Research paper

**CAROTENOID CLEAVAGE DIOXYGENASE 7 modulates plant growth, reproduction, senescence, and determinate nodulation in the model legume *Lotus japonicus***

Junwei Liu1, Mara Novero2, Tatsiana Charnikhova3, Alessandra Ferrandino1, Andrea Schubert1, Carolien Ruyter-Spira3, Paola Bonfante2, Claudio Lovisolo1, Harro J. Bouwmeester3 and Francesca Cardinale1,*

1 Department of Agriculture, Forestry and Food Sciences, University of Turin, via Leonardo da Vinci 44, 10095 Grugliasco (TO), Italy
2 Department of Life Sciences and Systems Biology, University of Turin, viale Mattioli 25, 10025 Turin, Italy
3 Laboratory of Plant Physiology, Wageningen University, Droevendaalsesteeg 1, NL-6708 PB Wageningen, The Netherlands

*To whom correspondence should be addressed. E-mail: francesca.cardinale@unito.it

Received 21 November 2012; Revised 25 January 2013; Accepted 14 February 2013

Abstract

Strigolactones (SLs) are newly identified hormones that regulate multiple aspects of plant development, infection by parasitic weeds, and mutualistic symbiosis in the roots. In this study, the role of SLs was studied for the first time in the model plant *Lotus japonicus* using transgenic lines silenced for **CAROTENOID CLEAVAGE DIOXYGENASE 7 (LjCCD7)**, the orthologue of *Arabidopsis More Axillary Growth 3*. Transgenic LjCCD7-silenced plants displayed reduced height due to shorter internodes, and more branched shoots and roots than the controls, and an increase in total plant biomass, while their root:shoot ratio remained unchanged. Moreover, these lines had longer primary roots, delayed senescence, and reduced flower/pod numbers from the third round of flower and pod setting onwards. Only a mild reduction in determinate nodule numbers and hardly any impact on the colonization by arbuscular mycorrhizal fungi were observed. The results show that the impairment of CCD7 activity in *L. japonicus* leads to a phenotype linked to SL functions, but with specific features possibly due to the peculiar developmental pattern of this plant species. It is believed that the data also link determinate nodulation, plant reproduction, and senescence to CCD7 function for the first time.

Key words: Arbuscular mycorrhizal fungi (AMF), carotenoid cleavage dioxygenase, determinate nodulation, leaf senescence, *Lotus japonicus*, reproduction, shoot and root branching; strigolactone.

Introduction

Plant development is plastic and environmentally sensitive, and is regulated by hormones acting as long-range signals to integrate developmental, genetic, and environmental inputs. In the control of shoot and root branching, for example, large variations in plant architecture can be generated in a single genotype in response to a number of cues (Domagalska and Leyser, 2011; Müller and Leyser, 2011). The role of classic plant hormones, such as auxin and cytokinins, in the regulation of shoot and root branching and in the maintenance of coordinated growth between root and shoot has been extensively studied (Sachs, 2005; Hwang et al., 2012).

Recently, strigolactones (SLs) have been identified as a new class of branch-inhibiting hormones that seem to fine-tune the regulation of shoot branching further (Gomez-Roldan et al., 2008; Umehara et al., 2008; Xie et al., 2010). In the past decade, genotypes affected in the SL pathway were identified, and include *ramosus* (*rms*) mutants in pea, *decreased apical dominance* (*dad*) in petunia, *more axillary branch* (*max*) in *Arabidopsis*, and *high-tillering dwarf* (*htd* or *d*) in rice (Beveridge and Kyozuka, 2009).

Besides regulating shoot architecture, SLs contribute to shaping the root system, by affecting primary root length,
adventitious root formation, lateral root initiation and subsequent outgrowth, and root hair elongation (Koltai, 2011, 2012; Ruyter-Spira et al., 2011; Rasmussen et al., 2012). Moreover, a broad range of developmental roles was postulated for SLs in the alleviation of secondary seed dormancy induced by high temperature (Toh et al., 2012), hypocotyl elongation (Hu et al., 2010; Tsuchiya et al., 2010), secondary growth (Agusti et al., 2011), light harvesting (Mayzlish-Gati et al., 2010; Tsuchiya et al., 2010), reproductive development (Snowden et al., 2005; Kohlen et al., 2012), and leaf senescence (Snowden et al., 2005; Ledger et al., 2010). SLs seem to be specifically involved in the establishment of the symbiosis with nitrogen-fixing bacteria in legumes, namely in the formation of indeterminate nodules (Soto et al., 2010; Foo and Davies, 2011; Foo et al., 2013). Finally, besides their hormonal role, SLs exuded into the rhizosphere stimulate the germination of parasitic plants (Yoneyama et al., 2010; Lechat et al., 2012) and the branching of arbuscular mycorrhizal fungi (AMF) (Akiyama et al., 2005; Bouwmeester et al., 2007; Yoshida et al., 2012). A recent report suggests that the hormonal effect on the elongation of rhizoids (the equivalent of root hairs in higher plants) in ancestral species of the green lineage pre-dates the exogenous function as signalling molecules in the rhizosphere (Delaux et al., 2012).

