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BOTANICAL BRIEFING

Strigolactones: Chemical Signals for Fungal Symbionts and Parasitic Weeds in Plant Roots

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• *Aims* Arbuscular mycorrhizae are formed between >80 % of land plants and arbuscular mycorrhizal (AM) fungi. This Botanical Briefing highlights the chemical identification of strigolactones as a host-recognition signal for AM fungi, and their role in the establishment of arbuscular mycorrhizae as well as in the seed germination of parasitic weeds.
• *Scope* Hyphal branching has long been described as the first morphological event in host recognition by AM fungi during the pre-infection stages. Host roots release signalling molecules called 'branching factors' that induce extensive hyphal branching in AM fungi. Strigolactones exuded from host roots have recently been identified as an inducer of hyphal branching in AM fungi. Strigolactones are a group of sesquiterpenes, previously isolated as seed germination stimulants for the parasitic weeds *Striga* and *Orobanche*. Parasitic weeds might find their potential hosts by detecting strigolactones, which are released from plant roots upon phosphate deficiency in communication with AM fungi. In addition to acting as a signalling molecule, strigolactones might stimulate the production of fungal symbiotic signals called 'Myc factors' in AM fungi.
• *Conclusions* Isolation and identification of plant symbiotic signals open up new ways for studying the molecular basis of plant–AM-fungus interactions. This discovery provides a clear answer to a long-standing question in parasitic plant biology: what is the natural role for germination stimulants? It could also provide a new strategy for the management and control of beneficial fungal symbionts and of devastating parasitic weeds in agriculture and natural ecosystems.

Key words: Sesquiterpene lactone, *Lotus japonicus*, *Gigaspora margarita*, root exudate, *Striga*, *Orobanche*, phosphate nutrition.

INTRODUCTION

Mycorrhizae are symbiotic associations between soil fungi and plant roots (Smith and Read, 1997). Arbuscular mycorrhizae, formed between >80 % of land plants and arbuscular mycorrhizal (AM) fungi belonging to the Glomeromycota (Schüssler *et al*., 2001), are the most common and widespread symbiosis on our planet (Fig. 1A) (Gianinazzi-Pearson, 1996; Harrison, 2005). AM fungi are obligate symbionts incapable of completing their life cycle in the absence of a host root. The fungi penetrate and colonize plant roots, where they differentiate into highly branched structures known as arbuscules, which are thought to be the principal sites of nutrient exchange between the two organisms. Concomitant development of extra-radical hyphae outside the plant roots allows the fungi to supply the host with essential nutrients such as phosphate, nitrate and other minerals from the soil. In return, AM fungi receive carbohydrates derived from photosynthesis in the host. AM symbiosis also confers resistance to the plant against pathogens and environmental stresses. Species composition and richness of AM fungi have been shown to contribute greatly to plant biodiversity as well as to the variability and productivity of natural ecosystems (van der Heijden *et al*., 1998). The fossil records from the Ordovician and Devonian eras indicate the existence of AM symbioses over 460 million years ago, suggesting that the fungi played a crucial role in facilitating the colonization of land by plants (Remy *et al*., 1994; Redecker *et al*., 2000). Despite the central importance of AM symbiosis in both agriculture and natural ecosystems, the mechanisms for the formation of a functional symbiosis between plants and AM fungi are largely unknown. A major factor hampering studies on AM fungi is their obligately biotrophic nature; the fungi have not been cultured in the absence of a plant host.

The plant–AM-fungus interaction is initiated by mutual signal exchange between the two partners during pre-infection stages (Harrison, 2005). Host roots release signal molecules called 'branching factors' (BFs) that induce extensive hyphal branching in AM fungi. AM fungi have long been postulated to produce signal molecules called 'myc factors' (MFs) that induce the molecular and cellular responses leading to successful root colonization by AM fungi. Neither of these signals had been isolated and chemically identified until recently when a BF was isolated from the root exudates of the model legume *Lotus japonicus*, and was identified as a strigolactone, 5-deoxystrigol (Akiyama *et al*., 2005). Strigolactones are a group of sesquiterpene lactones, previously isolated as seed-germination stimulants for the root-parasitic weeds *Striga* and *Orobanche* (Bouwmeester *et al*., 2003). It now turns out that the same compounds are detected by beneficial fungal symbionts and by devastating parasitic weeds as host-derived signals.

