Plant Growth Regul (2011) 65:495–504
DOI 10.1007/s10725-011-9620-z

ORIGINAL PAPER

Characterization of strigolactones exuded by Asteraceae plants

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Received: 8 March 2011 / Accepted: 4 August 2011 / Published online: 12 August 2011

Abstract Strigolactones (SLs), originally characterized as germination stimulants for root parasitic weeds, are now recognized as hyphal branching factors for symbiotic arbuscular mycorrhizal fungi and as a novel class of plant hormones inhibiting shoot branching. In the present study, SLs in root exudates of 13 Asteraceae plants including crops, a weed, and ornamental plants were characterized. High performance liquid chromatography/tandem mass spectrometry (LC–MS/MS) analyses revealed that all the Asteraceae plants examined exuded known SLs and, except for sunflower (*Helianthus annuus*), high germination stimulant activities at retention times corresponding to these SLs were confirmed. The two major SLs exuded by these Asteraceae plants were orobanchyl acetate and orobanchol. 5-Deoxystrigol and 7-hydroxyorobanchyl acetate were detected in root exudates from several Asteraceae species examined in this study.

Keywords Asteraceae · Germination stimulant · Root parasitic weed · Strigolactone

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Abbreviations

AM	Arbuscular mycorrhizal
HPLC	High performance liquid chromatography
LC–MS/MS	Liquid chromatography/tandem mass spectrometry
SL	Strigolactone

Introduction

Root parasitic weeds hamper growth of host plants by obtaining nutrients and water from them, and consequently, negatively affect agricultural production globally. In temperate regions, achlorophyllous broomrapes (*Orobanche* and *Phelipanche* spp.) are widely distributed and parasitize dicotyledonous crops like legumes and vegetables. The areas threatened by broomrapes, as estimated in 1991, are 16 million ha in the Mediterranean and west Asia (Parker 2009). Witchweeds (*Striga* spp.), which prevail throughout tropical regions, have functional chloroplasts but cannot complete their life-cycle without parasitizing their hosts, which are mainly monocots. In sub-Saharan Africa, 50 million ha of crop fields are infested by *Striga* with annual losses of 10 billion dollars (Ejeta and Gressel 2007). Feasible and cost-effective methods to adequately control these root parasites have not yet been established.

Seeds of these parasites germinate only when they perceive germination stimulants released from plant roots. Strigolactones (SLs) are representative germination stimulants and have been isolated from root exudates of various plants. To date, more than 15 SLs have been characterized and all of these natural SLs are composed with a tricyclic lactone (ABC part) that connects via an enol ether bridge to a butenolide group (D ring; Xie et al. 2010). The enol ether

bridge, the C-D ring moiety, which is easily cleaved under alkaline conditions, was shown to be an essential structure to induce seed germination of root parasites (Zwanenburg et al. 2009). Although over a hundred SLs have been predicted to exist in the plant kingdom, such an unstable feature of SLs makes isolation and characterization of novel SLs difficult (López-Ráez et al. 2008; Xie et al. 2010).

High performance liquid chromatography (HPLC)/tandem mass spectrometry (LC–MS/MS), in particular, multiple reaction monitoring (MRM), is a rapid and highly sensitive analytical method to identify and quantify known SLs (Sato et al. 2003). However, peaks in MRM chromatograms are not necessarily SLs and may be other compounds that happen to have similar fragmentations under the analytical conditions employed. Therefore, LC–MS/MS analyses should be combined with germination assays using seeds of root parasitic plants, e.g., *Orobanche minor*. It should be noted, however, that only major germination stimulants may be detected in these germination assays.

By using LC–MS/MS analyses coupled with germination assays, monocots such as sorghum, maize, and millet, representative host crops of *Striga*, were shown to produce SLs including sorgomol, sorgolactone, and 5-deoxystrigol (Awad et al. 2006). In dicots, several Fabaceae plants (Yoneyama et al. 2008), Solanaceae tomato (López-Ráez et al. 2008) and tobacco (Xie et al. 2007), Cruciferae *Arabidopsis* (Goldwasser et al. 2008; Kohlen et al. 2011), Cucurbitaceae cucumber and Linaceae flax (Xie et al. 2009b) were found to produce several known SLs and novel germination stimulants, possibly SLs. To date, there are no clear differences in SL production between monocots and dicots. Not only higher plants, but also mosses have been found to produce SLs (Proust et al. 2011). Although precise data have not yet been published, we have already confirmed that trees including *Pinus* spp. and *Eucalyptus* spp. also produce SLs (Xie et al. 2010). All of the plants examined so far produce and exude mixtures of several SLs (Xie et al. 2010).

