New Phytologist

Research

Low levels of strigolactones in roots as a component of the systemic signal of drought stress in tomato

Ivan Visentin[1], Marco Vitali[1], Manuela Ferrero[1], Yanxia Zhang[2], Carolien Ruyter-Spira[2], Ondřej Novák[3], Miroslav Strnad[3], Claudio Lovisolo[1], Andrea Schubert[1] and Francesca Cardinale[1]

[1]Laboratory of Plant Physiology, DISAFA – Turin University, Grugliasco, 10095, TO, Italy; [2]Laboratory of Plant Physiology, Wageningen University, 6708, PB Wageningen, the Netherlands; [3]Laboratory of Growth Regulators, Institute of Experimental Botany ASCR & Palacky University Olomouc, Olomouc, Czech Republic

Author for correspondence:
Francesca Cardinale
Tel: +390116708875
Email: francesca.cardinale@unito.it

Received: *19 May 2016*
Accepted: *4 August 2016*

New Phytologist (2016)
doi: 10.1111/nph.14190

Key words: abscisic acid (ABA), drought, strigolactones (SL), systemic signalling, tomato (*Solanum lycopersicum*).

Summary

• Strigolactones (SL) contribute to drought acclimatization in shoots, because SL-depleted plants are hypersensitive to drought due to stomatal hyposensitivity to abscisic acid (ABA). However, under drought, SL biosynthesis is repressed in roots, suggesting organ specificity in their metabolism and role. Because SL can be transported acropetally, such a drop may also affect shoots, as a systemic indication of stress.

• We investigated this hypothesis by analysing molecularly and physiologically wild-type (WT) tomato (*Solanum lycopersicum*) scions grafted onto SL-depleted rootstocks, compared with self-grafted WT and SL-depleted genotypes, during a drought time-course.

• Shoots receiving few SL from the roots behaved as if under mild stress even if irrigated. Their stomata were hypersensitive to ABA (likely via a localized enhancement of SL synthesis in shoots). Exogenous SL also enhanced stomata sensitivity to ABA.

• As the partial shift of SL synthesis from roots to shoots mimics what happens under drought, a reduction of root-produced SL might represent a systemic signal unlinked from shootward ABA translocation, and sufficient to prime the plant for better stress avoidance.

Introduction

Drought stress counts among the most recurrent and limiting environmental conditions for plant development and full productivity; under water scarcity, phytohormones interact cooperatively to allow resource optimization (Christmann *et al.*, 2006). Abscisic acid (ABA) biosynthesis is strongly and rapidly increased by drought, and prevents water loss mainly by driving stomata closure, thus controlling transpiration. Also, root-synthesized ABA is, in some plants, a systemic stress signal, travelling shootward to prevent, among others effects, the negative consequences of soil water deficit (Comstock, 2002). However, in plants such as *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*), ABA produced by roots under water deprivation is unnecessary for shoot responses, leaving uncertainty on the chemical nature of the systemic drought stress signal (Holbrook *et al.*, 2002; Christmann *et al.*, 2007). Additionally, it was shown in tomato that ABA travels from shoots to roots under long-term drought, thus inverting the original hypothesis (Manzi *et al.*, 2015). Other signals, such as hydraulic, electrical and chemical signals, including other phytohormones and changes in xylem sap pH, therefore are also thought to contribute (reviewed by Huber & Bauerle, 2016). It is argued, however, that positive chemical signals alone cannot account for the initial stomatal responses to root drying, because of the relatively low xylem transport velocity (Huber & Bauerle, 2016).

