The tomato **CAROTENOID CLEAVAGE DIOXYGENASE8** (S/CCD8) regulates rhizosphere signaling, plant architecture and affects reproductive development through strigolactone biosynthesis

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**Summary**

- Strigolactones are plant hormones that regulate both above- and belowground plant architecture. Strigolactones were initially identified as rhizosphere signaling molecules. In the present work, the tomato (**Solanum lycopersicum**) **CAROTENOID CLEAVAGE DIOXYGENASE 8** (S/CCD8) was cloned and its role in rhizosphere signaling and plant physiology assessed by generating knock-down lines.
- Transgenic **S/CCD8** plants were generated by RNAi-mediated silencing. Lines with different levels of strigolactone reduction – confirmed by UPLC-MS/MS – were selected and their phenotypes investigated.
- Lines exhibiting reduced **S/CCD8** levels displayed increased shoot branching, reduced plant height, increased number of nodes and excessive adventitious root development. In addition, these lines exhibited reproductive phenotypes such as smaller flowers, fruits, as well as fewer and smaller seeds per fruit. Furthermore, we show that strigolactone loading to the xylem sap is possibly restricted to orobanchol.
- Infestation by **Phelipanche ramosa** was reduced by 90% in lines with a relatively mild reduction in strigolactone biosynthesis and secretion while arbuscular mycorrhizal symbiosis, apical dominance and fruit yield were only mildly affected. This demonstrates that reduction of strigolactone biosynthesis could be a suitable tool in parasitic weed management. Furthermore, our results suggest that strigolactones are involved in even more physiological processes than so far assumed.

**Introduction**

Strigolactones are a group of carotenoid-derived plant hormones (**Matusova et al., 2005; López-Ráez et al., 2008a**). They were initially identified as germination stimulants for root parasitic plants of the Orobanchaceae (**Cook et al., 1966; Bouwmeester et al., 2003**) and pre-symbiotic signals inducing hyphal branching in arbuscular mycorrhizal (AM) fungi (**Akiyama et al., 2005; Besserer et al., 2006; Bouwmeester et al., 2007**). Strigolactones have been detected in the root extracts and exudates of both monocot and dicot plant species (**Yoneyama et al., 2007; Goldwater et al., 2008; Gomez-Roldan et al., 2008; López-Ráez et al., 2008a; Umehara et al., 2008**) and were identified to be the branching/tillering inhibiting signal (**Gomez-Roldan et al., 2008; Umehara et al., 2008**). This graft-transmissible signal was postulated to exist over 15 years ago and to originate – at least to a large extent – from the roots (**Beveridge et al., 1994; Napoli, 1996; Turnbull et al., 2002**). However, intergrafting of hypocotyl tissue is sufficient to restore shoot branching of strigolactone biosynthesis mutants in pea and **Arabidopsis thaliana** (**Arabidopsis**) to near wild-type levels, indicating that – in addition to roots – other tissues can contribute to the production of strigolactones that inhibit the outgrowth of axillary buds (**Foo et al., 2001**). Nevertheless, strigolactones are transported acropetally to the parts of the plant where they exert their function. This is supported by the fact that in both **Arabidopsis** and **Solanum lycopersicum** (tomato) at least one
Strigolactone production is induced under phosphate-limiting conditions in several plant species (Yoneyama et al., 2007; López-Ráez et al., 2008a; Umehara et al., 2008; Kohlen et al., 2011a), likely to stimulate the establishment of AM symbiosis (Akiyama et al., 2005; Bouwmeester et al., 2007). More recently, it was proposed that these elevated strigolactone concentrations might also serve an additional function in planta, as they could be involved in reducing shoot branching under unfavorable conditions (Umehara et al., 2010; Kohlen et al., 2011a). In agreement with this, the concentration of orobanchol in the xylem sap of Arabidopsis is elevated under phosphate deficiency (Kohlen et al., 2011a). This could explain why strigolactone biosynthesis in Arabidopsis—a plant species which is not mycorrhizized—shows a similar response to phosphate starvation (Kohlen et al., 2011a).

In addition to regulating shoot architecture, novel biological functions for strigolactones are being discovered at a rapid pace, showing that they play a broader role in plant development. A small-molecule screen identified several putative functions for strigolactones in Arabidopsis, ranging from seed germination to hypocotyl elongation (Tsuchiya et al., 2010). Strigolactones were also identified as positive regulators of secondary stem growth by stimulating cambium development in several species (Agustí et al., 2011). In addition, strigolactones have been shown to be involved in the regulation of plant architecture belowground affecting the root system architecture. It was demonstrated that manipulation of the strigolactone concentration in the root—either by mutation or exogenous application of the synthetic strigolactone analog GR24—leads to changes in the primary root length, root hair development and lateral root initiation (Koltai et al., 2010a; Kapulnik et al., 2011; Ruyter-Spira et al., 2011). Similarly, strigolactones were recently shown to repress adventitious root development in Arabidopsis and *Pisum sativum* (pea; Rasmussen et al., 2012). Interestingly, in most—in not all—of these newly discovered strigolactone functions a cross-talk with auxin seems to play a predominant role (Bennett et al., 2006; Agustí et al., 2011; Ruyter-Spira et al., 2011; Rasmussen et al., 2012).

