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The tomato MAX1 homolog, SlMAX1, is involved in the biosynthesis of tomato strigolactones from carlactone

Yanxia Zhang¹, Xi Cheng¹*, Yanting Wang¹*, Carmen Diez-Simón¹, Kristyna Flokova¹, Andrea Bimbo¹, Harro J. Bouwmeester¹,² and Carolien Ruyter-Spira¹

¹Laboratory of Plant Physiology, Wageningen UR, PO Box 658, 6700 AR Wageningen, the Netherlands; ²Present address: Plant Hormone Biology, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, the Netherlands

Summary

• Strigolactones (SLs) are rhizosphere signalling molecules exuded by plants that induce seed germination of root parasitic weeds and hyphal branching of arbuscular mycorrhiza. They are also phytohormones regulating plant architecture. MORE AXILLARY GROWTH 1 (MAX1) and its homologs encode cytochrome P450 (CYP) enzymes that catalyse the conversion of the strigolactone precursor carlactone to canonical strigolactones in rice (Oryza sativa), and to an SL-like compound in Arabidopsis. Here, we characterized the tomato (Solanum lycopersicum) MAX1 homolog, SlMAX1.

• The targeting induced local lesions in genomes method was used to obtain Slmax1 mutants that exhibit strongly reduced production of orobanchol, solanacol and didehydro-orobanchol (DDH) isomers. This results in a severe strigolactone mutant phenotype in vegetative and reproductive development.

• Transient expression of SIMAX1 – together with SID27, SICCD7 and SICCD8 – in Nicotiana benthamiana showed that SIMAX1 catalyses the formation of carlactonoic acid from carlactone.

• Plant feeding assays showed that carlactone, but not 4-deoxy-orobanchol, is the precursor of orobanchol, solanacol and two of the three DDH isomers. Inhibitor studies suggest that a 2-oxoglutarate-dependent dioxygenase is involved in orobanchol biosynthesis from carlactone and that the formation of solanacol and DDH isomers from orobanchol is catalysed by CYPs.

Introduction

Strigolactones (SLs) were originally discovered as rhizosphere signalling molecules secreted by plants into the soil that stimulate seed germination of root parasitic plants of the Orobanchaceae (Striga, Phelipanche and Orobanche genera) (Cook et al., 1966). Many years later they were demonstrated to promote hyphal branching of beneficial arbuscular mycorrhizal fungi (Cook et al., 1966; Akiyama et al., 2005) and to represent a new class of phytohormones regulating plant architecture, including shoot branching and several other aspects of plant and root development (Gomez-Roldan et al., 2008; Umehara et al., 2008; Ruyter-Spira et al., 2011; Kapulnik & Koltai, 2014; Sun et al., 2015). Interestingly, in and between plant species there is extensive variation in the decoration of the typical SL structure, the backbone of which consists of a butenolide D-ring attached to a tricyclic ABC-lactone ring. The SLs are distributed into two groups: the orobanchol- and the strigol-type SLs (Zwanenburg & Pospisil, 2013). More recently, noncanonical SL-LIKE structures with a noncyclized BC-ring were discovered in Arabidopsis and sunflower, methyl carlactonoate (MeCLA) and heliolactone (Abe et al., 2014; Ueno et al., 2014). The different SL and SL-LIKE molecules may all display different activities with regard to the stimulation of parasitic plant seed germination, arbuscular mycorrhizal fungi hyphal branching or the inhibition of plant axillary bud outgrowth (Akiyama et al., 2010; Boyer et al., 2012; Nomura et al., 2013; Zwanenburg & Pospisil, 2013). However, to better understand the biological significance of all the different SLs, manipulation of their content would be desirable, for which knowledge of their biosynthetic pathway is required.

The biosynthesis of SLs has been partially unravelled, with the identification of a number of enzymes that were characterized in several plant species. More than a decade ago, genetics studies in Arabidopsis showed that MORE AXILLARY GROWTH 1 (MAX1), MORE AXILLARY GROWTH 3 (MAX3) and MORE AXILLARY GROWTH 4 (MAX4) are required for the biosynthesis of a shoot-branching inhibiting signal, which was later shown to be SL (Stirnberg et al., 2002; Sorefan et al., 2003; Booker et al., 2004, 2005; Gomez-Roldan et al., 2008; Umehara et al., 2008). The homologues of MAX3 and MAX4 were characterized also in rice (Oryza sativa – DWARF 17 and DWARF 10), pea (Pisum sativum – RAMOSUS 5 and RAMOSUS 1) and petunia...
(DECREASED APICAL DOMINANCE 3 and DECREASED APICAL DOMINANCE 1) (Morris et al., 2001; Sorefan et al., 2003; Zou et al., 2006; Arite et al., 2007; Simons et al., 2007; Drummond et al., 2009). MAX3 and MAX4 encode CAROTENOID CLEAVAGE DIOXYGENASEs 7 and 8 (CCD7 and CCD8) respectively. Together with a β-carotene isomerase, encoded by DWF27 (D27), these three enzymes catalyse the conversion of β-carotene to the C19 SL precursor, carlactone (CL) (Lin et al., 2009; Alder et al., 2012). Recent studies showed that CL can be oxidized by MAX1 homologues from Arabidopsis (AtMAX1) and rice (Os900) to carlactonoic acid (CLA) and 4-deoxyorobanchol (4DO) respectively (Abe et al., 2014; Seto et al., 2014; Zhang et al., 2014). Intriguingly, MAX1 in monocots and certain dicot species usually occurs in multiple copies (Challis et al., 2013). For example, there are five MAX1s in rice, with Os1400— one of the most divergent members— encoding the orobanchol synthase catalysing orobanchol formation from 4DO (Zhang et al., 2014). The major function of other rice MAX1 homologues is not entirely clear, and they might be involved in the formation of the other SLs reported in rice or SL precursors (Jamil et al., 2011; Abe et al., 2014; Seto et al., 2014; Zhang et al., 2014). By contrast, in many dicot species, such as Arabidopsis, MAX1 exists as a single copy (Challis et al., 2013).

