Tomato strigolactones are derived from carotenoids and their biosynthesis is promoted by phosphate starvation

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Summary

• Strigolactones are rhizosphere signalling compounds that mediate host location in arbuscular mycorrhizal (AM) fungi and parasitic plants. Here, the regulation of the biosynthesis of strigolactones is studied in tomato (*Solanum lycopersicum*).

• Strigolactone production under phosphate starvation, in the presence of the carotenoid biosynthesis inhibitor fluridone and in the abscisic acid (ABA) mutant *notabilis* were assessed using a germination bioassay with seeds of *Orobanche ramosa*; a hyphal branching assay with *Gigaspora* spp; and by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis.

• The root exudates of tomato cv. MoneyMaker induced *O. ramosa* seed germination and hyphal branching in AM fungi. Phosphate starvation markedly increased, and fluridone strongly decreased, this activity. Exudates of *notabilis* induced approx. 40% less germination than the wild-type. The LC-MS/MS analysis confirmed that the biological activity and changes therein were due to the presence of several strigolactones; orobanchol, solanacol and two or three didehydro-orobanchol isomers.

• These results show that the AM branching factors and parasitic plant germination stimulants in tomato root exudate are strigolactones and that they are biosynthetically derived from carotenoids. The dual activity of these signalling compounds in attracting beneficial AM fungi and detrimental parasitic plants is further strengthened by environmental conditions such as phosphate availability.

Key words: carotenoids, *Orobanche ramosa*, phosphate, strigolactones, tomato (*Solanum lycopersicum*).

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Introduction

Parasitic plants of the *Striga* and *Orobanche* genera are some of the most damaging agricultural pests. These obligate root parasites attach themselves to the roots of many plant species and acquire nutrients and water from their host through an organ called an haustorium. These parasitic plants cause considerable crop losses in many parts of the world (Joel, 2000; Press *et al.*, 2001; Shen *et al.*, 2006). The seeds of these parasitic plants contain few nutrient reserves. They can survive for only a few days after germination unless they reach a host root and establish a xylem connection (Bouwmeester *et al.*, 2003; Shen *et al.*, 2006). Although *Striga* and *Orobanche* spp. parasitize different hosts in different parts of the world, their lifecycles are broadly similar and involve germination, radicle growth towards the host root, haustorium formation and connection to the host vascular elements. The interaction between host and parasite begins with the secretion of secondary

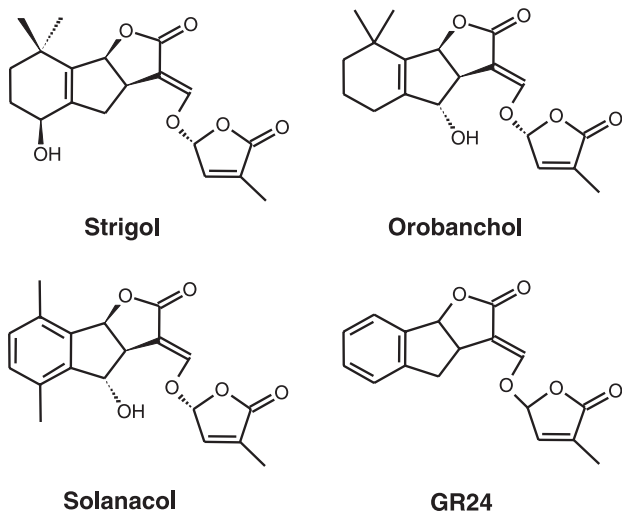

Fig. 1 Structures of some strigolactone germination stimulants and the synthetic strigolactone analogue GR24.

metabolites from the roots of the host (or some false (non)hosts) that induce the germination of the parasite's seeds and are called germination stimulants (Bouwmeester *et al.*, 2003; Humphrey *et al.*, 2006).

The *Orobanche* spp. (broomrapes; Orobanchaceae) are holoparasites and parasitize important agricultural crops around the globe such as legumes, crucifers, sunflower, hemp, tobacco and tomato (Joel, 2000; Press *et al.*, 2001). Tomato (*Solanum lycopersicum*) is an important vegetable crop in Southern Europe, the Americas, the Middle East and India, with production increasing also in China, Japan, and South East Asia (van Eck *et al.*, 2006). The production of tomato is highly susceptible to infestation by *Orobanche ramosa* and *Orobanche aegyptiaca*, which can cause severe yield losses of up to 75% (Delavault & Thalouarn, 2002; Mauromicale *et al.*, 2005; Radi *et al.*, 2006).

Several germination stimulants for *Orobanche* and *Striga* spp. have been identified in root exudates of their hosts and also in nonhosts. Most of them belong to the strigolactones (Bouwmeester *et al.*, 2003; Matusova *et al.*, 2005; Awad *et al.*, 2006). Of these, orobanchyl acetate (formerly called alectrol), orobanchol and a third unidentified germination stimulant have already been isolated from the root exudates of red clover (Yokota *et al.*, 1998; Xie *et al.*, 2008), and sorgolactone, 5-deoxystrigol, sorgomol and strigol from sorghum (Hauck *et al.*, 1992; Awad *et al.*, 2006) (Fig. 1).

Interestingly, the strigolactones do not just act as germination stimulants for root parasitic plants. Recently, Akiyama and coworkers (2005) isolated the strigolactone 5-deoxystrigol from the root exudates of *Lotus japonicus* and identified it as the compound responsible for the induction of hyphal branching in arbuscular mycorrhizal (AM) fungi, which is a critical step in host recognition. Other strigolactones, including strigol and sorgolactone, and the synthetic strigolactone analogues GR24 and GR7 have also been shown to induce hyphal branching and/or spore germination of the AM fungi (Akiyama *et al.*, 2005; Besserer *et al.*, 2006). AM fungi are obligate symbionts that establish a symbiosis with vascular plants which depends on the carbon provided by the host plant to complete their developmental cycle. The successful colonization of a host plant by AM fungi relies on the establishment of a network of connections between the host plant roots and the fungal hyphae. This colonization is preceded by extensive hyphal branching in response to the secretion of strigolactones by the host roots (Akiyama *et al.*, 2005; Harrison, 2005; Humphrey *et al.*, 2006). However, AM fungi also help the plant by improving the uptake of inorganic phosphate (Pi) and other minerals, and hence improve agricultural production in areas with limited mineral nutrition. Inorganic phosphate plays a pivotal role in carbohydrate metabolism in plants, has a significant impact on plant growth and plant health and contributes to plant biodiversity and ecosystem productivity.

