JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Susceptibility of the Tomato Mutant *High Pigment-2^{dg}* (*hp-2^{dg}*) to *Orobanche* spp. Infection

JUAN ANTONIO LÓPEZ-RÁEZ,[†,‡] TATSIANA CHARNIKHOVA,[†,‡] PATRICK MULDER,[§] WOUTER KOHLEN,[†,‡] RAOUL BINO,[‡] ILAN LEVIN,[||] AND HARRO BOUWMEESTER*[,†,‡]

Plant Research International, P.O. Box 16, 6700 AA Wageningen, The Netherlands, Laboratory for Plant Physiology, Wageningen University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands, RIKILT, Institute of Food Safety, Bornsesteeg 45, 6708 PD Wageningen, The Netherlands, and Institute of Plant Sciences, The Volcani Center, P.O. Box 6, 50250 Bet Dagan, Israel

The consumption of natural products with potential health benefits has been continuously growing, and enhanced pigmentation is of major economic importance in fruits and vegetables. The tomato *hp-2^{dg}* is an important mutant line that has been introgressed into commercial tomato cultivars marketed as lycopene rich tomatoes (LRT) because of their enhanced fruit pigmentation, attributed to higher levels of carotenoids, including lycopene. Strigolactones are signaling compounds that mediate host finding in root parasitic plants and are biosynthetically derived from carotenoids. Considering the high carotenoid content of the *hp-2^{dg}* mutant, we studied its susceptibility to the root parasite *Orobanche*. In a field experiment, the average number of *Orobanche aegyptiaca* plants growing on *hp-2^{dg}* was surprisingly significantly reduced compared with its isogenic wild-type counterpart. *In vitro* assays and LC-MS/MS analysis showed that this reduction was associated with a lower production of strigolactones, which apparently renders the high-carotenoid *hp-2^{dg}* mutant less susceptible to *Orobanche*.

KEYWORDS: ***hp-2^{dg}* mutant; tomato; strigolactones; carotenoids; *Orobanche***

INTRODUCTION

Fruits constitute a major component of our diet, providing fiber, vitamins, minerals, pigments, and additional phytonutrients thought to promote or at least maintain good health. The consumption of natural products with potential health benefits has been continuously growing, and enhanced pigmentation is of major economic importance in fruits and vegetables (*1, 2*). Fleshy fruits such as tomatoes (*Solanum lycopersicum*) contain high levels of several of these compounds and hence are a good source of vitamins, carotenoids, and antioxidants (*2, 3*). The tomato *high pigment* (*hp*) lines include a number of tomato mutants with single-point mutations with increased levels of such phytonutrients (*4*). Because of their impact on fruit lycopene content, *hp* mutations have been introgressed into commercial tomato cultivars marketed as lycopene rich tomatoes (LRT) (*5, 6*).

The tomato mutant *hp-2^{dg}* (*high pigment-2^{dg}*), formerly named *dark green*, is mutated in the *DEETIOLATED1* (*DET1*) gene. *DET1* encodes a regulatory protein that represses several signaling pathways controlled by light (*5, 7*). As a consequence, *hp-2^{dg}* plants display higher fruit pigmentation due to an elevated level of chlorophylls in immature fruits and of carotenoids, including lycopene, throughout fruit ripening from very early breaker to over-ripe stages (*5, 7, 8*). In addition to increased carotenoids, fruits of *hp-2^{dg}* mutants are also characterized by increased levels of other functional metabolites such as vitamins C and E, as well as several flavonoids (*4, 7*).

Tomato is an important fruit crop in Southern Europe, the Americas, the Middle East, and India, with increasing production in China, Japan, and Southeast Asia (*9*). However, the production of tomatoes is highly susceptible to infection by *Orobanche ramosa* and *Orobanche aegyptiaca* that cause severe yield losses of up to 75% (*10–13*). The parasitic plant *Orobanche* spp. (broomrapes; Orobanchaceae) is a holoparasite and parasitize important agricultural crops around the globe, such as legumes, crucifers, sunflower, hemp, tobacco, and tomato, causing large crop losses (*14–16*). These obligate root parasites attach to the roots of many plant species and acquire nutrients and water from their host through an organ called haustorium. The interaction between host and parasite begins with the secretion of secondary metabolites from the roots of the host that induce the germination of seeds of the parasite, which are called strigolactones (**Figure 1**) (*17, 18*). Strigolactones have also been identified as the compounds responsible for the induction of hyphal branching

* Corresponding author. Tel: +31317480528. Fax: +31317418094. E-mail: harro.bouwmeester@wur.nl.
[†] Plant Research International.
[‡] Wageningen University.
[§] Institute of Food Safety.
[||] The Volcani Center.

10.1021/jf800760x CCC: $40.75
Published on Web 07/09/2008

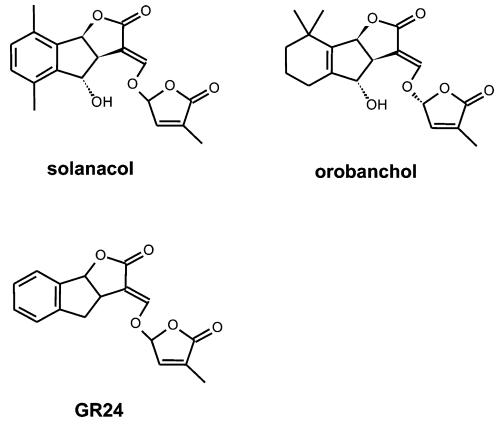

Figure 1. Structures of some strigolactone germination stimulants and the synthetic strigolactone analogue GR24.

in arbuscular mycorrhizal (AM) fungi, which is a critical step in host recognition (*19–21*). Using both mutants and isoprenoid pathway inhibitors, we have demonstrated that strigolactones are carotenoid derived through the action of a carotenoid cleavage enzyme (*22, 23*).

In the present article, the susceptibility of the commercially important tomato line *hp-2^{dg}*, with enhanced production of carotenoids and other functional metabolites in fruits, to *Orobanche* infection is studied. Moreover, the link between this susceptibility and the production/exudation of strigolactones is analyzed.

