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Does ABA affect strigolactone biosynthesis?

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Key Words:	strigolactones , abscisic acid, hormone regulation, tomato, mutants, inhibitors



1 **Does ABA affect strigolactone biosynthesis?**

2

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1 **SUMMARY**

2

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

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

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

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

20

21

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

23

1 INTRODUCTION

2

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

12 Strigolactones have been detected in the root exudates of a wide range of mono- and
13 dicotyledonous plant species. The strigolactones discovered so far all have a similar chemical structure
14 suggesting that they are all derived from the same biosynthetic pathway (Bouwmeester *et al.*, 2007;
15 Yoneyama *et al.*, 2008). Indeed, we have previously demonstrated that the ABC-part of the
16 strigolactones (Fig. 1) is derived from carotenoids through oxidative cleavage by carotenoid cleavage
17 dioxygenases, hence classifying the strigolactones as apocarotenoids (Matusova *et al.*, 2005; López-
18 Ráez *et al.*, 2008a; Rani *et al.*, 2008). In addition, we have postulated how, after carotenoid cleavage,
19 further enzymatic conversions are likely to lead to the production of all the strigolactones known to
20 date (Matusova *et al.*, 2005; Rani *et al.*, 2008). Indeed, it was recently demonstrated that two
21 carotenoid cleavage dioxygenases (CCDs), CCD7 and CCD8 - which were already proposed to be
22 responsible for the biosynthesis of the elusive shoot branching inhibiting signal (Sorefan *et al.*, 2003;
23 Booker *et al.*, 2004) - are directly involved in the biosynthesis of strigolactones (Gomez-Roldan *et al.*,
24 2008; Umehara *et al.*, 2008). The latter papers showed that mutants of pea [*ramosus5* (*rms5*) and

1 *ramosus1 (rms1)*] and rice [*high-tillering dwarf1* or *dwarf17 (htd1* or *d17)* and *dwarf 10 (d10)*] for
2 CCD7 and CCD8, respectively, produce significantly less strigolactones than the corresponding wild-
3 types. In pea, the *rms1* mutation reduced mycorrhizal symbiosis which could be restored by
4 exogenously applied synthetic strigolactone (Gomez-Roldan *et al.*, 2008). Moreover, root exudates of
5 *rms5* and *rms1* induced less AM fungal hyphae branching and less germination of *Orobanche* seeds
6 (Gomez-Roldan *et al.*, 2008). Similarly in rice, the orthologous mutants were less infected by *Striga*
7 *hermonthica* (Umehara *et al.*, 2008).

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

1 bioactive ABA (Fig. 1) (Schwartz *et al.*, 1997; Taylor *et al.*, 2005). In addition to *notabilis*, in tomato
2 two more ABA-deficient mutants - *sitiens* and *flacca* - have been characterized. *Sitiens* has been shown
3 to be mutated in the enzyme AAO and *flacca* has a mutation in a molybdenum cofactor (MoCo) which
4 is required for the activity of the enzyme AAO (Fig. 1) (Cornish & Zeevaart, 1988; Taylor *et al.*, 1988;
5 Sagi *et al.*, 2002).

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

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

23

24

1 MATERIALS AND METHODS

2

3 Plant material and chemicals

4

5 Seeds of tomato (*Solanum lycopersicum*) *sitiens* (LA0574) and its parental isogenic cv. Rheinlands
6 Ruhm, and *flacca* (LA3613) and corresponding parental isogenic cv. Ailsa Craig, were obtained from
7 the Tomato Genetics Resource Center (TGRC) at the University of California, Davis, CA, USA. Seeds
8 of cv. Ailsa Craig and *notabilis* (LA3614) were kindly provided by Wim Vriezen (Department of Plant
9 Cell Biology, Radboud University, Nijmegen, The Netherlands). Seeds of tomato cv. MoneyMaker
10 were purchased at a local garden centre. *O. ramosa* seeds were kindly provided by Maurizio Vurro
11 (Istituto di Scienze delle Produzioni Alimentari, Bari, Italy). The synthetic strigolactone analogue
12 GR24 was kindly provided by Binne Zwanenburg (Department of Organic Chemistry, Radboud
13 University, Nijmegen, The Netherlands). The strigolactone standards orobanchol and solanacol were
14 kindly provided by Koichi Yoneyama (Weed Science Center, Utsunomiya University, Japan). The
15 inhibitor abamineSG was kindly provided by Tadao Asami (RIKEN, Saitama, Japan). [²H₆]-*cis*, *trans*-
16 ABA was purchased at Olchemlm Ltd (Czech Republic).

17

18

19 Growth conditions and experiments

20

21 Tomato seeds were sterilized in 4% (v/v) sodium hypochlorite containing 0.02% (v/v) Tween 20,
22 rinsed thoroughly with sterile water, and then germinated for 48 h on moistened filter paper at 25°C in
23 darkness. Subsequently, tomato seedlings were grown in a greenhouse as described before (López-Ráez
24 *et al.*, 2008b). Phosphate (Pi) starvation promotes the production of strigolactones (Yoneyama *et al.*,

1 2007; López-Ráez *et al.*, 2008a). Therefore, one week before root exudate collection the substrate
2 (sand:vermiculite; 1:1, v/v) in the pots was rinsed with 1.5 l (2 times the pot volume) of modified half-
3 strength Hoagland solution without Pi to remove the strigolactones accumulated. Then plants were
4 watered (twice a week) with modified half-strength Hoagland nutrient solution without Pi. For root
5 exudate collection, the substrate in the pots was first rinsed as describe above to remove the
6 strigolactones accumulated. After another 5h, 0.7 l of modified half-strength Hoagland solution without
7 Pi was applied to the pots and the root exudate collected. Roots from each pot were then collected
8 separately and frozen in liquid nitrogen and stored at -80°C until use. Purification of the root exudates
9 and the germination bioassay were carried out as described before (López-Ráez *et al.*, 2008b).

12 **Treatment with inhibitors and ABA**

13
14 Half-strength Hoagland solution without Pi and with or without 50 µM of the inhibitors abamineSG or
15 D2 were applied to 4 weeks-old tomato plants which were then grown for an additional 3 or 7 d. To
16 maintain the effect of the inhibitors, after 3 d plants for the 7 d treatment were watered with fresh
17 nutrient solution containing the inhibitors. Root exudates and roots were collected on day 3 and day 7
18 as described above. In an attempt to rescue the strigolactone exudation phenotype of the ABA mutants,
19 mutants were grown as described above. Half-strength Hoagland solution without Pi and with or
20 without 0.5 µM ABA was applied to 4 weeks-old plants and grown for an additional 7 d. To maintain
21 the ABA levels, after 3 d plants were watered with fresh nutrient solution containing ABA. Root
22 exudates and roots were collected after 7 d for analyses.