Recently, the hormones strigolactones (SL) also have been proposed as signal mediators under environmental stress. SL have pervasive roles in development from germination and reproduction, to root and shoot architecture; at various levels, they also promote the interaction with beneficial root symbionts as well as with detrimental (micro)organisms (reviewed by Ruyter-Spira *et al.*, 2013). SL and ABA share their biosynthetic precursor, both being carotenoid-derived terpenoid lactones (Matusova *et al.*, 2005). Several enzymes act sequentially in SL biosynthesis: DWARF 27 (D27) is a β-carotene isomerase, CCD7 and CCD8 are carotenoid-cleavage dioxygenases (CCD) and MORE AXYLLARY GROWTH 1 (MAX1) is a class III cytochrome P450 that, with its orthologues and paralogues and the recently characterized LATERAL BRANCHING OXIDOREDUCTASE (LBO) (Brewer *et al.*, 2016), is thought to contribute to the oxidation of the SL precursor carlactone and to the chemical diversification of SL family members (reviewed by Al-Babili & Bouwmeester, 2015). The core enzyme set is mostly active in roots; root-produced SL are then exported out of the producing cell by ABC_G transporter protein(s) such as PhPDR1 (Kretzschmar *et al.*, 2012; Sasse *et al.*, 2015), both to be exuded in soil and to travel shootward, as shown in Arabidopsis and tomato (Kohlen *et al.*, 2011). Although transcripts of SL-related genes, and final metabolites, are mostly not or barely detectable in shoots, biosynthesis in aboveground tissues is known to occur, possibly at specific spots. In fact, wild-type (WT) shoots grafted onto SL-

depleted rootstocks do not display the typical morphological phenotype of SL-depleted plants (Foo *et al.*, 2001; Sorefan *et al.*, 2003).

Recently, SL metabolism and physiological effects in plants under osmotic stress conditions have been analysed. SL-depleted *A. thaliana* and *Lotus japonicus* (Liu *et al.*, 2013) are hypersensitive to drought at the shoot level, a feature linked to the hyposensitivity of their stomata to endogenous and exogenous ABA. This finding supports a positive role for SL in the acclimatization to drought in aboveground organs (Ha *et al.*, 2014; Liu *et al.*, 2015). Consistent with this idea, the transcript of SL biosynthetic genes is increased by drought in Arabidopsis leaves (Ha *et al.*, 2014). However, transcription of biosynthetic and SL transporter-encoding genes is repressed along with the accumulation of SL in nonmycorrhizal *L. japonicus* and tomato roots under drought (Liu *et al.*, 2015; Ruiz-Lozano *et al.*, 2016). This is surprising *per se,* because roots are the main SL production site under normal conditions, and suggests different dynamics for shoot- and root-derived SL. A negative correlation between ABA and SL levels was observed in nonmycorrhizal, water-stressed roots of *L. japonicus* and tomato (Liu *et al.*, 2015; Ruiz-Lozano *et al.*, 2016). Because drought stress-triggered ABA accumulation is hampered by exogenous SL in *L. japonicus* roots, the drop in SL biosynthesis in roots under drought might have a role in allowing an increase of local ABA and possibly, also, of its levels in the xylem sap, leading to systemic responses to a dropping root water potential in plants that rely also on ABA for chemical signalling of drought (Liu *et al.*, 2015). However, the possibility exists also that such a drop has a direct physiological effect on shoots, namely as a systemic indication of stress at the root level, because root-produced SL can also be transported to the whole plant (Kohlen *et al.*, 2011). This, and the fact that SL are needed locally in stressed shoots for efficient control of water loss by transpiration (Ha *et al.*, 2014; Liu *et al.*, 2015), led us to hypothesize that WT scions grafted onto SL-depleted rootstocks may behave as if stressed even in the absence of stress, at least in some respects, and perform differently under stress than if grafted onto WT rootstocks.

In this work, we investigated the possible systemic significance of the SL decrease in roots under drought, by analysing molecularly and physiologically WT scions grafted over SL-depleted (*CCD7*-silenced) tomato rootstocks, compared with self-grafted WT and SL-depleted genotypes, both under normal and stress conditions. The results proved that indeed stomata of shoots receiving less SL from the roots are hypersensitive to ABA also in the absence of stress, possibly through an enhancement of local SL synthesis. This is likely to mimic what normally happens under drought conditions and suggests that root-derived SL – or better, a reduction thereof – might be a component of the systemic signal of stress in tomato.