Although the strigolactones so far identified have been isolated from a wide variety of (host) plant species and induce germination of a range of parasitic plant species, their chemical structure is strikingly similar and they are evidently derived from the same biosynthetic pathway (Bouwmeester *et al.*, 2003). The strigolactones were originally identified as sesquiterpene lactones (Siame *et al.*, 1993; Butler, 1995; Yokota *et al.*, 1998; Akiyama *et al.*, 2005). However, using both mutants and isoprenoid pathway inhibitors we have demonstrated that, in maize, sorghum and cowpea, the ABC-part of these germination stimulants is derived from carotenoids through the action of a carotenoid cleavage enzyme, carotenoid cleavage dioxygenase (CCD) or 9-*cis*-epoxycarotenoid dioxygenase (NCED), in a way similar to the plant hormone abscisic acid (ABA), which is derived from *cis*-neoxanthin through the action of an NCED (Matusova *et al.*, 2005).

In the investigations reported here, we studied whether the germination stimulants/hyphal branching factors of tomato are also strigolactones, whether they are derived from the carotenoid pathway; and whether Pi limitation and/or ABA affects. Both bioassays and LC-MS/MS analysis were used.

Materials and Methods

Plant material and chemicals

Seeds of tomato (*S. lycopersicum* L.) cv. MoneyMaker were kindly provided by Fien Meijer (Laboratory for Plant Breeding, Wageningen University, the Netherlands). The tomato ABA mutant *notabilis* (in background 'Ailsa Craig') and wild-type seeds were obtained from Wim Vriezen (Department of Plant Cell Biology, Radboud University, Nijmegen, the Netherlands) and *O. ramosa* seeds (collected from a tomato field) were kindly provided by Maurizio Vurro and Angela Boari (Instituto di Scienze delle Produzioni Alimentari, Bari, Italy). The carotenoid

pathway inhibitor fluridone was obtained from Duchefa (Duchefa Biochemie B.V., Haarlem, the Netherlands). The synthetic germination stimulant, strigolactone analogue GR24, was kindly provided by Binne Zwanenburg (Department of Organic Chemistry, Radboud University, Nijmegen, The Netherlands). The natural germination stimulants orobanchol and solanacol were kindly provided by Koichi Yoneyama (Weed Science Center, Utsunomiya University, Japan).

Growth conditions and root exudate collection

Tomato seeds were sterilized in 4% sodium hypochlorite containing 0.02% (v : v) Tween 20, rinsed thoroughly with sterile water and then germinated for 48 h on moistened filter paper at 25°C in darkness. Subsequently, tomato seedlings were grown hydroponically in 0.7-l glass containers containing a modified half-strength Hoaglands nutrient solution (2.4 mM KNO_3, 1.6 mM $Ca(NO_3)_2$, 0.2 mM KH_2PO_4, 0.8 mM $MgSO_4$, 0.18 mM $FeSO_4$, 0.1 mM Na_2EDTA, 4.5 μM $MnCl_2$, 23 μM H_3BO_3, 0.3 μM $CuSO_4$, 1.5 μM $ZnCl_2$ and 0.1 μM Na_2MoO_4) in a glasshouse at 20°C : 18°C with 16 h : 8 h photoperiod and 70% humidity. The nutrient solution was replaced twice a week. Experimental treatments (see later) were approximately applied *c.* 3 wk after sowing. After treatment, plants were carefully removed from the hydroponics system to collect root exudates. This was performed by placing the roots for 5 h in fresh nutrient solution of the same composition that was used for the experimental treatment to avoid stress responses. For this, each tomato plant was placed in *c.* 10 ml of nutrient solution in aluminium-foil covered (to exclude light) plastic 10-ml Greiner tubes in the glasshouse at 20°C (same conditions as used for growing). After collection of the root exudates, the root fresh weight (FW) of each seedling was determined. Subsequently, within each experiment the exudates were diluted to the same ratio of root fresh weight per ml of root exudate medium before being used to assess the induction of *O. ramosa* seed germination.

Plant treatments

Tomato cv. MoneyMaker seedlings were grown hydroponically for 3 wk on half-strength Hoaglands with Pi (0.2 mM) before starting treatment. For each experiment, six to eight individual plants were used. The root exudates were collected from each plant individually, and used for the bioassays. After collection of the root exudates, the roots from each experiment were also collected separately, frozen in liquid nitrogen and stored at −80°C until analysis.

Fluridone treatment Half-strength Hoaglands solution without Pi and containing 0, 0.01, 0.1 or 1 μM fluridone was applied to 3-wk-old tomato seedlings in which they were then grown for an additional 4 d. To maintain the effect of the inhibitor during root exudate collection, plants from all inhibitor treatments were placed on fresh nutrient solution containing 0.01 μM fluridone. Root exudates of control plants were collected under the same conditions but without fluridone.

Phosphate starvation For the Pi starvation time-course experiment, half-strength Hoaglands solution without Pi was applied to 3-wk-old seedlings. At each time-point (0, 6, 24 and 96 h after removal of Pi from the nutrient solution), 12 plants were harvested, of which six were used to collect root exudates (in fresh nutrient solution) and six to harvest roots that were frozen in liquid nitrogen and stored at −80°C. For one set of 12 plants that were Pi-starved for 96 h, Pi (0.2 mM) was restored and the plants were grown for an additional 72 h. For all time-points, control plants were grown under the same conditions but in the continuous presence of 0.2 mM Pi.

Mutant analysis Ten seeds of the tomato mutant *notabilis* and its corresponding wild-type ('Ailsa Craig') were prepared and grown as already described for cv. MoneyMaker. After 4 wk on half-strength Hoaglands with Pi (0.2 mM), the seedlings were transferred to fresh nutrient solution without Pi and grown for an additional 5 d. Root exudates were then collected from each seedling individually. The roots were harvested, frozen in liquid nitrogen and stored at −80°C.

Extraction of germination stimulants from roots

In addition to root exudate collection, pools of roots from the Pi starvation time-course experiment were used to extract the germination stimulants from the tomato roots. For this, 0.5 g of roots from each time-point were ground in a mortar with liquid nitrogen and then extracted with 1 ml of ethyl acetate in a 3 ml glass tube. The tubes were vortexed and sonicated for 10 min in a Branson 3510 ultrasonic bath (Branson Ultrasonics, Danbury, CT, USA). Samples were centrifuged for 10 min at 1350 *g* in an MSE Mistral 2000 centrifuge (Mistral Instruments, Leicester, UK), and the organic phase were carefully transferred to 1 ml glass vials and stored at −20°C until use in the germination bioassays.