23

24

1 **Extraction of ABA and strigolactones from roots and shoots**

2

3 For ABA and strigolactone analysis, 0.5 g of root or shoot tissue was ground in a mortar with liquid
4 nitrogen. The samples were extracted with 2 ml of cold ethyl acetate containing [²H₆]-ABA as internal
5 standard (0.025 nmol or 0.25 nmol for root or shoot tissue, respectively) in a 10 ml glass vial. The vials
6 were vortexed and sonicated for 10 min in a Branson 3510 ultrasonic bath (Branson Ultrasonics,
7 Danbury, CT, US). Samples were centrifuged for 10 min at 2500 g in an MSE Mistral 2000 centrifuge
8 (Mistral Instruments, Leicester, UK) after which the organic phase was carefully transferred to a 4 ml
9 glass vial. The pellets were re-extracted with another 2 ml of ethyl acetate. The combined ethyl acetate
10 fractions were dried under a flow of N₂ and the residue dissolved in 250 µl of acetonitrile:water:formic
11 acid (25:75:0.1, v/v/v). Before analysis, samples were filtered through Minisart SRP4 0.45 µm filters
12 (Sartorius, Germany) and LC-MS/MS was performed as described below.

13

14

15 **Strigolactone and ABA detection and quantification by liquid chromatography-tandem mass** 16 **spectrometry (LC-MS/MS)**

17

18 Analysis of strigolactones in tomato exudates and root extracts was conducted by comparing retention
19 times and mass transitions with those of available strigolactone standards as described before (López-
20 Ráez *et al.*, 2008b). ABA analysis was performed by LC-MS/MS using a published protocol with some
21 modifications (Saika *et al.*, 2007). Analyses were carried out on a Waters Micromass Quattro Premier
22 XE tandem mass spectrometer (Waters, Milford, MA, USA) equipped with an ESI source and coupled
23 to an Acquity UPLC system (Waters, USA). Chromatographic separation was achieved using an
24 Acquity UPLC BEH C₁₈ column (150 x 2.1 mm, 1.7 µm) (Waters, USA), applying a water/acetonitrile

1 gradient, starting at 0% acetonitrile for 2.0 min, raised to 50% (v/v) acetonitrile in 8.0 min, followed by
2 a 1.0 min gradient to 90% (v/v) acetonitrile which was then maintained for 0.1 min and followed by a
3 0.2 min gradient back to 0% acetonitrile before the next run. The column was then equilibrated at this
4 solvent composition for 2.8 min. Total run time was 15 min. The column was operated at 50°C with a
5 flow-rate of 0.4 ml min⁻¹ and sample injection volume was 30 µl. The mass spectrometer was operated
6 in positive electrospray ionization (ESI) mode. The nebuliser and desolvation gas flows were 50 and
7 800 l h⁻¹, respectively. The capillary voltage was set at 2.7 kV, the cone voltage at 10 V, the source
8 temperature at 120°C and the desolvation gas temperature at 450°C. Fragmentation was performed by
9 collision induced dissociation with argon at 3.0 x 10⁻³ mbar. Multiple reaction monitoring (MRM) was
10 used for ABA quantification. Parent-daughter transitions were set according to the MS/MS spectra
11 obtained for the standards ABA and [²H₆]-ABA. Transitions were selected based on the most abundant
12 and specific fragment ions for which the collision energy (CE) was optimized. For ABA, the MRM
13 transitions *m/z* 265>229 at a CE of 10 eV and 265>247 at 5 eV; and for [²H₆]-ABA, the transitions *m/z*
14 271>234 at 10 eV and 271>253 at 5 eV were selected. ABA was quantified using a calibration curve
15 with known amount of standards and based on the ratio of the summed area of the MRM transitions for
16 ABA to those for [²H₆]-ABA. Data acquisition and analysis were performed using MassLynx 4.1
17 software (Waters, USA). The summed area of all the corresponding MRM transitions was used for
18 statistical analysis.

19

20

21 **RNA isolation and first strand cDNA synthesis**

22

23 Total RNA from tomato roots was extracted using Tri-Pure reagent (Roche, Germany) according to the
24 manufacturer's protocol. The RNA was sequentially treated with DNase I (Invitrogen, The

1 Netherlands) at 37°C for 15 min in order to remove the remaining genomic DNA. Before cDNA
 2 synthesis, the RNA was purified through a silica column using the RNeasy RNA Cleanup kit (Qiagen,
 3 Germany). The first strand cDNA was synthesized with 1 µg of purified total RNA using the iScript
 4 cDNA Synthesis kit (Bio-Rad, The Netherlands) according to the manufacturer's instructions.

5
 6
 7 **Gene expression analysis by real time quantitative RT-PCR (qPCR)**

8
 9 For gene expression analysis by real time quantitative RT-PCR (qPCR) the iCycler iQ5 system (Bio-
 10 Rad, The Netherlands) was used (Spinsanti *et al.*, 2008) using specific primers. *LeActin*: 5'-
 11 TCCCAGGTATTGCTGATAGAA-3' and 5'-TGAGGGAAGCCAAGATAGAG-3'; *LeNCED1*: 5'-
 12 ACCCACGAGTCCAGATTTC-3' and 5'-GGTTCAAAAAGAGGGTTAGC-3'; *LeNCED4*: 5'-
 13 ACAACATCGAAAATGAAGCCG-3' and 5'-GGCGAAAAGTTTACCTCCA-3'; *LeCCD1-B*: 5'-
 14 AGAACAGCGTGACGGTTTCACA-3' and 5'-AGTGTAGTTCTCGTTGATCCGTG-3'; *LeCCD7*:
 15 5'-AGCCAAGAATTCGAGATCCC-3' and 5'-GGAGAAAGCCCACATACTGC-3'; *LeCCD8*: 5'-
 16 CAGGACAATGGCACATAGGT-3' and 5'-GCGTCCGATTCGATTTG-3'; *SICYP7070A1*: 5'-
 17 TGTCCAGGGAATGAACTTGC-3' and 5'-CAATGGGACTGGGAATGGTC-3'; *Le4*: 5'-
 18 ACTCAAGGCATGGGTACTGG-3' and 5'-CCTTCTTTCTCCTCCCACCT-3'. Three independent
 19 biological replicates were used and each PCR reaction was done in triplicate. Relative quantification of
 20 mRNA amount was performed using the comparative C_t method (Livak & Schmittgen, 2001). These
 21 values were then normalized using the C_t value for the tomato household gene *LeActin*. All the values
 22 were used to determine the change in gene expression according to the following calculation: fold-
 23 change = $2^{-\Delta(\Delta C_t)}$, where $\Delta C_t = C_t(\text{target}) - C_t(\text{household})$ and $\Delta(\Delta C_t) = \Delta C_t(\text{treatment}) - \Delta C_t(\text{control})$.
 24 Down-regulation of expression is shown as negative values.

