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2′-Epi-orobanchol and Solanacol, Two Unique Strigolactones, Germination Stimulants for Root Parasitic Weeds, Produced by Tobacco

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Germination stimulants for root holoparasitic weeds broomrapes (*Orobanche* and *Phelipanche* spp.) produced by tobacco (*Nicotiana tabacum* L.) were purified and characterized. The root exudates of tobacco contained at least five different stimulants, and LC-MS/MS analyses revealed that four of them were strigolactones; a tetradehydrostrigol isomer, a didehydrostrigol isomer, and two strigol isomers. The two isomers of strigol were identified as (+)-orobanchol and its 2′-epimer by comparison of NMR and GC- and LC-MS data with those of synthetic standards. The structure of the tetradehydrostrigol isomer, the major stimulant of the bright yellow tobacco cultivars, was determined as 4-α-hydroxy-5,8-dimethyl-GR24 [(*E*)-4-α-hydroxy-5,8-dimethyl-3-(4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy)methylene)-3a,4-dihydro-3*H*-indeno[1,2-*b*]furan-2(8b*H*)-one] and named solanacol. 2′-Epi-orobanchol and solanacol are the first natural strigolactones having a 2′-epi stereochemistry and a benzene ring, respectively.

KEYWORDS: Strigolactone; germination stimulant; parasitic weeds; tobacco; 2′-epi-orobanchol; orobanchol; solanacol

INTRODUCTION

Witchweeds (*Striga* spp.) and broomrapes (*Orobanche* and *Phelipanche* spp.) are root parasitic weeds causing enormous losses of agricultural production (*1*). The seeds of these parasites germinate when they perceive chemical signals released from their host and some nonhost plants (*2*). Three different classes of plant secondary metabolites, dihydrosorgoleone, sesquiterpene lactones, and strigolactones, are known to induce seed germination of these parasites (*3*). Among these germination stimulants, strigolactones appear to be of primary importance because >80% of land plants produce and release strigolactones as host recognition signals for arbuscular mycorrhizal (AM) fungi from which plants benefit (*4*, *5*).

To date, six strigolactones have been characterized from root exudates of different plant species; strigol and strigyl acetate from cotton (*6*, *7*), alectrol (orobanchyl acetate, see below) from cowpea (*8*), sorgolactone from sorghum (*9*), orobanchol from red clover (*10*), and 5-deoxystrigol from *Lotus japonicus* (*4*) and gramineous plants including sorghum, maize, and pearl millet (*11*). Recently, alectrol was identified as orobanchyl acetate (*12*). In addition to these known strigolactones, there are at least several novel strigolactones including sorgomol (formally named sorghumol) (*11*), an isomer of strigol found in sorghum root exudates (*13*).

Tobacco (*Nicotiana tabacum* L.) is a host of *Phelipanche ramosa* L. (*P. ramosa*), but germination stimulants produced by tobacco plants have not been elucidated. In the present paper, characterization of germination stimulants produced by four different cultivars of tobacco and structural elucidation of two strigolactones, 2′-epi-orobanchol and solanacol (**Figure 1**), are described along with qualitative and quantitative differences in strigolactone exudations among these tobacco cultivars.

MATERIALS AND METHODS

Chemicals. (+)-Strigol, (+)-orobanchol, and (+)-2′-epi-orobanchol were generously provided by Emeritus Prof. Kenji Mori (The University of Tokyo, Japan). GR24 was supplied by Prof. Binne Zwanenburg (Nijmegen University, The Netherlands). Other chemicals of analytical grade and HPLC solvents were obtained from Kanto Chemical Co. Ltd. (Tokyo, Japan) and Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Chromatographic Materials. Wakogel C-300 (Wako Pure Chemical Industries Ltd.) was used for silica gel column chromatography. The ODS columns (Mightysil RP-18, 10 × 250 mm, 10 μm; 4.6 × 250 mm, 5 μm; 2.1 × 250 mm, 5 μm; Kanto Chemical Co. Ltd.), an ODS-CN column (Develosil CN-UG-5, 4.6 × 250 mm, 5 μm; Nomura

