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Complete List of Authors:	Lopez-Raez, Juan Antonio; Wageningen University, Laboratory of Plant Physiology; Estacion Experimental del Zaidin-CSIC, Soil Microbiology and Symbiotic Systems Kohlen, Wouter; Wageningen University, Laboratory of Plant Physiology Charnikhova, Tatsiana; Wageningen University, Laboratory of Plant Physiology Mulder, Patrick; RIKILT - Institute of Food Safety, Analysis and Development Undas, Anna; Wageningen University, Laboratory of Plant Physiology; Wageningen University, Centre for Biosystems Genomics Sergeant, Martin; University of Warwick, Warwick-HRI Verstappen, Francel; Wageningen University, Laboratory of Plant Physiology; Wageningen University, Centre for Biosystems Genomics Bugg, Timothy; University of Warwick, Department of Chemistry Thompson, Andrew; University of Warwick, Warwick-HRI Ruyter-Spira, Carolien; Wageningen University, Laboratory of Plant Physiology Bouwmeester, Harro; Wageningen University, Laboratory of Plant Physiology; Wageningen University, Centre for Biosystems Genomics
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Does ABA affect strigolactone biosynthesis?

Juan A. López-Ráez[1,2*], Wouter Kohlen[1*], Tatsiana Charnikhova[1], Patrick Mulder[3], Anna K. Undas[1,4], Martin J. Sergeant[5], Francel Verstappen[1,4], Timothy D. H. Bugg[6], Andrew J. Thompson[5], Carolien Ruyter-Spira[1], Harro Bouwmeester[1,4φ]

[1] Laboratory of Plant Physiology, Wageningen University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

[2] Present address: Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín (CSIC), Prof. Albareda 1, 18008 Granada, Spain

[3] RIKILT, Institute of Food Safety, Bornsesteeg 45, 6708 PD Wageningen, The Netherlands

[4] Centre for Biosystems Genomics, P. O. Box 98, 6700 AB Wageningen, The Netherlands

[5] Warwick-HRI, Wellesbourne, University of Warwick, Warwickshire, CV35 9EF, UK

[6] Department of Chemistry, University of Warwick, Coventry, CV4 7AL, UK.

* These authors contributed equally to this work

[φ] Corresponding author

Corresponding author:

e-mail address: harro.bouwmeester@wur.nl

Telephone: +31317480528

Fax: +31317418094

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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). [$^2H_6$]-*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 25ºC 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.*,

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 [$^2H_6$]-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_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_{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 [$^2H_6$]-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 [$^2H_6$]-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 [$^2H_6$]-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'-GGCGAAAAGTTTACCTCCA-3'; *LeCCD1-B*: 5'-AGAACAGCGTGACGGTTTCACA-3' and 5'-AGTGTAGTTCTCGTTGATCCGTG-3'; *LeCCD7*: 5'-AGCCAAGAATTCGAGATCCC-3' and 5'-GGAGAAAGCCCACATACTGC-3'; *LeCCD8*: 5'-CAGGACAATGGCACATAGGT-3' and 5'-GCGTCCGATTCGATTTG-3'; *SlCYP7070A1:* 5'-TGTCCAGGGAATGAACTTGC-3' and 5'-CAATGGGACTGGGAATGGTC-3'; *Le4*: 5'-ACTCAAGGCATGGGTACTGG-3' and 5'-CCTTCTTTCTCCTCCCACCT-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 = $2^{-\Delta(\Delta Ct)}$, where $\Delta C_t = C_t$ (target) - C_t (household) and $\Delta(\Delta C_t) = \Delta C_t$ (treatment) - ΔC_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[squareroot(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 didehydro-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 *SlCYP707A1*, 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_2N) 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.

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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

Adie BAT, Perez-Perez J, Perez-Perez MM, Godoy M, Sanchez-Serrano J-J, Schmelz EA, Solano R. 2007. ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in Arabidopsis. *Plant Cell* **19**(5): 1665-1681.

Akiyama K, Matsuzaki K, Hayashi H. 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* **435**(7043): 824-827.

Alder A, Holdermann I, Beyer P, Al-Babili S. 2008. Carotenoid oxygenases involved in plant branching catalyse a highly specific conserved apocarotenoid cleavage reaction. *Biochemical Journal* **416**: 289-296.

Aroca R, Alguacil MD, Vernieri P, Ruiz-Lozano JM. 2008. Plant responses to drought stress and exogenous ABA application are modulated differently by mycorrhization in tomato and an ABA-deficient mutant (*sitiens*). *Microbial Ecology* **56**(4): 704-719.

Auldridge ME, McCarty DR, Klee HJ. 2006. Plant carotenoid cleavage oxygenases and their apocarotenoid products. *Current Opinion in Plant Biology* **9**(3): 315-321.

Baindridge K, Sorefan K, Ward S, Leyser O. 2005. Hormonally controlled expression of the Arabidopsis MAX4 shoot branching regulatory gene. *Plant Journal* **44**(4): 569-580.

Bethke PC, Lonsdale JE, Fath A, Jones RL. 1999. Hormonally regulated programmed cell death in barley aleurone cells. *Plant Cell* **11**(6): 1033-1045.

Booker J, Auldridge M, Wills S, McCarty D, Klee H, Leyser O. 2004. MAX3/CCD7 is a carotenoid cleavage dioxygenase required for the synthesis of a novel plant signaling molecule. *Current Biology* **14**(14): 1232-1238.