1

2

3 **Statistical analysis**

4

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

10

11

12 **RESULTS**

13

14 **Germination stimulatory activity of *notabilis*, *sitiens* and *flacca* root exudates**

15

16 We previously demonstrated that ABA-deficient maize (*vp14*) and tomato (*notabilis*) mutants with a
17 mutation in NCED exhibit a decreased strigolactone production by the roots (Matusova *et al.*, 2005;
18 López-Ráez *et al.*, 2008a). It has been suggested that NCEDs are the key enzymes in the ABA
19 biosynthetic pathway (Fig. 1). To assess whether the reduction in strigolactone biosynthesis in the
20 NCED mutants is caused directly by reduced NCED action or indirectly because of its effect on the
21 ABA content of these mutants, in addition to *notabilis*, the tomato ABA-deficient mutants *sitiens* and
22 *flacca* and their parental isogenic lines were studied. *Sitiens* and *flacca* are blocked in the final step of
23 the ABA biosynthetic pathway, where the enzyme AAO catalyzes the oxidation of abscisic aldehyde to
24 ABA (Fig. 1) (Schwartz *et al.*, 2003; Taylor *et al.*, 1988). *Sitiens* is known to have a mutation in the

1 AAO enzyme and mutant leaves contain only about 11% of the wild-type ABA levels (Cornish &
2 Zeevaart, 1988; Taylor *et al.*, 1988). The mutant *flacca* has a mutation in a MoCo cofactor required for
3 the activity of AAO and mutant leaves contain about 33% of the wild-type ABA levels (Cornish &
4 Zeevaart, 1988; Sagi *et al.*, 2002).

5 The mutants *notabilis*, *sitiens* and *flacca* showed the characteristic wilted phenotype (Taylor *et al.*,
6 1988; Thompson *et al.*, 2000b). Root exudates of the three mutants and their corresponding wild-types
7 were collected for a germination bioassay with *O. ramosa* seeds. The synthetic germination stimulant
8 GR24, as a positive control, always induced the germination of preconditioned *O. ramosa* seeds (up to
9 about 85%). Water, used as a negative control, only induced 2% germination (Fig. 2). As we described
10 before (López-Ráez *et al.*, 2008a), root exudates of *notabilis* induced about 40% less germination than
11 the corresponding wild-type (Fig. 2). The germination stimulatory activity of *sitiens* and *flacca*
12 exudates was about 52 and 48%, respectively, lower than for those of the corresponding wild-types
13 (Fig. 2). In addition to the differences between the mutants and corresponding wild-types, there was
14 some variation between the germination stimulatory activities of the wild-type exudates (Fig. 2). For
15 example, the wild-type for *sitiens* (cv. Rheinlands Ruhm) induced lower germination than the wild-type
16 for *flacca* and *notabilis* (cv. Ailsa Craig) (Fig. 2).

17

18

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

20

21 To assess whether the lower germination stimulatory activity of the ABA-deficient tomato mutants was
22 due to a decrease in the production of strigolactones, LC-MS/MS analysis was performed to compare
23 the levels of strigolactones in the root exudates of the mutants and corresponding wild-types. All the
24 three major strigolactones detected - solanacol and the two didehydro-orobanchol isomers - were

1 significantly ($P < 0.01$) reduced in the *notabilis*, *sitiens* and *flacca* root exudates compared with the
2 wild-types (Fig. 3A). The other strigolactone present in tomato - orobanchol - was also detected, but its
3 concentration was too low for accurate quantification. The concentration of strigolactones in the root
4 extracts showed a similar trend as for the root exudates (Fig. 3B), indicating that there is a reduction in
5 strigolactone biosynthesis in the mutants rather than just a decrease in the exudation. Interestingly, in
6 the roots the decrease in didehydro-orobanchol 1 in the ABA mutants was larger than the decrease in
7 solanacol and the didehydro-orobanchol 2 isomer, whereas in exudates the decrease in their
8 concentration was similar (Fig 3). Overall, the level of strigolactones in the exudates of *notabilis*,
9 *sitiens* and *flacca* was about 40, 47 and 52%, respectively, lower than the corresponding wild-types,
10 which correlates well with the reduction in the germination stimulatory activity of the mutant exudates
11 (40%, 52% and 48%, respectively). In an attempt to rescue the phenotype of the mutants, ABA (0.5
12 μM) was exogenously applied by irrigation to all the three mutants. However, no effect on
13 strigolactone biosynthesis was observed in comparison with the untreated plants (data not shown). The
14 same pattern was observed when higher ABA concentrations (1 and 10 μM) were applied to *sitiens* and
15 its corresponding wild-type (Fig. S1). Although no increase in ABA was detected in the roots after
16 exogenous ABA application, expression analysis of the ABA-responsive gene *Le4* (Kahn *et al.* 1993)
17 and *SICYP707A1*, an ABA-8'-hydroxylase involved in ABA catabolism (Taylor *et al.*, 2005, Nitsch *et*
18 *al.*, 2009), showed a 250- and 15-fold increase, respectively upon ABA treatment (Fig. S2), indicating
19 that ABA was effectively taken up by the roots but is also effectively catabolised.

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

24

1

2 **ABA quantification by LC-MS/MS**

3

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

14

15

16 **Specific carotenoid cleavage enzyme inhibitors**

17

18 To further investigate the involvement of NCEDs in strigolactone biosynthesis an experiment with the
19 specific NCED inhibitor abamineSG (Fig. 1) was carried out. In parallel, the effect of another inhibitor
20 - D2 - specific for the other class of carotenoid-cleaving enzymes CCD7 and CCD8, which are
21 involved in strigolactone biosynthesis (Fig. 1), was also tested. AbamineSG is a tertiary amine
22 derivative acting as a competitive inhibitor of the NCEDs (Kitahata *et al.*, 2006). D2 is a derivative of a
23 hydroxamic acid (aryl-C₂N) and is a potent inhibitor of CCD enzymes cleaving at the 9,10 position of
24 carotenoids (Sergeant *et al.*, 2009), but is considerably less active against NCEDs (11,12 cleavage) *in*

1 *vitro*. It has been postulated that CCD7 cleaves β -carotene at the 9,10 position to produce the
2 apocarotenoid 10-apo- β -carotene which is then further cleaved by CCD8 in the pathway leading to the
3 strigolactones (Alder *et al.*, 2008; Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008).

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

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

1 catabolism in shoots were induced upon phosphorus stress, while ABA content in both roots and shoots
2 was not affected (Jeschke *et al.*, 1997).

3
4
5 **Gene expression analysis for ABA and strigolactone biosynthetic genes**

6
7 Expression analysis by quantitative real time RT-PCR (qPCR) was performed to check expression of
8 tomato genes encoding different carotenoid cleavage enzymes. As the tomato genome is not completely
9 sequenced, only some of the genes coding for enzymes from this family are known. The genes studied
10 in the present work are *LeCCD1-B*, *LeCCD7*, *LeCCD8*, *LeNCED1* and *LeNCED4*. *LeCCD1-A* and *-B*
11 are involved in the production of the flavour volatiles β -ionone, pseudo-ionone and geranylacetone
12 (Simkin *et al.*, 2004). *CCD7* and *CCD8* have been described to be involved in the biosynthesis of
13 strigolactones in pea, rice and *Arabidopsis* (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). More
14 recently, *CCD7* from tomato was cloned and characterized and shown to be involved in strigolactone
15 biosynthesis, shoot branching and formation of mycorrhiza-induced apocarotenoids (Vogel *et al.*,
16 2009). In *A. thaliana* all NCEDs, except *AtNCED4* are known to be involved in ABA production
17 (Auldridge *et al.*, 2006; Bouwmeester *et al.*, 2007).