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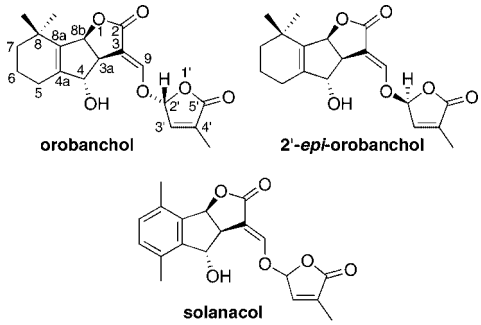

Figure 1. Chemical structures of strigolactones produced by tobacco plants.

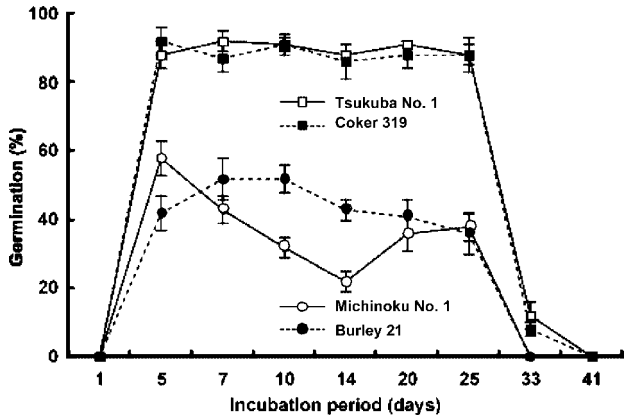

Figure 2. Time course of characteristics of germination stimulation activity of root exudates from four cultivars of tobacco plants grown hydroponically.

Chemical Co. Ltd., Seto, Japan), and an ODS-Phenyl column (Inertsil Ph, 2.1 × 250 mm, 5 μm; GL Science Inc., Tokyo, Japan) were used for preparative and analytical HPLC.

Instrumentation. HPLC analysis and purification were conducted on a Hitachi L-2130 low-pressure gradient system and a Hitachi L-7100 high-pressure gradient system equipped with a UV and PDA detector, respectively. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ (δ_H 7.26, δ_C 77.0) on a JEOL Lambda 400 spectrometer. (For those of solanacol, see the Supporting Information.) The standard pulse sequence and phase cycling were used for HMQC, HMBC, and NOE spectra. CD spectra were recorded with a JASCO J-720W spectropolarimeter in MeCN. EI/GC-MS spectra were obtained with a JEOL JMX-500 and a JOEL JMS-Q1000GC/K9 on a DB-5 (J&W Scientific, Agilent) capillary column (4 or 5 m × 0.25 mm) using a He carrier gas (3 mL min^{-1}). The operating conditions were the same as reported earlier (*10*). ESI-LC-MS analyses were performed using a Quattro LC tandem MS instrument from Micromass (Manchester, U.K.). HPLC separation and LC-MS/MS analytical conditions were essentially the same as in refs *14* and *15*.

Source of Seeds. Seeds of four cultivars of tobacco [*Nicotiana tabacum* L. cv. Burley 21 and Michinoku No. 1 (burley tobaccos), and Tsukuba No. 1 and Coker 319 (bright yellow tobaccos)] that are widely cultivated in Japan were generous gifts of Japan Tobacco Inc. *Orobanche minor* Sm. seeds were collected from mature plants that parasitized red clover (*Trifolium pratense* L.) in the Watarase basin of Tochigi Prefecture, Japan. *Phelipanche ramosa* L. (formally called *O. ramosa*) seeds collected from mature plants that parasitized tomato were kindly supplied by Prof. A. G. T. Babiker (ARC, Sudan).

