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RESEARCH PAPER

A tomato strigolactone-impaired mutant displays aberrant shoot morphology and plant interactions

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Abstract

Strigolactones are considered a new group of plant hormones. Their role as modulators of plant growth and signalling molecules for plant interactions first became evident in Arabidopsis, pea, and rice mutants that were flawed in strigolactone production, release, or perception. The first evidence in tomato (*Solanum lycopersicon*) of strigolactone deficiency is presented here. *Sl-ORT1*, previously identified as resistant to the parasitic plant *Orobanche*, had lower levels of arbuscular mycorrhizal fungus (*Glomus intraradices*) colonization, possibly as a result of its reduced ability to induce mycorrhizal hyphal branching. Biochemical analysis of mutant root extracts suggested that it produces only minute amounts of two of the tomato strigolactones: solanacol and didehydro-orobanchol. Accordingly, the transcription level of a key enzyme (CCD7) putatively involved in strigolactone synthesis in tomato was reduced in *Sl-ORT1* compared with the wild type (WT). *Sl-ORT1* shoots exhibited increased lateral shoot branching, whereas exogenous application of the synthetic strigolactone GR24 to the mutant restored the WT phenotype by reducing the number of lateral branches. Reduced lateral shoot branching was also evident in grafted plants which included a WT interstock, which was grafted between the mutant rootstock and the scion. In roots of these grafted plants, the *CCD7* transcription level was not significantly induced, nor was mycorrhizal sensitivity restored. Hence, WT-interstock grafting, which restores mutant shoot morphology to WT, does not restore mutant root properties to WT. Characterization of the first tomato strigolactone-deficient mutant supports the putative general role of strigolactones as messengers of suppression of lateral shoot branching in a diversity of plant species.

Key words: CCD7, CCD8, grafted plants, mycorrhiza, shoot branching, strigolactones, tomato.

Introduction

Today, strigolactones—or their biosynthetic metabolites—are considered to be a new group of plant hormones. They are suggested to play a pivotal role in the regulation of above-ground plant architecture (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008).

Strigolactones are suggested to be synthesized mainly in the lower parts of the stem and in roots (reviewed by Dun *et al.*, 2009), but their biosynthetic pathway in plants is not fully understood. Strigolactones are suggested to be derived from the carotenoid pathway (synthesized in the plastids; Naik *et al.*, 2003; Matusova *et al.*, 2005), and to involve novel branching of the carotenoid cleavage pathway, via the activity of different oxygenases (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008; Floss and Walter, 2009; reviewed by Dun *et al.*, 2009); the involvement of additional, unknown enzymes in the synthesis of this complex molecule has been suggested.

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Strigolactones have been shown to have a role belowground as well, in plant interactions with the parasitic plants *Orobanche* and *Striga*; strigolactones have been suggested as important signalling factors for *Orobanche* and *Striga* seed germination (Joel *et al.*, 1995, 2007; Yokota *et al.*, 1998; Goldwasser *et al.*, 2008; Matusova *et al.*, 2005; Bouwmeester *et al.*, 2007; Xie *et al.*, 2007, 2008). Strigolactones have also been suggested to be inducers of hyphal branching and of mitochondrial metabolism and mitotic activity of arbuscular mycorrhizal fungi (AMF; Akiyama *et al.*, 2005; Akiyama and Hayashi, 2006; Besserer *et al.*, 2006, 2008; Gomez-Roldan *et al.*, 2008; Yoneyama *et al.*, 2008); AMF are soil micro-organisms that establish mutual symbioses with higher plants and promote plant growth under suboptimal growth conditions (reviewed by Koltai *et al.*, 2009).

The role of strigolactones, both as modulators of plant growth and as signalling molecules for plant interactions, became evident from studies of *Arabidopsis*, pea, and rice mutants, flawed in strigolactone production or perception (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). Additional studies have suggested two key hypotheses regarding the role that strigolactones may play in determining shoot architecture: either strigolactones serve as auxin-promoted secondary messengers that move up into buds to repress their outgrowth (Brewer *et al.*, 2009; Ferguson and Beveridge, 2009; reviewed by Dun *et al.*, 2009), or they act primarily by reducing the capability of polar auxin transport from the apical meristem, thereby inhibiting polar auxin transport from buds, resulting in restrained bud outgrowth (Bennett *et al.*, 2006; Mouchel and Leyser, 2007; Ongaro and Leyser, 2008; Leyser, 2009).

In the present research, a tomato (*Solanum lycopersicon*) strigolactone-deficient mutant, *Sl-ORT1*, previously demonstrated to be resistant to the parasitic plant *Orobanche* was characterized; this is the first reported strigolactone-deficient mutant in tomato. The mutant's interaction with AMF, its shoot morphology, and its reversion to WT phenotype via exogenous addition of a synthetic strigolactone, GR24, to roots are characterized here. WT interstock grafting also led to a reduction in shoot branching; however, it did not lead to changes in the grafted plant roots, in either transcription of the key enzymes of strigolactone biosynthesis or AMF sensitivity. Hence, WT interstock grafting, which restores mutant shoot morphology to WT, does not restore mutant root properties to WT; rather, it may act on the shoot to suppress lateral branching.

Materials and methods

Plant mutant and WT strains

Tomato (*Solanum lycopersicon)* cv. M82 seeds (WT; Eshed *et al.*, 1992) were obtained from Tarsis Agricultural Chemicals Ltd. (Petah Tikva, Israel). The mutant *Sl-ORT1* was induced by irradiation with fast-neutron irradiation mutagenesis and selected for *Orobanche* resistance, as described in Dor *et al.* (2010). *Sl-ORT1* was self-propagated for another five generations for homogeneity.

Arbuscular mycorrhizal strain and assays for mycorrhizal susceptibility

All mycorrhizal studies were conducted with the AMF *Glomus intraradices* (LPA 8). The initial screening studies were performed in the greenhouse. *G. intraradices* was used as a sand-based inoculum. The different types of inoculum, prepared as described previously by David-Schwartz *et al.* (2001), were: 'spores only' (200 spores per plant), and 'whole inoculum' (30 propagules per plant, including both spores and colonized roots); grafted plants were inoculated with 'spores only'.

Experiments were carried out in a randomized block design with six replicates for each treatment, and in each replicate 10 plants were examined. Infectivity potential of these inocula was determined using the most probable number (MPN) method (Haas and Krikun, 1985). Means and standard error were determined for all replicates; means of replicates were subjected to statistical analysis by multiple-range test ($P \leq 0.05$), using the JMP statistical package (SAS, Cary, NC).

Roots from all experiments were stained with trypan blue solution (Phillips and Hayman, 1970) and mycorrhization was enumerated using the gridline intersection method (Giovannetti and Mosse, 1980).