Fractionation of root exudates

The crude exudates from the 96 h Pi starvation treatment and 1 μM fluridone-treated plants and their corresponding controls were fractioned using reverse-phase chromatography on a C_{18} SEPAK cartridge (Octadecyl 500 mg; JT Baker, Deventer, the Netherlands). For this, 50 ml of the exudate was loaded onto the pre-equilibrated column. Subsequently, 3 ml fractions were eluted using increasing concentrations of acetone (from 0–100% in 10% increments). In parallel to the fractionation of the root exudates, we fractioned a solution of 0.5 μM orobanchol standard in water using the same procedure. Before germination bioassays were performed, the acetone

was removed from the samples by first adding a corresponding volume of demineralized water and then evaporating the solvent *in vacuo* in a SpeedVacuum SC100 (Savant Instruments, Holbrook, NY, USA).

Germination bioassay

Germination bioassays with *O. ramosa* seeds were conducted as reported previously (Matusova *et al.*, 2005). Seeds of *O. ramosa* require preconditioning (or warm stratification) for a certain period of time at a suitable temperature before the seeds become responsive to germination stimulants (Matusova *et al.*, 2004). Aliquots (50 µl) of exudate, SEPAK fraction or root extract were added to triplicate discs bearing approximately 100 preconditioned seeds. The synthetic germination stimulant GR24 (10^{-9} M) and demineralized water were included as positive and negative controls in each bioassay. After 7 d, the germinated and ungerminated seeds were counted using a binocular microscope. Seeds were considered germinated when the radicle had protruded through the seed coat.

Branching bioassay

Spores of *Gigaspora gigantea* and *Gigaspora rosea* were used for the branching bioassay. Before branching bioassays were performed, the samples were dried under vacuum and resuspended in 10 : 90 (v : v) acetonitrile–water. The assays were carried out according to Buee *et al.* (2000). Spores were germinated in atmosphere of 2% CO_2 at 30°C, on 0.1% $MgSO_4$ solidified with 0.5% Phytagel (Sigma-Aldrich, St Quentin Fallavier, France) (*G. gigantea*) or 'M' medium (described by Buee *et al.*, 2000), supplemented with 10 µM quercetin and solidified with 0.5% Phytagel (*G. rosea*). Six days after inoculation, each spore had produced a single germ tube. Two small wells were made in the gel on each side of the growing hyphae and 5 µl of the solution to test were deposited in each well. Hyphal branching was recorded quantitatively 48 h later by counting newly formed hyphal branches. Ten to 20 spores were used for each treatment. The synthetic germination stimulant GR24 (10^{-8} M) and 10 : 90 (v : v) acetonitrile–water were used as positive and negative controls, respectively.

Carotenoid analysis by high-pressure liquid chromatography (HPLC)-photodiode array (PDA)

Carotenoid extraction and analysis from tomato roots were performed using HPLC with PDA detection as described by Bino *et al.* (2005) with some modifications. Frozen root powder (2 g) was extracted with 4.5 ml methanol/chloroform (5 : 4, v : v) containing 0.1% butylated hydroxytoluene (BHT). Samples were shaken and incubated on ice for 10 min. Then, 2.5 ml of 1 M NaCl in Tris-HCl buffer pH 7.4 was added and extracts were incubated for another 10 min on ice and then centrifuged for 10 min at 1350 *g* at room temperature. The samples were re-extracted with 1 ml chloroform containing 0.1% BHT. The chloroform fractions were combined, dried under a flow of N_2 and the residue was taken up in 200 µl ethyl acetate containing 0.1% BHT. An HPLC analysis was performed as described by Bino *et al.* (2005).

Strigolactone analysis using liquid chromatography-tandem mass spectrometry

Characterization of strigolactones in tomato root exudates was performed by comparing retention times and MS transitions of germination stimulants with those of strigolactone standards (sorgolactone, strigol, orobanchol, 5-deoxystrigol, solanacol and orobanchyl acetate), using ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS), essentially as described by Sato *et al.* (2005). Analyses were performed using a Waters Micromass Quattro Premier XE tandem mass spectrometer (Waters, Mildorf, MA, USA) equipped with an electrospray ionization (ESI) source and coupled to an Acquity UPLC system (Waters). Chromatographic separation was achieved using an Acquity UPLC BEH C_{18} column (100 × 2.1 mm, 1.7 µM; Waters), applying a water–methanol gradient. Separation started at 30% methanol for 1 min, followed by a 5 min gradient to 80% methanol, which was then maintained for 1 min, followed by a 0.2 min gradient back to 30% methanol. The column was equilibrated at this solvent composition for 1.8 min before the next run. Total run time was 9 min. The column was operated at 50°C with a flow-rate of 0.4 ml min^{-1} (sample injection volume of 30 µl). The mass spectrometer was operated in positive ESI mode. The nebulizer and desolvation gas flows were 50 l h^{-1} 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 120°C and the desolvation gas temperature at 450°C. Fragmentation was performed by collision induced dissociation with argon at 3.0×10^{-3} mbar. The collision energy was optimized for each compound. Multiple reaction monitoring (MRM) was used to search for strigolactones. The MRM channels were set according to the transitions of the sodium adduct ion $[M + Na]^+$ to the fragment ion corresponding to the neutral loss of the D-ring $[M + Na]^+ - 97$ (Sato *et al.*, 2003, 2005). Data acquisition and analysis were performed using MASSLYNX 4.1 software (Waters).

Statistical analysis

Data were analysed using GENSTAT for Windows (9th edition) using a generalized linear model, a generalized linear mixed model or analysis of variance (ANOVA). For the analysis of the mutant *notabilis* and wild-type data, ANOVA after arcsine (squareroot(×)) transformation was performed.