SLs are a family of carotenoid-derived terpenoid lactones mostly produced in roots (Matusova et al., 2005). The precursor all-trans-β-carotene is converted by the recently characterized β-carotene isomerase D27 into 9-cis-β-carotene (Alder et al., 2012). The isomerized substrate is then cleaved by two double bond-specific cleavage enzymes (carotenoid cleavage dioxygenases, CCDs), CCD7 and CCD8. The 9°, 10° bond of 9-cis-β-carotene is cleaved by CCD7, yielding β-ionone (C15) and 10°-apo-β-carotenal (C27). The latter compound is subsequently cleaved and cyclized by CCD8 into a bioactive SL precursor named carlactone (Booher et al., 2004; Schwartz et al., 2004; Alder et al., 2012). Several orthologues of these two CCDs have been characterized in Arabidopsis, pea, petunia, rice, and tomato (Morris et al., 2001; Sorefan et al., 2003; Booher et al., 2004; Snowden et al., 2005; Zou et al., 2006; Arite et al., 2007; Drummond et al., 2009; Vogel et al., 2010; Kohlen et al., 2012). CCD8 orthologues are also characterized in kiwifruit, chrysanthemum, maize, and the moss Physcomitrella patens (Ledger et al., 2010; Liang et al., 2010; Proust et al., 2011; Guan et al., 2012). MAX1, a class-III cytochrome P450 protein of Arabidopsis, has also been proposed to act in the SL biosynthetic pathway, namely converting carlactone into 5-deoxystrigol, but its biochemical action still needs to be resolved experimentally (Booher et al., 2005; Alder et al., 2012). Other genes putatively involved in SL biosynthesis have been identified in several species (SIORT1 and AtPPDS5), but their exact function is so far unknown (Koltai et al., 2010b; Roose et al., 2011). No orthologues of any of the above biosynthetic genes have been characterized in Lotus japonicus yet.

Lotus japonicus is a perennial legume of temperate climates, and a model plant for several developmental processes and interactions with soil (micro)organisms (Handberg and Stougaard, 1992; Lohar and Bird, 2003); the first SL molecule described as a branching factor for AMF, 5-deoxystigrol, was isolated from this plant (Akiyama et al., 2005). Additionally, L. japonicus has a phyllotaxis distinct from that of other model plants such as Arabidopsis, pea, petunia, tomato, and rice. In fact, all of its cotyledonary axillary buds develop immediately into lateral shoots even in very young seedlings. This, and the regular, proliferative accessory (axillary) meristem initiation and immediate development of axillary shoots, makes L. japonicus an attractive experimental model to study the regulation of axillary meristem initiation and development (Alvarez et al., 2006). Namely, how SLs specifically affect the architecture of a plant with this kind of phyllotaxis is not known at present. Also, L. japonicus develops determinate nodules, differently from pea and Medicago sativa, the two species investigated so far for the role of SLs in nodulation.

In this study, the cloning of the Lotus orthologue of CCD7 (LjCCD7), and an overall characterization of its role in the regulation of plant architecture, reproductive development, senescence, and root symbiosis are reported. The results also link CCD7 expression with the regulation of determinate nodulation, reproduction, and senescence in L. japonicus. These latter phenotypes have not been reported so far for any CCD7 orthologue.

Materials and methods

RNA isolation, cDNA synthesis, and quantitative reverse transcription–PCR (RT–qPCR)

For transcript quantification and rapid amplification of cDNA ends (RACE) cloning purposes, RNA was isolated from freshly harvested tissues of wild-type L. japonicus ecotype Gifu B-129 and transgenic lines in the same background with Tripure reagent (Roche). On-column DNase digestion was performed with an RNase-free DNase kit, and total RNA was further purified by an RNasy Plant Mini Kit (both Qiagen). RNA quality and integrity were checked by NanoDrop ND-2000 and standard gel electrophoresis. A 1 μl aliquot of total RNA was reverse transcribed to cDNA with an iScript cDNA synthesis kit (Bio-Rad). RT–qPCRs were set up in 20 μl using the IQ SYBR Green Supermix on the IQ5 Real-Time PCR system (Bio-Rad). LjCCD7-specific primers are listed in Supplementary Table S1 available at JXB online. Ubiquitin (LjUBI) transcript was used as a normalizer (Yokota et al., 2009). Quantification followed the 2-ΔΔCT method.

5′- and 3′-RACE

The putative LjCCD7 gene was identified based on the EST (expressed sequence tag) in the Kazusa database (http://www.kazusa.or.jp/lotus/) aligning best under BlastP default settings with query sequences from Arabidopsis (GI: 330255400) and pea (GI: 90019042). The SMART RACE cDNA amplification kit (Clontech) was used to amplify the unknown ends of the coding sequence in a cDNA pool from Lotus roots, and cloning was performed using the Advantage 2 PCR enzyme system (Clontech) and primers UPM with GSP1 or GSP2 (Supplementary Table S1 at JXB online). PCR conditions for both cDNA ends were five cycles at 94 °C for 30 s, 72 °C for 3 min; five cycles at 94 °C for 30 s, 70 °C for 30 s, 72 °C for 3 min; and 25 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. RACE products were electrophoresed, tested with primers NestF and NestR (Supplementary Table S1 at JXB online), cloned in the pGEM-T easy vector (Promega), and sequenced (BMR...
Plasmid constructs
For expression in *Escherichia coli* of glutathione S-transferase (GST–LjCCD7), an EcoRI fragment was subcloned from *pDONR-LjCCD7* into pGEX-SX-3. To generate the LjCCD7 silencing vector, a 250bp region (from 1451bp downstream of the transcription start site to the stop codon, within the last exon) was amplified with *Pfu* polymerase (Promega) and primers RNAiF and RNAiR (Supplementary Table S1 at *JXB* online). The PCR product was directly sequenced and cloned in pDONR221 to generate the entry clone *pDONR-LjCCD7*. Finally, a single-step Gateway-based reaction was performed with *pDONR-LjCCD7* and the destination vector *pTKO2* (*Snowden et al., 2005*). In parallel, the negative control (NC) construct *pTKO2_pENTR* was generated with the empty entry vector *pENTR4* and the destination vector *pTKO2*. Both *pTKO2_LjCCD7* and *pTKO2_pENTR* constructs were sequenced with primer pairs 572F/1133R and 1388F/1847R (Supplementary Table S1 at *JXB* online).

