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Does ABA affect strigolactone biosynthesis?

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SUMMARY

• Strigolactones are considered a novel class of plant hormones that in addition to their endogenous signaling function are also exuded into the rhizosphere acting as a signal to stimulate hyphal branching of arbuscular mycorrhizal (AM) fungi and germination of root parasitic plant seeds. Considering the importance of the strigolactones and their biosynthetic origin (from carotenoids), we investigated the relationship with the plant hormone abscisic acid (ABA).

• Strigolactone production and ABA content in the presence of specific inhibitors of oxidative carotenoid cleavage enzymes and in several tomato ABA-deficient mutants were analyzed by LC-MS/MS. In addition, the expression of two genes involved in strigolactone biosynthesis was studied.

• The carotenoid cleavage dioxygenase (CCD) inhibitor D2 reduced strigolactone but not ABA content of roots. However, in abamineSG treated plants, an inhibitor of 9-cis-epoxycarotenoid dioxygenase (NCED), and the ABA mutants notabilis, sitiens and flacca, ABA and strigolactones were greatly reduced. The reduction in strigolactone production correlated with the down-regulation of LeCCD7 and LeCCD8 genes in all three mutants.

• The results show a correlation between ABA levels and strigolactone production, and suggest a role for ABA in the regulation of strigolactone biosynthesis.

Key Words: strigolactones; abscisic acid; hormone regulation; tomato; mutants; inhibitors
INTRODUCTION

Strigolactones are important signalling molecules that were first described as germination stimulants for the seeds of parasitic plants of the genera *Striga* and *Orobanche* (Cook et al., 1972; Bouwmeester et al., 2003). Later, they were also described as hyphal branching factors for germinating spores of the symbiotic arbuscular mycorrhizal (AM) fungi (Akiyama et al., 2005). Therefore, strigolactones play a dual and important role in the rhizosphere as host detection signals for AM fungi and root parasitic plants (Akiyama et al., 2005; Harrison, 2005; Paszkowski, 2006; Bouwmeester et al., 2007). In addition to their important role as rhizosphere signalling molecules, it has recently been demonstrated that strigolactones also act as a new hormone class that inhibits shoot branching in plants and hence regulates above-ground plant architecture (Gomez-Roldan et al., 2008; Umehara et al., 2008).

Strigolactones have been detected in the root exudates of a wide range of mono- and dicotyledonous plant species. The strigolactones discovered so far all have a similar chemical structure suggesting that they are all derived from the same biosynthetic pathway (Bouwmeester et al., 2007; Yoneyama et al., 2008). Indeed, we have previously demonstrated that the ABC-part of the strigolactones (Fig. 1) is derived from carotenoids through oxidative cleavage by carotenoid cleavage dioxygenases, hence classifying the strigolactones as apocarotenoids (Matusova et al., 2005; López-Ráez et al., 2008a; Rani et al., 2008). In addition, we have postulated how, after carotenoid cleavage, further enzymatic conversions are likely to lead to the production of all the strigolactones known to date (Matusova et al., 2005; Rani et al., 2008). Indeed, it was recently demonstrated that two carotenoid cleavage dioxygenases (CCDs), CCD7 and CCD8 - which were already proposed to be responsible for the biosynthesis of the elusive shoot branching inhibiting signal (Sorefan et al., 2003; Booker et al., 2004) - are directly involved in the biosynthesis of strigolactones (Gomez-Roldan et al., 2008; Umehara et al., 2008). The latter papers showed that mutants of pea *ramosus5* (*rms5*) and
ramosus1 (rms1)] and rice [high-tillering dwarf1 or dwarf17 (htd1 or d17) and dwarf 10 (d10)] for CCD7 and CCD8, respectively, produce significantly less strigolactones than the corresponding wild-types. In pea, the rms1 mutation reduced mycorrhizal symbiosis which could be restored by exogenously applied synthetic strigolactone (Gomez-Roldan et al., 2008). Moreover, root exudates of rms5 and rms1 induced less AM fungal hyphae branching and less germination of Orobanche seeds (Gomez-Roldan et al., 2008). Similarly in rice, the orthologous mutants were less infected by Striga hermonthica (Umehara et al., 2008).

In addition to the reduced production of strigolactones by the ccd7 and ccd8 mutants, it was previously shown that exudates of the mutants viviparous14 (vp14) in maize and notabilis in tomato, with a null mutation in the genes ZmNCED and LeNCED1 and encoding for 9-cis-epoxycarotenoid dioxygenases, also induced less germination of S. hermonthica and Orobanche ramosa seeds, respectively (Matusova et al., 2005; López-Ráez et al., 2008a). Moreover, by LC-MS/MS analysis it was demonstrated that in the case of the tomato mutant this reduction in germination stimulatory activity correlates closely with a reduction in the production of strigolactones, suggesting that NCED enzymes are involved, either directly or indirectly, in the biosynthesis of these signaling molecules (López-Ráez et al., 2008a). NCEDs belong to the family of carotenoid cleavage dioxygenase enzymes - to which also CCD7 and CCD8 belong - that form a small family composed of nine different members in Arabidopsis and twelve in rice of which five and six, respectively, belong to the NCED subgroup (Tan et al., 2003; Auldridge et al., 2006; Bouwmeester et al., 2007). NCEDs catalyze a critical step in the regulation of the biosynthesis of the phytohormone abscisic acid (ABA) in higher plants. 9'-Cis-neoxanthin and 9-cis-violaxanthin have been proposed to be the precursors for ABA biosynthesis (Li & Walton, 1990; Rock & Zeevaart, 1991; Parry et al., 1992). Cleavage of these molecules by NCED enzymes leads to the formation of xanthoxin that is converted to ABA-aldehyde by a short-chain alcohol dehydrogenase ABA2. Finally, an aldehyde oxidase (AAO) transforms ABA-aldehyde into the
bioactive ABA (Fig. 1) (Schwartz et al., 1997; Taylor et al., 2005). In addition to *notabilis*, in tomato
two more ABA-deficient mutants - *sitiens* and *flacca* - have been characterized. *Sitiens* has been shown
to be mutated in the enzyme AAO and *flacca* has a mutation in a molybdenum cofactor (MoCo) which
is required for the activity of the enzyme AAO (Fig. 1) (Cornish & Zeevaart, 1988; Taylor et al., 1988;
Sagi et al., 2002).