MATERIALS AND METHODS

Plant Material and Chemicals. Seeds of tomato (*Solanum lycopersicum*) of the open-pollinated cultivar Manapal, homozygous for the *hp-2^{dg}* mutation (LA2451) and its fully isogenic wild-type counterpart (wild-type) (LA3007), were originally provided by R.T. Chetelat (Tomato Genetics Cooperative, UC Davis, USA). Seeds of the open-pollinated tomato cv. Moneymaker were purchased at a local market. *Orobanche 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 synthetic germination stimulant strigolactone analogue GR24 was kindly provided by Binne Zwanenburg (Department of Organic Chemistry, Radboud University, Nijmegen, The Netherlands). The strigolactone standard sorgolactone was obtained from Soizic Rochang (Laboratory for Cell Surfaces and Signaling in Plants, University-CNRS, France), strigol from Yukihiro Sugimoto (Faculty of Agriculture, Kobe University, Japan), 5-deoxystrigol from Kohki Akiyama (Osaka Prefecture University, Japan), and orobanchol, solanacol, and orobanchyl acetate were kindly provided by Koichi Yoneyama (Weed Science Center, Utsunomiya University, Japan).

Field Experiment. Tomato seeds of the *hp-2^{dg}* mutant and corresponding wild-type were sown in seedling trays on April 12, 2007 and grown in a greenhouse until May 11, 2007 when they were transplanted to the field. The field chosen was known to be highly infected with *O. aegyptiaca* and is located in Mevo Hama, Southern Golan Heights, Northern Israel.

Tomato plants were planted in a randomized block design (6 blocks), each block containing 1 double row plot for each genotype. The final experimental layout was slightly imbalanced, mainly due to plant survival, with more mutant *hp-2^{dg}* than wild-type plants in the corresponding plots of each block. The final number of surviving *hp-2^{dg}* mutant plants was 37, 39, 40, 40, 43, and 40, while the final number of the corresponding wild-type plants was 36, 37, 36, 35, 37, and 34 in blocks 1, 2, 3, 4, 5, and 6, respectively.

Greenhouse Experiment and Root Exudate Collection. Tomato seeds of the *hp-2^{dg}* mutant, the wild-type (cv. Manapal), and the cultivar Moneymaker 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 in 0.5 L pots with sand/vermiculate (1:1) for 4 weeks in a greenhouse at 21/18 °C with 16/8 h photoperiod and 70% humidity. For each tomato genotype, 6 pots containing 3−4 plants per pot were used. Plants were watered twice a week with a modified, half-strength Hoaglands nutrient solution (*23*). Root exudates were collected from each pot individually (see below) and used for the bioassay and analysis by LC-MS/MS.

Phosphate (Pi) starvation promotes the production of the germination stimulant strigolactones (*23, 24*). Therefore, one week before root exudate collection, plants were watered (twice a week) with modified half-strength Hoaglands nutrient solution without Pi. For root exudate collection, the substrate in the pots was rinsed with 1.5 L (3 times the pot volume) of modified half-strength Hoaglands solution without Pi to remove the strigolactones accumulated. After another 5 h, 0.3 L of modified half-strength Hoaglands solution was applied to the pots and the root exudates collected. Roots from each pot were then collected separately and frozen in liquid nitrogen and stored at −80 °C until use.

Root Exudate Purification and Germination Bioassay. The crude exudates were concentrated and purified by solid phase extraction on a C_{18} SEPAK cartridge (Octadecyl 500 mg, J.T. Baker, Deventer, The Netherlands). Hereto, 0.3 L of the exudate solution was loaded onto the pre-equilibrated column. Subsequently, the column was washed with 5 mL of 30% acetone/water, and the active fraction was eluted with 5 mL of 60% acetone/water (*23*). Within each experiment, the exudates were diluted to the same ratio of root fresh weight per mL of root exudate before analysis.

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 bioassays with *O. ramosa* seeds were conducted as reported before (*23*). To avoid saturation of the germination response, we used a series of dilutions of the purified exudate in each germination bioassay.

Carotenoid Analysis. Carotenoid extraction and analysis on tomato roots were performed as described before (*7*) with minor modifications using high performance liquid chromatography (HPLC) with photo diode array (PDA) detection. Frozen powdered roots (2 g) were extracted with 4.5 mL of methanol/chloroform (5:4, v/v) containing 0.1% butylated hydroxytoluene (2,6-di-*t*-butyl-4-methylphenol, BHT). Samples were shaken and incubated on ice for 10 min. Then, 2.5 mL of 1 M NaCl in Tris-HCl buffer at pH 7.4 was added, and extracts were incubated for another 10 min on ice and then centrifuged for 10 min at 1500 rpm at room temperature. The samples were re-extracted with 1 mL of chloroform containing 0.1% BHT. The chloroform fractions were combined and dried under N_2. The residue was taken up in 200 μL of ethyl acetate containing 0.1% BHT. HPLC analysis was performed as described by Bino et al. (*7*).

Strigolactone Analysis Using Liquid Chromatography-Tandem Mass Spectrometry. Analysis of strigolactones in tomato root exudates was performed by comparing retention times and mass transitions with those of available 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 López-Ráez et al. with a number of modifications (*23*). Analyses were performed using a Waters Micromass Quattro Premier XE tandem mass spectrometer (Waters, Milford, MA, USA) equipped with an ESI source and coupled to an Acquity UPLC system (Waters, USA). Chromatographic separation was achieved using an Acquity UPLC BEH C_{18} column (150 − 2.1 mm, 1.7 μm) (Waters, USA), applying a water/acetonitrile gradient, starting at 0% acetonitrile for 0.5 min, raised to 25% acetonitrile in 0.5 min, followed by a 6.5 min gradient to 45% acetonitrile, followed by a 4.5 min gradient to 70% acetonitrile which was then maintained for 0.1 min and followed by a 0.1 min gradient back to 0% acetonitrile before the next run. The column was then equilibrated at this solvent composition for 2.8 min. Total run time was 15 min. The column was operated at 50 °C with a flow-rate of 0.4 mL min^{-1}, and the sample injection volume was 20 μL. The mass spectrometer was operated in positive electrospray ionization (ESI) mode. The nebulizer and desol-

Table 1. Carotenoid Content of the Roots of the tomato *hp-2^{dg}* Mutant and corresponding Wild-Type (WT) (cv. Manapal), and the Cultivar Moneymaker (MM)[a]

	μg g^{-1} fresh weight root			
	neoxanthin	violaxanthin	lutein	β-carotene
hp-2^{dg}	3.74 ± 0.41 a	0.86 ± 0.13 a	0.30 ± 0.04 a	0.14 ± 0.01 a
WT	2.59 ± 0.24 b	0.57 ± 0.05 b	0.23 ± 0.02 b	0.08 ± 0.01 b
MM	2.17 ± 0.64 b	0.48 ± 0.13 b	0.15 ± 0.06 c	0.08 ± 0.02 b