18 Expression of *LeCCD7* and *LeCCD8* was clearly reduced in all three ABA-deficient mutants -
19 *notabilis*, *sitiens* and *flacca* - compared with the corresponding wild-types, whereas the expression of
20 the other carotenoid cleavage genes was not affected (Fig. 5A). Moreover, this reduction was more
21 evident in *sitiens* and *flacca*, which had a lower ABA content than *notabilis* (Table 1). In *notabilis*,
22 with a null mutation in *LeNCED1* caused by a single A/T base pair deletion (Burbidge *et al.*, 1999), the
23 expression of this gene was not significantly reduced, in agreement with previous observations that
24 expression of *LeNCED1* was not regulated by ABA (Thompson *et al.*, 2000a). The other NCED gene

1 so far described in tomato - *LeNCED4* - was significantly reduced although to a lesser extent than
2 *LeCCD7* and *LeCCD8* (Fig. 5A). When gene expression was assessed in roots upon 7 d application of
3 the inhibitors abamineSG and D2, no significant changes were detected in the expression levels of any
4 of the selected genes (Fig. 5B). None of the genes was significantly affected by Pi starvation (data not
5 shown).

6

7

8 **DISCUSSION**

9

10 ABA is an important phytohormone playing many physiological roles in plants (Zeevaart & Creelman,
11 1988; Davies *et al.*, 2005; De Smet *et al.*, 2006; Adie *et al.*, 2007; Jiang & Hartung, 2008). ABA is an
12 apocarotenoid produced via oxidative cleavage of epoxycarotenoids through action of NCEDs, which
13 catalyze the rate-limiting step in ABA biosynthesis (Parry & Horgan, 1992; Thompson *et al.*, 2000a;
14 Taylor *et al.*, 2005) (Fig. 1). We have previously demonstrated that in the ABA-deficient mutants *vp14*
15 and *notabilis* in maize and tomato, respectively - having a mutation in an NCED and therefore showing
16 a lower ABA content - the production of strigolactones was decreased by about 40% (Matusova *et al.*,
17 2005; López-Ráez *et al.*, 2008a). From these results it was concluded that the NCEDs either have a
18 direct role in strigolactone biosynthesis or that ABA has a regulatory role in this process (Matusova *et*
19 *al.*, 2005; López-Ráez *et al.*, 2008a). We show here that the corresponding decrease in ABA and
20 strigolactone production also occurs in two other well characterized tomato ABA-deficient mutants
21 *sitiens* and *flacca* that are mutated in the last step of ABA biosynthesis - transformation of ABA
22 aldehyde into ABA by an AAO - (Fig. 1) (Schwartz *et al.*, 2003; Taylor *et al.*, 1988). The decrease in
23 ABA content in the roots of these mutants was stronger than for *notabilis*, being 60 and 65% lower
24 than the corresponding wild-types, respectively. This reduction in ABA content in the roots and also in

1 the shoots of *sitiens* and *flacca* is somewhat lower than previously reported, when 89 and 67%
2 reduction, respectively were detected in leaves (Cornish & Zeevaart, 1988). This discrepancy is likely
3 due to differences in the conditions under which the plants were grown and/or the analytical method
4 used. In the present study ABA content as well as strigolactones were measured using the same
5 methodology - LC-MS/MS in MRM mode - and using the same plant material. The levels of
6 strigolactones produced by the roots of *sitiens* and *flacca* were also lower than for *notabilis* (Fig. 3A).
7 A strong correlation was observed between the reduction of ABA and strigolactone content in the roots
8 when comparing a mutant with its corresponding wild-type, but across cultivars and mutants there was
9 no correlation between ABA and strigolactone levels. Strigolactone-deficient mutants are characterized
10 by a shoot branching phenotype (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008), but this phenotype
11 was not observed for *notabilis*, *sitiens* and *flacca*, even though the strigolactone content of these
12 mutants is reduced. Apparently, the reduced strigolactone level is still high enough to maintain normal
13 shoot architecture. In a recent study on tomato expressing an *SICCD7* antisense construct it was shown
14 that an 80% reduction in strigolactone level induced only a weak branching phenotype, whereas in two
15 other lines with over 90% reduction in strigolactone level branching was strongly increased (Vogel *et*
16 *al.*, 2010).

17 The fact that ABA-deficient mutants with mutations in enzymes of the biosynthetic pathway other
18 than the NCEDs also produce less strigolactones seems to indicate that NCED is not directly involved
19 in strigolactone biosynthesis, but that its biosynthetic product ABA might be involved somehow in
20 regulating strigolactone biosynthesis. ABA has previously been associated to AM symbiosis as well
21 (Herrera-Medina *et al.*, 2007; Aroca *et al.*, 2008). These authors showed that the mutant *sitiens* was
22 less prone to colonization by AM fungi than the wild-type, but that continuous exogenous application
23 of ABA could not significantly compensate this effect in the mutant. Herrera-Medina and co-workers
24 proposed that ABA increases the susceptibility of tomato to AM colonization and that this is necessary

1 for a proper AM establishment. The authors also suggest that ABA may play a role in the development
2 of the arbuscule and in regulating its functionality (Herrera-Medina *et al.*, 2007). We show here that in
3 *sitiens*, as well as in the other two ABA-deficient mutants *notabilis* and *flacca*, the production of
4 strigolactones is significantly reduced compared with the corresponding wild-types (Figs. 2 and 3).
5 Therefore, the reduction in AM colonization observed in *sitiens* may also be due to a reduction in the
6 production of strigolactones by this mutant, although an additional role of ABA in the establishment of
7 AM symbiosis cannot be discarded. Indeed, root exudates of the pea strigolactone-deficient mutants
8 *rms1* and *rms5*, with a mutation in the *CCD8* and *CCD7* genes respectively, have been shown to have a
9 significantly reduced activity in promoting AM fungal hyphae branching when compared with wild-
10 type exudates (Gomez-Roldan *et al.*, 2008). Interestingly, when ABA was exogenously applied to
11 *sitiens* no effect on strigolactone production was observed. The failure to complement the strigolactone
12 phenotype of *sitiens* by exogenous ABA application is in line with the above-mentioned failure to
13 complement the AM-colonization phenotype of *sitiens* by exogenous ABA application (Aroca *et al.*,
14 2008). The same lack of effect was observed when ABA was applied to the other two mutants *notabilis*
15 and *flacca*. These results suggest that endogenous ABA may be required for strigolactone production in
16 specific root cells or tissues and hence for AM colonization and development. Apparently this
17 requirement cannot be replaced by exogenous ABA application. That exogenous ABA application may
18 not be suitable to replace endogenous ABA is also clear from a study on drought-stress induced ABA
19 using luciferase ABA-reporter plants. Exposure of Arabidopsis seedlings to exogenous ABA resulted
20 in a uniform pattern of reporter expression, whereas reporter expression in response to drought stress
21 was predominantly confined to the vasculature and stomata (Christmann *et al.*, 2005). ABA is known
22 to stimulate its own degradation via the ABA hydroxylases in order to control its homeostasis (Cutler *et*
23 *al.*, 1997). Indeed, we observed induction of the gene encoding ABA hydroxylase upon ABA
24 application.