In tomato (Solanum lycopersicum), there is also only one MAX1 homologue present (hereafter called SIMAX1) according to the public tomato genomic database (Sol Genomics Network: https://solgenomics.net/), but the enzymatic function of the enzyme encoded by SIMAX1 is uncharacterized. On the basis of the biochemical function of MAX1 in rice and Arabidopsis (Abe et al., 2014; Zhang et al., 2014), we hypothesized that SIMAX1 is required for the oxidation of the SL precursor CL. However, there are great differences between Arabidopsis and tomato in the reported SL- or CL-derivative profiles (Abe et al., 2014). In tomato, many different SLs, such as orobanchol, solanacal, several didehydro-orobanchol (DDH) isomers, orobanchyl acetate, 7-oxoorobanchol and 7-hydroxyorobanchol, were identified (Koltai et al., 2010; Vogel et al., 2010; Kohlen et al., 2012, 2013). The two basic SL pathway enzymes CCD7 and CCD8 have been characterized in tomato (Vogel et al., 2010; Kohlen et al., 2012). However, the biochemical mechanism by which SL diversification in tomato is created remains elusive. In this study, we use a tomato max1 mutant to show that SIMAX1 is required for the oxidation of CL to form orobanchol, likely via CLA. In addition, we provide evidence that DDH1, one of the main DDH isomers, is a strigol-type SL— implying that there are both orobanchol-type and strigol-type SLs present in tomato—and that solanacal and one of the other predominant DDH isomers (DDH2) are formed from orobanchol.

Materials and Methods

Plant growing conditions and treatments

The targeting induced local lesions in genomes (TILLING) method was carried out to obtain the mutant of SIMAX1 – Slmax1 as described by using ethyl methanesulfonate mutagenesis (Kurowska et al., 2011). M2 seeds were used for screening of targeted point mutations in SIMAX1. A mutation carrying a G → T mutation (at position +466 bp downstream of the transcription start site) in the tomato (S. lycopersicum) genomic sequences was selected. The mutation of SIMAX1 and homozygosity of Slmax1 mutants were confirmed by using the primers and probe in Supporting Information Table S1. Two M3 Slmax1 homozygous plants, 13539-02 and 13539-03, carrying this mutation were used for further study.

For phenotype characterization of young seedlings, germinated wild-type and Slmax1 seeds were grown in trays filled with vermiculite. After 10 d, photos were taken of the roots of these young seedlings to perform further image analysis of hypocotyl and root phenotypes.

To characterize the phenotype of adult plants, pre-germinated plants were transferred to pots containing a mixture of soil and vermiculite (2:1) and grown under 16 h: 8 h photoperiod at 25°C (60% humidity) in the glasshouse. After 10 wk, number and length/diameter of nodes, shoot branches, flowers and fruits were scored and measured.

To examine the SL levels, pre-germinated wild-type and Slmax1 seeds were grown on moistened vermiculites for 1 wk under a 12 h: 12 h photoperiod at 22°C. Young seedlings were then grown hydroponically for another week to get sufficient root development before they were moved to an X-stream 20 aeroponic system (Nutriculture, Lancashire, UK) in the glasshouse (16 h 25°C: 8 h 22°C photoperiod, 60% humidity). The plants were continuously supplied with half-strength Hoagland solution for 18 d, after which phosphorus (P) deficiency (by using half-strength Hoagland solution without P) was applied to induce the production of SLs (Lopez-Raez et al., 2008). Root exudates of wild-type plants and the Slmax1 mutant were collected every day during P-deficiency treatment. After 15 d of P starvation, plant roots were harvested, frozen in liquid nitrogen and stored at −80°C for further analysis. Four plants were pooled as one biological replicate.

For gene expression analysis, wild-type and Slmax1 tomato seeds were pre-germinated in darkness at 25°C for 4 d. Seedlings were transferred to Rock wool or water–agar blocks in an Eppendorf vial from which the bottom was cut and grown in hydroponic trays supplied with half-strength Hoagland solution under 16 h: 8 h photoperiod at 25°C in the glasshouse for 3 wk followed by a 1 wk P-deficiency treatment. During this treatment, roots, and several other tissues were collected at different time intervals (days 3, 5 and 7) for further analysis.

Biosynthetic intermediate feeding assays

Biosynthetic intermediate feeding assays with 4DO, CL and orobanchol isomers (orobanchol and ent-2′-epi-orobanchol) were carried out on 18-d-old plants according to a previously published protocol with modifications (Motonami et al., 2013). The plants were pre-grown in half-strength Hoagland solution (under 16 h: 8 h photoperiod, 25°C, 60% humidity) for 14 d. Then they were grown in 100 ml tap water (to mimic the P starvation)
supplemented with 1 μM fluridone (the inhibitor of phytoene desaturase, a key-step in carotenoid biosynthesis) (Matusova et al., 2005), which effectively inhibits SL biosynthesis. After 3 d, the plants were transferred to fresh tap water (containing again 1 μM fluridone) supplemented with the CL or SLs (at the required concentration). The plant root exudates (100 ml) were collected after 24 h feeding and concentrated through a C18-fast column (Grace, 500 mg 3 ml−1). All substrate SLs, CL, 4DO and two orobanchol isomers (orobanchol and ent-2’-epi-orobanchol) were applied with the same concentrations (0.05 μM) separately to plants in the same developmental stage. Enzyme inhibitors, uniconazole-P and prohexadione, were applied at 50 μM. All chemicals and SLs were prepared in a master stock in acetone with the exception of uniconazole-P, which was dissolved in dimethyl sulfoxide, before they were diluted for the treatment and the control plants were treated with an equal amount of acetone/dimethyl sulfoxide. Three to five biological replicates were used for each treatment. Two plants were pooled as one biological replicate.