ABA plays a regulatory role in many physiological processes in all higher and lower plants
(Zeevaart & Creelman, 1988). It mediates plant responses to different kinds of abiotic stress such as
drought stress and is involved in long distance signalling in plants. ABA is the key signal regulating
stomatal aperture (Davies et al., 2005; Jiang & Hartung, 2008). In seeds, ABA promotes seed
development, embryo maturation, synthesis of storage products (proteins and lipids), desiccation
tolerance, and is involved in apoptosis and maintenance of dormancy (inhibition of germination)
(Zeevaart & Creelman, 1988; Bethke et al., 1999). In concert with other plant signalling molecules,
ABA is also implicated in mediating responses to pathogens and wounding (Adie et al., 2007).
Moreover, ABA also affects plant architecture, including root growth and morphology, and root-to-
shoot ratios (De Smet et al., 2006). In line with its important role as a phytohormone, ABA
concentrations in the plant are controlled by a tightly regulated balance between biosynthesis,
inactivation and degradation (Zeevaart & Creelman, 1988).

In the present study, the production of strigolactones in tomato mutants affected in ABA
biosynthesis at different steps of the pathway such as *notabilis*, *flacca* and *sitiens* was assessed.
Moreover, the effect of specific inhibitors of different oxidative carotenoid cleavage enzymes such as
abamineSG (NCED specific) and D2 (CCD7 specific) was also analyzed. The role of the
phytohormone ABA in regulating the production of strigolactones in plants is discussed.
MATERIALS AND METHODS

Plant material and chemicals

Seeds of tomato (*Solanum lycopersicum*) *sitiens* (LA0574) and its parental isogenic cv. Rheinlands Ruhm, and *flacca* (LA3613) and corresponding parental isogenic cv. Ailsa Craig, were obtained from the Tomato Genetics Resource Center (TGRC) at the University of California, Davis, CA, USA. Seeds of cv. Ailsa Craig and *notabilis* (LA3614) were kindly provided by Wim Vriezen (Department of Plant Cell Biology, Radboud University, Nijmegen, The Netherlands). Seeds of tomato cv. MoneyMaker were purchased at a local garden centre. *O. ramosa* seeds were kindly provided by Maurizio Vurro (Instituto di Scienze delle Produzioni Alimentari, Bari, Italy). The synthetic strigolactone analogue GR24 was kindly provided by Binne Zwanenburg (Department of Organic Chemistry, Radboud University, Nijmegen, The Netherlands). The strigolactone standards orobanchol and solanacol were kindly provided by Koichi Yoneyama (Weed Science Center, Utsunomiya University, Japan). The inhibitor abamineSG was kindly provided by Tadao Asami (RIKEN, Saitama, Japan). [\(^2\)H\(_6\)]-cis, trans-ABA was purchased at Olchemlm Ltd (Czech Republic).

Growth conditions and experiments

Tomato seeds were sterilized in 4% (v/v) sodium hypochlorite containing 0.02% (v/v) Tween 20, rinsed thoroughly with sterile water, and then germinated for 48 h on moistened filter paper at 25°C in darkness. Subsequently, tomato seedlings were grown in a greenhouse as described before (López-Ráez *et al.*, 2008b). Phosphate (Pi) starvation promotes the production of strigolactones (Yoneyama *et al.*, 2008).
2007; López-Ráez et al., 2008a). Therefore, one week before root exudate collection the substrate (sand:vermiculite; 1:1, v/v) in the pots was rinsed with 1.5 l (2 times the pot volume) of modified half-strength Hoagland solution without Pi to remove the strigolactones accumulated. Then plants were watered (twice a week) with modified half-strength Hoagland nutrient solution without Pi. For root exudate collection, the substrate in the pots was first rinsed as describe above to remove the strigolactones accumulated. After another 5h, 0.7 l of modified half-strength Hoagland solution without Pi was applied to the pots and the root exudate collected. Roots from each pot were then collected separately and frozen in liquid nitrogen and stored at -80°C until use. Purification of the root exudates and the germination bioassay were carried out as described before (López-Ráez et al., 2008b).

**Treatment with inhibitors and ABA**

Half-strength Hoagland solution without Pi and with or without 50 µM of the inhibitors abamineSG or D2 were applied to 4 weeks-old tomato plants which were then grown for an additional 3 or 7 d. To maintain the effect of the inhibitors, after 3 d plants for the 7 d treatment were watered with fresh nutrient solution containing the inhibitors. Root exudates and roots were collected on day 3 and day 7 as described above. In an attempt to rescue the strigolactone exudation phenotype of the ABA mutants, mutants were grown as described above. Half-strength Hoagland solution without Pi and with or without 0.5 µM ABA was applied to 4 weeks-old plants and grown for an additional 7 d. To maintain the ABA levels, after 3 d plants were watered with fresh nutrient solution containing ABA. Root exudates and roots were collected after 7 d for analyses.
Extraction of ABA and strigolactones from roots and shoots

For ABA and strigolactone analysis, 0.5 g of root or shoot tissue was ground in a mortar with liquid nitrogen. The samples were extracted with 2 ml of cold ethyl acetate containing [\textsuperscript{2}H\textsubscript{6}]-ABA as internal standard (0.025 nmol or 0.25 nmol for root or shoot tissue, respectively) in a 10 ml glass vial. The vials were vortexed and sonicated for 10 min in a Branson 3510 ultrasonic bath (Branson Ultrasonics, Danbury, CT, US). Samples were centrifuged for 10 min at 2500 g in an MSE Mistral 2000 centrifuge (Mistral Instruments, Leicester, UK) after which the organic phase was carefully transferred to a 4 ml glass vial. The pellets were re-extracted with another 2 ml of ethyl acetate. The combined ethyl acetate fractions were dried under a flow of N\textsubscript{2} and the residue dissolved in 250 µl of acetonitrile:water:formic acid (25:75:0.1, v/v/v). Before analysis, samples were filtered through Minisart SRP4 0.45 µm filters (Sartorius, Germany) and LC-MS/MS was performed as described below.