1 In addition to the differences between the ABA-deficient mutants and corresponding wild-types, we
2 also observed significant differences in the germination stimulatory capacity for *O. ramosa* seeds of
3 root exudates from the wild-type cultivars Ailsa Craig and Rheinlands Ruhm (Fig. 2). LC-MS/MS
4 analysis confirmed that the activity differences correlate with differences in the level of strigolactones
5 in the root exudates (Fig. 3A), and these correlated to the strigolactone levels in root extracts (Fig. 3B).
6 The results show that there is genetic variation for the production of strigolactones in different tomato
7 cultivars, as we previously observed for two other tomato cultivars MoneyMaker and Manapal (López-
8 Ráez *et al.*, 2008b). The genetic variation in strigolactone production indicates that selection of tomato
9 cultivars producing low levels of germination stimulants - strigolactones - may be a strategy to breed
10 tomato varieties resistant or less susceptible to *Orobanche* (López-Ráez *et al.*, 2009). Selection for low
11 germination stimulant germplasm has been successfully used in other crops such as sorghum in order to
12 produce *Striga* resistant varieties (Ejeta, 2007). The selection process in sorghum was based on the use
13 of germination bioassays, but with the advent of extremely sensitive analytical methods such as LC-
14 MS/MS in MRM mode described in the present paper, an analytically-supported selection process
15 seems now feasible.

16 Besides ABA-deficient mutants, specific inhibitors for different carotenoid cleaving enzymes were
17 used in the present study (Fig. 1). The inhibitor abamineSG - a specific inhibitor for NCEDs and
18 therefore for ABA biosynthesis (Kitahata *et al.*, 2006) - reduced the production of the three major
19 strigolactones present in tomato, solanacol and the two didehydro-orobanchol isomers in plants grown
20 under Pi limited conditions (Fig. 4). After 7 d treatment, the reduction in strigolactone production in the
21 abamineSG-treated plants was similar to the reduction observed in the mutants *sitiens* and *flacca*.
22 Moreover, this reduction in strigolactones was accompanied by a minor but significant decrease in
23 ABA content in the roots of the treated-plants, again suggesting a regulatory effect of ABA in
24 strigolactone biosynthesis. The other members of the carotenoid cleavage enzyme family are the CCDs,

1 involved in the formation of apocarotenoid compounds such as flavour volatiles, cyclohexanone and
2 mycorradicin derivatives (the yellow pigment formed in host roots upon arbuscular mycorrhizal
3 colonization) and strigolactones (Simkin *et al.*, 2004; Strack & Fester, 2006; Sun *et al.*, 2008). CCD7
4 and CCD8 are involved in the control of plant architecture because they are involved in the production
5 of the strigolactones (Sorefan *et al.*, 2003; Booker *et al.*, 2005; Gomez-Roldan *et al.*, 2008; Umehara *et al.*,
6 2008). It was shown that AtCCD7 can convert C40 carotenoids into C27 apocarotenoids (Schwartz
7 *et al.*, 2004) and that, subsequently, CCD8 can cleave the C27 into a C18 apocarotenoid, the probable
8 precursor of the plant branching inhibitor (Alder *et al.*, 2008). D2 specifically inhibits these CCDs, so
9 also strigolactone biosynthesis and was included to investigate whether low strigolactone levels also
10 affect ABA levels. Recently, it was shown that D2 showed selectivity *in vitro* towards CCDs that
11 cleave at the 9,10 position - such as CCD7 - rather than towards NCEDs that cleave at the 11,12
12 position of C40 *cis*-carotenoids (Sergeant *et al.*, 2009). In the present study, D2 indeed caused a
13 reduction in strigolactone production suggesting that this inhibitor did indeed inhibit either CCD7 or
14 CCD8 or both (Fig. 4). However, no effect on ABA content was observed in the roots of D2-treated
15 plants, confirming that NCEDs are not significantly inhibited *in vivo*, thus supporting the observed *in*
16 *vitro* selectivity of the inhibitor (Sergeant *et al.*, 2009). These results also show that a short-term
17 reduction in strigolactone levels does not lead to a reduction in ABA. The results are also in agreement
18 with those observed previously where continuous application of D2 increased the number of side
19 branches from the rosette nodes of Arabidopsis, mimicking the Arabidopsis *max3 (ccd7)* bushy
20 phenotype, presumably by inhibiting one or more of the CCDs involved in strigolactone biosynthesis
21 (Sergeant *et al.*, 2009). However, in that study it was not analytically assessed whether that phenotype
22 was caused by an inhibition of strigolactone biosynthesis. Here we show that application of D2 indeed
23 inhibits strigolactone production, and thus it is a useful and selective inhibitor for use in *in vivo* studies
24 on strigolactones.

1 When the expression of *LeCCD7* and *LeCCD8* was checked by real time qPCR, a clear decrease in
2 expression for both genes was observed in all three mutants *notabilis*, *sitiens* and *flacca* (Fig. 5A). This
3 reduction was most clear in *sitiens* and *flacca*, the mutants with the strongest reduction in ABA content
4 (Table 1). No differences were observed in the expression of the other carotenoid-cleaving enzymes
5 known in tomato (Fig. 5A). Although a reduction in gene expression levels does not necessarily imply
6 a reduction in the enzyme activity (Fraser *et al.*, 2007; Carbone *et al.*, 2009), the results shown here
7 confirm the involvement of *CCD7* and *CCD8* in strigolactone biosynthesis in tomato (Vogel *et al.*,
8 2010). In contrast, the reduction in strigolactone level by abamineSG treatment did not correlate with a
9 down-regulation of *LeCCD7* and *LeCCD8* (Fig. 5B). This suggests that these genes are not
10 transcriptionally regulated by ABA in the short-term. Post-transcriptional regulation of *CCD8* by auxin
11 was postulated to occur in *Arabidopsis* (Bainbridge *et al.*, 2005). In *sitiens* the levels of IAA in the
12 roots have been reported to be lower than in the corresponding wild-type (Dunlap & Binzel, 1996).
13 Therefore, the decrease in strigolactone production in the tomato ABA-mutants may be mediated by a
14 decrease in auxin levels in the roots which negatively affects *LeCCD7* and *LeCCD8* expression and
15 hence, the production of strigolactones in these mutants. A short-term decrease in ABA through
16 abamineSG application does not lead to a similar reduction in *LeCCD7* and *LeCCD8* expression, even
17 though strigolactone production is decreased, suggesting that a short-term response of strigolactone
18 production to ABA is not mediated through a decrease in auxin levels and its negative effect on
19 *LeCCD7* and *LeCCD8* expression.

20 It has been shown that Pi starvation promotes strigolactone biosynthesis (Yoneyama *et al.*, 2007;
21 López-Ráez *et al.*, 2008a). Despite the fact that the strigolactones are carotenoid derived, the amount of
22 carotenoids is not increased in roots under Pi starvation (López-Ráez *et al.*, 2008a) and no changes in
23 the expression of genes encoding enzymes involved in carotenoid biosynthesis were observed under Pi
24 deprivation using microarray studies (Wasaki *et al.*, 2003; Misson *et al.*, 2005; Hernandez *et al.*, 2007;

1 López-Ráez & Bouwmeester, 2008). Here we observed that Pi starvation, like ABA application, does
2 also not affect the expression of the strigolactone biosynthetic genes *LeCCD7* and *LeCCD8*. This
3 suggests that the effect of phosphorous deficiency on strigolactone production might be at the post-
4 transcriptional level or on an as yet unknown step in the strigolactone biosynthetic pathway. Our results
5 suggest that ABA could be involved in this regulation.