Collection of Root Exudates from Tobacco. Tobacco seeds were surface-sterilized in 70% ethanol for 1 min and then in 1% sodium hypochlorite for 1 min. After thorough rinsing with sterile distilled water, they were sown in plastic containers (28.5 × 23.5 × 11 cm, W × L × H) filled with autoclaved vermiculite. The plants were grown in a growth chamber with a 16/8 h photoperiod at 250 μmol of photons m^{-2} s^{-1} at 27/23 °C. The plants were watered with tap water as required, and a 1000-fold diluted liquid fertilizer (Hyponex, Hyponex Japan, Osaka, Japan) was supplied weekly. Twenty-one days after sowing, 50 seedlings each were selected for uniformity and transferred to a hydroponic culture system. Hydroponic culture using plastic containers was conducted as reported in ref *16* except that tap water was used as growth medium. The growth media containing root exudates collected daily (800 mL) were extracted three times with equal volumes 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.

For isolation and purification of novel strigolactones, root exudates were collected by using activated charcoal cartridges installed in aquarium water pumps according to Akiyama et al. (*4*). In these experiments, two of the strainers carrying about 500 seedlings each growing in a 5 cm layer of rockwool were transferred to a larger container (53.5 × 33.5 × 14 cm, W × L × H) containing 12 L of tap water. The tap water medium was continuously circulated with a pump through a nylon mesh (pore size 48 μm) containing 2 g of charcoal (activated carbon for column chromatography, Wako Pure Chemical Industries Ltd.). The charcoal cartridges were exchanged every 3 days and the root exudates absorbed on charcoal were eluted with acetone. After evaporation of acetone in vacuo, the residue was dissolved in 50 mL of 0.2 M K_2HPO_4 (pH 8.3) and extracted three times with 50 mL of ethyl acetate. The ethyl acetate solutions were combined, dried over anhydrous $MgSO_4$, and concentrated in vacuo to afford root exudate samples.

Germination Assays. Germination assays using *O. minor* and *P. ramosa* seeds were conducted as reported previously (*16*, *17*).

Isolation and Identification of 2′-Epi-orobanchol and Orobanchol. In total about 5000 seedlings of tobacco (Michinoku No. 1) were grown hydroponically during 5 months and the root exudates collected as described in the previous section. The combined crude extract (785 mg) was subjected to silica gel (250 × 30 mm) column chromatography using a gradient of *n*-hexane/ethyl acetate (100:0–0:100, 10% step) as eluting solvent system to give fractions 1–11. Germination stimulation activities on *O. minor* seeds were detected in fractions 3 (20% ethyl acetate), 4 (30% ethyl acetate), and 7–11 (60–90% ethyl acetate). Fractions 7–11 were combined (149 mg) and chromatographed on a silica gel column (300 × 20 mm) using *n*-hexane/ethyl acetate (45:55) as a mobile phase. Fractions were collected every 5 mL. Two active fractions designated TM-1 (fractions 37–46) and TM-2 (fractions 61–73) were found to contain an isomer of orobanchol and orobanchol, respectively, by LC-MS/MS analysis. TM-1 (48 mg) was purified with a 250 × 10 mm Mightysil RP-18 (ODS) semipreparative HPLC column eluted with MeCN/H_2O (40–100% MeCN over 40 min) at a flow rate of 3 mL min^{-1}. The strong germination stimulant activities were eluted between 10 and 15 min with a distinct UV-absorbing peak at 12.9 min (detection at 246 nm). The active fractions were combined and further purified on an HPLC using a 250 × 4.6 mm Mightysil RP-18 column eluted isocratically with 40% MeCN in water at a flow rate of 1 mL min^{-1} to afford 0.35 mg of (+)-2′-epi-orobanchol. (+)-Orobanchol (0.15 mg) was obtained from TM-2 in a similar manner. The identities of these compounds were confirmed with LC-MS/MS and GC-MS analyses using the synthetic standards. The ^{1}H and ^{13}C NMR spectroscopic data for these compounds were identical to those reported in the literature (*18*). The CD spectrum of (+)-2′-epi-orobanchol showed a typical pattern for 2′-epi-strigolactones as reported by Welzel et al. (*19*).