Table 1 Carotenoid content of roots of tomato (*Solanum lycopersicum*) plants

	Neoxanthin	Violaxanthin	Lutein	β-Carotene
	(μg g^{-1} FW root)			
Treatments				
+Pi	1.39 ± 0.20[a]	0.49 ± 0.10[a]	1.01 ± 0.15[a]	0.23 ± 0.03[ab]
–Pi	1.37 ± 0.43[a]	0.50 ± 0.05[a]	1.32 ± 0.24[a]	0.28 ± 0.05[b]
–Pi +Flu	0.56 ± 0.26[b]	0.26 ± 0.09[b]	0.88 ± 0.31[a]	0.19 ± 0.03[a]
Mutant				
notabilis	3.92 ± 0.32[c]	0.81 ± 0.05[c]	0.16 ± 0.02[b]	0.19 ± 0.02[a]
Wild-type *notabilis*	2.70 ± 0.43[d]	0.78 ± 0.05[c]	0.28 ± 0.03[c]	0.18 ± 0.02[a]

Tomato plants were exposed to normal inorganic phosphate (+Pi), Pi starvation (–Pi) and Pi starvation plus the carotenoid inhibitor fluridone (1 μM) (–Pi +Flu). Carotenoid content of the roots of these plants and the abscisic acid (ABA)-deficient mutant *notabilis* and corresponding wild-type were subsequently analysed using high-pressure liquid chromatography (see the Materials and Methods section). Numbers represent the average of three independent replicates ± SD. Different superscript letters indicate statistically significant differences between means ($P < 0.05$) for each compound.

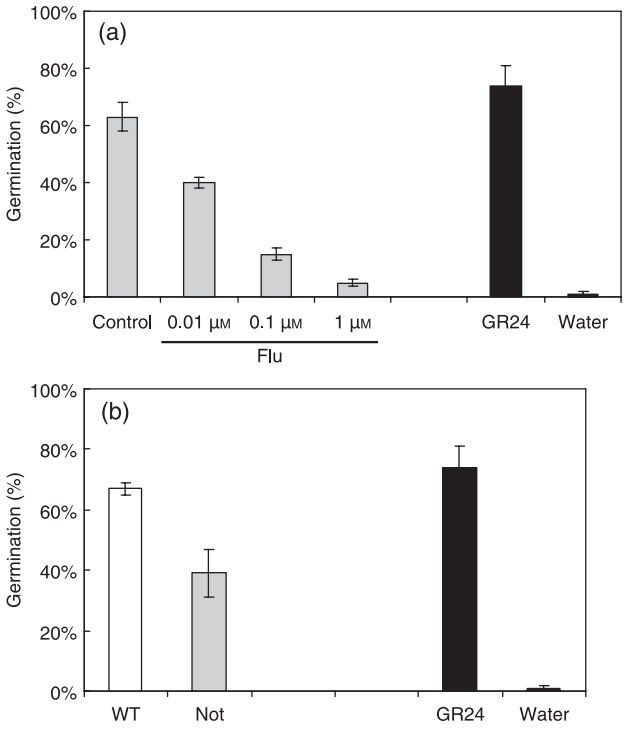

Fig. 2 Germination of *Orobanche ramosa* seeds induced by root exudates of tomato (*Solanum lycopersicum*). (a) Effect of the treatment of tomato (cv. MoneyMaker) with the inhibitor fluridone (Flu) at 0.01, 0.1 and 1 μM. (b) Germination of *O. ramosa* seeds induced by the root exudates of the tomato mutant *notabilis* (Not) compared with the corresponding wild-type (WT). GR24 (10^{-9} M) and demineralized water were used as positive and negative controls, respectively. Within each experiment, the concentrations of the root exudates were equalized by dilution to the same ratio of volume of exudate to root fresh weight. Numbers represent the average of three independent replicates ± SE.

Results

Biosynthetic origin of the tomato strigolactones

To assess the biosynthetic origin of the germination stimulants in tomato plants, we investigated the induction of *O. ramosa* seed germination by root exudate of Pi-starved tomato seedlings treated with the carotenoid inhibitor fluridone and of the tomato mutant *notabilis*. The synthetic germination stimulant GR24 (10^{-9} M), used as a positive control, always induced germination of preconditioned *O. ramosa* seeds (up to *c*. 80%). Water alone, used as a negative control, did not induce any germination. Leaves of tomato seedlings grown in the presence of 0.01 μM fluridone did not show any phenotype. However, at the higher concentrations (0.1 μM and 1 μM), the newly formed leaf tissues were white, indicating chlorophyll bleaching (Kim *et al.*, 2004). The germination of *O. ramosa* seeds induced by the root exudates of untreated tomato plants was lower than that induced by the positive control GR24, indicating that saturation of the germination response did not occur at the root exudate dilutions used in the bioassays (Fig. 2a). To exclude a possible direct effect of fluridone on germination, a dose–response curve of GR24 plus different concentrations of fluridone were included in the germination bioassays to show that at the concentration used for the bioassays, fluridone did not affect germination (data not shown).

Germination induced by the root exudates of tomato plants was significantly lower in fluridone-treated plants in a dose-dependent manner (Fig. 2a). The maximum inhibition in germination (*c*. 98%) was detected after treatment with 1 μM fluridone. The content of all carotenoids analysed, except lutein, was significantly ($P < 0.05$) lower in the roots of 1 μM fluridone-treated plants (Table 1).

In addition to the carotenoid biosynthesis inhibitor fluridone, we also tested the ABA-deficient tomato mutant *notabilis* for

its capacity to induce germination of *O. ramosa*. This mutant is believed to be a null mutation in the gene *LeNCED1*, encoding an important enzymatic step in the abscisic acid biosynthetic pathway (Burbidge *et al.*, 1999; Thompson *et al.*, 2004). This mutant is analogous to the maize *vp14* mutant, which was used to clone the first carotenoid cleavage gene (Tan *et al.*, 1997) and which was shown to induce less germination of the seeds of the parasitic plant *Striga hermonthica* (Matusova *et al.*, 2005). Indeed, *notabilis* also induced significantly (Fprob = 0.008) less germination, *c.* 40%, than the corresponding wild-type (Fig. 2b). Interestingly, in this mutant only the level of the carotenoid neoxanthin, the substrate for the enzyme NCED, was slightly but significantly ($P < 0.05$) increased compared with the wild type, while lutein was significantly decreased (Table 1).

Role of phosphate in strigolactone production

To evaluate the effect of the plant's Pi status on the production and/or exudation of germination stimulants by tomato roots, plants were grown hydroponically under normal Pi conditions and then transferred to media with or without Pi. Plants under adequate Pi nutrition grew faster and their root system was bigger, whereas in plants grown under limited-Pi conditions the roots were smaller and more branched (data not shown). In addition, the leaves of plants grown in media without Pi for 96 h showed the characteristic purple colour owing to overproduction of anthocyanins under Pi starvation (Marschner, 1995).

