi is not unlikely as it would result in auto-regulation of host roots already colonised by AM fungi. Work is in progress to further underpin this hypothesis.

Concluding remarks

We have cloned a carotenoid cleavage enzyme (*ZmCCD1*) from maize roots that cleaves carotenoids at the 9, 10 /9'10' position, which constitutes a likely step in the formation of the yellow pigment (cyclohexenone and mycorradicin derivatives) in mycorrhizal maize roots. Mycorrhizal maize roots

display enhanced *ZmCCD1* expression and at the same time induce lower germination of *Striga* possibly via depleted carotenoid pools for strigolactone formation Our future work will be to overexpress and knockout *ZmCCD1* in maize to study the importance of the cyclohexenone and mycorradicin derivatives for the symbiotic interaction of plants with AM fungi and to study whether root-directed *CCD1* overexpression without mycorrhizal colonisation also leads to reduced strigolactone formation. If this is true, it could potentially be used to develop maize varieties with improved *Striga* resistance through a lower production of germination stimulants.

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Chapter 3

Silencing of NCEDs reduces the formation of *Striga hermonthica* germination stimulants in maize

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Abstract

The parasitic weed *Striga* causes a serious problem for cereal crop production in Africa. Breeding programs have tried to make crops, such as sorghum, maize and millet that produce less germination stimulants, compounds that are responsible for the induction of seed germination of the parasite and that are produced in and secreted from the host roots. In earlier work we showed that the strigolactone germination stimulants biosynthetically originate from the carotenoids, probably through the action of carotenoid cleaving enzymes, such as the 9-cis-epoxycarotenoid cleavage dioxygenases (NCEDs). In the present paper, we have silenced the maize *NCED* family using RNAi technology with root specific and universal promoters. The root exudates from transgenic maize plants were analysed using *Striga* germination bioassays. Our results show that the RNAi constructs can efficiently block known *NCED* genes (and possibly laso unknown ones) and that silencing of *NCEDs* leads to a strong reduction of germination stimulant production in transgenic maize. These results confirm that the NCEDs are involved directly or indirectly in the formation of germination stimulants in maize and that the germination stimulants are mainly produced in the maize root. This technology could be an important component of a long-term strategy for *Striga* control.

Key Words: *Striga hermonthica*, germination stimulants, NCEDs, RNAi, maize

Introduction

Parasitic weeds of the genus *Striga* (*Orobanchaceae*) result in a tremendous yield losses for major food crops in Africa, such as maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L.), pearl millet (*Pennisetum glaucum*), upland rice (*Oryza sativa*) and cowpea (*Vigna unguiculata* L.) (Ejeta 2005; Joel et al. 1995; Musselman et al. 2001; Parker and Riches 1993). It was estimated that two-thirds of the 70 million hectares of cereals and legumes in Sub-Saharan Africa are heavily infected with *Striga* spp. and this is affecting the livelihood of some 300 million Africans in 25 countries (M'Boob 1989; M'Boob 1994). These data show that the parasitic weed *Striga* spp. are among the most important constraints to agricultural development on the African continent.

Many potential control methods have been developed against the *Striga* problem including physical, cultural, chemical and biological methods (Joel 2000). So far these methods however have had a limited impact on controlling the menace of *Striga* and there is no single control method that can effectively solve this problem (Ejeta 2005; Joel 2000; Oswald 2005). For example, chemical herbicides have been applied to reduce *Striga hermonthica* and can reduce infestation to some degree in maize and sorghum (Babiker et al. 1996; Kanampiu et al. 2003), and were more cost-effective than other methods (Traore et al. 2001). Some herbicides, however, such as for instance ALS (acetolactate synthase)-inhibitors, pose a risk to the biotic soil suppressiveness, thus they may not be a sustainable solution (Ahonsi et al. 2004). Other methods can not be used because they are either too expensive or have a too low efficiency due to our limited understanding of the host-parasite biology (Ejeta 2005). New methods for effective control of *Striga* should be both economically and practically feasible, and potentially the most effective methods can be developed based on a thorough knowledge of the host plant-*Striga* interaction. A potential target for new control methods are the chemical signals that are exchanged between the host and *Striga* and coordinate the *Striga* life cycle. One of the most important signals in the early developmental stage of *Striga* is the chemical signal that induces germination. These germination stimulants have been identified in host and non-host root exudates and mostly belong to a class of structurally closely related compounds, called strigolactones (Bouwmeester et al. 2003). Strigol, the first strigolactone identified, was isolated from the false host cotton (*Gossypium hirsutum*) (Cook et al. 1972) and later from the true *Striga* hosts maize, sorghum and pearl millet (Butler 1995; Hauck et al. 1992; Siame et al. 1993). Recently, 5-deoxystrigol, and an isomer of strigol, tentatively named sorghumol also have been identified in maize (Awad et al. 2006). Control methods that affect *Striga* seed germination are expected to be more effective than those affecting later stages of development because they prevent parasitism prior to crop damage. Breeding for lower germination stimulants could be a cost effective and easy applicable approach. Indeed, several research groups have used traditional approaches in breeding for lower germination stimulants in different plant species. For example, in sorghum a breeding program for low-germination stimulant formation has resulted in low-stimulant sorghum varieties with improved resistance to *Striga* (Ejeta et al. 2000; Mutengwa et al. 1999; Ramaiah et al. 1990). Similarly in maize, by traditional selection for the existing high-yielding maize cultivars, root exudates from two cultivars, IWD STR Co and *Zea diploperennis* BC4C2, stimulated relatively little germination of *Striga asiatica*, indicating tolerance to *S .asiatica* infection (Pierce et al. 2003). In addition, Kiruki and his colleagues developed non-stimulating maize mutants by azide-based mutagenesis (Kiruki et al. 2006) and these 'non-stimulant' materials could be crossed to susceptible varieties of agronomic interest to create new cultivars with a level of resistance to *Striga*. However the use of traditional approaches in breeding for low-germination on maize varieties

has been hampered by the lack of knowledge about the genetic background of germination stimulant formation. Because recently increased knowledge about the physiology, biochemistry and molecular biology of the parasitic weed and plant interaction has been reported, transgenic maize could be used to speed up the breeding progress for lower germination stimulants varieties (Sun et al. 2007). Using pathway inhibitors and maize mutants, we demonstrated that the strigolactones are derived from the carotenoid pathway (Matusova et al. 2005). *Striga* germination bioassays with the exudates of the maize mutant *vp14* having a mutation in 9-cis-epoxycarotenoid dioxygenase (*NCED*), an important regulatory step in the abscisic acid (ABA) biosynthetic pathway (Schwartz et al. 1997) and with maize plants treated with the NCED inhibitor naproxen (Lee and Milborrow 1997) both reduced *Striga* germination by about 40% (Matusova et al. 2005). This suggests that an NCED is involved in strigolactone formation in maize either directly or indirectly through ABA. The incomplete inhibition of *Striga* seed germination in for example *vp14* is likely due to the fact that more than one NCED exists in the maize genome. Therefore, silencing all or more than one *NCED* could lead to a more efficient reduction in the formation of strigolactones.

NCEDs are a group of enzymes that catalyze carotenoid cleavage at the 11, 12 double bond of 9-*cis*-epoxycarotenoids leading to ABA biosynthesis. In *Arabidopsis*, five highly conserved *NCEDs* have been found in the genome (Tan et al. 2003) and in rice three putative highly conserved *NCEDs* were predicted by blasting *Arabidopsis NCEDs* to the rice genome (Chapter 5). Although the first *NCED* in plants was identified by analysis of the maize *viviparous 14* (*vp14*) mutant (Schwartz et al. 1997; Tan et al. 1997), other NCED genes in maize have not been identified. However, Southern Blot analysis using *VP14* as a probe suggests that there are 4 to 6 NCEDs in the maize genome (Tan et al. 1997). In order to knock out all or as many as possible maize *NCEDs*, we decided to use RNA interference (RNAi) technology. RNAi is a process in which double-stranded RNA acts as a signal to trigger the degradation of a homologous messenger sequence-specific mRNA (Guo et al. 2003). The RNAi technology has been successfully used in monocot plants. For example in rice the *OsRAC* gene family of seven members was effectively suppressed by a single RNAi construct (Miki et al. 2005). In addition, RNAi technology usually yields transgenic plants with a range of remaining expression levels of the target gene such that a lethal phenotype by completely blocking ABA biosynthesis can be prevented. To further reduce the risk of lethality, root specific promoters are also included in the constructs. In this paper, we tested if *NCED* RNAi constructs can efficiently block the maize NCED enzyme activity in maize and we also examined whether silencing NCEDs suppressed the germination stimulants strigolactone formation.

Materials and Methods

Plant materials and chemicals

The NCED fragment to be used for making the RNAi constructs was cloned from maize cultivar LG21 which was kindly provided by Vicky Child of Long Ashton Research Station, Bristol, UK. For maize transformation, maize (genotype A188 x H99) was obtained from the maize seed stock center (http//www.maizegdb.org/). Plants were grown in 1-l plastic containers containing potting compost under greenhouse conditions with a day/night temperature of 25ºC (16 h) / 18ºC (8h). Ten to twelve days after pollination, immature embryos to be used for transformation (see below) were isolated. For germination bioassays, transgenic maize plants (T1) were grown in 1-l plastic containers containing perlite for the first two weeks under greenhouse conditions with a day/night temperature of 25ºC (16 h) / 18ºC (8h). The

plants were only given tap water. After two weeks, exudates were collected from the seedlings for germination bioassay (see below) and subsequently the maize plants were transferred to 2-l plastic containers containing potting compost and placed in the same greenhouse to obtain progeny through selfing. For RNA isolation, LG21, transgenic T_0, and T_1 maize plants were first grown for 8 weeks on potting compost. Subsequently some maize roots and leaves from each maize plant were harvested non-destructively, ground in liquid nitrogen and stored at -80°C until used. The *Striga hermonthica* seeds used in the bioassay were collected from a maize field in Kibos, Kenya in 1994 and were kindly provided by Vicky Child of Long Ashton Research Station, Bristol, UK. The synthetic germination stimulant GR24 was obtained from B. Zwanenburg, University of Nijmegen, The Netherlands.

Construction of the RNAi vectors

Three RNAi constructs were made in pUbicas (Christensen et al. 1992) (Fig. 1A) derived from plasmid pUC18. The three constructs were the same except for the promoter and therefore the vectors were named based on the promoters that were used: pUbi (maize ubiquitin promoter), pMtPT1 (*Medicago truncatula* phosphate transporter promoter) and pHM62 (unknown rice gene promoter). The cloning procedure is summarised in Fig. 1. The constructs were made by first inserting a rice intron into the vector pUbicas (containing the ubiquitin promotor and nos terminator) (Fig. 1). The rice intron was isolated by PCR using forward primer (intron 5'-onwards oligo introducing *BamHI* site): 5' GGATCCCTCCTGGGTCTCTGAGAT 3' and reverse primer (intron 5'-onwards oligo introducing *KpnI* site): 5' GGTACCCCAGCCACACCCTCCTTT 3') from the rice *OsSH1* gene (kindly provided by Andy Pereira, Plant Research International, the Netherlands). The resulting PCR fragment of 196bp was digested with *BamHI/KpnI* and cloned into pUbicas to yield the intermediate construct pUbi-intron (Fig. 1B). To determine which *NCED* fragment to use for the inverted-repeat, we did an alignment among the maize *NCED* (*VP14*, U95953) and two predicted rice *NCEDs* (AY838899 and AY838901) using the online program Clustal X (http://www.cluster-x.org). A 331 bp fragment containing several highly conserved regions of more than 20 identical bp corresponding to nucleotides (nt) 1126-1457 of the maize VP14 was amplified by RT-PCR using two specific pairs of primers: forward (sense fragment 5'-onwards oligo introducing *BamHI* site) 5' GGATCCCCACGATGATCCACGACTTC 3' and reverse (sense fragment 5'-onwards oligo introducing *BglII* site) 5' AGATCTATCTCGGTCAGCACGCTCTC 3'; and forward (antisense fragment 5'-onwards oligo introducing Sal I site) 5' GTCGACCCACGATGATCCACGACTTC 3' and reverse (antisense fragment 5'-onwards oligo introducing Kpn I site): 5' GGTACCATCTCGGTCAGCACGCTCTC 3'. Maize LG21 cDNA was used as a template. Subsequently, the sense fragment was digested by *BamHI/BgLII* and then cloned into pUbi-intron to yield intermediate construct pUbi-intron-sense (Fig. 1C); the antisense fragment was digested by *KpnI/SacI* and then was cloned into pUbi-intron-sense completing RNAi construct pUbi (Fig. 1D). Subsequently, the ubiqutin promoter in construct pUbi was replaced by root specific promoters to create the RNAi construct pMtPT1 and pHM62. *MtPT1* was obtained from Zeng-YU Wang (Xiao et al. 2006). The promoter of *MtPT1* was amplified with specific primers: forward (MtPT1 promoter 5'-onwards oligo introducing *HindIII* site) 5' GTGATAAGCTTGCTGGAGTTCGAATCCCGAACAC 3' and reverse (MtPT1 promoter 5'-onwards oligo introducing *BamHI* site) 5' GTGATGGATCCCTGAATTTGTTACCTAGTTTTTCCCTGCATA 3' and using maize cDNA as a template. The resulting fragment was digested with *HindIII/BamHI* and was cloned into pUbi to create RNAi construct pMtPT1 (Fig. 1E); *pHM62* was obtained from Hiromi Higo (Plant Functional Genomics

Co. Ibaraki, Japan). The promoter was amplified with specific primers forward (pHM62 promoter 5'-onwards oligo introducing *HindIII* site) 5' GTGATAAGCTTGGTAGTCCCGTAACCGTAGCA 3' and reverse (pHM62 promoter 5'-onwards oligo introducing *BamHI* site) 5' GTGATGGATCCGACGAACAGCGCGTGGTTGA 3' and using maize cDNA as a template. The obtained fragment was digested by HindIII/BamHI and then cloned into pUbi to create RNAi construct pHM62 (Fig. 1F). The orientation and junctions of the resulting constructs were confirmed by DNA sequencing and restriction enzyme digestions (data not shown).

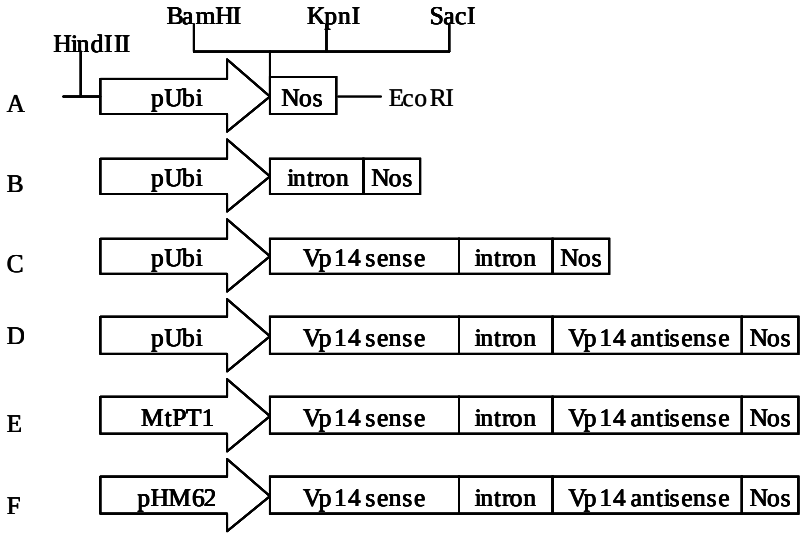


Fig. 1 A-F. Schematic representation of maize transformation/expression constructs. A, empty vector pUbicas; B, pUbi-intron: rice SHN1 gene intron was cloned into vector A; C, pUbi-sense-intron: a 331bp sense DNA fragment of VP14 was cloned into B; D, pUbi RNAi construct: a 331bp antisense DNA fragment of VP14 was inserted into C; E, pMtPT1 RNAi construct; a root specific promoter pMtPT1 was cloned into D instead of the constitutive promoter pUbi (ubiquitin); F, pHM62 RNAi construct: a root specific promoter pHM62 was cloned into D instead of the pUbi promoter. Nos; a nos-terminator.

Transformation of maize by particle bombardment and selection of transgenic plants

The detailed procedure of the biolistic transformation of maize has been described before (Brettschneider et al. 1997). Briefly, immature maize embryos were isolated from cobs that were harvested 10-12 days after pollination and the explants were cultured with the scutellar side facing upward on MScas (Zhong et al. 1992) solid medium (0.8% agarose) supplemented with 2 $mg.l^{-1}$ 2,4-D for 7 days before bombardment (Brettschneider et al. 1997). Four hours before bombardment the explants were treated with an osmoticum and this was continued for 20 to 24 h after bombardment for better transformation efficiency.

Bombardment was carried out with a PDS 1000/He gun (BioRad, Munich, Germany). Two and a half µg plasmid DNA (pUbi, pMtPT1 and pHM62) was coated onto gold particles with an average size of 0.4 -1.2 µm (Heraeus, Karlsruhe, Germany) following a protocol described by BioRad (Munich, Germany) and modified according to Brettschneider (Brettschneider et al. 1997). The RNAi constructs were co-bombarded with equimolar amounts of plasmid containing a *pat* gene under the control of a CaMV35S promoter which was used as the selection marker. In addition, plasmid containing *GFP* under the control of a CaMV35S promoter was also co-transformed with the above plasmids. As a quick check for

transformation efficiency, GFP fluorescence of maize calli was detected with a fluorescence stereomicroscope (Zeiss, Germany) under UV epifluorescent light (425/60nm). After shoot regeneration, transgenic plants were selected using phosphinotricin (PPT), the commercial herbicide Basta. Details of plant regeneration and selection have been described before (Brettschneider et al. 1997).

Southern Blot hybridisation

Total genomic DNA was isolated from the roots of maize primary transformants and non-transformed maize using the protocol of Dellaporta (Dellaporta et al. 1983). The analysis was performed with 15 µg DNA, digested with EcoRI and HindIII restriction enzymes. The restricted DNA was separated by electrophoresis and transferred to a Hybond N membrane (Amersham, England). Filters were prehybridised with salmon sperm and then hybridised with a PCR generated digoxigenin-labelled rice *OsSH1* intron probe using a modified protocol of the non-radioactive digoxigenin chemiluminescent method (Neuhaus-Url and Neuhaus 1993).

Real-Time PCR

For real-time RT-PCR analysis, 1µg total RNA was first treated using DNase I (Invitrogen, US) with 1µl 10x DNase I reaction buffer, 1µl DNase I and 7ul MilliQ water before the RT-PCR reactions. RT reactions were performed by using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA) following the product instruction. SYBR Green PCR Master Mix (Applied Biosystems) and ABI PRISM 7700 sequence detector (Applied Biosystems) were used according to the manufacturer's instructions. As internal standard, the elongation factor gene (Q41803) was used for normalization. Primers designed using program express software (Applied Biosystems) are as follows: for elongation factor, forward primer 5’ CTCTCCAGGAGGCCCTTCC 3’, reverse primer 5’ ACATTCTTCACGTTGAAGCCAA 3’. For VP14, Forward primer 5’ ACTTCGCCATCACCGAGAAC 3’, Reverse primer 5’ GGAGCTTGAACACCACCTGG 3’. The PCR conditions were 50°C for 2 minutes, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Root exudate collection and germination bioassay

For maize root exudate collection, the two-week maize plants grown on perlite were gently washed with tap water and transferred to 25 ml glass tubes containing 20 ml demineralised water. The size of the maize plants was rated into three categories for later correction of germination percentage. Root exudates were collected from each single plant separately after 24 hours and were tested using a germination bioassay. Hereto, *Striga* seeds were preconditioned by surface-sterilising seeds in 2% sodium hypochlorite containing 0.1 % Tween 20 for 5 minutes. Subsequently, seeds were rinsed three times with sterile demineralised water, and excess water was removed by filtration over a Büchner funnel. The sterile seeds were allowed to air dry for 2 hours and subsequently approximately 50-100 seeds were sprinkled on 9-mm diameter glass fibre filter paper (Sartorius Germany) discs. Twelve discs were placed in 9-cm diameter Petri-dishes with a filter paper (Waterman, UK) moistened with 3 ml demineralised water. The Petri dishes were sealed with parafilm and wrapped in aluminum foil and placed in a growth chamber (30ºC) for 10 days. Before applying root exudates, the discs with the seeds were dried on the sterile filter paper for 3 minutes and then transferred to a new Petri dish with a filter paper ring (diameter of 9 cm), moistened with 1 ml sterile demineralised water to keep a moist environment inside the Petri dishes. Fifty µl of the root exudates to be tested were applied to triplicate discs. GR24 (0.1 $mg.l^{-1}$) was used as a

positive control and sterile demineralised water was as a negative control in each germination assay. Seeds then were incubated at 30 ºC in darkness. After 2 days, germination was scored using a binocular microscope. Seeds were considered to be germinated when the radicle protruded through the seed coat (Mangnus and Zwanenburg 1992).

Statistical analysis

The Chi model method was used for statistical analysis of all germination bioassay results using GenStat Release 9.2 (PC/Windows XP), VSN international Ltd, UK.

Results

Maize transformation

To test the efficiency of bombardment for maize transformation, maize scutellar tissue of embryogenic calli (Fig. 2A, Appendix Chapter 3 Fig 2A in color) were co-bombarded with NCEDs RNAi constructs and a GFP cDNA to monitor and trace the transformation event because GFP has its advantage of a non-destructive method. High numbers of GFP expression signals were obtained in transformed transgenic calli cells already at 2 days after bombardment (Fig. 2 B, Appendix Chapter 3 Fig 2B in color). In total about 190 embryos were bombarded at two different times with each of the three RNAi constructs. All selected calli were highly regenerable in the beginning, but a lot of embryogenic structures turned brown

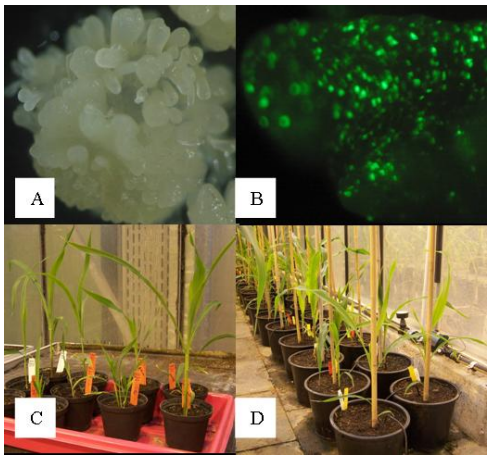


Fig. 2. Regeneration of transgenic maize plants from bombarded scutellar tissue. A, embryogenic callus of A188 x H99 after 7 day's isolation. B, GFP transient expression in bombarded A188 x H99 embryo cells. C, transgenic regenerants in small pots in the greenhouse have been sprayed with a Basta solution (250 mg^{-1} PPT, 0.1% Tween). D, transgenic regenerants in big pots in the greenhouse after being sprayed twice with a Basta solution (250 mg^{-1} PPT, 0.1% Tween).

on selection medium in the Petri dish or afterwards when regenerants were transferred to selection medium in boxes in the climate room (Fig. 2C, Appendix Chapter 3 Fig 2C in color). Moreover, 50% of the regenerated plantlets did not survive herbicide spraying in the greenhouse that is very unusual because normally only 10 to 20% of the sprayed regenerants die due to escapes from the tissue culture selection. In the end only 12 healthy herbicide resistant T_0 transgenic plants were recovered in the greenhouse (Fig. 2D). Among them, seven plants were transformed with construct pHM62, five plants with construct pUbi and only one plant with construct pMtPT1. These 12 plants were successfully grown into plants in the

greenhouse and were then self-fertilized or backcrossed to A188 (when a plant had no pollen). All 12 plants were fertile and did not have precocious seed germination as do ABA deficient mutants in maize, such as *vp14* (Tan et al. 1997). Except for some somatic variation due to tissue culture and regeneration from callus (also seen in controls), the plants did not show any phenotype that could be associated to ABA deficiency such as wilting, except perhaps that some plants had a dwarf phenotype and underdeveloped tassels and ears.

Molecular analyses of transformed maize

To test if the constructs were integrated into the T_0 transgenic plants that survived in the herbicide selection procedure, Southern Blot analyses were performed (Fig. 3). Because the constructs differ in their promoter size, the intron probe hybridised to different size EcoRI /BamHI fragments in the three RNAi constructs. Fig. 3 shows that the regenerated maize plants contained the expected hybridization fragment of about 2.75 kb for pUbi, 2.7 kb for pMtPT1 and 1.8 kb for pHM62. The number of bands obtained indicates the number of construct copies and integration sites in the maize genome and varied between one to eight copies or integration sites (Fig 3). The Southern blot analysis suggests that plants 75 and 76 and 82 and 87 are clonal regenerants of the same transgenic cells. Other transgenic plants showed characteristic hybridization patterns, indicating that they are independent transformation events. Wildtype control 77 has no band (Fig. 3). Based on these results, six transgenic lines (68, 73, 86, 69, 80 and 75) and non-transformed plant (77) were selected for further analysis in the next generation (T1).