In the rhizosphere, SLs released from plant roots also work as a host recognition signal for symbiotic arbuscular mycorrhizal (AM) fungi. SLs induce extreme hyphal branching of AM fungi and this morphological change is considered to be a host recognition response (Giovannetti et al. 1993; Akiyama et al. 2005). AM colonization enhances host plant growth by increasing uptake of nutrients, especially phosphate (P) and nitrogen (N), through widespread network of fine hyphae (Harrison 2005; Smith and Read 2008). In fact, plants under P (Yoneyama et al. 2007a; López-Ráez et al. 2008; Umehara et al. 2010) and N (Yoneyama et al. 2007b) deficiencies significantly promote SL production and exudation to commence symbiosis with AM fungi.

SLs also play a pivotal role as a novel class of plant hormones inhibiting shoot branching. Exogenously applied GR24, a synthetic SL, suppressed shoot outgrowth in SL deficit mutants of *Arabidopsis*, pea, and rice plants (Gomez-Roldan et al. 2008; Umehara et al. 2008). Therefore, SLs are prominent signaling compounds which regulate parasitism of root parasites, symbiosis of AM fungi, and plant aboveground architecture. In addition, effects of SLs on the regulation of root system architecture and development have recently been unveiled (Koltai et al. 2010; Kapulnik et al. 2010; Ruyter-Spira et al. 2011). However, the biosynthetic pathway of SLs remains elusive except that they are derived from carotenoids (Matusova et al. 2005; López-Ráez et al. 2008; Xie et al. 2010).

The Asteraceae is one of the largest plant families and has a global distribution. This family includes important host crops of broomrapes such as lettuce, sunflower, and safflower (Musselman and Parker 1982). Petunia, an Asteraceae plant, has been used as a model plant in the study on shoot branching-inhibiting plant hormones. So far, however, there is no report on SLs produced by Asteraceae plants.

In the present study, characterization of SLs in the root exudates from 13 Asteraceae plants including crops, a weed, and ornamental plants grown hydroponically was conducted by comparing retention times of germination stimulants on reversed phase HPLC with those of natural and synthetic SL standards and by using LC–MS/MS. In addition, qualitative and quantitative differences of SLs produced by different varieties and cultivars of lettuce were examined and their susceptibilities to clover broomrape (*O. minor*) parasitism were determined by pot tests.

Materials and methods

Chemicals

(+)-Orobanchol and (+)-orobanchyl acetate were purified from red clover root exudates (Yokota et al. 1998; Xie et al. 2008b). 7-Hydroxyorobanchol (mixture of 7α and 7β isomers) and their acetates (also mixture), 7-oxoorobanchol, and 7-oxoorobanchyl acetate were purified from flax root exudates (Xie et al. 2009b). Solanacol, sorgomol, and fabacyl acetate were purified from root exudates of tobacco, sorghum, and pea, respectively (Xie et al. 2007, 2008a, 2009a). Fabacol was recently purified from pea root exudates. (+)-Strigol, sorgolactone, and 5-deoxystrigol along with their 2′-epimers were generous gifts of Emeritus Prof. Kenji Mori (The University of Tokyo), Prof. Yukihiro Sugimoto (Kobe University), and Assoc. Prof. Kohki Akiyama (Osaka Prefecture University), respectively. Strigyl acetate was prepared from strigol (Sato et al. 2005). The other chemicals of analytical grade and HPLC solvents

were obtained from Kanto Chemical Co. Ltd. and Wako Pure Chemical Industries Ltd.

Plant material

Orobanche minor Sm. (clover broomrape) seeds were collected from mature plants that parasitized *Trifolium pratense* L. grown in the Watarase basin of Tochigi Prefecture, Japan. Seeds of *Actium lappa* L. (edible burdock), *Carthamus tinctorius* L. (safflower), *Chrysanthemum coronarium* L. (garland chrysantemum), *Cosmos bipinnatus* Cav. (cosmos), *Helianthus annuus* L. (sunflower), *Lactuca sativa* L. (lettuce), *Petunia hybrida* Vilm. (petunia), *Tagetes erecta* L. (African marigold), and *T. patula* L. (French marigold) were obtained from a local supplier. Seeds of *Hedypnois rhagodioloides* L. (cretanweed) were generously supplied by Dr. John Matthews (University of Adelaide, Australia).

Hydroponic culture

Hydroponic culture was conducted as reported previously (Yoneyama et al. 2008) with minor changes. Plant seeds were surface-sterilized in 70% ethanol for 2 min and then 1% NaClO for 2 min. After thorough rinsing with sterile distilled water, seeds were soaked in water at room temperature for 2 days. Germinated seeds (n = 10) were transferred to a strainer (28 × 23 × 9 cm, width × length × height (W × L × H)) lined with a sheet of gauze moistened by placing it in a slightly larger container (28.5 × 23.5 × 11 cm, W × L × H) containing 1 l of tap water as the culture medium in a growth chamber with a 14:10 h photoperiod at 120 μmol photons m^{-2} s^{-1} at 23:20°C. The plants were grown in tap water for 7 days and then transferred to 1/2 Tadano and Tanaka medium (Tadano and Tanaka 1980) without phosphate to promote SL production and exudation (Yoneyama et al. 2007a, b). Culture media were refreshed every 2 days and in 10 days, root exudates released into culture medium were adsorbed by activated charcoal using circulation pumps (Akiyama et al. 2005; Yoneyama et al. 2008) for 2 days.