Protein expression and in vitro enzyme assays
Cultures of *E. coli* BL21 (600ml) harbouring pGEX_LjCCD7 or the empty vector in 2×YT medium (per litre: 16g of tryptone, 10g of yeast extract, and 5g of NaCl) were grown at 30 °C until A-abs=0.5. Recombinant protein expression was induced by 0.2mM isopropyl-β-D-thiogalactopyranoside at 18 °C for 24h. *Escherichia coli* cells were harvested by centrifugation at 4 °C, resuspended in cold STE buffer (100mM NaCl, 10mM TRIS, 1mM EDTA, pH 8.0) containing 100 μg ml⁻¹ lysozyme, incubated on ice for 15min, and then lysed by sonication. The recombiant protein was purified with glutathione-Sepharose 4B (GE Healthcare), visualized by 10% SDS-PAGE, and electrotransferred to a blotting membrane (Fluorotrans, Fluka). Western blot analysis was performed by blocking with 5% non-fat dehydrated milk in TBST buffer (10mM TRIS-HCl, 150mM NaCl, and 0.05% Tween-20), followed by incubation with polyclonal antibodies against GST and then against alkaline phosphatase (Sigma) diluted respectively at 1:5000 and 1:10 000 in TBST buffer with 0.5% milk. Extensive washing in TBST buffer was followed by nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyolphosphate staining (Sigma).

For *in vitro* enzymatic assays, reactions were performed as previously reported (Schwartz et al., 2004; Marasco et al., 2006) with minor modifications. Soluble proteins were quantified with the Bradford reagent (Bio-Rad). Aliquots of the affinity-purified GST–LjCCD7 protein (50–100 μg in 100 μl of glutathione elution buffer) were brought to 400 μl with 100mM TRIS buffer, pH 7.0 containing 300mM NaCl, 0.5mM FeSO₄, 10mM dithiorthreitol (DTT), 5mM ascorbate, and 0.05% Triton X-100. After 20min of equilibration, 80 μl of substrate (0.5mM Type II β-carotene in acetonitrile; presumably all-trans, Sigma C4592) were added. Note that even if 9-cis-β-carotene is the real substrate of CCD7 (*Aldex et al., 2012*), 9-cis-β-carotene can also be cleaved (though not as efficiently), and is the commercially available isomer. Reaction tubes were gently shaken in the dark at 28 °C for 5h before being quenched with 50 μl of 33% formamide for 10min at 37 °C. A 600 μl aliquot of acetonitrile was added to each tube and the organic layer was saved for HPLC analysis. A Perkin Elmer series 200 HPLC system equipped with a diode array was used for detection. Separation was performed using a C18 reversed-phase column (250 mm×4.6 mm internal diameter, 5 μm particles; YMC Europe) with the solvent system methanol:water (70:30, v/v) containing 0.1% ammonium acetate (B) and methanol (A) as described (Marasco et al., 2006).
and P16) and analysed as reported (Ni et al., 2009). From the third round of seed setting until plants were 11 months old, flowers were counted on three plants for each of the three RNAi lines and NCs, and pods were harvested. The definitions used to describe Lotus phylloclades are as in Alvarez et al. (2006).

SL extraction and LC-MS/MS analysis

Two-week-old seedlings of transgenic line P16 and transgenic NC (15 each) were transplanted into an X-stream 20 aeroponic system (Nutriculture) and supplied with 5 litres of modified half-strength Hoagland solution (Hoagland and Arnon, 1950) refreshed twice a week, and irrigated with B&D nutrient solution (Broughton and Arnon 1950) refreshed twice a day. After 5 h incubation in the presence of β-carotene (C\textsubscript{40}) as substrate, two compounds were detected in the presence of GST–LjCCD7 and not with GST alone. One of them had a β-9', 10'-bond of β-ionone and its apparent size (~98 kDa) was assumed to derive from their product. The predicted polypeptide was 74% identical to RMS5 and 58% identical to MAX3. A phylogenetic tree (Supplementary Fig. S1 at JXB online) was produced from an alignment of several CCD7 homologues from land plants and moss, as well as of some putative homologues identified in cyanobacteria (Cui et al., 2012). The CCD7 proteins clustered into several clades, with LjCCD7 in the subclade of predicted leguminous homologues within the dicot clade, as expected (Supplementary Fig. S1 at JXB online).