Isolation, Purification, and Structure Determination of Solanacol. The crude extract (398 mg) collected from in total about 3000 seedlings of tobacco (Coker 319) grown hydroponically was fractionated by silica gel column chromatography (250 × 20 mm) using a gradient of *n*-hexane/ethyl acetate (100:0–0:100, 10% step) to give fractions 1–11. The active fractions 7–11 (60%–90% ethyl acetate) were combined (121 mg) and subjected to silica gel column chromatography (300 × 20 mm) eluted with 60% ethyl acetate in *n*-hexane. Fractions were collected every 5 mL. Weak and strong activities were detected in fractions 41–48 (TC-1) and 54–73 (TC-2), respectively. TC-1 and TC-2 were found to contain 2′-epi-

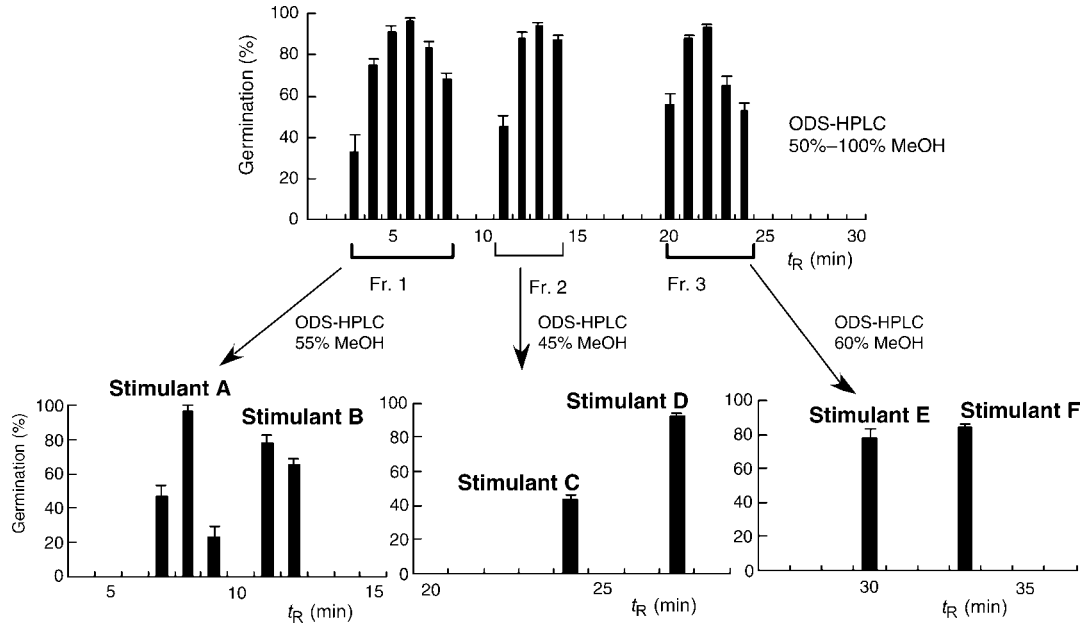

Figure 3. Separation of stimulants A–F in the root exudates from tobacco Coker 319 by ODS-HPLC.

orobanchol and, a tetradehydro-strigol isomer, a didehydro-strigol isomer, and orobanchol, respectively, by LC/MS/MS analyses. TC-2 (53 mg) was purified with a 250 × 10 mm Mightysil RP-18 HPLC column eluted with MeCN/H_2O (40–100% MeCN in 40 min) at a flow rate of 3 mL min^{-1}. The fractions eluted between 5 and 18 min with strong germination stimulant activities were combined (9.2 mg), and purified again on HPLC using the same column eluted isocratically with 30% MeCN in water at a flow rate of 3 mL min^{-1}. The tetradehydro-strigol isomer and didehydro-strigol isomer were eluted between 34 and 41 min, and orobanchol at 43–48 min. The former active fraction (2.4 mg) was further separated with a 250 × 4.6 mm ODS-CN HPLC column eluted isocratically with 30% MeCN in water at a flow rate of 1 mL min^{-1}. The tetradehydro-strigol isomer (t_R = 15.35 min) was finally purified on the same ODS-CN HPLC column with 25% MeCN in water as eluting solvent to afford pure compound and named solanacol (0.7 mg, t_R = 25.22 min).