Extraction of root exudates

Extraction of root exudates was conducted as reported previously (Yoneyama et al. 2008). Root exudates adsorbed onto the charcoal were eluted with acetone. After the acetone was evaporated in vacuo, the residue was dissolved in 50 ml of water and extracted 3 times with 50 ml of ethyl acetate. The ethyl acetate extracts were combined, washed with 0.2 M K_2HPO_4 (pH 8.3), dried over anhydrous $MgSO_4$, and concentrated in vacuo. These crude extracts were stored in sealed glass vials at 4°C until use.

Identification of SLs by LC–MS/MS

Identification of SLs by LC–MS/MS was conducted as reported previously (Yoneyama et al. 2008). HPLC separation was conducted with a U980 HPLC instrument (Jasco, Tokyo, Japan) fitted with an ODS (C_{18}) column (Mightysil RP-18, 2 × 250 mm, 5 μm, Kanto Chemicals Co., Ltd., Tokyo, Japan). The crude extracts were dissolved in 60% methanol and filtered through spin columns (Ultra-Free MC, 0.45 μm pore size, Millipore, Tokyo, Japan), and 10 μl was injected. The mobile phase was 60% methanol in water and was changed to 100% methanol 30 min after injection. The column was then washed with 100% methanol for 20 min. The flow rate was 0.2 ml min^{-1} and the column temperature was set to 40°C. Mass spectrometry was performed with a Quattro LC mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray source. The drying and nebulizing gas was nitrogen generated from pressurized air in an N2G nitrogen generator (Parker-Hanifin Japan, Tokyo, Japan). The nebulizer gas flow was set to approx. 100 l h^{-1}, and the desolvation gas flow to 500 l h^{-1}. The interface temperature was set to 400°C, and the source temperature to 150°C. The capillary and cone voltages were adjusted to orobanchol and to the positive ionization mode. MS/MS experiments were conducted using argon as the collision gas and the collision energy was set to 16 eV. The collision gas pressure was 0.15 Pa. For the detection of known SLs, we used ten-channel MRM. The nine transitions of *m/z* 339 > 242, 353 > 256, 365 > 268, 367 > 270, 369 > 272, 383 > 286, 385 > 288, 411 > 254, 425 > 268, and 427 > 270 were monitored for sorgolactone, 5-deoxystrigol, solanacol, didehydro-orobanchol (didehydro-strigol), strigol (orobanchol and sorgomol), 7-oxoorobanchol, orobanchyl acetate (strigyl acetate), 7-hydroxyorobanchol and fabacol, 7-oxo-orobanchyl acetate, and fabacyl acetate and 7-hydroxyorobanchyl acetate, respectively. Retention times of SLs under these analytical conditions were; 7-hydroxyorobanchol, 3.3 min; 7-oxoorobanchol, 3.4 min; fabacol, 3.5 min; 7-hydroxyorobanchyl acetate, 4.2 min; 7-oxoorobanchyl acetate, 4.8 min; solanacol, 5.8 min; two didehydro-orobanchol isomers, 6.9 min and 7.2 min; orobanchol, sorgomol, and strigol, 8.0 min; fabacyl acetate, 12.3 min; orobanchyl acetate, 15.7 min; strigyl acetate, 16.5 min; sorgolactone, 25.5 min; 5-deoxystrigol, 27.5 min. For separation of orobanchol, strigol, and sorgomol, a Phenyl column (Intertsil Ph, 2.1 × 250 mm, 5 μm, GL Sciences Inc., Tokyo, Japan) was used with 50% acetonitrile as the eluting solvent. The low rate was 0.2 ml min^{-1} and the column temperature was set to 40°C. Retention times of orobanchol, strigol, and sorgomol on the Phenyl column were 9.9, 10.9, and 11.6 min, respectively. Data acquisition and analysis were performed with the MassLynx software (ver. 4.1).

Germination assay

Germination assay was conducted as reported previously (Yoneyama et al. 2008). A portion of the ethyl acetate extracts dissolved in 60% methanol was fractionated by reversed-phase HPLC operated under the same conditions as for LC–MS/MS analyses and the fractions collected every minute were examined for *O. minor* seed germination stimulation. The amounts of crude extracts used for germination assays were 1/100 that used for LC–MS/MS analyses.