Materials and Methods

Plant material and growth conditions

The tomato (*Solanum lycopersicum* L.) *SlCCD7*-silenced line 6936, hereafter called SL−, and its wild-type (WT) genotype M82 were a kind gift by Dr H. J. Klee (University of Florida). Seeds were sterilized in 4% (v/v) sodium hypochlorite containing 0.02% (v/v) Tween 20, rinsed thoroughly with sterile water, and then germinated for 48 h on moistened filter paper at 25°C in darkness. Subsequently, seedlings were grown in inert substrate (sand : vermiculite; 1 : 1, v/v) and the pots watered with Hoagland solution twice per week. The three grafted lines were produced by the clamp grafting technique on plants at the 2/4-leaf stage and with stem diameter of *c.* 1.5–2 mm. Water stress was applied to plants 4 wk after grafting by withholding water starting at day zero (T0); shoots and roots were collected 0, 1, 3 and 5 d after the beginning of the stress (T0 to T5, respectively; three plants per line and sampling point) and stored at −80°C. At T5, three plants per line were watered and collected after two additional days to give the rehydrated (recovery) samples. The experiment was repeated twice. Supporting Information Fig. S1 shows how relative water content and soil water potential were dropping during the course of one drought experiment. Relative soil water content was determined gravimetrically by collecting daily *c.* 10 ml of soil from three different points and depths in each pot (at 5, 10 and 15 cm depth with 120° of angular separation between each of the respective sample points). The soil was weighed, oven-dried at 100°C for 24 h and then reweighed to assess water content. At the same time, the soil water retention curve was assessed with pressure plate measurements of the potting substrate according to Tramontini *et al.* (2014).

Gene transcript quantification

Total RNA from tomato roots and shoots was extracted as described (Gambino *et al.*, 2008) and treated with DNase I (ThermoScientific) at 37°C for 30 min to remove residual genomic DNA. First-strand cDNA was synthesized from 3 μg of purified total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Monza, Italy) according to the manufacturer's instructions. For transcript quantification of *SlCCD7*, *SlCCD8* and *SlNCED1* by quantitative reverse-transcription PCR (qRT-PCR), the StepOne system (Applied Biosystems) was used, with the transcript of the Elongation factor 1α (*SlEF-1*α gene) as a reference; primers used are reported as in Table S1. Three independent biological replicates were analysed and each qRT-PCR reaction was run in technical triplicates. Transcripts of the target genes were quantified by the $2^{-\Delta\Delta Ct}$ method.

Physiological measurements

Leaf water potential, stomatal conductance and net carbon assimilation were measured daily between 10:00 and 12:00 h on at least three plants per grafted line and independent experiment, as reported by Liu *et al.* (2015). Briefly, stomatal conductance and net carbon assimilation rate were measured with a portable gas exchange system (GFS-3000; Walz GmbH, Effeltrich, Germany) by clamping the most apical leaves of a shoot in the leaf chamber, where photosynthetically active radiation (1200 μmol photons m^{-2} s^{-1}), air flow (750 μmol s^{-1}) and temperature (25°C) were kept constant. Environmental conditions of CO_2

(450 ppm) and vapour pressure deficit (2.3 kPa) were stable during the 10-d experiments. Leaf water potential was measured with a pressure chamber (Scholander *et al.*, 1965) on one leaf per plant, immediately after gas exchange quantification. For the quantification of responses to ABA, stomatal conductance was measured as previously at 30-s intervals before and during ABA treatment. This was accomplished by cutting leafy twigs while submerged in filtered water (one leaf each, from three plants per grafted line, treatment and experiment), by letting stomatal conductance stabilize with the twig dipped in water and then by adding ABA to 5, 20 or 50 μM final concentration, while continuously recording both stomatal conductance and transpiration rates every 30 s, as detailed earlier. For treatment with exogenous SL, WT plants were sprayed with a 5 μM solution of *rac*GR24 (StrigoLab SrL, Turin, Italy) 24 h before treatment with ABA 5 μM and stomatal conductance recording as earlier.