Determining the effects of root exudates on AMF hyphal branching

Roots of WT and *Sl-ORT1* plants grown in pots were gently removed and transferred to sterilized Erlenmeyer flasks (500 ml capacity). The culture solution containing root exudates was decanted and filtered through a polycarbonate filter to remove sloughed root debris. The hyphal branching assay was performed as described previously by Nagahashi and Douds (2000) and Gadkar *et al.* (2003) using *G. intraradices* (DAOM 181602) as the test AMF. Hyphal branching was determined following 7 d of incubation with plant extract or controls (GR24—Johnson *et al.*, 1981—as a positive control and sterile distilled water as a negative control). Two experiments were conducted, with 20 replicates each; in each replicate, branching of at least two spores was examined. Means and standard error were determined for all replicates; means of replicates were subjected to statistical analysis by multiple-range test ($P \leq 0.05$), using the JMP statistical package.

LC-MS analysis

For LC-MS analysis of root extracts, whole roots were cut from 5 week hydroponically grown plants. For hydroponic plant growth, *SL-ORT1* and WT seeds were surface-sterilized in 70% ethanol for 0.5 min and then in 1% sodium hypochlorite containing 0.02% (v: v) Tween 20 for 2.5 min. After rinsing three times with an excessive amount of sterile distilled water, the seeds were placed on moistened rock wool (approximately 500 tomato seeds per container). Two containers were used for the seeds of each line. The containers were placed in growth chamber at 25 °C with 16/8 h (day/night) and the nutrient solution was replaced twice a week. For root extraction, tomato roots were blended in a blender at top speed for 1 min in acetone (w/v=1:2). The acetone was evaporated under reduced pressure at 35 °C.

Root extracts were then fractionated to isolate strigolactones according to Yoneyama *et al.* (2007) and analysed by LC–MS as described in Yoneyama *et al.* (2007) with modifications. Briefly, HPLC separation was conducted with a U980 HPLC instrument (Jasco, Tokyo, Japan) fitted with an ODS (C18) column (Mightysil RP-18, 2×250 mm, 5 μm; Kanto Chemicals, Tokyo, Japan). The mobile phase was 60% methanol in water (v/v) and was switched to 100% methanol 15 min after injection. The column was then washed with 100% methanol for 20 min at a flow rate of 0.2 ml min^{-1} and the column temperature set to 40 °C.

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MS was performed with a Quattro LC mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray source. Both the drying and nebulizing gas was nitrogen generated from pressurized air in an N2G nitrogen generator (Parker-Hanifin Japan, Tokyo, Japan). The nebulizing gas flow was set to approximately 100 l h^{-1}, and the desolvation gas flow was set to 500 l h^{-1}. The interface temperature was set to 400 °C, and the source temperature to 150 °C. MS/MS experiments were conducted using argon as the collision gas and a collision energy of 16 eV. The collision gas pressure was 0.15 Pa. The following *m/z* transitions were monitored: 367>270 for didehydro-orobanchol and 365>268 for solanacol. Data acquisition and analysis were performed with MassLynx software (ver. 3.2). Solanacol was quantified using a standard purified from cowpea root exudates (Xie *et al.*, 2007). The root extract sample was dissolved in 50% aqueous methanol and filtered through a spin column (Ultra-free MC, 0.45-μm pore size, Millipore, Billerica, MA). An aliquot of the filtered solution was diluted with a volume of either pure 50% methanol or 50% methanol containing known amounts of solanacol. The increase in peak area on the chromatogram corresponded to the amounts of solanacol added, enabling estimation of the amounts of this compound in a sample. The identity of didehydro-orobanchol was not verified using a standard. Determination of this compound was based on the *m/z* 367>270 transition and retention time. All peaks corresponding to known strigolactones were confirmed by bioassay on broomrape seeds. Two replicates were performed for each line, WT or *Sl-ORT1*. Each replicate contained approximately 500 individual plants.

Determination of shoot branching

WT and *Sl-ORT1* seeds were surface-sterilized and allowed to germinate and grow in styrofoam seedling trays in soil:vermiculite (1:1, v/v). GR24 was added at a concentration of 0.027 μM as water solution to the styrofoam trays for 3 weeks. Four-week-old seedlings were transferred to 3.0 l pots (one plant per pot) with a 1:1 mixture of soil and vermiculite. Plants were grown in a greenhouse under natural light conditions supplemented with artificial light (100 μmol m^{-2} s^{-1}) to maintain a 16 h photoperiod at 28/24 °C (day/night). Number and weight of lateral shoots were measured for each plant after 45 d: eight plants were examined for each WT and *Sl-ORT1* strain. Unsuppressed branching was counted as lateral branches of more than 0.3 g FW and more than 5 cm length. The experiment was repeated four times. Means and standard deviations were determined for all replicates; means of replicates were subjected to statistical analysis by multiple-range test ($P \leqslant 0.05$), using the JMP statistical package.

Grafting experiments

Grafted plants were prepared by Hishtil nursery (Nehalim, Israel). Briefly, sterile-grown seedlings, 21 d post-germination, were sectioned within the hypocotyl region and the combinations of scion and rootstock were aligned to form a graft union.

For interstock grafting, 5 cm long epicotyl segments with slanted cuts were grafted to the rootstock and, simultaneously, to the scion. Seedlings were incubated in a controlled chamber at 25/20 °C (day/night temperatures), with relative humidity of 75% for 3 weeks. Following healing of the graft union, seedlings were transferred to 1.5 l pots filled with a mixture of sterile sand and vermiculite (1:1 v/v), with or without the presence of AMF spores (as described above) and allowed to grow under greenhouse conditions, with 16 h light. Plants were examined following 8 weeks of greenhouse growth. Unsuppressed branching was counted as lateral branches with more than 0.3 g FW and more than 5 cm length.

For hypocotyl grafting experiments, WT and *Sl-ORT1* were reciprocally grafted. For interstock grafting, four combinations of WT and *Sl-ORT1* plants were used—WT/WT/WT: WT_{scion}-$WT_{interstock}$-WT_{stock}; O/O/O: $Sl\text{-}ORT1_{scion}$-$Sl\text{-}ORT1_{interstock}$-$Sl\text{-}ORT1_{stock}$; WT/O/WT: WT_{scion}-$Sl\text{-}ORT1_{interstock}$-$WT_{stock}$; O/WT/O: $Sl\text{-}ORT1_{scion}$-$WT_{interstock}$-$Sl\text{-}ORT1_{stock}$. Non-grafted WT and *Sl-ORT1* plants were used as controls. Shoot lateral branching, AMF colonization and *Sl-CCD7* and *Sl-CCD8* transcription levels were determined as described above and below, respectively.

For all grafting experiments, eight replicates (plants) were used for each grafting combination and controls, and the experiment was repeated three times. Means of replicates were subjected to statistical analysis by multiple-range test ($P \leqslant 0.05$), using the JMP statistical package.