(*E*)-4-α-Hydroxy-5,8-dimethyl-3-(4-methyl-5-oxo-2,5-dihydrofuran-2-yloxymethylene)-3a,4-dihydro-3*H*-indeno[1,2-*b*]furan-2(8b*H*)-one, solanacol: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.05 (t, 3H, J = 1.5 Hz, 4′-CH_3), 2.30 (s, 3H, 8-CH_3), 2.37 (s, 3H, 5-CH_3), 3.81 (ddd, 1H, J = 7.3, 3.4 and 2.0 Hz, 3a-*H*), 5.25 (s, 1H, 4-*H*), 6.15 (d, 1H, J = 7.3 Hz, 8b-*H*), 6.22 (t, 1H, J = 1.5 Hz, 2′-*H*), 6.99 (t, 1H, J = 1.5 Hz, 3′-*H*), 7.16 and 7.23 (AB quartet, 2H, J = 7.8 Hz, 5-*H*, 6-*H*), 7.55 (d, 1H, J = 2.4 Hz, 9-*H*).

Purification of the didehydrostrigol isomer (t_R = 16.89 min, 30% MeCN) did not afford enough pure sample for structural elucidation.

RESULTS AND DISCUSSION

Characterization of Germination Stimulants in Tobacco Root Exudates by ODS-HPLC Separation/Bioassays and by LC-MS/MS. The root exudates collected from the four tobacco cultivars that had been grown hydroponically with tap water for 25 days still elicited moderate to high germination of *O. minor* seeds as shown in **Figure 2**. In this figure, germination stimulation activities of the root exudate samples collected on the designated days were plotted. In general, at this dilution (20-fold dilution; 1 mL of assay solutions contained root exudate samples corresponding to 0.05 mL of the growth medium), the root exudates from the burley tobaccos (Michinoku No. 1 and Burley 21) showed slightly weaker activities as compared to those from the bright yellow tobaccos (Tsukuba No. 1 and Coker 319). When the plants were grown hydroponically with tap water for more than 30 days, they turned yellow, and the germination stimulation activity of the root exudate samples decreased significantly.

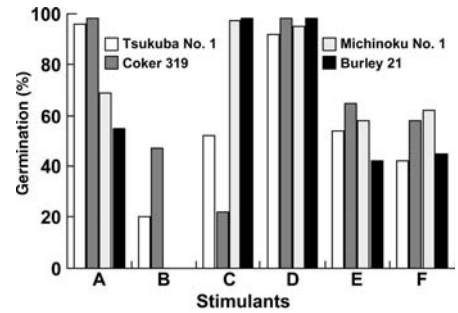

Figure 4. Relative activities of stimulants A–F in the root exudates collected from 50 seedlings on the 25th day of hydroponic culture.

Then, characterization of strigolactones in the root exudates from the tobacco plants was conducted by comparing retention times of germination stimulants on ODS-HPLC with those of synthetic standards and by using LC-MS/MS. For this, a part of crude extract was subjected to an ODS-HPLC eluted with MeOH/H_2O (50–100% MeOH over 40 min) at a flow rate of 1 mL min^{-1} and the fractions collected every minute were examined for their germination stimulation activity on *O. minor* seeds. Under the HPLC conditions, retention times (t_R) for synthetic standards of 2′-epi-orobanchol, orobanchol, strigol, strigyl acetate, and sorgolactone were 12.3, 12.7, 14.7, 17.1, and 20.8 min, respectively. For example, the extract from Coker 319 afforded three active fractions (fractions 1–3), and then fractions 1–3 were further purified with ODS-HPLC eluted with 55, 45, and 60% MeOH, respectively, to give six different stimulants A–F (**Figure 3**). The root exudate from Tsukuba No. 1 was found to contain the same six stimulants. By contrast, the root exudates from the burley tobaccos (Burley 21 and Michinoku No. 1) appeared to lack stimulant B and therefore contained five stimulants.