Strigolactone and ABA detection and quantification by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Analysis of strigolactones in tomato exudates and root extracts was conducted by comparing retention times and mass transitions with those of available strigolactone standards as described before (López-Ráez et al., 2008b). ABA analysis was performed by LC-MS/MS using a published protocol with some modifications (Saika et al., 2007). Analyses were carried out on a Waters Micromass Quattro Premier XE tandem mass spectrometer (Waters, Milford, MA, USA) equipped with an ESI source and coupled to an Acquity UPLC system (Waters, USA). Chromatographic separation was achieved using an Acquity UPLC BEH C\textsubscript{18} column (150 x 2.1 mm, 1.7 µm) (Waters, USA), applying a water/acetonitrile
gradient, starting at 0% acetonitrile for 2.0 min, raised to 50% (v/v) acetonitrile in 8.0 min, followed by a 1.0 min gradient to 90% (v/v) acetonitrile which was then maintained for 0.1 min and followed by a 0.2 min gradient back to 0% acetonitrile before the next run. The column was then equilibrated at this solvent composition for 2.8 min. Total run time was 15 min. The column was operated at 50ºC with a flow-rate of 0.4 ml min\(^{-1}\) and sample injection volume was 30 µl. The mass spectrometer was operated in positive electrospray ionization (ESI) mode. The nebuliser and desolvation gas flows were 50 and 800 l h\(^{-1}\), respectively. The capillary voltage was set at 2.7 kV, the cone voltage at 10 V, the source temperature at 120ºC and the desolvation gas temperature at 450ºC. Fragmentation was performed by collision induced dissociation with argon at 3.0 x 10\(^{-3}\) mbar. Multiple reaction monitoring (MRM) was used for ABA quantification. Parent-daughter transitions were set according to the MS/MS spectra obtained for the standards ABA and \([^2\text{H}_6]\)-ABA. Transitions were selected based on the most abundant and specific fragment ions for which the collision energy (CE) was optimized. For ABA, the MRM transitions \(m/z\) 265>229 at a CE of 10 eV and 265>247 at 5 eV; and for \([^2\text{H}_6]\)-ABA, the transitions \(m/z\) 271>234 at 10 eV and 271>253 at 5 eV were selected. ABA was quantified using a calibration curve with known amount of standards and based on the ratio of the summed area of the MRM transitions for ABA to those for \([^2\text{H}_6]\)-ABA. Data acquisition and analysis were performed using MassLynx 4.1 software (Waters, USA). The summed area of all the corresponding MRM transitions was used for statistical analysis.

RNA isolation and first strand cDNA synthesis

Total RNA from tomato roots was extracted using Tri-Pure reagent (Roche, Germany) according to the manufacturer’s protocol. The RNA was sequentially treated with DNase I (Invitrogen, The
Netherlands) at 37ºC for 15 min in order to remove the remaining genomic DNA. Before cDNA synthesis, the RNA was purified through a silica column using the RNeasy RNA Cleanup kit (Qiagen, Germany). The first strand cDNA was synthesized with 1 µg of purified total RNA using the iScript cDNA Synthesis kit (Bio-Rad, The Netherlands) according to the manufacturer’s instructions.

Gene expression analysis by real time quantitative RT-PCR (qPCR)

For gene expression analysis by real time quantitative RT-PCR (qPCR) the iCycler iQ5 system (Bio-Rad, The Netherlands) was used (Spinsanti et al., 2008) using specific primers. LeActin: 5’-TCCCAGGTATTGCTGATAGAA-3’ and 5’-TGAGGGAAGCCAAGATAGAG-3’; LeNCED1: 5’-ACCCACGAGTCCAGATTTC-3’ and 5’-GGTTCAAAAAGAGGGTTAGC-3’; LeNCED4: 5’-ACAACATCGAAAATGAAGCCG-3’ and 5’-GGCGAAAAAGTTTACCTCCA-3’; LeCCD1-B: 5’-AGAACACGTGACGGTTTACA-3’ and 5’-AGTGTAGTTCTCGTTGATCCGTG-3’; LeCCD7: 5’-AGCCAAGAATTGAGCCCG-3’ and 5’-GGGAAAAAGTTTACCTCCA-3’; LeCCD8: 5’-CAGGACAATGGCAGATCCC-3’ and 5’-GGGAAAAAGTTTACCTCCA-3’; SlCYP7070A1: 5’-TGTCCAGGGAATGAACTTGC-3’ and 5’-CAATGGGACTGGGAATGGTC-3’; Le4: 5’-ACTCAAGGCATGGGTACTGG-3’ and 5’-CCTTCTTTCTCCTCCACCT-3’. Three independent biological replicates were used and each PCR reaction was done in triplicate. Relative quantification of mRNA amount was performed using the comparative C_t method (Livak & Schmittgen, 2001). These values were then normalized using the C_t value for the tomato household gene LeActin. All the values were used to determine the change in gene expression according to the following calculation: fold-change = 2^{-\Delta(\Delta C_t)}, where \Delta C_t = C_t (target) - C_t (household) and \Delta(\Delta C_t) = \Delta C_t (treatment) - \Delta C_t (control). Down-regulation of expression is shown as negative values.
Statistical analysis

Data for ABA and strigolactone content of tomato roots and strigolactone content in tomato root exudates were subjected to one-way analysis of variance (ANOVA) using GenStat for Windows (9th edition). To analyze the results of germination bioassays, ANOVA after arcsine[sqrt(X)] transformation was used. When appropriate, data were subjected to the Duncan’s honestly significant difference test.

RESULTS

Germination stimulatory activity of notabilis, sitiens and flacca root exudates

We previously demonstrated that ABA-deficient maize (vp14) and tomato (notabilis) mutants with a mutation in NCED exhibit a decreased strigolactone production by the roots (Matusova et al., 2005; López-Ráez et al., 2008a). It has been suggested that NCEDs are the key enzymes in the ABA biosynthetic pathway (Fig. 1). To assess whether the reduction in strigolactone biosynthesis in the NCED mutants is caused directly by reduced NCED action or indirectly because of its effect on the ABA content of these mutants, in addition to notabilis, the tomato ABA-deficient mutants sitiens and flacca and their parental isogenic lines were studied. Sitiens and flacca are blocked in the final step of the ABA biosynthetic pathway, where the enzyme AAO catalyzes the oxidation of abscisic aldehyde to ABA (Fig. 1) (Schwartz et al., 2003; Taylor et al., 1988). Sitiens is known to have a mutation in the
AAO enzyme and mutant leaves contain only about 11% of the wild-type ABA levels (Cornish & Zeevaart, 1988; Taylor et al., 1988). The mutant flacca has a mutation in a MoCo cofactor required for the activity of AAO and mutant leaves contain about 33% of the wild-type ABA levels (Cornish & Zeevaart, 1988; Sagi et al., 2002).