Booker J, Sieberer T, Wright W, Williamson L, Willett B, Stirnberg P, Turnbull C, Srinivasan M, Goddard P, Leyser O. 2005. *MAX1* encodes a cytochrome p450 family member that acts downstream of max3/4 to produce a carotenoid-derived branch-inhibiting hormone. *Developmental Cell* **8**(3): 443-449.

Bouwmeester HJ, Matusova R, Zhongkui S, Beale MH. 2003. Secondary metabolite signalling in host-parasitic plant interactions. *Current Opinion in Plant Biology* **6**(4): 358-364.

Bouwmeester HJ, Roux C, López-Ráez JA, Bécard G. 2007. Rhizosphere communication of plants, parasitic plants and AM fungi. *Trends in Plant Science* **12**(5): 224-230.

Burbidge A, Grieve TM, Jackson A, Thompson A, McCarty DR, Taylor IB. 1999. Characterization of the ABA-def.icient tomato mutant *notabilis* and its relationship with maize *Vp14*. *Plant Journal* **17**(4): 427-431.

Carbone G, Preuss A, de Vos RCH, D'Amico E, Perrotta G, Bovy AG, Martens S, Rosati C. 2009. Developmental, genetic and environmental factors affect the expression of flavonoid genes, enzymes and metabolites in strawberry fruits. *Plant Cell and Environment* **32**: 1117-1131.

Cook CE, Whichard LP, Wall ME, Egley GH, Coggon P, Luhan PA, McPhail AT. 1972. Germination stimulants. 2. The structure of strigol, a potent seed germination stimulant for witchweed *(Striga lutea* Lour.). *Journal of the American Chemical Society* **94**(17): 6198-6199.

Christmann A, Hoffmann T, Teplova I, Grill E, Muller A. 2005. Generation of active pools of abscisic acid revealed by *in vivo* Imaging of water-stressed Arabidopsis. *Plant Physiology* **137**(1): 209-219.

Cornish K, Zeevaart JAD. 1988. Phenotypic expression of wild-type tomato and three wilty mutants in relation to abscisic acid accumulation in roots and leaflets of reciprocal grafts. *Plant Physiology* **87**(1): 190-194.

Cutler AJ, Squires TM, Loewn MK, Balsyich JJ. 1997. Abscisic acid induces abscisic acid 8'-hydroxylase in corn cells. *Plant Physiology* **114**(3): 795-795.

Davies WJ, Kudoyarova G, Hartung W. 2005. Long-distance ABA signaling and its relation to other signaling pathways in the detection of soil drying and the mediation of the plant's response to drought. *Journal of Plant Growth Regulation* **24**(4): 285-295.

De Smet I, Zhang HM, Inze D, Beeckman T. 2006. A novel role for abscisic acid emerges from underground. *Trends in Plant Science* **11**(9): 434-439.

Dunlap JR, Binzel ML. 1996. NaCl reduces indole-3-acetic acid levels in the roots of tomato plants independent of stress-induced abscisic acid. *Plant Physiology* **112**: 379-384.

Ejeta G. 2007. Breeding for *Striga* resistance in sorghum: Exploitation of an intricate host-parasite biology. *Crop Science* **47**(S3): S216–S227.

Fraser PD, Enfissi EMA, Halket JM, Truesdale MR, Yu D, Gerrish C, Bramley PM. 2007. Manipulation of phytoene levels in tomato fruit: Effects on isoprenoids, plastids, and intermediary metabolism. *Plant Cell* **19**(10): 3194-3211.

Gomez-Roldan V, Fermas S, Brewer PB, Puech-Pagés V, Dun EA, Pillot JP, Letisse F, Matusova R, Danoun S, Portais JC, Bouwmeester H, Bécard G, Beveridge CA, Rameau C, Rochange SF. 2008. Strigolactone inhibition of shoot branching. *Nature* **455**(7210): 189-194.

Harrison MJ. 2005. Signaling in the arbuscular mycorrhizal symbiosis. *Annual Review of Microbiology* **59**: 19-42.

Hernandez G, Ramirez M, Valdes-Lopez O, Tesfaye M, Graham MA, Czechowski T, Schlereth A, Wandrey M, Erban A, Cheung F, Wu HC, Lara M, Town CD, Kopka J, Udvardi MK, Vance CP. 2007. Phosphorus stress in common bean: Root transcript and metabolic responses. *Plant Physiology* **144**(2): 752-767.

Herrera-Medina MJ, Steinkellner S, Vierheilig H, Bote JAO, Garrido JMG. 2007. Abscisic acid determines arbuscule development and functionality in the tomato arbuscular mycorrhiza. *New Phytologist* **175**(3): 554-564.

Jeschke WD, Peuke AD, Pate JS and Hartung W. 1997. Transport, synthesis and catabolism of abscisic acid (ABA) in intact plants of castor bean (*Ricinus communis* L.) under phosphate deficiency and moderate salinity. *Journal of Experimental Botany* **48**(314): 1737-1747.

Jiang F, Hartung W. 2008. Long-distance signalling of abscisic acid (ABA): The factors regulating the intensity of the aba signal. *Journal of Experimental Botany* **59**(1): 37-43.