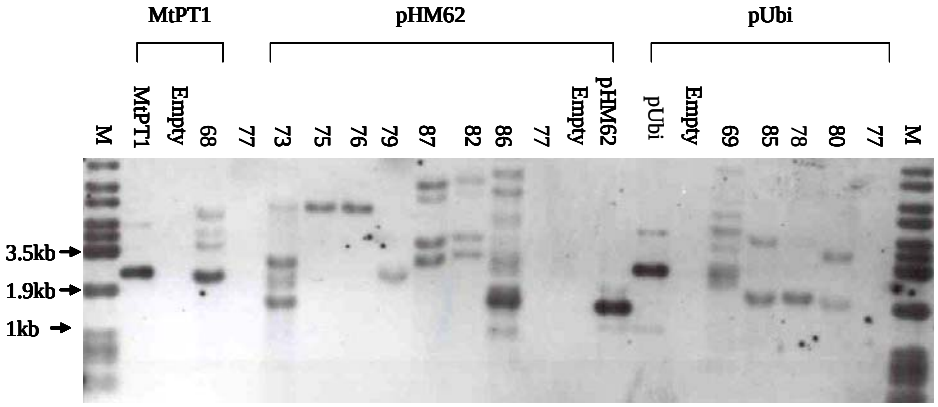


Fig. 3. Southern Blot analysis of genomic DNA from putative transgenic maize lines with NCED RNAi constructs (see Fig. 1). Fifteen µg of genomic DNA from untransformed control A188 X H99 plant (77), 12 transgenic regenerants and three vectors of constructs were digested with EcoRI and HindIII restriction enzymes and separated on a 0.8 % agarose gel. The filter bound DNA was hybridised with a DIG-labelled rice-intron fragment in the constructs. Hybridization fragments (pUbi 2.75kb, pMtPT1 2.7 kb and pHM62 1.8 kb) are indicated. Numbers on the lanes represent independent transgenic lines (pMtPT1: 68; pHM62: 73, 75, 76, 79, 82, 86 and 87; pUbi: 69, 78, 80, and 85), non-transformed control 77 and three vectors of constructs (pMtPT1, pHM62, pUBi). The DIG labelled MVII marker (Boehringer Mannheim; Germany) was used as size standard (M).

Suppression of maize NCEDs in T_0 transgenic maize root

To quantify mRNA levels in the roots of transgenic maize plants in the T_0 generation, a transcript-specific assay based on fluorescent real-time reverse transcriptase-polymerase chain reaction (Q-PCR) was

developed. Gene-specific primers were designed for maize *VP14*. *NCEDs* transcripts were detected at a dramatically lower level in most of the transgenic plants compared with that of the control except one transformant 78, in which the transcript were expressed at similar levels (Fig. 4). Possibly in 78 only a partial construct was integrated into the maize genome (Fig. 3). The results also showed that the level of transcript varied in different transgenic plants.

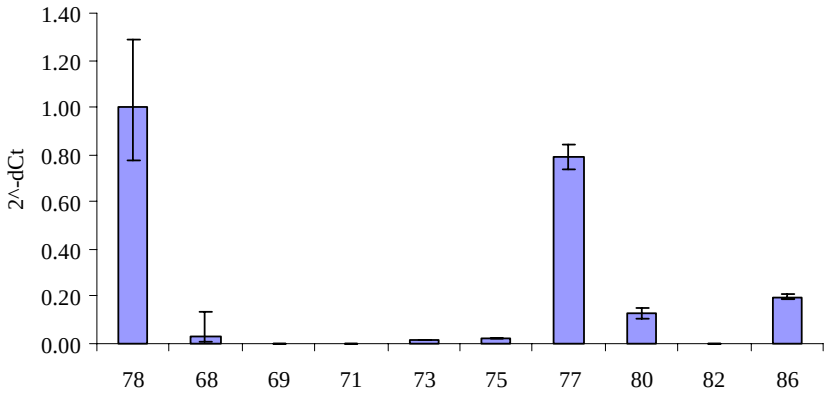


Fig. 4. Expression of *NCEDs* in the roots of T_0 transgenic maize lines. Levels of the expression were determined by quantitative RT-PCR. Equal amounts of total RNA were used in each reaction. All *NCED* expression values are normalized to expression of the elongation factor (Q41803) that was used as an internal control and then normalized to values in the wild type. Error bars represent the range for 2^-dCt from independent replicate of the transgenic lines.

Striga germination bioassay

Germination bioassays with the transgenic maize plants started from the second generation (T_1) because the first generation (T_0) plants are relatively weak after transfer from the tissue culture. Root exudates from T_1 transgenic maize plants with RNAi constructs pMtPT1, pUBi and pHM62 induced different germination percentages of *Striga* seeds (Fig. 5). For the transgenic line 68 containing pMtPT1, thirteen of twenty plants were grown (the other 7 plants did not germinate) in the greenhouse. Except for one plant (6), root exudates collected from the other 12 plants seemed to induce lower seed germination of *Striga* than that of the non-transgenic maize lines (Fig. 5A). Of the transgenic lines containing construct pHM62, for line 86 13 of the 20 plants sown survived, of which, nine plants seemed to induce less germination than the non-transgenic control plants (Fig. 5B). The other four plants (1, 5, 8 and 9) were similar to the non-transgenic plants (Fig. 5B). Of line 73 of pHM62 4 out of 28 plants seemed to induce decreased germination of *Striga* seeds (6,11, 17 and 18) compared with non-transgenic plants (Fig. 5C). For line 75 of pHM62, six (3, 4, 5, 7, 13 and 21) of twenty-one plants seemed to induce a lower seed germination of *Striga* than non-trangenic plants (Fig. 5D). For lines containing pUbi, line 69 had about 50% plants (1, 3, 4, 6, 8, 10, 12, 15, 17 and 18) that seemed to induce less germination than non-transgenic plants (Fig. 5E). A similar result was found for line 80 of which 9 plants (1, 2, 8, 9, 13, 14, 15, 16 and 20) of 21 plants seemed to induce less germination (Fig. 5F). In all assays, GR24 (0.1 mg^{-1}) induced high 72 % seed germination, whereas tap water did not induce any germination.

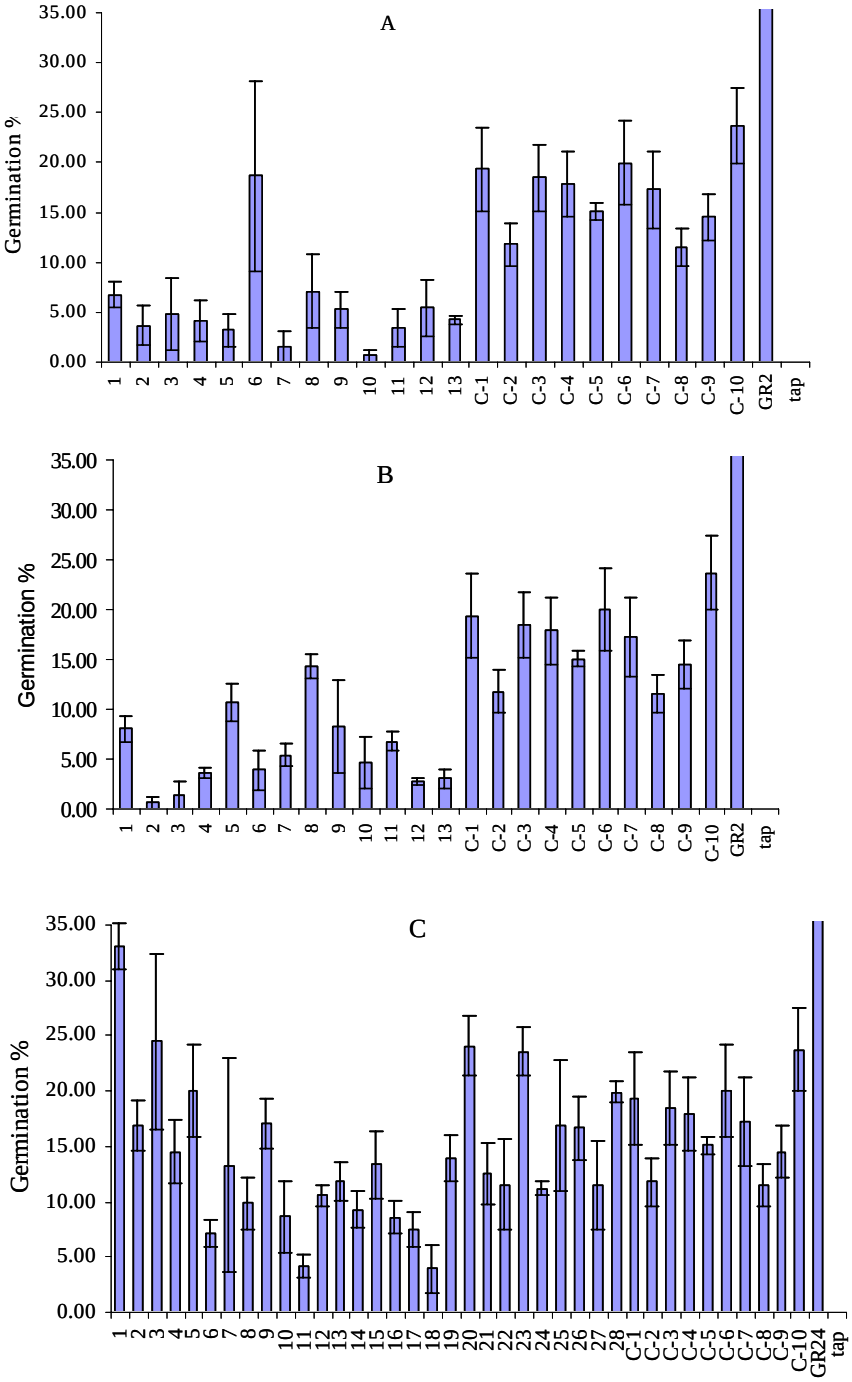
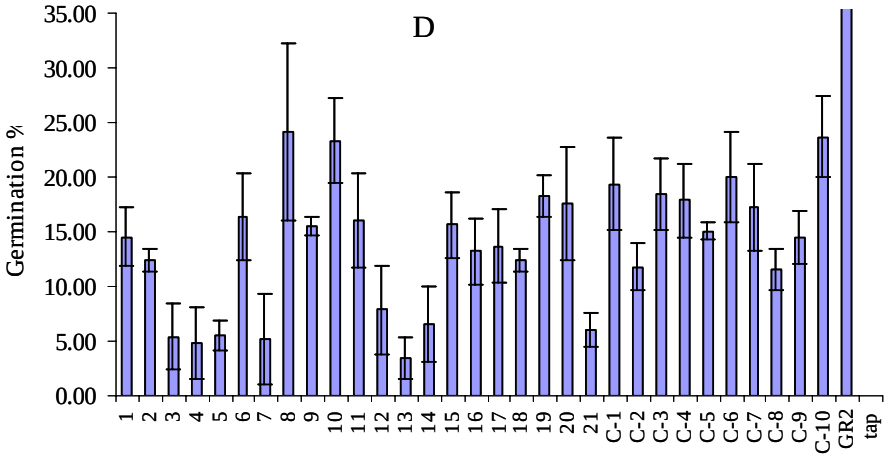


Fig. 5. Next two pages. Germination bioassay with *Striga hermonthica* seeds with exudates of maize plants. Of each transgenic line, 20-30 seeds were grown in pots with perlite for two weeks, giving only water. Then the plants were transferred to 50 ml glass tubes containing 25 ml water. Root exudates were collected after 24 hours and applied to preconditioned *S. hermontica* seeds. Seed germination was assessed after two days using a binocular microscope. A, T_1 transgenic maize line 68 (construct pMtPT1); B, T_1 transgenic maize line 86 (construct pHM62); C, T_1 transgenic maize line 73 (construct pHM62); D, T_1 transgenic maize line 75 (construct pHM62); E, T_1 transgenic maize line 69 (construct pUbi); F, T_1 transgenic maize line 80 (construct pUbi). C-1 to C-10 are wild type line 77; GR24 is a synthetic germination stimulants used as a positive control for *Striga* seed germination and induced >70% germination (bars not completely shown) and water is used as a negative control. Bars represent means of *Striga* seed germination of 3 replicate disks and error bars represent the standard error of the mean.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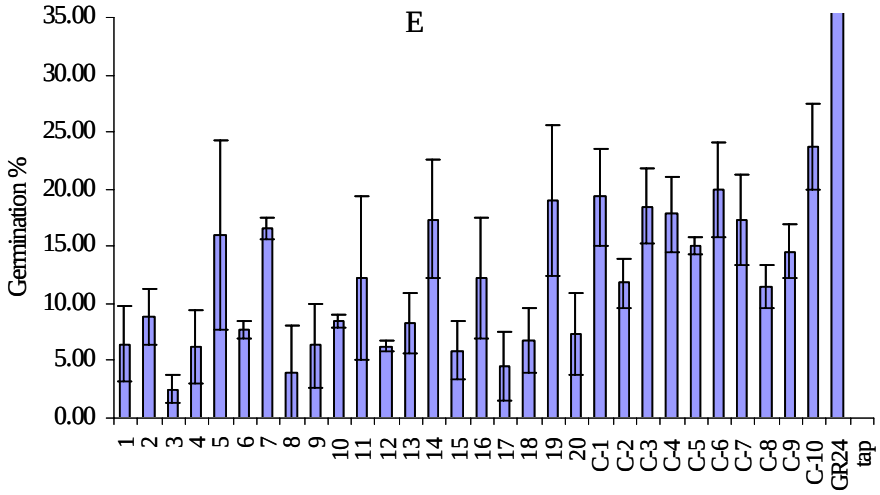
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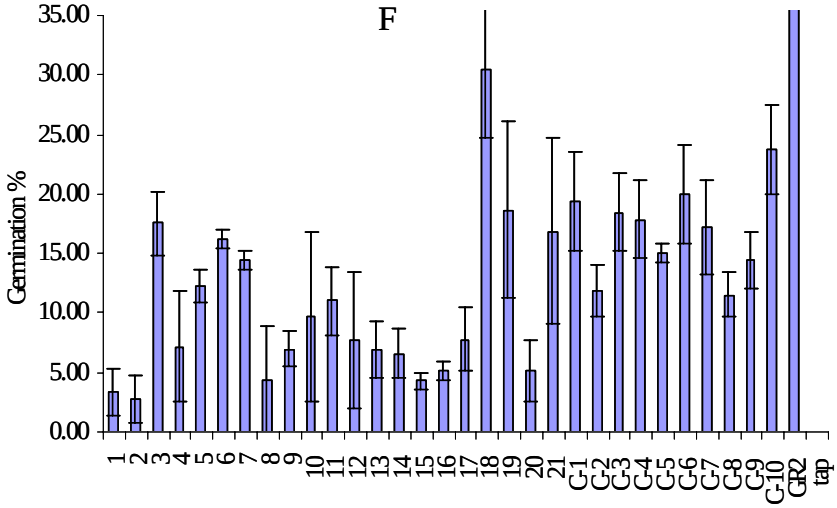
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Discussion

Although our bioassay results showed that root exudates from putative transgenic *NCED* RNAi maize plants (T1) induced less seed germination of parasitic weed *S. hermontica* compared with the root exudates from non-transformed maize roots, we still need to confirm the integration of the constructs in the chromosome, the expression of the *NCED* genes and the ABA content. Nevertheless, this result indicates that these transgenic plants probably produce less germination stimulants (strigolactones) and therefore maize *NCEDs* are directly or indirectly (through ABA) involved in the formation of germination stimulants. This result agrees with the one reported by Matusova who demonstrated that the root exudates of the maize mutant *vp14* induced about 40% lower *Striga* seeds germination compared with the root exudates from wildtype maize plants (Matusova et al. 2005). A similar reduction in the induction of germination of another parasitic plant, *Orobanche ramosa,* was found with the root exudates of the ABA-deficient tomato mutant *notabilis*, which is a null mutation in the gene *LeNCED1*, encoding a 9-cis-epoxycarotenoid dioxygenase involved in ABA biosynthesis (López-Ráez et al. 2007). Some of the present putative transgenic plants exhibited a much stronger reduction in the induction of germination of *Striga* seeds than *vp14* (Fig. 5). Possibly other maize *NCED* family members partly compensate for the NCED activity lost in *vp14*. This also suggests that more than one *NCED* gene is involved in the formation of germination stimulants, directly because they cleave carotenoids *en route* to the formation of strigolactones, or indirectly because a lower ABA production leads to an increase in strigolactone secretion.

Transformation efficiency

In total approximately 560 embryos were initially subjected to microprojectile bombardment and only 12 transgenic regenerants were recovered. Obviously the transformation efficiency of about 2% is very low because normally the transformation efficiency of this protocol with other constructs is from 5% to 10% or even higher (Bretschneider unpublished result). The low frequency may be attributable to a number of different factors that affect the transformation process. For example, it could be that simply the experiments performed this time were not that efficient as before although the cultures looked in good conditions. But the most possible reason is that the RNAi constructs used for the transformation are responsible for the low efficiency. The *NCED* family of genes are involved in the biosynthesis of the important plant hormone ABA. Blocking ABA production may interfere with important physiological processes. Indeed, ABA has been found to play an important role in maize somatic embryogenesis and regenerability (Duncan et al. 2003). Lack of ABA could also cause plant leaf wilting by affecting stomatal regulation in leaves (Tan et al. 1997). Surviving transgenic plants did not show any obvious phenotype that could be related to ABA shortage such as wilting or precocious germination. Therefore we expect that analysis of the expression level of the *NCEDs* of the selected transgenic plants (T_0) will show that only mild knock-downs (relatively low *NCED* silencing) have survived. The possible solution to improve the embryo and regenerant growing is feeding ABA to the selection medium or spraying plants at later stages in the greenhouse, but on the other hand this can probably not lead to plants suitable to be used in agriculture.

Segregation, stability and inheritance in selfed progeny

Southern Blot analysis on T_0 transgenic plants showed that one to eight copies have been integrated into the maize genome (Fig. 3). The introduction of multiple copies of the same gene often leads to

co-suppression (Iyer et al. 2000). Thus the selection of transgenic plants with one or a few copies is desirable. In our study, a number of lines already have a low-copy number integration of the transgenes but we still need to check if the inserted transgenes were successfully transferred in a Mendelian fashion to the progeny in the next generation. For example, transformant 75 (pHM62) has only one copy to produce fertile herbicide-resistant progeny in the first generation, whereas transformant 80 (pUbi) has two insertions but one insertion is a partial vector integrated into the genome. Similar to 75, this transformant also produces fertile herbicide-resistant progeny in the first generation. We are currently working on the second generation from these two primary transformants. Several other primary transformants (68, 73, 69, 86) that contain multiple copies of whole vectors in the genome are also investigated in the second generation.

Effects of the promoters

The initial idea of using three promoters (one universal promoter and two root specific promoters) was to investigate if a root specific promoter (silencing only root-expressed *NCEDs*) would be enough to block the formation of germination stimulants. On one hand this could be beneficial for the transgenic plants (we only block ABA biosynthesis in the roots), on the other hand, it could give us information about the localisation of strigolactone biosynthesis. If germination stimulants are still produced in root-specifically silenced plants, this indicates that germination stimulants could also be produced in the plant leaves and then be transported to the roots. In this case using the universal promoter (silencing *NCEDs* both in root and leaves) should reduce the germination stimulant production. With regard to phenotype there were no differences between the surviving transgenic plants with the three different promoters (T_0). However, the transformation efficiency was different. Only one transgenic regenerant was obtained from about 190 bombarded calli with root promoter *pMtPT1*, seven transgenic regenerants with root specific promoter *pHM62* and four from universal promoter *pUbi*. This suggests that *pMtPT1* is the most active one among the three promoters or is expressed in a critical part of the roots, and that pHM62 and pUbi are relatively mild. Moreover, a root specific promoter was sufficient to suppress NCED activity and reduce very much the formation of germination stimulants (Fig 5) in transgenic maize roots. The results indicate that the germination stimulants are probably only produced in the maize roots and/or they (or their precursors) are not transported from the leaves to the roots (if the leaves also produce it). Finally, RNAi signals may also be transported within the plant (Tournier et al. 2006). *NCED* expression analysis in roots and shoots of the transgenic plants equipped with a root specific promoter should show if this is indeed the case.

Outlook

In our experiment, the high expression of green fluorescent protein (GFP) in the calli from all three constructs suggests that the vectors are introduced into the chromosome stably. Real-time PCR showed that expression of *NCED* was suppressed using highly conserved regions of three *NCED* family genes (*VP14* and two rice *NCED* genes) in transgenic maize plants (T_0) (Fig. 4) with variable efficiencies. We anticipate that our constructs also block other maize *NCED* family members but these genes have not been identified yet and thus we cannot check that. It has been shown before that one single RNAi construct can efficiently and specifically suppress multiple genes in a gene family in maize with high sequence similarity, but in some cases the reduction of mRNA levels were not detectably which indicates that RNAi-induced silencing is gene dependent (McGinnis et al. 2005; McGinnis et al. 2007). In addition, direct delivery of dsRNA to other cereals is also found to lead to a rapid and sequence-specific

interference with gene and gene family function. For example, in rice Daisuke Miki and colleagues found that using a highly conserved region of two members of the *OsRac* gene family, all seven members of the gene family were suppressed with variable efficiencies and suppression efficiency was generally correlated with the level of homology between the trigger and target sequence (Miki et al. 2005). These results clearly indicate that using single RNAi is an effective approach to detect the function of gene family in maize involved in the germination stimulant formation of parasitic weed *Striga*.

Conclusions

In summary, we have demonstrated that knocking out *VP14* and possibly other *NCED* family members in maize using RNAi technology effectively suppressed the formation of germination stimulants of *Striga*. Potentially these transgenic maize plants could substantially decrease the infection of *Striga* in maize in a field situation. Moreover, if the silencing of *NCEDs* would be combined with other (natural) resistance genes (stacking) this would provide an even more efficient and probably also more durable approach to a long-term control of the parasitic weed *Striga* in maize.

Future work

We are currently characterising the second and third generation of transgenic plants using molecular techniques. Also the root exudates from these plants will be used to do germination bioassays with *Striga* and will analysed using LC-MS/MS (see Chapters 4 and 5) to quantify strigolactone and ABA content. These results will hopefully uncover the mystery of how maize *NCED* genes are involved in the germination stimulant formation of the parasitic weed *Striga*.

Acknowledgements

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Chapter 4

Arabidopsis thaliana germination stimulants for the root parasitic plant *Orobanche ramosa*

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Abstract

Germination stimulants are early signals produced by host plants in the interaction between plants and parasitic plants and these signal molecules have been identified in many plant species. *Arabidopsis thaliana* is the most important model in plant research and is a host of parasitic plants; its germination stimulants however are unknown. In our study, we aimed to identify the germination stimulants in the *Arabidopsis* root exudates for the parasitic plant *Orobanche ramosa* and elucidate their biosynthetic origin. Hereto we used *Arabidopsis* plants (wildtype and several mutants) and hairy root cultures to study the effect of inhibitors and phosphate on the formation of germination stimulants. This was analysed using a germination bioassay and an arbuscular mycorrhizal (AM) fungi branching assay to collect evidence for the existence of strigolactones. To investigate whether *Arabidopsis* also produces other signal molecules, we fractioned *Arabidopsis* root exudates and assessed their germination stimulating activity. Finally LC-MS/MS was used to directly detect the germination stimulants in the root exudates. Our results show that orobanchol is one of the germination stimulants in *Arabidopsis* root exudates but that these root exudates also contain several other molecules with germination inducing activity for *O. ramosa* seeds. Moreover, phosphate starvation promoted and fluridone inhibited orobanchol formation but both treatments did not affect the production of these other germination stimulants. Results with inhibitors and mutants suggest that these other stimulants are at least partly derived from the plastidic isoprenoid pathway.

Key words: Germination stimulants, orobanchol, *Orobanche ramosa*, *Arabidopsis*, inhibitors

Introduction

The root parasitic *Orobanche* spp. (*Orobanchaceae*) are obligate holoparasites that rob their plant host from water, minerals and nutrients and cause tremendous yield losses to the infested host plants (Parker and Riches 1993). The most commonly occurring species, *Orobanche ramosa*, infects a broad range of host plants including potato, tomato, eggplant, tobacco, melon, watermelon, cucumbers, and crucifers (Fernández-Aparicio et al. 2007), and also the model plant *Arabidopsis thaliana* (Goldwasser et al. 2000; Westwood 2000).

The germination of the seeds of *Orobanche* spp. is induced by chemical signals - so called germination stimulants - that are secreted into the rhizosphere by host plant roots (Bouwmeester et al. 2003). Of the germination stimulants for *Orobanche spp*. and the closely related *Striga* spp., the strigolactones have been most thoroughly studied. Strigolactones induce the germination of seeds of *Orobanche* as well as *Striga* spp. For example, strigol is the first identified strigolactone germination stimulant of *Striga hermonthica* from the non-host crop cotton (Cook et al. 1972) and was later also isolated from maize, sorghum and millet (Bouwmeester et al. 2003). Alectrol, now renamed to orobanchyl acetate, was identified in cowpea and red clover and is the germination stimulant of *Striga gesneroides* and *Orobanche minor* (Muller et al. 1992; Yokota et al. 1998). Orobanchol is the germination stimulant of *O. minor* and was identified in red clover root exudates (Yokota et al. 1998). Although these strigolactones are structurally related, their biosynthetic origin is still a mystery. Originally it was proposed that strigol and its analogs are terpenoids (Vail et al. 1990) or more specifically sesquiterpene lactones (Akiyama et al. 2005; Bradow et al. 1988). However, recently our group showed that the strigolactones are derived from the carotenoid pathway in maize, cowpea and sorghum (Matusova et al. 2005). This was concluded from studies using maize carotenoid mutants and the carotenoid biosynthesis inhibitors fluridone and amitrole.

