Strigolactones: host recognition signals for arbuscular mycorrhizal fungi

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Abstract

Arbuscular mycorrhizal (AM) fungi form mutualistic, symbiotic associations with the roots of more than 80% of terrestrial plants (Smith & Read, 1992). This symbiosis began 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). 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 signaling molecules called ‘branching factors’ (BFs) that induce extensive hyphal branching in AM fungi (Mosse & Hepper, 1975; Giovannetti et al., 1993; Giovannetti et al., 19994). For the first time, we have successfully isolated a BF from the roots exudates of Lotus japonicus and identified it as a strigolactone, 5-deoxystrigol, by spectroscopic analysis and chemical synthesis. Strigolactones have been identified as seed germination stimulants for the parasitic weeds Striga and Orobanche (Bouwmeester et al., 2003), which are among the most damaging agricultural pests in large parts of the world. The natural strigolactones including 5-deoxystrigol, sorgolactone, and strigol together with a synthetic analogue, GR24, induced extensive hyphal branching in germinating spores of the AM fungus Gigaspora margarita at very low concentrations. 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.

Keywords: arbuscular mycorrhizal fungi, branching factor, germination stimulant, Lotus japonicas, strigolactones

Introduction

AM fungi are ancient, obligate symbionts in the phylum Glomeromycota (Schussler et al., 2001). The critical developmental step in their life cycle is hyphal branching, which helps them to ensure contact with the host root and the establishment of symbiosis. Branching factor (BF) is hypothesized to be a plant signal molecule needed to trigger the hyphal morphogenesis that precedes successful root colonization (Giovannetti et al., 1996; Buee et al., 2000). Dialysis membranes have been used to determine that the BF exuded from growing roots of Ocimum basilicum, which elicits hyphal branching of the AM fungus Glomus mosseae, is a low-molecular-weight compound (less than 500 Da) (Giovannetti et al., 1996). The development of an in vitro bioassay for hyphal branching in germinating spores of the genus Gigaspora (Nagahashi & Douds, 1999) has facilitated the analysis of the chemical characteristics and distribution of BF in the plant kingdom (Buee et al., 2000; Nagahashi & Douds, 2000). BF was present in root exudates of all the mycotrophic plants tested, but absent in those of non-host plants. The discovery that the active compound is partitioned into ethyl acetate from an aqueous root exudate (Buee et al., 2000), and is retained on C18 reverse-phase resin (Nagahashi & Douds, 2000), indicates that BF is a lipophilic compound. Root exudates from plants grown under phosphate (Pi)-limited conditions are more active than those from plants with sufficient Pi nutrition, suggesting that the production of BF in roots and its exudation are regulated by Pi availability (Nagahashi & Douds, 2000). The identity of BF has been the subject of a considerable amount of research, but purification of the root metabolites has been hampered by the extremely low concentrations produced and exuded by host roots (Tamasloukht et al., 2003) and their relative instability (Akiyama and Hayashi, unpublished data). Here we report the isolation and structural identification of BF from root exudates of the model legume L. japonicus.

Materials and Methods

Hyphal branching assay. Effects on the hyphal branching of AM fungi were evaluated in vitro by the paper disk diffusion method. Spores of G. margarita Becker & Hall (CGC1411, Central Glass Co.), surface-sterilized with 0.2% NaClO and 0.05% Triton X-100, were inserted into a 0.2% Phytagel gel (Sigma-Aldrich) containing 3 mM MgSO4 in 60-mm plastic Petri dishes. The dishes were incubated vertically for 5–7 days in a 2% CO2 incubator at 32 °C. Secondary hyphae emerging from a primary hypha, which grew upward in a negative geotropic manner in the gel, were used for assay. Test samples were first dissolved in acetone then diluted with 70%
ethanol in water. The concentration of test sample solutions of natural 5-deoxystrigol was adjusted with reference to the calibration of synthetic (±)-5-deoxystrigol in HPLC analysis. Paper disks (6 mm in diameter; ADVANTEC) loaded with 15 µL of test sample solution were placed in front of the tips of the secondary hyphae. The control was 70% ethanol in water. The hyphal branch patterns were observed 24 h after treatment. The sample was scored as positive for hyphal branching if new hyphal branches formed from the treated secondary hyphae or primary hyphae located proximal to the paper disks. The assay was repeated at least twice, using between three and five dishes for each concentration.