The Pi starvation resulted in a significant, gradual increase in time (Fprob < 0.0001) in germination stimulant activity of the root exudates compared with the control (Fig. 3a). The largest difference was observed after 96 h of treatment, when the exudates of the Pi-starved plants induced about threefold higher germination than the control exudates. The observed difference in germination stimulation between Pi-starved and control plants was similar to the difference between plants treated with 1 μM fluridone and control plants (both grown under Pi-limited conditions) (Fig. 2a). Interestingly, when Pi supply was restored in the nutrient solution and plants were grown for an additional 3 d, the germination stimulant activity decreased again, approaching the level induced by exudates of control plants continuously grown in the presence of Pi (Fig. 3a). This result suggests that the increase in the germination stimulant activity of the tomato root exudates results from Pi withdrawal itself and not from indirect effects. Under Pi starvation the lutein level increased significantly whereas the other carotenoids did not change significantly (Table 1).

To investigate further whether the increase in the germination stimulant activity under Pi starvation may be caused by an increase in the exudation by the roots or by *de novo* production of germination stimulants, a germination bioassay was performed with root extracts (Fig. 3b). The increase in germination stimulation by Pi starvation, as observed in root exudates, was also found with root extracts, suggesting that the increase in germination stimulant activity is mainly caused by *de novo* biosynthesis of strigolactones rather than by an increase in the exudation only.

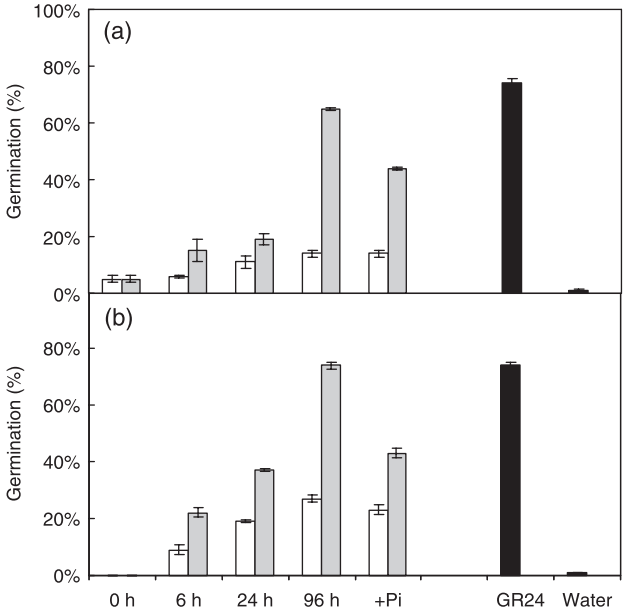

Fig. 3 Effect of inorganic phosphate (Pi) availability on the production of germination stimulants by tomato (*Solanum lycopersicum*) roots. Plants were grown for 3 wk on half-strength Hoaglands nutrient solution and then transferred to the same solution with 0.2 mM Pi (open bars) or without Pi (tinted bars) and grown for an additional 0, 6, 24 or 96 h. After 96 h, Pi was added back to the remaining plants of the Pi starvation treatment (+Pi). Germination bioassays with *Orobanche ramosa* seeds were carried out using (a) root exudates or (b) root extracts. GR24 (10^{-9} μ) and demineralized water were used as positive and negative controls, respectively. Within each experiment, the concentrations of the root exudates were equalized by dilution to the same ratio of volume of exudate to root fresh weight. Bars represent the average of three independent replicates ± SE (a) or the average of three replicate discs ± SE (b).

Fractionation of tomato root exudates

Because exudates of plants may contain more than one strigolactone and possibly also germination stimulants from other metabolite classes (Bouwmeester *et al.*, 2003; Scervino *et al.*, 2005), tomato root exudates were fractionated by solid-phase extraction and the germination stimulant activity of the resulting fractions tested. Root exudates of fluridone-treated (1 μM), Pi-starved (during 96 h) and corresponding control plants (see above) were fractioned by eluting them from a C_{18} column with increasing concentrations of acetone in water. The 50% acetone fraction was the most active (Fig. 4a,b), although considerable fronting occurred particularly in the Pi starvation experiment. This could indicate the presence of additional, different germination stimulants but may also

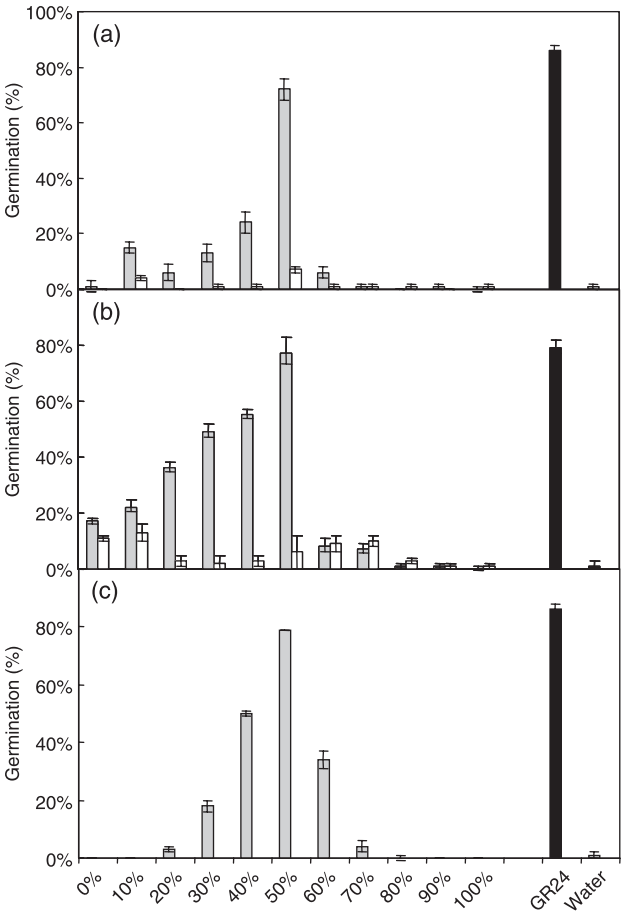

Fig. 4 Effect of fractionation of tomato (*Solanum lycopersicum*) root exudates on *Orobanche ramosa* germination. The root exudates were fractioned on a SEPAK C_{18} column with increasing concentrations of acetone as described in the Materials and Methods section. (a) Tomato seedlings grown under inorganic phosphate (Pi) starvation for 96 h were treated with the inhibitor fluridone (open bars) or without fluridone (tinted bars). (b) Plants grown with Pi (open bars) compared with plants under Pi starvation (tinted bars). (c) Germination results with an orobanchol standard that was fractioned in the same way. GR24 (10^{-9} M) and demineralized water were used as positive and negative controls, respectively. Within each experiment, the concentrations of the root exudates were equalized by dilution to the same ratio of volume of exudate to root fresh weight. Bars represent the average of three replicate discs ± SE.