Extraction and quantification of SL and ABA

Solanacol, orobanchol and didehydro-orobanchol were quantified in the roots of the three grafted lines, whereas ABA was quantified in both roots and shoots. For SL extractions, three plants per line and time-point were pooled, whereas two independent biological assays were run. For SL quantification, samples (0.5 g each) were ground manually in liquid nitrogen and extracted with 2 ml of cold ethyl acetate containing D6-*epi*-5 deoxystrigol as internal standard (0.05 nmol ml^{-1}) in 10-ml glass vials. Standards for didehydro-oronbanchol isomers were not available, so quantities for this SL were expressed as percentage ratio with respect to WT root tissues in the absence of stress (T0); the isomer reported in Fig. S2(c) is the one with retention time of 4 min 6 s in our conditions. The extraction and quantification procedures for SL were performed as reported previously (Lopez-Raez *et al.*, 2010). For ABA extraction, two biological replicates of two pooled plants each were sampled per line and time-point, whereas two independent biological assays were run. For ABA quantification, labelled internal standard was added ($[^2H]_6$-ABA, 20 pmol) to each sample (20–25 mg homogenized in 1 ml of cold 10% MeOH in H_2O, v/v) and subsequently extracted and analysed as detailed in Flokova *et al.* (2014).

Results

WT shoots transpire and dehydrate less when grafted onto SL-depleted roots

In order to investigate the systemic meaning of SL decrease in stressed roots, we sought to reproduce such a condition in the absence of stress. To this purpose, rootstocks of the SL-depleted line (6936) (Vogel *et al.*, 2010) were joined to shoots of the corresponding WT (M82) to give WT/SL- hetero-grafts. Two sets of control plants were also generated, that is, self-grafts of SL- and WT rootstocks and scions (SL−/SL− and WT/WT, respectively). The physiological, transcriptional and metabolic responses to water stress were examined at different time-points for these three sets of individuals. As a preliminary check, SL content in roots was quantified, confirming that the 6936 genotype was indeed defective in SL production (*c.* 20-fold less orobanchol, solanacol and one of the didehydro-orobanchol isomers under unstressed conditions). The three SL metabolites decreased under stress, already 1 d after water withdrawal, both in WT and SL− roots, irrespectively of the scion genotype (Fig. S2a–c), confirming what observed in polyethylene glycol treated *L. japonicus* roots (Liu *et al.*, 2015).

Measuring stomatal conductance and leaf water potential confirmed that in tomato, as in Arabidopsis and *Lotus*, whole-plant SL depletion increases stomatal conductance and decreases leaf water potential in the absence of stress; under the same conditions, however, WT/SL− plants showed significantly lower stomatal conductance than WT/WT (Fig. 1a, and T0 in Fig. S3a). Accordingly, leaf water potential values were significantly less negative in WT leaves grafted onto SL− than WT roots (Fig. S3b). Photosynthesis of WT scions grafted over SL− rootstocks was only slightly and nonsignificantly affected by the reduced gas exchange of hetero-grafts compared with self-grafted WT plants, whereas both displayed significantly lower values than SL− shoots (Figs 1b, S3c).

Under stress, the three grafted lines followed a similar trend of stomatal conductance and net carbon assimilation decrease, although starting from different values (Fig. 1a,b). Under severe stress, gas exchange in leaves of WT/SL− plants was comparable to the WT, even if leaf water potential was less negative than in the latter; WT/SL− leaves also performed photosynthesis significantly better than WT/WT (Fig. S3a–c). SL−/SL− plants confirmed their hypersensitivity to drought for all parameters tested. These data indicated that SL depletion at the root level reduces stomatal conductance and attenuates the drop in leaf water potential in WT shoots under drought, whereas SL depletion in shoots has opposite effects. After rehydration (recovery; closed symbols in Fig. 1a, b, R in Fig. S3a–c), the physiological parameters of all three lines returned to levels similar to those observed in the absence of stress.