As mentioned above, strigolactones are derived from the carotenoids and therefore belong to a chemical class called the apocarotenoids. Indeed, recently 9-cis-β-carotene was identified as the substrate for strigolactone biosynthesis (Alder et al., 2012). Moreover, three of the four enzymes previously demonstrated to be involved in their biosynthesis (Gomez-Roldán et al., 2008; Umehara et al., 2008; Lin et al., 2009; Kohlen et al., 2011a) have now been functionally identified (Alder et al., 2012). All-trans-β-carotene is first isomerised to 9-cis-β-carotene by DWF27, and is subsequently sequentially cleaved by CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7) and CAROTENOID CLEAVAGE DIOXYGENASE 8 (CCD8) to give rise to carlactone (Alder et al., 2012). This apocarotenoid closely resembles strigolactones as it already has the D-ring and enol ether bridge characteristic for strigolactones. Alder and co-workers have also postulated that the fourth biosynthetic enzyme, with so far unknown catalytic activity—the cytochrome P450 MORE AXILLARY GROWTH1 (MAX1; Stirnberg et al., 2002; Booker et al., 2005)—might catalyse the conversion of carlactone to 5-deoxystrigol (Alder et al., 2012), proposed to be the first real strigolactone in the biosynthetic pathway (Matusova et al., 2005; Rani et al., 2008). Strigolactone biosynthesis seems to be tightly regulated as it was demonstrated that despite increased carotenoid accumulation in the tomato mutant high pigment-2darkgreen (hp-2d) strigolactone biosynthesis and secretion into the rhizosphere were significantly reduced. As a result, this mutant was less susceptible to the root parasitic plant *Phelipanche aegyptiaca* (López-Ráez et al., 2008b). Also the *Orobanche*-resistant and AM-deficient tomato mutant—SL-ORT1—with a mildly branched phenotype was shown to produce fewer strigolactones (Koltai et al., 2010b). However, the gene underlying SL-ORT1 and its function in regulating strigolactone biosynthesis so far remains unknown. This demonstrates that controlling strigolactone biosynthesis might be a good strategy to control these crop-damaging parasitic weeds as previously suggested (Bouwmeester et al., 2003; López-Ráez et al., 2009; Cardoso et al., 2011). Remarkably, AM symbiosis in tomato also leads to a reduced germinating stimulatory activity for *Phelipanche ramosa* seeds, a reduction caused by a decrease in strigolactone production and exudation (López-Ráez et al., 2011a).

Tomato has become an important model in strigolactone research and its major strigolactone composition (solanacol, orobanchol and didehydro-orobanchol isomers 1 and 2) has been elucidated (López-Ráez et al., 2008a,b). Therefore, having full insight into the strigolactone biosynthetic pathway in tomato is vital, as this would make it an excellent model for combining both analytical and molecular tools for strigolactone research in an agronomically important crop. The first strigolactone biosynthetic gene characterised in tomato was *SICCD7* (Vogel et al., 2010), and the authors demonstrated that strigolactones also regulate the outgrowth of axillary bud in tomato. In the present study, we set out to clone the tomato *SICCD8* gene, and characterise its role in strigolactone biosynthesis and tomato plant development using knock-down transgenic lines. Our results provide insight into a possible new function for strigolactones in reproductive development of tomato.

**Materials and Methods**

**Plant material, growth conditions and chemicals**

Seeds of tomato (*Solanum lycopersicum* L. cv Craigella (LA3247)) and the three independent transgenic *SICCD8* knock-down lines (L16, L04, and L09) were surface-sterilised in 4% sodium hypochlorite containing 0.02% (v/v) Tween 20, rinsed thoroughly with sterile water and germinated for 48 h on moistened filter paper at 25°C in darkness. For strigolactone analysis, germinated
seeds were pre-grown on perlite for 7 d. Then, all perlite was removed from the roots and ten seedlings per biological replicate were transferred to an X-stream 20 aeroponics system (Nurtriculture, Lancashire, UK) operating on 5 l modified half-strength Hoagland nutrient solution (López-Ráez et al., 2008a) as previously described (Liu et al., 2011). To induce strigolactone production, a 7 d phosphate-starvation stress was applied to the plants by replacing the nutrient solution by modified half-strength Hoagland solution without phosphate (López-Ráez et al., 2008a; Liu et al., 2011). Prior to exudate collection the nutrient solution was refreshed in order to remove all accumulated strigolactones. The exudates were collected 24 h later; purified and concentrated within 2 h. Roots were quick frozen in liquid nitrogen and stored at −80°C for further analysis. For phenotypical analysis, plants were grown in soil-filled pots for 8 wk and irrigated with 300 ml of tap water twice a week and once with 300 ml of full-strength Hoagland solution (Hoagland & Arnon, 1950). For complementation studies and analysis of adventitious root primordia, pre-germinated tomato seeds were transferred to an in vitro system using 200-ml half-strength MS medium supplemented with 1 × Gamborg’s B5 vitamin mix, 1% agar, and either 0 (mock) or 5 μM GR24 (synthetic strigolactone analog). Cutting experiments were performed using the top 4–5 primary stem nodes of 4-wk-old tomato seedlings. All plants were grown under controlled conditions in a glasshouse at with 16/8 h photoperiod, 23/20°C, and 60% relative humidity. Additional light was provided when needed to achieve a 250 μmol m−2 s−2 minimum light intensity.

IAA was purchased from Sigma-Aldrich (St. Louis, USA), GR24 was kindly provided by Prof. dr. Zwanenburg (Radboud University, Nijmegen, The Netherlands).