An aliquot of either root exudate samples in 60% methanol or a methanol solution of the synthetic SL, GR24 (10^{-6} M), as positive control was added to a 5-cm Petri dish lined with a filter paper. The solvent was allowed to evaporate before the discs carrying the conditioned seeds were placed on the filter paper and treated with sterile Milli-Q water (650 µl). The Petri dishes were sealed, enclosed in polyethylene bags, and placed in the dark at 23°C for 5 days. Seeds were considered germinated when the radicle protruded through the seed coat.

Pot test

Lettuce seeds were surface sterilized by soaking in 70% ethanol for 2 min and then 1% NaClO for 2 min and planted in sterilized soil mixture [vermiculite:sand and soil (typical Japanese volcanic ash soil) = 1:1:1 v/v/v] with *O. minor* seeds (0.8 mg l^{-1} soil). As fertilizers, NH_4NO_3 (N, 200 kg ha^{-1}), K_2SO_4 (K, 200 kg ha^{-1}) and $NaHPO_4$ (P, 100 kg ha^{-1}) were mixed with soils before planting. One individual seedling was grown in a 1 l pot. On day 70 after planting, lettuce plants were carefully pulled out from the pots, roots were washed to remove soil, and the number of *O. minor* attachments was counted.

Quantification of SLs from root exudates of lettuce plants

Sterilized lettuce seeds were soaked for 2 days in tap water and germinated seeds were transferred to a stainless steel sieve lined with a sheet of gauze moistened by placing it on the cup (9.5 cm in diameter, 17 cm deep, ca. 550 ml in volume) containing 500 ml of tap water. The plants were grown hydroponically with tap water for 5 days and in 1/2 Tadano-Tanaka medium for another 3 days in a growth chamber with a 14/10-h photoperiod at 120 µmol photons m^{-2} s^{-1} at 23/20°C. Then, plants were subjected to—P conditions. After 10 days of acclimatization under P deficiency, the growth media containing root exudates (plus washings) collected at 24-h intervals (approximately 450 ml) were extracted three times with an equal volume of ethyl acetate. The ethyl acetate solutions were combined, washed with 0.2 M K_2HPO_4 (pH 8.3), dried over anhydrous $MgSO_4$, and concentrated in vacuo to afford root exudate samples. Quantification of SLs by LC–MS/MS was conducted as described before.

Results and discussion

Characterization of SLs exuded by Asteraceae plants

Different plants may produce different sets of SLs at different levels. In addition, SL exudation from a plant is strongly affected by growth conditions, in particular by nutrient availability (Yoneyama et al. 2007a, b), and may change with growth stages (Xie et al. 2010). In the present study, therefore, we did not try to compare quantitative differences in SL exudation among Asteraceae plants tested.

SLs identified in root exudates from the 13 Asteraceae plants are shown in Fig. 1 and Table 1. Neither strigol, sorgolactone, solanacol, 7-hydroxyorobanchol, fabacol, strigyl acetate nor 7-oxoorobanchol was detected in root exudates from the Asteraceae plants examined in this study (Table 1). Strigol, which had been isolated from root exudates of cotton along with strigyl acetate (Cook et al. 1966) and later identified in gramineous crops sorghum, maize, and proso millet (Siame et al. 1993), and sorgolactone, which had been isolated from sorghum root exudates (Hauck et al. 1992), were also not detected in root exudates from 11 Fabaceae species (Yoneyama et al. 2008). Fabacol was recently identified in pea root exudates (Xie et al. unpublished result). These SLs that were not detected from the Asteraceae plants may be relatively minor ones in dicots grown hydroponically under the experimental conditions. Solanacol, which was isolated from tobacco root exudates (Xie et al. 2007) and also identified in tomato root exudates (López-Ráez et al. 2008), seems to be absent or produced at a low level in the Asteraceae plants. Among SLs identified so far, 7-hydroxyorobanchol and 7-oxoorobanchol are less stable than orobanchol, and therefore these SLs might have been decomposed during purification steps in this study.

Orobanchyl acetate was detected in root exudates from all the Asteraceae plants examined and distinct germination stimulation activity on *O. minor* seed was observed at the retention time on the HPLC corresponding to that of orobanchyl acetate except for safflower and sunflower, demonstrating that orobanchyl acetate is one of the major SLs in the Asteraceae plants. The corresponding hydroxy-SL, orobanchol, was detected by both LC–MS/MS and HPLC separation–germination tests in most of the Asteraceae plants examined except for garland chrysanthemum, cosmos, sunflower, and petunia. 5-Deoxystrigol, 7-oxoorobanchyl