To verify whether the phylogenetic prediction corresponded to a conserved enzymatic function, the carotenoid cleavage activity of LjCCD7 was tested \textit{in vitro}. To do so, recombinant GST–LjCCD7 was affinity purified from \textit{E. coli} cells, and its apparent size (~98 kDa) checked by SDS–PAGE and western blotting (Supplementary Fig. S2A, B at JXB online). After 5 h incubation in the presence of β-carotene (C\textsubscript{40}) as substrate, two compounds were detected in the presence of GST–LjCCD7 and not with GST alone. One of them had a visible spectrum peak and retention time similar to β-ionone (C\textsubscript{15}; Supplementary Fig. S2C). The second was deduced to be 10'-apo-β-carotenal (Supplementary Fig. S2D); that is, the complementary C\textsubscript{37} that would result from the cleavage of the 9', 10'-bond of β-carotene by CCD7. These results agreed with previous work in \textit{Arabidopsis} and tomato (Booker et al., 2004; Schwartz et al., 2004; Vogel et al., 2010) and proved that the cloned cDNA corresponds to a protein endowed with carotenoid cleavage activity \textit{in vitro}. With the outcome of phylogenetic analysis shown in Supplementary Fig. S1, they support the notion that the identified gene encodes the \textit{L. japonicus} CCD7 orthologue.

To study the expression pattern of \textit{LjCCD7}, RT–qPCR was performed on RNA samples from a range of vegetative tissues from 8-week-old wild-type plants. As in \textit{Arabidopsis} (Booker et al., 2004), pea (Johnson et al., 2006), petunia (Drummond et al., 2009), and tomato (Vogel et al., 2010), \textit{LjCCD7} was predominantly expressed in roots, ~2-fold more than in leaves and the shoot apex. \textit{LjCCD7} transcript was evaluated in three plants and at least 160 cm of roots under an optical microscope (Trouvelot et al., 1986).

**Results**

Cloning of \textit{LjCCD7} and \textit{in vitro} confirmation of its enzymatic activity

MAX3 from \textit{Arabidopsis} and RMS5 from pea were used to interrogate the EST and genomic database at the Kazusa DNA Research Institute. The EST aligning best to the queries (LjSGA_131670.1, 873 bp) was assumed to derive from \textit{bona fide} Lotus orthologue, designated \textit{LjCCD7}. Based on the partial coding sequence available, the missing cDNA ends of \textit{LjCCD7} were obtained by 5‘- and 3‘-RACE followed by direct amplification of the full-length coding sequence. The predicted polypeptide was 74% identical to RMS5 and 58% identical to MAX3. A phylogenetic tree (Supplementary Fig. S1 at JXB online) was produced from an alignment of several CCD7 homologues from land plants and moss, as well as of some putative homologues identified in cyanobacteria (Cui et al., 2012). The CCD7 proteins clustered into several clades, with LjCCD7 in the subclade of predicted leguminous homologues within the dicot clade, as expected (Supplementary Fig. S1 at JXB online).

Rhizobia and AMF infection assays

\textit{Mesorhizobium loti} strain R7A was used for nodulation assays. Slightly lignified shoots were cut off 4-month-old plants (line P16), and set for root induction in rockwool. When roots were ~0.5–1.0 cm long (1.5–2.0 weeks), plants were transferred to perlite for another week, and irrigated with B&D nutrient solution (Broughton and Dilworth, 1971). Each plant was inoculated with 30 ml of bacterial culture grown at 28 °C in YMB medium (per litre: 0.4 g of yeast extract, 10.0 g of mannitol, 5.0 g of K\textsubscript{2}HPO\textsubscript{4}, 0.2 g of MgSO\textsubscript{4}, 0.1 g of NaCl, 0.5 g of sucrose) and diluted to a final A\textsubscript{600} of ~0.05. Nodules and lateral roots were counted 2 weeks post-inoculation.

AMF colonization assessment was carried out on plants infected with \textit{Gigaspora margarita} spores in ‘Millipore sandwiches’ (Novero et al., 2002). Briefly, seeds sterilized with sulphuric acid were pre-germinated on 0.6% water agar. Roots were placed between two nitrocellulose membranes (5 cm diameter, 0.45 μm pores; Millipore) with 20 fungal spores sterilized by chloramine T (3%) and streptomycin sulphate (0.3%). After 4 weeks of co-culture, roots were sampled and stained with cotton blue (0.1% in lactic acid) to visualize fungal structures. For each genotype, intraradical colonization was evaluated in three plants and at least 160 cm of roots under an optical microscope (Trouvelot et al., 1986).

**Statistical analysis**

One-way analysis of variance (ANOVA) was performed on all data sets by using GenStat for Windows. If needed, data were also subjected to Student’s t-test.
also detected in stipules, although 13 times less abundantly than in roots (Fig. 1A).

**Generation and molecular characterization of transgenic lines impaired in LjCCD7 expression**

To elucidate the biological function of **LjCCD7 in planta**, >40 independent RNAi lines were generated. Three of them (PG, P9, and P16) displayed ~70% less **LjCCD7** transcript in roots relative to corresponding transgenic control plants (Fig. 1B), and were selected for further analysis. **CCD7** is a key biosynthetic gene for SLs, so the presence of all known SLs in the root exudates and extracts of wild-type *Lotus* was initially checked (not shown); 5-deoxystrigol was the only one detected, in accordance with previous work (Sugimoto and Ueyama, 2008). 5-Deoxystrigol was therefore quantified in both root exudates and extracts of wild-type *L. japonicus* and transgenic seedlings (line P16). In all samples, one distinct peak was detected that had the retention time and transitions corresponding to 5-deoxystrigol. 5-Deoxystrigol levels were higher in root exudates than in root extracts, both in wild-type seedlings and in the **CCD7**-silenced line. Metabolite abundance in RNAi plants was decreased by 81% and 73% in root exudates and extracts, respectively, relative to the non-silenced control seedlings (Fig. 1C).