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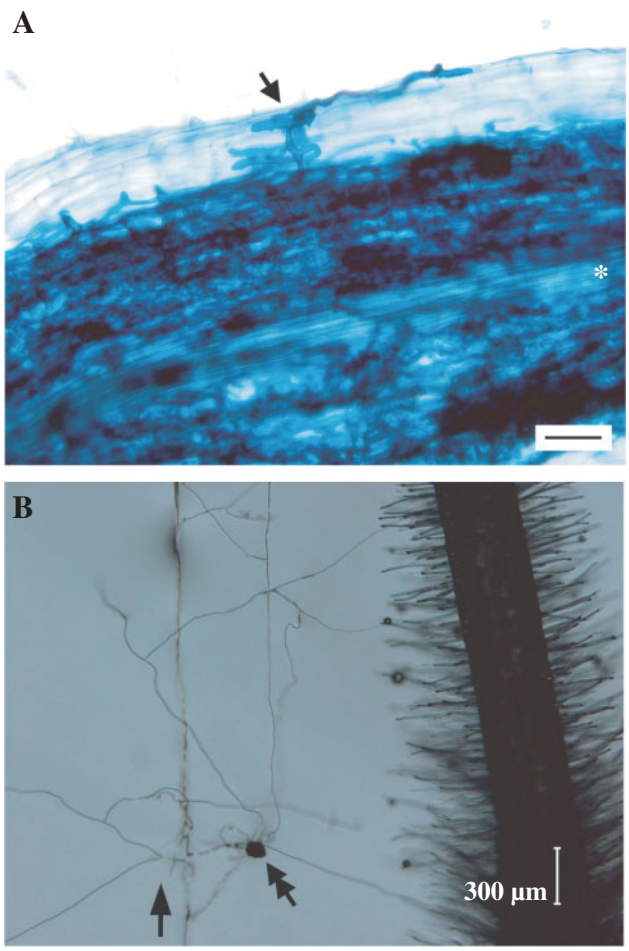

FIG. 1. Symbiotic interactions between the model legume *Lotus japonicus* and the AM fungus *Gigaspora margarita*. (A) Arbuscular mycorrhizae formed between *L. japonicus* and *G. margarita*. Fungal hyphae are stained with trypan blue after the root has been cleared by heating in KOH. Arrow, appressorium formed on root surface; asterisk, root's stele. Scale bar = 60 µm. (B) Hyphal branching of *G. margarita* in the vicinity of roots of *L. japonicus*. Secondary, tertiary, fourth and fifth hyphae emerge from primary hypha. Arrow, direction of growth of primary hypha (germ tube); double arrow, auxiliary cells of fungus. Scale bar = 300 µm.