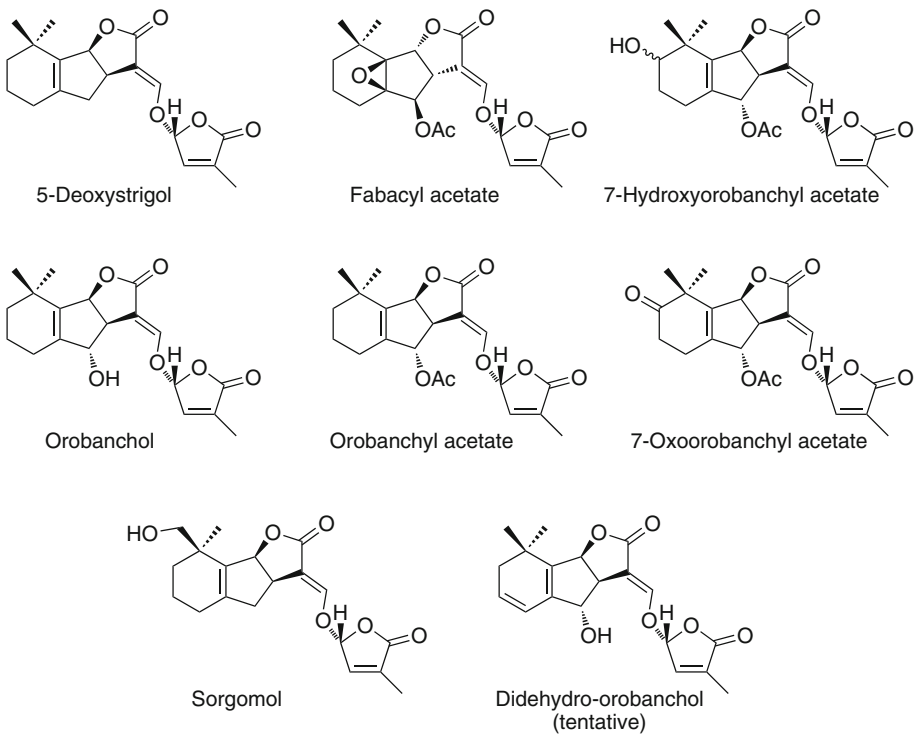

Fig. 1 Structures of SLs identified from 13 Asteraceae root exudates. A tentative structure for didehydro-orobanchol is also shown

Table 1 Distribution of SLs in Asteraceae plants

Plant name	7-Hydroxyorobanchyl acetate	7-Oxoorobanchyl acetate	Orobanchol	Sorgomol	Fabacyl acetate	Orobanchyl acetate	5-Deoxystrigol
A. lappa (edible burdock)	–	MS	MS/G	–	MS/G	MS/G	MS/G
C. tinctorius (safflower)	–	MS	MS/G	–	MS/G	MS	MS/G
C. coronarium (garland chrysanthemum)	–	MS/G	–	–	–	MS/G	MS
C. bipinnatus (cosmos)	–	MS/G	–	MS/G	–	MS/G	MS/G
H. annuus (sunflower)	–	–	–	–	–	MS	MS
H. rhagodioloides (cretan weed)	–	–	MS/G	–	MS/G	MS/G	MS/G
L. sativa (lettuce) var. capiata	MS/G	MS/G	MS/G	–	–	MS/G	–
var. crispa cv. Greenweave	MS/G	MS/G	MS/G	–	–	MS/G	MS/G
var. crispa cv. Redfire	MS/G	MS/G	MS/G	–	–	MS/G	–
P. hybrida (petunia)	MS	MS/G	–	–	–	MS/G	–
T. erectra (African marigold)	MS	MS/G	MS/G	–	–	MS/G	–
T. patula (French marigold)							
cv. Bonanza	MS	–	MS/G	–	–	MS/G	–
cv. Supersensation	MS	–	MS/G	–	–	MS/G	–

SLs were characterized by using LC–MS/MS and by comparing retention times of germination stimulation activity on reversed-phase HPLC with those of natural and synthetic standards. MS indicates that the strigolactone was detected by LC–MS/MS. G indicates that germination stimulation activity on *Orobanche minor* seed was observed at the retention time corresponding to that of the SL. It should be noted that only major germination stimulants could be detected in the germination assays

acetate, and 7-hydroxyorobanchyl acetate were found in root exudates of several Asteraceae plants examined. Sorgomol, originally isolated and characterized from sorghum (Xie et al. 2008a) and later identified in root exudates from white lupin and Chinese milk vetch (Yoneyama et al. 2008), was detected only from cosmos. Therefore, among monohydroxy-SLs,

orobanchol seems to be most widely distributed in the Asteraceae as well as in the Fabaceae plants (Yoneyama et al. 2008). Fabacyl acetate, isolated from pea root exudates (Xie et al. 2009a), was found in edible burdock, safflower, and cretanweed root exudates, suggesting that this may be minor SL in Asteraceae plants. Fabacol was not detected in root exudates from the plants that exuded fabacyl acetate, indicating that the conversion of fabacol to fabacyl acetate proceeds rapidly in these plants. Since 7-hydroxyorobanchyl acetate, 7-oxoorobanchyl acetate, and fabacyl acetate have been isolated after our previous characterization of SLs produced by the Fabaceae plants (Yoneyama et al. 2008), distribution of these SLs in the Fabaceae plants needs to be examined.