Molecular cloning

The cDNA sequences of SLD27, SICCD7, SICCD8 and SIMALXI were obtained from SGN (Sol Genomics Network: https://solgenomics.net/) by using protein sequences of the Arabidopsis homologues as baits (tBLASTN) or from previously published reports (Table S2) (Kohlen et al., 2012). Primers were designed to clone the coding sequences of the genes from cDNA of tomato cultivar M82 (Table S3). Cloning for agro-infiltration was conducted as previously described into a pBIN19-plus binary vector (Zhang et al., 2014). Primers were designed with restriction sites included (Table S1).

Gene expression analysis

Total RNA was extracted using TriPure isolation reagent (Sigma) combined with a Qiagen RNeasy mini kit following the manufacture’s manual. For all samples, 800 ng total RNA was used to synthesize cDNA using the iScript cDNA Synthesis kit (Bio-Rad). Real-time quantitative PCR (qPCR) was performed with the CFX Connect Real-Time PCR Detection System (Bio-Rad) using primers as shown in Table S1. Tomato housekeeping genes were selected based on the stability as previously described (Dekkers et al., 2012). Relative expression of transcripts in different plant tissues was normalized to the average expression level of two housekeeping genes as listed in Table S1. To compare gene expression in wild-type and mutants/transgenic lines (Slmax1 or CCD8-RNAi line), the expression levels were normalized to the expression levels in the wild-type plants.

Transient expression in Nicotiana benthamiana

For transient expression, 4-wk-old Nicotiana benthamiana plants were used for agro-infiltration. The preparation of the Agrobacterium (AGL0) strains (OD = 0.5) was performed as previously described (Zhang et al., 2014). To compensate for differences in numbers of constructs per treatment, strains carrying empty vectors were used. The TBSV P19 gene was co-infiltrated to prevent gene silencing (Voinnet et al., 2003). The bacterial suspension was injected into the abaxial side of the leaf by using a 1 ml syringe without needle. After 6 d, the infiltrated leaves were harvested and frozen in liquid nitrogen and stored at −80°C until further analysis. Six biological replicates were used for each gene combination.

Strigolactone extraction and measurement

To analyze SL levels in the root exudates, the nutrient solution from aeroponically or hydroponically grown plants was concentrated using C18 columns (Grace, C18-fast/5000 mg) as previously described (Kohlen et al., 2012). CL and SLs were extracted from root tissue or N. benthamiana leaves and further analysed by multiple reaction monitoring (MRM)–LC–MS/MS as previously described (Lopez-Raez et al., 2011; Zhang et al., 2014). The mass spectrometer was operated in positive electrospray ionization mode. Cone and desorption gas flows were set to 50 l h−1 and 1000 l h−1 respectively. The capillary voltage was set at 3.0 kV, the source temperature was 150°C, and the desolvation temperature was 650°C. The cone voltage was optimized for each standard compound using the Waters IntelliStart MS Console. MRM was used for identification of CLs and SLs by comparing retention times and MRM mass transitions with standard compounds (CL and 12 major SLs). Chromatographic separation was achieved on a nonchiral Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm; Waters) by applying a water–acetoniitrile gradient (with 0.1% formic acid) to the column. The set-up for the ultra-performance LC gradients and conditions was carried out as previously described (Zhang et al., 2014).

For CLA analysis in N. benthamiana leaves, 500 mg fresh N. benthamiana leaves were ground and extracted with 4 ml ethyl acetate. Subsequently, the ethyl acetate was evaporated with a SpeedVac until dryness, and then the sample was redissolved in 100 μl 50% acetonitrile (in Milli-Q water) before injection. Then LC–MS profiling of crude plant extracts was performed as previously described (De Vos et al., 2007), using an LC–Orbitrap Fourier transform MS (FTMS) instrument consisting of an Acquity HPLC with photodiode array detection (Waters) interfaced to a linear trap quadrupole ion trap/Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ionization source (van der Hooft et al., 2012). The sample injection volume was 5 μl. A Luna RP-C18 analytical column (2.0 mm diameter, 150 mm length, 100 Å pore size and spherical particles of 3 μm, Phenomenex, USA) was used for chromatographic separation. The mobile phase consisted of a binary eluent solvent system of degassed ultra-pure water (solvent A) and acetonitrile (solvent B), both containing 0.1% v/v formic acid, with a flow rate of 0.19 ml min−1 and a column temperature of 40°C. The high-performance LC gradient started at 5% B and linearly increased to 75% B across a period of 45 min. The column was re-equilibrated for 15 min following the separation of each sample.
Software and statistics for data analysis

Image analysis of root phenotypes was performed by using ImageJ. Gene expression data analysis was done with Bio-Rad CFX MANAGER 3.0 combined with Microsoft Excel. Masslynx 4.1 software and XCALIBUR software (combined with Microsoft Excel) were used for compound identification, semi-quantification or quantification using MRM-LC–MS/MS and LC–Orbitrap-FTMS data respectively. PCR efficiencies of qPCR were calculated using LinREG PCR software (Dekkers et al., 2012). Statistical analysis was performed using one-way ANOVA of PRISM v.6.0 or Student’s t-test of Microsoft Excel.