Kahn TL, Fender SE, Bray EA and Oconnell MA. 1993. Characterization of expression of drought and abscisic acid-regulated tomato genes in the drought-resistant species *Lycopersicon pennellii*. *Plant Physiology*, **103**: 597-605.

Kitahata N, Han SY, Noji N, Saito T, Kobayashi M, Nakano T, Kuchitsu K, Shinozaki K, Yoshida S, Matsumoto S, Tsujimoto M, Asami T. 2006. A 9-cis-epoxycarotenoid dioxygenase inhibitor for use in the elucidation of abscisic acid action mechanisms. *Bioorganic & Medicinal Chemistry* **14**(16): 5555-5561.

Li Y, Walton DC. 1990. Violaxanthin is an abscisic acid precursor in water-stressed dark-grown bean leaves. *Plant Physiology* **92**(3): 551-559.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2_ddc$_t$ method. *Methods* **25**(4): 402-408.

López-Ráez JA, Bouwmeester H. 2008. Fine-tuning regulation of strigolactone biosynthesis under phosphate starvation. *Plant Signaling & Behavior* **3**(11): 963-965.

López-Ráez JA, Charnikhova T, Gómez-Roldán V, Matusova R, Kohlen W, De Vos R, Verstappen F, Puech-Pages V, Bécard G, Mulder P, Bouwmeester H. 2008a. Tomato strigolactones are derived from carotenoids and their biosynthesis is promoted by phosphate starvation. *New Phytologist* **178**(4): 863-874.

López-Ráez JA, Charnikhova T, Mulder P, Kohlen W, Bino R, Levin I, Bouwmeester H. 2008b. Susceptibility of the tomato mutant *high pigment-2^{dg}* (*hp-2^{dg}*) to *Orobanche* spp infection. *Journal of Agricultural and Food Chemistry* **56**(15): 6326-6332.

López-Ráez JA, Matusova R, Cardoso C, Jamil M, Charnikhova T, Kohlen W, Ruyter-Spira C, Verstappen F, Bouwmeester H. 2009. Strigolactones: Ecological significance and use as a target for parasitic plant control. *Pest Management Science* **64**(5): 471-477.

Matusova R, Rani K, Verstappen FWA, Franssen MCR, Beale MH, Bouwmeester HJ. 2005. The strigolactone germination stimulants of the plant-parasitic *Striga* and *Orobanche* spp are derived from the carotenoid pathway. *Plant Physiology* **139**: 920-934.

Misson J, Raghothama KG, Jain A, Jouhet J, Block MA, Bligny R, Ortet P, Creff A, Somerville S, Rolland N, Doumas P, Nacry P, Herrerra-Estrella L, Nussaume L, Thibaud MC. 2005. A genome-wide transcriptional analysis using *Arabidopsis thaliana* affymetrix gene chips determined plant responses to phosphate deprivation. *Proceedings of the National Academy of Sciences of the United States of America* **102**(33): 11934-11939.

Nitsch LMC, Oplaat C, Feron R, Ma Q, Wolters-Arts M, Hedden P, Mariani C Vriezen WH. 2009. Abscisic acid levels in tomato ovaries are regulated by *LeNCED1*and *SlCYP707A1*. *Planta* 229:1335–1346.

Parry AD, Griffiths A, Horgan R. 1992. Abscisic acid biosynthesis in roots. 2. The effects of water stress in wild-type and abscisic acid-deficient mutant (*notabilis*) plants of *Lycopersicon esculentum* mill. *Planta* **187**(2): 192-197.

Parry AD, Horgan R. 1992. Abscisic acid biosynthesis in roots .1. The identification of potential abscisic acid precursors, and other carotenoids. *Planta* **187**(2): 185-191.

Paszkowski U. 2006. Mutualism and parasitism: The yin and yang of plant symbioses. *Current Opinion in Plant Biology* **9**(4): 364-370.

Rani K, Zwanenburg B, Sugimoto Y, Yoneyama K, Bouwmeester HJ. 2008. Biosynthetic considerations could assist the structure elucidation of host plant produced rhizosphere signalling compounds (strigolactones) for arbuscular mycorrhizal fungi and parasitic plants. *Plant Physiology and Biochemistry* **46**(7): 617-626

Rock CD, Zeevaart JAD. 1991. The ABA mutant of *Arabidopsis thaliana* is impaired in epoxy-carotenoid biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America* **88**(17): 7496-7499.

Sagi M, Scazzocchio C, Fluhr R. 2002. The absence of molybdenum cofactor sulfuration is the primary cause of the *flacca* phenotype in tomato plants. *Plant Journal* **31**(3): 305-317.

Saika H, Okamoto M, Miyoshi K, Kushiro T, Shinoda S, Jikumaru Y, Fujimoto M, Arikawa T, Takahashi H, Ando M, Arimura S, Miyao A, Hirochika H, Kamiya Y, Tsutsumi N, Nambara E, Nakazono M. 2007. Ethylene promotes submergence-induced expression of *Osaba8ox1*, a gene that encodes ABA 8 '-hydroxylase in rice. *Plant Cell and Physiology* **48**(2): 287-298.

Schwartz SH, Qin X, Zeevaart JAD. 2003. Elucidation of the indirect pathway of abscisic acid biosynthesis by mutants, genes and enzymes. *Plant Physiology* **131**(4): 1591-1601.