Isolation of Sl-CCD8 *gene fragment and determination of* Sl-CCD8 *and* Sl-CCD7 *transcription levels using semi-quantitative PCR*

The *Sl-CCD8* gene fragment was isolated from WT cDNA (H Koltai, unpublished results; see Supplementary Fig. S3 at *JXB* online) using primers designed based on conserved gene regions. A sequence of a fragment of *Sl-CCD7* gene was kindly provided by Drs Klee and Vogel. RNA was extracted from whole roots of plants that were allowed to grow under greenhouse conditions, with 16/8 h (day/night) for 8 weeks, as described in Gal *et al.* (2006). Semi-quantitative PCR was performed by amplifying 140 bp and 150 bp fragments from each of the genes; *Sl-CCD7* and *Sl-CCD8*, respectively, using forward primer 5′-TGGGAAGGTGGTGATCCTTA-3′ and reverse primer 5′-TAGCTGAGCAGCAACATCCA-3′ for *Sl-CCD7* and forward 5′-CAATCACAGCGGTAACTCTTCCA-3′ and reverse primer 5′-GCATCCTGATTCTAAAGCATTT-3′ for *Sl-CCD8*. Tomato ribosomal *18S* (accession no. AY552528) served as the reference gene for the amount of RNA, and was amplified using the forward primer 5′-TTGATTACGTCCCTGCCCTTTGTACAC-3′ and the reverse primer 5′-AGGTTCACACCTACGGAAACCTTGTTAC-3′. PCR amplification was performed for 30 cycles under the following conditions: 94 °C denaturation for 30 s, 58.4 °C annealing for 30 s and 72 °C extension for 30 s. The resulting PCR products were separated by electrophoresis on a 1.4% agarose gel, and quantified using TotalLab TL120 (Nonlinear Dynamics Ltd., Newcastle, UK). The amount of amplification of the gene of interest was determined relative to that of the *18S* ribosomal gene. The experiment was performed in eight replicates; in each replicate, the resulting amount-of-amplification product values were normalized to that of the WT. Means and standard error were determined from all replicates; means of replicates were subjected to statistical analysis by multiple-range test ($P \leqslant 0.05$), using the JMP statistical package.

Results

The Sl-ORT1 *mutant is impaired in its interaction with the arbuscular mycorrhizal fungus* Glomus intraradices

The *Solanum lycopersicon Orobanche-Resistant Trait 1* (*Sl-ORT1*) mutant was isolated as described in Dor *et al.* (2010); *Sl-ORT1* was demonstrated to be resistant to *Orobanche* under both field and greenhouse conditions as compared to its parental WT line.

Since it has previously been suggested that the parasitic *Orobanche* and the symbiotic AMF respond to similar signals for their interaction with host plants (Akiyama and Hayashi, 2006; Bouwmeester *et al.*, 2007; Yoneyama *et al.*, 2008), the ability of the *Sl-ORT1* mutant to be infected by AMF was determined. *Sl-ORT1* plants were shown to have reduced *G. intraradices* colonization rates, by either whole inoculum or spores, relative to WT plants. AMF whole inoculum was significantly more infective to *Sl-ORT1* than

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spores, whereas both spores and whole inoculum were similarly infective to the WT (Fig. 1A).

To determine a possible cause of *Sl-ORT1* resistance to AMF, the ability of *Sl-ORT1* and WT root exudates to induce AMF hyphal branching was determined. A reduced level of hyphal branching was evident in the presence of *Sl-ORT1* versus WT exudates; GR24 (Johnson *et al.*, 1981), which is a synthetic strigolactone analogue previously shown to have biological activities (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008) served as a positive control, and distilled sterile water served as a negative control (Fig. 1B; Table 1). Hence, the resistance of *Sl-ORT1* to AMF may result from its inability to promote hyphal branching.

The Sl-ORT1 *mutant is deficient in strigolactone production*

Since strigolactones are prominent signalling molecules for both plant–AMF and plant–*Orobanche* interactions, the *Sl-ORT1* mutant's ability to produce strigolactones was analysed by determining the level of strigolactones in the WT and *Sl-ORT1* mutant root extracts.

In the WT root extracts, two peaks corresponding to previously identified tomato strigolactones were examined (López-Ráez *et al.*, 2008); these were detected in different channels of tandem mass spectrometry (MS/MS) analysis of WT root extracts (Fig. 2; Table 2). One, corresponding to solanacol, was detected in the 365 >268 transition with a retention time of 5.73 min (Fig. 2; Table 2). The MS/MS analysis and the addition of solanacol as an internal standard, together with its ability to induce broomrape seed germination in a bioassay (not shown), confirmed its identity as solanacol. The second peak, corresponding to a putative didehydro-orobanchol isomer(s), was detected in the 367 >270 transition with a retention time of 6.81 min (Fig. 2). The identity of this compound was not verified using a standard (due to its unavailability), but was

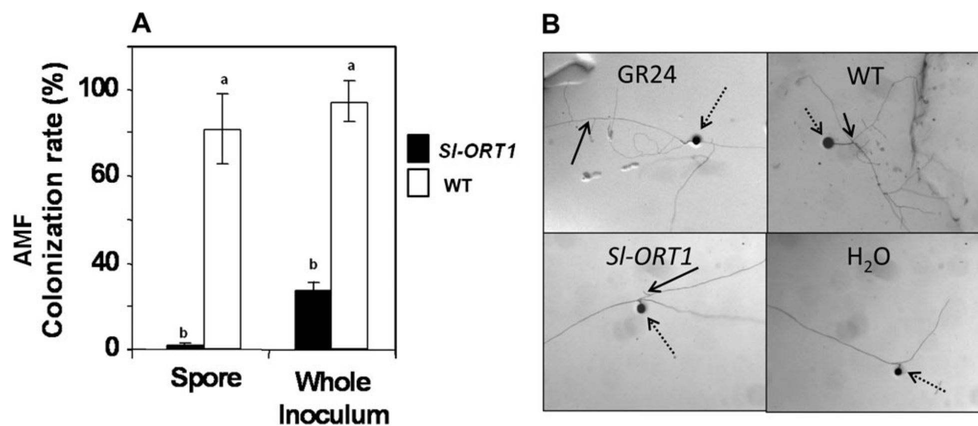

Fig. 1. (A) Colonization rate by arbuscular mycorrhizal fungi of WT (*Solanum lycopersicon* cv. M82) and *Sl-ORT1* plants. Six replicates were done for each treatment, and in each replicate 10 plants were examined. Means and standard error were determined for all replicates; means of replicates were subjected to statistical analysis by multiple-range test. (B) Examples of arbuscular mycorrhiza hyphal branching following exposure to WT or *Sl-ORT1* root exudates, or to GR24 as a positive control or sterile distilled water as a negative control. Two experiments were conducted, with 20 replicates each; in each replicate, branching of at least two spores was examined. Means and standard error were determined for all replicates; means of replicates were subjected to statistical analysis by multiple-range test. Different lowercase letters (a, b) above bars represent significantly different means ($P \leqslant 0.05$). Dashed arrows point to AMF spores; black arrows point to AMF hyphal branching sites.