In order to further investigate the biosynthesis of these host derived signal molecules, it would be better to use a model plant. *Arabidopsis* is a well known model for studying many aspects of plant biology, including the interactions between plants and pathogens, viruses, bacteria and nematodes (Baker et al. 1997; Buell 2002). Therefore it seems that *Arabidopsis* would also be an ideal candidate to explore the interaction of plants with *Orobanche* spp. Indeed, some years ago it was demonstrated that *Arabidopsis* can serve as a host for *Orobanche aegyptica*, *O. ramosa* and *O. minor* (Goldwasser et al. 2000; Westwood and Foy 1998). Since then several groups have used *Arabidopsis* as a model host to study *Orobanche* parasitation. For example, roots of *Arabidopsis* infected by *O. ramosa* were used to study the host plant response to parasitic plant attack and it was found that *O. ramosa* induces most of the general defense signalling pathways in a transient manner except salicylic acid-dependent defense (Vieira Dos Santos et al. 2003). *Arabidopsis* has also been used as a model to study the induction of germination by host root exudates. For example, 303 *Arabidopsis* ecotypes representing populations from 23 different countries were screened for their ability to induce germination of *O. aegyptiaca* (Goldwasser et al. 2002; Westwood and Foy 1998). All ecotypes tested induced seed germination of *O. aegyptiaca* and surprisingly there were no significant differences in the germination percentages that were induced by these ecotypes (Goldwasser et al. 2002; Westwood and Foy 1998). Moreover, in an *O. ramosa* germination screen of 13000 *Arabidopsis* M2 fast neutron mutants not a single consistent knock-down phenotype could be recovered (Goldwasser et al. 2002; Goldwasser and Yoder 2001). Finally, also a screen of a large number of flavonoid defective *Arabidopsis* mutant lines revealed no differences in the ability to stimulate germination (Westwood 2000).

All in all, the *Arabidopsis* work has not helped us forward very much in our understanding of germination stimulant biosynthesis. What could perhaps help us back on track is a solid hypothesis about what to expect. Considering that strigolactone germination stimulants have been reported to occur in the exudates of many different plants species, ranging from graminaea to dicots, and from tropical to more temperate climate plant species it is tempting to assume that also *Arabidopsis* is producing strigolactones. However, on the other hand, it was recently discovered that the strigolactones are host-finding signals for AM fungi (Akiyama et al. 2005; Besserer et al. 2006) and *Arabidopsis* just like other *Brassicaceae* is not a host of these symbiotic organisms (Glenn et al. 1985). The latter would suggest that *Arabidopsis* is perhaps less likely to produce strigolactones. This could also imply that *Arabidopsis* is unlikely to respond to phosphate starvation in the same way as hosts of AM fungi such as red clover and tomato do (Bouwmeester et al. 2007; López-Ráez et al. 2007; Yoneyama et al. 2007). That is, these species produce much higher concentrations of strigolactones under phosphate limited conditions, with the biological rationale being that under these conditions the attraction of AM fungi is even more important (Bouwmeester et al. 2007; Yoneyama et al. 2007).

Therefore the objective of our study was to answer the following questions: does the production of *Arabidopsis* germination stimulants respond to phosphate starvation and can the production of the *Arabidopsis* germination stimulants be blocked by carotenoid biosynthesis inhibitors. If this is the case they could be strigolactones and we would like to proof that using analytical techniques. If the answer to these two questions is negative, then what is the identity of the germination stimulant in *Arabidopsis*? And what is (are) the biosynthetic origin(s) of these other signalling molecules? We tried to answer these questions by taking different approaches. We treated intact *Arabidopsis* plants but also *Arabidopsis* hairy root cultures with chemical inhibitors, exposed them to phosphate starvation and investigated mutants in isoprenoid pathways. From all these treatments we collected root exudates which we used for column fractionation, after which the exudates and fractions were characterised using germination and/or AM hyphal branching assay and LC-MS/MS analysis. Our results show that *Arabidopsis* is indeed a suitable model for the study of this early stage in the host-parasite interaction provided the right conditions are used.

Materials and Methods

Plant materials

Arabidopsis thaliana seeds (ecotype Wassilewskija) were kindly provided by Dr. Andy Pereira (Plant Research International, the Netherlands) for intact plant bioassays. *Arabidopsis* seeds to initiate hairy root cultures (ecotypes Wassilewskija and Columbia) were obtained from Dr. Rene Geurts (Wageningen University, the Netherlands). Seeds of *Arabidopsis* T-DNA mutant *dxr* (DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase) and its background ecotype Columbia were obtained from NASC, UK. *Arabidopsis AtMECT* (4-diphosphocytidyl-2C-methyl-D-erythritol synthase) antisense transgenic plants and its background ecotype Columbia were obtained from Dr Okada, Kazunori (Tokyo Gakugei University, Japan). *O. ramosa* seeds were obtained from Dr. Jean-Pierre Palleau (Surgeres, Cetiom, France) and collected from heavily infected rapeseeds field in France in 2003. The seeds were cleaned, air-dried and stored in a glass vial at room temperature until used.

Chemicals

Orobanchol was kindly provided by Koichi Yoneyama (Utsunomiya University, Japan). GR24 was obtained from Binne Zwanenburg (Univerisity of Nijmegen, the Netherlands). Inhibitors fluridone and mevastatin were purchased from Duchefa (Haarlem, the Netherlands) and Sigma (St. Louis, USA) and fosmidomycin was from Leiden University (The Netherlands). C18 Sep-Pak columns were purchased from Waters (USA).

Growth conditions

For the fluridone experiment of intact plants and AM fungi branching assay, *Arabidopsis* seeds (Wassilewskija) were surface sterilized in 2 ml Eppendorf tubes with 1 ml 70 % ethanol for 30 seconds followed by washing immediately with sterile demineralised water. Then seeds were rinsed in 2% sodium hypochlorite plus 0.02 % Tween 20 for 15 minutes. Subsequently, the seeds were thoroughly washed three times in sterile demineralised water to clean out the detergent and sprinkled on moistened filter paper (Whatman UK) in 9 cm Petri-dishes and incubated at 4 ºC in the dark. Three days later, the Petri-dishes were transferred to a climate room with a 16/8-h photoperiod at 28 $\mu mol.m^{-2}.s^{-1}$ at 25 ºC for 2 days. The seedlings were grown in hydroponics using standard nutrient solution as described (Tocquin et al. 2003).

For hairy root culture, prior to germination on 0.5 MS with 0.2% top agar, the seeds of *Arabidopsis* ecotype Wassilewskija and Columbia were surface sterilised (5 minutes in 4% bleach), followed by four 10-min rinses in distilled water. The seeds were allowed to air dry and subsequently spread evenly on the surface of the 0.5 MS medium with top agar in the plates. After that the plates were incubated for 3 days at 4ºC in darkness. Then the plates were transferred to a growth chamber at 25ºC (Tocquin et al. 2003) under 16h photoperiod (800-900 Lux) and ambient relative humidity. The seedlings were grown in the chamber for 6 days and then 10 seedlings were transferred to a new Petri-dish (9cm) on 0.5 MS medium with a half round filter paper on the medium. Ten seedlings were distributed evenly in the middle of the plates and the root of seedlings placed on the filter paper. Subsequently the hypocotyls of each plant was cut by using a scalpel carrying *Agrobacterium rhizogenes* MSU440 strain transformed with the pRed root binary vector, which contains dsRED1 (Clontech-Takara Bio Europe, France) under control of the *Arabidopsis* UBQ10 promoter within the T-DNA borders (kindly provided by Dr. Rene Geurts, Laboratory for Molecular Biology, Wageningen University, the Netherlands). Then the roots were removed and the plates were partly (2/3) closed with parafilm to enable aeration. Plates were incubated in a vertical position in a growth chamber at 21ºC (16 hours light, 8 hours dark). Three days later, the seedlings were transferred to 0.5 MS plate containing 300 $ug.ml^{-1}$ cefotaxime. Transgenic roots (showing dsRED fluorescence) were observed under the Leica MZIII fluorescence stereomicroscope with appropriate filter setting after 7 days.

Root cultures were made by excising a piece of 1 – 2 cm of transformed roots on solid B5 media (B5 medium with vitamin, 30 $g.l^{-1}$ sucrose, pH 5.8, 0.8% agar) containing 300 $\mu g.ml^{-1}$ cefotaxime in the dark at 21ºC for 2 weeks. Then one piece of transformed root was inoculated in liquid ARC medium (MS salts without vitamin, 30 $g.l^{-1}$ sucrose, 0.8 % agar, $2ml.l^{-1}$ vitamix stock (vitamix: 100 mg myo-inositol, 5 mg vitamin B1, 0.5 mg vitamin B6, 0.5 mg nicotinic acid, 1 mg glycine, 0.05 mg biotin per liter) (Márton and Browse 1991) in 250 ml Erlenmeijer flasks. Roots were subcultured by transferring 0.2 g of roots into 100 ml fresh ARC medium. The medium was refreshed every three to four weeks.

For the experiments with *Arabidopsis* mutants, the homozygous antisense *AtMECT* mutant and heterozygous T-DNA knockout mutant (*dxr*) and their controls (Columbia) were first grown in the green house and seeds of all lines were collected simultaneously. Homozygous DXR mutants were identified by PCR reactions using forward primer: CTTTAATCGCAGGTGGTCCT and reverse primer: AAAGCTCCACCAGATGCAGT. Briefly, about 100 *Arabidopsis* mutant seeds for each mutant and their wildtypes were used. Seed sterilization, dormancy relief and germination and plating followed the method described before (Goldwasser and Yoder 2001).

Root exudate collection

For *Arabidopsis* root exudate collection, phosphate starvation and fluridone treatments of intact plants and AM fungi branching assay, *Arabidopsis* seedlings were first grown hydroponically under normal phosphate condition for two weeks to insure plants produced normal healthy roots, and then plants were gently washed with tap water and treated in four different nutrition solutions in new containers: 130 µM phosphate, without phosphate, 130 µM phosphate plus 0.01 µM fluridone and without phosphate plus 0.01 µM fluridone. Six plants were used for each treatment. After 5 days, plants were transferred to 10 ml glass tubes with 10 ml demineralised water (and 0.001 µM fluridone for the fluridone treatment to ensure the inhibition of carotenoid biosynthesis is retained during root exudates collecting). Root exudates were collected from single plants separately for all the treatments after 24 hours. To test any inhibitory effect of the low concentration fluridone used during exudates collection on the germination of *O. ramosa*, two extra controls were used in the germination bioassay: a combination of root exudate of control plants (in several dilutions) and fluridone (0.001 µM) and a combination of fluridone (0.001 µM) and GR24 (0.00001mg.l^{-1}). The sub-optimal concentration of GR 24 ensures that any negative inhibitory effect of the fluridone on the germination of *O. ramosa* seeds can be detected with great sensitivity.

Thirty ml of these *Arabidopsis* root exudates were further fractionated using C18 Sep-Pak (Water USA) columns. Sep-PAK columns were first conditioned by washing them with 5 ml of methanol, 5 ml of acetone and 5 ml demineralised water. Then 30 ml of crude undiluted root exudate from each sample was loaded onto the columns. The columns were washed with 5 ml of demineralised water to remove water soluble compounds. The stimulants were eluted using 3 ml of water with increasing concentration of acetone (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%). Half a millilitre of each sample was used to prepare the germination assay. Since we found that acetone can inhibit *O. ramosa* seed germination, we removed actetone by first adding the same amount of demineralised water according to the amount of acetone present in each sample and then using a vacuum centrifuge to remove the acetone in the samples. GR24 (0.0001 mg.l^{-1}) was mixed with an equal volume of acetone and was used as a control to check for acetone removal during the vacuum evaporation. After that all the elutions were diluted 10 times to the original exudate volume for the germination assay.

For the experiment of inhibitors using hairy root culture, hairy roots (0.2 g) were first subcultured into fresh ARC medium and grown for 5 days. These cultures were then transferred to fresh ARC medium with inhibitors (fosmidomicin 100 µM, mevastatin 5 µM or fluridone 5 µM). Each treatment and control (non-treated root cultures) consisted of three replicates. After 5 days, root cultures were transferred again into fresh ARC medium. Exudates were collected after 2 hours for all the treatments and controls. Before testing on *O. ramosa* seeds, root exudates were diluted 2-, 4-, 8- and 16-fold to avoid saturation of the

germination response. The positive control GR24 (0.0001 $mg.l^{-1}$) and the negative control fresh ARC medium were also applied.

For LC-MS/MS analysis, the cultures were treated without phosphate and with and without fluridone (0.1 µM). Each treatment and control (non-treated root cultures) consisted of two replicates. The samples were collected after 2 hours. One hundred millilitre root exudate from each treatment was purified and concentrated 40-fold using a C18 Sep-Pak (Water USA) columns with the same procedure as above but using 100% acetone as a one-step eluent.

Germination assay

Root exudates obtained from all the experiments were assayed using a germination bioassay. The seeds of *O. ramosa* require preconditioning for a certain period of time at optimal temperature and moisture before the seeds response to the germination stimulants (Joel et al. 1995). Preconditioning start from surface sterilizing seeds by soaking in 2% sodium hypochlorite plus 0.1 % Tween 20 for 5 minutes. Seeds were then washed three times with sterile demineralised water, and excess water was removed by filtration through a Büchner funnel. The sterile seeds were allowed to air dry for 2 hours and subsequently approximately 50-100 seeds were sprinkled on 9-mm diameter glass-fibre filter paper (Sartorius Germany) discs. These discs were placed in 9-cm diameter Petri-dishes with a filter paper (Waterman, UK) moistened with 2.7 ml demineralised water in the bottom. The Petri dishes were sealed with parafilm and wrapped in aluminum foil and placed in a growth chamber (21ºC) for 12 days. Before applying root exudates, the discs with the seeds were dried on sterile filter paper for 3 minutes and transferred to a new Petri dish with a filter paper ring (diameter of 9 cm), moistened with 0.9 ml sterile demineralised water to keep a moist environment inside the Petri dishes during the germination bioassay. Fifty µl of the samples to be tested were applied to triplicate discs. GR24 (0.0001 mg/ l) was used as a positive control and the sterile ARC medium or demineralised water (depending on the experiment) as a negative control in each germination assay. Seeds then were incubated at 25 ºC in darkness for 7 days. After 7 days, germination was scored using a binocular microscope. Seeds were considered to be germinated if the radical protruded through the seed coat (Mangnus and Zwanenburg 1992). In addition, although we obtained more or less the same fresh weight of roots for the different treatments in plants and hairy root cultures, germination rates were corrected for root fresh weight (germination / weight ratio).

For the T-DNA (*dxr*) mutant and antisense mutant (*AtMECT*), twenty-four seedlings for each mutant and its wildtype were selected and placed in 96 well tissue culture plates (Greiner bio-one, Germany) including two extra controls plates (only preconditioned *O. ramosa* seeds without host plants or with 50 µl 0.0001 $mg.l^{-1}$ GR24). The details of this method were described by Goldwasser and Yoder (Goldwasser and Yoder 2001).

Mycorrhizal branching assay

For the AM fungi branching assay, based on a protocol developed by (Buee et al. 2000), samples were purified by using a Sep-Pak column with 100% acetone elution. The eluted samples were dilute to 50% acetone with distilled water to prevent the inhibition of the AM fungi branching by 100% acetone. GR24 (10^{-9} M) was used as a positive control and 50% acetone was used as negative control in the experiments.

UPLC-MS/MS analysis

The germination stimulants for root parasitic plants *Orobanche* produced by *Arabidopsis* hairy root cultures were examined using Ultra Performance Liquid Chromatography coupled to tandem Mass Spectrometry (UPLC-MS/MS). Analyses were performed using a Waters Micromass Quattro Premier XE tandem mass spectrometer (Waters, USA) equipped with an ESI source and coupled to an Acquity UPLC system (Waters, USA). Chromatographic separation was performed on an Acquity UPLC BEH C18 column (100 x 2.1 mm, 1.7 µm) (Waters, USA), applying a water/acetonitrile gradient. The gradient started at 20% acetonitrile for 1 min, followed by a 5-min ramp to 70% acetonitrile which was maintained for 1 min, followed by a 0.20 min ramp back to 20% acetonitrile and the column was equilibrated at this solvent composition for 1.8 min. Total run time was 9 min. The column was kept at 50°C and run at a flow-rate of 0.400 $ml.min^{-1}$ (sample injection volume: 30 µl). The mass spectrometer was operated in positive electrospray ionization (ESI) mode. The nebuliser and desolvation gas flows were 50 and 800 $l.h^{-1}$, respectively. The capillary voltage was set at 2.7 kV, the cone voltage at 20 V, the source temperature at 120ºC and the desolvation gas temperature at 450ºC. Fragmentation was performed by collision induced dissociation with argon at $3.0x10^{-3}$ mbar. The collision energy was optimized for each compound. Multiple reactions monitoring (MRM) method was used for detection of strigolactones in *Arabidopsis* hairy roots exudates. The conditions of MRM were optimized according to the setting of known strigolactones. Data acquisition and analysis were performed using MassLynx 4.1 software (Waters, US).

Statistcal analysis

All the experiments were performed in a completely randomized design. In the inhibitor experiment in *Arabidopsis* plants, individual treatments were replicated 6 times, whereas in the inhibitor treatment in *Arabidopsis* hairy root culture, Individual treatments were replicated 3 times under normal phosphate conditions and two times under phosphate starvation. Germination data from all experiments were transformed by taking the arcsine of the square root of the proportion of germinated seeds and then were subjected to analysis of variance (ANOVA) to determine main effects. Where significant differences were detected, treatment means were separated using an LSD test. For the experiments of *Arabidopsis* mutants, mutants and wildtypes were replicated 24 times and the Chi-square method was used for statistical analysis. All results were analyzed using GenStat Release 9.2 (PC/Windows XP), VSN international Ltd, UK.

Results

Effects of phosphate starvation and fluridone treatments

Phosphate starvation of the host plant has been reported to promote *O. minor* seed germination in red clover root exudates and *O. ramosa* germination in tomato root exudate (López-Ráez et al. 2007; Yoneyama et al. 2007). Thus, we were curious to know if there is the same effect on *O. ramosa* seed germination if *Arabidopsis* plants are grown in the absence of phosphate. Under these conditions, *Arabidopsis* plants were smaller than the plants grown under normal phosphate conditions. In addition, leaves were light green and roots increased in the length and frequency of root hairs. *O. ramosa* seeds were very sensitive to *Arabidopsis* root exudates and the exudates required 16-fold dilution to avoid saturation of the germination response. The positive control GR24 (0.0001 $mg.l^{-1}$) induced seed

germination up to 82% and the negative control (demineralised water) did not induce any germination (Fig. 1). Root exudates collected from the phosphate starvation treatment induced significantly higher germination (average 49%) of *O. ramosa* seed germination than the normal phosphate control (average 21%) ($P<0.01$) (Fig. 1). The effect of phosphate was still observed when *Arabidopsis* plants were treated with fluridone. *Orobanche* seed germination significantly increased from 11 % in the normal phosphate control to 28% under phosphate starvation ($P<0.05$) (Fig. 1). Within both phosphate treatments, fluridone significantly reduced the exudate induced germination of *Orobanche* seeds by 43% in the normal phosphate control ($P<0.05$) and 48% under phosphate starvation ($P<0.05$) (Fig. 1).

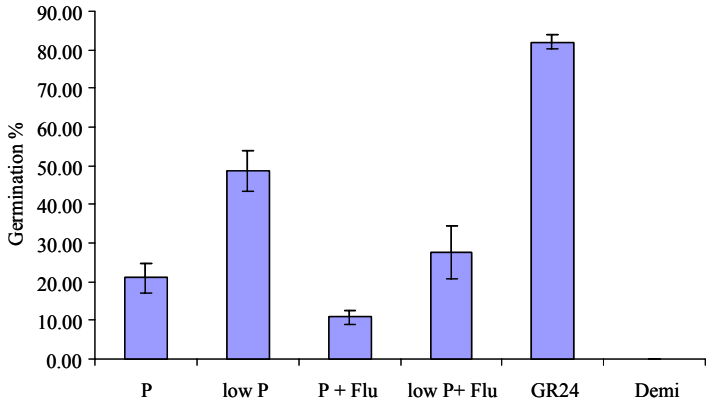

Fig. 1 Effect of phosphate starvation and fluridone treatments in *Arabidopsis* plants grown hydroponically (ecotype Wassilewskija) on the induction of germination of *O. ramosa* seeds by root exudates. The *Arabidopsis* plants were first grown two weeks hydroponically then treated with 0.01 µM fluridone. After 5 days, the root exudates were collected for 24 hours in demineralised water. The root exudates were diluted 4-, 8- and 16-fold before being assayed in a seed germination bioassay for which three discs of approximately 50-100 *O. ramosa* seeds per replicate were used. P, normal phosphate; low P, phosphate starvation; P + Flu, combination of normal phosphate and fluridone; low P + Flu, combination of phosphate starvation and fluridone; GR24 ($0.0001 mg.l^{-1}$), the synthetic germination stimulant used as positive control; Demi demineralised water; Bars represent means of 6 plants and error bars represent the standard error of the means.

Fractioning of Arabidopsis *plant root exudates*

To investigate the presence of more than one germination stimulating compounds, *Arabidopsis* plants again were treated with phosphate starvation and fluridone. Root exudates from all four treatments (zero phosphate, normal phosphate, plus or minus fluridone) were then separated into 10 fractions obtained by eluting the exudates from a Sep-Pak C18 Column using a stepwise-gradient of acetone in water. The positive control GR24 ($0.0001 mg.l^{-1}$) induced high germination, up to 80% (Fig. 2). High germination (80%) was also obtained with the GR24/acetone control confirming that the acetone was effectively removed from the root exudate fractions by vaccum centrifugation. The negative control (demineralised water) did not induce any germination (Fig. 2). Six (from 10% to 60% acetone) from the total of ten fractions induced germination of *O. ramosa* seeds for all treatments including normal phosphate (Fig. 2A), low phosphate (Fig. 2B), low phosphate with fluridone (Fig. 2C). Within these six peaks, three (40%, 50%, 60%) showed a clear reduction of *O. ramosa* seed germination in phosphate and fluridone treated root exudates (Fig. 2A, 2C). Interestingly the 40%, 50% and 60% fractions fit well to the one generated by using a standard of orobanchol and following the same fractioning procedure (Fig. 2D). These results

suggest that orobanchol or a compound with similar chemical properties could be one of the germination stimulants in *Arabidopsis* root exudates.

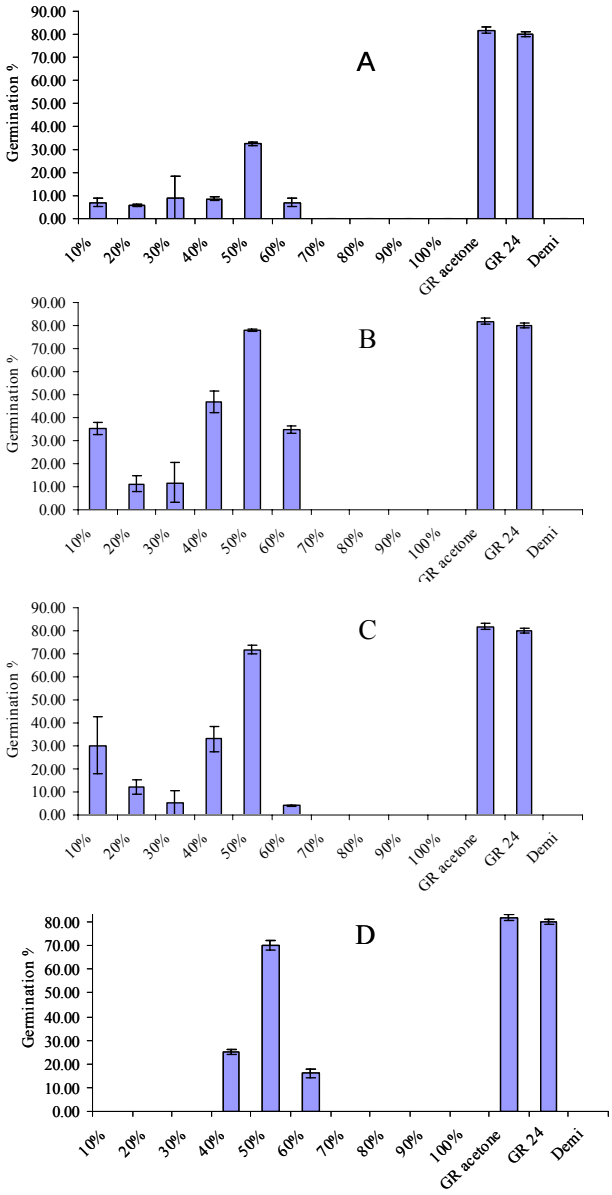

Fig. 2 Germination of *O. ramosa* seeds as induced by C18 Sep-Pak fractioned *Arabidopsis* root exudates. A, from normal phosphate plants B, from phosphate starved plants, C, from phosphate-starved and fluridone treated plants, D, standard orobanchol. Root exudates were fractionated under the Sep-Pak column conditions and assayed in a germination bioassay as described in the materials and methods. The synthetic germination stimulant GR24 ($0.0001 mg^{-1}$) was used as a positive control. GR24 with acetone was used as a control to monitor if the acetone in the root exudates fractions had been removed completely after vacuum centrifugation. Demi represents demineralised water. Error bars represent the standard error of the mean (n = 3 triplicate discs with *O. ramosa* seeds).