Both drought and depletion of SL in the roots induce transcript accumulation for SL biosynthetic genes in the shoots

In order to assess whether the change in metabolite abundance is regulated at the gene transcription level, two SL biosynthetic genes (*SlCCD7* and *SlCCD8*) were profiled by qRT-PCR in roots and shoots of the three grafted lines under irrigated and drought stress conditions, in the same plant material used for SL quantification.

The analysis confirmed that in roots, the transcript amount of both genes inversely correlated with stress severity for all grafted lines (Figs 2a,b, S4a,b). In the shoots of the same sets of plants, however, transcripts of both biosynthetic genes followed an opposite trend compared with roots and accumulated under drought, as reported previously in Arabidopsis and postulated in *Lotus* (Ha *et al.*, 2014; Liu *et al.*, 2015) (Figs 2c,d, S4d,e). It must be noted, however, that in terms of relative transcript abundance, values in shoots remained much lower (about one hundredth;

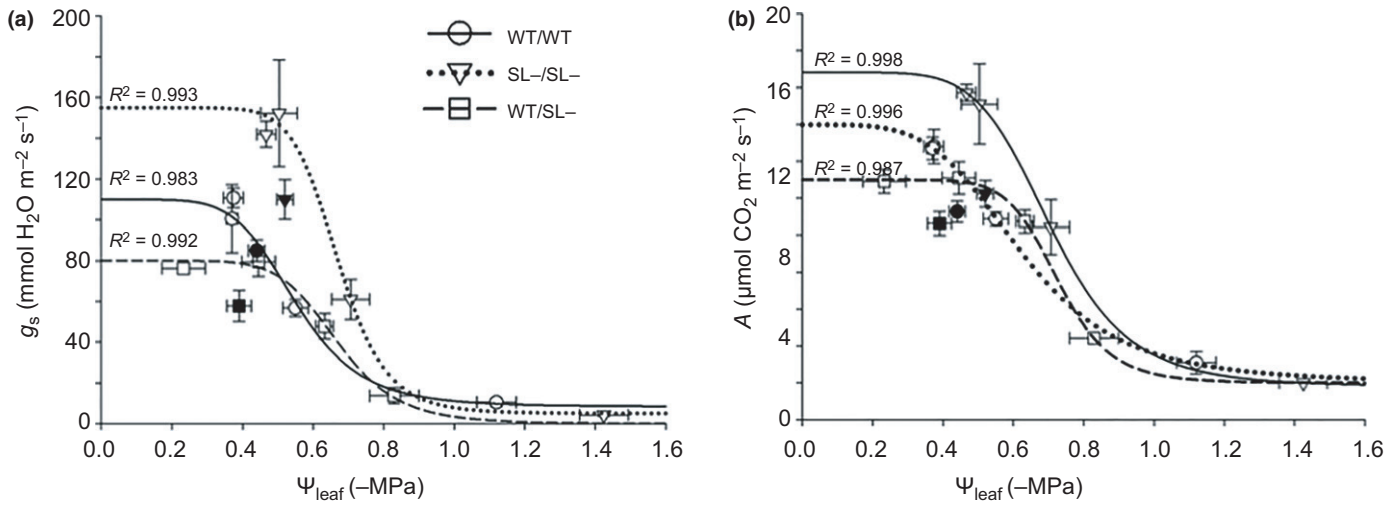

Fig. 1 Physiological performance of the grafted lines in the absence and presence of stress. (a) Stomatal conductance (g_s), and (b) mean carbon assimilation rate (A) as a function of leaf water potential (Ψ_{leaf}) of grafted tomato plants (WT/WT, SL−/SL− and WT/SL−) along a water-deprivation time-course. Closed symbols in each series indicate rehydrated samples (recovery). Data represent the mean and SEM of $n = 6$ biological replicates from two independent experiments. WT, wild-type; SL, strigolactones.