[a] Carotenoids were analyzed using HPLC (see Materials and Methods). Numbers represent the average of 6 independent replicates ± SD. Different letters indicate statistically significant differences between the means ($P < 0.01$) for each compound.

vation gas flows were 50 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 3.6×10^{-3} mbar. Multiple reaction monitoring (MRM) was used to search for strigolactones. The MRM transitions were set according to the MS/MS spectra obtained for the standards. Protonated molecular ions $[M + H]^+$ were more abundant in the full-scan mass spectra obtained from the standard strigolactones than the sodium ion adducts $[M + Na]^+$; therefore, they were selected as parent ion for the transitions. Two or 3 parent-daughter transitions were selected for each strigolactone, according to the most abundant and/or specific fragment ions for which the collision energy (CE) was optimized. For solanacol, the MRM transitions *m/z* 343 > 183 at a CE of 14 eV, and 343 > 97 at 18 eV were selected; for orobanchol, the transitions *m/z* 347 > 233 at 10 eV, 347 > 205 at 15 eV and 347 > 97 at 18 eV; and for the didehydro-orobanchol isomers the transitions *m/z* 345 > 203 at 16 eV, 345 > 175 at 18 eV and 345 > 97 at 18 eV were selected.

Data acquisition and analysis were performed using MassLynx 4.1 software (Waters, USA). For each compound, the summed area of all the corresponding MRM transitions was used for statistical analysis.

Statistical Analysis. Data for the carotenoid content of tomato roots and for the strigolactone content in tomato root exudates were subjected to one-way analysis of variance (ANOVA) using GenStat for Windows (ninth edition). For the analysis of the germination bioassays, ANOVA after arcsine[squareroot(x)] transformation was performed. For the field experiment, statistical analyses were carried out with the JMP Statistical Discovery software (SAS Institute, Cary, NC, USA).

RESULTS

Carotenoid Content in Roots. The tomato mutant *high pigment-2^{dg}* (*hp-2^{dg}*) is characterized by its higher carotenoid content in fruits (*7*). The carotenoid content in the roots of this mutant has not been reported before. In the present work, we have analyzed the levels of carotenoids in the roots of this mutant and corresponding wild-type, and this shows that the carotenoids neoxanthin (*hp-2^{dg}*/wt ratio 1.44), violaxanthin (*hp-2^{dg}*/wt ratio 1.51), lutein (*hp-2^{dg}*/wt ratio 1.30), and β-carotene (*hp-2^{dg}*/wt ratio 1.75) are also significantly ($P < 0.01$) higher in the roots of the *hp-2^{dg}* mutant than in the wild-type (**Table 1**). As expected, the concentrations of most carotenoids observed in the roots were much lower than the values reported for fruits (*7*), but there were differences for the different compounds analyzed. Violaxanthin (1.5-fold), lutein (13-fold), and β-carotene (80-fold) were lower in roots compared to red fruit, but neoxanthin was about 20-fold higher in the roots (*7*). For the first time, we have now shown that the elevated levels of carotenoids in the *hp-2^{dg}* mutant extent also to the roots.

In addition to *hp-2^{dg}* and wild-type Manapal, the carotenoid content in the roots of Moneymaker was measured. In this cultivar, the carotenoid levels were comparable to those observed for the wild-type Manapal (**Table 1**).

***Orobanche aegyptiaca* Infection in the Field.** To check the susceptibility of the tomato mutant *hp-2^{dg}* to *Orobanche aegyptiaca*, a field experiment was performed as described in Materials and Methods. After sowing, the field was visited 5 times throughout the season. The first *O. aegyptiaca* appeared between June 6, 2007 and June 24, 2007, i.e., 26−44 days after planting. *O. aegyptiaca* plants were counted for 4 consecutive weeks at 1- to 2-wk intervals until July 15, 2007 (65 days after planting) when *O. aegyptiaca* plants started to senesce.

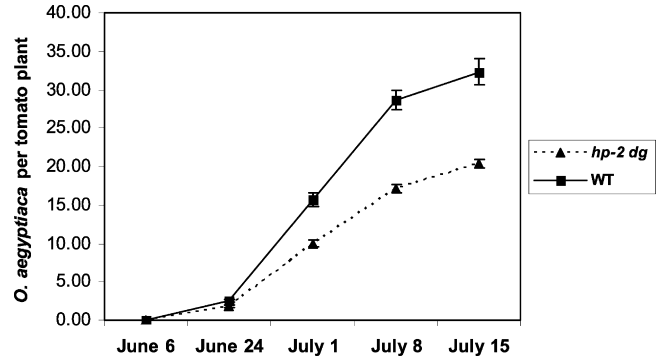

Figure 2. Time course for *O. aegyptiaca* plants growing in a tomato crop. Number of *O. aegyptiaca* plants growing on individual tomato plants for the *hp-2^{dg}* mutant and corresponding wild-type (WT). Numbers represent the average of 6 independent replicates ± SE.

On average, the number of *O. aegyptiaca* plants counted in plots of the *hp-2^{dg}* mutant was lower than in plots of the wild-type plants in the corresponding blocks. On June 24, the average number of *O. aegyptiaca* plants counted in the mutant plots was 19% lower than in the wild-type plots, but statistically not significant [$P(\chi^2) > 0.05$, $P(F) > 0.05$)]. The average number of *O. aegyptiaca* plants continued to increase for both genotypes, but the difference between them also increased and became statistically significant from July 1 onward. The average number of *O. aegyptiaca* plants counted in the mutant plots was 30%, 34%, and 30% lower than in plots containing the wild-type on July 1, 8, and 15, respectively [$P(\chi^2) < 0.05$, $P(F) < 0.05$)].

The final experimental layout was slightly imbalanced with more mutant *hp-2^{dg}* than wild-type plants in the corresponding plots of each block. Therefore, the results better distinguish between the genotypes when analyzed as the average number of *O. aegyptiaca* plants per tomato plant, showing again lower average numbers for the mutant (**Figure 2**). The average number of *O. aegyptiaca* plants per tomato plant in the mutant plots was about 28%, 36%, 41%, and 37% lower than in plots containing wild-type plants on June 24 and July 1, 8, and 15, respectively (**Figure 2**). These differences were statistically significant on the latter 3 dates [$P(\chi^2) < 0.05$, $P(F) < 0.05$)].