Schwartz SH, Qin XQ, Loewen MC. 2004. The biochemical characterization of two carotenoid cleavage enzymes from Arabidopsis indicates that a carotenoid-derived compound inhibits lateral branching. *Journal of Biological Chemistry* **279**(45): 46940-46945.

Schwartz SH, Tan BC, Gage DA, Zeevaart JAD, McCarty DR. 1997. Specific oxidative cleavage of carotenoids by *vp14* of maize. *Science* **276**(5320): 1872-1874.

Sergeant MJ, Li JJ, Fox C, Brookbank N, Rea D, Bugg TDH, Thompson AJ. 2009. Selective inhibition of carotenoid cleavage dioxygenases: Phenotypic effects on shoot branching. *Journal of Biological Chemistry* **284**(8): 5257-5264.

Simkin AJ, Schwartz SH, Auldridge M, Taylor MG, Klee HJ. 2004. The tomato *carotenoid cleavage dioxygenase 1* genes contribute to the formation of the flavor volatiles beta-ionone, pseudoionone, and geranylacetone. *Plant Journal* **40**(6): 882-892.

Sorefan K, Booker J, Haurogne K, Goussot M, Bainbridge K, Foo E, Chatfield S, Ward S, Beveridge C, Rameau C, Leyser O. 2003. *MAX4* and *rms1* are orthologous dioxygenase-like genes that regulate shoot branching in Arabidopsis and pea. *Genes & Development* **17**(12): 1469-1474.

Spinsanti G, Panti C, Marsili L, Casini S, Fossi MC. 2008. Selection of reference genes for quantitative RT-PCR studies in striped dolphin (*Stenella coeruleoalba*) skin biopsies. *Marine Environmental Research* **66**(1): 141-141.

Strack D, Fester T. 2006. Isoprenoid metabolism and plastid reorganization in arbuscular mycorrhizal roots. *New Phytologist* **172**(1): 22-34.

Sun Z, Hans J, Walter MH, Matusova R, Beekwilder J, Verstappen FWA, Ming Z, van Echtelt E, Strack D, Bisseling T, Bouwmeester HJ. 2008. Cloning and characterisation of a maize carotenoid cleavage dioxygenase (*ZmCCD1*) and its involvement in the biosynthesis of

apocarotenoids with various roles in mutualistic and parasitic interactions. *Planta* **228**(5): 789-801.

Tan BC, Joseph LM, Deng WT, Liu LJ, Li QB, Cline K, McCarty DR. 2003. Molecular characterization of the Arabidopsis 9-cis epoxycarotenoid dioxygenase gene family. *Plant Journal* **35**(1): 44-56.

Taylor IB, Linforth RS, Al-Naieb RJ, Bowman WR, Marples BA. 1988. The wilty tomato mutants *flacca* and *sitiens* are impaired in the oxidation of ABA-aldehyde to ABA. *Plant, Cell & Environment* **11**(8): 739-745.

Taylor IB, Sonneveld T, Bugg TDH, Thompson AJ. 2005. Regulation and manipulation of the biosynthesis of abscisic acid, including the supply of xanthophyll precursors. *Journal of Plant Growth Regulation* **24**(4): 253-273.

Thompson AJ, Jackson AC, Parker RA, Morpeth DR, Burbidge A, Taylor IB. 2000a. Abscisic acid biosynthesis in tomato: Regulation of zeaxanthin epoxidase and *9-cis-epoxycarotenoid dioxygenase* mRNAs by light/dark cycles, water stress and abscisic acid. *Plant Molecular Biology* **42**(6): 833-845.

Thompson AJ, Jackson AC, Symonds RC, Mulholland BJ, Dadswell AR, Blake PS, Burbidge A, Taylor IB. 2000b. Ectopic expression of a tomato *9-cis-epoxycarotenoid dioxygenase* gene causes over-production of abscisic acid. *Plant Journal* **23**(3): 363-374.

Umehara M, Hanada A, Yoshida S, Akiyama K, Arite T, Takeda-Kamiya N, Magome H, Kamiya Y, Shirasu K, Yoneyama K, Kyozuka J, Yamaguchi S. 2008. Inhibition of shoot branching by new terpenoid plant hormones. *Nature* **455**(7210): 195-200.

Vogel JT, Walter MH, Giavalisco P, Lytovchenko A, Kohlen W, Charnikhova T, Simkin AJ, Goulet C, Strack D, Bouwmeester HJ, Fernie AR, Klee HJ. 2010. *SlCCD7* controls strigolactone biosynthesis, shoot branching and mycorrhiza-induced apocarotenoid formation in tomato. *Plant Journal* **61**(2): 300-311.

Wasaki J, Yonetani R, Kuroda S, Shinano T, Yazaki J, Fujii F, Shimbo K, Yamamoto K, Sakata K, Sasaki T, Kishimoto N, Kikuchi S, Yamagishi M, Osaki M. 2003. Transcriptomic analysis of metabolic changes by phosphorus stress in rice plant roots. *Plant Cell and Environment* **26**(9): 1515-1523.

Yoneyama K, Xie XN, Sekimoto H, Takeuchi Y, Ogasawara S, Akiyama K, Hayashi H, Yoneyama K. 2008. Strigolactones, host recognition signals for root parasitic plants and arbuscular mycorrhizal fungi, from Fabaceae plants. *New Phytologist* **179**(2): 484-494.