Hydroponic culture. Seeds (~2,000) of *L. japonicus* B-129 Gifu were sown on vermiculite in a plastic tray. Purification of BF (5-deoxystrigol). 5-Deoxystrigol: UV (acetonitrile) λ<sub>max</sub> 234 nm; IR (KBr) ν<sub>max</sub> 1784, 1744, 1683, 1340, 1024 cm<sup>-1</sup>; ¹H-NMR (400 MHz, CDCl₃) δ 1.08 (3H, s, H-9 or H-10), 1.10 (3H, s, H-9 or H-10), 2.01 (3H, t, J=1.5 Hz, H-7), 5.49 (1H, br.d. J=8.0 Hz, H-8b), 6.12 (1H, m, H-2'), 6.90 (1H, m, H-3'), 7.39 (1H, d, J=2.7 Hz, H-6'); EIMS 70 eV, m/z (rel. int) 330 [M]+(4), 315 (2), 233 (16), 216 (9), 215 (18), 205 (7), 201 (9), 187 (18), 97 (100); CD (acetonitrile) λ<sub>max</sub> (Δε) 262 (-2.86), 230 (21.2) nm.

Synthesis of (±)-5-deoxystrigol. The racemic ABC ring was synthesized, starting from 2,2-dimethyl-cyclohexanone. Ester condensation of the racemic ABC ring with ethyl formate followed by alkylation with racemic 4-bromo-2-methyl-2-buten-4-olide provided (±)-5-deoxystrigol and its 2′-epimer. (±)-5-deoxystrigol was purified by a silica gel column (Wakogel C-200, n-hexane-ethyl acetate stepwise) and semi-preparative HPLC (Inertsil ODS-3, 70% acetonitrile in water).

Results and Discussion

Plants were grown hydroponically under low-Pi conditions (35 µM). Lipophilic compounds released into the hydroponic solution were extracted with ethyl acetate and then tested for hyphal branching activity in germinating spores of *G. margarita*. Using the paper disk diffusion method, the ethyl acetate extract elicited strong hyphal branching at 15 µg per disk (Fig. 1). The extract showed activity at concentrations as low as 1.9 µg per disk. The ethyl acetate extract was further divided into ethyl acetate-soluble acidic, neutral and basic fractions. The activity was found only in the neutral fraction, indicating that BF from *L. japonicus* is a neutral compound.

Figure 1 Hyphal branching of *G. margarita* induced by lipophilic fraction from root exudates of *L. japonicus* using the paper disk diffusion method. a. Control hypha (70% ethanol in water. b. Hyphal branching from a secondary hypha upon treatment with the ethyl acetate extracts (15 µg per disk). Arrows indicate direction of growth of primary hyphae.

To isolate the neutral active compound, we used activated charcoal to extract BF from the hydroponic solution. The solution was continuously circulated
through an activated charcoal cartridge. Active compounds adsorbed on the charcoal were eluted with acetone. The ethyl acetate-soluble, neutral fraction prepared from the acetone eluate was first chromatographed on a silica gel column by eluting stepwise from n-hexane to ethyl acetate. Activity was found in 40% ethyl acetate eluate. The active fraction was further purified by high-performance liquid chromatography (HPLC) on a semi-preparative C18 column, resulting in the isolation of a BF eluted as a single peak at 22.3 min.