***O. ramosa* Germination Assay.** To assess whether the results observed in the field are due to differences in the induction of germination, tomato *hp-2^{dg}* mutant and corresponding wild-type plants were grown in the greenhouse, and their root exudates were collected for a germination bioassay with *O. ramosa* seeds. The synthetic germination stimulant GR24 (10^{-9} M) (**Figure 1**), used as a positive control, always induced the germination of preconditioned *O. ramosa* seeds (up to about 80%). Water alone, used as a negative control, did not induce any germination. The *hp-2^{dg}* mutant displayed the shorter and darker phenotype characteristic for this mutant (*25*) (data not shown). No differences in germination of *O. ramosa* seeds induced by the purified exudates were observed after 100-fold dilution. However, when diluted 500- and 1000-fold, the germination activity of the *hp-2^{dg}* mutant was about 40 and 38%, respectively, lower than that for the corresponding wild-type (**Figure 3**). In addition to the activity of the root exudates of the two

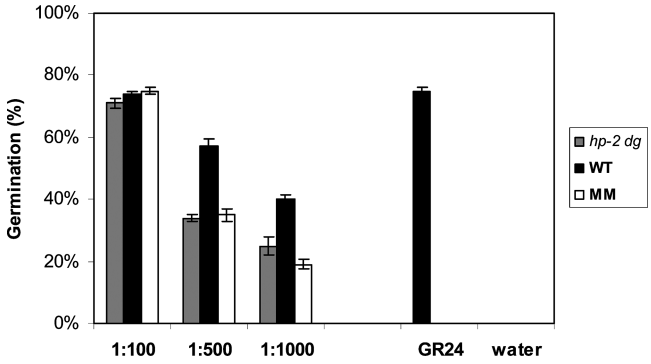

Figure 3. Germination of *O. ramosa* seeds induced by the root exudates of the tomato *hp-2^{dg}* mutant compared with the corresponding wild-type (WT) (cv. Manapal) and the cultivar Moneymaker (MM). Serial dilutions of the root exudates (1:100, 1:500 and 1:1000) were used. GR24 (10^{-9} M) and demineralized 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 the volume of exudate to root fresh weight. Numbers represent the average of 6 independent replicates ± SE.

genotypes of Manapal, the exudate of Moneymaker was also tested for its capacity to induce germination of *O. ramosa* seeds. To our surprise, the germination stimulatory activity of Moneymaker was similar to that of the *hp-2^{dg}* mutant, being 39 and 53%, respectively, lower than wild-type Manapal (**Figure 3**).

LC-MS/MS Analysis of Strigolactones in Tomato Root Exudates. To further investigate whether the lower germination stimulatory activity of the tomato *hp-2^{dg}* mutant was due to a decrease in the strigolactone production, LC-MS/MS analysis was performed to compare the levels of strigolactones in the root exudates of the mutant and corresponding wild-type. The SPE purified exudates were analyzed by LC-MS/MS in positive ESI mode. An acetonitrile/water gradient was applied to improve separation of the two major didehydro-orobanchol isomers, which are not well resolved using methanol/water (*23*). Interestingly, acetonitrile as organic modifier, affected the ratio between protonated molecular ions and the corresponding sodium adducts, the former being much more prominent with acetonitrile. Protonated molecular ions of strigolactones fragment differently than sodium adducts. Loss of the lactone ring (M-97) is by far the most prominent fragmentation route for sodium adducts of strigolactones, while other fragments are formed with rather low abundance. In contrast, protonated molecular ions produce a larger number of abundant fragment ions (**Figure 4**). These fragments are quite specific for individual strigolactones, a feature which can be used for identification. For each compound, the collision energy was optimized for two or three major parent to daughter transitions, which were incorporated into a MRM method. Using the LC-MS/MS in MRM mode, the tomato exudates were then screened for the presence of strigolactones. In the LC-MS/MS chromatograms, three single intense peaks were detected at R_t 6.50, 7.27, and 7.40 min in the different channels (**Figure 5A**). Comparison of the R_t and corresponding mass transitions with those of standards suggested that the three peaks are solanacol (**Figure 1**) (transtitions *m/z* 343 > 183, 343 > 97) and two didehydro-orobanchol isomers (transitions *m/z* 345 > 203, 345 > 185 and 345 > 97), respectively (**Figure 5A**). In addition, a small peak eluting at R_t 8.13 corresponding to the strigolactone orobanchol (**Figure 1**) was detected (transitions *m/z* 347 > 233, 347 > 205 and 347 > 97). However, its concentration was too low for quantification. The presence of solanacol and orobanchol in root exudates of tomato plants was further confirmed by standard addition experiments.

All the three major strigolactones detected were significantly ($P < 0.01$) reduced in the *hp-2^{dg}* mutant root exudates compared to the wild-type (**Figures 5B** and **6**). The amount of solanacol and the two didehydro-orobanchol isomers (according to the peak area) were 63, 58, and 57%, respectively, lower in the mutant ($P < 0.01$) than that in the wild-type. The levels of strigolactones in the exudates from the cultivar Moneymaker were comparable to those of the *hp-2^{dg}* mutant (**Figures 5B** and **6**). The levels of solanacol and the two didehydro-orobanchol isomers were 57, 52, and 54%, respectively, lower than those in the wild-type Manapal. Standard addition of solanacol and orobanchol to the samples confirmed that the observed reduction in peak areas in the *hp-2^{dg}* mutant and Moneymaker exudates is real and not caused by ion suppression during ionization in the ion source of the mass spectrometer.

DISCUSSION

Among the different *Orobanche* species, *O. ramosa* and the very similar *O. aegyptiaca* have the widest host range and cause enormous damage to numerous important agricultural crops such as tomato, potato, tobacco, and sunflower in different parts of the world, especially in Mediterranean countries (*14, 15, 26*). We have previously demonstrated that the germination stimulants (viz. strigolactones) of maize, cowpea, and sorghum are derived from carotenoids probably through the action of a carotenoid cleavage enzyme (*22*). Recently, we have shown that this is also true for the germination stimulants of tomato, indicating that the carotenoid origin of the strigolactones is a general phenomenon in the plant kingdom (*23*).