Strigolactone standards

A CL standard was obtained from Dr Salim Al-Babili (King Abdullah University of Science and Technology); it was produced using biochemical methods as described previously (Alder et al., 2012). CLA standard was obtained from Dr Gavin R. Flematti (School of Chemistry and Biochemistry, The University of Western Australia). All other SL standards were obtained as described previously (Zhang et al., 2014).

Results

The cloning and characterization of SlMAX1

A homology search approach was employed to get the DNA sequence of SlMAX1. Hereto, the protein sequence of Arabidopsis MAX1 was BLASTed against the SGN (Sol Genomics Network). The DNA sequence of SlMAX1 is 3300 bp long and contains a coding region of 1560 bp (Table S3) that encodes a protein of 519 aa (Table S3). A sequence alignment shows that SlMAX1 has 72%, 62% and 64% amino acid identity with AtMAX1 and the two functionally characterized rice MAX1 homologues Os900 and Os1400 respectively (Fig. S1a). SlMAX1 clusters into the dicot MAX1 clade in a phylogenetic tree with MAX1 homologues from other plant species including monocots and dicots plants (Fig. 1).

To characterize its biological function, we generated an SlMAX1 mutant using TILLING. This method allows the detection of point mutations, usually introduced through ethyl methanesulfonate treatment, in the genome sequence of target genes, in this case SlMAX1. A Slmax1 mutant was identified that carried a G → T mutation (at position +466 bp) (Fig. S1b). This point mutation results in a glutamate (E156) → premature stop codon in the SlMAX1 amino acid sequence (Fig. S1b). Two M3 homozygous mutants for this SlMAX1 mutation were identified and used for further experiments. The Slmax1 homozygous mutants exhibited an average 90% reduction in SlMAX1 transcripts level compared with wild-type plants (Fig. S1c).

The Slmax1 mutant produces less strigolactones

MAX1 homologues in both Arabidopsis and rice have been shown to be involved in the production of noncanonical (MeCLA) and canonical SLs (4DO and orobanchol) respectively (Abe et al., 2014; Zhang et al., 2014). To gain insight into whether SlMAX1 is playing a role in the biosynthesis of SLs in tomato, we analysed the SL content of both wild-type plants and the Slmax1 mutant. We pre-grew both genotypes on aeroponics with normal P and then applied a P-starvation treatment to induce the production of SLs. SL levels in the root exudates were then analysed for several days, starting from the 11th until the 15th day of P starvation. In the root exudates and root extracts of wild-type plants, solanacol, orobanchol and three isomers of DDH were detected (Figs 2a,b, S2a), while there were only trace levels of these SLs in both root extracts and root exudates of the Slmax1 mutant (Fig. 2a,b), demonstrating that SlMAX1 is a key enzyme in the biosynthesis of tomato SLs.

Consistent with what was previously described (Kohlen et al., 2013), the three DDH isomers – DDH1, DDH2 and DDH3 – exhibit a
different pattern in the root exudates and root extracts (Fig. S2a). DDH1 was the major SL present in the root exudate (Fig. S2b), while in the root extract DDH2 was the major SL (Fig. S2c). Only trace amount of DDH3 were observed in both root exudate and root extract (Fig. S2b,c).

The Slmax1 mutant displays a plant architecture and development phenotype

To examine the effect of the SIMAX1 mutation on plant architecture and development, plant phenotypic data at different developmental stages were recorded for the Slmax1 mutant and corresponding wild-type plants. Growth of the Slmax1 mutant was impaired compared with the wild-type tomato plants (Fig. 3a). The Slmax1 mutant had a significantly higher number of branches (5.75- to 7.25-fold higher than that in the wild-type plant) and average lateral branch length (27.8- to 58-fold longer) compared with wild-type plants (Fig. 3b,c). To gain more insights into how SIMAX1 regulates the shoot architecture of tomato, the stem length and number of internodes were scored. Wild-type plants showed significantly longer stem length (1.9- to 2.7-fold longer than in Slmax1 mutants) with fewer internodes (10% less than in Slmax1 mutants) (Figs 3d, S3a). The Slmax1 mutant exhibited a reduction in total root length (decreased by 23%) and average lateral root length (decreased by 30%) during the seedling stage (Fig. S3b,c). There is no difference between wild-type plant and Slmax1 mutant in number of lateral roots (data not shown). The mutation in SIMAX1 also caused defects in the reproductive stage of the plants and resulted in reduced flower length and fruit size in the Slmax1 mutant (Figs 3e, S3d).

SIMAX1 expression is induced by phosphate starvation

The expression of SL biosynthetic genes was shown to respond to P deficiency in several plant species (Umehara et al., 2010; Liu et al., 2015; van Zeijl et al., 2015). We analysed the expression of SIMAX1 and the upstream SL biosynthetic genes (SID27, SICCD7, SICCD8) under P starvation in tomato. Initially, we checked the expression of the phosphate starvation marker gene
LePS2 (Lycopersicon esculentum phosphate starvation-induced gene) at different time intervals during the P-deficiency treatment (3, 5 and 7 d), which reveals the P-starvation status of the plant (Baldwin et al., 2001). After 7 d of P starvation, we observed a strong increase in LePS2 expression in all the tissues tested (root, adventitious root, leaf and leaf axil) except the flower buds, which indicates the success of the P-starvation treatment (Fig. S4a,b). At this time point, SIMAX1 transcripts were 1.8-fold upregulated by P starvation in roots but not in other plant tissues tested (Fig. S4c). The expression of SIMAX1 was at least threefold lower in the leaves and flower buds than in the root and other tissues tested (adventitious root, stem and leaf axil) under normal P conditions (Fig. S4c). The expression of the three SL biosynthetic genes upstream of SIMAX1 was also induced by P starvation in the root, and their expression was low or absent in leaf and flower bud (Fig. S4d–f). However, unlike SIMAX1, expression of these three genes was also increased by P starvation in adventitious roots and the stem (Fig. S4d–f). In the leaf axil, the expression of SIDD27 and SICC7D was induced by P starvation, while the expression of SIMAX1 and SICC8D was not (Fig. S4c–f).