Figure 4 compares germination stimulation activity of stimulants A–F in the root exudates collected on the 25th day from the 50 seedlings grown hydroponically and separated by ODS-HPLC. The average shoot and root lengths of the seedling on the 25th day were about 10 and 6 cm, respectively. In the

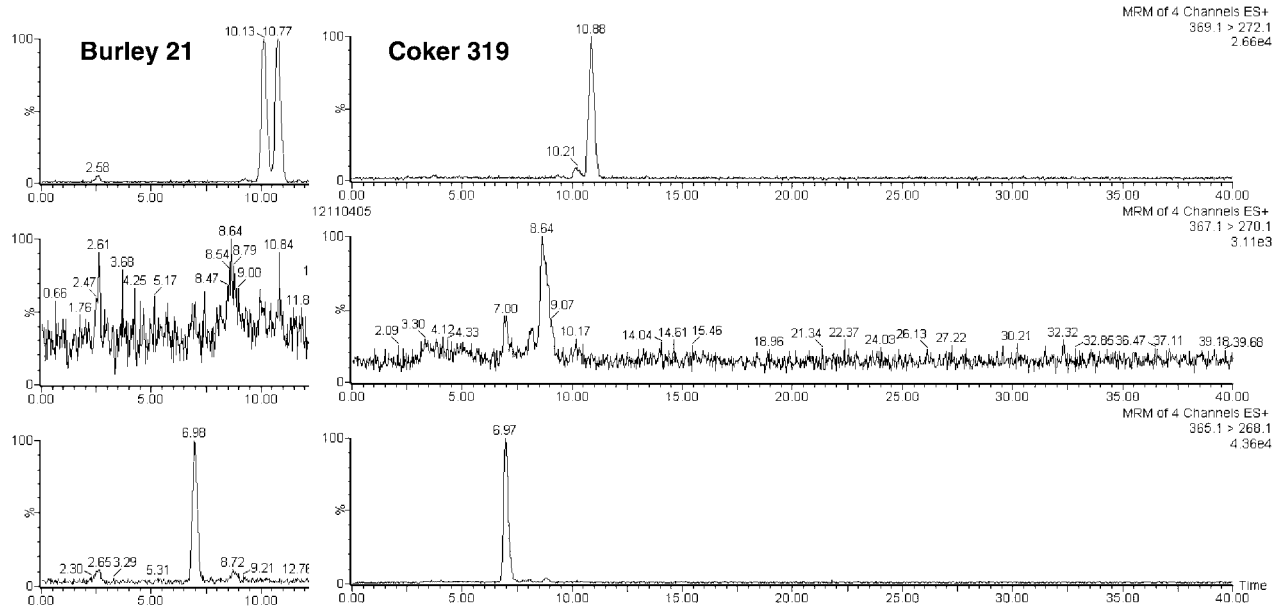

Figure 5. MRM chromatograms of Burley 21 and Coker 319 tobacco root exudates. The upper, middle, and lower channels are for monitoring the transitions of *m*/*z* 369 > 272, 367 > 270, and 365 > 268, for the detection of strigol isomers, didehydrostrigol isomers, and tetradehydrostrigol isomers, respectively.

root exudates from the bright yellow tobaccos, stimulants A and D were two major ones. By contrast, in the case of burley tobaccos, stimulants C and D were major ones, and activity of stimulant B was not detected at this dilution. Similar ratios of activities of individual stimulants were obtained for the root extract samples collected on the 30th day. These ratios may not be related to the actual amounts of individual stimulants, and there may be possible losses during HPLC purifications. Although stimulants E and F were separable by HPLC, purification of these compounds was not successful because of their scarcity.