Cloning of the full-length SlCCD8

A 1233-bp partial SlCCD8 coding sequence was amplified from tomato (cv Moneymaker) by reverse-transcriptase polymerase chain reaction (RT-PCR) using primers (forward, 5′-GCTGAGTGGCACGTACCTAA-3′; reverse, 5′-TCTCATCTTTCTCGGTGTGCAC-3′) designed to a highly conserved region of plant CCD8s. The SlCCD8 5′- and 3′-cDNA ends were obtained using the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA; 5′-RACE, 5′-GGTGCCATCTC-3′ and 3′ RACE, 5′-TCCTGCTTATTTAGGCAAG-3′). The complete SlCCD8 coding sequence (1674 bp) was PCR amplified from root cDNA (forward, 5′-ATGGCTTCTTTGCTCA TTCA TGTC-3′; reverse, 5′-CTATTTCTTTGGAACCCAGC-3′). Finally, the amplified cDNA fragment was modified using the A-tailing procedure and cloned into pGEM-T Easy vector (Promega) according to the manufacturer instructions, and both strains sequenced.

RNAi-mediated silencing of SlCCD8 and tomato transformation

The silencing of SlCCD8 in tomato was carried out by means of a 349 bp fragment plus the gateway CACC directional cloning sequence that was PCR amplified using specific primers (forward, 5′-CAACCAGGACATGGGCACATTAGGT-3′; reverse, 5′-TCTCT AGGGTTTCCGGATCACA-3′). The PCR fragment was cloned into the pENTR/D vector (Gateway Technology; Invitrogen) and then introduced into the binary destination vector pHellsgate8 (Hellwell et al., 2002) by recombination using the LR clonase II (Invitrogen). The pHellsgate8::CCD8 RNAi construct was transferred to Agrobacterium tumefaciens strain LB4404 and used to transform tomato (cv Craigella) plants as previously described (van Roekel et al., 1993).

RNA isolation and gene expression analysis by real time quantitative RT-PCR (qPCR)

Total RNA was extracted using Tri-Reagent (Sigma-Aldrich St. Louis, USA) according to the manufacturer’s instructions. The RNA was treated with RQ1 DNase (Promega, Madison, WI, USA), purified through a silica column using the NucleoSpin RNA Clean-up kit (Macherey-Nagel, Düren, Germany) and stored at −80°C until use. For gene expression analysis by real-time quantitative PCR (qPCR) the iCycler iQ5 system (Bio-Rad Hercules, CA, USA) was used (Spinsanti et al., 2006) using specific primers. For the tomato elongation factor-1α (household gene) SlEF, 5′-GATTGGTGTTATGGAACACTGTC-3′ and 5′-AGGGTT GTGGTGACATCTC-3′, for SlCCD7: 5′-AGCCCAAGATTCTCG AGATCCC-3′ and 5′-GGGAGAAGGCGCCACATCTGC-3′; for SlCCD8: 5′-CCAATCGCTGTATAGTTCC-3′ and 5′-GCCTTTACGACGATGTC-3′. The first-strand cDNA was synthesized with 1 μg of purified total RNA using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. Three independent biological replicates were used and each PCR reaction was done in triplicate. Relative quantification of mRNA level was performed using the comparative Ct method (Livak & Schmittgen, 2001). Values were normalised using the Ct value for the tomato household gene SlEF (Rotenberg et al., 2006). Values were used to determine the change in gene expression according to the following calculation: fold-change = 2−ΔΔCt, where ΔCt = Ct (target) − Ct (household) and Δ(ΔCt) = ΔCt (treatment) − ΔCt (control).

Strigolactone and auxin analysis by multiple reaction monitoring liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)

Root exudates were purified and concentrated as previously described (López-Ráez et al., 2008b, 2010) with some modifications. Five liters of root exudates were loaded onto a pre-equilibrated C18 column (GracePure C18-Fast 5000 mg 20 ml−1). Subsequently, columns were washed with 50 ml of demineralised water, and 50 ml of 30% acetone/water. Strigolactones were eluted with 50 ml of 60% acetone/water. All exudates were collected within 2 h and stored at −20°C before measurements. Strigolactones were extracted from root material as previously described (López-Ráez et al., 2010). Xylem sap was collected and purified as previously described (Kohlen et al., 2011a). Analysis and quantification of strigolactones were performed using UPLC-MS/MS, as previously described (Kohlen et al., 2011a). Auxin (IAA) was
extracted, purified and analysed as previously described (Ruyter-Spira et al., 2011).

**Phelipanche ramosa** germination assay and infection study

Germination assays with *Phelipanche ramosa* (L.) Pomel seeds were conducted as previously reported by Matusova et al. (2005). GR24 (10^{-7} M) and demineralised water were included as positive and negative controls, respectively. To perform the *P. ramosa* infection assay, 3-l pots were filled with a soil:sand mixture (3 : 1) and seeds were added following the procedure previously described by Kroschel (2001) with some modifications. *P. ramosa* seeds were sown in a layer of c. 3–10 cm below the surface at a density of 15 mg · pot^{-1}. In order to keep a moist environment, pots were watered daily with 60 ml of tap water for 12 d. Then, 5-d-old tomato seedlings were introduced into the pots and watered for an additional 5 d as described before. Subsequently, pots were irrigated with 300 ml of tap water twice a week and once with 300 ml of full-strength Hoagland solution (Hoagland & Arnon, 1950). Emerged *P. ramosa* shoots were counted 10 wk after tomato planting.