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

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16
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2 seeds, and Harry Klee and Jonathan Vogel for *LeCCD7* sequence information.

3

4

5 **FIGURE AND TABLE LEGENDS**

6

7 **Figure 1.**

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

13

14 **Figure 2.**

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

22

23 **Figure 3.**

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

8
 9 **Figure 4.**

10 Effect of Pi starvation and treatment with inhibitors on strigolactone production in tomato. The
 11 amounts (according to the peak area and corrected to 1 g root fresh weight) of the strigolactones
 12 solanacol, and the didehydro-orobanchol isomers 1 and 2 (Didehydro-1 and Didehydro-2) in the root
 13 exudates of tomato (cv. MoneyMaker) plants under Pi starvation (-Pi) and under Pi starvation plus
 14 treatment with 50 μ M of abamineSG (-Pi +A-SG) or D2 (-Pi + D2) for 3 and 7 days. The SEPAK C₁₈
 15 purified exudates were analysed using LC-MS/MS (see Materials and Methods). Bars represent the
 16 average of 5 independent replicates \pm SE. Bars with a different letter differ significantly ($P < 0.01$)
 17 according to Duncan's significant difference test.

18
 19 **Figure 5.**

20 Gene expression analysis by real time qPCR for the carotenoid cleaving genes *LeCCD7*, *LeCCD8*,
 21 *LeCCD1-B*, *LeNCED1* and *LeNCED4* in tomato roots. **A**, gene expression of the tomato mutants
 22 *notabilis* (white bars), *sitiens* (black bars) and *flacca* (grey bars). **B**, gene expression in roots of tomato
 23 (cv. MoneyMaker) plants grown under Pi starvation (-Pi; grey bars) and under Pi starvation plus
 24 treatment with 50 μ M of abamineSG (-Pi +A-SG; black bars) or D2 (-Pi + D2; white bars) for 7 days.

1 Real time qPCR was based on the C_t values as described in Materials and Methods. C_t values were
2 normalized using the household gene *LeActin*. The expression for each gene in the different mutants is
3 given relative to the expression of the same gene in the corresponding wild-type. Bars represent mean
4 values \pm SE of 3 independent biological replicates.

5

6 **Table 1.**

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

11

12 **Table 2.**

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

19

20

21 **SUPPLEMENTAL MATERIAL**

22

23 **Figure S1.**

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

7 **Figure S2.**

8 Gene expression analysis by real time qPCR of *Le4* (A) and ABA-8'-hydroxylase (*SICYP7070A1*) (B)
 9 upon ABA application (10 μ M) to *sitiens* (black bars) and corresponding wild-type (Reinlands Rhum,
 10 WT) (grey bars) for 48 hours. Real time qPCR was based on the C_t values as described in Materials and
 11 Methods. C_t values were normalized using the gene *LeActin* as an endogenous control gene. The
 12 expression for each gene is given relative to the expression of the same gene in plants not treated with
 13 ABA. Bars represent mean values \pm SE of 3 independent biological replicates.