Next, we assessed whether there is feedback regulation in the expression of the three upstream biosynthetic genes in the Simax1 mutant. The expression of SIDD27 and SICC7D, but not that of SICC8D, was upregulated in the Simax1 mutant by 90% and 153% respectively compared with wild-type plants after 7 d of P starvation (Fig. 4). Furthermore, we examined SIMAX1 expression in the CCD8-RNAi line and its corresponding wild-type (Kohlen et al., 2012), and this showed that SIMAX1 is significantly induced in the CCD8-RNAi line possibly because of feedback upregulation (Fig. S4g). These data further support the involvement of SIMAX1 in tomato SL biosynthesis together with SIDD27, SICC7D and SICC8D.

SIMAX1 is involved in the oxidation of carlactone in vivo

To further assess the role of SIMAX1 in the SL biosynthetic pathway of tomato, we reconstituted the tomato SL biosynthetic pathway in N. benthamiana. Hereto, we used the published sequence of SICC7D and SICC8D and we identified the putative tomato D27 – SIDD27 – based on its homology with Arabidopsis D27 (Table S3) (Waters et al., 2012). Subsequently, we transformed the coding regions of these genes individually to Agrobacterium tumefaciens and co-infiltrated these into the leaves of N. benthamiana for transient expression as described before (Zhang et al., 2014). After 6 d of transient expression, we analysed the level of CL and SLs in leaf extracts of N. benthamiana. When expressing SIDD27, SICC7D and SICC8D simultaneously, there was a strong accumulation of CL in the N. benthamiana leaves (Fig. 5a). When SIMAX1 was co-infiltrated with SIDD27, SICC7D and SICC8D the level of CL greatly decreased, suggesting that SIMAX1 catalyses the conversion of CL to something else (Fig. 5a). Since in Arabidopsis AtMAX1 catalyses the conversion of CL to the SL intermediate CLA (Abe et al., 2014), we analysed leaf extracts for the presence of possible CL derivatives (19-hydroxy-CL, 19-oxo-CL or CLA) by LC–Orbitrap–FTMS. CLA was present in the samples in which SIMAX1 was co-infiltrated with SIDD27, SICC7D and SICC8D. These results indicate that tomato SIMAX1 is catalysing the oxidation of CL to CLA.

To assess if any canonical SLs were produced, we used MRM–LC–MS/MS analysis on N. benthamiana leaves after co-infiltration of SIMAX1 with the three CL biosynthetic pathway genes. This showed that trace amounts of 4DO and 5-deoxystrigol (5DS) were produced, but no other known tomato SLs (Fig. S5a,b). This is consistent with the fact that SIMAX1 is required for the formation of CLA from CL (Fig. 5) and also similar to what we observed for co-infiltration of some of the rice MAX1 homologues (such as Os5100 and Os1900) and AtMAX1 individually with the CL biosynthetic genes (Zhang et al., 2014). In the latter study, Os900 and Os1400, two other rice MAX1 homologues, were shown to sequentially catalyse the conversion of CL to 4DO and 4DO to orobanchol respectively (Zhang et al., 2014). Orobanchol is one of the major SLs in tomato (Fig. 2), yet neither orobanchol nor its direct precursor 4DO were formed in any appreciable amount by SIMAX1 from CL. To investigate whether SIMAX1 perhaps plays a role in the conversion of 4DO to orobanchol, we produced 4DO as substrate for SIMAX1 in N. benthamiana by co-infiltrating rice Os900 with the tomato CL biosynthetic genes. As expected, the latter resulted in a strong production of 4DO (Fig. S5c). However, coexpression of SIMAX1 did not result in the production of orobanchol nor a significant reduction in amount of 4DO (Fig. S5c). Taken together, these results show that SIMAX1 catalyses the oxidation of CL to CLA, but not to 4DO, and that 4DO is not the substrate of SIMAX1 for the production of orobanchol.

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**Fig. 4** Expression of tomato (Solanum lycopersicum) strigolactone biosynthetic genes upstream of SIMAX1 in the Simax1 mutant. Relative expression of strigolactone biosynthetic genes (SIDD27, SICC7D and SICC8D) in the roots of Simax1 mutants after 7 d of phosphorus starvation (n = 3). The gene expression level in wild-type plants was set to 1. Error bars represent means ± SE. *, P < 0.01. SGN-U584254 and SGN-U563892 are the two housekeeping genes used for normalization of target genes (Dekkers et al., 2012).
Carlactone, but not 4-deoxyorobanchol, is the preferred direct precursor for strigolactone formation in tomato.