The mutants notabilis, sitiens and flacca showed the characteristic wilty phenotype (Taylor et al., 1988; Thompson et al., 2000b). Root exudates of the three mutants and their corresponding wild-types were collected for a germination bioassay with O. ramosa seeds. The synthetic germination stimulant GR24, as a positive control, always induced the germination of preconditioned O. ramosa seeds (up to about 85%). Water, used as a negative control, only induced 2% germination (Fig. 2). As we described before (López-Ráez et al., 2008a), root exudates of notabilis induced about 40% less germination than the corresponding wild-type (Fig. 2). The germination stimulatory activity of sitiens and flacca exudates was about 52 and 48%, respectively, lower than for those of the corresponding wild-types (Fig. 2). In addition to the differences between the mutants and corresponding wild-types, there was some variation between the germination stimulatory activities of the wild-type exudates (Fig. 2). For example, the wild-type for sitiens (cv. Rheinlands Ruhm) induced lower germination than the wild-type for flacca and notabilis (cv. Ailsa Craig) (Fig. 2).

**LC-MS/MS analysis and quantification of strigolactones**

To assess whether the lower germination stimulatory activity of the ABA-deficient tomato mutants was due to a decrease in the production of strigolactones, LC-MS/MS analysis was performed to compare the levels of strigolactones in the root exudates of the mutants and corresponding wild-types. All the three major strigolactones detected - solanacol and the two dihydro-orobanchol isomers - were
significantly ($P < 0.01$) reduced in the *notabilis*, *sitiens* and *flacca* root exudates compared with the wild-types (Fig. 3A). The other strigolactone present in tomato - orobanchol - was also detected, but its concentration was too low for accurate quantification. The concentration of strigolactones in the root extracts showed a similar trend as for the root exudates (Fig. 3B), indicating that there is a reduction in strigolactone biosynthesis in the mutants rather than just a decrease in the exudation. Interestingly, in the roots the decrease in didehydro-orobanchol 1 in the ABA mutants was larger than the decrease in solanacol and the didehydro-orobanchol 2 isomer, whereas in exudates the decrease in their concentration was similar (Fig 3). Overall, the level of strigolactones in the exudates of *notabilis*, *sitiens* and *flacca* was about 40, 47 and 52%, respectively, lower than the corresponding wild-types, which correlates well with the reduction in the germination stimulatory activity of the mutant exudates (40%, 52% and 48%, respectively). In an attempt to rescue the phenotype of the mutants, ABA (0.5 µM) was exogenously applied by irrigation to all the three mutants. However, no effect on strigolactone biosynthesis was observed in comparison with the untreated plants (data not shown). The same pattern was observed when higher ABA concentrations (1 and 10 µM) were applied to *sitiens* and its corresponding wild-type (Fig. S1). Although no increase in ABA was detected in the roots after exogenous ABA application, expression analysis of the ABA-responsive gene *Le4* (Kahn *et al.* 1993) and *SICYP707A1*, an ABA-8’-hydroxylase involved in ABA catabolism (Taylor *et al.*, 2005, Nitsch *et al.*, 2009), showed a 250- and 15-fold increase, respectively upon ABA treatment (Fig. S2), indicating that ABA was effectively taken up by the roots but is also effectively catabolised.

Corresponding to the differences in germination stimulatory activity (see above), differences in strigolactone concentration between the different wild-types were observed, with the background of *notabilis* and *flacca* (cv. Ailsa Craig) producing more strigolactones than the background of *sitiens* (cv. Rheinlands Ruhm) (Fig. 3A).
ABA quantification by LC-MS/MS

The tomato mutants *notabilis*, *sitiens* and *flacca* have previously been characterized to have a lower ABA content mainly in the leaves (Cornish & Zeevaart, 1988; Thompson *et al*., 2000b). Since in the current work we wished to study the relationship between ABA and the root-produced strigolactones, we have also analyzed the levels of ABA in the roots of these three mutants. The concentration of ABA detected in the roots of *notabilis*, *sitiens* and *flacca* was about 45, 60 and 65%, respectively, lower than in the corresponding wild-types (Table 1). ABA levels in the aerial part (stems and leaves combined) showed similar reductions as in the roots, although their levels were much higher (about 25-fold) than in the roots (Table 1). Just as for the concentration of the strigolactones and the germination stimulatory activity, significant ($P < 0.01$) differences between the different wild-types in the ABA content were observed (Table 1).

Specific carotenoid cleavage enzyme inhibitors

To further investigate the involvement of NCEDs in strigolactone biosynthesis an experiment with the specific NCED inhibitor abamineSG (Fig. 1) was carried out. In parallel, the effect of another inhibitor - D2 - specific for the other class of carotenoid-cleaving enzymes CCD7 and CCD8, which are involved in strigolactone biosynthesis (Fig. 1), was also tested. AbamineSG is a tertiary amine derivative acting as a competitive inhibitor of the NCEDs (Kitahata *et al*., 2006). D2 is a derivative of a hydroxamic acid (aryl-C$_2$N) and is a potent inhibitor of CCD enzymes cleaving at the 9,10 position of carotenoids (Sergeant *et al*., 2009), but is considerably less active against NCEDs (11,12 cleavage) in
vitro. It has been postulated that CCD7 cleaves β-carotene at the 9,10 position to produce the apocarotenoid 10-apo-β-carotene which is then further cleaved by CCD8 in the pathway leading to the strigolactones (Alder et al., 2008; Gomez-Roldan et al., 2008; Umehara et al., 2008).