Yoneyama K, Yoneyama K, Takeuchi Y, Sekimoto H. 2007. Phosphorus deficiency in red clover promotes exudation of orobanchol, the signal for mycorrhizal symbionts and germination stimulant for root parasites. *Planta* **225**(4): 1031-1038.

Zeevaart JAD, Creelman RA. 1988. Metabolism and physiology of abscisic acid. *Annual Review of Plant Physiology and Plant Molecular Biology* **39**: 439-473.

Figure 1

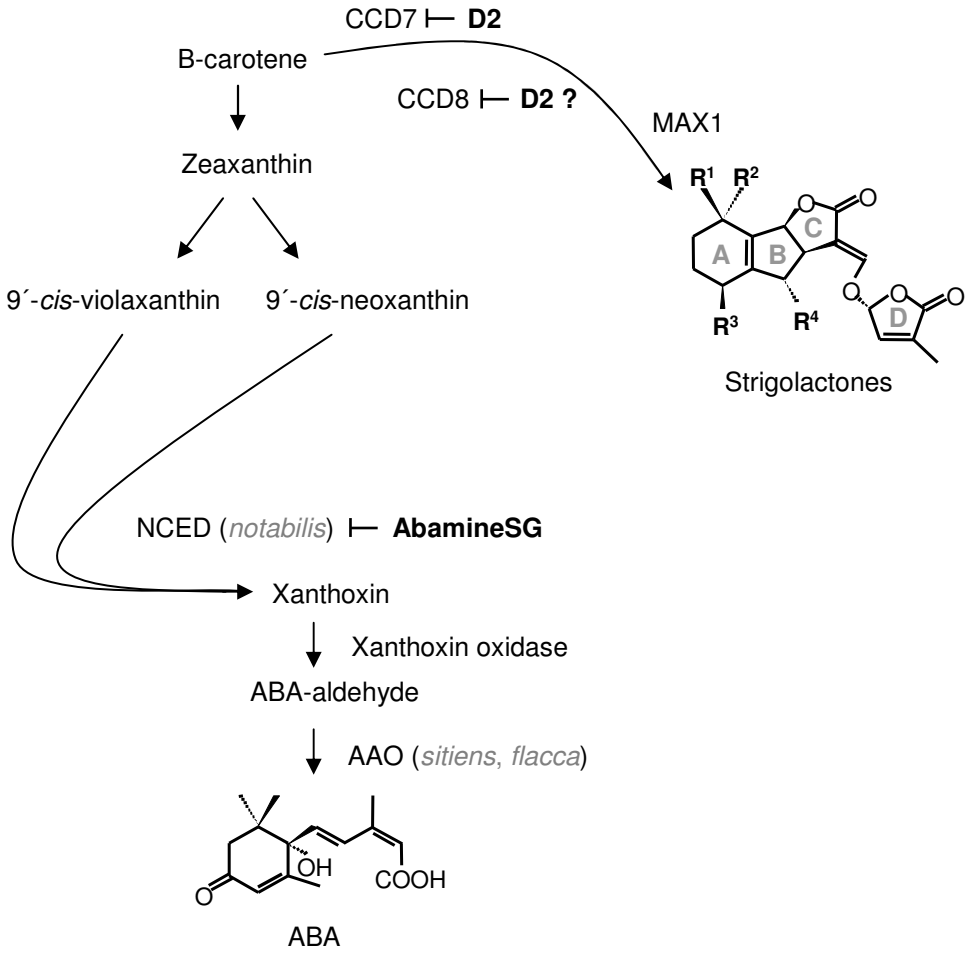

CCD7 ⊢ D2
B-carotene
CCD8 ⊢ D2 ?
MAX1
Zeaxanthin
R1 R2
A
B
C
D
R3
R4
9´-cis-violaxanthin
9´-cis-neoxanthin
Strigolactones
NCED (notabilis) ⊢ AbamineSG
Xanthoxin
Xanthoxin oxidase
ABA-aldehyde
AAO (sitiens, flacca)
OH
COOH
O
ABA