HYPHAL BRANCHING: HOST RECOGNITION RESPONSE OF AM FUNGI

The critical developmental step in the life cycle of AM fungi is hyphal branching. Although AM fungi cannot complete their life cycle in the absence of a host root, their spores can germinate independently of host plants as long as some physical and physiological conditions are fulfilled. However, the hyphal growth is very limited, and ceases long before consumption of spore reserves if a host root is not present in the environment (Bécard and Piché, 1989). In the presence of host roots, the hyphae of AM fungi differentiate into specific morphological structures characterized by extensive hyphal branching, which help the fungi to ensure contact with the root and the establishment of symbiosis (Fig. 1B). These hyphal branching phenomena were first described by Mosse and Hepper (1975) in the first report of the *in vitro* co-culture of a clover root and an AM fungus. They called these structures 'arbuscule-like branches'. Powell (1976) found that hyphae, recently germinated from spores, branch repeatedly to form septate 'fan-like structures' in the close vicinity of onion roots before appressorium formation, and suggested that the fan-like pre-infection hyphae are the site of cytological changes necessary before hyphae from spores become physiologically infective. Giovannetti *et al*. (1993, 1994) used a membrane sandwich system to show that this differential hyphal morphogenesis is a host recognition response of AM fungi. AM fungal hyphae, grown on membranes overlying AM host roots, exhibited extensive hyphal branching, while no morphogenetic event was elicited by the roots of non-hosts (including hosts of ecto, arbutoid and ericoid mycorrhizae as well as non-mycorrhizal plants). These findings strongly indicated the existence of chemical signals, emitted exclusively by host roots, acting as early cues for differential hyphal branching.

BRANCHING FACTORS: LIPOPHILIC LOW-MOLECULAR-WEIGHT COMPOUNDS EXUDED FROM HOST ROOTS

The identity of BFs, exuded from host roots, has been the object of considerable research. Dialysis membranes have been used in the sandwich system described above to determine that the BF exuded from growing roots of common basil (*Ocimum basilicum*), which elicits hyphal branching in *Glomus mosseae*, is a low-molecular-weight compound <500 Da (Giovannetti *et al*., 1996). The development of an *in-vitro* bioassay for hyphal branching in germinating spores of the genus *Gigaspora* (Nagahashi and Douds, 1999) has facilitated the analysis of the chemical characteristics and distribution of BF in the plant kingdom (Buee *et al*., 2000; Nagahashi and Douds, 2000). BF was present in root exudates of all host plants of AM fungi, but absent in those of non-hosts. The discovery that BF is partitioned into ethyl acetate from an aqueous root exudate (Buee *et al*., 2000), and is retained on a C_{18} reverse-phase resin (Nagahashi and Douds, 2000), indicates that BF is a lipophilic compound. The presence of several BFs in host plants was suggested by C_{18} reverse-phase column and preparative thin-layer chromatography (Nagahashi and Douds, 2000). Root exudates from plants grown under phosphate-limited conditions are more active than those from plants with sufficient phosphate nutrition, suggesting that the production of BF in roots and its exudation are regulated by phosphate availability (Nagahashi and Douds, 2000).

Phenolics including flavonoids have been strong candidates for BF because these compounds play an active role in the regulation of symbiotic and pathogenic interactions with microbes (Peters and Varma, 1990). In fact, some flavonoids such as quercetin have been reported to promote spore germination, hyphal elongation and hyphal branching (Gianinazzi-Pearson *et al*., 1989; Tsai and Phillips, 1991; Bécard *et al*., 1992). However, flavonoids have been ruled out as BF candidates because root exudates

of maize mutants deficient in chalcone synthase, necessary for the biosynthesis of flavonoids, showed comparable activity to those of the wild type in the hyphal branching assay (Buee *et al*., 2000). This is further supported by the evidence that the same maize mutants are equally colonized by AM fungi as the wild-type maize (Bécard *et al*., 1995) and that quercetin shows no activity in the branching bioassay (Buee *et al*., 2000; Nagahashi and Douds, 2000). In addition, significant amount of quercetin, myricetin and kaempferol, flavonoids with strong stimulatory activity on AM fungi, have been detected in non-mycorrhizal plants such as *Arabidopsis thaliana* (Burbulis *et al*., 1996).