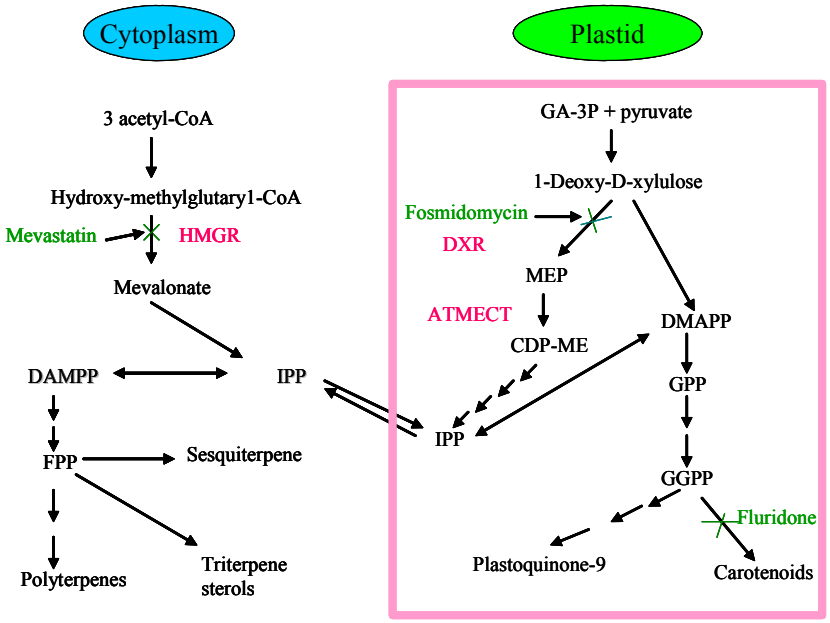

Fig. 3 Schematic representation of isoprenoid biosynthesis pathways in plants with inhibitors and knockout mutants used in the present paper indicated. Mevastatin specifically inhibits the cytosolic enzyme HMGR (hydroxyl-mehylglutaryl-CoA reductase); fosmidomycin inhibits the plastidic enzyme DXR (1-deoxy-D-xylulose 5-phosphate reductoisomerase) and fluridone inhibits the production of carotenoids by blocking the enzyme phytoene desaturase. DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosophate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate, MEP, 2-C-methyl-D-erythritol 4-phosphate, CDP-ME, 4-(cytidine 5-diphospho)-2-C-methyl-D-erythritol); AtMECT, 4-diphosphocytidyl-2C-methyl-D-erythritol synthase. Redrawn from (Okada et al. 2002; Schwender et al. 2001).

Effect of inhibitors in Arabidopsis *hairy roots*

The above results show that some germinating molecules were not regulated by phosphate nor inhibited by fluridone (Figs 1,2). These molecules should belong to another class of germination stimulating compounds but not the strigolactones. Since the isoprenoid biosynthetic pathways produces many biologically active compounds and these compounds could be the precursors for the formation of germination stimulants, we used three inhibitors: fosmidomycin, which specifically blocks the enzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) in the plastidic pathway; mevastatin, which specifically blocks the enzyme (hydroxyl-mehylglutaryl-CoA reductase (HMGR) in the cytosolic pathway; fluridone, which specifically blocks carotenoid biosynthesis in the plastids (Fig.3). These three inhibitors themselves (concentrations used in the experiments) do not have any effect on *O. ramosa* seed germination as was checked by mixing GR24 and the inhibitors and scoring germination (data not shown). Fosmidomycin treatment of the hairy root cultures showed a significant decrease of *O. ramosa* seed germination compared with untreated root cultures in all dilutions ($P<0.01$) (Fig. 4A). This effect was not visible in the undiluted root exudate because germination was saturated (Fig. 4A). Fluridone treated root exudates did not show any change of *O. ramosa* seed germination (Fig. 4B). Finally, mevastatin treated root cultures showed a significant increase in *O. ramosa* seed germination ($P<0.01$) (Fig. 4C). Fig. 4 also showed that GR24 (0.0001 $mg.l^{-1}$) induced seed germination up to 77% and the negative control (ARC medium) virtually not (average 1%) (Fig. 4).

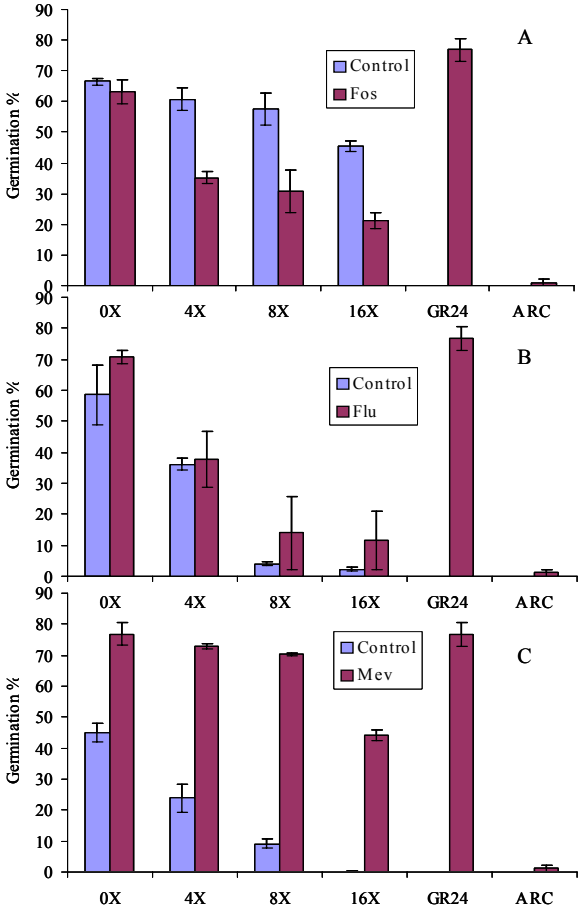

Fig. 4 The effect of inhibitor treatments in *Arabidopsis* hairy root cultures (ecotype Columbia) on exudate-induced germination of *O. ramosa* seeds. *Arabidopsis* hairy root cultures (about 0.2 g fresh weight) were first grown 5 days after inoculation in ARC medium under normal phosphate conditions and then treated with A, 100 µM fosmidomicin, B, 5 µM mevastatin or C, 5 µM fluridone. After 5 days, the root cultures were transferred to fresh ARC medium and the root exudates were collected for 2 hours. After collection, the root exudates were diluted 4-, 8- and 16-fold before being assayed in an *O. ramosa* seed germination-bioassay. The synthetic germination stimulant GR24 (0.0001mgl^{-1}) was used as a positive control. ARC is the culture medium and was used as a negative control. Error bars represent the standard error of the means of three independent cultures.

Effect of fluridone on Arabidopsis *hairy roots under phosphate starvation*

Above we showed that phosphate starvation promotes *O. ramosa* seed germination in the root exdates from *Arabidopsis* plants and the above experiment showed that there was no difference in seed germination of *O. ramosa* between the cultures treated with fluridone and non-treated cultures under normal phosphate conditions. The following experiment was to test if under phosphate starvation, fluridone would inhibit the production of germination stimulants in *Arabidopsis* hairy root culture. Fig. 5 shows that root exudates collected from fluridone treatment induced significantly lower *O. ramosa* seed

germination than that of the control treatment when the root cultures were diluted 8- or 16-fold (P<0.01).

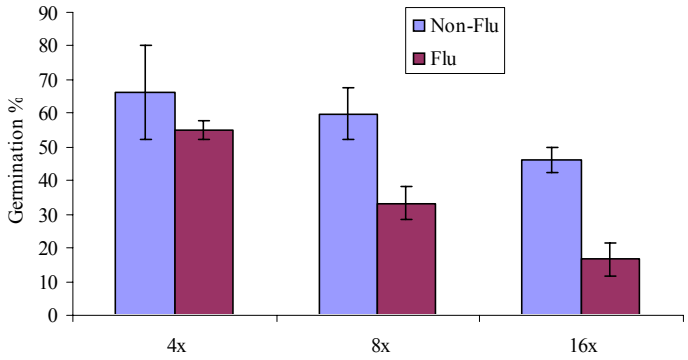

Fig. 5 The effect of fluridone treatment in *Arabidopsis* hairy root cultures (ecotype Columbia) on exudate-induced germination of *O. ramosa* seeds. *Arabidopsis* hairy root cultures (about 0.2 g fresh weight) were first grown 5 days after inoculation in ARC medium under normal phosphate conditions and then transferred to fresh medium without phosphate and with/without 0.1 µM fluridone. After 5 days, the root cultures were transferred into fresh medium and the root exudates were collected for 2 hours. After collection, the root exudates were diluted 4-, 8- and 16-fold before being assayed in an *O. ramosa* seed germination-bioassay. The synthetic germination stimulant GR24 ($0.0001 mgl^{-1}$) was used as a positive control. ARC is the culture medium and was used as a negative control. Error bars represent the standard error of the means of two independent cultures.

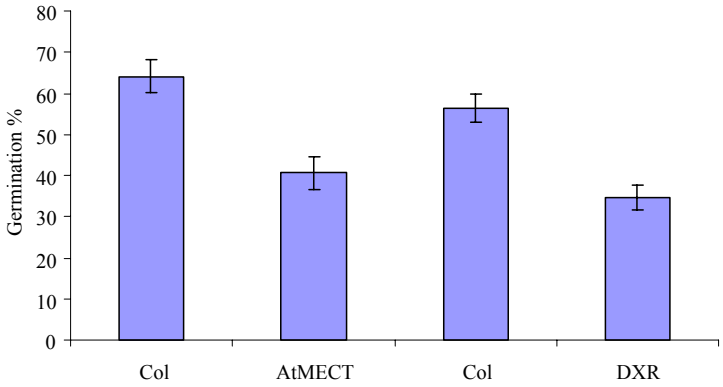

Fig. 6 Induction of *O. ramosa* seed germination in a 96-well plate assay by *Arabidopsis* wild type and mutants. *Arabidopsis* wild type Columbia and antisense *AtMECT* plants and *DXR* were first grown in agar for 6 days after three days cold treatment and then transferred to 96 wells where preconditioned *O. ramosa* seeds were placed on the bottom. After 7 days, the germination percentage of *O. ramosa* seeds was determined. Two wildtypes were used as controls (col), the left one is for *AtMECT* and the right one is for *DXR*. Bars represent means of 24 replicates and error bars represent the standard error of the means.

Arabidopsis mutants in the plastidic isoprenoid pathway

Because fosmidomycin negatively affected the germination stimulating activity in *Arabidopsis*, a number of existing plastidic pathway T-DNA knockout *Arabidopsis* mutants were tested. Of these, a 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase antisense mutant (*AtMECT*) (ecotype Columbia) (Okada et al. 2002) (Fig. 3) showed a significant reduction of the seed germination of *O. ramosa* compared with the wild type (P<0.01) (Fig. 6). In addition, a T-DNA insertion *DXR* mutant (Fig. 3) also induced significantly less seed germination of *O. ramosa* than the wildtype (P<0.01) (Fig. 6).

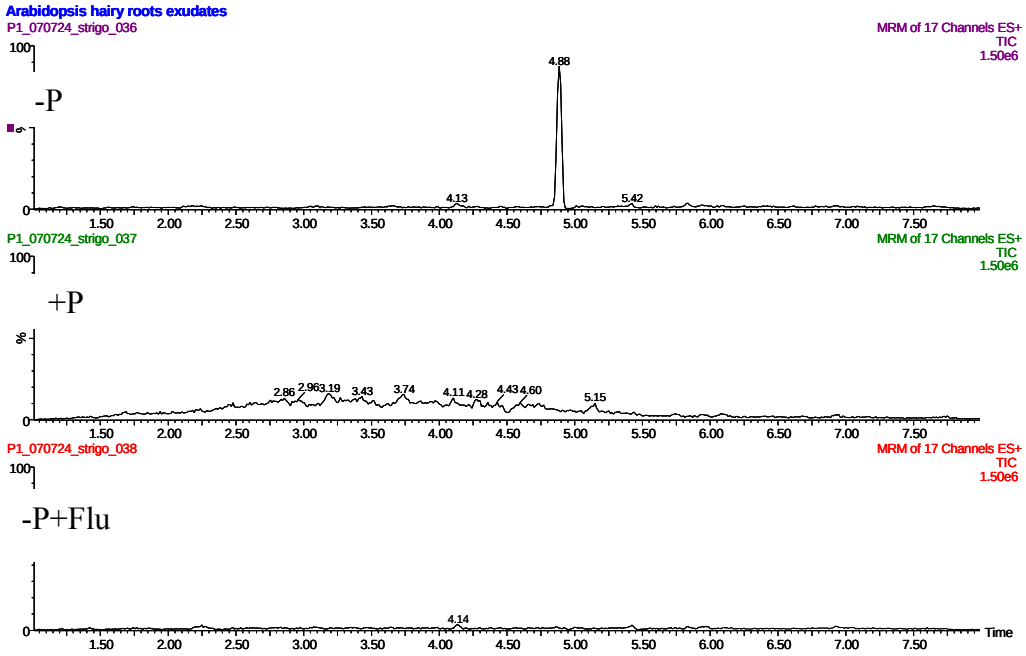

Fig. 7. A, LC-MS/MS analysis using multiple reaction monitoring (MRM) of *Arabidopsis* hairy root exudates growing under phosphate starvation (-P), with sufficient phosphate (+P) and under phosphate starvation/fluridone-treated (-P + Flu).

Identification of orobanchol using LC/MS/MS

Multiple reaction monitoring (MRM) in positive ESI mode was used to search for strigolactones in *Arabidopsis* hairy roots root exudates. The MRM transitions were set according the MS-MS spectrum of standard strigolactones. The sodium adduct ion and the "protonated" ion $[M+H]^+$ were the most abundant in full-scan positive mass spectrums of known strigolactones used as standards. Therefore, those ions were selected as precursor ions for collision-induced degradation (CID). In the MS-MS spectrum of the sodium adduct ion $[M + Na]^+$ with *m/z* 369 and the parent ion $[M+H]^+$ with *m/z* 347 of the standard orobanchol the fragment ions at *m/z* 272 $[M+Na\text{-D ring}]^+$, *m/z* 233 $[M\text{-D ring} – 16]^+$ and *m/z* 97 (D-ring) were the most abundant at the collision energy 15, 10 and 18 eV, respectively (data not shown). Therefore, for the multiple reaction monitoring (MRM) the transitions of *m/z* 369>272; 347>233; 347>97 were selected for orobanchol. In the MRM chromatograms of phosphate-starved *Arabidopsis* hairy root an intense peak was detected for channels *m/z* 369>272; 347>233; 347>97 at the retention time 4.88 min which matches with the retention time of an orobanchol standard (Fig. 7A-B). Standard addition of authentic orobanchol to the samples confirmed that this compound in phosphate-starved *Arabidopsis* hairy root exudates was indeed orobanchol (data not shown). The identity of orobanchol was also confirmed by comparison of MS/MS spectra of the compound eluted at 4.88 min with the corresponding standard (data not shown).

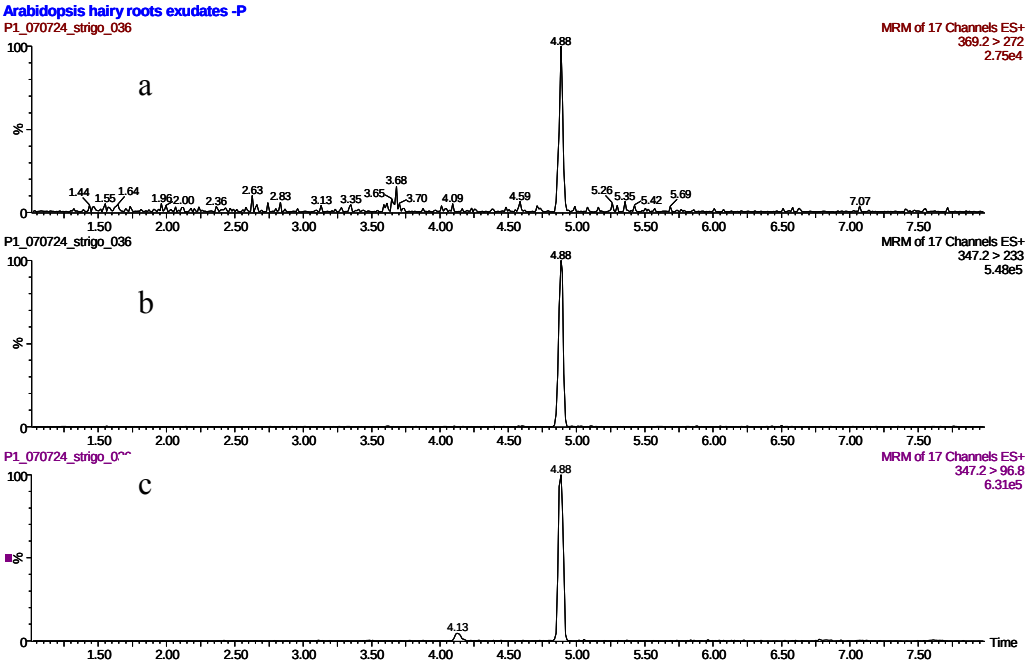

Fig. 7. B, Multiple reaction monitoring (MRM) chromatograms of hairy root exudates from plants growing without phosphate. The chromatograms a, b and c are for monitoring the transitions of m/z 369>272, m/z 347>233 and m/z 347>97, respectively, for the detection of orobanchol.

Arabidopsis *root exudates did not induce AM hyphal branching*

Germination stimulant strigolactones can also induce AM hyphal branching (Akiyama et al. 2005). Therefore it will provide an indication of the presence of strigolactones if *Arabidopsis* root exudates would have this activity. Root exudates collected from different treatments like above were applied to the AM germinated spores. As expected, the positive control GR24 (10^{-9} M) induced much higher AM branching than the negative control (data not known). Unfortunately, the expected increased in AM hyphal branching was not observed in the phosphate starvation treatment compared with normal phosphate conditions (data not shown). Also fluridone treatment did not show any decrease of hyphal branching numbers compared with the control. (data not shown).

Discussion

Our results show that orobanchol (stigolactones) is one of the germination stimulants for *O. ramosa* in *Arabidopsis* roots. Its was detected in hairy root culture exudate once, using UPLC-MS/MS (Fig. 7). Germination bioassays suggest it is also present in plant root exudates (Fig. 1) and other hairy root culture experiments (Fig. 5, 7) but so far at too low concentrations to be detected by UPLC-MS/MS. Orobanchol has also been identified from red clover root exudates stimulating *O.* minor seed germination (Yokota et al. 1998). All these evidences indicate that orobanchol is probably a natural germination stimulant for *Orobanche* spp. and may be present in other plant species. Furthermore, strigol, another strigolactone germination stimulant for *Striga* spp., was isolated from both root culture of *Menispermum dauricum* (Yasuda et al. 2003) and root exudates of different plant species (cotton, maize, sorghum, and proso millet) (Butler 1995; Cook et al. 1972; Hauck et al. 1992; Siame et al. 1993). Thus strigolactones seem to

be a class of general germination stimulating compounds that are biosynthesised in and secreted from plant roots for parasitic *Striga* and *Orobanche* spp.

Interestingly, under the phosphate starvation, the germination of *O. ramosa* seeds was promoted suggesting that low phosphate conditions can enhance the orobanchol formation in *Arabidopsis* (Fig. 1, 5). Our results agree with the ones from Yoneyama et al and Lopez-Raez et al, who demonstrated that phosphate deficiency increased the orobanchol concentration in red clover root and tomato exudates (López-Ráez et al. 2007; Yoneyama et al. 2007). Moreover, the fact that we did not detect the orobanchol by UPLC-MS/MS in the root culture under normal phosphorus conditions (data not shown) also supports the above conclusion. This conclusion could probably explain that some researchers did not find indications for the presence of strigolactones in the *Arabidopsis* root exudates, because they used normal phosphate conditions (Denev et al. 2007). The mechanism of how phosphate regulates strigolactone formation is still unknown. Possibly, the phosphate-starvation induced morphogenetic changes in the *Arabidopsis* roots that we observed (increase in the length and frequency of root hairs) enhance strigolactones production in the root exudates. In addition, clearly, under the normal phosphorus conditions only and with fluridone treatments *O. ramosa* seeds can still germinate to some extent (average 21% and 11% respectively) (Fig. 1). The logical explanation of these results is either phosphorus and fluridone concentration are not high enough to completely inhibit strigolactone formation in the plant roots or plant roots can produce other germinating molecules which phosphorus and fluridone have no effect or fluridone also has some effect on these unknown molecules production.

The strigolactones have been identified as important rhizosphere plant signals that induce hyphal branching in AM fungi (Akiyama et al. 2005; Besserer et al. 2006). Although AM fungi can form an intimate symbiotic association with about 80% of land plant species, *Arabidopsis* is not a host plant of AM fungi. *Arabidopsis* also lacks a DMI3 homolog, a class of genes which is well conserved among host plants that interact with mycorrhizal fungi (Mitra et al. 2004) and the lack of a Ca^{2+} response as intracellular messenger is essential for the establishment of the AM symbiosis (Navazio et al. 2006). In spite of being a non host plant, *Arabidopsis* still produces a strigolactone (Fig. 7). Nevertheless, we did not detect AM fungi branching using *Arabidopsis* root exudates that were produced under phosphate starvation. Possibly, strigolactone concentration produced in *Arabidopsis* root exudates are too low to evoke the AM fungi branching, or there are branching inhibitors in the root exudates. Subsequently the interesting question is why *Arabidopsis* produces this supposed AM fungi signalling compound? Possibly, strigolactones have other unknown biological functions in the whole plant kingdom.

Fractioning of *Arabidopsis* root exudates demonstrated an additional peak that also has germination inducing activity for *O. ramosa* seeds (Fig. 2), which indicate that there are one or more additional germination stimulants in the *Arabidopsis* root exudate besides orobanchol. The results were supported by our chemical inhibitor experiments. In these experiments, three inhibitors were used in two metabolic pathways for isoprenoid biosynthesis: the cytosolic mevalonate (MVA) pathway and the plastidic non-mevalonate (MEP) pathway. We found that *O. ramosa* seed germination were inhibited to some extent by fosmidomycin (Fig. 4A); no effect by fluridone (Fig. 4B); however promoted by mevastatin (Fig. 4C) under normal phosphorus conditions. The results indicate that these active molecules are not derived from the carotenoid pathway but somewhere upstream in the plastidic pathway. A surprising result was the strong increase of seed germination after mevastatin treatment of the hairy root culture (Fig. 4C). This can possibly be explained by the fact that exchange of isoprenoid precursors (IPP has been

postulated) occurs between the MVA and MEP pathways (Fig. 3). It has been reported that blocking of the MVA pathway in the cytosol by mevastatin will stimulate the activity of the MEP pathway in the plastids to compensate for the lack of isoprenoid precursor in the cytosol (Hemmerlin et al. 2003). Apparently, the upregulation of the MEP pathway leads to a higher production of non-strigolactone germination stimulant. This metabolic compensation between the two pathways has also been found in tobacco bright yellow-2 cells, in which two inhibitors (mevinolin with the same effect as mevastatin and fosmidomycin) were used to block the MVA and MEP pathways. Incorporation studies showed that sterols, normally derived from MVA, will be synthesized from precursors derived from the MEP pathway when treated with mevinolin. The reverse was also detected by incorporation studies in which plastoquinone, representative of the plastidial isoprenoids, is produced from MVA pathway precursors when cells are treated with fosmidomycin (Hemmerlin et al. 2003). Under normal phosphate conditions, this metabolite exchange between cytosolic and plastidic pathways of isoprenoid biosynthesis in *Arabidopsis* is unidirectional transport of intermediates across the chloroplast envelope membrane to the cytosol (Bick and Lange 2003; Laule et al. 2003). This unidirectional compensation was also found in snapdragon flowers where transfer of plastid-produced IPP occurred undirectionally from the plastids to the cytosol (Dudareva et al. 2005). Our work on the *Arabidopsis* mutants *DXR* and the antisense *AtMECT* plants confirmed that an early block in the plastidic isoprenoid pathway decreases the production of this unknown germination stimulant (Fig. 6). Moreover other research groups were also using the same approach (inhibitors mevinolin like mevostatin in cytosol, fosmidomycin, and norflurazon like fluridone in *Arabidopsis* plants) and predicted that *Arabidopsis* germination stimulants are produced from an earlier intermediate of the plastidic pathway (Denev et al. 2007). We however have to keep in mind that this is only half of the truth because these experiments were performed under normal phosphate condition and therefore there are no or less strigolactones produced. Finally, Goldwasser and Yoder concluded that the *Arabidopsis* germination stimulants could be more than one molecule when they had screened a large collection of *Arabidopsis* mutants and a number of ecotypes for mutants in the ability to induce germination of *Orobanche* seeds, and did not find a single consistent mutant (Goldwasser et al. 2002). This conclusion indicates that using single *Arabidopsis* knockout mutant is not a good strategy to detect the relevant genes in the biosynthesis of strigolactones, unless a quick and reproducible method can be designed to separate strigolactones from other bioactive molecules (if a germination bioassay is used). A screen using LC-MS/MS (which would be selective for the strigolactones) is currently impossible due to the extremely low production by *Arabidopsis*.