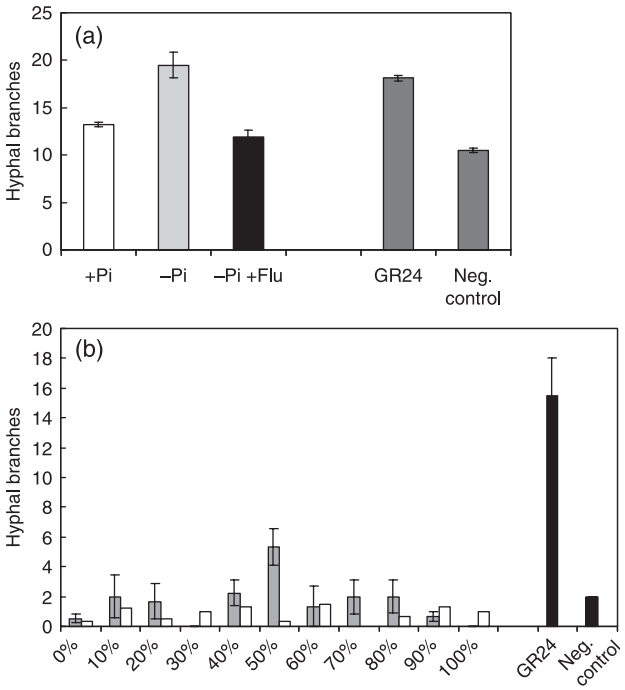

Fig. 5 *Gigaspora rosea* and *Gigaspora gigantea* hyphal branching as induced by root exudates of tomato (*Solanum lycopersicum*) seedlings. (a) Effect of sufficient inorganic phosphate (+Pi), Pi starvation (–Pi) and Pi starvation/fluridone-treatment (–Pi +Flu). (b) Effect of fractioned Pi-starved tomato root exudates. The root exudates were fractioned on a SEPAK C_{18} column with increasing concentrations of acetone as described in the Materials and Methods section. GR24 (10^{-8} M) was used as a positive control and 10 : 90 (v : v) acetonitrile/water as a negative control (Neg. control). Bars represent the average of three independent replicates ± SE (each replicate using 10–20 spores per sample).

be indicative of overloading of the column (see later). The germination stimulant activity eluting in the 50% acetone fraction (and the fractions preceding it) was strongly increased under Pi starvation (*c.* 10-fold higher in the most active fraction; Fig. 4b) and was almost completely abolished by fluridone treatment (Fig. 4a). Earlier work on tomatoes demonstrated the presence of the strigolactones orobanchol, solanacol and other strigol(orobanchol) isomers and derivatives in root exudates (Y. Goldwasser and K. Yoneyama, pers. comm.). Therefore, an aqueous solution of an orobanchol standard was fractioned under the same conditions, showing the highest activity also in the 50% acetone fraction (Fig. 4c). As was the case with root exudates, with a pure orobanchol standard there was fronting (and tailing) of germination stimulant activity upon C_{18} fractionation. It is not possible conclude yet whether the other active fractions of the tomato exudates (10–40% acetone) also contain orobanchol, but the fact that their germination activity were all increased upon Pi starvation and inhibited by fluridone strongly suggests that they contain strigolactones, possibly orobanchol and possibly other more polar strigolactones.

Arbuscular mycorrhizal fungi branching assay

Because strigolactones have hyphal branching activity in AM fungi (Akiyama *et al.*, 2005), we also tested the ability of the tomato root exudates to induce hyphal branching in *G. rosea*. Root exudates from plants grown for 96 h under Pi starvation were significantly ($P < 0.05$) more active than those from plants under sufficient Pi supply and even more active than the positive control GR24 at 10^{-8} M (Fig. 5a). Moreover, when plants grown under Pi starvation were treated with the

inhibitor fluridone their branching activity was significantly reduced (Fig. 5a). The exudates of plants grown in the presence of Pi or treated with fluridone induced similar hyphal branching as the negative control (water) (Fig. 5a).

All the acetone fractions from the Pi starvation experiment that were tested in the germination assay were also assayed for their hyphal branching activity with *G. gigantea*. Again, the 50% acetone fraction was the most active, whereas most other fractions did not induce more hyphal branching than the negative control (Fig. 5b). This shows that the 50% acetone fraction contains one or more compounds, induced by Pi starvation and decreased upon fluridone treatment, which can induce hyphal branching of AM fungi (Fig. 5b) as well as germination of the parasitic plant *O. ramosa* (Fig. 4b), most likely (a) strigolactone(s).

LC-MS/MS analysis of strigolactones in tomato root exudates

The combined 40–60% acetone C_{18} SEPAK fractions were analysed by LC-MS/MS using MRM in positive ESI mode to confirm that the results obtained in the bioassays can be attributed to strigolactones. Three single intense peaks were detected at different channels. For orobanchol, the sodium adduct ion $[M + Na]^+$ with *m/z* 369, was the most abundant, as was previously described by Sato *et al.* (2003). In the transition 369 > 272 (loss of the D-ring (97)), an intense peak was detected at a retention time of 5.30 min. This peak was present in the Pi-starved root exudates and was clearly reduced in the sufficient Pi and not detectable in the Pi-starved/fluridone-treated plant root exudates (Fig. 6a). The MS/MS analysis (data not shown) and standard addition of synthetic orobanchol to the sample confirmed that this compound in root exudates of the Pi-starved plants was indeed orobanchol (Fig. 6a). In addition to orobanchol, two other strigolactones were detected by MRM. One of these corresponds to solanacol (Fig. 1), which was detected in the transition 365 > 268 at a retention time of 4.56 min, and was more intense than orobanchol (Fig. 6b). The MS/MS analysis (data not shown) and spiking confirmed that this compound was solanacol (Fig. 6b). Just as orobanchol, solanacol was induced by Pi starvation and not detectable in the Pi-starved/fluridone-treated plant root exudates (Fig. 6b). Orobanchol and solanacol standard addition experiments showed that the reduction in the strigolactone peak area in the Pi and fluridone treatments was not caused by ion suppression in the LC-MS/MS (data not shown).

Solanacol is one of the major strigolactones present in some cultivars of tobacco (Xie *et al.*, 2007). It is quite a unique strigolactone as it contains an aromatic ring. The third peak corresponds to (a) putative didehydro-orobanchol isomer(s) and was detected in the transition 367 > 270 at a retention time of 4.96. This peak is quite broad suggesting that it is composed of more than one, possibly even up to three, isomer(s) (Fig. 6c). It showed the same behaviour as orobanchol and solanacol upon Pi starvation and fluridone treatment (Fig. 6c). The identity of this compound has not been verified using a standard.