IDENTIFICATION OF STRIGOLACTONES AS INDUCERS OF HYPHAL BRANCHING IN AM FUNGI

Purification of the BFs has been severely hampered by the extremely low concentrations produced and exuded by roots as well as their chemical instability. For the first time, a BF has been successfully isolated from the root exudates of *L. japonicus* and identified (Akiyama *et al*., 2005). BF from *L. japonicus* grown hydroponically under low-phosphate conditions is a lipophilic, neutral compound as revealed by solvent partition between ethyl acetate and acidic or basic aqueous solutions. To obtain sufficient amounts of BF for spectroscopic analysis, a BF-enrichment procedure was developed, in which the hydroponic solution containing BF was continuously pumped through an activated charcoal cartridge. The BF adsorbed to the surface of activated charcoal was eluted with acetone, and was partitioned to yield an ethyl acetate-soluble neutral fraction. Hyphal branching assay-guided purification of the neutral fraction by column chromatography over silica gel and semi-preparative C_{18} reverse-phase HPLC resulted in the isolation of a BF. The BF was identified as a strigolactone, 5-deoxy-strigol, by spectroscopic analysis and chemical synthesis (Fig. 2A). Natural 5-deoxy-strigol induced extensive hyphal branching in germinating spores of *Gigaspora margarita* even if only 30 pg were applied per disc (Fig. 3A, B). The hyphal branching activity of racemic 5-deoxy-strigol (prepared by chemical synthesis) was comparable to that of the natural compound, confirming that the activity of the natural 5-deoxy-strigol is not due to contaminants in the purified sample.

Strigolactones are a group of sesquiterpene lactones, previously isolated as seed-germination stimulants for the parasitic weeds *Striga* and *Orobanche* spp. The known natural strigolactones (sorgolactone and strigol; Fig. 2B) and a synthetic analogue (GR24; Fig. 2D) induced hyphal branching at picogram to nanogram levels in the assay. Orobanchol (Fig. 2B) is also highly active on *G. margarita* (K. Akiyama and H. Hayashi, unpublished results). Thus, surprisingly, strigolactones are revealed to be BFs. Recently, Bécard *et al*. (2005) also reported the stimulatory effects of the synthetic strigolactone analogues GR24 and GR7 (Fig. 2E) on hyphal branching in *Gigaspora rosea*. They also found that these two analogues activate cellular respiration in *G. rosea* and *Glomus intraradices*.

STRIGOLACTONES: GERMINATION STIMULANTS FOR PARASITIC WEEDS

The parasitic weeds *Striga* and *Orobanche* are among the most damaging agricultural pests in large parts of the world (Bouwmeester *et al*., 2003). The roots of parasitic weeds can attack the roots of their plant hosts and rob them of water and nutrients. The lives of millions of people in Africa, India and the Middle East are affected by severe harvest reductions due to heavy infestations of susceptible crops with these parasites. Most species are obligate parasites incapable of completing their life cycle in the absence of a host. The first important step in their life cycle is seed germination. Seeds of the parasites remain dormant until germination is stimulated by a chemical produced and exuded by host roots. For development of a control programme, considerable efforts have been made in the identification of the germination stimulants. Thus, to date, five natural strigolactones have been isolated, and a number of structural analogues have been synthesized (Bouwmeester *et al*., 2003). 5-Deoxy-strigol is the sixth natural strigolactone; this compound has been prepared by organic synthesis as a derivative of strigol, but has not been isolated from any natural source (Frischmuth *et al*., 1991). This strigolactone was reported to be approximately one-third as active as (+)-strigol on *Orobanche crenata* seed germination (Bergmann *et al*., 1993).

Strigolactones are highly active on parasitic weeds, inducing 50 % seed germination at picomolar concentrations. The connection of the C and D rings to each other via an enol ether bond (see chemical structure shown in Fig. 2B) was shown to be necessary for germination stimulation (Mangnus and Zwanenburg, 1992). The inherent instability of strigolactones is principally due to easy cleavage of the enol ether bond by nucleophilic agents including water. Taken with the observations that all strigolactones tested were highly active on AM fungi, and that their activity drastically decreased after concentration or storage of a solution of strigolactones in nucleophilic solvents such as pure or aqueous methanol, it appears that the C–D part is also essential for the effect of strigolactones on AM fungi (K. Akiyama and H. Hayashi, unpubl. res.). The chemical lifetime of strigolactones under natural soil conditions can be very short, enabling these chemicals to convey positional information about the roots of living host plants to AM fungi (and also parasitic weeds). Given the facts that production of strigolactones by red clover roots is stimulated under low phosphate conditions, and that parasitic weeds prevail in areas with limited phosphate availability in the soil (Yoneyama *et al*., 2001), it is tempting to speculate that parasitic weeds might find their potential hosts by detecting strigolactones, which are released from plant roots upon phosphate deficiency in communication with AM fungi.