Accordingly, orobanchol, orobanchyl acetate, and 5-deoxystrigol seem to be major SLs in the Asteraceae as well as in the Fabaceae plants. Moreover, the Asteraceae and the Fabaceae plants produce and exude similar mixtures of SLs, suggesting that in these plants SLs may be synthesized through a common or similar pathway. In addition, in these plants, SL biosynthesis and metabolism appear to be regulated in a similar manner.

Between the two cultivars of French marigold, there were no qualitative differences in SL exudation, whereas distinct differences existed between the two species in the same genus. African marigold exuded 7-oxoorobanchyl acetate which was not detected in the root exudates from the two French marigolds.

Difference in SL exudation and susceptibility to clover broomrape of lettuce cultivars

5-Deoxystrigol was detected by LC–MS/MS and strong germination stimulant activity corresponding to the retention time of 5-deoxystrigol was observed from root exudates of *L. sativa* var. *crispa* cv. Greenweave (Table 1, Fig. 2). By contrast, 5-deoxystrigol was detected neither from *L. sativa* var. *capiata* nor *L. sativa* var. *crispa* cv. Redfire (Table 1, Fig. 2). In the case of sorghum, *Striga* susceptible cultivars produced larger amounts of 5-deoxystrigol than did *Striga* resistant cultivars (Yoneyama et al. 2010). Thus, production of 5-deoxystrigol which is more stable SL than hydroxy-SLs (including orobanchol) appeared to be related to susceptibility to the root parasitic weed. Therefore, if this is applicable to lettuce, *L. sativa* var. *crispa* cv. Greenweave would be more susceptible to root parasitic weeds than the other two lettuce species. These three lettuce species were then examined for their susceptibilities to *O. minor* parasitism by the pot test conducted in a greenhouse. However, all lettuce species were equally susceptible and parasitized by each 6 to 10 *O. minor* seedlings under P deficient conditions, indicating that difference in 5-deoxystrigol exudation did not influence susceptibility to *O. minor* (data not shown). This is probably because *O. minor* is more sensitive to orobanchol and orobanchyl acetate than to 5-deoxystrigol (Kim et al. 2010) and the three lettuce cultivars exuded relatively large amounts of both orobanchol and orobanchyl acetate (Fig. 2). Accordingly, orobanchol and orobanchyl acetate but not 5-deoxystrigol may be major germination stimulants for *O. minor* seeds in root exudates of these lettuce species. These results imply that it is important to identify which SL in the root exudates contributes more to seed germination stimulation of root parasitic weeds. Alternatively, since plants produce and exude mixtures of SLs, additive, synergistic, and/or antagonistic effects among SLs in root exudates may determine germination stimulation potency on seeds of root parasites. In addition, plants may produce and exude germination inhibitors as well.

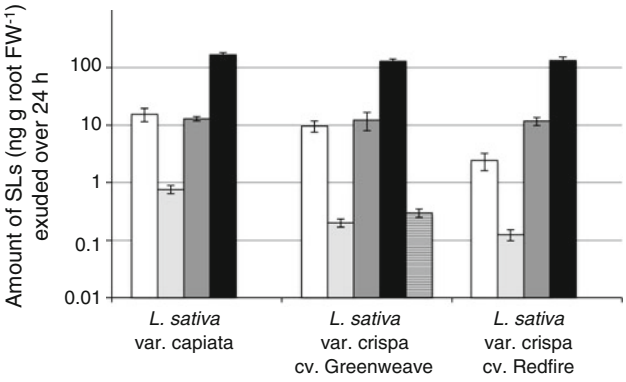

Fig. 2 Exudation of 7-hydroxyorobanchyl acetate (*white bars*), 7-oxoorobanchyl acetate (*gray bars*), orobanchol (*dark gray bars*), orobanchyl acetate (*black bars*) and 5-deoxystrigol (*hatched bars*) by three lettuce species grown hydroponically under P deficiency for 10 days. The experiments were repeated three times. Values represent the means ± SE

SLs exuded by sunflower

Complex interactions between SLs and also the presence of germination inhibitors in root exudates would explain why the fractions corresponding to the retention times of the SLs, orobanchyl acetate and 5-deoxystrigol, detected by LC–MS/MS in sunflower root exudates were not active in the germination test (Table 1). Although germination assays are in general more sensitive than LC–MS/MS in the detection of SLs, presence of germination inhibitors would mask germination stimulation activities of SLs as described before. Furthermore, two active fractions with retention times different from those of known SLs were detected in the sunflower root exudates, indicating that sunflower produces at least two novel germination stimulants (Fig. 3a). Sunflower is a host of *O. cumana* whose germination was not induced by the synthetic SL GR24.