Discussion

In the work reported here we show that the *O. ramosa* germination stimulants produced by tomato cv. Moneymaker roots are principally strigolactones and that these are biosynthetically derived from carotenoids. We have demonstrated this through a number of different approaches such as using the carotenoid biosynthesis inhibitor fluridone and the ABA-deficient tomato mutant *notabilis*, using germination bioassays with seeds of *O. ramosa*, a branching factor assay with AM fungi and LC-MS/MS using MRM. The present work is in line with our earlier results that demonstrated that *S. hermonthica* germination stimulants from maize, cowpea and sorghum are derived from the carotenoids (Matusova *et al.*, 2005) and shows that this can be extended to the germination stimulants of the broomrapes.

In tomato root exudates, orobanchol, solanacol and other strigol/orobanchol isomers and derivatives have been detected (Y. Goldwasser and K. Yoneyama, pers. comm.). Here we show that the tomato strigolactones can be fractioned using a reverse-phase C_{18} SEPAK column, and that the 50% acetone fraction contains most of the stimulant activity. This activity was clearly reduced after treatment of the tomato plants with the inhibitor fluridone (Fig. 4a) and strongly induced in plants grown under Pi starvation (Fig. 4b). Interestingly, we also found that this fraction was the most active in inducing hyphal branching of germinating spores of AM fungi (Fig. 5b). Moreover, the strigolactone orobanchol used as a standard also eluted predominantly in the 50% acetone fraction (Fig. 4c). We have also shown by LC-MS/MS that the main known strigolactones present in the combined 40–60% acetone fraction are orobanchol and the recently characterized solanacol (Fig. 6a,b), the first strigolactone with an aromatic ring (Xie *et al.*, 2007). Solanacol has been shown to be one of the major strigolactones present in some cultivars of tobacco (Xie *et al.*, 2007). In addition to orobanchol and solanacol, another peak corresponding to a didehydro-orobanchol isomer was detected in this fraction, and showed the same behaviour as the other strigolactones (Fig. 6c). All three strigolactones were induced by Pi starvation and clearly repressed by fluridone treatment (Fig. 6b). Our fractionation results also show that, although there may be compounds from other metabolite classes present in the tomato exudates, which also have germination stimulant activity, these are quantitatively of minor importance (Fig. 4a,b).

In the present work, fluridone dramatically inhibited the germination stimulatory activity of tomato root exudates (Figs 2a and 4a). This reduction is larger than that observed for maize and cowpea where fluridone treatment reduced the

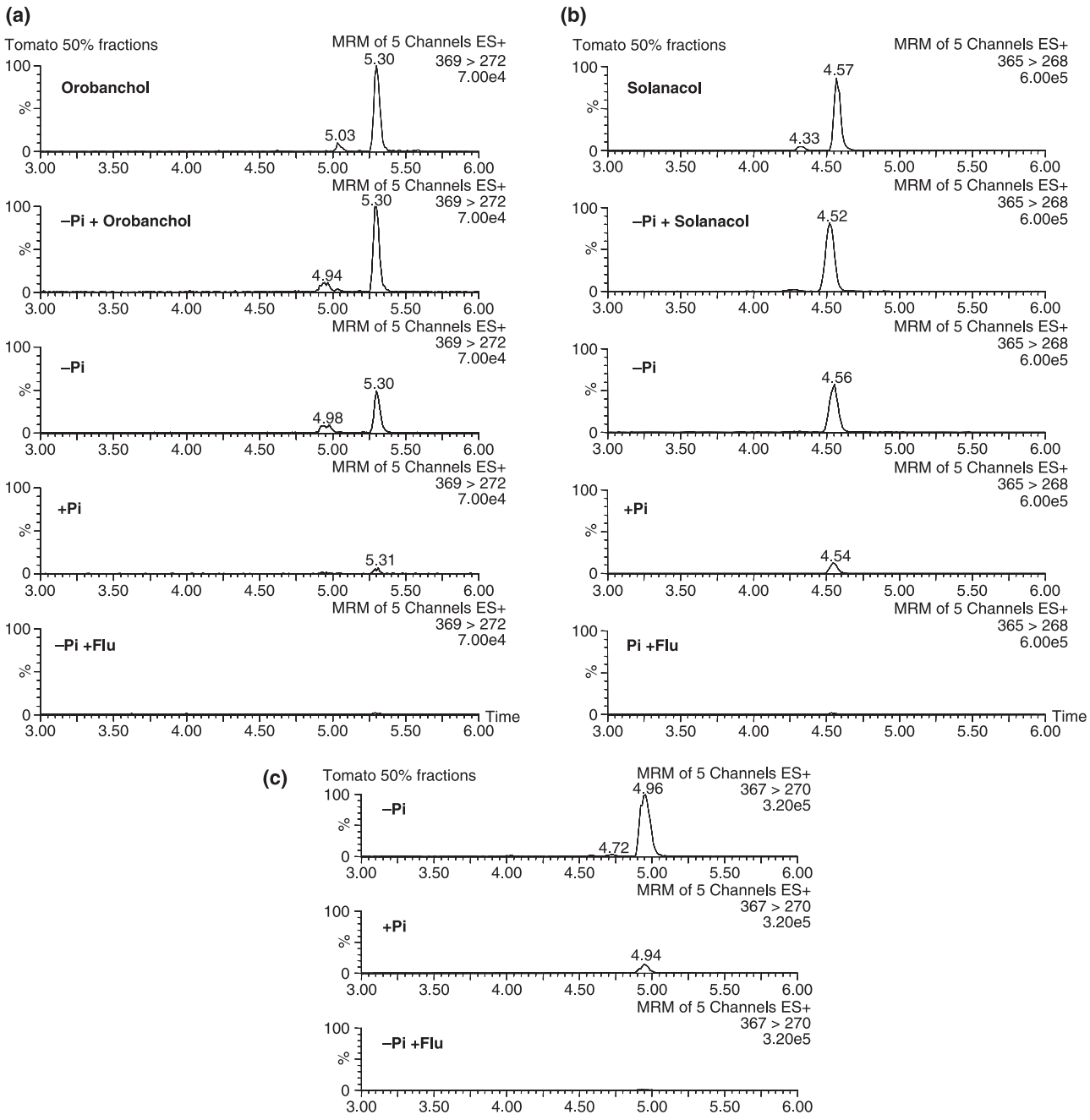

Fig. 6 Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis using multiple reaction monitoring (MRM) of the combined SEPAK C_{18}-separated 40–60% acetone fractions of tomato (*Solanum lycopersicum*) root exudates from plants grown with sufficient inorganic phosphate (+Pi), under Pi starvation (–Pi) or Pi starvation/fluridone treatment (–Pi +Flu) conditions. Chromatograms of (a) transition 369 > 272 for orobanchol, and (b) transition 365 > 268 for solanacol. (c) Chromatograms for transition 367 > 270 for the didehydro-orobanchol isomer(s). For the orobanchol (a) and solanacol (b) transitions chromatograms of authentic standards and coinjection of these standards with Pi starved tomato root exudates are also shown.