The tomato *hp-2^{dg}* is an important mutation that has been introgressed into commercial tomato cultivars marketed as lycopene rich tomatoes (LRT) because it imparts significantly higher levels of carotenoids, including lycopene, and other functional metabolites such as vitamins C and E, and several flavonoids in fruits (*5–7*). Because germination stimulants (strigolactones) are derived from carotenoids (*22, 23*), and considering the increasing problems with *Orobanche* in tomato cultivation, we assumed that *hp-2^{dg}* mutant plants could potentially display higher root carotenoid levels, leading to higher levels of strigolactones and hence to higher susceptibility to *Orobanche*. In accordance with this expectation, we showed for the first time that the carotenoid content in the roots of tomato plants carrying the *hp-2^{dg}* mutation is also higher than that in the corresponding wild-type (**Table 1**). The carotenoid levels in roots of the mutant were about 1.5-fold higher than that in the roots of the wild-type. In fruits, this difference was larger at about 4-fold (*7*). In addition, we also showed that the concentrations of the carotenoids violaxanthin, lutein, and β-carotene are lower in roots than in fruits, whereas neoxanthin is higher in roots.

Surprisingly, in a field experiment the *hp-2^{dg}* mutant showed to be significantly less susceptible to *O. aegyptiaca* infection than the corresponding wild-type (36%, 41% and 37% lower on July 1, 8, and 15, respectively) (**Figure 2**). An *in vitro* germination bioassay with *O. ramosa* seeds showed a reduction in the germination stimulatory activity of the *hp-2^{dg}* mutant root exudates that was very similar to the decrease in susceptibility to *O. aegyptiaca* observed in the field (about 40%) (**Figures 2** and **3**). In addition, these results were further confirmed by LC-MS/MS. We have previously shown by LC-MS/MS analysis that in the root exudates of tomato cultivar Moneymaker the major strigolactones are solanacol, orobanchol, and at least two

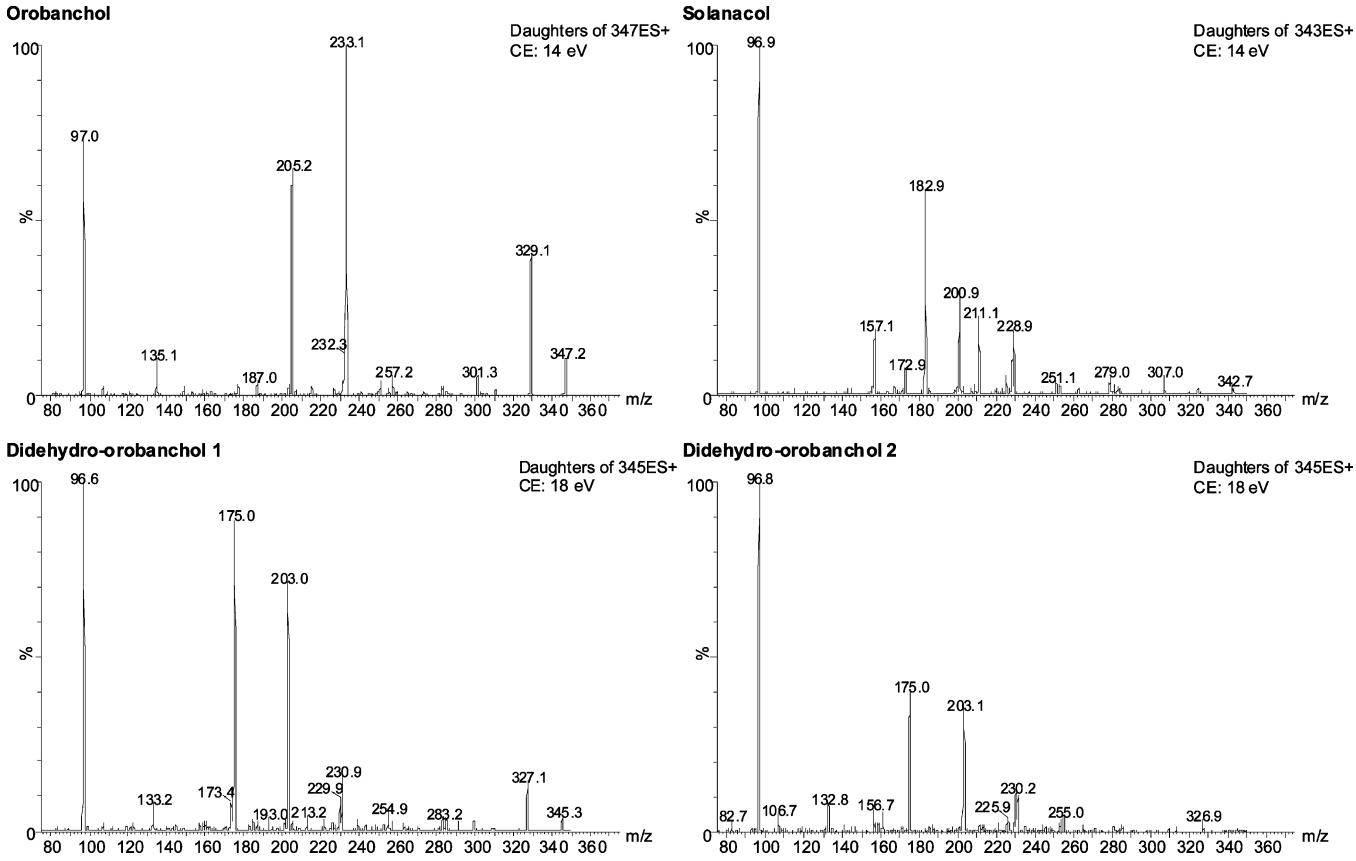

Figure 4. MS/MS fragmentation spectra of the protonated molecular ions of orobanchol, solanacol, and the two didehydro-orobanchol isomers. The MS/MS spectra of the didehydro-orobanchol isomers and solanacol were recorded during online separation of a tomato *hp-2^{dg}* exudate, and that of orobanchol was recorded by direct infusion of a standard solution.