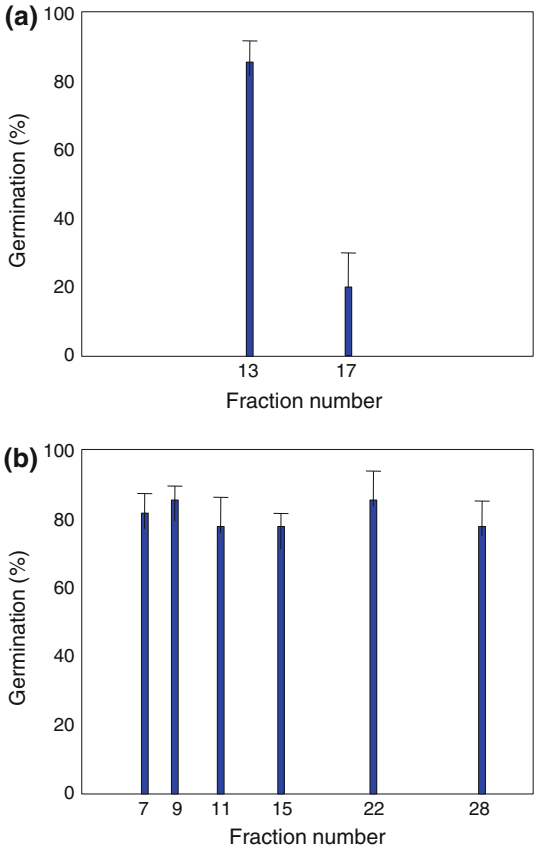

Fig. 3 Distribution of germination stimulation activity on *O. minor* after reversed-phase high-performance liquid chromatography (*HPLC*) separation of the root exudates of sunflower (**a**) and cretanweed (**b**). All the fractions were tested for germination activity but only the numbers of active fractions are presented. Data are means ± SE ($n = 3$)

Recently, dehydrocostus lactone has been identified from sunflower root exudates as a germination stimulant which elicits germination of *O. cumana* but not that of *Phelipanche ramosa* (Joel et al. 2011).

SLs exuded by cretanweed

As shown in Fig. 3b, cretanweed was found to exude orobanchol (fraction 9), fabacyl acetate (fraction 11), orobanchyl acetate (fraction 15), and 5-deoxystrigol (fraction 28). In addition to these SLs, didehydro-orobanchol (fraction 7), and at least one novel germination stimulant (fraction 22) were detected in cretanweed root exudates. The novel stimulant in the fraction 22 showed a distinct peak in the channel for monitoring the transition of *m/z* 353 > 256 for the detection of 5-deoxystrigol but its retention time (22.5 min) was different from those of 5-deoxystrigol and its 2′-epimer (both 27.5 min), suggesting that this novel stimulant may be an isomer of 5-deoxystrigol. The fractions corresponding to the retention times of these SLs and the novel stimulant induced high germination rates (> 80%) of *O. minor* seeds (Fig. 3b). Such a wide distribution of germination stimulation activity after HPLC separation of root exudate samples was unique to cretanweed, because, in general, only one or two fractions were highly active and the other fractions induced no or only low germination rates as in the case of sunflower (Fig. 3a). Therefore activities of minor SLs were sometimes not detectable as shown in Table 1. Cretanweed is a rampant weed even in nutrient-poor soils and a major host of *Phelipanche ramosa* in South Australia. These results suggest that this weed has fitted well to the nutrient deficient soils because of its high productivity of SLs which facilitate AM symbiosis.

Didehydro-orobanchol isomers

Didehydro-orobanchol isomers have been identified from tobacco (Xie et al. 2007), tomato (López-Ráez et al. 2008), and several legumes (Yoneyama et al. 2008), however, their structures still remain to be elucidated. One possible structure for didehydro-orobanchol isomers is shown in Fig. 1. These SLs were detected from root exudates of cosmos and cretanweed and their germination stimulant activities were quite high (e.g., fraction 7 in Fig. 3b). Since cosmos and cretanweed exuded sorgomol and orobanchol, there are possibilities that they exude didehydro-sorgomol and didehydro-orobanchol, respectively.

Monohydroxy-SLs and their acetates

In root exudates from petunia, garland chrysanthemum, and sunflower, monohydroxy-SLs were below detection limits of LC–MS/MS. Monohydroxy-SLs are highly active in seed germination stimulation of *O. minor* (Kim et al. 2010). In addition, orobanchol exhibited a distinct hyphal branching activity in *Gigaspora margarita*, and strigol and sorgomol were moderately active branching factors (Akiyama et al. 2010). Structural requirements of SLs for hyphal branching in AM fungi and for germination stimulation in root parasites are similar but not identical (Akiyama et al. 2010). Although essential structural features for inhibition of shoot branching appear to be similar to those for AM fungi hyphal branching and parasite seed germination, there would be distinct differences in the SL structures optimal for each activity (Xie et al. 2010).