To further confirm the function of SlMAX1 and gain more insight into the origin of the tomato SLs, we performed plant feeding assays with SLs or SL precursors while inhibiting endogenous SL production with the carotenoid pathway inhibitor fluridine (Matusova et al., 2005; Motonami et al., 2013). In rice, it was shown that orobanchol is derived from 4DO by the orobanchol synthase (Os1400) (Zhang et al., 2014). However, 4DO has never been detected in tomato plants to date. To investigate whether 4DO is an intermediate in the biosynthesis of tomato SLs (orobanchol, solanacol and DDH isomers), we fed plants with an equal concentration (0.05 μM) of 4DO or the SL precursor CL. Upon CL feeding, there was a significant production of orobanchol in the root exudates, while with 4DO only a very small amount of orobanchol was detected (Fig. 6a). Similar results were observed in root extracts (Fig. S6a). Addition of the cytochrome P450 (CYP) inhibitor uniconazole-P in the feeding assay was able to suppress the conversion of CL to orobanchol, likely through inhibition of the activity of SlMAX1 or (an)other CYP(s) that might be involved in this multistep conversion (Fig. 6b). Upon CL feeding, we also detected a small amount of epi-orobanchol, likely ent-2’-epi-orobanchol (the other naturally occurring orobanchol stereoisomer) (Fig. S6b,c), which has not been identified in tomato as a natural SL to date. ent-2’-epi-Orobanchol has been reported to be present in other Solanaceae, such as tobacco (Xie et al., 2013), so it is not unlikely that it can also be produced in tomato.

It is well known that 2-oxoglutarate and iron(II)-dependent dioxygenase (2-OGD) family enzymes are involved in various oxidation and hydroxylation reactions in the plant kingdom (Kawai et al., 2014). To investigate whether this type of enzyme is also involved in tomato SL production, we supplemented the 2-OGD inhibitor prohexadione during the CL feeding assay. Intriguingly, in the tomato root exudates, the level of orobanchol derived from CL feeding was reduced by prohexadione, but to a lower extent than by the same concentration of CYP inhibitor (Fig. 6b). Although prohexadione did not cause a significant reduction in orobanchol amount in the root extracts (Fig. S6d), we cannot exclude the possibility that a 2-OGD enzyme contributes to the biosynthesis of orobanchol from CL in tomato. In our feeding assays, the conversion of 4DO to a trace amount of orobanchol was not decreased by CYP or 2-OGD inhibitor (Fig. S6e). These results are in line with the results from the heterologous expression experiments using *N. benthamiana* (Fig. S5c), suggesting that SlMAX1 uses CL as a substrate to produce CLA and that (an)other enzyme(s) is (are) required for biosynthesis of orobanchol, directly from CLA but not through 4DO.
Fig. 6  Strigolactone quantification in plant feeding assays. (a) Oroban chol production in the tomato root exudates after feeding of carlactone (CL) and 4-deoxy-orobanchol (4DO) in the same molar concentration (0.05 μM). (b) Oroban chol levels in the root exudates of tomato plants supplied with CL after supplementation of enzyme inhibitors for cytochrome P450s (uniconazole-P) or 2-oxoglutarate-dependent dioxygenases (prohexadione). (c) Solanacol accumulation in the root exudates of tomato plants supplied with CL or 4DO with or without the application of uniconazole-P. (d) Quantification of total amount of didehydro-orobanchol (DDH) isomers (1–3) in tomato plants after feeding of 4DO or CL with or without the presence of uniconazole-P. The total level of all three DDH isomers was quantified by summing their peak areas together. (e) Accumulation of solanacol in the tomato root exudates after feeding with orobanchol isomers (orobanchol or ent-2'-epi-orobanchol) with or without the presence of uniconazole-P. (f) Quantification of DDH2 in tomato root exudates after feeding of orobanchol stereoisomers with or without the presence of uniconazole-P. (g) Abundance of DDH1 in tomato root exudates after feeding of orobanchol stereoisomers with or without the addition of uniconazole-P. Grey and black bars: (a, b) multiple reaction monitoring (MRM) transitions of m/z 347.2 > 97 and 347.2 > 233.15 for orobanchol; (c, e) MRM transitions of m/z 343.16 > 96.97 and 343.16 > 183.02 for solanacol; (d, f, g) MRM transitions of m/z 345.16 > 96.96 and 345.16 > 203 for DDH isomers. Error bars in (a–g) represent means ± SE (n = 3–5). Statistical significance was determined by one-way ANOVA performed in Graphpad Prism 6, P < 0.01. F represents the carotenoid pathway inhibitor fluridone in (a–g). Lowercase letters in (a–g) indicate significant differences between treatments.
Cytochrome P450 enzymes are involved in the tomato strigolactone decoration