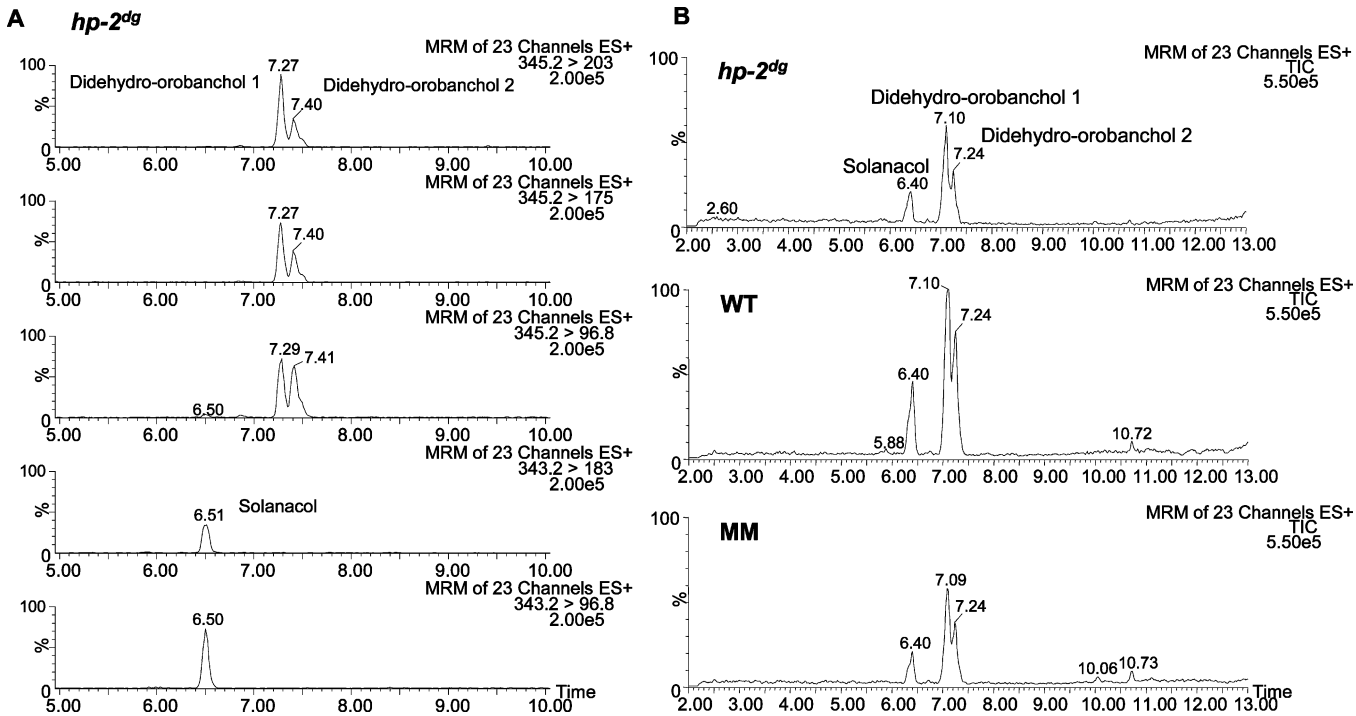

Figure 5. LC-MS/MS analysis using multiple reaction monitoring (MRM) of the SEPAK C_{18} purified tomato root exudates. The MRM transitions for didehydro-orobanchol isomers (3 transitions) and solanacol (2 transitions) obtained for the *hp-2^{dg}* mutant exudate are shown as example **A**. (**B**) Comparison of the root exudates of the *hp-2^{dg}* mutant plants and corresponding wild-type (WT) (cv. Manapal) and the cultivar Moneymaker (MM); peak areas represent the total of all transitions. Six independent samples for each plant genotype were pooled before analysis.

didehydro-orobanchol isomers (*23*). In the root exudates of the *hp-2^{dg}* mutant, the amounts (according to the peak area) of solanacol and the two didehydro-orobanchol isomers were 63, 58, and 57%, respectively, lower in the mutant than those in the wild-type (**Figures 5B** and **6**). This suggests a direct relationship between the amount of strigolactones produced by

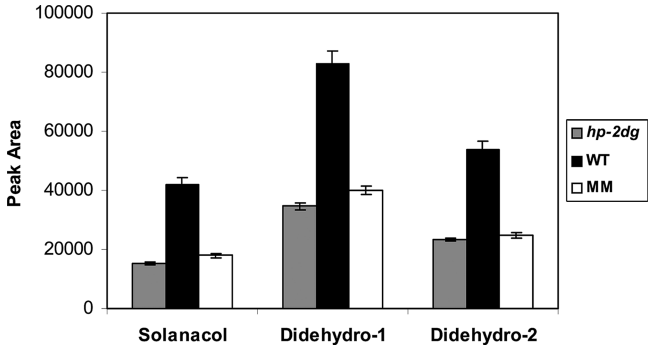

Figure 6. Strigolactone content in tomato root exudates. The amount (according to the peak area) of the strigolactone solanacol and the didehydro-orobanchol isomers 1 and 2 (didehydro-1 and didehydro-2) in the root exudates of the *hp-2^{dg}* mutant plants and corresponding wild-type (WT) (cv. Manapal), and the cultivar Moneymaker (MM) was quantified. The SEPAK C_{18} purified exudates were analyzed using LC-MS/MS (see Materials and Methods). Numbers represent the average of 6 independent replicates ± SE.

the host plant, the germination stimulatory activity for *Orobanche* spp. seeds, and the infection of *Orobanche* spp. in the field. As far as we know, this is the first time that a parasitic plant infection experiment in the field is correlated with *in vitro* germination analysis and confirmed by LC-MS/MS characterization and quantification of the responsible compounds for such activity.

The fact that the root exudates of *hp-2^{dg}*, which has a higher carotenoid content, contain significantly less strigolactones than the corresponding wild-type genotype seems to be contradictory. However, there are other examples for this mutant where the increase in carotenoids is not related with an increase in carotenoid derived compounds. The carotenoid breakdown products β-ionone and β-damascenone were not increased in fruits harvested from the mutant despite their significantly higher carotenoid levels (*7*). Moreover, in a number of plant species an increase in carotenoid content in the roots upon mycorrhizal colonization is not accompanied by an increase in strigolactone production. The strigolactones also act as hyphal branching factors for AM fungi (*19–21*), and it has been demonstrated that after AM colonisation carotenoid biosynthesis is activated in mycorrhizal roots (*27, 28*). Nevertheless, a clear reduction in the parasitic plant *Striga hermonthica* infection after AM fungal inoculation in maize and sorghum has been described (*29*) and evidence given that this is caused by a reduction in the production of strigolactones (*30*). Considering the reported role of strigolactones in mycorrhizal colonization (*19, 20*), it would be interesting to investigate whether *hp-2^{dg}* plants also exhibit less AM colonization and, if this is the case, whether this has consequences for crop performance of LRT-type tomatoes under low soil fertility conditions. Whatever the effects of the *hp-2^{dg}* mutation on mycorrhizal colonization, this phenomenon could have important implications for *Orobanche* management.