CHEMICAL DIVERSITY AND DISTRIBUTION OF STRIGOLACTONES AMONG PLANTS

Strigolactones have been isolated from root exudates of a variety of plants, including the monocots sorghum, maize

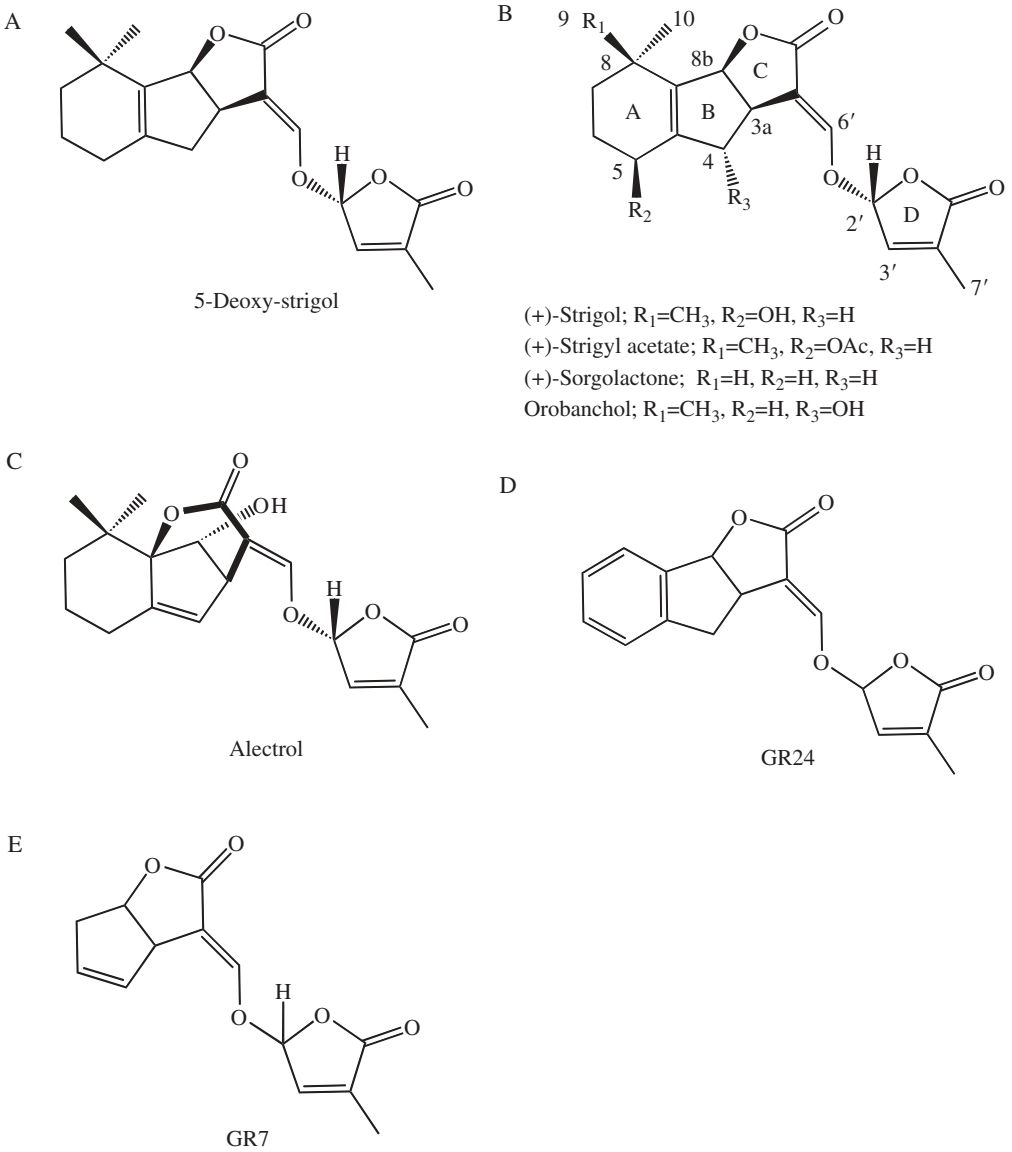

FIG. 2. Chemical structures of natural strigolactones and synthetic analogues: (A) 5-deoxy-strigol; (B) four natural strigolactones; (C) alectrol (tentative structure); (D) synthetic analogue GR24; (E) synthetic analogue GR7. The absolute configuration of the natural 5-deoxy-strigol (A), (+)-strigol and (+)-sorgolactone (B) is 3a(*R*), 8b(*S*), 2'(*R*).

and proso millet, and the dicots cotton, cowpea, red clover, *Menispermum dauricum* and *Lotus japonicus* (Fig. 2A–C) (Cook *et al*., 1966, 1972; Hauck *et al*., 1992; Müller *et al*., 1992; Siame *et al*., 1993; Yokota *et al*., 1998; Yasuda *et al*., 2003; Akiyama *et al*., 2005). The isolation of (+)-strigol from an aseptic root organ culture of *M. dauricum* has unambiguously demonstrated that this strigolactone is of plant origin (Yasuda *et al*., 2003). Although strigolactones are suggested to be more widely distributed in the plant kingdom, the isolation and characterization of strigolactones in root exudates, as in the case of this BF, have been hampered by the extremely low concentrations produced and exuded by host roots as well as their relative instability. Theoretical considerations based on the ubiquitous occurrence of AM symbiosis suggest that strigolactones are produced by almost all plants, including angiosperms, gymnosperms, pteridophytes including psilotophytes and lycopods and some mosses, although all the strigolactones identified so far have been isolated exclusively from herbaceous higher plants. It is noteworthy that *Arabidopsis thaliana*, belonging to the non-mycotrophic family Brassicaceae, has been found to produce seed germination stimulants, but at much lower concentrations than in the crop plants carrot and tobacco, which are hosts of AM fungi (Westwood, 2000). This broad distribution of strigolactones in the plant kingdom and their levels in plant root exudates are consistent with the host specificity of AM fungi.

Given that the C–D part of strigolactones is essential for hyphal branching activity, and that modifications in the A and B rings do not appear to affect their ability to induce hyphal branching in AM fungi, >100 strigolactone derivatives can be predicted to exist in the plant kingdom. This is