Table 1. Results of two experiments studying hyphal branching of arbuscular mycorrhiza (*Glomus intradices*) following exposure to WT or mutant (*Sl-ORT1*) or to GR24 as a positive control or sterile distilled water as a negative control

Six replicates were done for each treatment, in each replicate 10 plants were examined. Means and standard error were determined for all replicates.

	2° branching	3° branching	4° branching	5° branching		Total branching
WT	7.02±1.08	3.99±0.61	0.83±0.28	0.19±0.02		12.03
Sl-ORT1	4.02±0.74	1.76±0.45	0.13±0.13			5.91
GR24	4.81±0.52	2.92±0.63	0.40±0.31			8.13
H_2O	2.71±0.39	0.88±0.36	0.25±0.25			3.84
	2° branching	**3° branching**	**4° branching**	**5° branching**	**6° branching**	**Total branching**
WT	6.23±0.88	3.84±1.73	1.93±0.94	0.65±0.40	0.23±0.23	12.89
Sl-ORT1	2.46±0.53					2.46
GR24	3.68±0.71	1.66±0.43	0.31±0.20	0.14±0.14		5.79
H_2O	3.28±0.58	0.68±0.29				3.96

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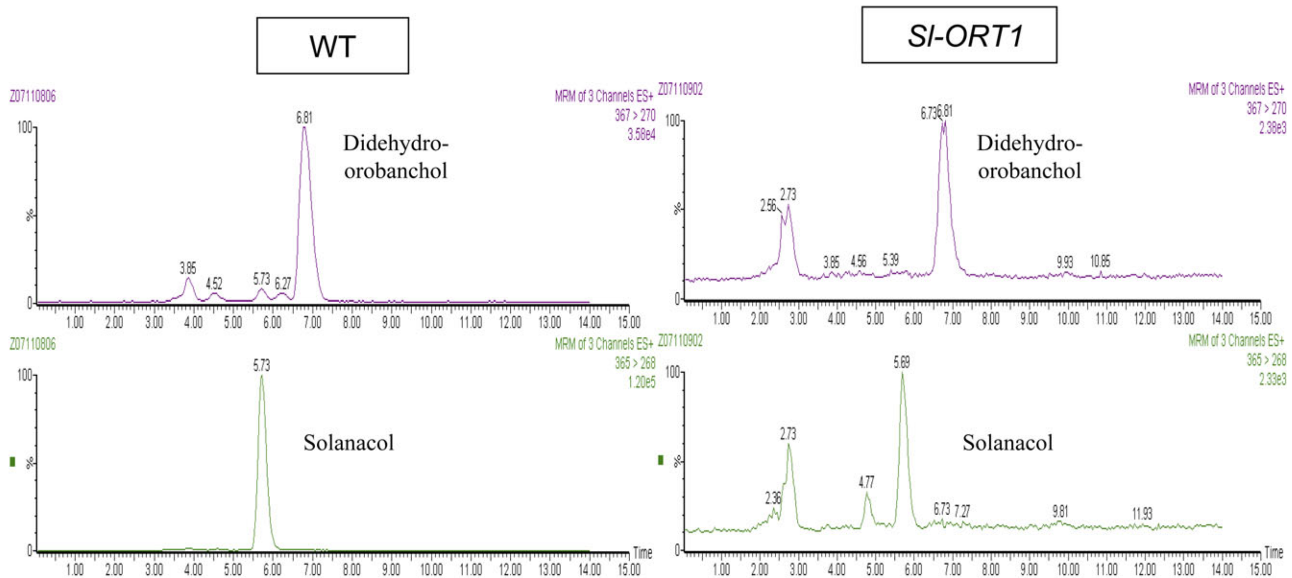

Fig. 2. Results of LC-MS analysis of strigolactones in WT (*Solanum lycopersicon* cv. M82) and *Sl-ORT1* tomato root extracts. Two replicates were performed for each line, WT or *Sl-ORT1*. Each replicate contained approximately 500 individual plants. One of the replicates is presented in the figure, whereas the second replicate demonstrated similar data.

Table 2. Content of strigolactones solanacol and didehydro-orobanchol in root extracts obtained from hydroponically grown tomato plants following 5 weeks of growth

Two replicates were performed for each line, WT or *Sl-ORTl*. Each replicate contained approximately 500 individual plants; means for two replicates are presented.

Tomato line	WT	*Sl-ORT1*
Solanacol		
concentration (ng g^{-1} root)	6.76	0.61
Didehydro-orobanchol		
peak area	13 048	408

confirmed by MS/MS analysis and broomrape seed-germination bioassay (not shown).

In *Sl-ORT1* root extracts the two peaks detected in the different channels correspond to strigolac-335 tones were demonstrated. Solanacol was detected as a peak in the 365 >268 transition with a retention time of 5.69 min, as confirmed by the addition of solanacol as an internal standard. Didehydro-orobanchol isomer(s) was detected as a peak in the 367 >270 transition at a retention time of 6.81 min (Fig. 2). However, both solanacol and didehydro-orobanchol isomer(s) were found to be reduced in *Sl-ORT1* as compared to the WT (Table 2). These results suggested that the *Sl-ORT1* mutant produces only minute amounts of the two strigolactones relative to the WT.

Mutant characterization

Sl-ORT1 mutant has aberrant shoot morphology compared to the WT: Shoot morphology of *Sl-ORT1* and WT plants was examined. In tomato WT, apical dominance is maintained up until the formation of 7–11 metamers (reviewed by McSteen and Leyser, 2005; Schmitz and Theres, 2005). Then the primary shoot apical meristem is converted into an inflorescence, and in determined plants (such as WT, the progenitor of *Sl-ORT1*; Pnueli *et al.*, 1998), shoots acquire a sympodial architecture.

A higher number of unsuppressed side branches emerged in *Sl-ORT1* plants than in the WT, prior to acquisition of sympodial architecture (Fig. 3A, B, C). Lack of suppression of side branches was evident in *Sl-ORT1* from the first to seventh (±2) metamer (n=48). Further up in the metamers and inflorescence, a WT-like architecture resumed. In *Sl-ORT1*, primary side branches did not display multiplicity of unsuppressed secondary side branches and were similar to those of the WT. Upon exogenous application of 0.027 μM GR24, *Sl-ORT1* shoot morphology was restored to that of the WT: the hyper-branching phenotype was suppressed, as evidenced by both the number of lateral branches and their fresh weight (Fig. 3B, C).

Phenotype complementation in WT and mutant grafted plants: *Sl-ORT1* shoots grafted to WT roots exhibited reduced lateral branching, and were similar to WT/WT (WT_{scion}-WT_{stock}) plants. Also WT shoots grafted to *Sl-ORT1* roots exhibited a phenotype similar to that of WT/WT (Fig. 4). Notably, in addition to a lower level of lateral shoot branching, O/WT (*Sl-ORT1*$_{scion}$-WT_{stock}) and WT/O (WT_{scion}-*Sl-ORT1*$_{stock}$) plants exhibited a postponement in the appearance of lateral branching, such that unlike in *Sl-ORT1* or O/O (*Sl-ORT1*$_{scion}$-*Sl-ORT1*$_{stock}$) plants, and similarly to WT or WT/WT (WT_{scion}-WT_{stock}) plants, nodes 1–3 did not develop lateral branches (see Supplementary Fig. S1 at *JXB* online).