Analysis of AM colonization of **SlCCD8** knock-down lines

The AM fungus *Glomus intraradices* N.C. Schenck & G.S. Sm (BEG 121) was maintained as a soil:sand based inoculum containing a mix of diverse fungal propagules (spores, hyphae and chopped mycorrhizal roots). Tomato seeds of the *SlCCD8* knock-down lines and corresponding wild-type (cv Craigella) were surface-sterilised and germinated for 3 d on a container with sterile vermiculite at 25°C in darkness. Subsequently, individual seedlings were transferred to 0.5-l pots with a sterile sand:soil (4 : 1) mixture. Pots were inoculated by adding 10% (v : v) *G. intraradices* inoculum. The same amount of soil: sand mix but free from AM was added to control plants. For each treatment five replicate plants were used. Plants were randomly distributed and grown in a glasshouse at 24/16°C with 16/8 h photoperiod and 70% humidity and watered three times a week with Long Ashton nutrient solution (Hewitt, 1966) containing 25% of the standard phosphorous concentration. Plants were harvested after 8 wk of growth. Roots were stained with trypan blue (Phillips & Hayman, 1970) and examined using a Nikon Eclipse 50i microscope (Nikon Corporation, Tokyo, Japan) under bright-field conditions. The percentage of root length colonised by the AM fungus was determined by the gridline intersection method (Giovannetti & Mosse, 1980).

**Sucrose analysis by high-performance liquid chromatography (HPLC)**

Samples were extracted and analysed as previously described (Sergeeva et al., 2000).

**Statistical analysis**

Data for strigolactone and auxin content were subjected to one-way analysis of variance (ANOVA) using GenStat for Windows (9th edition). To analyse the results of germination bioassays, ANOVA after arcsine(square root(X)) transformation was used. When appropriate, data were subjected to the Student’s *t*-test.

**Results**

**SlCCD8 cloning and characterization**

A search of the available tomato EST libraries failed to identify ESTs with homology to any published *CCD8* sequence. Therefore, a PCR-based approach using primers designed against highly conserved regions in known plant *CCD8* s in combination with RACE was used to isolate the full-length coding sequence of the putative tomato *CCD8/MAX4*, hereafter designated as *SlCCD8* (IF831532). *SlCCD8* has an open reading frame (ORF) of 1674 bp (Supporting Information Table S1). A BLAST search of the *SlCCD8* sequence was performed on the tomato genome (Bombarely et al., 2011) and the sequence aligned with a 4100-bp region located on chromosome 8. *SlCCD8* is predicted to contain six exons (Fig. 1a). The ORF encodes a 557 amino acid protein (Table S2) with 89% and 66% homology to petunia *CCD8/DAD1* (PhCCD8/DAD1 (Snowden et al., 2005)) and Arabidopsis *CCD8/MAX4* (ArCCD8/MAX4 (Sorefan et al., 2003)) proteins, respectively (Fig. 1b, Table S3). In a phylogenetic alignment, *SlCCD8* clustered closely together with *PhCCD8/DAD1* in what seems to constitute a sub-clade of dicot *CCD8* s (Fig. 1c). Monocot *CCD8* s of maize, rice and sorghum clustered separately from dicot *CCD8* s. *SlCCD8* expression was detected at low levels in all plant tissues. However, it was primarily expressed in tomato roots and stems with the highest expression in roots (Fig. 2a).

In order to address the biological function of *SlCCD8*, an RNA interference (RNAi) construct containing a specific region of 349 bp of the gene was created and introduced into tomato (cv Craigella) through *Agrobacterium tumefaciens*-mediated transformation. Three independent *SlCCD8* RNAi lines (L16, L04 and L09) displaying 64%, 91% and 97% reduction in *SlCCD8* mRNA levels in the roots, respectively (Fig. 2b), were selected and propagated to T3 generation. Transcript levels of the other closely related carotenoid cleavage dioxygenase described in tomato — *SlCCD7* (Vogel et al., 2010) — were not affected in any of the transgenic lines (Fig. S1), demonstrating the specificity of the RNAi construct for *SlCCD8*.

**Role of SlCCD8 in rhizosphere signaling**

The concentrations of all strigolactones previously reported in tomato (López-Raíez et al., 2008a,b) were reduced in the exudates of all three transgenic lines compared with wild-type plants. In L16 strigolactone concentrations were reduced by 52% and in L04 and L09 by 95% (Fig. 3a), correlating with the reduction in *SlCCD8* transcript (Fig. 2b). Colonisation by the mutualistic AM fungus *Glomus intraradices* in L16, L04 and L09 was reduced by 27%, 44% and 65%, respectively, compared to wild-type plants (Fig. 3b). This reduction in AM symbiosis correlated to some extent with the decrease in strigolactone exudation (Fig. 3a). In addition, a 90% reduction in shoot emergence of *Phelipanche ramosa* was observed...
for all three transgenic lines (Fig. 3c). *Phelipanche ramosa* seed germination using root exudates was significantly lower for all transgenic lines (Fig. 3d), and this decrease correlated well with the reduction in strigolactone exudation (Fig. 3a).

Strigolactone concentrations in root extracts of L16, L04 and L09 were also analysed. As in exudates, a clear reduction in the concentration of all strigolactones was detected for all three transgenic lines (Fig. 4a). On average, the strigolactone concentration in root extracts of L16, which showed the mildest reduction in *SlCCD8* expression, was reduced by 53%, whereas strigolactone concentrations in L04 and L09 were reduced by 92% and 94%, respectively (Fig. 4a). This decrease in strigolactone production was almost identical to the reduction observed in the root exudates (Fig. 3a).