In the multiple reaction monitoring (MRM) chromatograms of Coker 319 and Burley 21 tobacco root exudates, where the transitions of the sodium adduct ions of strigolactones $[M + Na]^+$ to the product ions formed by the neutral loss of the D ring moiety $[M - 97]^+$ were used for the detection, two peaks in the upper channel for strigol isomers (*m*/*z* 369 > 272), one peak in the mid channel for didehydrostrigol isomers (*m*/*z* 367 > 270), and one peak in the lower channel for tetradehydrostrigol isomers (*m*/*z* 365 > 268) were observed as shown in **Figure 5**. No peaks were detected in the channels for sorgolactone isomers (*m*/*z* 339 > 242) or 5-deoxystrigol isomers (*m*/*z* 353 > 256). Cochromatography using synthetic standards on ODS and ODS-Phenyl columns suggested that the two isomers of strigol were 2′-epi-orobanchol and orobanchol.

By comparison of t_R with those of synthetic standards and the data of LC-MS/MS analyses, stimulants A, B, C, and D were suggested to be tetradehydrostrigol isomer, didehydrostrigol isomer, 2′-epi-orobanchol, and orobanchol, respectively. To confirm these tentative assignments, larger amounts of root exudates were collected to obtain pure stimulants for structural determination. Because burley tobaccos were found to exude relatively large amounts of stimulant C, probably 2′-epi-orobanchol, Burley 21 tobacco was used to collect root exudates for the purification of stimulants C and D. For the purification of stimulants A and B, Coker 319 was used, because it was expected to exude both stimulants at relatively large amounts.

Identification and Structural Determination of Strigolactones. Stimulants C and D, the two isomers of strigol, were purified from the root exudates of Burley 21 tobacco plants and identified as (+)-2′-epi-orobanchol and (+)-orobanchol, respectively, by comparison of their NMR and GC- and LC-MS data with those of synthetic standards. This is the first report on the isolation of natural 2′-epi-strigolactones. Although the 2′-stereochemistry has been reported to be important for germination stimulation activity (*20*, *21*), 2′-epi-orobanchol was slightly more active than orobanchol on *O. minor* and *P. ramosa* seed germination as shown in **Table 1**.

Table 1. Germination Stimulation Activity of Strigolactones Identified in Tobacco Root Exudates, (+)-Strigol, and GR24 [a]

		% germination ± SE	
strigolactone	concn (M)	*O. minor*	*P. ramosa*
(+)-orobanchol	10^{-9}	93 ± 2.0	84 ± 1.4
	10^{-10}	90 ± 1.2	76 ± 2.1
	10^{-11}	54 ± 4.6	32 ± 3.4
(+)-2′-epi-orobanchol	10^{-9}	96 ± 1.5	82 ± 1.9
	10^{-10}	94 ± 2.5	81 ± 2.5
	10^{-11}	86 ± 2.1	58 ± 4.4
solanacol	10^{-9}	90 ± 3.5	82 ± 2.5
	10^{-10}	96 ± 1.4	83 ± 2.1
	10^{-11}	14 ± 5.0	14 ± 3.5
(+)-strigol	10^{-9}	90 ± 2.5	79 ± 2.6
	10^{-10}	92 ± 1.9	80 ± 3.2
	10^{-11}	18 ± 5.1	12 ± 2.1
GR24	10^{-6}	97 ± 1.2	83 ± 2.0
	10^{-7}	55 ± 4.8	24 ± 5.2
	10^{-8}	9 ± 3.2	0
distilled water		0	0

[a] Data presented are the mean ± SE of one representative experiment.