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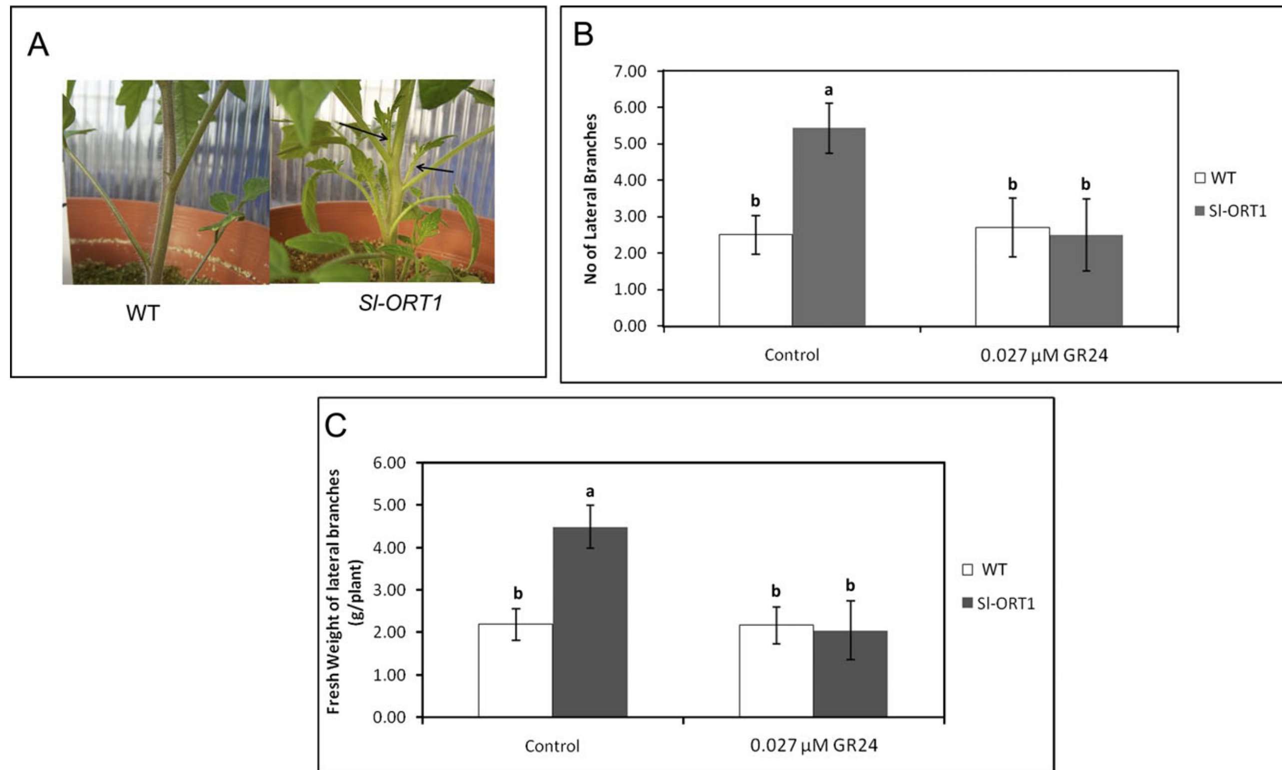

Fig. 3. Morphological analysis of shoots of WT (*Solanum lycopersicon* cv. M82) and *Sl-ORT1* plants. (A) An example of *Sl-ORT1* and WT shoots. Arrows denote sites of lateral shoot branching (B) Lateral shoot number per plant, with or without (control) exogenous application of GR24 (0.027 μM). (C) Lateral shoot fresh weight, with or without (control) exogenous application of GR24 (0.027 μM). Number and weight of lateral shoots were measured for eight plants for each WT and *Sl-ORT1* strains. The experiment was repeated four times. Means and standard deviations are shown; means of replicates were subjected to statistical analysis by multiple-range test. Different lowercase letters (a, b) above bars represent significantly different means ($P \leq 0.05$).

However, mycorrhizal sensitivity was not restored to that of the WT in WT-scion grafted to *Sl-ORT1* roots (WT/O), whereas WT roots grafted to *Sl-ORT1* scion (O/WT) were sensitive to mycorrhizae (Table 3). No differences were observed in shoot fresh weights of the different grafted plant combinations (Fig. 4).

Restoration of *Sl-ORT1* shoot morphology to that of the WT also appeared in interstock-grafted plants: WT interstock that was grafted between *Sl-ORT1* stock and *Sl-ORT1* scion (O/WT/O plants) suppressed *Sl-ORT1* hyper-branching and restored the WT phenotype, as evidenced by both the number and fresh weight of the lateral branches (Fig. 5). Similar to hypocotyl grafting, in addition to the lower level of lateral shoot branching, both WT/O/WT (WT_{scion}-$Sl\text{-}ORT1_{interstock}$-$WT_{stock}$) and O/WT/O ($Sl\text{-}ORT1_{scion}$-$WT_{interstock}$-$Sl\text{-}ORT1_{stock}$) plants exhibited a postponement in lateral branch appearance, such that unlike in *Sl-ORT1* or O/O/O ($Sl\text{-}ORT1_{scion}$-$Sl\text{-}ORT1_{interstock}$-$Sl\text{-}ORT1_{stock}$) plants, and similar to WT and WT/WT/WT (WT_{scion}-$WT_{interstock}$-WT_{stock}) plants, nodes 1–3 did not develop lateral branches (see Supplementary Fig. S2 at *JXB* online). Shoot fresh weight, however, was higher in *Sl-ORT1* and O/O/O plants, probably reflecting the marked increase in lateral branching compared to the WT (Fig. 5). However, in O/WT/O roots, mycorrhizal sensitivity was not restored to that of the WT (Table 4). Hence, also with regard to mycorrhizal sensitivity, in O/WT/O plants, WT interstock may not have restored WT-characteristics to *Sl-ORT1*-originated roots.

To characterize the effect of interstock grafting on roots further, the level of transcription of CCD8 and CCD7 was determined. CCD7/MAX3 and CCD8/MAX4 have been suggested to be key enzymes of carotenoid cleavage leading to strigolactone synthesis (Booker *et al.*, 2004; Schwartz *et al.*, 2004; Umehara *et al.*, 2008), and to be expressed mainly in roots and lower stems (Booker *et al.*, 2004; Bainbridge *et al.*, 2005; reviewed by Dun *et al.*, 2009).