As mentioned above, strigolactone production is induced under phosphate deficiency (Yoneyama *et al.*, 2007; López-Raéz *et al.*, 2008a), just as the concentration of orobanchol in the xylem sap of *Arabidopsis* (Kohlen *et al.*, 2011a). Indeed, a five-fold increase in the concentration of orobanchol was also detected in the xylem sap of phosphate-starved wild-type tomato plants (Fig. 4b, Fig. S2). The three knock-down lines L16, L04 and L09 showed a clear reduction in xylem sap orobanchol concentrations (26%, 55% and 84%, respectively) compared with wild-type plants (Fig. 4c). Interestingly, neither solanacol nor any of the

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**Fig. 1** Tomato (*Solanum lycopersicum*) *CCD8* (*SlCCD8*). (a) The postulated intron/exon structure for *SlCCD8* (4100 nt). (b) Alignment of the putative *SlCCD8* amino acid sequence with those from known *CCD8* proteins; Sl, *Solanum lycopersicum*; Ph, Petunia hybrida; At, *Arabidopsis thaliana*. Identical and similar amino acids are shaded in black and grey, respectively (c) Phylogenetic tree of known *CCD1*, *CCD7* and *CCD8* nucleotide sequences; Sl, *Solanum lycopersicum*; Ph, *Petunia hybrida*; At, *Arabidopsis thaliana*; Ps, *Pisum sativum*; Os, *Oryza sativa*; Zm, Zea mays; Mt, *Medicago truncatula*; Sb, *Sorghum bicolor* (*AtCCD1* (AT3G63520), OsCCD1 (Os12g0640600), PhCCD1 (AY576003), *SlCCD1a* (AY576001), *SlCCD1b* (AY576002), *ZmCCD1* (GRMZM2G057243), *AtCCD7* (AT2G44990), *MtCCD7* (Medtr7g040830), *OsCCD7* (Os04g0550600), *PsCCD7* (DQ403160), *PhCCD7* (FJ790878), *SlCCD7* (GQ468556), *ZmCCD7* (GRMZM2G158657), *AtCCD8* (AT4G32810), *MtCCD8* (Medtr3g127920), *OsCCD8a* (Os01g0566500), *OsCCD8b* (Os01l0746400), *PsCCD8* (AY557342), *PhCCD8* (AY743219), *SbCCD8* (Sb03g034400), *SlCCD8* (JF831532), *ZmCCD8* (GRMZM2G446858).

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**Fig. 2** *SlCCD8* transcript accumulation (normalised to the tomato (*Solanum lycopersicum*) household gene *SlEF*). (a) Relative gene expression of *SlCCD8* in tomato cv Craigella (WT) in different plant tissues: R, root; S, stem; L, leaf; F, flower; MG, mature green fruit. (*n* = 3) (b) Relative gene expression of *SlCCD8* in the roots of cv Craigella (WT) and three independent *SlCCD8* RNAi lines (L16, L04, and L09) The expression in wild-type tomato is set at 100% (*n* = 3). Error bars represent means ± SE. Bars with different letters differ significantly at *P* < 0.05.

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**Fig. 3** Strigolactone concentrations in root extracts of L16, L04 and L09 were also analysed. As in exudates, a clear reduction in the concentration of all strigolactones was detected for all three transgenic lines (Fig. 4a). On average, the strigolactone concentration in root extracts of L16, which showed the mildest reduction in *SlCCD8* expression, was reduced by 53%, whereas strigolactone concentrations in L04 and L09 were reduced by 92% and 94%, respectively (Fig. 4a). This decrease in strigolactone production was almost identical to the reduction observed in the root exudates (Fig. 3a).

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didehydro-orobanchol isomers were detected in the xylem sap. A second strigolactone – orobanchyl acetate – was also detected in tomato xylem sap (Fig. S2), but its concentration was too low to accurately quantify its reduction in the transgenic lines or to assess the effect of phosphate starvation on the concentration of this compound in the xylem. No sucrose was detected in any of the xylem sap samples analysed (Fig. S3), showing the samples are not contaminated with phloem sap. Furthermore, hypocotyls were also analysed for their strigolactone content, but strigolactones were undetectable in these samples (Fig. S4). These data confirm that the orobanchol detected is from the xylem sap and not from contaminating phloem sap or hypocotyl tissue.

Effect of decreased SLCCD8 expression on shoot architecture

In order to assess the consequence of the reduced strigolactone concentrations on shoot architecture, L16, L04 and L09 lines were grown in pots for 8 wks and their phenotypes compared with wild-type plants (Fig. 5a). All three knock-down lines were significantly more branched, displaying a 1.7-, 4.4- and 7.3-fold increase in lateral shoot branch number, respectively (Fig. 5b), which inversely correlated with the level of SLCCD8 transcript (Fig. 2b) and strigolactone production (Figs 3a, 4a). GR24 application complemented the branching phenotype (Fig. 5c), further confirming its relation to strigolactone depletion. To get a more detailed insight into the effect of the reduction in SLCCD8 expression on branching, the distribution of first- and second-order branches was analysed. In wild-type plants c. 50% of the primary stem nodes were carrying a visible lateral branch, most of which were shorter than 5 cm (Fig. S5). All SLCCD8 knock-down lines had a significantly (P < 0.05) higher number of branches of the first order (Fig. S5). Moreover, these branches were longer than in the wild-type plants (Fig. S5). No branches of the second order were observed in any of the wild-type plants at this stage of development (Figs 5d, S5), whereas all knock-down lines displayed multiple lateral branches of the second order (Figs 5e, S5). The transgenic lines – L16, L04 and L09 – also displayed a reduction in the primary stem height of 19%, 52% and 60%, respectively, compared with wild-type plants (Fig. 5f). In addition, the total number of nodes in the transgenic lines increased slightly, but significantly (P < 0.05; Fig. 5g).