The biosynthetic origin of *Arabidopsis* germination stimulants is still not yet completely understood. The reason could be on one hand the amount of germination stimulants from *Arabidopsis* root exudates is significantly lower than the exudates of better known plants, like carrot and tobacco (Humphrey et al. 2006), on the other hand, in general germination stimulants are often unstable for example, strigolactones, which makes the isolation and characterization of these compounds quite complicated (Sato et al. 2005). The strigolactones from many plant species have been intensively studied by several research groups (Akiyama and Hayashi 2006; Besserer et al. 2006; Humphrey et al. 2006; López-Ráez et al. 2007; Matusova et al. 2005; Yokota et al. 1998; Yoneyama et al. 2007), the natural precursors of these compounds so far however have not been determined. Our group demonstrated that (+)-strigol is derived from the carotenoid biosynthetic pathway by using carotenoid inhibitors and carotenoid metabolism-impaired maize mutants (Matusova et al. 2005). If this conclusion is right, orobanchol must be produced from the carotenoid pathway. The most likely explanation will be that strigolactones firstly

are synthesized in the plastids from carotenoids and later – likely after cleavage –are translocated to the cytosol where their biosynthesis is completed (Humphrey et al. 2006). Our earlier work also suggested that carotenoid cleavage dioxygenases must be involved in germination stimulant biosynthesis and we have already postulated how, after carotenoid cleavage, further enzymatic conversions may lead to the production of all strigolactones known to date (Matusova and Bouwmeester 2006; Matusova et al. 2005).

Of the other germination stimulants that we observed in *Arabidopsis* exudate we know even less than of the strigolactones. By using inhibitors and mutants we showed that these chemical signals could be derived from an early plastidic pathway intermediate but also here the exact natural precursors are unknown. Moreover, these compounds still need to be chemically characterised. We are currently using further fractionation and untargeted metabolite analysis using LC-MS/MS in combination with our sensitive bioassay to try to identify these unknown active chemical signalling compounds for *Orobanche* seed germination in *Arabidopsis* roots

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Chapter 5

Rice germination stimulants for the root parasitic plant *Striga hermonthica*

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Abstract

The root parasitic *Striga* spp. causes serious losses in upland rice in the Africa continent. Control methods that interrupt the *Striga* infection process in an early stage are desirable but these require a thorough understanding of the early steps of the *Striga* lifecycle. Here we have studied the identity and biosynthetic origin of the *Striga* germination stimulants of rice. Germination of *Striga* seeds and mycorrhizal branching are strongly induced by rice root exudates of plants grown under phosphate starvation. Inhibitor experiments showed that fluridone treatments significantly reduced the production of these germination stimulants and mycorrhizal branching factor. All this suggests that rice produces strigolactones and indeed we could demonstrate, using LC-MS/MS analysis, that rice root exudates contain orobanchol and an isomer of 5-deoxystrigol. Using a bioinformatics approach, we discuss genes, for example rice CCDs, NCEDs and their co-expressed P450s that may be involved in the formation of germination stimulants in rice and other plant species. The possible involvement of strigolactones in determining radicle growth direction and the use of carotenoid biosynthesis inhibiting herbicides for *Striga* control are discussed.

Key words: rice, germination stimulants, strigolactones, *Striga hermontica*

Introduction

Rice (*Oryza sativa* L.) is by far the most important cereal crop in the developing world, and feeds more than two billion people as a staple food (Datta 2004). Also in Sub-Saharan Africa, rice is one of the major crops, especially in West Africa, where rice consumption has increased by about 6% per year since 1970 (Johnson et al. 1997). More than half of the rice grown in Africa is grown under upland conditions (Terry et al. 1994). Upland rice is often grown on poor arable land without the use of (inorganic) fertilizer (Johnson et al. 1997) and quite often the grain production is seriously suffering from the parasitic weed genus *Striga* (*Orobanchaceae*) (Adagba et al. 2002; Dugje et al. 2006; Johnson et al. 1997). Of the 28 *Striga spp*., which are found in Africa, *Striga asiatica*, *Striga aspera*, and *Striga hermonthica* are able to parasitise upland rice and other cereal crops such as maize (*Zea mays L*.), sorghum (*Sorghum bicolour L*) and pearl millet (*Pennisetum typhoideum L*) (Johnson et al. 1997; Oswald 2005; Parker and Riches 1993). Unlike other weeds, which compete for water and nutrients, *Striga* spp. as root parasites, compensate for the lack of their own root system by penetrating the roots of other host plants and diverting essential nutrients from them. This results in host plants with stunted growth and yields are greatly reduced (Joel et al. 1995; Musselman 1980; Nickrent and Musselman 2004; Press and Graves 1995). In some areas, the problem of *Striga* is so bad that farmers have been forced to abandon their land (Butler 1995; Oswald 2005). It is reported that *Striga* infests about two-thirds of the 70 million hectares devoted to cereal crop production in the Africa continent, which results in crop losses of up to 70% among subsistence farmers and thus negatively affects the lives of some 300 million people (Cherfas 2002; Kim et al. 2002; Press et al. 2001). Therefore *Striga* is considered to be one of the greatest obstacles to food production in Africa, particularly in the Sahel region.

Over the years, finding ways of controlling *Striga* has been the dream of many research groups, but success has been limited. So far there is no single effective and economically feasible *Striga* control method available to the small scale African farmer (Butler 1995; Ejeta 2005; Oswald 2005). Although integrated weed management has been encouraged in recent years, the results are not ideal. One of the important reasons is that the lifecycle of *Striga* spp. is complicated. Control methods developed on the basis of this lifecycle and particularly the earlier stages are expected to be more effective and efficient than those affecting later stage of development because they prevent parasitism prior to crop damage. This includes, for example, control methods that are based on the fact that *Striga* spp. require germination stimulants before they can attach to a host root. Germination stimulants are the first chemical signal derived from host or non-host plants and *Striga* seeds will not germinate without receiving this signal compound (Bouwmeester et al. 2003; Press and Graves 1995). Germination stimulants have been reported in many different plant species. For example, three classes of compounds have been described to have germination activities inducing: dihydrosorgoleone, sesquiterpene lactones and strigolactones (Bouwmeester et al. 2003). The best explored germination stimulants are the strigolactones which are a class of structurally closely related compounds (Akiyama and Hayashi 2006; Awad et al. 2006; Bouwmeester et al. 2007; Yoneyama et al. 2006). For example, strigol has been identified in cotton (*Gossypium hirsutum*), maize, pearl millet and sorghum (Awad et al. 2006; Butler 1995; Cook et al. 1972; Siame et al. 1993). Recently an isomer of strigol, named sorghumol, was identified in sorghum (Awad et al. 2006). Other strigolactone compounds, for example, sorgolactone was identified in sorghum (Hauck et al. 1992); alectrol, which has now been shown to be orobanchyl acetate (Xie et al. 2007) in cowpea (*Vigna unguiculata*) (Muller et al. 1992) and red clover (*Trifolium pratense*) (Yokota et al. 1998);

orobanchol in red clover and tomato (*Lycopersicon esculentum*) (López-Ráez et al. 2007; Yokota et al. 1998); 5-deoxystrigol is reported to be one of major germination stimulants of gramineous plants and has been identified in the root exudates of maize, pearl millet and sorghum (Awad et al. 2006). Recently, it was shown that the strigolactones are not only host-finding factors for parasitic plants but also induce hyphal branching in arbuscular mycorrhizal (AM) fungi, a process essential for the host root colonisation process of these symbiotic fungi (Akiyama et al. 2005).

Although many strigolactone compounds have been identified in many different plant species, the exact biosynthetic origin of these germination stimulants is as yet unknown. Our group has demonstrated that strigolactones are derived from the carotenoid pathway in maize, sorghum and cowpea (Matusova et al. 2005). It was postulated that carotenoid cleaving enzymes such as carotenoid cleavage dioxygenases (CCD) or 9-*cis*-epoxycarotenoid cleavage dioxygenases (NCED) are in involved in the cleavage of a carotenoid precursor. The product of this cleavage reaction is subsequently further converted to the strigolactones (Matusova et al. 2005). For example, we have shown that root exudates of the maize *vp14* mutant (mutated in an NCED involved in ABA biosynthesis) reduced strigolactone production by about 40% (Matusova et al. 2005). When the activity of VP14 (and homologous NCEDs) was reduced through an RNAi approach, strigolactone biosynthesis was also dramatically decreased in maize (Chapter 3). However, the real carotenoid precursor and the further enzymatic conversions remain unknown. The major disadvantage of the use of maize lies in the lacking genome sequence and the technical difficulty of transformation and thus it is difficult for using the genetic approach to unravel the metabolic network of germination stimulant biosynthesis. For *Arabidopsis*, currently the best-studied model plant, we have shown that although it produces very low levels of strigolactones, it also produces germination stimulants that are not carotenoid derived but from other isoprenoid pathways (Chapter 4). This makes *Arabidopsis* a less suitable model for the dissection of this biological process. Therefore we decided to study another plant model, rice, that has an advantage over other cereals because its genome has been sequenced and partially annotated and a well established, relatively easy transformation system has been developed (Nishimura et al. 2007). In addition, the *Striga* weed problem occurs mainly in cereal crops including rice, thus rice would be a good entry point also for other cereals for characterizing the genes involved in the formation of germination stimulants. A disadvantage of rice may be that the germination stimulants have so far not been identified. However, rice is a host of AM fungi (Gao et al. 2007) and considering that strigolactones are essential for the symbiosis between plants and AM fungi - and the most important class of *Striga* germination stimulants especially in cereal crops - it is likely that rice also produce strigolactones. Therefore the objectives of this study were (i) to identify the germination stimulants in rice, (ii) to investigate their involvement in *Striga* germination and (iii) to investigate their biosynthetic origin in rice.

Materials and Methods

Chemicals

The synthetic germination stimulant GR24 was obtained from Binne Zwanenburg, University of Nijmegen, The Netherlands. Standards of orobanchol and 5-deoxystrigol were kindly provided by Koichi Yoneyama (Utsunomiya University, Japan) and Kohki Akiyama (Osaka Prefecture University, Japan), respectively. Fluridone was purchased from Duchefa (Haarlem, The Netherlands). C18 Sep-Pak columns were purchased from Waters Chromatography B.V. (Etten-Leur, The Netherlands).

Plant materials and growth conditions

Rice (*O. sativa* L., cv. TN1 and cv. Nipponbare) seeds were surface sterilized in 2% sodium hypochlorite containing two drops of Tween-20 for 15 min followed by three washes in sterile water. For pot experiments, sterilized TN1 seeds were germinated in Petri dishes on moist filter paper at 30°C under dark conditions for 1 d. After germination, the seeds were then grown in washed river sand in plastic pots (24.5 height x 10.5 cm diameter). Three seeds were sown in each pot and seedlings were thinned to 1 plant per pot 10 days after sowing. Seedlings were grown in a greenhouse chamber set for a daily regime of 28°C day (16 h) and 25°C night (8 h) with relative humidity 85%. For the pot experiment, the seedling were watered with 0.4-strength Long Ashton solution (Hewitt 1966) in the first two weeks in order to produce healthy plants. For LC-MS-MS analysis, sterilized Nipponbare seeds were germinated in Petri dishes on moist filter paper at 28°C under dark conditions for 2 d. Eighteen germinated seeds were grown in 4Kg washed river sand in plastic pots (25 height x 20 cm diameter). The plants were grown in a greenhouse under the same conditions as described above and watered with 100 ml of 0.5-strength Hoagland's nutrient solution (Gamborg and Wetter 1975) every two days for the first two weeks and 250 ml for the third and fourth week.

Striga hermonthica seeds used in the bioassays were (i, sample A) collected from a maize field in Kibos, Kenya in 1994 for pot experiment (kindly provided by Vicky Child of Long Ashton Research Station, Bristol, UK) or (ii, sample B) collected from a sorghum field in Sudan in 1995 (kindly provided by Bob Vassey, University of Sheffield, Sheffield, U.K.). *S. hermonthica* seeds need a preconditioning period to break their dormancy before the seeds are able to response to the germination stimulants. Briefly, the preconditioning is started by surface-sterilising seeds in 2% sodium hypochlorite containing 0.1 % Tween 20 for 5 minutes. Seeds were then washed three times with sterile demineralised water, and excess water was removed by filtration through a Buchner funnel. The sterile seeds were allowed to air dry for 2 hours and subsequently approximately 15 mg seeds were evenly distributed on 47-mm diameter glass filter paper disks (Sartorius Germany). These disks were placed in a 9-cm diameter Petri-dishes on filter paper (Waterman, UK) moistened with 3 ml demineralised water. The Petri-dishes were sealed with parafilm and wrapped in aluminum foil and placed in a growth chamber (30ºC) for 10 days.

Root exudates collection and germination bioassay

To collect exudates for mycorrhizal branching factor assays, rice plants were grown in pots with normal phosphate nutrition. After two weeks, the pots were thoroughly washed with water to remove any phosphate in the sand as much as possible, then rice plants were grown under normal phosphate (500 μM) and without phosphate in 0.4-strength Long Ashton solution. Six plants were used for each treatment. After one week, plants were transferred to 25 ml glass tubes with 15 ml demi water for root exudate collection. Root exudates were collected from each single plant separately for all the treatments after 12 and 24 hours. Root exudates were purified and concentrated using Sep-pak columns before mycorrhizal branching factor assay (see below).

For the pot experiment of fluridone treatment, rice plants were grown in pots supplemented with 0.4-strength Long Ashton nutrition solution. After two weeks, half of the plants were transferred to 0.4-strength Long Ashton nutrition solution without phosphate. After one week half of the control and half of the phosphate-starved rice seedlings were treated with 0.1 μM fluridone (dissolved in 0.4-strength Long Ashton nutrient solution with or without phosphate, respectively). After 5 days, each pot was

inoculated with about 30 mg of preconditioned *S. hermonthica* seeds (sample A). This was achieved by carefully removing sand from the roots of the seedlings, depositing the seeds – suspended in water - onto the root system using a syringe and subsequently replacing the sand. Control plants were treated similarly but with water without *Striga* seeds. Germinated and/or attached seeds were assessed after 10 days under a dissecting microscope. To exclude that fluridone was not only affecting the production of germination stimulants but also the attachment success, in a similar experiment, the experimental procedures were the same as above except two additional experimental treatments were added: *Striga* seeds were pre-germinated with GR24 and then added to fluridone-treated and control plants. The attached seeds to the rice roots were counted in three weeks under binocular. The pre-germination of *Striga* seeds was achieved by treating the preconditioned seeds using GR24 0.1µM for 10 hours. For the rice seedling fluridone spraying experiment, rice plants were grown in pots supplemented with 0.4-strength Long Ashton nutrition solution. After two weeks, the plants were transferred to 0.4-strength Long Ashton nutrition solution without phosphate and then sprayed daily on plant leaves for 5 days with 10 ml (per plant) of 0.001 µM, 0.01 µM, 0.1 µM or 1 µM fluridone in water supplemented with Tween 20 (0.01%). After that, preconditioned *Striga* seeds were applied to the pot and germination and attachment assessed using the procedure described above.

For LC-MS/MS analysis, rice seeds (cv. Nipponbare) were grown as described above. After four weeks, two treatments were applied: Hoagland nutrient solution without phosphate and with 0.1µM Fluridone (-P+Flu), and Hoagland nutrient solution without phosphate (-P). The control plants were maintained with the complete phosphate nutrition of 0.5 strength Hoagland solution (+P). Before applying the treatments, the pots were washed with 3 l (3 fold of the field capacity) of 0.5 strength Hoagland nutrient solution without phosphate to remove the phosphate from the sand, and then 1 l of the respective treatments were applied. The pots of the control plants were mock treated by applying the same volume of Hoagland nutrient solution with the normal phosphate. Each treatment was made in 3 replicates and each replicate was 18 plants in one pot. After three days, the pots were washed with 2 l of the respective treatments to remove any accumulated germination stimulants. After 24 hours, the root exudates were collected by applying 1 l of the respective treatments and recovered the run through from the pot. The exudates were then concentrated using a C18 column with 1.5 g of SPE Bulk Sorbent (Alltech, US) and eluted in 9 ml of 100% acetone. The obtained samples were used for germination bioassay and LC-MS/MS analysis. For the bioassay, the exudates were prepared by mixing 400 µl of the concentrated exudates in acetone with water in a 1:1 ratio and evaporating the acetone in a vacuum centrifuge.

Before the germination bioassay, the *S. hermonthica* seeds were preconditioned. Hereto, seeds were surface-sterilized in 2% sodium hypochlorite containing 0.1 % Tween 20 for 5 minutes. Then seeds were rinsed three times with sterile demineralised water, and excess water was removed by filtration through a Büchner funnel. The sterile seeds were allowed to air dry for 2 hours and subsequently approximately 50 to 100 seeds were placed on 9-mm diameter glass filter paper (Sartorius, Germany) discs. Twelve discs were placed in 9-cm diameter Petri-dishes with a filter paper (Whatman, UK) moistened with 3 ml demineralised water. The Petri dishes were sealed with parafilm and wrapped in aluminium foil and placed in a growth chamber at 30ºC for 10 days. Before applying root exudates, the discs of the seeds were dried on sterile filter paper for 3 minutes and transferred to a new Petri dish with a 1-cm wide filter paper ring (outer diameter of 9 cm), moistened with 1 ml sterile demi water to keep a moist environment inside the Petri dish. Fifty µL of the root exudates to be tested were applied to triplicate discs. The synthetic strigolactone analogue GR24 (0.1 $mg.l^{-1}$) was used as a positive control and sterile

demineralised water as a negative control in each germination assay. Seeds were then incubated at 30ºC in darkness for 2 days. After 2 days, germination was assessed using a binocular microscope. Seeds were considered to be germinated if the radical protruded through the seed coat.

Mycorrhizal branching factor assay

For the AM fungi branching factor assay, 40 ml of combined rice root exudates in all treatments were purified using C18 Sep-Pak (Water USA) columns. The columns were first conditioned by washing them with 5 ml of methanol, 5 ml of acetone and 5 ml of demineralised water. Then the combined 60 ml of crude undiluted root exudates (from 6 rice plants) from each treatment was loaded onto the columns. The columns were washed with 5 ml of demineralised water to remove any remaining water soluble compounds and then eluted using 3 ml of 100% acetone. The eluted samples were diluted to 50% acetone with demineralised water because 100% acetone inhibits AM fungi branching. GR24 (10^{-9} M) was used as a positive control and 50% acetone in water as negative control in the experiments. Branching factor activity of the root exudates was tested as described before (Buee et al. 2000; López-Ráezı et al. 2007).

Strigolactones analysis using liquid chromatography-tandem mass spectrometry.

Analysis was performed using a Waters Micromass Quattro Premier XE tandem mass spectrometer (Waters, USA) equipped with an ESI source and coupled to an Acquity UPLC system (Waters, USA). Two hundred and fifty µL of the acetone eluent of the Sep-Pak columns were diluted with the same volume of water and analyzed directly by UPLC-MS/MS (sample injection volume: 30 µL). Chromatographic separation was performed on an Acquity UPLC BEH C18 column (100 x 2.1 mm, 1.7 µm) (Waters, USA), applying a water/acetonitrile gradient. The gradient started at 20% acetonitrile for 1 min, followed by a 5-min ramp to 70% acetonitrile which was maintained for 1 min, followed by a 0.20 min ramp back to 20% acetonitrile and the column was equilibrated at this solvent composition for 1.8 min. Total run time was 9 min. The column was kept at 50°C and run at a flow-rate of 0.4. ml min^{-1}. The mass spectrometer was operated in positive electrospray ionization (ESI) mode. The nebuliser and desolvation gas flows were 50 and 800. l h^{-1}, respectively. The capillary voltage was set at 2.7 kV, the cone voltage at 20 V, the source temperature at 120ºC and the desolvation gas temperature at 450 ºC. Fragmentation was performed by collision induced dissociation with argon at 3.0 x 10^{-3} mbar. The collision energy was optimized for each compound. Multiple reactions monitoring (MRM) method was used for the detection of strigolactones. The MRM transitions were set according the MS/MS spectrum of standard strigolactones or based on analogy with standards for unknown strigolactones and strigolactones for which standards were not available. The "protonated" ion $[M+H]^+$ was the most abundant in full-scan positive mass spectrums of known strigolactones used as standards. Therefore, that ion was selected as precursor ion for collision-induced degradation (CID). In the MS-MS spectrum of the parent ion $[M+H]^+$ with *m/z* 347 of the standard strigolactone orobanchol, the fragment ions at *m/z* 233 $[M\text{-D ring} - 16]^+$, *m/z* 205 $[M\text{-D ring-}CH_2O_2]^+$ and *m/z* 97 (D-ring) were the most abundant at the collision energy 14, 18 and 20 eV, respectively (data not shown). Therefore, for the multiple reaction monitoring (MRM) the transitions of *m/z* 347>233; 347>205; 347>97 were selected for orobanchol. The fragment ions at *m/z* 234 $[M+H\text{-D ring}]^+$, *m/z* 217 $[M+H\text{-D ring-}H_2O]^+$ and *m/z* 97 (D-ring) were the most abundant in the MS/MS spectrum of a standard of 5-deoxystrigol (data not shown) and therefore the MRM transitions of *m/z* 331>234, 331>217, 331>97 at the collision energy 10 and 18 eV were used for the detection of

5-deoxystrigol. Data acquisition and analysis were performed using MassLynx 4.1 software (Waters, USA).

Statistical analysis

All the experiments were performed at least twice with the exception of the LC-MS/MS analysis and corresponding germination bioassay experiment that was done only once. In the experiment of fluridone irrigation on rice plants in pots, individual treatments were replicated 3 times, for fluridone spraying 5 times. Attachment and germination data from these experiments were transformed by taking the arcsine of the square root of the proportion of germinated seeds and then were subjected to analysis of variance (ANOVA) to determine main effects. For the experiments of mycorrhizal branching assay, T-test was used for statistical analysis. All results were analysed using GenStat Release 9.2 (PC/Windows XP), VSN international Ltd, UK.

Coexpression analysis between CCDs or NCEDs and P450s

The rice CCDs and NCEDs were identified from a BLAST search of the orthologous genes in *Arabidopsis* in the rice database (NCBI and TIGR). Rice CCDs and NCEDs that showed co-expression with rice P450s were then selected from 22 libraries (http://mpss.udel.edu/rice/). The Spearman rank correlation coefficient (>0.7) was used as a cut off because the Pearson correlation coefficient was not sensitive to relatively low expression values. Based on expression specificity (in roots) and co-expression with P450s, a number of promising candidate genes were detected and will be discussed.

Results

Effect of fluridone and phosphate treatments in rice plant roots on Striga hermonthica *seed germination*

To test if the rice germination stimulants are derived from the carotenoid pathway, irrigation with the carotenoid pathway inhibitor fluridone was used to treat rice plants in pots. In addition, phosphate starvation has been reported to promote strigolactone formation in red clover (Yoneyama et al. 2007), thus rice plants were phosphate starved to see if this also applies to rice. New rice leave tips showed bleaching after 3-5 days of fluridone treatment (0.1 μM). *In vitro* germination bioassay results showed that the root exudates of fluridone treated rice plants induced significantly lower *Striga* seeds germination than that of control plants ($P < 0.01$) (Fig. 1). In addition, root exudates from rice plants grown under phosphate starvation induced significantly higher *Striga* seed germination than that of control plants grown with normal phosphate ($P < 0.01$) (Fig. 1). The positive control (GR24 0.01 μM) induced a similar germination percentage as root exudates from phosphate-starved plants, whereas the negative control (tap water) did not induce any germination (Fig. 1). *In vivo* germination bioassays (where preconditioned *Striga* seeds were applied directly along the roots) also showed that the number of germinated and attached *Striga* seeds in fluridone treated rice plants reduced significantly compared with that of non-treated plants ($P < 0.01$) (Fig. 2).

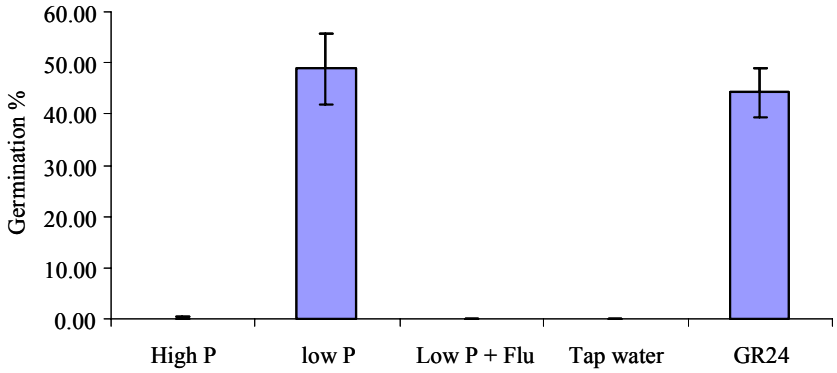

Fig. 1 Effect of fluridone and phosphorus treatments in rice (cv, Nipponbare) on the exudate induced germination of *Striga hermonthica* seeds (batch B). Rice plants were grown in sand in pots and were watered with half strength Hoagland nutrient solution. Four-week old rice plants were then treated with phosphate starvation alone or in combination with 0.1µM fluridone or left untreated (control). After three days, root exudates of each treatment were collected and assessed for germination stimulant activity as described in the Material and Methods. High P , 24-hour rice root exudates under normal phosphate (330 µM); Low P, 24-hour rice root exudates without phosphate; GR24, synthetic strigolactone as a positive control (0.1 µM);Tap water, negative control; Error bars represent the standard error of the means of 6 individual plants .