**Decreased LjCCD7 transcript correlates with changes in shoot and root morphology**

Since SLs regulate plant architecture, experiments were conducted to assess whether the reduction in **LjCCD7** transcript and SL content was associated with altered morphology in RNAi line P16; lines P9 and PG, in which **LjCCD7** was silenced comparably, were also evaluated. From 3 weeks after germination, the RNAi lines exhibited a clearly stunted and bushy phenotype (Fig. 2A). Shoot branches were counted at two time points. Eight-week-old transgenic plants were significantly more branched than controls transformed with the empty vector: RNAi lines displayed 5.3, 2.1, and 4.0 times more cotyledonary, primary, and secondary aerial branches, respectively (Fig. 2B). When 6 months old, plants of the PG, P9, and P16 lines displayed 6.4, 6.6, and 5.7 times more total shoot branches relative to the controls, respectively (Fig. 2C). No branches of secondary or higher order occurred in the

---

**Fig. 1.** **LjCCD7** transcription pattern, and molecular and metabolic characterization of **CCD7**-silenced lines. (A) **LjCCD7** transcript abundance in various vegetative tissues relative to root expression level in 8-week-old wild-type *L. japonicus*. Values are the average of *n* biological and three technical replicates ±SE, normalized to **LjUBI** transcript levels. *n*=6 for root and shoot apex, *n*=5 for expanded or developing leaf, *n*=4 for stipule. (B) Relative **LjCCD7** transcript amounts in roots of RNAi lines PG, P9, and P16 (grey bars), compared with a line transformed with the empty vector (negative control, NC; black bar). Values are normalized to **LjUBI** transcript amounts, and displayed as means of *n* biological and three technical replicates ±SE (*n*=3 for each RNAi line and *n*=5 for controls, all at the T1 generation). The asterisks indicate statistically significant differences for *P* < 0.01. (C) LC-MS/MS analysis of 5-deoxystrigol content in root exudates and extracts of 6/7-week-old, aeroponically grown plants of the wild type (black bars) and of the **CCD7**-knockdown line P16 at generation T1 (grey bars). Twenty-four hours before collection of root exudates, the nutrient solution was refreshed for 15 plants. In root extracts, three independent samples were used and each consisted of five plants. Error bars represent the SE for three analytical replicates. The asterisks indicate a statistically significant difference for *P* < 0.01.
transgenic control plants at this or later stages, in contrast to RNAi lines (Supplementary Fig. S3A, B at JXB online). RNAi plants also had a significantly reduced main stem height (39, 40, and 29% shorter than the transgenic NC line, respectively; Fig. 3A). Reduced plant height was not due to a reduced number of nodes in the RNAi lines (not shown). Rather, internodes were significantly shorter in silenced plants compared with the corresponding control, particularly in high-order nodes. In fact, internodes IV–VII of the main stems were ~30–40% shorter in line P16 than in the wild type (Fig. 3B). The same was observed for most internodes above node III in secondary shoot branches (Fig. 3C). SLs have been shown to control the thickening of stems and roots (Agusti et al., 2011). As expected, RNAi line P16 displayed an ~39% reduction of secondary shoot diameter compared with the control (Fig. 3D, E).

SLs affect root development, including lateral root formation, and primary root and root hair development (Koltai, 2011; Ruyter-Spira et al., 2011). To assess the function of SLs in Lotus root development, plants of RNAi line P16 were grown either in aeroponic tanks or in pots. Under all conditions, the RNAi line consistently showed more lateral roots and longer primary roots (Fig. 4A). Five-week-old plants grown in aeroponic tanks were used for quantitative analysis: RNAi plants had a 1.6–2.0 times higher total root length, area, and volume than controls (Fig. 4B, C). Notably, the primary root of the RNAi line was significantly longer than in the corresponding controls grown either in the aeroponic system or in pots.

Effect of LjCCD7 silencing on reproduction

Lotus japonicus ecotype ‘Gifu’ flowers profusely and, in subsequent rounds, yields thousands of seeds within months after pollination (Handberg and Stougaard, 1992). We observed that seed production was severely impaired in RNAi plants compared with controls, particularly after two rounds of seed settings (representative individuals are shown in Supplementary Fig. S4A at JXB online). For a statistical assessment, all flowers from the third round on were counted and pods were harvested separately. An at least 7-fold reduction of flower and pod numbers was observed in RNAi lines relative to the NC (Fig. 5). After the above-ground part of 11-month-old plants was lopped off, ~40 times fewer pods
Phenotyping of strigolactone-deficient *Lotus* | 1973

Fig. 3. Shoot morphology of SL-depleted versus control plants. (A) Plant height of 6-month-old T₁ transgenic negative control (NC, black bar) and RNAi lines PG, P9, and P16 (grey bars). Error bars represent the SD of the means (n = 10 and n=3 for NC and each RNAi line, respectively). Asterisks indicate statistically significant differences between each RNAi line and the NC, for *P* < 0.01.

(B and C) Comparison of internode length between 2.5-month-old wild-type plants (black bars) and RNAi line P16, T₁ generation (grey bars). The internode above the cotyledons was set as the first node in the acropetal direction, so internode I lies between collar and cotyledonary node I, and so forth. Both the internodes from the main stem (B) and basal secondary shoots (C) were used for analysis. Data are means ±SD (n ≥ 6). Asterisks indicate statistically significant differences for *P* < 0.05 and **P** < 0.01.

(D) Representative display of secondary stem width in SL-depleted (RNAi) and NC plants. The picture was taken on 3-month-old T₁ plants of RNAi line P16 and transgenic plants transformed with the empty vector. Bar scale=1 cm.