Tomato plants were treated with the inhibitors abamineSG or D2 by irrigation (see Materials and Methods) and the amount of strigolactones produced by the plants was measured by LC-MS/MS. Pi starvation clearly induced the production of the tomato strigolactones solanacol, didehydro-orobanchol 1 and didehydro-orobanchol 2 compared with control plants grown under adequate Pi nutrition in a time-dependent manner (Fig. 4). Again, orobanchol was also detected, but its concentration was too low for accurate quantification. In plants treated with the inhibitor abamineSG (ABA inhibitor), the increase in strigolactone production induced by Pi starvation was significantly ($P < 0.01$) reduced (Fig. 4), suggesting again an involvement of NCEDs in strigolactone biosynthesis. This inhibition was more evident after 7 d of treatment, when the production and/or exudation of solanacol and the two didehydro-orobanchol isomers was reduced by 58, 77 and 59%, respectively. When plants were treated with D2 (strigolactone inhibitor), similarly as after treatment with abamineSG, a clear reduction in strigolactone production was observed in a time-dependent manner (Fig. 4). In this case, the decrease in the production of solanacol and the two didehydro-orobanchol isomers after 7 d was 54, 70 and 62%, respectively.

To check the effect of these two inhibitors on ABA biosynthesis, the ABA content in the roots and shoots after 7 d treatment was quantified. Here, a low but significant ($P < 0.01$) reduction of about 27% was only observed in the roots of plants treated with abamineSG, whereas there was no effect on ABA content after treatment with the inhibitor D2 (Table 2). When ABA levels were analyzed in the shoots, no effect of either of the two inhibitors was observed. Pi starvation alone did not affect ABA content neither in the roots nor in the shoots (Table 2), which is in line with previous findings in castor bean (Ricinus communis) where it was shown that ABA synthesis in roots, ABA xylem transport and ABA
catabolism in shoots were induced upon phosphorus stress, while ABA content in both roots and shoots was not affected (Jeschke et al., 1997).

Gene expression analysis for ABA and strigolactone biosynthetic genes

Expression analysis by quantitative real time RT-PCR (qPCR) was performed to check expression of tomato genes encoding different carotenoid cleavage enzymes. As the tomato genome is not completely sequenced, only some of the genes coding for enzymes from this family are known. The genes studied in the present work are LeCCD1-B, LeCCD7, LeCCD8, LeNCED1 and LeNCED4. LeCCD1-A and -B are involved in the production of the flavour volatiles β-ionone, pseudo-ionone and geranylacetone (Simkin et al., 2004). CCD7 and CCD8 have been described to be involved in the biosynthesis of strigolactones in pea, rice and Arabidopsis (Gomez-Roldan et al., 2008; Umehara et al., 2008). More recently, CCD7 from tomato was cloned and characterized and shown to be involved in strigolactone biosynthesis, shoot branching and formation of mycorrhiza-induced apocarotenoids (Vogel et al., 2009). In A. thaliana all NCEDs, except AtNCED4 are known to be involved in ABA production (Auldridge et al., 2006; Bouwmeester et al., 2007).

Expression of LeCCD7 and LeCCD8 was clearly reduced in all three ABA-deficient mutants - notabilis, sitiens and flacca - compared with the corresponding wild-types, whereas the expression of the other carotenoid cleavage genes was not affected (Fig. 5A). Moreover, this reduction was more evident in sitiens and flacca, which had a lower ABA content than notabilis (Table 1). In notabilis, with a null mutation in LeNCED1 caused by a single A/T base pair deletion (Burbidge et al., 1999), the expression of this gene was not significantly reduced, in agreement with previous observations that expression of LeNCED1 was not regulated by ABA (Thompson et al., 2000a). The other NCED gene
so far described in tomato - *LeNCED4* - was significantly reduced although to a lesser extent than *LeCCD7* and *LeCCD8* (Fig. 5A). When gene expression was assessed in roots upon 7 d application of the inhibitors abamineSG and D2, no significant changes were detected in the expression levels of any of the selected genes (Fig. 5B). None of the genes was significantly affected by Pi starvation (data not shown).

**DISCUSSION**

ABA is an important phytohormone playing many physiological roles in plants (Zeevaart & Creelman, 1988; Davies *et al.*, 2005; De Smet *et al.*, 2006; Adie *et al.*, 2007; Jiang & Hartung, 2008). ABA is an apocarotenoid produced via oxidative cleavage of epoxycarotenoids through action of NCEDs, which catalyze the rate-limiting step in ABA biosynthesis (Parry & Horgan, 1992; Thompson *et al.*, 2000a; Taylor *et al.*, 2005) (Fig. 1). We have previously demonstrated that in the ABA-deficient mutants *vp14* and *notabilis* in maize and tomato, respectively - having a mutation in an NCED and therefore showing a lower ABA content - the production of strigolactones was decreased by about 40% (Matusova *et al.*, 2005; López-Ráez *et al.*, 2008a). From these results it was concluded that the NCEDs either have a direct role in strigolactone biosynthesis or that ABA has a regulatory role in this process (Matusova *et al.*, 2005; López-Ráez *et al.*, 2008a). We show here that the corresponding decrease in ABA and strigolactone production also occurs in two other well characterized tomato ABA-deficient mutants *sitiens* and *flacca* that are mutated in the last step of ABA biosynthesis - transformation of ABA aldehyde into ABA by an AAO - (Fig. 1) (Schwartz *et al.*, 2003; Taylor *et al.*, 1988). The decrease in ABA content in the roots of these mutants was stronger than for *notabilis*, being 60 and 65% lower than the corresponding wild-types, respectively. This reduction in ABA content in the roots and also in
the shoots of *sitiens* and *flacca* is somewhat lower than previously reported, when 89 and 67% reduction, respectively were detected in leaves (Cornish & Zeevaart, 1988). This discrepancy is likely due to differences in the conditions under which the plants were grown and/or the analytical method used. In the present study ABA content as well as strigolactones were measured using the same methodology - LC-MS/MS in MRM mode - and using the same plant material. The levels of strigolactones produced by the roots of *sitiens* and *flacca* were also lower than for *notabilis* (Fig. 3A). A strong correlation was observed between the reduction of ABA and strigolactone content in the roots when comparing a mutant with its corresponding wild-type, but across cultivars and mutants there was no correlation between ABA and strigolactone levels. Strigolactone-deficient mutants are characterized by a shoot branching phenotype (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008), but this phenotype was not observed for *notabilis*, *sitiens* and *flacca*, even though the strigolactone content of these mutants is reduced. Apparently, the reduced strigolactone level is still high enough to maintain normal shoot architecture. In a recent study on tomato expressing an *SlCCD7* antisense construct it was shown that an 80% reduction in strigolactone level induced only a weak branching phenotype, whereas in two other lines with over 90% reduction in strigolactone level branching was strongly increased (Vogel *et al.*, 2010).