Figure 2

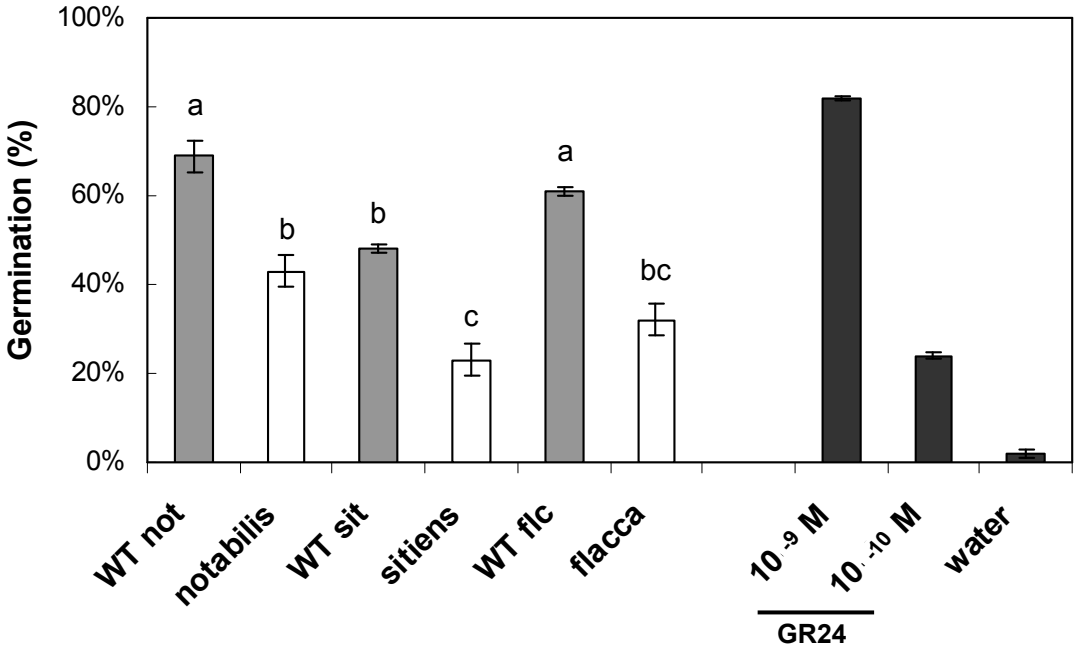
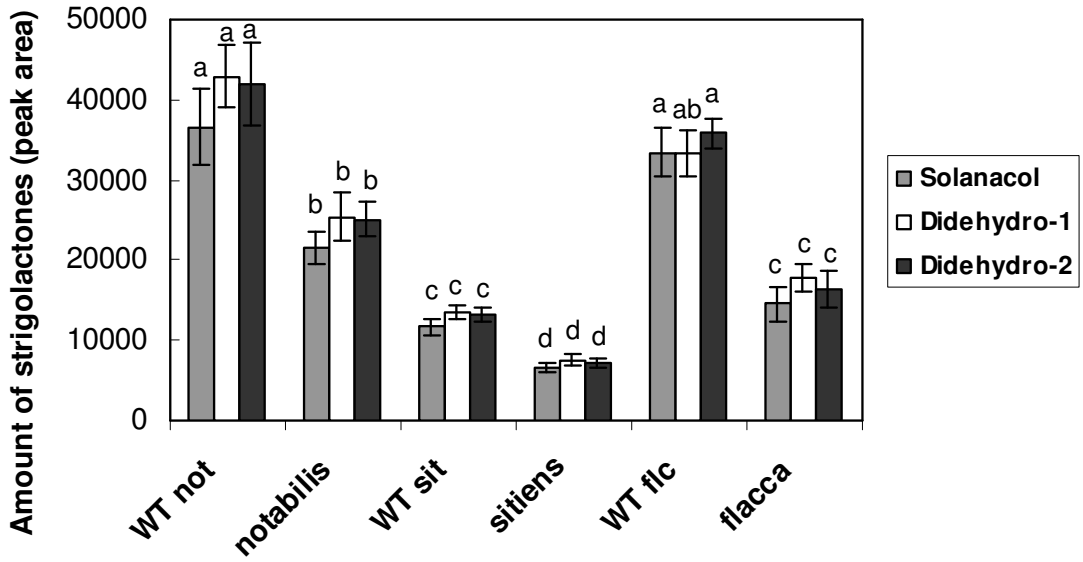

100%
80%
60%
40%
20%
0%
Germination (%)
a
b
b
c
a
bc
WT not
notabilis
WT sit
sitiens
WT flc
flacca
10 -9 M
10 -10 M
water
GR24

Figure 3

A


Amount of strigolactones (peak area)
50000
40000
30000
20000
10000
0
a a a
b b b
c c c
d d d
a ab a
c c c
WT not
notabilis
WT sit
sitiens
WT flc
flacca
Solanacol
Didehydro-1
Didehydro-2

B

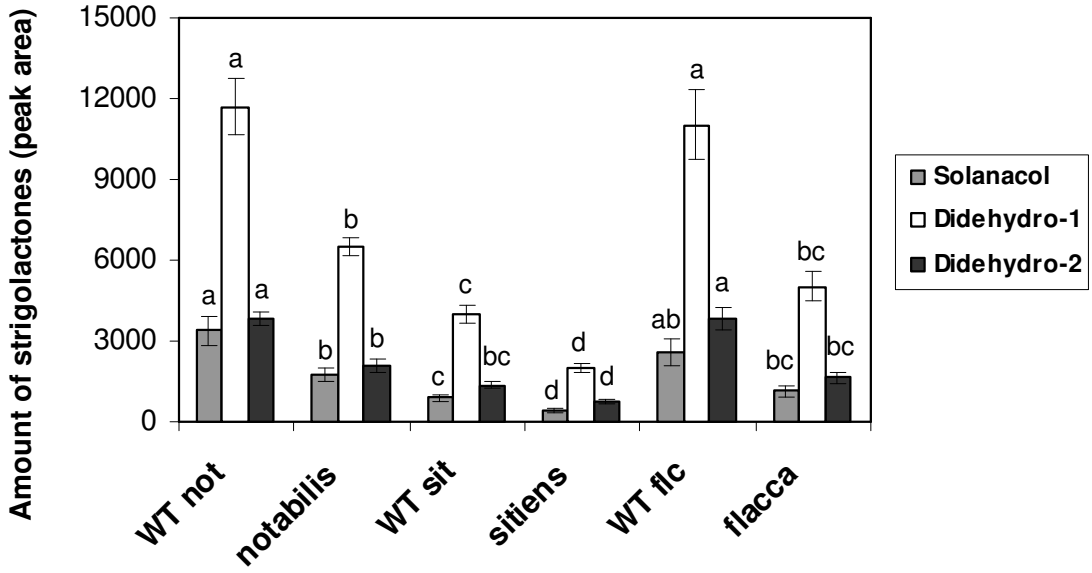

Amount of strigolactones (peak area)
15000
12000
9000
6000
3000
0
a a a
b b b
c c bc
d d d
ab a a
bc bc bc
WT not
notabilis
WT sit
sitiens
WT flc
flacca
Solanacol
Didehydro-1
Didehydro-2