Stimulants A and B, tetradehydro- and didehydrostrigol isomers, were purified from the root exudates of Coker 319. ^{1}H NMR analyses of stimulant A revealed that stimulant A contained the common structural feature for the known strigolactones, the C/D ring moiety. The two methyl singlet signals at 2.30 and 2.37 ppm and the AB quartet signals at 7.16 and 7.23 ppm (2H, J = 7.8 Hz) showed that there was a 1,4-dimethylbenzene fragment in the molecule. The presence of a benzene ring was also supported with the downfield shifts of H-4 and H-8b (benzylic position) by ca. 0.6 ppm as

compared to those of orobanchol. In particular, these data were in good agreement with those for 8-methyl-GR24 (*22*). The singlet signal of H-4 indicated the presence of an α-hydroxyl group at this position. The 2D-NMR data supported these assignments. Consequently, stimulant A was determined as 4-α-hydroxy-5,8-dimethyl-GR24 [(*E*)-4-α-hydroxy-5,8-dimethyl-3-(4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy)methylene-3a,4-dihydro-3*H*-indeno[1,2-*b*]furan-2(8b*H*)-one] and named solanacol. This is the first natural strigolactone containing a benzene ring. The C-2′ configuration of solanacol could not be estimated from its CD spectra; the sign of the CD changed from negative to positive around 270 nm (*19*). Unfortunately, the amount of pure stimulant B was not enough for structural determination.

As already suggested by Wigchert and Zwanenburg for 8- and 6-methyl-GR24 (*22*), the introduction of a methyl group on the A ring does not affect germination stimulation activity. Therefore, higher activity of 4-α-hydroxy-5,8-dimethyl-GR24, solanacol, as compared to GR24, may be attributable to the positive effect of the hydroxyl group on the B ring (**Table 1**). Of course, such an effect may be different in different bioassays.

In summary, the bright yellow and the burley tobacco cultivars produce at least six and five different germination stimulants, respectively, including four strigolactones. These are solanacol, didehydrostrigol isomer, 2′-epi-orobanchol, and orobanchol. In particular, solanacol and 2′-epi-orobanchol are the first natural strigolactones having a benzene ring and the 2′-epi stereochemistry, respectively.

As shown in **Figure 2**, the bright yellow tobaccos (Tsukuba No. 1 and Coker 319) seemed to exude larger amounts germination stimulants than the burley tobaccos (Michinoku No. 1 and Burley 21). Then, parts of the root exudate samples used for the germination assays shown in **Figure 4** were analyzed, without any purifications, by LC-MS/MS for quantification of orobanchol and 2′-epi-orobanchol. The bright yellow tobacco cultivars, Tsukuba No. 1 and Coker 319, were found to exude ca. 570 and 390 pg of orobanchol plant^{-1} day^{-1}, respectively, whereas exudations by the burley tobaccos, Michinoku No. 1 and Burley 21, were 230 and 240 pg of orobanchol plant^{-1} day^{-1}. By contrast, the exudation of 2′-epi-orobanchol by the bright yellow tobaccos was only $^{1}/_{25}$ that of the burley tobaccos that exuded ca. 350 pg of 2′-epi-orobanchol plant^{-1} day^{-1}. Solanacol production was quantified for the root exudate samples from Coker 319 and Burley 21, being 580 and 120 pg of solanacol plant^{-1} day^{-1}, respectively. Therefore, the total amounts of strigolactones in the root exudates may be very similar among these tobacco cultivars. Although the growth stages and sizes of the plants were different, tobacco plants appeared to produce relatively large amounts of strigolactones as compared to red clover (∼10 pg of orobanchol plant^{-1} day^{-1}) (*14*) and cotton (∼30 pg of strigol plant^{-1} day^{-1}) (*15*) when grown hydroponically with tap water.

Further study is needed to clarify why tobacco plants produce various strigolactones and how each strigolactone contributes to the host recognition by root parasites and by AM fungi. Synthetic study to clarify the stereochemistry of solanacol is in progress.

ACKNOWLEDGMENT

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Supporting Information Available: ^{1}H NMR and CD spectra of solanacol. This material is available free of charge via the Internet at http://pubs.acs.org.

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