SLCCD8 reduction alters adventitious rooting

In addition to these phenotypes, all transgenic plants, but not the wild-type, displayed a larger number of root primordia and adventitious roots on their stems (Fig. 6a,b). The severity of this phenotype was less pronounced in L16 than in L04 and L09, correlating with the stronger reduction in strigolactone biosynthesis in these latter two lines (Fig. 4a). Cuttings from the SLCCD8 knock-down lines produced 34%, 77% and 89% (L16, L04 and L09, respectively) more adventitious roots compared with wild-type plants (Fig. 6c). Moreover, application of GR24 also reduced the number of adventitious roots (Fig. 6c) and adventitious root primordia (Fig. 6d), a phenotype that could be rescued by GR24 application (Fig. 6c). In addition, the total number of nodes in the transgenic lines increased slightly, but significantly (P < 0.05; Fig. 5g).

Effect of SLCCD8 knock-down on tomato reproductive development

An unexpected phenotype observed in the SLCCD8 knock-down lines was that their flowers were significantly smaller than in
wild-type plants (Fig. 7a). In order to quantify this effect and elucidate a possible role of strigolactones in flower development, the length of sepals, petals and anthers were measured at anthesis. The average length of all these organs was significantly ($P < 0.05$) reduced in all three transgenic lines (Fig. 7b). The diameter of the ovaries was also significantly reduced (Fig. 7c). This effect persisted throughout fruit development (Fig. 7d), leading to significantly smaller fruits in all transgenic lines compared with the wild-type in different ripening stages, mature green (40 DAP), breaker and ripe red (Fig. 7e,f). Although fruit size of all transgenic lines was reduced, only in L04 and L09 was the total fruit yield slightly decreased (Fig. 7g).

Fig. 4 MRM-LC-MS/MS quantification of strigolactone content in planta of tomato (Solanum lycopersicum) cv Craigella (WT) and three independent SlCCD8 RNAi lines (L16, L04, and L09). (a) Quantification of the major tomato strigolactones (solanacol, didehydro-orobanchol isomers 1 and 2, and orobanchol) in root extracts of 5-wk-old plants according to the peak area ($n = 3$). (b) Quantification of the effect of treatment with sufficient phosphate (+ Pi) and limiting phosphate (− Pi) on orobanchol content in tomato xylem sap ($n = 3$). (c) Quantification of orobanchol in tomato xylem sap of 8-wk-old plants ($n = 5$). Error bars represent means ± SE. Bars with different letters differ significantly ($P < 0.05$).

Fig. 5 Analysis of plant architecture of tomato (Solanum lycopersicum) cv Craigella (WT) and three independent SlCCD8 RNAi lines (L16, L04, and L09). (a) Photograph of 4-wk-old wild type and transgenic SlCCD8 RNAi line L09 plants. (b) Average number of visible branches (1st and 2nd order, > 2 mm) on 8-wk-old plants ($n = 5$). (c) The effect of GR24 on branching in 4-wk-old plants ($n = 5$). Light grey bars, 5 μM GR24; dark grey bars, control. (d) Close-up of 8-wk-old wild-type primary branch. (e) Close-up of 8-wk-old L09 primary branch, arrows indicate secondary branches. (f) Average main stem length of 8-wk-old plants ($n = 5$). (g) Average number of nodes per main stem of 8-wk-old plants ($n = 5$). Error bars represent means ± SE. Bars with different letters differ significantly ($P < 0.05$).
As fruit size usually correlates with seed quantity (Mapelli et al., 1978), the effect of SlCCD8 knock-down on seed set was investigated. A reduction of about 65% in seed quantity (Fig. 7h) and a reduction in seed size (Fig. S6) was observed in all three knock-down lines. Despite their reduced number and size, the quality of the seeds appeared to be unaffected because no obvious differences in germination rate were observed in any of these lines when germinated under normal conditions (Fig. S7).

Auxin plays an important role in several strigolactone-related phenotypes and it has been shown to be one of the major regulators of fruit development (Mapelli et al., 1978; Gillaspy et al., 1993). Moreover, it has recently been demonstrated that during fruit development an auxin gradient between developing seeds, placenta and the pericarp is established during phase III, with relatively high auxin concentrations in the developing seeds (Pattison & Catalá, 2012). For this reason the concentration of free auxin was assessed in fruits harvested during this phase of fruit development. Indeed, in wild-type fruits a 4.5-fold higher concentration of IAA was detected in the fruit parts containing the seeds compared with the pericarp (Fig. 7i). This auxin gradient between the different tissues was absent in the SlCCD8 knock-down fruits (Fig. 7i).

Discussion

In the present study we identified and characterised a second strigolactone biosynthetic gene in tomato – SlCCD8 – encoding a carotenoid cleavage dioxygenase. SlCCD8 showed highest homology to the petunia PhDAD1/CCD8, belonging to a dicot subclade within the CCD8 cluster, clearly separated from the monocots. SlCCD8 is expressed in all tissues examined, but predominantly in root and stem tissue. This expression pattern is in agreement with CCD8 expression in petunia, Arabidopsis, pea and rice (Napoli, 1996; Sorefan et al., 2003; Bainbridge et al., 2005; Arite et al., 2007), indicating a conserved pattern across plant species. In the Arabidopsis max4/CCD8 mutant, MAX3/CCD7 expression was shown to be downregulated (Mashiguchi et al., 2009). This feedback seems not to be present in tomato as the expression of SlCCD7 was not reduced. It has been reported that CCD7 is involved in processes other than strigolactone biosynthesis such as the production of mycorradacin in mycotrophic plants such as tomato (Walter et al., 2010). Therefore, the regulation of CCD7 may differ between tomato and other nonmycorrhizal plant species. By generating SlCCD8 knock-down lines with a reduction in strigolactone levels, we showed that SlCCD8, through strigolactone biosynthesis, is involved in the regulation of multiple processes relevant for plant physiology and rhizosphere signaling.