S. hermonthica germination stimulant activity by *c*. 80%, but similar to that observed in sorghum (Matusova *et al*., 2005). The lower efficacy of fluridone in maize and cowpea may well be caused by these exudates tested by Matusova and co-workers, having been collected in water. It is likely that strigolactone biosynthesis was partly restored during the 24-h exudate collection period. By contrast, in the present experiments exudates of fluridone-treated plants were collected at low concentrations of fluridone (see the Materials and Methods section) to prevent biosynthesis being initiated again during exudate collection. The effectiveness of the fluridone treatments is clear from the carotenoid analysis (Table 1). A more direct

proof of the effect of fluridone on strigolactone biosynthesis is provided by the LC-MS/MS analysis (Fig. 6). In all cases, fluridone treatment reduced the production of all strigolactones that we observed to values below detection limits. This is also relevant for the discussion on the biosynthetic origin of solanacol and the didehydro-orobanchol isomer(s). Although a carotenoid origin of the aromatic A-ring cannot easily be envisaged, our results show that solanacol behaves similarly to the other strigolactones and hence, must also be carotenoid' derived. Moreover, it is likely that solanacol (which can also be named tetradehydro-orobanchol) is derived from orobanchol, with the unidentified didehydro-orobanchol isomer(s) being intermediate(s) in this conversion.

Taken together, these and previous results (Matusova *et al.*, 2005) indicate that the carotenoid origin of strigolactones is probably a general phenomenon in the plant kingdom. Although the branching point from the carotenoid pathway for strigolactone biosynthesis has not yet been identified, our previous results have suggested that the ABC-part of the strigolactones is derived from carotenoid cleavage by the action of NCED or CCD enzymes (Matusova *et al.*, 2005; Bouwmeester *et al.*, 2007). Carotenoid cleavage is a common biosynthetic reaction that occurs in a number of biosynthetic pathways, including the production of important plant signalling molecules, such as the plant hormone ABA through NCED action (Tan *et al.*, 2003; Thompson *et al.*, 2004; Taylor *et al.*, 2005). The fact that the tomato mutant *notabilis*, with a null mutation in the gene *LeNCED1*, produced less active root exudates than the wild-type (Fig. 2b) suggests that NCED1 is involved in the biosynthesis of strigolactones in tomato. However, the germination stimulant activity in the mutant was not completely abolished and was reduced by only *c.* 40%. This result agrees strikingly with our previous results with the maize mutant *vp14*, in which the reduction in germination stimulant activity of *S. hermonthica* was *c.* 44% (Matusova *et al.*, 2005). This reduction agrees again quite well with the decrease in ABA accumulation in maize embryos in *vp14* (Tan *et al.*, 1997). However, it is still unclear whether the NCED mutations have a direct effect on the production of strigolactones or through their effect on ABA production.

The strigolactones also play an important role as host detection signals for AM fungi, by inducing hyphal branching in germinating mycorrhizal spores (Akiyama *et al.*, 2005; Harrison, 2005; Besserer *et al.*, 2006; Paszkowski, 2006; Bouwmeester *et al.*, 2007). In the AM symbiosis, the fungi obtain carbon provided by the plant host and supply mineral nutrients, particularly Pi, to their host thus allowing the host to survive under various suboptimal growth conditions (Harrison, 2005; Akiyama & Hayashi, 2006). Here we show that tomato root exudates from plants grown under Pi limited conditions are more active in stimulating germination of *O. ramosa* (Fig. 3a) and inducing hyphal branching in AM fungi (Fig. 5a). That this effect results from a higher secretion of strigolactones was confirmed by LC-MS/MS analysis (Fig. 6). This is in accordance with the work of Yoneyama and coworkers (2007) who showed that, in red clover grown under limited supply of Pi, the exudation of the strigolactone orobanchol was significantly stimulated. We also report that this increase in strigolactone secretion under Pi starvation is most likely mainly due to *de novo* biosynthesis of strigolactones rather than just enhanced secretion (Fig. 3b), although an additional increase in exudation by the roots by alterations to the plasma membrane and to root morphology induced by Pi starvation cannot be excluded (Sanchez-Calderon *et al.*, 2005; Kobayashi *et al.*, 2006). The strong effect of Pi starvation on strigolactone formation is in agreement with the important role of AM fungi in the acquisition of phosphate. Indeed, without having identified strigolactones as the branching factor, it has already been shown that root exudates of plants grown under Pi limitation are more stimulatory to AM fungi than exudates produced under adequate Pi nutrition (Elias & Safir, 1987; Nagahashi & Douds, 2004; Harrison, 2005; Karandashov & Bucher, 2005; Malusa *et al.*, 2006; Weisskopf *et al.*, 2006). In addition, the application of phosphate to phosphate-deficient fields of clover and skeleton weed, significantly reduced the population of clover broomrape, and the application of phosphate to tomato plants grown in pots suppressed the infection by *O. aegyptiaca* (Jain & Foy, 1992; Yoneyama *et al.*, 2001).

The current study further confirms the double role of the strigolactones and shows that their production is tightly regulated by environmental conditions such as phosphate availability. The strigolactones are signalling molecules that host plants produce to facilitate the establishment of a symbiotic interaction with AM fungi to increase their chances of survival under nutrient deficient conditions (particularly Pi deficiency). At the same time the strigolactones also serve as host-presence signalling molecules for parasitic plants. Because of this intimate relationship between attracting the good and not being able to avoid the bad, a biotechnological approach for the control of parasitic plants, aimed at altering the production and/or exudation of the strigolactones by the roots of agricultural crops will require extensive fine-tuning. Clearly, further research is required to find and characterize the enzymes involved in the production of these signalling molecules and elucidate the mechanism by which their biosynthesis and bioactivity is regulated.

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