Figure 4

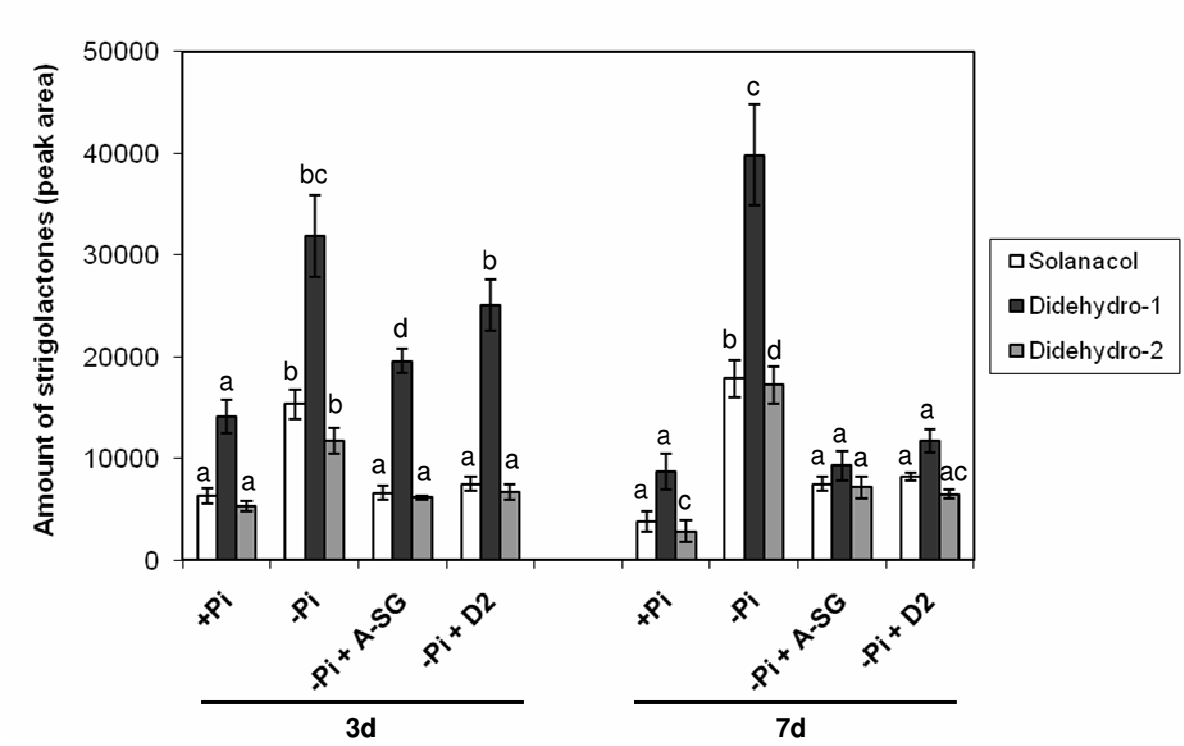
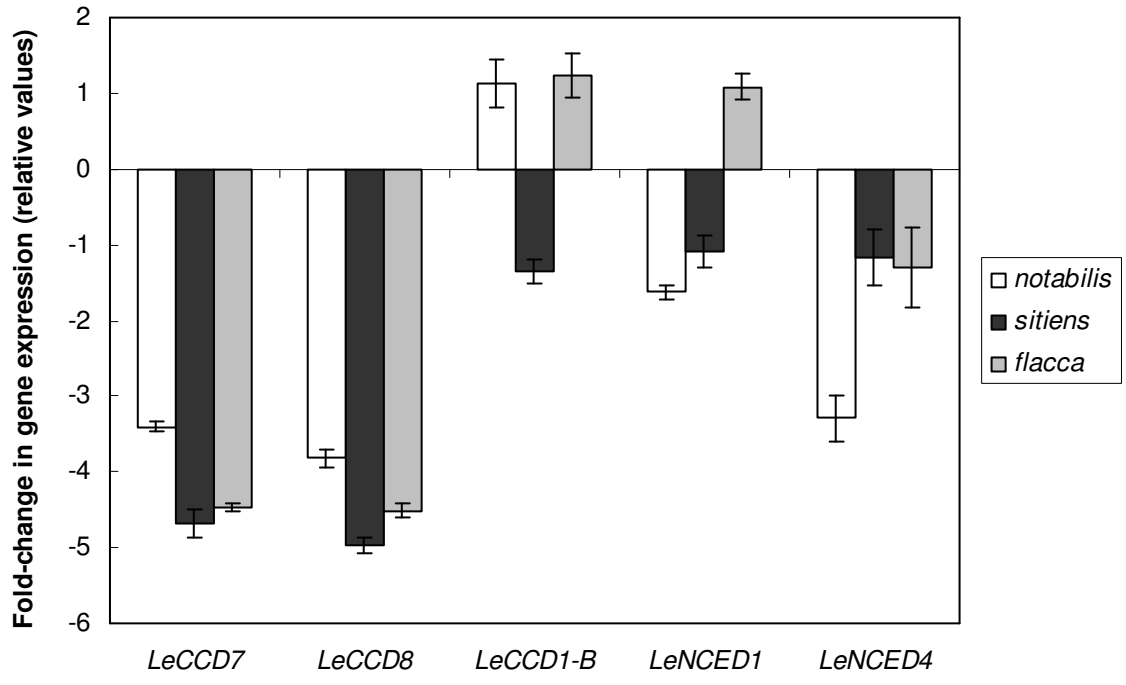