Strigolactones were initially identified as germination stimulants for root parasitic plants of the Orobanchaceae (Cook et al., 1966; Bouwmeester et al., 2003) and hyphal branching factors for AM fungi (Akiyama et al., 2005). As expected, reduced strigolactone concentrations in all the SlCCD8 knock-down tomato lines resulted in reduced AM colonisation (Fig. 3a,b). However, there was a nonlinear correlation between AM colonisation and strigolactone reduction, with the reduction in mycorrhization being less severe than the reduction in strigolactone concentrations. This is probably due to the use of a mixed inoculum containing mycelium, colonised roots and spores. Because strigolactones are hyphal branching factors for AM fungi that seem to be particularly important in germinating spores, AM colonisation could be more compromised by strigolactone reduction if only spores are used, as was previously demonstrated (Koltai et al., 2010b). In the present study a mixed inoculum was used as it more closely resembles the natural situation in the rhizosphere (Klironomos & Hart, 2002). Interestingly, the infection of all SlCCD8 knock-down lines by P. ramosa was reduced by 90% compared with wild-type plants (Fig. 3c). The observed reduction in emerging parasite shoots in L16 cannot be explained exclusively by its reduced strigolactone exudation (c. 60%), also considering that germination of P. ramosa seeds when using root exudates was only reduced by c. 50%. Possibly, the increased shoot branching of the host and the associated resource requirement inhibits P. ramosa development. Alternatively, it might be possible that strigolactones produced by the host plant are also required in later phases of the P. ramosa lifecycle after seed germination. Nevertheless, our results show that a mild reduction in strigolactone exudation could be sufficient to
significantly reduce parasitic weed infection without severely compromising AM symbiosis or apical dominance (Figs 3b, 5). Moreover, fruit yield of L16 was hardly affected, even though initial fruit set was delayed (Fig. 7i). These findings make strigolactone biosynthesis an attractive target for controlling root parasitism, as previously postulated (Bouwmeester et al., 2003; López-Ráez et al., 2009, 2011b; Cardoso et al., 2011). However, more research is needed to further assess the consequences of a mild reduction in strigolactone content on fruit quality and parasitic weed resistance under field conditions.

Fig. 7 Analysis of flowers, fruits and seeds of tomato (Solanum lycopersicum) cv Craigella (WT) and three independent SICCD8 RNAi lines (L16, L04, and L09). (a) Photograph of flowers at anthesis. (b) Flower organs length (n = 5). (c) Diameter of un-pollinated ovaries (n = 5). (d) Diameter of fruits at phase III tomato fruit development (14 DAP, days after pollination; n = 10). (e) Diameter of mature green (MG) fruits (40 DAP; n = 10). (f) Photograph of tomato fruits; mature green (MG), breaker (BR) and mature red (MR). (g) Fruit yield in grams fresh weight over a 10-wk period. (h) Number of seeds per fruit (n = 10). (i) Auxin distribution at phase III (14 DAP); PT, pericarp tissue; RT, remaining tissue. (n = 3). Error bars represent means ± SE. Bars with a different letter differ significantly (P < 0.05).
The concentrations of strigolactones in root extracts in all SlCCD8 knock-down lines were reduced to the same extent as in their exudates. This shows that, as expected, strigolactone biosynthesis and not secretion is compromised in these transgenic lines. All transgenic lines displayed a reduced primary stem height and an increase in the total number of lateral branches. Both phenotypes inversely correlated – to some extent – with the strigolactone concentrations. Surprisingly, in roots of L04 the reduction in SlCCD8 expression and strigolactone content were more severe than expected considering the moderate increase in lateral shoot outgrowth when compared with L09. Interestingly, a better inverse correlation with lateral branching across the knock-down lines was observed with the concentrations of orobanchol present in the xylem sap (Fig. 4c). It seems that the orobanchol concentration in the xylem sap was less affected than in the exudates, which suggests a preferential loading into the xylem instead of secretion to the rhizosphere. The ratio of orobanchol and the other strigolactones was much higher in root extracts than in root exudates. Furthermore, neither solanacol nor any of the didehydro-orobanchol isomers were detected in the xylem sap, whereas they were abundantly present in root exudates and extracts (Figs 3a, 4a). This suggests that a selective mechanism of localised strigolactone biosynthesis and/or transport ensures that orobanchol is less well secreted into the rhizosphere and is preferentially transported through the xylem to the shoot. Therefore, indicating that this strigolactone (and/or its derivatives) might be the one that is regulating shoot branching, while solanacol and the didehydro-orobanchol isomers could have a role as signaling molecules in the rhizosphere (Fig. 8). As strigolactones also co-regulate root system architecture (Kapulnik et al., 2011; Ruyter-Spira et al., 2011), it is possible that all are involved in this belowground function (Fig. 8). However, it is also possible that solanacol and the didehydro-orobanchol isomers have no function in the plant and merely act as rhizosphere signaling molecules. It was previously proposed that solanacol is derived from orobanchol through didehydro-orobanchol (Xie et al., 2010; Kohlen et al., 2011b). Therefore, orobanchol might play a double role as the transmissible signal regulating root and shoot architecture, and as the precursor to signaling molecules in the rhizosphere.