To examine the expression pattern of the tomato (*Sl*) CCD7 and CCD8, a 900 bp fragment of the *Sl-CCD8* gene was isolated (see Supplementary Fig. S3 at *JXB* online). A 400 bp fragment of the *Sl-CCD7* gene was provided by Drs Klee and Vogel. Semi-quantitative PCR suggested a significantly reduced level of *Sl-CCD7* in *Sl-ORT1* mutant roots relative to the WT (Fig. 6). This reduction may suggest a reduced level of strigolactone synthesis in *Sl-ORT1* roots. *Sl-CCD8*, which follows *Sl-CCD7* in the biogenesis pathway of strigolatones (Schwartz *et al.*, 2004; Umehara *et al.*, 2008), was not significantly changed in *Sl-ORT1* relative to the WT (Fig. 6).

In O/WT/O plants, *Sl-CCD7* transcription in roots was only slightly though not statistically significant induced in comparison to *Sl-ORT1* or O/O/O plants (Fig. 6). Hence, in

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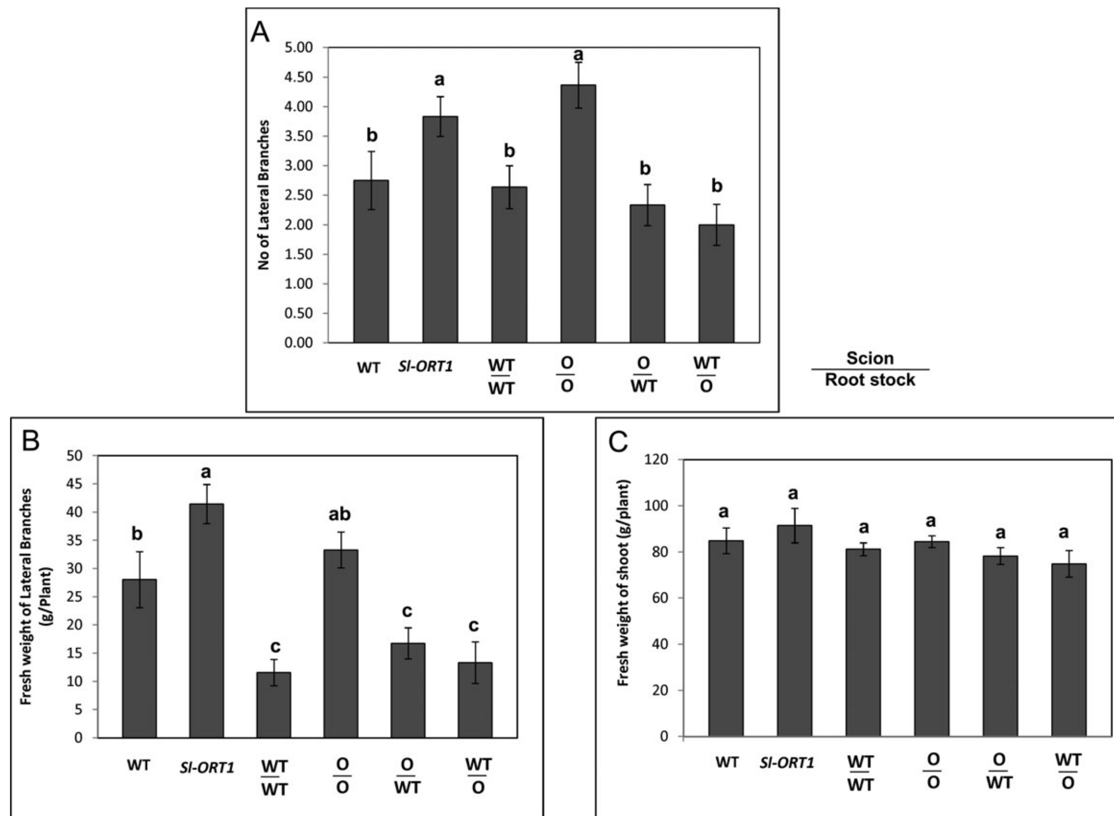

Fig. 4. Morphological analysis of WT (*Solanum lycopersicon* cv. M82), *Sl-ORT1* and hypocotyl grafted plants. (A) Lateral shoot number per plant. (B) Lateral shoot fresh weight. (C) Total (main and lateral) shoot fresh weight. WT/WT: WT_{scion}-WT_{stock}; O/O: *Sl-ORT1*$_{scion}$-*Sl-ORT1*$_{stock}$; WT/O: WT_{scion}-*Sl-ORT1*$_{stock}$; O/WT: *Sl-ORT1*$_{scion}$-WT_{stock}. Number and weight of lateral shoots were measured for eight plants for each WT and *Sl-ORT1* strain. The experiment was repeated three times. Means and standard deviations are shown; means of replicates were subjected to statistical analysis by multiple-range test. Different lowercase letters (a, b, c) above bars represent significantly different means ($P \leq 0.05$).

Table 3. Colonization rate by arbuscular mycorrhizal fungus (AMF) *Glomus intraradices* of WT, mutant (*Sl-ORT1*) and hypocotyl grafted plants

Six replicates were done for each treatment, in each replicate 10 plants were examined. Means and standard error were determined for all replicates; means of replicates were subjected to statistical analysis by multiple-range test.

Plant[a]	AMF colonization rate[b]
WT	80.3±10.0 a
Sl-ORT1	4.1±1.6 c
WT/WT	57.0±7.4 b
O/O	6.0±5.8 c
WT/O	5.9±5.1 c
O/WT	67.3±10.7 ab

[a] WT/WT: WT_{scion}-WT_{stock}; O/O: *Sl-ORT1*$_{scion}$-*Sl-ORT1*$_{stock}$; O/WT: *Sl-ORT1*$_{scion}$-WT_{stock}; WT/O: WT_{scion}-*Sl-ORT1*$_{stock}$.
[b] Different lowercase letters (a, b, c) represent significantly different means ($P \leq 0.05$).

O/WT/O plants, WT interstock may also not restore the WT characteristics to *Sl-ORT1*-originated roots with respect to *Sl-CCD7* transcription.

A significant reduction (albeit to a lesser extent than in O/WT/O plants) in mycorrhizal sensitivity was also recorded for WT/O/WT plants (Table 4). This reduced sensitivity was not accompanied by a reduction in *Sl-CCD7* transcription (Fig. 6) and may reflect the minor reduction in AMF sensitivity that occurs in most grafted plants (see also Table 3).

Discussion

The first tomato strigolactone-deficient mutant (*Sl-ORT1*) is reported here. The mutant displays several phenotypes, such as resistance to the parasitic plant *Orobanche* (Dor *et al.*, 2010), a lower level of mycorrhizal colonization and increased lateral shoot branching (this study). Also, WT interstock was found sufficient to rescue the hyper-branching phenotype of the tomato mutant. Similar properties of strigolactone-deficient mutants have been reported for other plant species (Napoli, 1996; Foo *et al.*, 2001; Booker *et al.*, 2004; Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008), supporting the notion of a general mechanism of strigolactone activity in plants (Klee, 2008; Leyser, 2009; Santner and Estelle, 2009; Schachtschabel and Boland, 2009).