Amount of strigolactones (peak area)
50000
40000
30000
20000
10000
0
a
a
a
b
bc
b
a
d
a
a
b
a
a
a
c
b
d
a
a
a
a
a
ac
+Pi
-Pi
-Pi + A-SG
-Pi + D2
+Pi
-Pi
-Pi + A-SG
-Pi + D2
3d
7d
Solanacol
Didehydro-1
Didehydro-2

Figure 5

A


Fold-change in gene expression (relative values)
2
1
0
-1
-2
-3
-4
-5
-6
LeCCD7
LeCCD8
LeCCD1-B
LeNCED1
LeNCED4
notabilis
sitiens
flacca

B

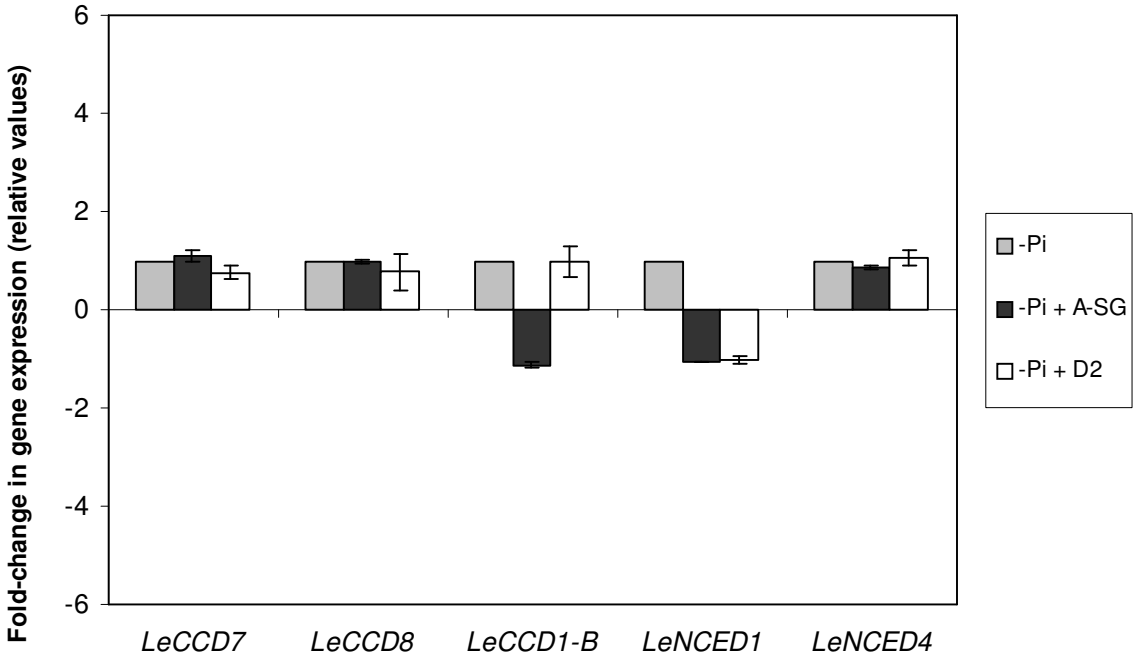

Fold-change in gene expression (relative values)
6
4
2
0
-2
-4
-6
LeCCD7
LeCCD8
LeCCD1-B
LeNCED1
LeNCED4
-Pi
-Pi + A-SG
-Pi + D2

Table 1

Genotype	[ABA] ng/g FW	
	Roots	**Shoots**
WT *notabilis*	9.51 ± 2.06[a]	203.91 ± 10.19[a]
notabilis	5.22 ± 1.06[b]	112.02 ± 11.42[b]
WT *sitiens*	6.26 ± 0.72[b]	180.92 ± 22.07[a]
sitiens	2.51 ± 0.60[c]	67.91 ± 7.14[c]
WT *flacca*	7.19 ± 0.51[ab]	198.63 ± 15.34[a]
flacca	2.54 ± 0.67[c]	66.60 ± 9.09[c]

Table 2

Treatment	[ABA] ng/g FW	
	Roots	Shoots
0h	2.37 ± 0.16[a]	133.75 ± 12.06[a]
+Pi	2.47 ± 0.19[a]	138.37 ± 11.98[a]
-Pi	2.44 ± 0.25[a]	130.40 ± 9.94[a]
-Pi + A-SG	1.79 ± 0.20[b]	126.92 ± 13.92[a]
-Pi + D2	2.37 ± 0.14[a]	131.67 ± 7.30[a]