The fact that ABA-deficient mutants with mutations in enzymes of the biosynthetic pathway other than the NCEDs also produce less strigolactones seems to indicate that NCED is not directly involved in strigolactone biosynthesis, but that its biosynthetic product ABA might be involved somehow in regulating strigolactone biosynthesis. ABA has previously been associated to AM symbiosis as well (Herrera-Medina *et al.*, 2007; Aroca *et al.*, 2008). These authors showed that the mutant *sitiens* was less prone to colonization by AM fungi than the wild-type, but that continuous exogenous application of ABA could not significantly compensate this effect in the mutant. Herrera-Medina and co-workers proposed that ABA increases the susceptibility of tomato to AM colonization and that this is necessary
for a proper AM establishment. The authors also suggest that ABA may play a role in the development
of the arbuscule and in regulating its functionality (Herrera-Medina et al., 2007). We show here that in
sitiens, as well as in the other two ABA-deficient mutants notabilis and flacca, the production of
strigolactones is significantly reduced compared with the corresponding wild-types (Figs. 2 and 3).
Therefore, the reduction in AM colonization observed in sitiens may also be due to a reduction in the
production of strigolactones by this mutant, although an additional role of ABA in the establishment of
AM symbiosis cannot be discarded. Indeed, root exudates of the pea strigolactone-deficient mutants
rms1 and rms5, with a mutation in the CCD8 and CCD7 genes respectively, have been shown to have a
significantly reduced activity in promoting AM fungal hyphae branching when compared with wild-
type exudates (Gomez-Roldan et al., 2008). Interestingly, when ABA was exogenously applied to
sitiens no effect on strigolactone production was observed. The failure to complement the strigolactone
phenotype of sitiens by exogenous ABA application is in line with the above-mentioned failure to
complement the AM-colonization phenotype of sitiens by exogenous ABA application (Aroca et al.,
2008). The same lack of effect was observed when ABA was applied to the other two mutants notabilis
and flacca. These results suggest that endogenous ABA may be required for strigolactone production in
specific root cells or tissues and hence for AM colonization and development. Apparently this
requirement cannot be replaced by exogenous ABA application. That exogenous ABA application may
not be suitable to replace endogenous ABA is also clear from a study on drought-stress induced ABA
using luciferase ABA-reporter plants. Exposure of Arabidopsis seedlings to exogenous ABA resulted
in a uniform pattern of reporter expression, whereas reporter expression in response to drought stress
was predominantly confined to the vasculature and stomata (Christmann et al., 2005). ABA is known
to stimulate its own degradation via the ABA hydroxylases in order to control its homeostasis (Cutler et
al., 1997). Indeed, we observed induction of the gene encoding ABA hydroxylase upon ABA
application.
In addition to the differences between the ABA-deficient mutants and corresponding wild-types, we also observed significant differences in the germination stimulatory capacity for *O. ramosa* seeds of root exudates from the wild-type cultivars Ailsa Craig and Rheinlands Ruhm (Fig. 2). LC-MS/MS analysis confirmed that the activity differences correlate with differences in the level of strigolactones in the root exudates (Fig. 3A), and these correlated to the strigolactone levels in root extracts (Fig. 3B). The results show that there is genetic variation for the production of strigolactones in different tomato cultivars, as we previously observed for two other tomato cultivars MoneyMaker and Manapal (López-Ráez *et al.*, 2008b). The genetic variation in strigolactone production indicates that selection of tomato cultivars producing low levels of germination stimulants - strigolactones - may be a strategy to breed tomato varieties resistant or less susceptible to *Orobanche* (López-Ráez *et al.*, 2009). Selection for low germination stimulant germplasm has been successfully used in other crops such as sorghum in order to produce *Striga* resistant varieties (Ejeta, 2007). The selection process in sorghum was based on the use of germination bioassays, but with the advent of extremely sensitive analytical methods such as LC-MS/MS in MRM mode described in the present paper, an analytically-supported selection process seems now feasible.