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Figure 1

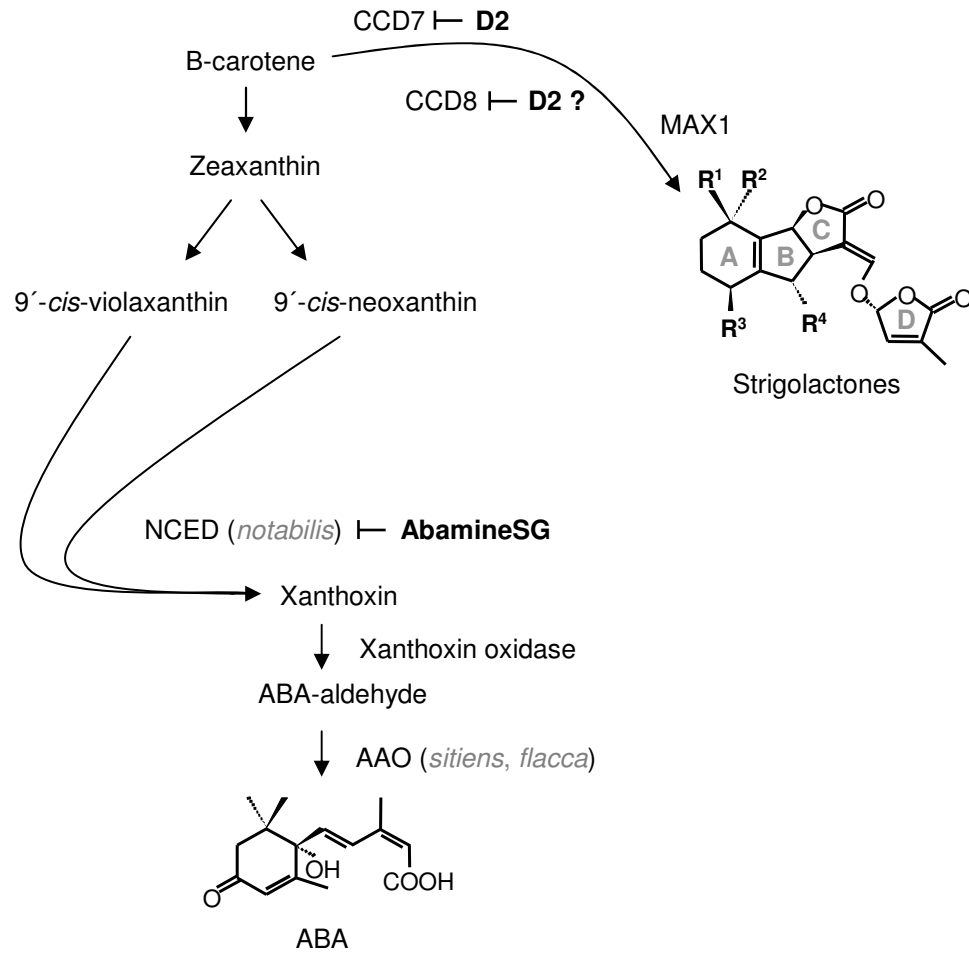


Figure 2

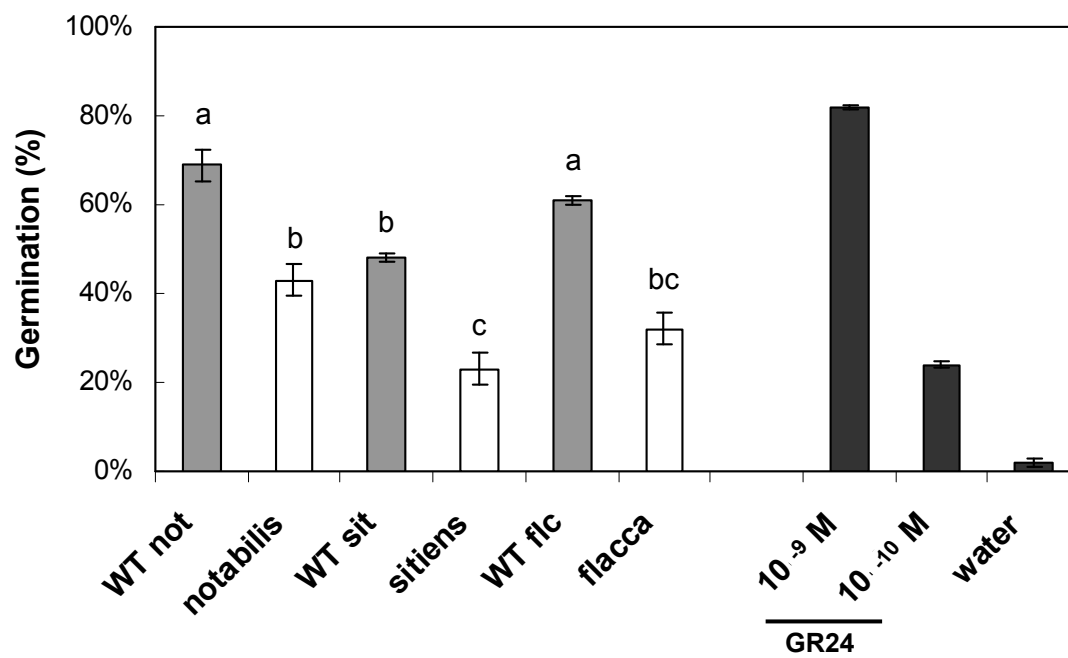
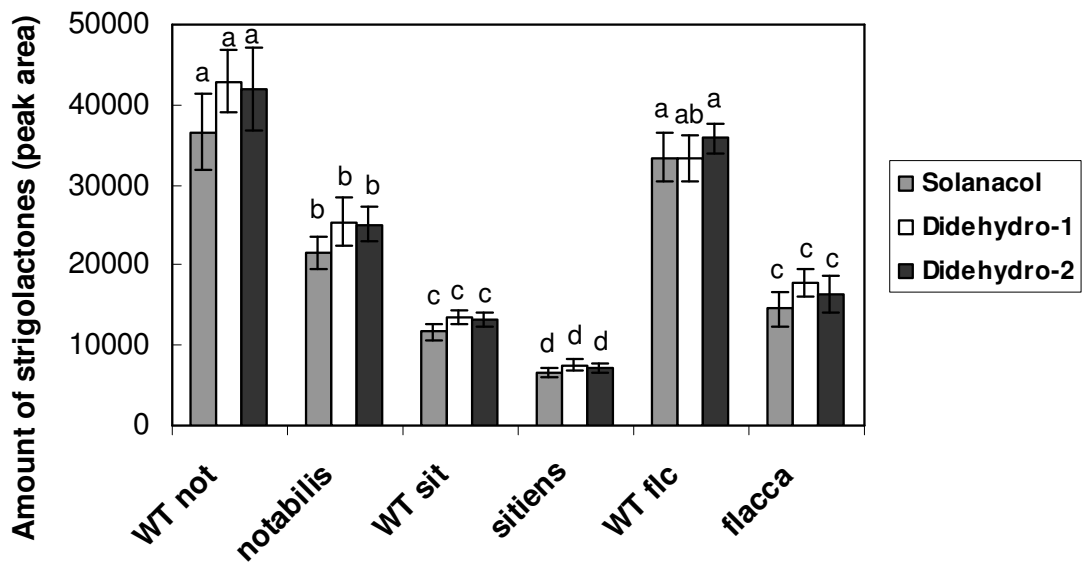