(E) Quantification of secondary stem width. Data were obtained on 3-month-old T₁ plants of RNAi line P16 and transgenic plants transformed with the empty vector. Data were obtained on 3-month-old T₁ plants and are presented as means ±SD (n=22 for NC, black bar; and n=45 for RNAi line P16, grey bar). The asterisks indicate significantly different values for *P* < 0.01. (This figure is available in colour at *JXB* online.)
Liu et al. were counted on the emergent branches of RNAi plants than on controls (not shown). Impairment of flower and seed setting was less severe for line P16 than lines PG and P9, which is why the first was used for most analyses. There were no significant changes with respect to size, shape, or colour of the flowers, pods, and seeds. Given the intimate cross-talk occurring between SLs and auxin during reproductive development (Kohlen et al., 2012), the levels of free indole acetic acid (IAA) were measured in flowers collected at anthesis. Only a mild but statistically non-significant reduction was observed in RNAi line P16 compared with controls (Supplementary Fig. S4B at JXB online).

Effect of decreased LjCCD7 transcript levels on plant senescence

Lotus is a deciduous perennial displaying progressive leaf senescence and abscission in temperate climates. Since a role for SLs in senescence had been proposed (Snowden et al., 2005; Ledger et al., 2010), the leaves of the transgenic plants were monitored for any obvious colour change compared with control, and chlorophyll in leaves of comparable physiological age was quantified. All three RNAi lines remained green for a longer time than the control (see Supplementary Fig. S5A at JXB online) and accumulated significantly more chlorophyll $a$ (Chl$a$) and Chl$b$ than the NC, with a cumulative 2-fold increase in total chlorophyll content when ~8 months old (Fig. 6A). At a younger stage (~3 months old), RNAi lines PG and P9 showed a significant increase in Chl$a$ and Chl$b$, respectively; however, total chlorophyll content was not significantly different from that of the NC (Fig. 6A). When the top and internal part of 8-month-old RNAi plants left to senesce spontaneously had slightly started to fade in colour, controls were already severely senescing throughout their aerial parts (Supplementary Fig. S5B at JXB online). Also, more new shoots and branches kept emerging in RNAi lines compared with NCs (Supplementary Fig. S3C at JXB online). Finally, the RNAi line had higher biomass relative to control plants when ~3.5 months old (Fig. 6B); the trend was maintained with plant ageing. However, the shoot-to-root ratio

Fig. 4. Phenotypic characterization of roots in SL-depleted and control plants. (A) Root morphology of 4-week-old plants representative of the wild type and RNAi line P16, T$_1$ generation (left panel). The plants in the picture were grown for 1 week in Petri dishes in a growth chamber, 1 week in perlite, and another 2 weeks in aeroponic tanks. Root shape of 3.5-month-old transgenic negative control (NC) and RNAi line P16, T$_2$ generation (right panel), cultivated in pots filled with Arabidopsis soil (Horticoop) and perlite (1:2). (B and C) Quantification of root parameters in 5-week-old wild-type (black bars) and RNAi plants (line P16 at T$_1$ generation, grey bars). Data are presented as means ±SD ($n > 6$). Asterisks indicate statistically significant differences for *$P < 0.05$ and **$P < 0.01$. (This figure is available in colour at JXB online.)
Phenotyping of strigolactone-deficient Lotus was largely unchanged (average ratio ±SD: 2.796 ± 0.204 for RNAi lines versus 2.591 ± 0.094 for controls; data in Fig. 6B).

LjCCD7-silenced Lotus nodulates less but is colonized by AMF as much as control plants

The influence of SLs on Lotus determinate root nodules was explored. There were no significant differences in root fresh weight of RNAi plants compared with the wild-type and NC plants (not shown). However, their adventitious roots were significantly more branched also in the specific culture conditions used for this test (Fig. 7A, B). Although this situation potentially offers a larger surface to rhizobial infection for the same root biomass, RNAi roots carried significantly fewer nodules per gram of fresh weight than the NC 2 weeks post-inoculation (~20% reduction; Fig. 7C). In contrast, when controls and RNAI lines P16 and P9 were inoculated with spores of the AMF G. margarita, no significant differences in the colonization parameters evaluated were detected (Fig. 7D). SL-depleted roots supported both extra- and intraradical mycelium, which did not differ in quantity and developmental pattern from control roots. Also, arbuscules were morphologically indistinguishable (not shown).

Discussion

The role of SLs in the regulation of plant architecture is conserved in L. japonicus, but with some peculiarities