Besides ABA-deficient mutants, specific inhibitors for different carotenoid cleaving enzymes were used in the present study (Fig. 1). The inhibitor abamineSG - a specific inhibitor for NCEDs and therefore for ABA biosynthesis (Kitahata *et al.*, 2006) - reduced the production of the three major strigolactones present in tomato, solanacol and the two didehydro-orobanchol isomers in plants grown under Pi limited conditions (Fig. 4). After 7 d treatment, the reduction in strigolactone production in the abamineSG-treated plants was similar to the reduction observed in the mutants *sitiens* and *flacca*. Moreover, this reduction in strigolactones was accompanied by a minor but significant decrease in ABA content in the roots of the treated-plants, again suggesting a regulatory effect of ABA in strigolactone biosynthesis. The other members of the carotenoid cleavage enzyme family are the CCDs,
involved in the formation of apocarotenoid compounds such as flavour volatiles, cyclohexanone and mycorradicin derivatives (the yellow pigment formed in host roots upon arbuscular mycorrhizal colonization) and strigolactones (Simkin et al., 2004; Strack & Fester, 2006; Sun et al., 2008). CCD7 and CCD8 are involved in the control of plant architecture because they are involved in the production of the strigolactones (Sorefan et al., 2003; Booker et al., 2005; Gomez-Roldan et al., 2008; Umehara et al., 2008). It was shown that AtCCD7 can convert C40 carotenoids into C27 apocarotenoids (Schwartz et al., 2004) and that, subsequently, CCD8 can cleave the C27 into a C18 apocarotenoid, the probable precursor of the plant branching inhibitor (Alder et al., 2008). D2 specifically inhibits these CCDs, so also strigolactone biosynthesis and was included to investigate whether low strigolactone levels also affect ABA levels. Recently, it was shown that D2 showed selectivity in vitro towards CCDs that cleave at the 9,10 position - such as CCD7 - rather than towards NCEDs that cleave at the 11,12 position of C40 cis-carotenoids (Sergeant et al., 2009). In the present study, D2 indeed caused a reduction in strigolactone production suggesting that this inhibitor did indeed inhibit either CCD7 or CCD8 or both (Fig. 4). However, no effect on ABA content was observed in the roots of D2-treated plants, confirming that NCEDs are not significantly inhibited in vivo, thus supporting the observed in vitro selectivity of the inhibitor (Sergeant et al., 2009). These results also show that a short-term reduction in strigolactone levels does not lead to a reduction in ABA. The results are also in agreement with those observed previously where continuous application of D2 increased the number of side branches from the rosette nodes of Arabidopsis, mimicking the Arabidopsis max3 (ccd7) bushy phenotype, presumably by inhibiting one or more of the CCDs involved in strigolactone biosynthesis (Sergeant et al., 2009). However, in that study it was not analytically assessed whether that phenotype was caused by an inhibition of strigolactone biosynthesis. Here we show that application of D2 indeed inhibits strigolactone production, and thus it is a useful and selective inhibitor for use in in vivo studies on strigolactones.
When the expression of *LeCCD7* and *LeCCD8* was checked by real time qPCR, a clear decrease in expression for both genes was observed in all three mutants *notabilis*, *sitiens* and *flacca* (Fig. 5A). This reduction was most clear in *sitiens* and *flacca*, the mutants with the strongest reduction in ABA content (Table 1). No differences were observed in the expression of the other carotenoid-cleaving enzymes known in tomato (Fig. 5A). Although a reduction in gene expression levels does not necessarily imply a reduction in the enzyme activity (Fraser et al., 2007; Carbone et al., 2009), the results shown here confirm the involvement of CCD7 and CCD8 in strigolactone biosynthesis in tomato (Vogel et al., 2010). In contrast, the reduction in strigolactone level by abamineSG treatment did not correlate with a down-regulation of *LeCCD7* and *LeCCD8* (Fig. 5B). This suggests that these genes are not transcriptionally regulated by ABA in the short-term. Post-transcriptional regulation of CCD8 by auxin was postulated to occur in Arabidopsis (Bainbridge et al., 2005). In *sitiens* the levels of IAA in the roots have been reported to be lower than in the corresponding wild-type (Dunlap & Binzel, 1996). Therefore, the decrease in strigolactone production in the tomato ABA-mutants may be mediated by a decrease in auxin levels in the roots which negatively affects *LeCCD7* and *LeCCD8* expression and hence, the production of strigolactones in these mutants. A short-term decrease in ABA through abamineSG application does not lead to a similar reduction in *LeCCD7* and *LeCCD8* expression, even though strigolactone production is decreased, suggesting that a short-term response of strigolactone production to ABA is not mediated through a decrease in auxin levels and its negative effect on *LeCCD7* and *LeCCD8* expression.

It has been shown that Pi starvation promotes strigolactone biosynthesis (Yoneyama et al., 2007; López-Ráez et al., 2008a). Despite the fact that the strigolactones are carotenoid derived, the amount of carotenoids is not increased in roots under Pi starvation (López-Ráez et al., 2008a) and no changes in the expression of genes encoding enzymes involved in carotenoid biosynthesis were observed under Pi deprivation using microarray studies (Wasaki et al., 2003; Misson et al., 2005; Hernandez et al., 2007;
López-Ráez & Bouwmeester, 2008). Here we observed that Pi starvation, like ABA application, does also not affect the expression of the strigolactone biosynthetic genes LeCCD7 and LeCCD8. This suggests that the effect of phosphorous deficiency on strigolactone production might be at the post-transcriptional level or on an as yet unknown step in the strigolactone biosynthetic pathway. Our results suggest that ABA could be involved in this regulation.

This is the first report in which a correlation is demonstrated between the levels of the phytohormone ABA and the new class of phytohormones strigolactones. Our results obtained with tomato ABA-deficient mutants blocked at different steps in the ABA biosynthetic pathway and the application of specific inhibitors for NCEDs and CCDs suggest that ABA is one of the regulators of strigolactone biosynthesis through an as yet unknown mechanism. Further research is required to elucidate the mechanism by which strigolactone biosynthesis is fine-tuning regulated and the hormone network behind this regulation.

ACKNOWLEDGMENTS

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Maurizio Vurro for supplying *O. ramosa* seeds, Wim Vriezen for supplying Ailsa Craig and *notabilis* seeds, and Harry Klee and Jonathan Vogel for *LeCCD7* sequence information.

**FIGURE AND TABLE LEGENDS**

**Figure 1.**
Abscisic acid (ABA) and strigolactone biosynthetic pathways. Mutants and inhibitors (→) used or discussed in this study are shown in italics and bold text, respectively. Abbreviations of enzyme names: NCED, 9-cis-epoxycarotenoid dioxygenase; AAO, aldehyde oxidase; CCD7 and CCD8, carotenoid cleavage dioxygenase 7 and 8; MAX1, corresponds to the cytochrome p450 shown to be involved in the biosynthesis of the branching inhibiting signal (Booker *et al.*, 2005).

**Figure 2.**
Germination of *O. ramosa* seeds induced by the root exudates of the tomato mutants *notabilis* (not), *sitiens* (sit) and *flacca* (flc) compared with the corresponding wild-types (WT). GR24 (10⁻⁹ and 10⁻¹⁰ M) and demineralized water were used as positive and negative controls, respectively. Within each experiment, the concentrations of the root exudates were equalized by dilution to the same ratio of volume of exudate to root fresh weight. Bars represent the average of 5 independent replicates ± SE. Bars with a different letter differ significantly (*P* < 0.01) according to Duncan’s significant difference test.

**Figure 3.**
Strigolactone content in tomato root exudates and extracts. The amount (according to the peak area) of the strigolactone solanacol and the didehydro-orobanchol isomers 1 and 2 (Didehydro-1 and Didehydro-2) in the root exudates (A), and in the root extracts (B) of the tomato mutants *notabilis*, *sitiens* and *flacca*, and corresponding wild-types (WT) was quantified. Strigolactone content was analysed using LC-MS/MS (see Materials and Methods). Bars represent the average of 5 independent replicates ± SE. Bars with a different letter differ significantly ($P < 0.01$) according to Duncan’s significant difference test.