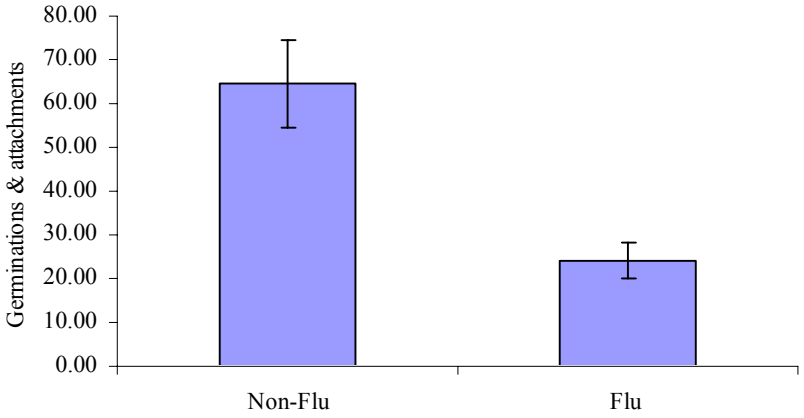

Fig. 2 Effect of fluridone treatments in rice (cv, Tn1) on the in situ germination and/or attachment of *Striga hermonthica* seeds. Rice plants were grown in pots supplemented with 0.4-strength Long Ashton nutrition solution. After two weeks, each plant was treated daily for 5 days with 100 ml of 0.1 µM fluridone (dissolved in 0.4-strength Long Ashton nutrient solution without phosphate) (Flu) or with nutrient solution without fluridone (Non-Flu). After that, each pot was inoculated with about 30 mg of preconditioned *S. hermonthica* seeds (sample A). Germinated and/or attached seeds were assessed after 10 days under a dissecting microscope. Error bars represent the standard error of the mean of three individual plants.

The above experiments demonstrate that fluridone irrigation can inhibit the germination stimulant formation in rice roots. To assess whether the inhibitor will also be transported from the shoot to the root, we also treated rice plant by spraying different concentration of fluridone. After 5 days of treatment with fluridone, the new rice leave tips did not show bleaching even with a concentration of 1 µM. However, the higher the concentration of fluridone applied, the lower the rate of *Striga* seed germination and/or attachment (from average 250 to average 75) was observed in the plant roots compared with control plant roots (Fig. 3). The reduction in *Striga* seed germination and/or attachment compared with control plants was significant for the treatments 0.1 µM ($P < 0.01$) and 1 µM ($P < 0.01$).

To exclude that our fluridone treatments were not only affecting germination but also attachment success, we pre-germinated *Striga* seeds before applying them to the rice roots with and without fluridone treatments. Fig. 4 shows that the number of attachments on fluridone treated rice roots (average 120) was not big different from that on non-fluridone treated control rice roots (average 128). In contrast, when using non-GR24 treated *Striga* seeds, the number of attachments was significantly higher on the control rice roots than on the fluridone treated roots ($P<0.01$). Pre-germination of the *Striga* seeds strongly and significantly increased the number of attachments on rice roots (Fig. 4).

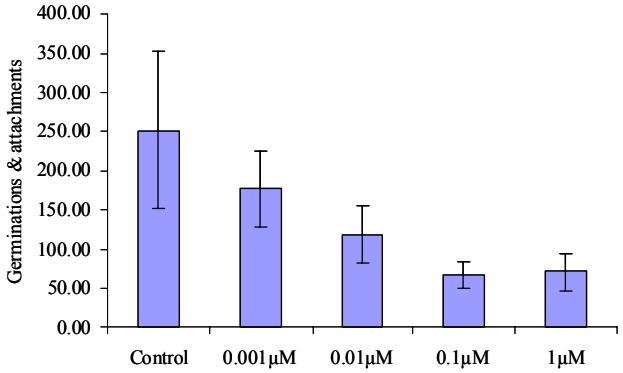

Fig. 3 Effect of fluridone spraying of rice (cv, Tn1) leaves on the induction of germination of *Striga hermonthica* seeds. Rice plants were grown in pots supplemented with 0.4-strength Long Ashton nutrition solution. After two weeks, the plants were transferred to 0.4-strength Long Ashton nutrition solution without phosphate and then sprayed on plant leaves daily for 5 days with 10 mL (each plant) of 0.001 µM, 0.01 µM, 0.1 µM or 1 µM fluridone in water supplemented with Tween 20 (0.01%). Then each pot was inoculated with about 30 mg of preconditioned *S. hermonthica* seeds (sample A). Germinated and/or attached seeds were assessed after 10 days under a dissecting microscope. Error bars represent the standard error of the mean of 6 individuals.

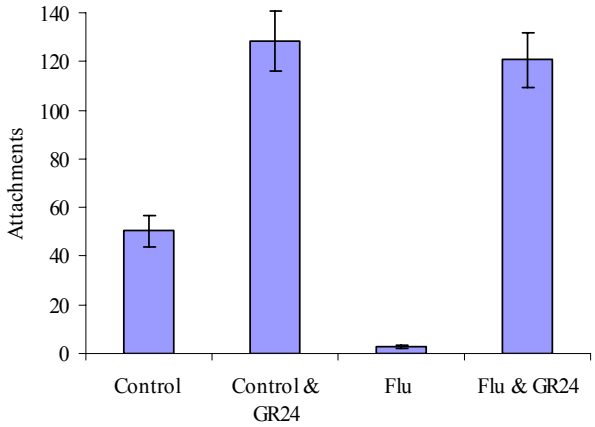

Fig. 4 Effect of fluridone treatments in rice plants (cv. Tn1) on *Striga hermonthica* attachments. Rice plants were grown in pots supplemented with 0.4-strength Long Ashton nutrition solution. After two weeks, plants were treated with 0.1 µM fluridone (dissolved in 0.4-strength Long Ashton nutrient solution without phosphate) for 5 days (Flu) or with nutrient solution without fluridone (Control). Then each pot was inoculated with about 30 mg of pre-germinated (preconditioned seeds treated with 0.1 mg. l^{-1} GR24 for 10 hours) or only preconditioned *S. hermonthica* seeds (sample A). The attached seeds were assessed after 3 weeks under a dissecting microscope. Error bars represent the standard error of the mean of 3 individual plants.

Rice root exudates induce mycorrhizal fungi hyphal branching

Strigolactones are not only germination stimulants of the parasitic *Striga* spp. but are also signal molecules that induce mycorrhizal fungi hyphal branching (Akiyama et al. 2005; Bouwmeester et al. 2007; Yoneyama et al. 2006). To investigate if rice root exudates contain compounds, such as strigolactones, that induce mycorrhizal branching, rice root exudates were collected from plants grown under normal phosphate or phosphate starvation. Mycorrhizal fungi hyphal branching was significantly increased in both 12 hours and 24 hours root exudates under phosphate starvation ($P<0.01$) (Fig. 5). Particularly in 12 hours root exudates, the activity was even higher than that of the positive control GR24 (10^{-9} M) ($P<0.05$). In contrast, under normal phosphate, the mycorrhizal fungi hyphal branching was similar to the negative control (50% acetone in water) (Fig. 5).

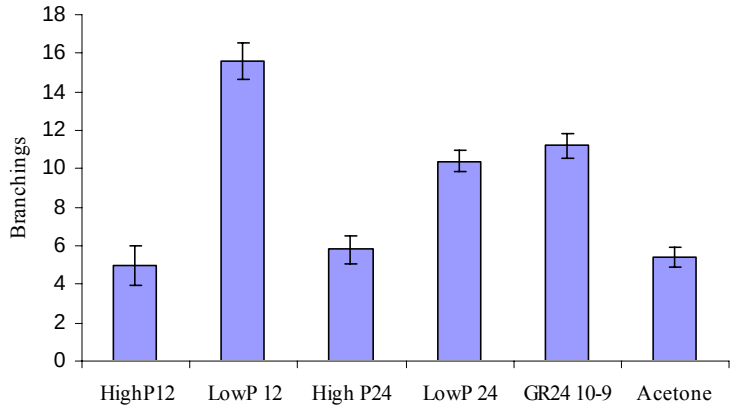

Fig. 5 Hyphal branching activity of rice root exudates on Gigaspora margarita spores. Rice plants were grown two weeks in pots with sand and were supplemented with 0.4-strength Long Ashton nutrient solution. Two week-old rice plants were then treated with normal nurtrient solution (330 µM phosphate) (HighP) or with nutrient solution without phosphate (LowP) for 7days. The root exudates were collected in 15 ml demineralised water for 12 and 24 hours and then were purified and concentrated using Sep-pak columns in 100% acetone solution. The eluted samples were diluted to 50% acetone with demineralised water before mycorrhizal branching factor assay. GR24, synthetic strigolactone as a positive control (10^{-9} M); Acetone, negative control (50% acetone in water); Bars represent means of 5 individual plants and error bars represent the standard error of the mean.

Identification of strigolactones using LC-MS-MS in rice root exudates

The presence of strigolactones in rice root exudates was examined using ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). In the MRM chromatograms of phosphate-starved rice roots exudates an intense peak was detected for MRM-channels *m/z* 347>233; 347>205; 347>97 at a retention time of 5.91 min matching with a standard of orobanchol (Fig. 6A). No transition for orobanchol was detected in the normal phosphate and in the phosphate-starved/fluridone-treated plant root exudates. Standard addition of authentic orobanchol to the samples confirmed that this compound in root exudates of the phosphate-starved plants was indeed orobanchol (Fig.6 B). The identity of orobanchol was also confirmed by comparison of the MS/MS spectra with the orobanchol standard (data not shown). The transitions *m/z* 331>234; 331>217; 331>97 (which were assigned for 5-deoxystrigol) in the MRM chromatograms of phosphate-starved rice root exudates showed a compound eluting at 11.12 min, which is slightly different form the retention time of a standard of 5-deoxystrigol that elutes at 11.23 min (Fig. 6A, B). This fact and the fragmentation in MS/MS spectra

with the spectra of authentic 5-deoxystrigol lead us to suggest that this compound is an isomer (probably 2'-epimer) of 5-deoxystrigol. Like orobanchol, the 5-deoxystrigol isomer was present only in exudates of phosphate starved plants and not detectable in the exudates of plants grown under normal phosphate or after fluridone treatment. These data show that the major known strigolactones in the exudates of rice cv. Nipponbare are orobanchol and an isomer of 5-deoxystrigol, possibly 2'-epi-5-deoxystrigol.

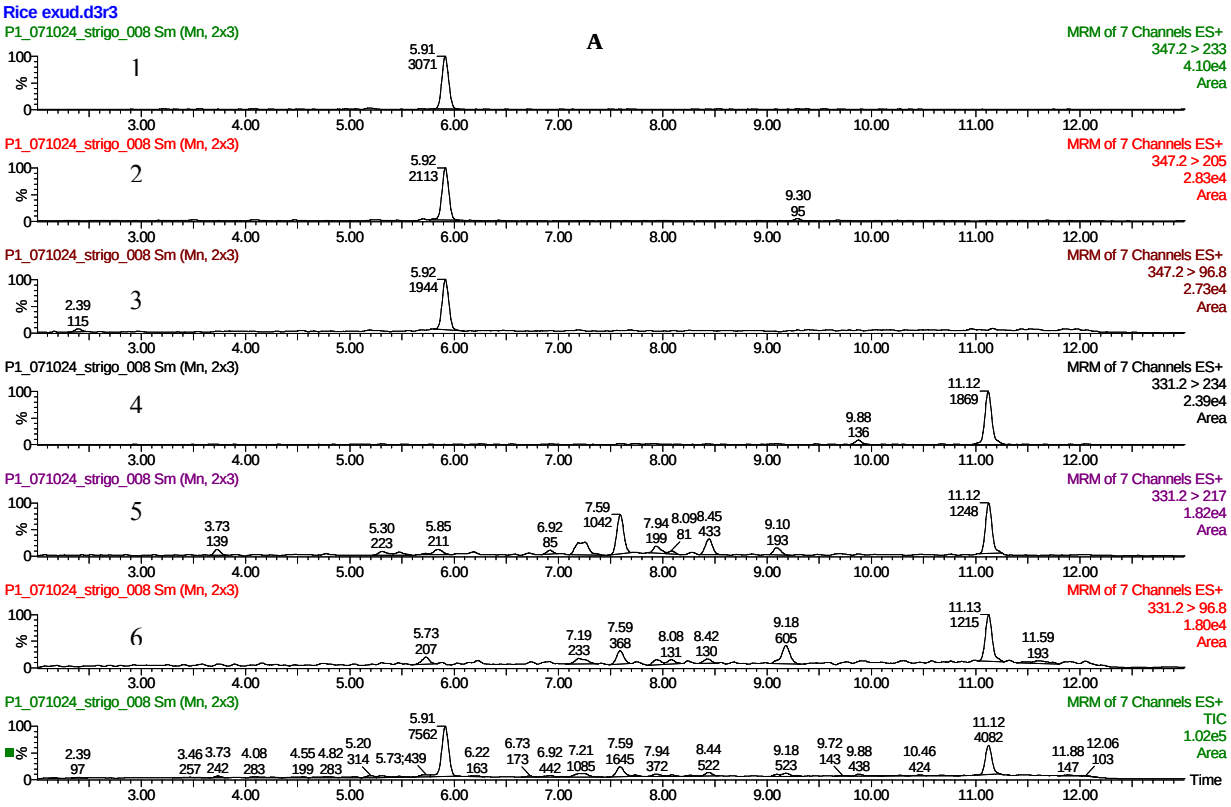

Fig. 6A LC-MS/MS analysis of strigolactones in rice root exudates. A: Multiple reaction monitoring (MRM) chromatograms of rice roots exudates from plants growing without phosphate (-P). The upper chromatograms 1, 2 and 3 are for monitoring the transitions of *m/z* 347>233, *m/z* 347>205 and *m/z* 347>97 for the detection of orobanchol. The MRM chromatograms 4, 5 and 6 are for monitoring the transitions of *m/z* 331>234, *m/z* 331>217 and *m/z* 331>97 for the detection of 5-deoxystrigol and its isomers.

Molecular analysis of rice carotenoid cleavage dioxygenase (OsCCDs and OsNCEDs)

In maize, sorghum, cowpea and now also rice it has been shown that the stigolactones are derived from the carotenoid pathway most likely through the action of a carotenoid cleavage dioxygenases (Matusova et al. 2005). Therefore, it is quite interesting to analyze this gene family in rice. Figure 7 is a CLUSTALX tree giving an overview of the family of carotenoid cleavage dioxygenase genes identified in the rice and *Arabidopsis* genomes. The rice genes are orthologs of *Arabidopsis CCDs* (*AtCCDs*) and *NCEDs* (*AtNCEDs*) and have been grouped into six classes. Four *OsNCEDs* (*OsNCED3*, *OsNCED5*, *OsNCED4* and a putative *OsNCED*) that are closely related to *VP14* of maize and 5 *Arabidopsis NCEDs* (*AtNCEDs*) cluster together and are most likely involved in ABA biosynthesis. These *NCEDs* are supposed to have 11,12 (11',12') 9-cis epoxycarotenoid cleavage activities based on what was found for the *Arabidopsis NCEDs* (Tan et al. 2003). The other three *NCEDs* (*OsNCED1*, *OsNCED2* and *OsNeoxanthin-putative*) cluster with AtCCD4 and are assumed to have the same (unknown) catabolic activity. The only characterised rice *CCDs/NCEDs* are *OsCCD7* (ortholog of *AtCCD7*) and *OsCCD8* (ortholog of *AtCCD8*)

(this cluster includes another ortholog, putative *Osdioxygenase*), which are involved in the regulation of the outgrowth of the axillary buds of rice (Zou et al. 2006). *OsCCD1* clusters with *AtCCD1* and is predicted to specifically cleave the 9,10 and 9'10'double bonds of a variety of carotenoid substrates to various ß-ionone-related C13 fragments and a C14 central fragment, based on the information from orthologs in *Arabidopsis* and maize (Schwartz et al. 2001; Sun et al. 2007). The last class has three members (*OsCrocetin-diadehyde-like*, *OsLignostilben-α,ß-dioxygenase* and *OsLignostilben-α1,ß-dioxygenase*) of which the catalytical functions are unknown.

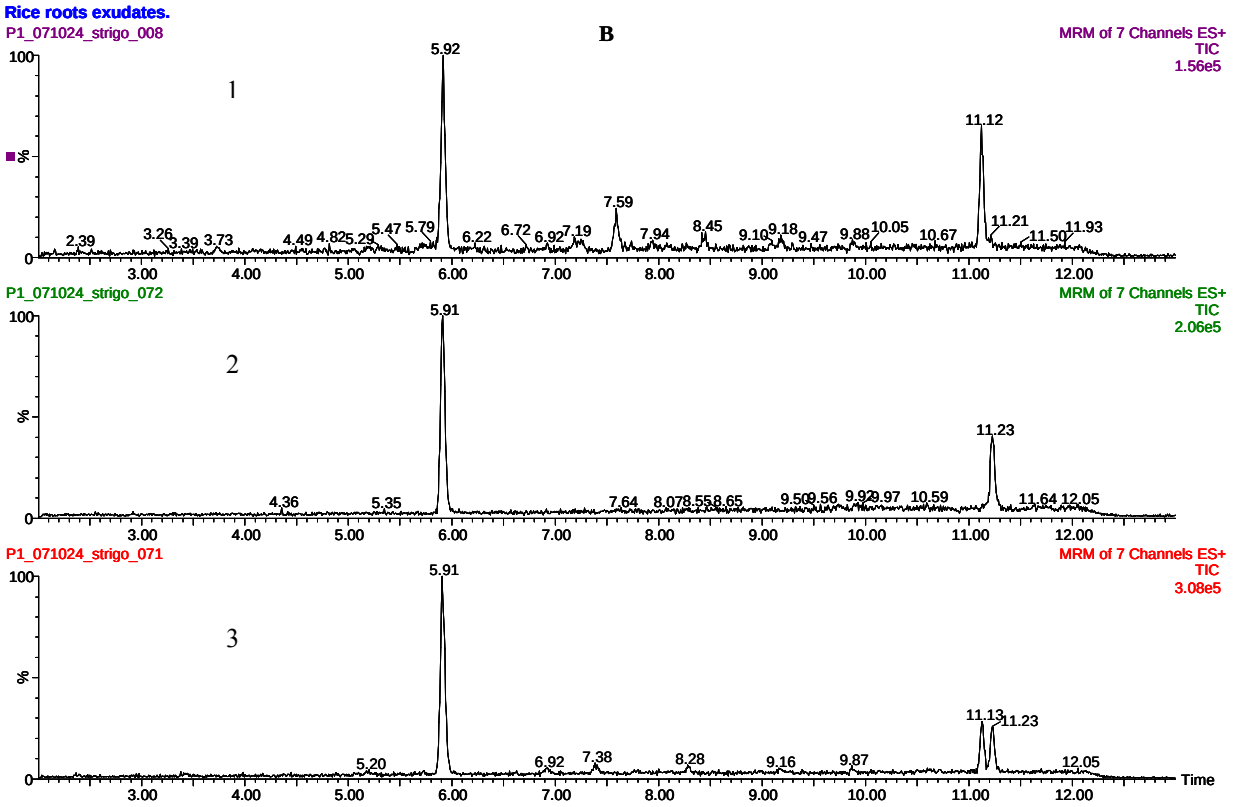

Fig. 6B LC/MS/MS analysis of strigolactones in rice root exudates. B: MRM chromatograms (TIC) of: 1. Rice roots exudates from plants growing without phosphate; 2. Mixture of standard orobanchol (60 ng.ml^{-1}) and 5-deoxystrigol (1 ng.ml^{-1}) in acetone/ water 1: 1. 3. Mixture of 1 and 2.

Molecular analysis of co-expressed P450s with carotenoid cleavage dioxygenases

Matusova et al. postulated that strigolactone formation needs further conversions, possibly catalysed by cytochrome P450s, after the cleavage action by CCDs or NCEDs (Matusova et al. 2005). This would be analogous to the cytochrome P450 that is supposed to be involved in conversion of the cleavage products of the *Arabidopsis* CCD7 and CCD8 and is co-expressed with these enzymes in the shoots (Booker et al. 2005). Therefore, co-expression of OsCCDs and OsNCEDs with P450s was studied in 22 expression libraries (MPSS database http://mpss.udel.edu/rice/) based on TIGR ID of each CCDs and NCEDs. We found 17 co-expressed P450s but only two of them were highly co-expressed with OsNCEDs that are expressed in rice roots (data not shown). One is a putative cytochrome P450 72A1 (locus name Os01g43710) and it is highly co-expressed with OsNCED5. The function of this gene in rice is unknown but a homolog converts loganin into secologanin in *Catharanthus roseus* (Irmler et al. 2000). The other one is a putative cytochrome P450 81E1 (locus name Os12g39310) and it is highly co-expressed with

OsNCED4. A close CYP81E1 homolog was found in *Glycyrrhiza echinata* (licorice) and this gene can catalyze the hydroxylation of isoflavones, daidzein and formononetin, to yield 2'-hydroxyisoflavones, 2'-hydroxydaidzein, and 2'-hydroxyformononetin, respectively (Akashi et al. 1997; Akashi et al. 1999).

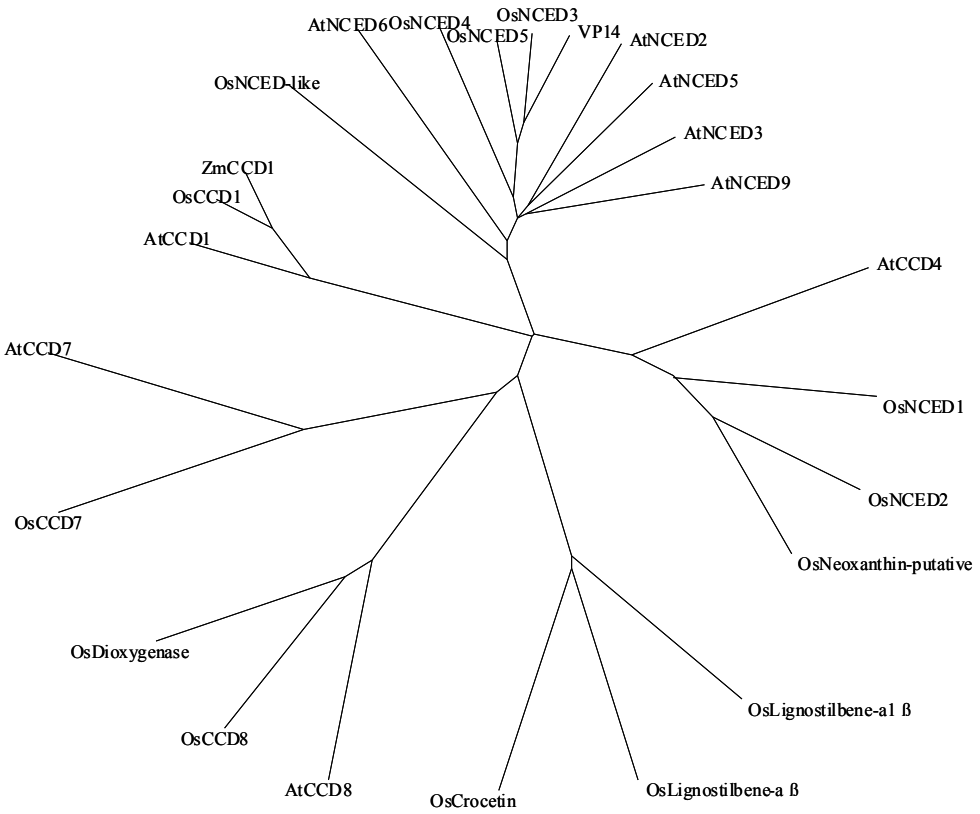

Fig. 7 Unrooted phylogenetic tree of plant carotenoid cleaving enzymes, carotenoid cleavage dioxygenases (CCDs) and 9-cis-epoxycarotenoid cleavage dioxygenases (NCEDs). The multiple sequences were aligned using the ClustalX program and the phylogenetic tree was created with the TreeView 3.2 program. Predicted full-length protein sequences for the following genes were used (with Gene bank Accession numbers): *OsCCD1* (DP000011), *OsNCED3* (AY838899), *OsNCED4* (AY838900), *OsNCED5* (AY838901), *OsNCED*-putative (AL663011), *OsNCED1* (AY838897), *OsNCED2* (AY838898), *OsNeoxhantin putative* (AC074355); *OsCCD7* (AL663000), *OsCCD8* (AP003141), *OsCrocetin* (AP005684), *OsDioxygenase* (AP003376), *OsLignostilben-α, ß-dioxygenase* (AP005298), *OsLignostilben-α1*, *ß-dioxygenase* (AP004647) , Os, *Oryza sativa*; *AtCCD1* (NM_116217), *AtCCD2* (NM_117945), *AtCCD3* (NM_112304), *AtCCD5* (NM_102749), *AtCCD6* (NM_113327), *AtCCD9* (NM_106486), *AtCCD4* (NM_118036), *AtCCD7* (NM_130064), *AtCCD8* (NP195007), At, *Arabidopsis thaliana*; *Vp14* (U95953), *ZmCCD1* (DQ539625), Zm (*Zea, mays*).

Discussion

Rice produces strigolactones

Using LC-MS/MS analysis we were able to demonstrate that rice produces the strigolactones orobanchol and an isomer of 5-deoxystrigol, possibly 2'-epi-5-deoxystrigol. We did not detect the other common cereal germination stimulants such as strigol and sorgolactone, but it has been reported that the germination stimulants may differ between different plant species and even between cultivars within the same species (Awad et al. 2006). The LC-MS/MS analysis showed that strigolactone secretion by rice plants is increased under phosphate starvation and can be blocked by fluridone treatment (Fig. 5). These

results were supported by pot experiments in which fluridone treated rice plants (by irrigation of the roots or spraying of the leaves) showed a strongly reduced induction of germination indicating that the rice germination stimulants are derived from the carotenoid pathway. This effect of fluridone on the germination stimulant production was also found in other cereal plants such as maize and sorghum (Matusova et al. 2005). Moreover, fluridone treatment of dicotyledonous plants also reduced germination stimulant production such as in *Arabidopsis* (Chapter 4), cowpea (Matusova et al. 2005) and tomato (López-Ráez1 et al. 2007). The branching factor assays for mycorrhizal fungi confirmed that phosphate starvation promotes germination stimulant / strigolactone formation in rice. This effect has also been found in other plant species such as red clover, tomato and *Arabidopsis* in which phosphate starvation promoted the formation of orobanchol (López-Ráez1 et al. 2007; Yoneyama et al. 2007). The branching activity is caused by strigolactones including 5-deoxy-strigol, sorgolactone and strigol (Akiyama et al. 2005; Buee et al. 2000). Later another strigolactone, orobanchol was also confirmed to have this ability (Yoneyama et al. 2007). Although the strigolactones were readily detectable by LC-MS/MS we could only get low *Striga* germination, and only after strong concentration of the samples. We assume this is due to the presence of inhibiting substances in rice exudates or to a low sensitivity of the *Striga* seeds (that were collected from sorghum and maize) to the strigolactone composition in the rice exudates. Indeed the combination of orobanchol and 2'-epi-5-deoxystrigol has not been reported before and definitely not in cereals.