In addition to increased carotenoids, in fruits harvested from *hp-2^{dg}* (a tomato *det1* mutant) other targeted and nontargeted compounds have been demonstrated to be enhanced, showing that this mutant is more active metabolically (*7*). These results are in agreement with recent transcriptional profiling experiments, carried out in *Arabidopsis* seedlings and in tomato fruits, showing that *det1* mutants are in general more transcriptionally active (*8, 31*). This is not unexpected since DET1 is considered a negative regulator of gene expression. In contrast, many other genes displayed reduced constitutive and/or developmental expression in *det1* mutant *Arabidopsis* seedlings and tomato fruits (*8, 31*). Therefore, DET1 may also act as a positive regulator of genes. Possibly this also holds for genes directly involved in strigolactone biosynthesis or exudation.

In addition, we also report here that the germination stimulatory capacity for *O. ramosa* seeds of Moneymaker root exudates was comparable to that observed for the *hp-2^{dg}* mutant and hence lower than the activity for the wild-type Manapal (**Figure 3**). LC-MS/MS analysis showed that this reduced activity also correlated with a decrease in the level of strigolactones present in the root exudates of Moneymaker (**Figures 5B** and **6**). The results indicate genetic variation for the production of strigolactones among different tomato cultivars. Strigolactone production will most likely not be the only factor determining *Orobanche* resistance in tomato as other resistance mechanisms could play a role such as haustorium formation and compatibility (*32, 33*). Genetic variation for the induction of *O. aegyptiaca* germination has already been described for tomatoes (*34*), but a correlation with strigolactone concentration was not investigated by these authors. Genetic variation in the induction of germination of parasitic plants has also been shown for other crops such as sorghum, where several cultivars with low *Striga* germination stimulant (LGS) production have been described (but also without investigating strigolactone production) (*35*). For sorghum, this genetic variation was used to breed *Striga* resistant varieties (*35*), and our results indicate that it may be possible to use similar approaches in tomato in order to reduce *Orobanche* susceptibility.

Our study shows that an agronomically important tomato mutant as *hp-2^{dg}*, introgressed into commercial tomato cultivars marketed as lycopene rich tomatoes (LRT), is less susceptible to *Orobanche* infection. Moreover, we demonstrate that this reduced susceptibility is a consequence of a reduction in the production and/or exudation of the strigolactones despite the fact that carotenoid levels in the roots of the *hp-2^{dg}* mutant are higher. This suggests that the conversion of carotenoids to strigolactones or their exudation is tightly regulated. Further research is required to understand how this occurs.

ACKNOWLEDGMENT

We thank Maurizio Vurro and Angela Boari for supplying *O. ramosa* seeds, Binne Zwanenburg for his kind help in supplying GR24, Kohki Akiyama, Soizic Rochang, Yukihiro Sugimoto for some strigolactone standards, and Koichi Yoneyama for his advice and the strigolactone standards orobanchol, solanacol, and orobanchyl acetate. We also wish to thank Uri Luchinsky and Shaol Graph for their assistance with the field trial.

LITERATURE CITED

(1) Schieber, A.; Stintzing, F. C.; Carle, R. By-products of plant food processing as a source of functional compounds - recent developments. *Trends Food Sci. Technol.* **2001**, *12* (11), 401–413.

(2) Willcox, J. K.; Catignani, G. L.; Lazarus, S. Tomatoes and cardiovascular health. *Crit. Rev. Food Sci. Nutr.* **2003**, *43* (1), 1–18.

(3) Willits, M. G.; Kramer, C. M.; Prata, R. T. N.; De Luca, V.; Potter, B. G.; Steffens, J. C.; Graser, G. Utilization of the genetic resources of wild species to create a non-transgenic high flavonoid tomato. *J. Agric. Food Chem.* **2005**, *53* (4), 1231–1236.

(4) Levin, I.; de Vos, C. H. R.; Tadmor, Y.; Bovy, A.; Lieberman, M.; Oren-Shamir, M.; Segev, O.; Kolotilin, I.; Keller, M.; Ovadia, R.; Meir, A.; Bino, R. J. *High pigment* tomato mutants - more than just lycopene (a review). *Israel J. Plant Sci.* **2006**, *54* (3), 179–190.