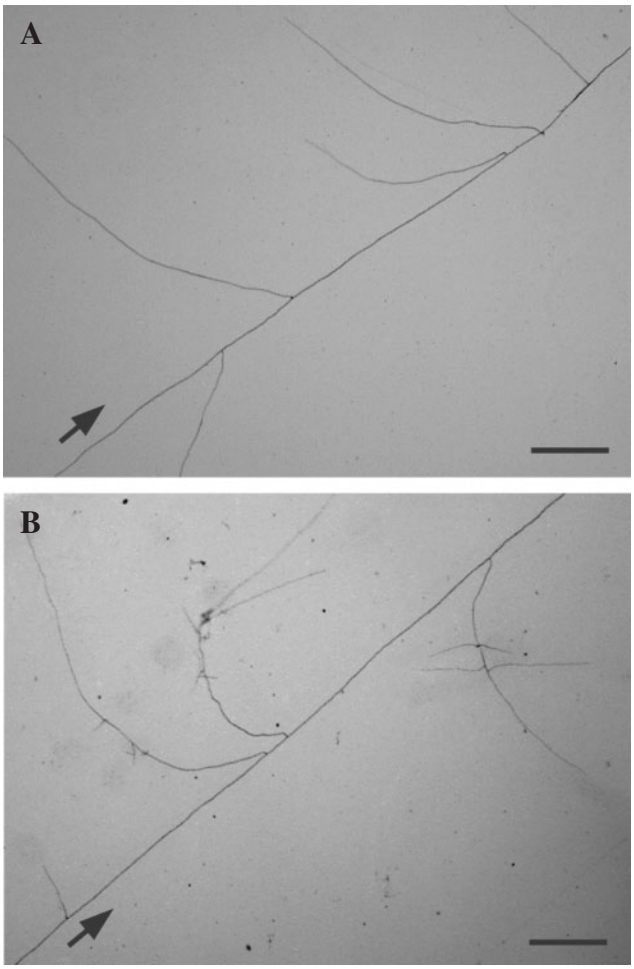

FIG. 3. Hyphal branching of *Gigaspora margarita* induced by 5-deoxy-strigol using the paper disc diffusion method: (A) control (70 % ethanol in water); (B) natural 5-deoxy-strigol (30 pg per disc). Extensive formation of hyphal branches from secondary hyphae is induced by treatment with 5-deoxy-strigol. Control hyphae, which treated with 70 % ethanol in water, form no hyphal branches from secondary hyphae. Arrows indicate direction of growth of primary hyphae. Scale bars = 1 mm.

not surprising if one considers the several hundred million years of co-evolution of plants and AM fungi. Recent development of an analytical method using HPLC connected to tandem mass spectrometry enables identification of known strigolactones, as well as the search for novel ones in root exudates from a relatively small number of plants (Sato *et al*., 2003, 2005). The enrichment procedure with activated charcoal, which was used for the isolation of 5-deoxy-strigol from *L. japonicus*, can provide sufficient amounts of strigolactone for spectroscopic analysis. Novel unidentified strigolactones can be isolated and identified by combining these two methods.

Very little is known about the biogenetic origin of strigolactones in plants, although they have been regarded to be sesquiterpenoids (isoprenoids consisting of three isoprene units). Isoprenoids are biosynthesized via two independent pathways: the cytosolic mevalonic acid pathway and the plastidic non-mevalonate, methylerythritol phosphate (MEP) pathway. The tricyclic ABC ring of strigolactones has recently been revealed to be formed by cleavage of C_{40}-carotenoids originating from the MEP pathway, as shown for the plant hormone abscisic acid (Matusova *et al*., 2005). Coupling of the D (methylbutenolide) ring to the ABC ring via the enol ether will lead to 5-deoxy-strigol, which itself is the first product in the strigolactone biosynthesis capable of acting both as a BF on AM fungi and as a germination stimulant on parasitic weeds. Isolated as a natural product for the first time, 5-deoxy-strigol could be further converted to strigol and orobanchol by hydroxylation at C-5 and C-4, respectively. Sorgolactone could also be biosynthesized from 5-deoxy-strigol via oxidative demethylation at C-9. Taken together, 5-deoxy-strigol is likely to be a branching point in strigolactone biosynthesis.