**Figure 4.**

Effect of Pi starvation and treatment with inhibitors on strigolactone production in tomato. The amounts (according to the peak area and corrected to 1 g root fresh weight) of the strigolactones solanacol, and the didehydro-orobanchol isomers 1 and 2 (Didehydro-1 and Didehydro-2) in the root exudates of tomato (cv. MoneyMaker) plants under Pi starvation (-Pi) and under Pi starvation plus treatment with 50 µM of abamineSG (-Pi +A-SG) or D2 (-Pi + D2) for 3 and 7 days. The SEPAK C$_{18}$ purified exudates were analysed using LC-MS/MS (see Materials and Methods). Bars represent the average of 5 independent replicates ± SE. Bars with a different letter differ significantly ($P < 0.01$) according to Duncan’s significant difference test.

**Figure 5.**

Gene expression analysis by real time qPCR for the carotenoid cleaving genes *LeCCD7, LeCCD8, LeCCD1-B, LeNCED1* and *LeNCED4* in tomato roots. **A**, gene expression of the tomato mutants *notabilis* (white bars), *sitiens* (black bars) and *flacca* (grey bars). **B**, gene expression in roots of tomato (cv. MoneyMaker) plants grown under Pi starvation (-Pi; grey bars) and under Pi starvation plus treatment with 50 µM of abamineSG (-Pi +A-SG; black bars) or D2 (-Pi + D2; white bars) for 7 days.
Real time qPCR was based on the C$_t$ values as described in Materials and Methods. C$_t$ values were normalized using the household gene *LeActin*. The expression for each gene in the different mutants is given relative to the expression of the same gene in the corresponding wild-type. Bars represent mean values ± SE of 3 independent biological replicates.

Table 1.

ABA quantification in ethyl acetate extracts from roots and shoots of the tomato mutants *notabilis*, *sitiens* and *flacca*, and corresponding wild-types (WT) by LC-MS/MS (see Materials and Methods). Numbers represent the average of 5 independent replicates ± SD. Different superscript letters indicate statistically significant differences between means (P < 0.01).

Table 2.

ABA quantification in the roots and shoots of tomato plants (cv. MoneyMaker) upon Pi starvation and after treatment with inhibitors for 7 d. ABA was analysed using LC-MS/MS (see Materials and methods) in extracts of tomato plants with normal Pi (+Pi), under Pi starvation (-Pi) and under Pi starvation plus treatment with 50 µM of abamineSG (-Pi +A-SG) or D2 (-Pi + D2) for 3 and 7 days. Numbers represent the average of 5 independent replicates ± SD. Different superscript letters indicate statistically significant differences between means (P < 0.01).

SUPPLEMENTAL MATERIAL

Figure S1.
Strigolactone content in the root exudates of the tomato ABA-deficient mutant sitiens (A), and corresponding wild-type (WT) (B) after ABA (10 μM) application. The amount (according to the peak area) of the strigolactone solanacol and the didehydro-orobanchol isomers 1 and 2 (Didehydro-1 and Didehydro-2) was quantified. Strigolactone content was analysed using LC-MS/MS. Bars represent the average of 4 independent replicates ± SE.

Figure S2.

Gene expression analysis by real time qPCR of Le4 (A) and ABA-8’-hydroxylase (SlCYP7070A1) (B) upon ABA application (10 μM) to sitiens (black bars) and corresponding wild-type (Reinlands Rhum, WT) (grey bars) for 48 hours. Real time qPCR was based on the C_t values as described in Materials and Methods. C_t values were normalized using the gene LeActin as an endogenous control gene. The expression for each gene is given relative to the expression of the same gene in plants not treated with ABA. Bars represent mean values ± SE of 3 independent biological replicates.

REFERENCES


Figure 1

B-carotene
\[ \downarrow \]
Zeaxanthin
\[ \downarrow \]
9'-cis-violaxanthin, 9'-cis-neoxanthin

NCED (notabilis) \[ \leftarrow \] AbamineSG
\[ \downarrow \]
Xanthoxin
\[ \downarrow \]
Xanthoxin oxidase
\[ \downarrow \]
ABA-aldehyde
\[ \downarrow \]
AAO (sitiens, flacca)

MAX1
\[ \leftarrow \]
CCD7 \[ \rightarrow \] D2

CCD8 \[ \leftarrow \] D2 ?

Strigolactones
Figure 2

The figure shows the germination percentage (%) at 0%, 20%, 40%, 60%, and 80% for different treatments. The treatments include WT notabilis, WT sitiens, WT flacca, 10⁻⁹ M, and 10⁻¹₀ M GR24. The bars are labeled with letters (a, b, c) and indicate significant differences in germination percentages among treatments.
Figure 3

A

B

Amount of strigolactones (peak area)

WT not  notabilis  WT sit  siliens  WT flc  flacca

WT not  notabilis  WT sit  siliens  WT flc  flacca

[Solanacol] [Didehydro-1] [Didehydro-2]
Figure 4

The figure shows the amount of strigolactones (peak area) measured at 3d and 7d after treatment. The data is presented for different conditions: 

- **+PI**: Treatment with PI alone.
- **+PI + ASG**: Treatment with PI and ASG.
- **+PI + D2**: Treatment with PI and D2.

The y-axis represents the amount of strigolactones, while the x-axis categorizes the different treatments. Bars with different letters indicate significant differences in the amount of strigolactones. The figure also includes legend for different types of strigolactones:

- **Solanacol**
- **Didehydro-1**
- **Didehydro-2**
Figure 5

A

Figure showing fold-change in gene expression (relative values) for LeCCD7, LeCCD8, LeCCD1-B, LeNCED1, and LeNCED4 across different treatments.

B

Figure showing fold-change in gene expression (relative values) for LeCCD7, LeCCD8, LeCCD1-B, LeNCED1, and LeNCED4 under different conditions.

Legend:
- notabilis
- sitiens
- flacca

Treatments:
- -Pi
- -Pi + A-SG
- -Pi + D2
Table 1

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Table 2

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