In root extracts of the *Sl-ORT1* mutant, two of the strigolactones previously identified in WT tomato, solanacol and didehydro-orobanchol (López-Ráez *et al.*, 2008), were present in minute amounts relative to the WT. This is in accordance with the reduced ability of *Sl-ORT1* root

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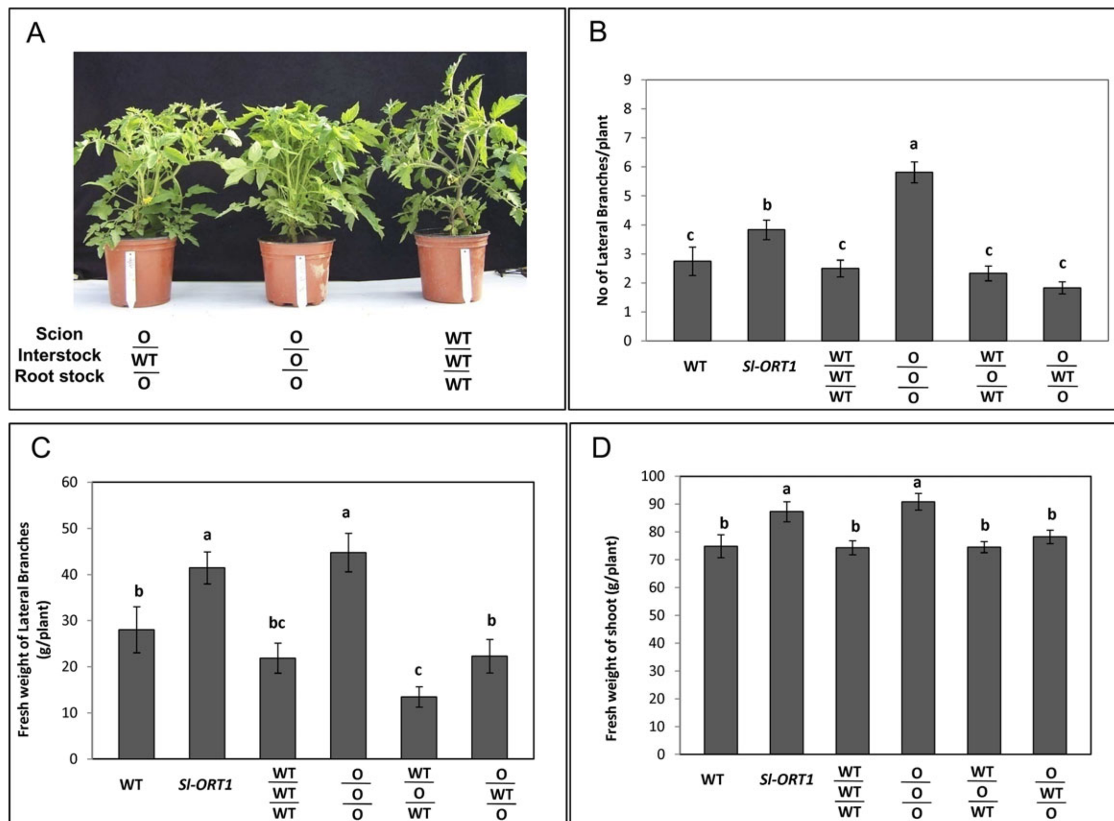

Fig. 5. Morphological analysis of WT (*Solanum lycopersicon* cv. M82), *Sl-ORT1* and interstock grafted plants. (A) An example of grafted plant morphology. (B) Lateral shoot number. (C) Lateral shoot fresh weight. (D) Total (main and lateral) shoot fresh weight. WT/WT/WT: WT_{scion}-$WT_{interstock}$-WT_{stock}; O/O/O: *Sl-ORT1*$_{scion}$-*Sl-ORT1*$_{interstock}$-*Sl-ORT1*$_{stock}$; WT/O/WT: WT_{scion}-*Sl-ORT1*$_{interstock}$-WT_{stock}; O/WT/O: *Sl-ORT1*$_{scion}$-$WT_{interstock}$-*Sl-ORT1*$_{stock}$. Number and weight of lateral shoots were measured for eight plants for each WT and *Sl-ORT1* strain. The experiment was repeated three times. Means and standard deviations are shown; means of replicates were subjected to statistical analysis by multiple-range test. Different lowercase letters (a, b, c) above bars represent significantly different means ($P \leqslant 0.05$).

Table 4. Colonization rate by arbuscular mycorrhizal fungus (AMF) *Glomus intraradices* of WT, mutant (*Sl-ORT1*) and interstock grafted plants

Six replicates were done for each treatment, in each replicate 10 plants were examined. Means and standard error were determined for all replicates; means of replicates were subjected to statistical analysis by multiple-range test.

Plant[a]	AMF colonization rate[b]
WT	86.2±4.8 a
Sl-ORT1	1.1±0.6 c
WT/WT/WT	84.8±2.4 a
O/O/O	2.1±1.0 c
O/WT/O	2.9±1.3 c
WT/O/WT	61.9±5.9 b

[a] WT/WT/WT: WT_{scion}-$WT_{interstock}$-WT_{stock}; O/O/O: Sl-ORT1_{scion}-$\text{Sl-ORT1}_{interstock}$-$\text{Sl-ORT1}_{stock}$; O/WT/O: Sl-ORT1_{scion}-$WT_{interstock}$-Sl-ORT1_{stock}; WT/O/WT: WT_{scion}-$\text{Sl-ORT1}_{interstock}$-$WT_{stock}$.
[b] Different lowercase letters (a, b, c) represent significantly different means ($P \leqslant 0.05$).

extracts to induce AMF hyphal branching (Akiyama *et al.*, 2005; Akiyama and Hayashi, 2006; Besserer *et al.*, 2006, 2008; Gomez-Roldan *et al.*, 2008; Yoneyama *et al.*, 2008). Hyphal branching of AMF is considered to be an important step that enhances the fungi's ability to reach a plant host root (Nagahashi and Douds, 2000). This step is probably more important for infection by spore inoculum than that by whole inoculum; the latter contains vegetative hyphae and colonized root segments in addition to spores. Hence, whole inoculum, which requires a lesser extent of hyphal branching, is more infective to *Sl-ORT1* than spore inoculum.

One key enzyme, CCD7, which has previously been identified as involved in strigolactone biosynthesis pathways in other plant species (Booker *et al.*, 2004; Schwartz *et al.*, 2004; Umehara *et al.*, 2008; reviewed by Dun *et al.*, 2009), exhibited reduced transcription levels in roots of the tomato mutant in comparison to the WT. Together, these results suggest a reduced level of strigolactone synthesis in the *Sl-ORT1* mutant compared to the WT, and a correlation between *Sl-CCD7* levels of transcription and strigolactone synthesis in tomato; this is also suggested for other plant species (Foo *et al.*, 2005; Johnson *et al.*, 2006; Dun *et al.*, 2009; Hayward *et al.*, 2009).