The absolute configuration of the C-ring in solanacol and medicocal (one of the DDH isomers isolated from Medicago truncatula) was reported to be the same as in orobanchol that is derived from 4DO in rice (Chen et al., 2010; Zwamenburg & Pospisil, 2013; Zhang et al., 2014; Tokunaga et al., 2015). Thus, we hypothesized that solanacol and DDH isomers in tomato share the same C-ring stereochemistry as 4DO, which might be the precursor of solanacol or DDH isomers. However, in our plant feeding experiment, solanacol was only detectable in root exudates and extracts after feeding with CL but not with 4DO; therefore, we could not determine its stereochemistry (Figs 6c, S6f). The total production of all three DDH isomers showed a strong increase after feeding of CL, while the induction after feeding of 4DO was much lower (Fig. 6d). Interestingly, the feeding of 4DO gave rise to a different DDH composition, showing predominantly an increase in DDH3, but not DDH1 and DDH2, suggesting 4DO, but not CL, is the substrate for DDH3 (Fig. S6g). After CL feeding, DDH2 is the most dominant peak, and it is probably masking the trace amount of DDH1 and/or DDH3 because of the overlapping of the peaks (Figs 6d, S6g). The production of orobanchol, solanacol and the DDH isomers from CL is inhibited by the CYP inhibitor (Fig. 6b–d). To further support the stereochemistry of solanacol and DDH isomers in tomato, we performed a plant feeding experiment with the two naturally occurring orobanchol stereoisomers – orobanchol and ent-2ʹ-epi-orobanchol (Fig. S7a). The production of solanacol was significantly induced by feeding of orobanchol (Figs 6c, S7b). Among the DDH isomers, the DDH2 peak was predominant after orobanchol feeding (Figs 6f, S7b,c), whereas DDH1 was induced (though at a low level) after feeding of ent-2ʹ-epi-orobanchol (Figs 6g, S7b,c). These results suggest that DDH1 is a strigol-type SL and is derived from ent-2ʹ-epi-orobanchol, while all other tomato SLs are orobanchol-type SLs. Solanacol biosynthesis and total production of DDH isomers from orobanchol was dramatically suppressed after the use of the CYP inhibitor (Figs 6d,e, S7b), suggesting that CYP enzymes are essential for the formation of solanacol and these DDH isomers from orobanchol or possibly other substrates (such as 4DO or ent-2ʹ-epi-orobanchol).

Discussion

In this study, we show that the MAX1 homologue in tomato, SIMAX1, catalyses the formation of CLA from CL (Fig. 5). In Arabidopsis, CLA is an intermediate in the production of SL-LIKE1 and is produced by the oxidation of CL by AtMAX1 (Abe et al., 2014). In this study, we did not detect the other two known intermediates in this triple oxidation reaction, 19-hydroxy-CL and 19-oxo-CL, in the N. benthamiana leaf samples co-expressing SlMAX1 with the CL biosynthetic genes using untargeted LC–Orbitrap-FTMS analysis (data not shown). Our results confirm that CL is an important intermediate in the production of canonical SLs (such as 4DO and orobanchol) as well as noncanonical SL-LIKE compounds, such as MeCLA in different plant species (Abe et al., 2014; Zhang et al., 2014; Brewer et al., 2016).

It has been postulated that 5DS and 4DO are the precursors for all canonical SLs of the strigol and orobanchol types respectively (Xie et al., 2010; Ruyter-Spira et al., 2013). Rice MAX1 homologue Os14000 was indeed demonstrated to act as orobanchol synthase, catalysing the conversion of 4DO to orobanchol (Zhang et al., 2014). However, multiple results from the present study are indicating that SIMAX1 does not produce 4DO from CL as an intermediate en route to orobanchol, but converts CL to CLA, which is then likely the precursor in the formation of orobanchol by an as yet unknown enzyme (Figs 5, 6a): feeding of CL, but not 4DO, to fluridone-treated plants results in orobanchol production (Fig. 6a). The conversion of CL to orobanchol is likely a multistep reaction mediated by multiple enzymes, including SIMAX1 (Fig. 7). Inhibition of orobanchol production by the 2-OGD inhibitor in the feeding assay suggests that a 2-OGD may play a role in this conversion (Figs 6b, 7). However, as this effect was not visible in root extracts (Fig. S6d), there may also be another CYP involved. The 2-OGD gene family is widely distributed in plants, microorganisms and mammals and is involved in the oxidation of organic substrates (Aravind & Koonin, 2001). In plants, this gene family has been reported to be essential for the biosynthesis and/or metabolism of several plant hormones, such as gibberellins, auxin and ethylene (Kawai et al., 2014). It is suggested that 2-OGDs prefer more hydrophilic substrates, such as those compounds that are obtained after hydroxylation by CYPs (Kawai et al., 2014). Therefore, oxidation catalysed by 2-OGDs usually occurs after the oxidation by CYPs or the glycosylation by uridine-diphosphate-sugar dependent glycosyltransferases. This has been proven to be true also in SL biosynthesis, in Arabidopsis, in which the 2-OGD, LATERAL BRANCHING OXIDOREDUCTASE (LBO), is involved in SL biosynthesis downstream of CYP AtMAX1 using MeCLA as a substrate (Brewer et al., 2016). However, we cannot exclude the involvement of a second CYP (Fig. 7).