Figure 3

A



B

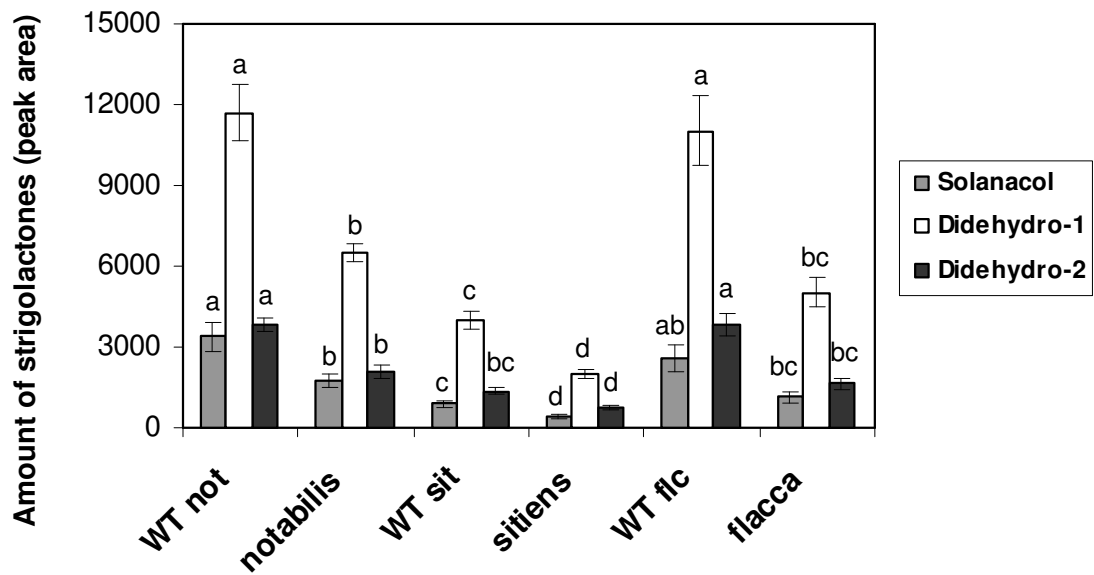


Figure 4

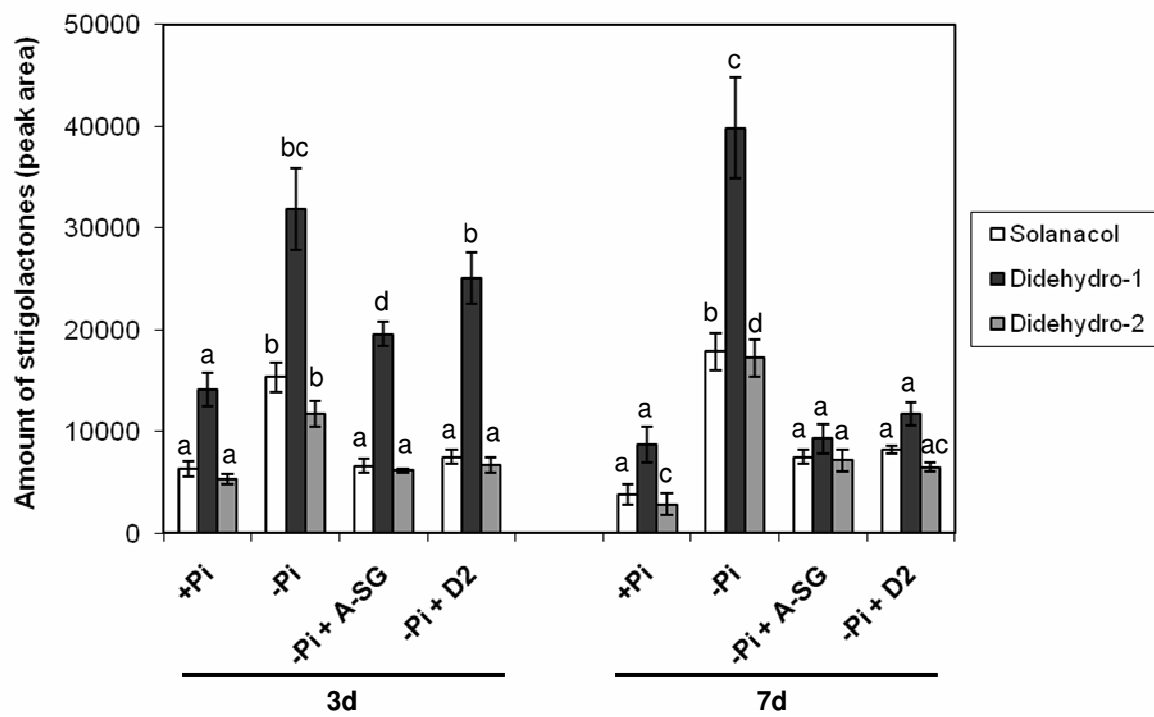
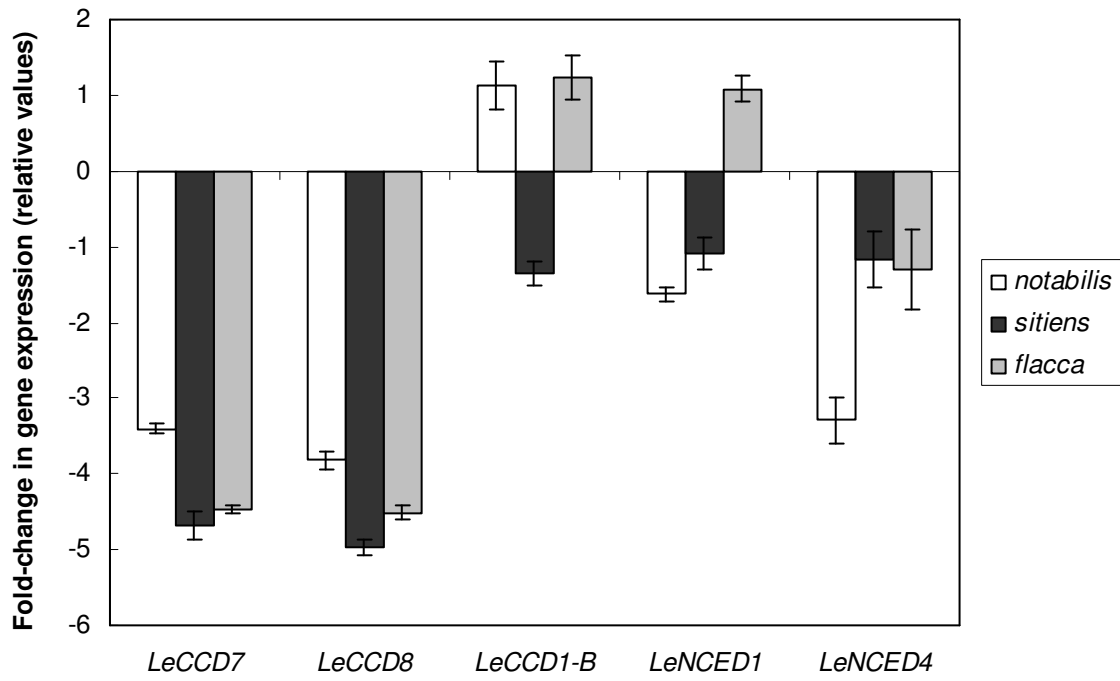


Figure 5

A



B

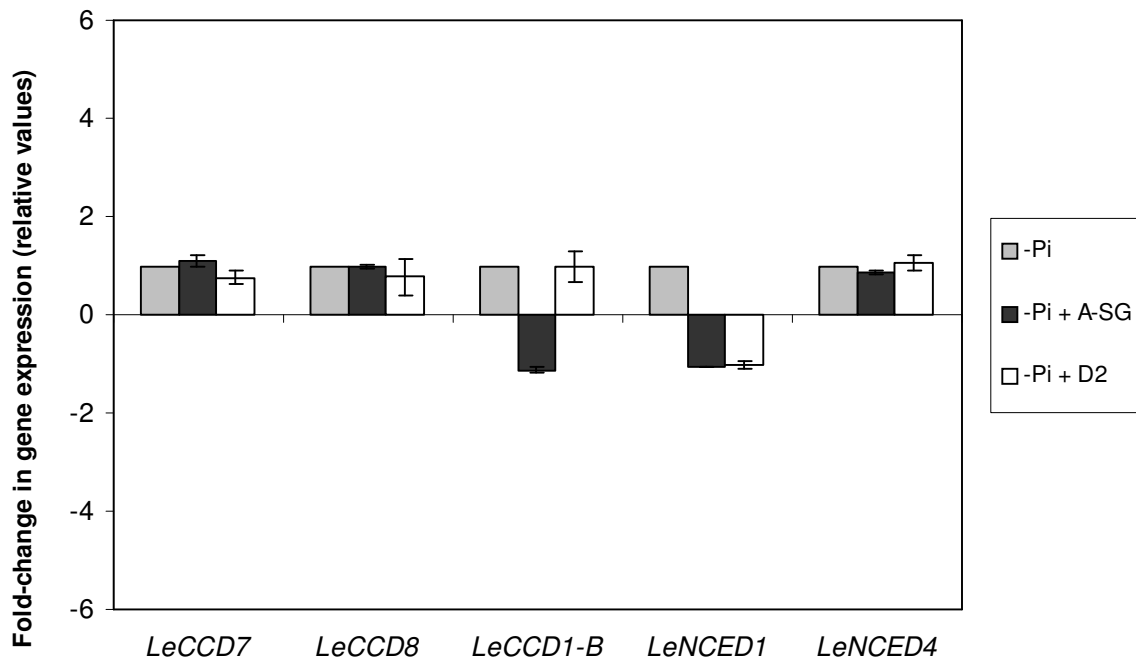


Table 1

Genotype	[ABA] ng/g FW	
	Roots	Shoots
WT <i>notabilis</i>	9.51 ± 2.06 ^a	203.91 ± 10.19 ^a
<i>notabilis</i>	5.22 ± 1.06 ^b	112.02 ± 11.42 ^b
WT <i>sitiens</i>	6.26 ± 0.72 ^b	180.92 ± 22.07 ^a
<i>sitiens</i>	2.51 ± 0.60 ^c	67.91 ± 7.14 ^c
WT <i>flacca</i>	7.19 ± 0.51 ^{ab}	198.63 ± 15.34 ^a
<i>flacca</i>	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

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