Molecular analysis of germination stimulant (strigolactone) biosynthesis in rice

Although we have postulated that the strigolactones are derived from the carotenoid pathway (Matusova et al. 2005), the exact position in the pathway where strigolactone biosynthesis branches off from the main pathway remain unknown so far. It is clear that carotenoid cleavage is involved in strigolactone biosynthesis because carotenoids have a 40 carbon skeleton, whereas the strigolactones have only 14 carbons in the molecule excluding the D-ring (Matusova et al. 2005). Thus, probably a carotenoid cleavage dioxygenases should be involved in strigolactone biosynthesis. By blasting 9 *Arabidopsis* CCDs and NCEDs to the rice genome, we retrieved 14 rice orthologs of AtCCDs and AtNCEDs that clustered into 6 groups (Fig. 6). The closely related *VP14*-like class of *OsNCEDs* (*OsNCED3*, *OsNCED5*, *OsNCED4* and a *putative OsNCED*) are most interesting so far because the maize *vp14* mutant and the homologous ABA mutant in tomato have been shown to produce about 40% less germination stimulants (López-Ráez1 et al. 2007; Matusova et al. 2005). Moreover, silencing the *VP14* cluster using an RNAi construct in maize also showed a decrease in strigolactone formation (Chapter 3). These results suggest that also OsNCEDs may be involved in strigolactone formation in rice roots. The other three NCEDs (*OsNCED1*, *OsNCED2* and *OsNeoxanthin-putative*) are in the same class of *AtCCD4* and supposed to have 11, 12 (11', 12') 9-cis epoxycartenoid cleavage activities based on the rice genome annotation (NCBI) but the functions are unknown. The ortholog *CmCCD4* in chrysanthemum has been reported to be expressed only in the flower petals and was not detected in other organs, such as the root, stem, or leaf (Ohmiya et al. 2006). Considering that germination stimulants are secreted from host roots, thus this class of genes in rice is not likely involved in strigolactone formation. Like *AtCCD7*, *OsCCD7* has been identified to be involved in the formation of a novel plant signalling molecule involved in the regulation of the outgrowth of the axillary buds of rice (Zou et al. 2006). It was confirmed to have the same biochemical function as *AtCCD7* since it can cleave multiple carotenoids at the 9,10 or 9'10' double bonds (Booker et al. 2004; Zou et al. 2006). In *Arabidopsis*, the production of SMS hormone requires the activity of both AtCCD8 and AtCCD7 (Booker et al. 2005). The *OsCCD8* gene cluster may have the

same function as *AtCCD8* but this is still unknown. It was also proposed that AtCCD7 cleaves ß-carotene asymmetrically and generate a C_{13}-aldehyde (ß-ionone) and a C_{27}-aldehyde (10'-apo-ß-carotenal), then AtCCD8 cleaves the C_{27} aldehyde at the 13, 14 double bond to yield the signal molecule (Auldridge et al. 2006; Schwartz et al. 2004). The identity of the signal molecule remains unknown. Preliminary results with the pea *CCD8* mutant *rms1* showed it had greatly reduced strigolactone production in the root exudates which suggests that *OsCCD8* could be involved in the strigolactone formation in rice roots (Rochange et al., in prep.). The *OsCCD1* gene cluster has only one member which is predicted to cleave the 9,10 and 9'10'double bonds of a variety of carotenoid substrates and its primary products of these reactions are perhaps various ß-ionone-related C_{13} fragments and C_{14} central fragment based on the information from orthologs in *Arabidopsis* and maize (Schwartz et al. 2001; Sun et al. 2007). Interestingly the ortholog in maize, *ZmCCD1*, was observed to be upregulated in maize roots colonised by AM fungi, whereas at the same time mycorrhizal maize roots induced lower germination of *Striga* seeds (Sun et al. 2007). This is probably because ZmCCD1 competes for substrates with another CCD/NCED which results in decreased strigolactone formation (Sun et al. 2007). The last class has three gene members (*OsCrocetin-diadehyde-like*, *OsLignostilben-α,ß-dioxygenase* and *OsLignostilben-α1,ß-dioxygenase*) and the functions are unknown. Interestingly, there are no orthologs of genes this cluster in *Arabidopsis* suggesting the diversification of the CCDs and NCEDs in different plant species. Until proven otherwise, they should also be regard as candidate genes for the biosynthesis of strigolactones.

The intermediates derived from the cleavage products from the carotenoid pathway need further enzymatic conversions to form strigolactones, for example hydroxylation by P450 enzymes (Matusova et al. 2005). Our results show that two putative P450s genes are highly co-expressed with OsNCEDs in rice roots. One of them, *cytochrome P45072A1*, is highly co-expressed with *OsNCED5* and both are highly expressed in rice roots. Unfortunately, this gene is not characterised in rice, and its closest homolog in *Catharanthus roseus* can convert loganin into secologanin a biosynthetic step in indole alkaloid biosynthesis (Irmler et al. 2000). A close *Arabidopsis* cytochrome P450 homolog, CYP72B1, has been implicated in brassinosteroid catabolism as well as photomorphogenesis in isoprenoid pathway (Turk et al. 2003). The other CCD/NCED co-expressed P450 is the putative *cytochrome P45081E1* which is highly co-expressed with *OsNCED4* and both also highly expressed in the rice roots. A close CYP81E1 homolog found in the *Glycyrrhiza echinata* (licorice) can catalyze the hydroxylation of isoflavones, daidzein and formononetin, to yield 2'-hydroxyisoflavones, 2'-hydroxydaidzein, and 2'-hydroxyformononetin, respectively (Akashi et al. 1997; Akashi et al. 1999). Since we expect a P450 that catalyses decarboxylation, these two P450s do not seem to be promising candidates. Nevertheless, as we know only little about the strigolactone biosynthetic pathway, we cannot rule out that they are involved in strigolactone formation. Moreover, we may not be able to find the right P450 using this approach. This is illustrated by a known P450 - CCD relation in *Arabidopsis*. The P450, MAX1, has been confirmed to further modify the apocarotenoid product of sequential carotenoid cleavage reactions by AtCCD7 and AtCCD8 to produce a new plants hormone (Booker et al. 2004; Booker et al. 2005). By analyzing the rice genome, five homologs of *MAX1* have been revealed (Nelson et al. 2004). Surprisingly we have not revealed these five genes in our co-expression result, although we may expect they show co-expression with *OsCCD7* and *OsCCD8*. It maybe the prediction through the bioinformatics is not correct in reality or the expression libraries we used are not big enough to reflect all the genes or they have different expression pattern in rice compared with *Arabidopsis*. In any case, if it is true that OsCCD8 is also

involved in the formation of strigolactone, these five P450s could be interesting. The reverse genetics with rice as a model plant and the availability of sensitive bioassays, for example *Striga* seed germination assay and mycorrhizal hyphal branching assay, and highly sensitive LC-MS/MS analysis could be an important tool to shed light on the relevant carotenoid cleavage dioxygenases and their co-expressed P450s in the strigolactone biosynthetic pathway.

Control of parasitic weed Striga *using herbicides*

Rice is one of the main food crops in Sub-Saharan Africa. The *Striga* problem in rice as well as in other major food crops such as maize and sorghum is increasing in recent years (Johnson et al. 1997; Oswald 2005). Using herbicides to limit the *Striga* infestation is one of the important approaches because selective herbicides could have a direct effect on the emerged *Striga* plants (Joel et al. 1995; Oswald 2005). However, most herbicides are not selective to the majority of susceptible crops and in addition control of the parasite after emergence is too late as much damage has already been done (Joel 2000). The alternative way is seed-dressing with selected herbicides to herbicide resistant crops. For example, Berthome and his co-workers used chlorthal-dimethyl, dicamba and pendimethalin which effectively inhibit germination of *S. hermonthica*, and *S. gesnerioides* seeds even in the presence of natural germination stimulants in vitro (Berthone et al. 1995). A seed coating with imazapyr and pyrithiobac of herbicide resistant maize was also found to be very effective for season-long *Striga* control and resulted in a 3–4-fold increased yield under high *Striga* density (Kanampiu et al. 2003). Moreover, some herbicides, such as clopyralid and linuron, are able to induce *Striga* seed germination and therefore could be used in a suicidal germination approach (Berthone et al. 1995). These herbicides in general have a lot of constrains (high investment, multiple applications, farm-training required and higher seed price) and thus the potential for adoption by African farmers is small (Oswald 2005). In addition, some herbicides (for example, imidazolinone and sulphonylurea) are effective in selectively controlling *Orobanche*, *Striga*, and *Alectra* spp. in some crops but the parasitic weeds rapidly evolve resistance to due to the heavy selection pressure (Gressel et al. 1996).The new method requires being feasible and effective. In the present study, with the application of fluridone in rice, we found that irrigation but also spraying with a low concentration of fluridone, a carotenoid biosynthetic inhibitor, significantly reduced the number of germinated/attached *Striga* seeds even with very low concentrations. Especially the concentrations of fluridone used for spraying were so low that we have not seen any phenotypic changes occurring such as chlorophyll bleaching. These results indicate that herbicides that inhibit carotenoid biosynthesis can possibly be an effective and cheap way to reduce the germination of parasitic seeds by spraying or irrigating plants. Our results also show that strigolactones are probably not the signal molecules to direct the growth of the radicle during the *Striga* rice interaction. So if we can find the inhibitors (herbicide) that can block these signal molecules, we can integrate such an inhibitor with a germination stimulant inhibitor to generate a more effective and durable way to control the parasite.

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Chapter 6

General discussion

The general discussion is partly adapted from Sun, Z., R. Matusova and H. Bouwmeester, 2007. Germination of *Striga* and chemical signaling involved: a target for control methods. In: Integrating new technologies for *Striga* control: Towards ending the witch-hunt, J. Gressel and G. Ejeta, Eds., World Scientific Publishing Co., Singapore, 47-60

Objectives:

The research presented in this thesis has focused on the biosynthetic origin of germination stimulants of the root parasitic plants, *Striga* spp. and *Orobanche* spp., as well as the genes that are involved, directly or indirectly, in germination stimulant formation. *Striga* spp. and *Orobanche* spp. have an increasing impact on cereal and other economically important crops in many regions of the world and are a big constraint on using the world's arable area to support the increasingly large numbers of people especially in Third-World countries (Kebreab and Murdoch 2001; Oswald 2005). The traditional control methods are not sufficient and therefore efficient and feasible control methods are urgently required. Control methods that target the early steps in the parasitic weed life cycle are of particular interest to us as they could prevent parasitic weed infection in an early stage. For example, the first chemical signalling between parasitic weeds and their host plant, through the germination stimulants, is obviously interesting as a target for control methods. However, development of such control methods needs to be guided by a thorough knowledge on the germination stimulant biosynthesis. Recent research on the germination stimulants has begun to elucidate their biosynthetic origin. For example, it is known that the best studied class of germination stimulants, the strigolactones, are derived from the carotenoid pathway (Matusova et al. 2005). This research was carried out with the non-model plants maize, sorghum and cowpea. The exact biosynthetic pathway and the genes involved in the formation of germination stimulant however remain unidentified. To further study the biosynthetic pathway of germination stimulants, I also started with the non-model plant maize but it turned out to be a quite difficult system due to the lack of genetic information and totally inefficient transformation system. Nevertheless, we managed to obtain interesting results with maize, including with transformed maize (see below) (Chapter 3). Using model plants may have a lot of advantages to dissect the germination stimulant biosynthetic pathway, but an important question was if these model plants are suitable. To answer this question we have to investigate if the model plants produce germination stimulants, what kind of germination stimulants and from which pathway. Below I will briefly discuss my results with the non-model maize, and then discuss the suitability as model for research on strigolactones of *Arabidopsis* and rice. In addition, I will discuss the results that I have obtained in my thesis work and can potentially be developed into new control methods.

Maize (Chapters 2 and 3)

It is known that strigolactones are the main germination stimulants in maize (Awad et al. 2006) and our group has demonstrated that the maize strigolactones are derived from the carotenoid pathway by using chemical inhibitors of carotenoid biosynthesis and carotenoid-defective mutants (Matusova et al. 2005).

To explain this carotenoid origin, carotenoid cleavage enzymes were predicted to be involved in the strigolactone formation in maize (Matusova et al. 2005). Therefore my research efforts were directed at cloning carotenoid cleavage enzymes. The first and only characterised carotenoid cleavage enzyme in maize so far is *VP14*. Southern Blot analysis using *VP14* as probe suggested that 4 to 6 *VP14* like genes (NCEDs) are present in the maize genome (Tan et al. 1997). In this thesis I have shown that knocking out the NCED (*VP14*) gene family in maize using RNAi technology suppressed germination stimulant formation leading to reduce *Striga* seed germination (Chapter 3). This result confirms the previous result in which the *vp14* mutant induced 40% lower *Striga* seed germination compared with the wild type (Matusova et al. 2005). In addition, also the tomato mutant, *notabilis*, with a mutation in tomato *NCED1* (*VP14* ortholog) showed a 40% reduction of *O. ramosa* seed germination (López-Ráez1 et al. 2007). These results indicate that NCEDs are directly involved in the strigolactone formation in maize or indirectly through the reduced formation of abscisic acid (ABA) by an as yet unknown mechanism.

In my thesis work I cloned a second, as yet unknown maize carotenoid cleavage dioxygenase, *ZmCCD1* (Chapter 2). I showed that ZmCCD1 is not involved in strigolactone formation but catalyses the formation of "yellow pigment" in mycorrhizal maize roots. Mycorrhizal maize roots show a clear reduction in germination stimulant formation (Chapter 2). The possible explanation is that ZmCCD1 competes with other carotenoid cleavage enzymes for a common carotenoid precursor. A higher activity of ZmCCD1 therefore leads to a decreased germination stimulant production in AM root. Indeed, in preliminary experiments we found that root exudates of mycorrhizal maize induced less germination than the exudates of non-mycorrhizal control plants (Chapter 2). These results suggest that the reduction of *Striga* infestation of maize by AM-fungi may be caused by a reduction in the formation of strigolactones.

Model plants

Model plants take advantage of the extensive genetic, biochemical and physiological information and can be genetically engineered more easily and rapidly. Two model plants were used to study the germination stimulant biosynthesis, the dicotyledonous model plant *Arabidopsis* and the monocotyledonous model plant rice. *Arabidopsis* is a host of several *Orobanche* spp. and rice can be infected by several *Striga* spp. Interestingly, *Arabidopsis* is not a host of mycorrhizal fungi, whereas rice is. The study that I did on these two model plants has opened new avenues for germination stimulant research that is currently being done in the research group.

Arabidopsis (Chapter 4)

Arabidopsis, a well-known model dicot plant for studying many aspects of plant biology, is also a host of *Orobanche aegyptica*, *Orobanche ramosa* and *Orobanche minor* (Goldwasser et al. 2000; Westwood and Foy 1998). The germination stimulants of *Arabidopsis* have so far not been identified but could be supposed not to be strigolactones because *Arabidopsis* is not a host of arbuscular mycorrhizal fungi. Nevertheless, my research has shown that orobanchol (a strigolactone) is present in the *Arabidopsis* root exudate and in a hairy root culture under phosphate starvation (Chapter 4) and this suggests that *Arabidopsis* is therefore a suitable model for studying the early stage of host-parasite interaction. However, the concentration of orobanchol found is much lower than in other plant species (compare Chapter 5) and, in addition to orobanchol, I also found several root exudate fractions containing unknown, most likely non-strigolactone, compounds in the *Arabidopsis* exudates, which have germination

stimulating activity on *O. ramosa* (Chapter 4). This could make it difficult to use fast screens to look for mutants as was experienced by (Goldwasser et al. 2002; Westwood 2000). The biosynthetic origin of these other germination stimulants remains unknown with the exception perhaps of one of these that I showed is probably derived from the early plastidic pathway (Chapter 4). The question remains why *Arabidopsis* produces strigolactones considering it is not a host of arbuscular mycorrhizal fungi. Is it because strigolactones are involved in other symbiotic associations? Also it is possible that the strigolactones have another physiological significance that we are currently not aware of.

Rice (Chapter 5)

For the host plants of *Striga*, strigolactones have been found in the root exudates of cereals in tropical areas such as maize, sorghum, and millet (Awad et al. 2006; Siame et al. 1993). The non-model plant, maize, is less suitable to analyse the biosynthetic pathway because of the lacking genome sequence, the difficult transformation and the long generation time. Therefore the cereal model rice could be a better candidate for our research. Indeed we were able to detect orobanchol and another strigolactone, possibly an epimer of 5-deoxystrigol, in the root exudates of rice grown under phosphate starvation. The production of these compounds was completely inhibited by fluridone. These results are confirmed by the germination bioassay, in which root exudates from fluridone treated rice induced much lower *Striga* seed germination than that of non-treated plants grown under phosphate starvation. In addition, in a mycorrhizal branching assay fluridone treated root exudates induced much lower branchings of mycorrhizal hyphae than the exudates of non-treated, phosphate-starved, plants. These results indicate that strigolactones are probably the major germination stimulants in rice root exudates. Thus, compared with *Arabidopsis*, rice has a great advantage to study the biosynthetic pathway of strigolactones. Currently we are trying to identify the NCEDs and/or CCDs involved in strigolactone production in rice. Hereto, rice mutants and transgenic plants will be analysed by molecular and analytical approaches and bioassays.

Control methods

Control methods based on the knowledge about germination stimulants that already existed before my work have been described in Chapter 1. My study in this thesis will add some new impacts on these control methods. The use of these methods, preferably in combination with other control methods may allow us to create an economical and effective *Striga* and *Orobanche* control strategy (Fig. 1).

Molecular breeding

Although currently high seed costs and political opposition to the imposition of GM technology may impede the speed to widespread adoption of the technology, in the long term, the creation of genetically modified crops with *Striga* and *Orobanche* resistance is certainly feasible. Once the strigolactone biosynthetic pathway has been elucidated, it would also become feasible to make low-stimulant producing plants through the inactivation of one or more steps in the pathway. For the time being, as suitable targets also enzymes of the primary carotenoid pathway could be used but preferably then these constructs should be expressed in an organ-specific and/or development-specific manner. In this way the inhibition of carotenoid/ABA biosynthesis is restricted to time and place necessary to obtain resistance against parasitic plants and possibly also avoiding a side-effect on mycorrhizal colonisation. Better targets

would be the dedicated pathway enzymes, i.e. the postulated enzymes involved in cleavage and further conversion of the cleavage product to the strigolactones (Matusova et al. 2005). For example, we have designed RNAi constructs to block the carotenoid cleavage dioxygenases (VP14 cluster enzymes) using both root specific and universal promoters and these constructs were transformed to maize and the transgenic plants induced lower germination of *Striga* seeds than wildtype maize plants (Chapter 3). As an alternative to knocking out enzymes from the germination stimulant pathway, overexpression of key-enzymes of competing pathways to channel away substrate can also be considered as a strategy to reduce germination stimulant formation. Possible candidate is the cleavage enzyme that is responsible for apocarotenoid formation upon mycorrhizal colonization, ZmCCD1 (Chapter 2).

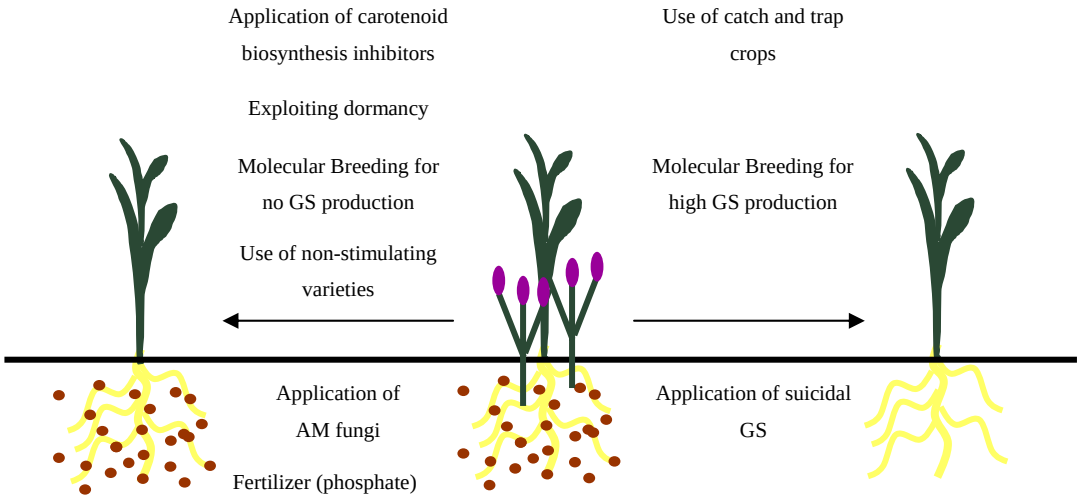

Fig. 1 Diagram showing the possible approaches to new control methods based on the knowledge about germination stimulants

Mycorrhiza

The fact that several groups have reported that AM fungi can reduce *Striga* infection of sorghum and maize (Gworgwor and Weber 2003; Lendzemo et al. 2005) in itself already warrants further research into the possibilities to use inoculation with AM fungi in a *Striga* control strategy (Lendzemo et al. 2005). The mechanism of this reduction was so far unknown, and therefore the possibilities to optimize and exploit this phenomenon for practical use were limited. However, in preliminary experiments, we have shown that this reduction is - in any case partly - due to a decrease in germination stimulant formation after mycorrhizal colonisation (Chapter 2). A possible explanation is that due to the formation of mycorrhiza-specific apocarotenoids or abscisic acid the formation of the *Striga* germination stimulant is reduced (Sun et al. 2007). Apocarotenoid formation may be competing for substrate with germination stimulant formation. Alternatively, mycorrhizal colonisation may be down-regulating the strigolactone production pathway directly. Therefore, research could now be aimed to optimize the use of AM fungi for controlling parasitic plants including *Orobanche* through reduced germination, for example through the selection of suitable AM fungus – host (variety) combinations.

Using chemicals

The results with the application of fluridone to maize, cowpea and sorghum in laboratory experiments have inspired us to look for the possibility to use carotenoid biosynthetic inhibitors to reduce infection with parasitic plants in situ (Chapter 5). In rice, we found that irrigation but also spraying with a low concentration of fluridone significantly reduced the number of germinated/attached *Striga* seeds even with very low concentrations (Chapter 5). In the spraying experiment, the concentration of fluridone used was so low that bleaching of the leaves did not occur. This effect of fluridone suggests that the rice germination stimulants are strigolactones and indeed we have confirmed that they are (Chapter 5). These experiments show that herbicides that inhibit carotenoid biosynthesis can be used to significantly reduce the germination of parasitic seeds and that spraying or irrigating plants with such herbicides at one or more time intervals may be an effective and cheap method to reduce parasitic-weed induced yield losses of crop plants.

Phosphate

Our research has shown that under increased phosphate levels, plants (maize, rice, *Arabidopsis*) produce less germination stimulants. This is also supported by Yoneyama and coworkers who reported that phosphorus deficiency in red clover and in sorghum promotes exudation of orobanchol and 5-deoxystrigol, respectively (Yoneyama et al. 2007a; Yoneyama et al. 2007b). Therefore increasing the phosphate level in deficient soils would probably inhibit *Striga* seed germination and hence seed production. However, strigolactones may not be the only germination stimulants of *Striga* and they are also the signal for mycorrhizal symbionts. Inhibition of production and exudation of strigolactones may also negatively effect the colonization by mycorrhizal fungi. Thus the effective control method should depend on the specific situation in the soil.

Conclusions

The most important conclusions of my research are the following. I have demonstrated that *ZmCCD1* is not involved in the germination stimulant strigolactone formation but in the formation of mycorradicin and cyclohexenone derivatives. Higher expression of *ZmCCD1* was found in mycorrhizal maize roots that at the same time produce less strigolactones. Moreover, by making transgenic knock-outs I also confirmed that VP14 in maize is directly or indirectly involved in strigolactone formation. I discovered that the germination stimulants in *Arabidopsis* do not only belong to the strigolactones but most likely also to other classes of molecules, whereas in rice, 2 strigolactones were identified in root exudates: orobanchol and a novel isomer, possibly epimer, of 5-deoxystrigol - and so far there are no indications that there are also other compound classes involved. My work has yielded several potential control methods of which the use of the chemical carotenoid inhibitor fluridone (or analogous compounds) is one of the most promising.