Generally, canonical SLs with the typical tricyclic lactone coupled to the D-ring have been classified into two groups according to the stereochemistry of the C-ring; namely, orobanchol type and strigol type (Xie et al., 2013; Zwamenburg & Pospisil, 2013). In rice, so far only orobanchol-type SLs (orobanchol and 4DO) have been identified (Xie et al., 2013), while in sorghum the strigol-type SLs (sorgomol, sorgolactone and 5DS) and orobanchol have been reported (Yoneyama et al., 2010). The solanaceous species tobacco has been shown to produce at least 11 SLs from both SL families, including two orobanchol isomers – orobanchol and ent-2ʹ-epi-orobanchol – and three putative DDH isomers (Xie et al., 2013). As a solanaceous species, tomato has been shown to produce orobanchol-type SLs, such as orobanchol and solanacol (Kohlen et al., 2012). In this study, we have further unravelled the biosynthetic origin of several tomato SLs. Orobanchol in tomato derives from CL – and not from 4DO – by the sequential oxidation of CL by SIMAX1 and possibly a 2-OGD or another CYP (Figs 6a,b, S6d, 7). Consistent with previously published results about the absolute stereochemistry of solanacol
(Chen et al., 2010), we have shown that solanacol derives from orobanchol but not from \textit{ent-2'-epi-}orobanchol; this reaction requires one or more CYPs (Figs 6f, 7). Unlike in tobacco, \textit{ent-2'-epi-}orobanchol has never been detected previously in tomato, but here we find that it can be produced by tomato after feeding of CL (Fig. S6b,c), implying that the enzyme(s) responsible for \textit{ent-2'-epi-}orobanchol production is (are) present in tomato, just as in tobacco (Xie et al., 2013).
In tomato, the predominant DDH isomers are DDH1 and DDH2 (Fig. S2a–c). These DDH isomers are contributing to the major tomato SL profile detected by MRM-LC–MS/MS (Fig. 2) (Liu et al., 2011; Kohlen et al., 2013). However, the stereochemistry and biosynthetic origin of these DDH isomers are unclear. In M. truncatula, a DDH isomer was identified as medicanol with the orobanchol-type stereochemistry (Tokunaga et al., 2015), which is in line with our results for the predominant production of DDH2 after orobanchol feeding (Fig. 6f). Feeding of CL also resulted in a dramatic induction of DDH2, which is obviously due to the production of orobanchol from CL and the subsequent further conversion to DDH2 (Fig. 6a,f). Our current study provides the first evidence that DDH1 may derive from a strigol-type SL, which is indicated by the accumulation of DDH1 after ent-2′-epi-orobanchol feeding (Figs 6g, S7c). Although ent-2′-epi-orobanchol has not been reported in tomato, it has been reported in the close relative tobacco (Xie et al., 2013). Our results show that DDH1 is derived from a strigol-type intermediate, such as ent-2′-epi-orobanchol. DDH3 is a minor DDH isomer as previously reported in tomato (Kohlen et al., 2013), and we show that it is produced from 4DO and thus is an orobanchol-type SL (Fig. S6g).

In our study, we also observed differences in the SL profiles between root exudates and root extracts. The DDH isomers have a relatively higher concentration in root exudates than in root extracts, compared with orobanchol and solanacol (Fig. 2). Moreover, the DDH isomer composition also exhibits differences between root exudates and root extracts. DDH1 is most abundant in root exudates and DDH2 in root extracts (Fig. S2). Perhaps this is related to differences in the unknown biological function of these DDH isomers and/or differences in the specificity of possible SL transporter(s). It is intriguing that tomato and several other plant species produce specific DDH isomers and secrete them differentially into the rhizosphere (Xie et al., 2013; Tokunaga et al., 2015). So far, however, there are no reports concerning the biological activity of the DDH isomers, let alone about differences in their activities. The structures of these DDH isomers in tomato have also not been elucidated yet; in this study, we provide hints on their possible stereochemistry. The biosynthesis of these DDH isomers does not seem to be simple considering the structure of the only characterized DDH isomer, medicanol (Tokunaga et al., 2015). We show here that CYPs are likely involved in their biosynthesis, as the CYP inhibitor greatly suppressed their production (Fig. 6f,g). The GRAS-type transcription factor NODULATION SIGNALING PATHWAY2 (NPS2) was demonstrated to regulate DDH isomer biosynthesis in M. truncatula (Liu et al., 2011). Perhaps, in tomato, a homologue of this transcription factor is involved in the regulation of the CYPs that catalyse the biosynthesis of the tomato DDH isomers. It would be interesting to further identify the structures of DDH isomers in tomato and investigate their biological significance.

Our study for the first time provides evidence that, in tomato, orobanchol originates from CL through oxidation by SlMAX1, without the formation of 4DO as an intermediate. A 2-OGD and/or other CYP is likely involved in this process. We also provide evidence that CYPs are involved in the conversion of orobanchol to solanacol and one of the DDH isomers (DDH2 in the present research). It will be of great interest to further unravel these biosynthetic relationships such that a better insight into the biological relevance of all these different SLs can be obtained. Although little is known about SL production in the above-ground tissues, the biological functions of SLs in shoot branching and leaf senescence have been well documented (reviewed by Al-Babili & Bouwmeester, 2015). The fact that, upon transient expression of SL biosynthetic genes in N. benthamiana leaves, SLs are produced confirms that the precursor of SLs (all-trans-β-carotene) is present in these tissues. The absence of SLs in the shoot of most plant species thus suggests that the production of SLs is limited by the low presence/absence of SL biosynthetic enzymes in such tissues (Figs 5, S5, S4c–f). The upregulation of SL production and their biosynthetic enzymes by P starvation will likely be a useful tool for further discovery of novel enzymes that are responsible for SL biosynthesis and structural diversification, including enzymes that may catalyse the formation of solanacol and DDH isomers in tomato.

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Author contributions


References


**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information tab for this article:

**Fig. S1** Alignment of SlMAX1 with *Arabidopsis* and rice MAX1 homologs and characterization of the mutation in Slmax1.

**Fig. S2** Identification of didehydro-orobanchol isomers in wild-type tomato plant after P-starvation.

**Fig. S3** Plant phenotypes of the Slmax1 mutant.

**Fig. S4** Expression of tomato strigolactone biosynthetic pathway genes in different tissues in response to P-starvation and SlMAX1 expression in the *CCD8-RNAi* line.

**Fig. S5** Biochemical characterization of SlMAX1 in transient expression assays.

**Fig. S6** Production of strigolactones in the plant feeding assay with carlactone (CL) and 4-deoxy-orobanchol (4DO).

**Fig. S7** Accumulation of strigolactones after feeding of two natural orobanchol isomers.

**Table S1** Primers used in this study

**Table S2** SlMAX1 homologous protein sequences from different plant species by using protein BLAST from NCBI

**Table S3** Gene sequences characterized in this study

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