The *Sl-ORT1* mutant had more side branches than the WT; this is in agreement with other studies that have described strigolactone-deficient mutants in *Arabidopsis*,

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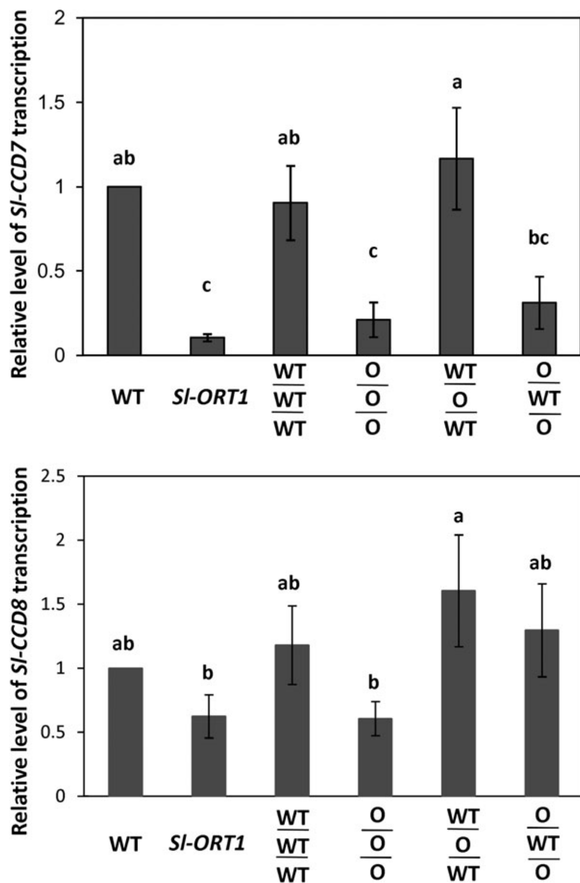

Fig. 6. Relative transcription level of *Sl-CCD7* and *Sl-CCD8* in WT (*Solanum lycopersicon* cv. M82), *Sl-ORT1* and interstock grafted plant whole-roots. WT/WT/WT: WT_{scion}-$WT_{interstock}$-WT_{stock}; O/O/O: *Sl-ORT1*$_{scion}$-*Sl-ORT1*$_{interstock}$-*Sl-ORT1*$_{stock}$; WT/O/WT: WT_{scion}-*Sl-ORT1*$_{interstock}$-WT_{stock}; O/WT/O: *Sl-ORT1*$_{scion}$-$WT_{interstock}$-*Sl-ORT1*$_{stock}$. The experiment was performed in eight replicates; in each replicate, the resulting amount-of-amplification product values were normalized to that of the WT. Means and standard error were determined from all replicates; means of replicates were subjected to statistical analysis by multiple-range test. Different lowercase letters (a, b) above bars represent significantly different means ($P \leq 0.05$).

pea, and rice, suggesting that strigolactones, or their derived substances, are important for shoot branching (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008; Brewer *et al.*, 2009; Ferguson and Beveridge, 2009). It was found that, in *Sl-ORT1*, the increase in lateral shoot branching prevailed in the metamers only prior to the acquisition of a sympodial architecture; after that, regular plant architecture resumes. Hence, in tomato, strigolactones may be involved in the control of lateral shoot branching during vegetative growth, prior to inflorescence formation. Reduced lateral shoot branching was restored to that of WT in *Sl-ORT1* following exogenous application of the synthetic strigolactone GR24, demonstrating that addition of strigolactone may complement the mutant phenotype, restoring it to that of the WT.

Sl-ORT1 shoots grafted to WT roots exhibited reduced lateral branching and restoration to a WT-like phenotype, indicating that, as in other plant species, WT roots may complement the shoot phenotype, probably via strigolactone supplementation (reviewed by Dun *et al.*, 2009). WT shoots grafted to *Sl-ORT1* roots exhibited a WT shoot phenotype, indicating that WT stems are sufficient to support lateral branching suppression, and suggesting that, as in other plant species, strigolactones are also synthesized to some extent in the shoots (reviewed by Dun *et al.*, 2009).

Reduced lateral shoot branching was also evident in interstock-grafted plants which included WT interstock grafted between a mutant rootstock and mutant scion (O/WT/O grafted plants), a phenomenon which has also been reported for other plant species (Napoli, 1996; Foo *et al.*, 2001; Booker *et al.*, 2004).

Due to findings suggesting that strigolactones are produced in shoots and roots, but moves in the root-to-shoot direction (Napoli, 1996; Beveridge *et al.*, 1997, 2000; reviewed by Dun *et al.*, 2009) and, accordingly, that roots are the main site of CCD7 and CCD8 expression (Booker *et al.*, 2004; Bainbridge *et al.*, 2005), the phenomenon of WT interstock complementation of mutant scions may suggest that the WT interstock itself may produce sufficient amounts of the necessary components (strigolactones or their derivatives) to confer a reduction in shoot branching.

Lack of both induction in the roots of *Sl-CCD7* transcription and sensitivity to AMF suggests that, in the O/WT/O plants, the ability of the WT interstock to restore the mutant shoot phenotype does not encompass the restoration of WT characteristics in mutant roots, such as the ability to synthesize strigolactones. The WT interstock probably produces a sufficient amount of components such as strigolactones, their metabolites, or other unknown secondary messengers that migrate towards the shoot apex, to confer a significant reduction in shoot branching; this is in accordance with the hypothesis raised by Foo *et al.* (2001), Brewer *et al.* (2009), and Ferguson and Beveridge (2009).

What might be the possible role of *Sl-ORT1* gene? Due to the reduced transcription level of *Sl-CCD7*, it might be that *Sl-ORT1* encodes *Sl-CCD7*. Another possibility is that *Sl-ORT1* works as a regulator of *Sl-CCD7* expression. *Sl-CCD8* expression level is not significantly affected in the *Sl-ORT1* mutant, whereas in other plant species (e.g. pea), *CCD8* (*RMS1*) expression is stimulated by a mobile feedback signal (probably strigolactones; Foo *et al.*, 2005; Beveridge *et al.*, 2009). The fact that in *Sl-ORT1* mutants, despite a reduction in *CCD7* transcription, and reduced levels of strigolactones, *CCD8* expression levels were not altered, may suggest an interaction between control of *CCD7* expression and feedback regulation of *CCD8* transcription.

To conclude, characterization of the first tomato strigolactone-deficient mutant supports the putative general role of strigolactones in a diversity of plant species as messengers of suppression of lateral shoot branching.

Supplementary data

Supplementary data are available at *JXB* online.

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Supplementary Fig. S1. Morphological analysis of WT (*Solanum lycopersicon* cv. M82), *Sl-ORT1*, and hypocotyl grafted plants, at each of the shoot nodes.

Supplementary Fig. S2. Morphological analysis of WT (*Solanum lycopersicon* cv. M82), *Sl-ORT1*, and interstock grafted plants, at each of the shoot nodes.

Supplementary Fig. S3. The tomato *CCD8* gene fragment sequence.

Acknowledgements

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