5-Deoxystrigol, the simplest SL without any substituents containing oxygen atoms in the AB ring moiety, is thought to be the common precursor of the other natural SLs. An allylic or homoallylic hydroxylation of 5-deoxystrigol leads to monohydroxy-SLs orobanchol and strigol, or sorgomol, respectively (Rani et al. 2008; Xie et al. 2010). These hydroxy-SLs are then acetylated and indeed

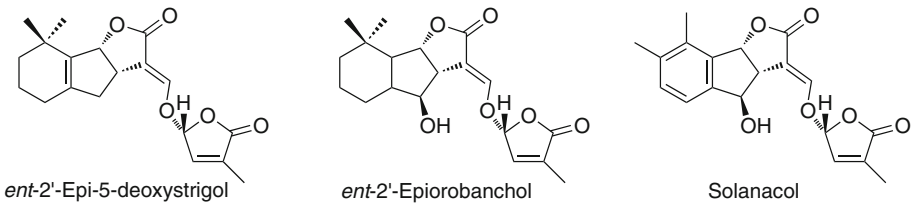

Fig. 4 Structures of *ent*-strigolactones

orobanchyl acetate and strigyl acetate have been identified from plant root exudates. Acetylation of sorgomol may occur, however, sorgomyl acetate has not been detected even in the root exudates of plants producing sorgomol such as cosmos. Sorgomol and orobanchyl acetate but not orobanchol were detected from cosmos root exudates. Similar results were obtained with white lupin and Chinese milk vetch as described in the previous paper (Yoneyama et al. 2008). These results suggest that orobanchyl acetate may be important as a pool conjugate of a highly active branching factor for AM fungi, orobanchol.

Biosynthesis of *ent*-strigolactones

Fabacol and fabacyl acetate are *ent*-SLs identified from pea root exudates (Xie et al. 2009a). So far, three other *ent*-SLs, i.e., *ent*-2′-epi-5-deoxystrigol, *ent*-2′-epiorobanchol, and solanacol, have been identified in plant root exudates or plant tissues (Fig. 4). In these *ent*-SLs, the configuration of the ring ABC is opposite that of other natural SLs. A rice cultivar (*Oryza sativa* cv. Nipponbare) has been shown to produce *ent*-2′-epi-5-deoxystrigol (Umehara et al. unpublished data). In addition, we recently isolated *ent*-2′-epi-5-deoxystrigol and *ent*-2′-epiorobanchol, the immediate precursor of fabacol and fabacyl acetate, from its root exudates, but we could detect neither fabacol nor fabacyl acetate (Xie et al. unpublished data). From a synthetic approach, solanacol was shown to be another *ent*-SL (Chen et al. 2010). These results suggest that plants produce both enantiomers of SLs and only those accumulated or produced at relatively larger amounts have been characterized so far. Therefore, it is preferable to confirm stereochemistry of SLs identified by LC–MS/MS, because they may be enantiomers of known SLs. For example, in the case of orobanchol, 3 of its 8 stereoisomers have been shown to be produced by plants; (+)-orobanchol, (+)-2′-epiorobanchol, and (–)-*ent*-2′-epiorobanchol. This may be done by using a chiral HPLC column for separation of enantiomers in LC–MS/MS analyses.

Conclusion

It is likely that all plants produce and exude SLs into rhizosphere, indicating that any organisms in the rhizosphere are continuously exposed to SLs and therefore SLs should have profound effects on the rhizosphere communications between plants and the other organisms. Furthermore, recent finding of SLs' effects on root system architecture imply that SLs are involved not only in belowground plant-root parasitic plant but also in plant–plant interactions (Kapulnik et al. 2010; Koltai et al. 2010; Ruyter-Spira et al. 2011). Therefore, it may be possible to optimize such belowground interactions by manipulating SL biosynthesis, metabolism, and exudation.

Acknowledgments We thank Emeritus Prof. Kenji Mori (The University of Tokyo, Japan), Prof. Yukihiro Sugimoto (Kobe University, Japan), and Assoc. Prof. Kohki Akiyama (Osaka Prefecture University, Japan) for the generous gifts of synthetic standards. Part of this work was supported by KAKENHI (18208010, 2109111) from the Japan Society for the Promotion of Science (JSPS) and by the Program for the Promotion of Basic and Applied Researches for Innovations in Biooriented Industry. Kaori Yoneyama and Xiaonan Xie were supported by JSPS Research Fellowship for Young Scientists and JSPS Post-Doctoral Fellowship for Foreign Researchers, respectively.

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