When strigolactone production was assessed under phosphate-limiting conditions, a strong increase in all tomato strigolactones was detected in root extracts (Fig. S8), which is in line with previous findings (López-Ráez et al., 2008a). In addition, we show that phosphate limitation also induced a five-fold increase in orobanchol concentrations in the xylem sap (Fig. 4b). Therefore, it seems that this upregulation under phosphate deficiency is a more conserved trade as it has also been demonstrated in Arabidopsis (Kohlen et al., 2011a).

A reduction in main stem height was also observed for all transgenic lines, again correlating with xylem sap orobanchol concentrations. The reduction in plant height inversely correlated to the increased number of shoot branches. This suggests that perhaps the observed dwarfism in the strigolactone-deficient knock-down lines is a secondary effect of the increased lateral shoot growth. However, the total number of internodes slightly increased in all transgenic lines (Fig. 5g). Our results show that the reduction in plant height in the SlCCD8 knock-down lines is due to shorter internodes maybe because of increased competition for nutrients between the primary stem and the axillary branches. However, the increase in node number suggests that strigolactones are not only involved in the regulation of bud outgrowth, but possibly also in the timing of organ development. Additional research will be needed to study this in more detail.

It has already been shown that strigolactones play a role in more plant development processes than just axillary bud outgrowth (Tsuchiya et al., 2010; Kapulnik et al., 2011; Ruyter-Spira et al., 2011). Strigolactones were also shown to co-regulate adventitious root development in pea (Rasmussen et al., 2012). Our results corroborate this new function because all transgenic lines showed a marked increase in adventitious root development and rooting of cuttings (Fig. 6). Interestingly, the increase in adventitious rooting in the transgenic lines correlated with elevated auxin concentrations in the lower region of the stem, possibly a result of elevated transport as was previously reported (Bennett et al., 2006). It is known that auxin induces lateral root formation (Klerk et al., 1999), therefore it is plausible that the observed repressive effect of strigolactones on adventitious root formation is a consequence of their assumed control over auxin transport. However, it was recently postulated that the increase in adventitious root initiation in pea and Arabidopsis is not due to elevated auxin concentrations but to increased auxin sensitivity (Rasmussen et al., 2012). The fact that the combined application of IAA and GR24 suppressed the increase in adventitious rooting induced by IAA (Fig. 6d) seems to support this hypothesis. If so, the elevated IAA concentrations detected in the lower part of the stem might be a secondary effect of reduced auxin sensitivity. Nevertheless, our results indicate that a link between strigolactones and auxin exists in adventitious root initiation. However, to determine the precise underlying mechanism of strigolactones-auxin cross-talk in relation to this phenotype further research will be needed.

A putative function for strigolactones in fruit development was already postulated as SlCCD7 is highly expressed in mature green and turning tomato fruits (Vogel et al., 2010), and the expression
of AcCCD7 and AcCCD8 in Actinidia chinensis (kiwifruit) was shown to be relatively high in young fruits and seeds (Ledger et al., 2010). The petunia ccd8/dad1 mutant was reported to have smaller flowers (Snowden et al., 2005). In agreement with these observations, we observed that in the SICCD8 knock-down lines sepal, petals, and anthers were smaller than in wild-type plants (Fig. 7), suggesting that strigolactone deficiency affects flower development. It might be that this phenotype is the result of increased competition for resources in these highly branched plants. However, the possibility that strigolactones have a more direct role in controlling flower development cannot be excluded. In addition to smaller flowers, the fruit size in the transgenic lines was also reduced, a reduction which correlated inversely with the strigolactone content in these lines. The relative reduction in fruit size was similar to the reduction in the diameter of un-pollinated ovaries. This implies that the initial reduction in ovary size is not compensated during later stages of fruit development, but also that the severity of the phenotype does not increase, which would be expected if the reduced fruit size is only caused by competition for resources. Moreover, as fruit yield was not strongly reduced in any of the transgenic lines, resource limitation seems not to be exclusively responsible for these reproductive phenotypes. It has been reported that auxin produced in developing seeds is required for fruit growth (Mapelli et al., 1978). Seed number, as well as seed size in the transgenic lines were clearly reduced compared with wild-type plants (Fig. 7g), and it is hence possible that this reduction – resulting in a lower auxin production – is responsible for the observed reduction in fruit size. However, it cannot explain the reduction in ovary size before pollination, because no auxin producing seeds are present at this stage. As mentioned above, cross-talk between strigolactones and auxin seems to play an important role in regulating various processes in plant development and rhizosphere signaling. We showed that a relatively small reduction in strigolactone secretion has a profound impact on P. ramosa infection, whereas AM interaction, apical dominance and fruit yield are only mildly affected, demonstrating that strigolactone reduction can be a suitable strategy against root parasitic weeds. In addition, specificity in strigolactone transport in and ex planta is proposed, as only orobanchol is preferentially loaded into the xylem. Finally, the phenotype of SICCD8 knock-down plants confirms a role for strigolactones in branching and adventitious root development and hints at a new role for strigolactones in reproductive development. Further research is required to establish whether this is a direct or an indirect effect.

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References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Relative SlCCD7 expression.

Fig. S2 MRM-LC-MS/MS analysis of tomato xylem sap.

Fig. S3 Analysis of sucrose in xylem sap.

Fig. S4 Strigolactone analysis in tomato root and hypocotyl tissue under limiting phosphate conditions.

Fig. S5 Distribution of lateral branches.

Fig. S6 Average seed size.

Fig. S7 Tomato seed germination.

Fig. S8 Strigolactone analysis in tomato root exudates under sufficient and limiting phosphate conditions.

**Table S1** SlCCD8 complete nucleotide sequence

**Table S2** SlCCD8 complete amino acid sequence

**Table S3** Sequence homology of SlCCD8

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