The phenotype of the SL-depleted L. japonicus plants characterized in this study was overall expected, on the basis of previously published work. RNAI plants were in fact stunted and their shoots more branched than controls, as were root apparatuses. More adventitious roots (not shown) were also observed, consistent with published data on Arabidopsis and pea (Rasmussen et al., 2012). Notwithstanding, the shoot morphology of L. japonicus is quite different from that of other model plant species, which makes it an attractive model to study SL function. In fact, in wild-type Lotus, the axillary buds in the cotyledonary node or in the leaf axes are already visible during the early vegetative stage. Once these buds are initiated, they develop directly into lateral branches without being inhibited by any factors released by the shoot apical meristems. Thereafter, numerous accessory meristems will potentially develop lateral branches in the cotyledonary node zone, but in the aerial part only a single bud in the leaf axis develops into a branch while the others remain dormant (Alvarez et al., 2006). This peculiar developmental pattern was reflected...
in some specific shoot features in the SL-depleted plants. In contrast to most SL-related phenotypes described so far, in fact, branching of secondary shoots was highly increased compared with controls. This phenotype has been reported, though to a milder extent, only in CCD7- and CCD8-silenced tomato plants (Vogel et al., 2010; Kohlen et al., 2012). In transgenic Lotus, this increase was most probably due to several of the accessory buds in the aerial axils developing into lateral shoots, in contrast to what happens in the wild type (Alvarez et al., 2006). Also, many more basal branches than in the wild type were produced from the activity of accessory buds in the cotyledonary axil, resulting in more numerous primary shoots (Supplementary Fig. S3C at JXB online).
plants compared with control plants because of a reduced internode length (as in petunia and maize) (Snowden et al., 2005; Guan et al., 2012) rather than number (as in tomato) (Kohlen et al., 2012).

LjCCD7-silenced plants differed slightly but significantly from other published SL mutants at the root level as well, in that they consistently exhibited longer primary roots than the controls. Results in Arabidopsis, rice, and maize point instead to a role for SLs in the promotion of primary root growth (Koltai et al., 2010a; Ruyter-Spira et al., 2011; Arite et al., 2012; Guan et al., 2012). In these species, SLs were proposed to alter the auxin gradient in the root tips, thus changing both the number and length of the cells located in the meristem and transition zone of primary roots. The results suggest that the role of SLs in primary root development differs from species to species, possibly in relation to varying endogenous hormonal levels.

Finally, the above- and below-ground mass of SL-depleted plants was increased, so that the shoot-to-root biomass ratio was comparable with that of controls. This observation differs from what has been reported for the petunia ccd7/dad3 mutant, which displays a higher shoot-to-root ratio than the wild type (Snowden et al., 2005). This discrepancy may be due to the different species, growth conditions (hydroponic for petunia), and/or physiological age of the plants.

**CCD7 affects reproduction and age-dependent senescence in L. japonicus**

A role for SLs was postulated in reproduction, considering the high expression of Arabidopsis MAX3 in siliques and seeds (Booker et al., 2004; Mashiguchi et al., 2009), of tomato SICCD7 in green immature fruits (Vogel et al., 2010), of AcCCD7 and AcCCD8 in young kiwifruits and seeds (Ledger et al., 2010), and of ZmCCD8 in maize shank and ear shoots of female inflorescences (Guan et al., 2012). To date, however, CCD7 activity has never been proven to affect reproduction, and, more specifically, the reduced flower, fruit, and seed numbers observed in L. japonicus have never been reported for SL-depleted plants of any species. Rather, the petunia dad1/ccd8 mutant is delayed in the setting of flowers, which also weigh less and are smaller than those of the wild type (Snowden et al., 2005). SICCD8-silenced tomato plants also have smaller floral organs, fruits, and seeds, as well as 60% fewer seeds relative to controls (Kohlen et al., 2012). Also a significant reduction in the size and diameter of ear and shank was observed in maize ZmCCD8 mutants (Guan et al., 2012).

In contrast, abnormal floral organs, altered size and colour of flowers, pods, and seeds, or seed patterning inside the pod were not observed in transgenic Lotus consistent with the fact that no significant differences in free IAA content could be detected in flowers of RNAi and wild-type plants. Therefore, it is proposed that the observed reduction in seed production in transgenic plants is mainly due to the reduced number of flowers. To form its pseudoraceme inflorescences, the shoot main apex of L. japonicus produces a flower branch, which is subtended in the compound leaves and is terminated when one or two flowers are developed (Guo et al., 2006). A possible explanation for the reduced number of flowers in RNAi compared with transgenic control plants, in spite of their higher number of branches, is that their potential to form new buds that can produce flower branches may be reduced compared with controls. This, in turn, could be linked to the fact that the dormancy of many accessory buds was broken to give higher order branches, before transition from vegetative growth to flowering. Alternatively, or additionally, fewer resources could be available to reproduction in the SL-depleted plants because of the more vigorous vegetative growth (see below).

LjCCD7-silenced Lotus lines lagged in leaf senescence and abscission; this the first report linking senescence to a functional defect in a CCD7 homologue. A correlation between SLs and senescence was instead reported in Arabidopsis, rice, and petunia plants lacking MAX2 (Woo et al., 2001; Yan et al., 2007; Drummond et al., 2012), and in the ccd8 mutants of petunia and kiwifruit (Snowden et al., 2005; Ledger et al., 2010). Transcripts of the Arabidopsis MAX3/CCD7 and MAX4/CCD8 genes accumulate during age-dependent leaf senescence, further suggesting that the role of SLs in the process may be direct (Breeze et al., 2011). However, mutation of the MAX2 orthologue in pea (RMS4) does not affect leaf senescence (Johnson et al., 2006), implying that this is not a conserved feature of SLs across plant species.

A link may be seen between the delayed leaf senescence and impaired flower and seed production in the RNAi plants used here. Reproductive development controls the timing of leaf senescence, with photosynthates translocated from leaves to reproductive organs before death. The negative correlation between senescence and reproduction could be even more evident in polycarpic plants such as L. japonicus, which sets numerous inflorescences in subsequent rounds. The iterative production of reproductive organs and seeds may require more resources at the expense of vegetative fitness (Munné-Bosch, 2008). In this scenario, LjCCD7-silenced plants would allocate fewer resources than control plants to reproduction, thus delaying senescence and allowing higher biomass. This interpretation agrees with the observation that chlorophyll content was higher in RNAi plants than in controls. Whether the higher allocation for SLs in RNAi plants to reproduction, or rather its effect, cannot be determined at this stage.