We subjected the BF, obtained as a colourless, amorphous solid, to spectroscopic analysis. The UV spectrum showed an absorption maximum at 234 nm, suggesting an α, β-unsaturated carbonyl chromophore. The IR spectrum exhibited strong absorption bands indicative of γ-lactones (1,784, 1,744 cm⁻¹) and enol ether (1,683 cm⁻¹). The EI-MS spectrum showed a weak molecular ion peak at m/z 330, with the base peak at m/z 97. The spectrum indicated cleavages from m/z 330 to peaks at m/z 315 [M-CH₃]⁻, 233 [M'-97], 215 [M'-97-H₂O], and 187 [M'-97-H₂O-CO] (Fig. 2). The ¹H-NMR spectrum displayed signals for two singlet methyls (δH 1.08, 1.10), an allylic methyl (δH 2.01, triplet, J = 1.5 Hz), an oxymethine (δH 5.49, broad doublet, J = 8.0 Hz), and an allylically coupled olefinic proton (δH 7.39, doublet, J = 2.7 Hz). Two methine protons were observed at δH 6.12 and 6.90 as a multiplet, respectively (Fig. 3). Taken together, these data strongly suggested that the BF is structurally closely related to strigolactones, a group of sesquiterpene lactones previously identified as seed germination stimulants for the parasitic weeds Striga and Orobanche. The spectral data of the BF were in good agreement with those reported for a synthetic derivative of strigol, (±)-5-deoxystrigol, which has not been isolated from any natural source (Bergmann et al., 1993). Strigol itself is the first natural strigolactone to be isolated from the false host cotton (Gossypium hirsutum) (Cook et al., 1966, 1972), and later identified from genuine hosts, sorghum (Sorghum bicolor), maize (Zea mays), and proso millet (Pennisetum glauccum) (Siame et al., 1993). To confirm the chemical structure of the BF, (±)-5-deoxystrigol was synthesized according to the previously reported procedures (Frischmuth et al., 1991; Sugimoto et al., 1998; Nakano et al., 1995). Retention times on HPLC and all the spectral data for the synthetic compound were identical to those of the natural compound. Thus, the BF isolated from L. japonicus was identified as 5-deoxystrigol. The CD spectrum of the natural compound was superimposable on that reported for the natural strigolactones, (+)-strigol and (+)-sorgolactone (Hauck et al., 1992), confirming that the absolute configuration of the natural 5-deoxystrigol is the same as that of the naturally occurring strigolactones (that is, 3α(R), 8b(S) and 2'(R)) (Frischmuth et al., 1991; Nakano et al., 1995; Brooks et al., 1985) (Fig. 4). Little is known about the biosynthetic pathway for strigolactones in plants owing to the extremely low concentrations of highly active compounds that are produced by and exuded from the roots. Isolated as a natural product for the first time, 5-deoxystrigol can be converted to strigol and orobanchol (Yokota et al., 1998) by hydroxylation at C-5 and C-4, respectively, suggesting that this compound is a branching point in the strigolactone biosynthesis.

Figure 2 EI mass spectra of BF from L. japonicus, identified as 5-deoxystrigol (top), and synthetic (±)-5-deoxystrigol (bottom).
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Figure 3 ¹H-NMR spectra of BF from L. japonicus, identified as 5-deoxystrigol (ca. 80 µg, top), and synthetic (±)-5-deoxystigol (ca. 3 mg, bottom).

Figure 4 Chemical structure of 5-deoxystrigol.