AM FUNGAL RESPONSES TO STRIGOLACTONES

It is clear that strigolactones exuded from host roots can trigger a cascade of molecular and cellular events leading to the formation of pre-infection hyphal branching structures in AM fungi. In the last few years, a growing number of studies has been conducted on the molecular changes occurring in AM fungi during pre-symbiotic stages (Jun *et al*., 2002; Breuninger and Requena, 2004). A semi-purified exudate from carrot root organ cultures was shown to induce the expression of mitochondrial-related genes and, in turn, fungal respiratory activity before intense hyphal branching (Tamasloukht *et al*., 2003). This cellular respiration is also activated by the chemically pure synthetic analogues GR24 and GR7 in *Gigaspora rosea* and *Glomus intraradices* (Bécard *et al*., 2005). These findings indicate that respiration is a primary target of AM fungal metabolism induced by the root factor. A slight induction of *GmarCuZnSOD* (*Gigaspora margarita* CuZn superoxide dismutase) gene expression was observed in germinated spores of *G. margarita* exposed to a semi-purified root exudate fraction from *L. japonicus* (Lanfranco *et al*., 2005). Strigolactones also appear to act as chemo-attractants: fungal hyphae of *Glomus mosseae* exhibited chemotropic growth towards roots at a distance of at least 910 μm in response to host-derived signals, possibly strigolactones (Sbrana and Giovannetti, 2005).

Strigolactones show potent activity at very low concentrations, suggesting a highly sensitive perception system for strigolactones present in AM fungi. Such a system should be a prerequisite for this obligate biotrophic organism to survive for over 460 million years under natural conditions. The induction of seed germination in parasitic weeds is thought to proceed via a receptor-mediated mechanism. A tentative molecular mechanism proposed for the stimulation of seed germination involves the addition of a nucleophilic species, present at a putative receptor site, to the enol ether carbon double bond in a Michael fashion, followed by elimination of the D ring (Mangnus and Zwanenburg, 1992). Labelled strigolactone analogues were synthesized for isolation and purification of the strigolactone receptor by affinity chromatography (Reizelman *et al*., 2003), though the receptor has not yet been isolated. Further study will

provide insights into the origin and evolution of the putative receptors in AM fungi and parasitic weeds.

Some solid evidence has been presented for AM fungal production of a long-hypothesized symbiotic signal, the MF, in response to the plant symbiotic signal strigolactones. Fungal hyphae of the genus *Gigaspora* growing in the vicinity of host roots, but separated from the roots by a membrane, release a diffusible substance that induces the expression of a symbiosis-specific gene, *MtENOD11* (*Medicago truncatula* early nodulin 11), in *Medicago truncatula* roots (Kosuta *et al*., 2003). This expression was correlated both spatially and temporally with the appearance of hyphal branching, and was not observed when hyphal branching was absent. These findings strongly suggest that strigolactones may be required for synthesis of the diffusible AM factor. Fungal exudates from *Gigaspora rosea*, *Gigaspora margarita* and *Glomus intraradices* were also found to stimulate lateral root formation (Oláh *et al*., 2005). Activation of the promoter of a symbiosis-specific gene, *LjCbp1* (*Lotus japonicus* calcium-binding protein 1), was observed not only in arbuscule-containing cells but also in cells which are not in contact with fugal hyphae, suggesting that this gene promoter can be used as a molecular marker to detect the diffusible AM factor (Kistner *et al*., 2005). With the aid of strigolactones, which might stimulate the production of the signal molecule in AM fungi in the absence of a host root, MF will be purified and characterized by bioassay based on these molecular and morphological responses in the near future.

CONCLUSIONS AND FUTURE PERSPECTIVES

Isolation and identification of plant symbiotic signals open up new ways for studying the molecular basis of plant–AM-fungus interactions. The model legume *L. japonicus*, which was used for identification of the chemical signals, enables a smooth transition of our chemical research results to molecular analysis of the signal molecule-mediated events in the AM symbiosis as well as the strigolactone biosynthetic pathway and its regulation in plants (Parniske, 2004). Availability of strigolactone and its analogues will facilitate basic research on AM fungal responses to this signal molecule, and also applied research on the development of novel techniques for *in vitro* propagation of AM fungi and increased AM colonization in plants. The discovery described in this paper also provides a clear answer to a long-standing question in parasitic plant biology: 'what is the natural role for germination stimulants?' Another implication is that crop protection strategies based on eliminating host production of strigolactones should be reconsidered so as to balance the disruption of parasite germination signalling with preservation of vital AM symbiosis.

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