(5) Levin, I.; Frankel, P.; Gilboa, N.; Tanny, S.; Lalazar, A. The.tomato dark green mutation is a novel allele of the tomato homolog of the *DEETIOLATED1* gene. *Theor. Appl. Genet.* **2003**, *106* (3), 454–460.
(6) Wann, E. V. Tomato germplasm lines T4065, T4099, T5019, and T5020 with unique genotypes that enhance fruit quality. *Hortscience* **1997**, *32* (4), 747–748.
(7) Bino, R. J.; de Vos, C. H. R.; Lieberman, M.; Hall, R. D.; Bovy, A.; Jonker, H. H.; Tikunov, Y.; Lommen, A.; Moco, S.; Levin, I. The light-hyperresponsive *high pigment-2(dg)* mutation of tomato: alterations in the fruit metabolome. *New Phytologist* **2005**, *166* (2), 427–438.
(8) Kolotilin, I.; Koltai, H.; Tadmor, Y.; Bar.Or, C.; Reuveni, M.; Meir, A.; Nahon, S.; Shlomo, H.; Chen, L.; Levin, I. Transcriptional profiling of *high pigment-2(dg)* tomato mutant links early fruit plastid biogenesis with its overproduction of phytonutrients. *Plant Physiology* **2007**, *145* (2), 389–401.
(9) Van Eck, J.; Kirk, D. D.; Walmsley, A. M. Tomato (*Lycopersicum esculentum*). *Methods Mol. Biol.* **2006**, *343*, 459–479.
(10) Delavault, P.; Simier, P.; Thoiron, S.; Veronesi, C.; Fer, A.; Thalouarn, P. Isolation of mannose 6-phosphate reductase cDNA, changes in enzyme activity and mannitol content in broomrape (*Orobanche ramosa*) parasitic on tomato roots. *Physiologia Plantarum* **2002**, *115* (1), 48–55.
(11) Mauromicale, G.; Lo Monaco, A.; Longo, A. M. G.; Restuccia, A. Soil solarization, a nonchemical method to control branched broomrape (*Orobanche ramosa*) and improve the yield of greenhouse tomato. *Weed Sci.* **2005**, *53* (6), 877–883.
(12) Radi, A.; Dina, P.; Guy, A. Expression of *sarcotoxin IA* gene via a root-specific *tob* promoter enhanced host resistance against parasitic weeds in tomato plants. *Plant Cell Rep.* **2006**, *25* (4), 297–303.
(13) Delavault, P.; Thalouarn, P. The obligate root parasite *Orobanche cumana* exhibits several rbcL sequences. *Gene* **2002**, *297* (1−2), 85–92.
(14) Joel, D. M. The long-term approach to parasitic weeds control: manipulation of specific developmental mechanisms of the parasite. *Crop Prot.* **2000**, *19* (8−10), 753–758.
(15) Press, M. C.; Scholes, J. D.; Riches, C. R. In *Current Status and Future Prospects for Management of Parasitic Weeds (Striga and Orobanche), the World'S Worst Weeds*; British Crop Protection Council: Farnham, UK, 2001; pp 71−90.
(16) Shen, H.; Ye, W.; Hong, L.; Huang, H.; Wang, Z.; Deng, X.; Yang, Q.; Xu, Z. Progress in parasitic plant biology: Host selection and nutrient transfer. *Plant Biol.* **2006**, *8* (2), 175–185.
(17) Bouwmeester, H. J.; Matusova, R.; Zhongkui, S.; Beale, M. H. Secondary metabolite signalling in host-parasitic plant interactions. *Curr. Opin. Plant Biol.* **2003**, *6* (4), 358–364.
(18) Humphrey, A. J.; Beale, M. H. Strigol: Biogenesis and physiological activity. *Phytochemistry* **2006**, *67* (7), 636–640.
(19) Akiyama, K.; Matsuzaki, K.; Hayashi, H. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* **2005**, *435* (7043), 824–827.
(20) Besserer, A.; Puech-Pages, V.; Kiefer, P.; Gómez-Roldán, V.; Jauneau, A.; Roy, S.; Portais, J. C.; Roux, C.; Bécard, G.; Sejalon-Delmas, N. Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. *PloS Biol.* **2006**, *4* (7), 1239–1247.
(21) Bouwmeester, H. J.; Roux, C.; López-Ráez, J. A.; Bécard, G. Rhizosphere communication of plants, parasitic plants and AM fungi. *Trends Plant Sci.* **2007**, *12* (5), 224–230.
(22) Matusova, R.; Rani, K.; Verstappen, F. W. A.; Franssen, M. C. R.; Beale, M. H.; Bouwmeester, H. J. The strigolactone germination stimulants of the plant-parasitic *Striga* and *Orobanche* spp are derived from the carotenoid pathway. *Plant Physiology* **2005**, *139*, 920–934.
(23) López-Ráez, J. A.; Charnikhova, T.; Gomez-Roldan, V.; Matusova, R.; Kohlen, W.; de Vos, C. H. R.; Verstappen, F.; Puech-Pages, V.; Bécard, G.; Mulder, P.; Bouwmeester, H. Tomato strigolactones are derived from carotenoids and their biosynthesis is promoted by phosphate starvation. *New Phytologist* **2008**, *178* (4), 863–874.
(24) Yoneyama, K.; Yoneyama, K.; Takeuchi, Y.; Sekimoto, H. Phosphorus deficiency in red clover promotes exudation of orobanchol, the signal for mycorrhizal symbionts and germination stimulant for root parasites. *Planta* **2007**, *225* (4), 1031–1038.
(25) Davuluri, G. R.; van Tuinen, A.; Fraser, P. D.; Manfredonia, A.; Newman, R.; Burgess, D.; Brummell, D. A.; King, S. R.; Palys, J.; Uhlig, J.; Bramley, P. M.; Pennings, H. M. J.; Bowler, C. Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes. *Nat. Biotechnol.* **2005**, *23* (7), 890–895.
(26) Parker, C.; Riches, C. R. , *Parasitic Weeds of the World: Biology and Control*; CAB International: Wallingford, UK, 1993; pp 111−163.
(27) Fester, T.; Wray, V.; Nimtz, M.; Strack, D. Is stimulation of carotenoid biosynthesis in arbuscular mycorrhizal roots a general phenomenon. *Phytochemistry* **2005**, *66* (15), 1781–1786.
(28) Strack, D.; Fester, T. Isoprenoid metabolism and plastid reorganization in arbuscular mycorrhizal roots. *New Phytologist* **2006**, *172* (1), 22–34.
(29) Lendzemo, V. W.; Kuyper, T. W.; Kropff, M. J.; van.Ast, A. Field inoculation with arbuscular mycorrhizal fungi reduces *Striga hermonthica* performance on cereal crops and has the potential to contribute to integrated *Striga* management. *Field Crops Res.* **2005**, *91* (1), 51–61.
(30) Lendzemo, V. W.; Kuyper, T. W.; Matusova, R.; Bouwmeester, H. J.; van Ast, A. Colonization by arbuscular mycorrhizal fungi of sorghum leads to reduced germination and subsequent attachment and emergence of *Striga hermonthica*. *Plant Signalling Behav.* **2007**, *2* (1), 58–62.
(31) Schroeder, D. F.; Gahrtz, M.; Maxwell, B. B.; Cook, R. K.; Kan, J. M.; Alonso, J. M.; Ecker, J. R.; Chory, J. *De-etiolated 1* and damaged DNA binding protein 1 interact to regulate Arabidopsis photomorphogenesis. *Curr. Biol.* **2002**, *12* (17), 1462–1472.
(32) Rispail, N.; Dita, M. A.; González-Verdejo, C; Pérez de Luque, A.; Castillejo, M. A.; Prats, E.; Román, B.; Jorrín, J.; Rubiales, D. Plant resistance to parasitic plants: molecular approaches to an old foe. *New Phytologist* **2007**, *173* (4), 703–712.
(33) Gurney, A. L.; Slate, J.; Press, M. C.; Scholes, J. D. A novel form of resistance in rice to the angiosperm parasite *Striga hermonthica*. *New Phytologist* **2006**, *169* (1), 199–208.
(34) El Halmouch, Y.; Benharrat, H.; Thalouarn, P. Effect of root exudates from different tomato genotypes on broomrape (*O. aegyptiaca*) seed germination and tubercle development. *Crop Prot.* **2006**, *25* (5), 501–507.
(35) Ejeta, G. Breeding for *Striga* resistance in *Sorghum*: exploitation of an intricate host-parasite biology. *Crop Sci.* **2007**, *47* (S216−S227), 216–227.

Received for review March 12, 2008. Revised manuscript received May 26, 2008. Accepted May 29, 2008. We acknowledge funding by the European Commission (Intra-European Marie Curie postdoctoral fellowship to J.A.L.; FP6-MEIF-CT-2005-024345) and The Netherlands Organisation for Scientific Research (NWO; VICI-grant to H.B., T.C., and W.K.).

JF800760X