Prospects

The further enzymatic conversions leading to the strigolactones are still not elucidated but have been postulated to include cytochrome P450 catalysed oxidation (Matusova et al. 2005). Also a novel apocarotenoid hormone, produced by CCD7 and CCD8 action in *Arabidopsis* roots, in the shoot is further modified by MAX1 P450 activity (Auldridge et al. 2006; Booker et al. 2005). MAX1 P450 belongs to

class III P450 family and interestingly also some other members of this class of enzymes (CYP711 and CYP74) act on substrates directly generated by dioxygenases (Booker et al. 2005). These results stimulate us to further work on all the NCEDs and CCDs and related P450s using analytic and genetic approaches in model plants *Arabidopsis* and rice and try to dissect the biosynthetic pathway in the formation of strigolactones.

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Summary

My research focused on the biosynthetic origin of germination stimulants of the root parasitic plants, *Striga* spp. and *Orobanche* spp., which have an increasing impact on cereal and other economically important crops in many regions of the world. The traditional control methods are not sufficient and therefore efficient and feasible control methods are urgently required. Control methods that target the early steps in the parasitic weed life cycle are of particular interest as they could prevent parasitic weed infection in an early stage. The first chemical signal exchange between the host and the parasitic plant, the secretion by the host of germination stimulants, is obviously interesting as a target for control methods. Recent research on the germination stimulants in our group has shown that the most important class of germination stimulants, the strigolactones, are derived from the carotenoid pathway. To explain this carotenoid origin, carotenoid cleavage enzymes were predicted to be involved in the strigolactone formation in maize. Therefore my research efforts were directed at cloning carotenoid cleavage enzymes from maize. The first and only characterised carotenoid cleavage enzyme in maize so far is *VP14* that belongs to a small family of related genes. In this thesis I have shown that knocking out the NCED (*VP14*) gene family in maize using RNAi technology suppressed germination stimulant formation leading to reduce *Striga* seed germination (Chapter 3). This result confirms the previous results with maize *vp14* that induced 40% lower *Striga* seed germination and the tomato mutant, *notabilis*, with a mutation in tomato *NCED1* (*VP14* ortholog) that also induced about 40% lower germination of *O. ramosa*. These results indicate that the NCEDs are directly involved in the strigolactone formation in maize or indirectly through the reduced formation of abscisic acid (ABA) through an as yet unknown mechanism.

In my thesis work I cloned a second, as yet unknown maize carotenoid cleavage dioxygenase, *ZmCCD1* (Chapter 2). I showed that ZmCCD1 is not involved in strigolactone formation but catalyses the formation of “yellow pigment” in mycorrhizal maize roots. Interestingly, mycorrhizal maize roots show a clear reduction in germination stimulant formation (Chapter 2). The possible explanation is that ZmCCD1 competes with other carotenoid cleavage enzymes for a common carotenoid precursor. A higher activity of ZmCCD1 therefore leads to a decreased germination stimulant production in AM root. These results suggest that the reduction of *Striga* infestation of maize by AM-fungi may be caused by a reduction in the formation of strigolactones.

Using model plants may have a lot of advantages to dissect the germination stimulant biosynthetic pathway, because they take advantage of the extensive genetic, biochemical and physiological information and can be genetically engineered more easily and rapidly. However, the first important question of course is if these model plants produce germination stimulants, what kind of germination stimulants and from which pathway. Two model plants were used to study the germination stimulant biosynthesis, the dicotyledonous model plant *Arabidopsis* and the monocotyledonous model plant rice. *Arabidopsis* is a host of several *Orobanche* spp. and rice can be infected by several *Striga* spp. Interestingly, *Arabidopsis* is not a host of mycorrhizal fungi, whereas rice is. *Arabidopsis* is a host of *Orobanche* aegyptica, *Orobanche ramosa* and *Orobanche minor*. The germination stimulants of *Arabidopsis* have so far not been identified but could be supposed not to be strigolactones because *Arabidopsis* is not a host of arbuscular mycorrhizal fungi. Nevertheless, my research has shown that orobanchol (a strigolactone) is present in the *Arabidopsis* root exudates and in a hairy root culture under phosphate starvation (Chapter 4) and this suggests that *Arabidopsis* is therefore a suitable model for

studying the early stage of host-parasite interaction. However, the concentration of orobanchol found is much lower than in other plant species (compare Chapter 5) and, in addition to orobanchol, I also found several root exudates fractions containing unknown, most likely non-strigolactone, compounds in the *Arabidopsis* exudates, which have germination stimulating activity on *O. ramosa* (Chapter 4). This could make it difficult to use fast screens to look for mutants. The biosynthetic origin of these other germination stimulants remains unknown with the exception perhaps of one of these that I showed is probably derived from the early plastidic pathway (Chapter 4). The question remains why *Arabidopsis* produces strigolactones considering it is not a host of arbuscular mycorrhizal fungi. Is it because strigolactones are involved in other symbiotic associations? Also it is possible that the strigolactones have another physiological significance that we are currently not aware of.

For the host plants of *Striga*, strigolactones have been found in the root exudates of cereals in tropical areas such as maize, sorghum, and millet. The cereal model rice could be good candidate for our research. Indeed we were able to detect orobanchol and another strigolactone, possibly an epimer of 5-deoxystrigol, in the root exudates of rice grown under phosphate starvation. The production of these compounds was completely inhibited by fluridone. These results are confirmed by the germination bioassay, in which root exudates from fluridone treated rice induced much lower *Striga* seed germination than that of non-treated plants grown under phosphate starvation. In addition, in a mycorrhizal branching assay fluridone treated root exudates induced much lower branching of mycorrhizal hyphae than the exudates of non-treated, phosphate-starved, plants. These results indicate that strigolactones are probably the major germination stimulants in rice root exudates. Thus, compared with *Arabidopsis*, rice has a great advantage to study the biosynthetic pathway of strigolactones.

My work has contributed new insight in the chemical regulation of the host-parasitic plant lifecycle and as such can contribute to the development of new control methods. Once the strigolactone biosynthetic pathway has been further elucidated, it would also become feasible to make low-stimulant producing plants through the inactivation of one or more steps in the pathway. For the time being, as suitable targets enzymes of the primary carotenoid pathway could be used but better targets would be the dedicated pathway enzymes, i.e. the postulated enzymes involved in cleavage and further conversion of the cleavage product to the strigolactones. For example, we have designed RNAi constructs to block the carotenoid cleavage dioxygenases (*VP14* cluster enzymes) using both root specific and universal promoters and these constructs were transformed to maize and the transgenic plants induced lower germination of *Striga* seeds than wildtype maize plants (Chapter 3). As an alternative to knocking out enzymes from the germination stimulant pathway, overexpression of key-enzymes of competing pathways to channel away substrate can also be considered as a strategy to reduce germination stimulant formation. Possible candidates are the cleavage enzymes that are responsible for apocarotenoid formation upon mycorrhizal colonization, ZmCCD1 (Chapter 2). Also the suppressive effect of AM fungi on *Striga* infection of sorghum and maize may be developed into a *Striga* control strategy. The mechanism of this reduction was so far unknown, and therefore the possibilities to optimize and exploit this phenomenon for practical use were limited. However, I have shown that this reduction is - in any case partly - due to a decrease in germination stimulant formation after mycorrhizal colonisation (Chapter 2). A possible explanation is that due to the formation of mycorrhiza-specific apocarotenoids or abscisic acid the formation of the *Striga* germination stimulant is reduced. Apocarotenoid formation may be competing for substrate with germination stimulant formation. Alternatively, mycorrhizal colonisation may be down-regulating the strigolactone production pathway directly. Therefore, research could now be aimed to

optimize the use of AM fungi for controlling parasitic plants including *Orobanche* through reduced germination, for example through the selection of suitable AM fungus – host (variety) combinations. In rice, we found that irrigation but also spraying with a low concentration of fluridone significantly reduced the number of germinated/attached *Striga* seeds even with very low concentrations (Chapter 5). In the spraying experiment, the concentration of fluridone used was so low that bleaching of the leaves did not occur. This effect of fluridone suggests that the rice germination stimulants are strigolactones and indeed we have confirmed that they are (Chapter 5). These experiments show that herbicides that inhibit carotenoid biosynthesis can be used to significantly reduce the germination of parasitic seeds and that spraying or irrigating plants with such herbicides at one or more time intervals may be an effective and cheap method to reduce parasitic-weed induced yield losses of crop plants. Our research has shown that under increased phosphate levels, plants (maize, rice, *Arabidopsis*) produce less germination stimulants. Therefore increasing the phosphate level in deficient soils would probably inhibit *Striga* infection. However, strigolactones may not be the only germination stimulants of *Striga* and they are also the signal for mycorrhizal symbionts. Inhibition of production and exudation of strigolactones may also negatively effect the colonization by mycorrhizal fungi. Thus the effective control method should depend on the specific situation in the soil.

Samenvatting

Mijn onderzoek heeft zich gericht op de biosynthese van kiemstimulantia van parasitaire planten, te weten *Striga* en *Orobanche* soorten, die een enorme negatieve impact hebben op graangewassen en andere economische belangrijke gewassen in grote delen van de wereld. De traditionele manieren om te proberen deze parasitaire planten onder controle te houden zijn niet toereikend en daarom zijn nieuwe beheersmaatregelen hard nodig. Beheersmaatregelen die zich richten op het begin van de levenscyclus van de parasitaire planten zouden extra aantrekkelijk kunnen zijn omdat ze al in een vroeg stadium de infectie zouden kunnen voorkomen. Het eerste signaal dat tussen waardplant en parasiet wordt uitgewisseld - de kiemstimulantia, verbindingen die door de wortel van de waardplant worden uitgescheiden en de kieming van het zaad van de parasiet kunnen induceren – zijn daarom een aantrekkelijk doelwit. Onderzoek in onze groep heeft aangetoond dat de belangrijkste klasse van kiemstimulantia, de strigolactonen, biosynthetisch afkomstig zijn van de carotenoiden. Recent is aangetoond dat de strigolactonen behalve kiemstimulant ook een belangrijke waardplant detectiefactor zijn voor de mycorrhiza schimmels, die tot wederzijds nut in en op plantenwortels kunnen groeien. Om van de grote C_{40} carotenoiden naar het C_{14} basisskelet van de strigolactonen te komen hebben wij gepostuleerd dat zogenaamde caroteen splitsende enzymen nodig zijn: “Carotenoid Cleavage Dioxygenases” (CCDs) of “9-*cis*-Epoxycarotenoid cleavage Dioxygenases” (NCEDs). Omdat dit onderzoek vooral met mais was gedaan heb ik me in mijn onderzoek in eerste instantie gericht op de clonering van caroteen splitsende enzymen uit mais. Het enige bekende enzym in mais dat in deze enzym familie thuishoort is VP14 (een NCED). In dit proefschrift laat ik zien dat het uitschakelen van VP14 in mais mbv RNAi leidt tot verminderde *Striga* kieming (Hoofdstuk 3). Dit resultaat bevestigt onderzoek in onze groep met de mais mutant *vp14* en de tomaten mutant *notabilis* (een ortholoog van VP14). En deze resultaten laten zien dat NCEDs betrokken zijn bij de productie van strigolactonen, ofwel direct (ze voeren de benodigde caroteen splitsende reactie uit) ofwel, meer waarschijnlijk, indirect doordat de abscissinezuur productie door deze enzymen wordt gereguleerd.

In mijn proefschrift beschrijf ik een tweede , tot nu toe onbekend, mais caroteen splitsend enzym, ZmCCD1 (Hoofdstuk 2). Ik heb laten zien dat dit enzym niet is betrokken bij de productie van strigolactonen maar dat het verantwoordelijk is voor de productie van de gele pigmenten die worden gevormd in de wortels van planten wanneer ze in symbiose leven met mycorrhiza schimmels (Hoofdstuk 2). De expressie van ZmCCD1 wordt in dat geval verhoogd. Het is intrigerend dat diezelfde door mycorrhiza schimmels gekoloniseerde planten minder kiemstimulantia produceren. We vermoeden dat dat komt doordat ZmCCD1 caroteen substraat wegneemt voor de vorming van het gele pigment waardoor er minder strigolactonen kunnen worden gemaakt. Vermoedelijk is dit effect verantwoordelijk voor de in het veld waargenomen verminderde infectie van sorghum en mais wanneer daaraan mycorrhiza schimmels zijn toegevoegd.

Het gebruik van model plantensoorten zou grote voordelen kunnen hebben boven het gebruik van mais voor het ophelderen en karakteriseren van de biosynthese route van de kiemstimulantia. Een belangrijke vraag is dan echter eerst of die planten wel strigolactonen produceren. In mijn proefschrift heb ik twee model plantensoorten bestudeerd, Arabidopsis en rijst, modellen voor respectievelijk de dicotylen en monocotylen. Arabidopsis is waardplant voor diverse *Orobanche* soorten en rijst kan worden geïnfecteerd door diverse *Striga* soorten. De kiemstimulantia van beide soorten zijn tot nu toe niet geïdentificeerd, maar gezien het feit dat Arabidopsis geen waardplant is voor mycorrhiza schimmels, zou het kunnen zijn dat de kiemstimulantia niet tot de strigolactonen behoren. Desalniettemin heb ik laten zien dat Arabidopsis “hairy root” cultures het strigolacton orobanchol produceren en dat Arabidopsis dus een geschikt model is (Hoofdstuk 4). Echter, de productie lijkt wel heel veel lager te zijn dan in andere plantensoorten (vergelijk met Hoofdstuk 5) en er blijken ook andere, niet-strigolacton, verbindingen in het exudaat te zitten die de kieming van *Orobanche* zaden kunnen induceren en waarvan er een vermoedelijk ook van de isoprenoiden biosynthese route afkomstig is (Hoofdstuk 4). Dit zou het gebruik van Arabidopsis voor een mutant screen lastig kunnen maken. De vraag blijft wel waarom Arabidopsis strigolactonen produceert (al is het veel minder) terwijl ze geen waardplant is voor mycorrhiza schimmels. Misschien is dit omdat strigolactonen een rol spelen in andere symbioses of omdat ze een andere tot nog toe onbekende fysiologische rol hebben.

Wat betreft de waardplanten van *Striga* soorten zijn strigolactonen gevonden in de wortelexudaten van tropische graangewassen zoals mais, sorghum en gierst. Het graan model rijst zou daarom een geschikt model kunnen zijn voor het onderzoek aan strigolactonen. Inderdaad hebben we orobanchol en nog een ander strigolacton, misschien een epimeer van 5-deoxystrigol, kunnen aantonen in de exudaten van rijst die aan fosfaat tekort zijn blootgesteld (Hoofdstuk 5). De productie van de strigolactonen werd volledig geremd door een behandeling met fluridon. Kiemings assays bevestigden deze resultaten: de kieming was vrijwel nul in exudaten van met fluridon behandelde planten. Ook in een assay met mycorrhiza schimmels bleek de biologische activiteit van het exudaat verloren na fluridon behandeling. Deze resultaten suggereren dat strigolactonen de belangrijkste (enige) kiemstimulanten zijn in de wortelexudaten van rijst. Potentieel maakt dat van rijst een veel betere kandidaat om de biosynthese van strigolactonen te bestuderen dan Arabidopsis.

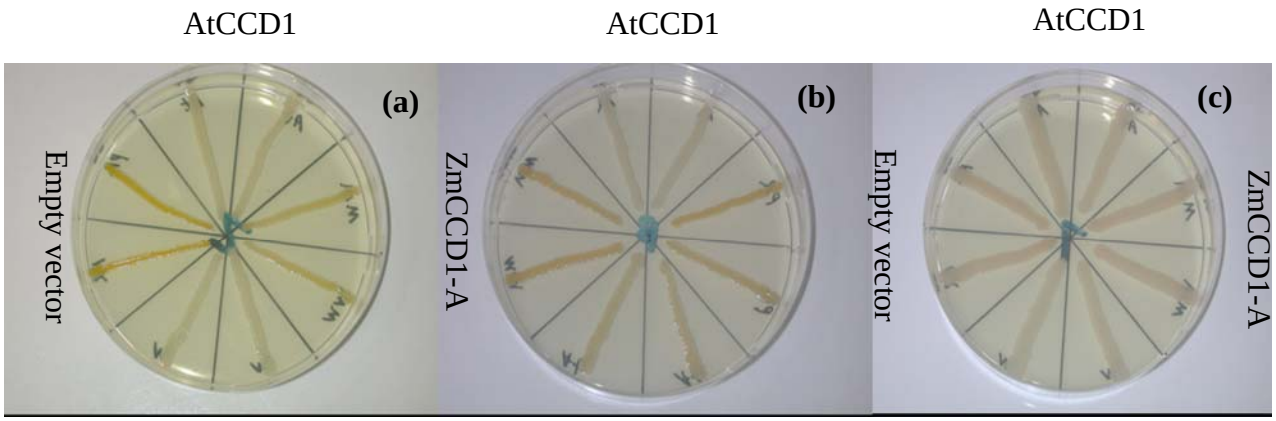
Mijn werk heeft bijgedragen aan nieuw inzicht in de chemische regulatie van de waardplant-parasitaire plant interactie en kan daarom wellicht ook bijdragen aan de ontwikkeling van nieuwe beheersmaatregelen (Hoofdstuk 6). Hoewel de strigolacton biosynthese route nog niet helemaal is opgehelderd zouden we nu al laag-stimulant producerende gewassen kunnen maken door bijvoorbeeld genen in de primaire carotenoid biosynthese uit te schakelen. Beter nog zou het zijn de gepostuleerde caroteen splitsende enzymen uit te schakelen. Ik heb dat bijvoorbeeld gedaan door met behulp van RNAi constructen de NCED VP14 met behulp van wortel-specifieke en constitutieve promoters uit te schakelen in mais. De transgene planten leken inderdaad minder *Striga* kieming te induceren (Hoofdstuk 3). Als alternatief voor het uitschakelen van genen uit de strigolacton biosynthese zouden we ook genen kunnen

activeren die verantwoordelijk zijn voor competerende routes. Een kandidaat hiervoor zou ZmCCD1 kunnen zijn, die verantwoordelijk is voor de vorming van de gele kleurstof in door mycorrhiza schimmels gekoloniseerde wortels (Hoofdstuk 2). Ook het remmende effect van mycorrhiza schimmels op de infectie van gewassen door *Striga* zou met de door mij gegenereerde achtergrondskennis – dat het effect van mycorrhiza in ieder geval deels wordt veroorzaakt door een verminderde kiemstimulant productie - tot nieuwe beheersmaatregelen kunnen worden ontwikkeld (Hoofdstuk 2). Hoe dit effect wordt veroorzaakt - competitie om caroteen substraat voor de vorming van de gele kleurstof en daardoor lagere strigolacton productie of een rechtstreeks down-regulerend effect van mycorrhiza op strigolacton vorming – is nog onbekend. Onderzoek dat er op gericht is dit effect te optimaliseren, potentieel ook voor *Orobanche*, bijvoorbeeld door te zoeken naar optimale waardplant soort - mycorrhiza soort combinaties, zou kunnen resulteren in nieuwe beheersmaatregelen voor parasitaire planten. In rijst heb ik aangetoond dat een behandeling met lage concentraties fluridon leidt tot een significant lagere infectie met *Striga* (Hoofdstuk 5). De gebruikte concentraties van fluridon waren zo laag dat de gebruikelijke chlorofyl ontkleuring achterwege bleef. Dit experiment laat zien dat herbicides, die de carotenoid biosynthese remmen, de kieming van parastaire planten reduceren en dat behandelingen met lage concentaries van die herbicides wellicht ontwikkeld kunnen worden tot een goedkoop en betrouwbare beheersmaatregel voor parasitaire onkruiden. Ons onderzoek heeft ook laten zien dat mais, rijst en Arabidopsis bij normale fosfaatgehaltes in het groeimedium veel minder strigolactonen produceren. Het verbeteren van de fosfaattoestand van arme gronden zou daarom wel eens een heel effectieve maatregel kunnen zijn om *Striga* problemen tegen te gaan. Wel moet worden bedacht dat de strigolactonen mogelijk niet altijd de enige kiemstimulanten zijn in het wortel exudaat van een bepaalde plantensoort en dat de strigolactonen ook het waardplant detectiesignaal zijn voor de symbiotische mycorrhiza schimmels. Maatregelen die getroffen worden om de strigolacton productie van een gewas te verlagen moeten dus altijd worden afgestemd op de specifieke situatie in de grond.

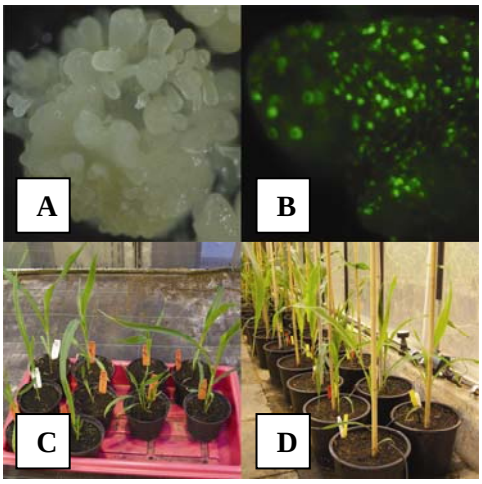
Appendix

Striga hermonthica on rice *Orobanche ramosa on tomato*

Chapter 1 Fig. 1



Chapter 2, Fig. 3



Chapter 3, Fig. 2

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About the Author

Zhongkui Sun was born on 10 February 1968 in Tianjin, China. Since 1990, having obtained a Bachelor of Science degree in horticulture from Shengyang Agricultural University in China, he had worked as a researcher in Tianjin Cucumber Research Institute of Tianjin Agricultural Academy of Science, where he gained experience in cucumber breeding, production and marketing.

A one-year experience as a visiting scholar at Plant Research International at Wageningen in The Netherlands from 1998 to 1999 convinced him to return to Wageningen University to pursuit a Master of Science degree in plant breeding and biotechnology in 2000. His success in master study has led to a Ph.D study at Group of Bioscience in Plant Research International starting from September 2002. This thesis is financed by Netherlands Organization for Scientific Research (NWO/WOTRO).

Since August 1, 2007, the author works as the China-based R & D manager for Rijk Zwaan.

Publications:

Sun Z, Hans J, Walter MH, Matusova R, Beekwilder J, Francel WA, Zhao M, Esther van Echtelt E, Strack D, Bisseling T, Bouwmeester HJ (2007) Cloning and characterization of a carotenoid cleavage dioxygenase from maize and its effect on germination stimulants of parasitic weeds. submitted to Planta.

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1) Start-up phase	date
► **First presentation of your project**	
Germination stimulants of the parasitic weeds Striga and Orobanche	Feb 13, 2003
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Secondary metabolite signalling in host-parasitic plant interactions	2003
Germination of Striga and chemical signaling involved	2007
► **MSc courses**	
► **Laboratory use of isotopes**	
Course 'Safe handling of radioisotopes'	Nov 10-15, 2002
Subtotal Start-up Phase	*9.0 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
PhD day 2003, Utrecht	Mar 27, 2003
PhD day 2006, Wageningen	Sep 19, 2006
► **EPS theme symposia**	
Theme II symposium 2003 "interactions between Plants and Biotic Agents Wageningen	Dec 12, 2003
Theme II symposium 2004 "interactions between Plants and Biotic Agents Utrecht	Sep 17, 2004
► **NWO Lunteren days and other National Platforms**	
13th ALW Meeting Secondary metabolism in plants and plant cells, Utrecht	Mar 14, 2003
► **Seminars (series), workshops and symposia**	
Workshop on Parasitic Plant Research, Wageningen	May 22-24, 2003
Workshop on COST Action 849, parasitic plant management Wageningen	Oct 13-15 2004
Workshop on COST Action 849, , Reading, U.K	Sep 15-17 2005
1st Int Symp Biology of non-weedy, hemiparasitic Scrophulariaceae, Wageningen	Apr 15-16, 2004
► **Seminar plus**	
► **International symposia and congresses**	
The Dutch-Chinese life science forum, Wageningen	Nov 12, 2003
The Dutch-Chinese life science forum, Utrecht	Oct 07, 2006
Terpnet 2005, Wageningen	Apr 20-23, 2005
► **Presentations**	
COST Action 849, Parasitic plant management Wageningen (Oral)	Oct 13-15,2004
13th ALW Meeting Secondary metabolism in plants and plant cells Utrecht (poster)	Mar 14, 2003
Workshop on Parasitic Plant Research, Wageningen (oral)	May 22, 2003
Chemical communication from gene to ecosystem Wageningen (oral)	Mar 19-23 2005
► **IAB interview**	Jun 03, 2005
Subtotal Scientific Exposure	*10.1 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
PhD course 'Disease Resistence in Plants', Wageningen	Oct 14-16, 2002
Bioinformation technology-1 Wageningen	May 12-21, 2003
EPS Theme 3 symposium, Molecular Basis of Microbe-Plant Interactions Leiden	Jun 05-07, 2003
Functional genomics: theory and hands-on data analysis Utrecht	Aug 25-28, 2003
Chemical Communication: from gene to ecosystem Wageningen	Mar 19-23 2005
Signaling in Plant Development and Defence: Wageningen	Jun 19-21, 2006
► **Journal club**	
► **Individual research training**	
RNAi technique and application in viral disease in plant and animals Wageningen	Sep 14-15, 2004
Subtotal In-Depth Studies	*8.7 credits**

4) Personal development	date
► **Skill training courses**	
Project and Time management Wageningen	Mar-May 2004
Media and mediators, messages and means Wageningen	Sep 09,10 &30, 2004
Teaching and Supervising Thesis student Wageningen	Jun 2005
Career Perspectives Wageningen	Oct-Dec 2006
► **Organisation of PhD students day, course or conference**	
EPS PhD day	Sep 16, 2006
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*6.3 credits**

TOTAL NUMBER OF CREDIT POINTS*	**34.1**

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 credits

** A credit represents a normative study load of 28 hours of study*