**Role of SL in the regulation of root symbiosis in L. japonicus**

The present data prove for the first time that SLs promote the formation of determinate nodules. The correlation between SLs and nodulation was previously investigated in M. sativa and pea, both forming indeterminate nodules (Soto et al., 2010; Foo and Davies, 2011; Foo et al., 2013). In L. japonicus as well, LjCCD7-silenced plants carried 20% fewer nodules than controls. As in the pea rms1 mutant, alterations in nodule development and morphology were not detected. SLs apparently have no direct influence on the
growth of rhizobia, but they do have a quantitative effect on nodulation, in spite of their negative effect on the total root length and surface available to infection (Foo and Davies, 2011). Even if nodules and lateral roots form from different founder cells (being initiated by cortical versus pericycle cell divisions, respectively), a balance was proposed long ago to exist between nodule and lateral root formation, with nodule primordia initiation dependent on the suppression of lateral root emergence (Nutman, 1949). Later, it was suggested that the balance of auxins, cytokinins, and abscisic acid (ABA) dictates the fate of pericycle cells and cortical cells, dictating whether lateral roots or nodules will be initiated (Ding and Oldroyd, 2009). Given the proven cross-talk of SLs with auxin (Crawford et al., 2010; Domagalska and Leyser, 2011; Ruyter-Spira et al., 2011; Kohlen et al., 2012) and ABA (Lechat et al., 2012; Toh et al., 2012), and the importance of both these hormones for normal frequency and development of determinate nodules in Lotus (Ding and Oldroyd, 2009), the phenotype observed in the SL-depleted plants could be auxin and/or ABA mediated, and be the outcome of an obviously defective suppression mechanism on lateral root emergence.

As proven for 5-deoxystrigol in root exudates of L. japonicus in the first place, SLs induce hyphal branching of AMF, increasing the chances of successful root colonization (Akiyama et al., 2005). A MAX2/D3-dependent, D14-independent effect on AMF colonization of roots was also observed very recently in rice (Yoshida et al., 2012). In an SICCD8-knockdown line of tomato, ~50% reduction of total SLs in roots correlated with a 27% decrease in colonization by Glomus intraradices (Kohlen et al., 2012). Interestingly, the RNAi lines used in the present study did not show a significant reduction in AMF colonization for any of the parameters tested. Among all natural and synthetic SLs, 5-deoxystrigol (the only SL found in Lotus) is the most active, inducing AMF branching at subnanogram concentrations (Akiyama et al., 2010; Xie et al., 2010). Therefore, it is possible that the residual 20–30% of 5-deoxystrigol in the RNAi lines used here is still above the required threshold to induce fairly normal hyphal branching of G. margarita in our inoculation system (clean roots placed in close contact with germinating AMF spores). Very recently, protocols enabling highly efficient insertion mutagenesis were set up for Lotus (Fukai et al., 2012; Urbanski et al., 2012). The availability of such reverse genetic tools, through which complete knockout of genes would be achievable, will allow this point to be solved.

In summary, this is the first report of an SL-related phenotype in the perennial herbaceous model L. japonicus. Evidence was presented that LjCCD7 is crucial for SL synthesis in Lotus, where the role of SLs is conserved in the regulation of root and shoot architecture, with some peculiarities; among them, the effects on primary root growth, internode length, number of higher order branches, and root-to-shoot biomass ratio. These apparent discrepancies are most probably specific features of SL action in the model plant used here, and, thus, just the reflection of biological diversity. Additional effects on reproduction and senescence were for the first time linked to an impairment in the expression of a CCD7 enzyme, and an effect of SLs on determinate nodulation was described. Further work is under way to unravel cross-talk of SLs with other hormones and their relative contributions to the observed phenotypes.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Phylogenetic analysis of CCD7 protein homologues.

Figure S2. LjCCD7 has carotenoid cleavage activity.

Figure S3. Representative comparison of high-order and basal shoot emergence between SL-depleted (RNAi) and negative transgenic control (NC) plants.

Figure S4. LjCCD7 influence on reproduction.

Figure S5. LjCCD7 impact on age-related senescence processes.

Table S1. List of primers used in this work.

Table S2. List of protein sequences used in phylogenetic analysis.

Acknowledgements

The authors wish to thank M. Chiurazzi (CNR, Italy) for suggestions about plant transformation, J. Stougaard (Aarhus University, Denmark) for Gifu B-129 seeds; S. Karunaiyetham (Plant & Food Research, New Zealand) and R. Geurts (Wageningen University, The Netherlands) for vector pTKO2 and Mesorhizobium loti, respectively; W. Kohlen and I. Haider for their guidance during JL’s stay in Wageningen. Research was funded by the BioBITs Project [Piedmont Region, Converging Technologies 2007] to FC, AC, CL, and PB, and by the Netherlands Organization for Scientific Research [Vici grant, 865.06.002, 834.08.001] to HJB; JL was funded by the China Scholarship Council [2008108168].

References


Ding Y, Oldroyd GE. 2009. Positioning the nodule, the hormone dictum. Plant Signaling and Behavior 4, 89–93.


Koltai H, LekKala SP, Bhattacharya C, et al. 2010b. A tomato strigolactone-impaired mutant displays aberrant shoot morphology
1980 | Liu et al.