To study structure–activity relationships of the BF, (±)-5-deoxystrigol, (±)-sorgolactone, (+)-strigol and the synthetic strigolactone analogue GR24 (Johnson et al., 1981) were tested for activity at concentrations ranging from 100 ng to 30 pg per disk. At all the concentrations tested (100 ng–30 pg per disk), (±)-5-deoxystrigol exhibited activity (Fig. 5). Sorgolactone is the third natural strigolactone to be isolated from sorghum root exudates (Hauck et al., 1992). Racemic sorgolactone prepared by organic synthesis was also highly active on the AM fungus (Fig. 5). The activity of this compound was comparable to that of (±)-5-deoxystrigol (100 ng–30 pg per disk). Isolated from an aseptic root culture of a Chinese medicinal plant Menispermum dauricum (Yasuda et al., 2003), (+)-strigol also induced hyphal branching at 1-100 ng per disk, though its activity at lower than 1 ng per disk has not yet been evaluated. GR24 was less active than the natural strigolactones (100–1 ng per disk), even considering that the GR24 used in this study was an equimolar mixture of two racemic diastereomers. Strigolactones are highly active on parasitic weeds, inducing 50% seed germination at picomolar concentrations. The CD rings of strigolactone molecules were shown to be responsible for stimulating germination (Magnus & Zwanenburg, 1992). A proposed molecular mechanism for germination stimulation 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. The inherent instability of strigolactones in the presence of nucleophilic agents can also be understood by this mechanism. Taken with our observations that all the strigolactones tested had a potent effect on the fungus, and that their activity drastically decreased after concentrating a solution of BF dissolved in nucleophilic solvents such as methanol and its aqueous mixture, it appears that the CD rings are also essential for the effect of strigolactones on the AM fungus. Recently, Bécard et al. also reported the stimulatory effect of the synthetic strigolactone analogues GR24 and GR7 on hyphal branching in Gigaspora rosea (Bécard et al., 2005). They also found that these two analogues activate cellular respiration in G. rosea and Glomus intraradices (Bécard et al., 2005).

Figure 5 Hyphal branching activity of BF and natural strigolactones on G. margarita. a, Natural 5-deoxystrigol (30 pg per disk). b, Synthetic (±)-5-deoxystrigol (3 ng per disk). c, Synthetic (±)-sorgolactone (10 ng per disk). d, Strigol isolated from M. dauricum root cultures (3 ng per disk).

Strigolactones have been isolated from the root exudates of a variety of plants, including the monocots sorghum, maize and proso millet, and the dicots cotton, cowpea, red clover and M. dauricum. The isolation of (+)-strigol from an aseptic root culture of M. dauricum has unambiguously demonstrated that this strigolactone is of plant origin (Yasuda et al., 2003). Although it has been suggested that strigolactones are 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 and their relative instability. The broad distribution of strigolactones in the plant kingdom and their levels in plant root exudates are consistent with the host specificity of AM fungi.

In addition to the above mentioned strigolactones, several derivatives have been isolated and identified (Fig. 6). Alectrol was purified from cowpea (Vigna unguiculata) root exudates (Müller et al., 1992) and recently identified as orobanchyl acetate (Xie et al., 2008a). The first described Orobanche germination stimulant, orobanchol, was isolated from red clover (Trifolium pratense) root exudates (Yokota et al., 1998). Recently, 2’-epiorobanchol and solanacol were characterized from root exudates of tabacco (Nicotiana tabacum), a host of Phelipanche ramosa (formally called Orobanche ramosa) (Xie et al., 2007). Sorgomol (formally named sorghumol) was identified in the root exudates of S. bicolor (Awad et al., 2006; Xie et al., 2008b).

Pi availability in the soil, it is tempting to speculate that parasitic weeds might find potential hosts by detecting strigolactones (which are released from plant roots under conditions of Pi deficiency in communication with AM fungi). The purification and characterization of plant symbiotic signals opens up new way to study the molecular basis of plant–AM fungus interactions. Availability of BF and its analogues will facilitate the detailed analysis of the molecular, physiological and morphological responses of AM fungi to this signal molecule. The use of the model legume L. japonicus (Parniske et al., 2004) will further our understanding of the BF biosynthetic pathway and its regulation. Molecular and chemical analysis of BF-mediated events during the development of the AM symbiosis between L. japonicus and G. margarita will provide insights into the chemical communication between plants and AM fungi. This could also provide a new strategy for the management and control of beneficial fungal symbionts and devastating parasitic weeds in both agriculture and natural ecosystems.

Conclusions

Hyphal branching has long been described as the first morphological event in host recognition by AM fungi during the pre-infection stages. Strigolactones exuded from host roots have been identified as an inducer of hyphal branching in AM fungi. Strigolactones are a group of sesquipiperenes, 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.

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References


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