not obvious in the normalized data of Fig. 2) of root values at T0, even in samples collected under very severe stress at T5. This justifies the fact that we were unable to detect the final metabolites in these shoot samples (data not shown). Relevantly here, expression of both biosynthetic genes in WT shoots was significantly higher when the mutant was used as rootstock (WT/WT vs WT/SL−, Figs 2c,d, S4c,d). This is a known pattern (Johnson *et al.*, 2006), consistent with the idea of a general negative feedback by the final metabolites on the SL biosynthetic pathway and supported by the repressive effect of exogenous SL on the same genes (see, e.g. Liu *et al.*, 2015). Overall, data on transcript of SL-biosynthetic genes indicated that the response of shoots to SL deficiency in roots overlaps with the response to osmotic stress. In fact, both drought stress and depletion of SL in the roots in the absence of stress induced transcript accumulation of SL biosynthetic genes in tomato shoots.

As an additional observation, *SlCCD7* transcripts in unstressed SL− (*CCD7*-silenced) rootstocks were more abundant in grafts bearing a WT instead of a SL- shoot (WT/SL− vs SL−/SL−; T0 of Fig. 2a). This correlated with a very slight increase of SL metabolites, especially orobanchol (see T0, Fig. S2a–c) and suggested that a SL-dependent, shoot-to-root signal feeding back on the transcription/transcript stability of this gene exists in tomato as in Arabidopsis and pea (Foo *et al.*, 2005; Johnson *et al.*, 2006), where it was shown to depend on the *RMS2* locus. Also, *SlCCD8* transcripts were more abundant in SL− than WT roots (as expected, given the already mentioned negative feedback of SL on the transcription of their biosynthetic genes; Fig. 2b); and in SL− roots, *SlCCD8* transcripts were more concentrated in the presence of a SL− than of a WT scion (Fig. 2b). In this sense, expression of *SlCCD7* and *SlCCD8* in the root seemed influenced oppositely by the ability of the shoot to produce SL. We may hypothesize that not only locally produced, but also shoot-synthesized SL may participate (directly or indirectly) in the negative feedback on *SlCCD8* expression in the root, and thus that in SL− roots, the presence of a WT scion may lead to less pronounced overexpression of *SlCCD8* than in the presence of a SL− scion. Finally, it is noteworthy that the concentration of *SlCCD8* transcript in WT shoots grafted onto SL− roots was as high as in SL− shoots in the absence of stress (T0, Fig. 2d) but remained stable along the time-course in the former, whereas it was further induced in the latter (Fig. S4d). We have no easy explanation for this pattern, which might, however, be due to the fact that leaves of WT/SL− plants dehydrate less and produce less ABA (see further on) along the time-course, than either self-grafted control line.

The low-transpiration phenotype of hetero-grafted, WT/SL− plants is not due to increased total free ABA

In order to determine whether the effects of SL depletion on WT shoots may be due to altered ABA metabolism, we set to quantify this hormone in roots and shoots of plants in the three grafted sets. Previous data in Arabidopsis and tomato leaves, and in *Lotus* roots and shoots, indicated no changes or slight decreases of ABA correlated with SL depletion in shoots, especially under stress (Ha *et al.*, 2014; Liu *et al.*, 2015); ABA content was reported to be lower than in WT under nonstressful conditions only in *CCD8*-silenced tomato shoots (Torres-Vera *et al.*, 2014).

Results showed that under normal conditions, WT roots contain less free ABA than SL− ones (WT/WT vs SL−/SL− and WT/SL− plants, T0 in Fig. S5a) per gram weight of fresh tissue. As stress increased, ABA started accumulating in roots of SL−/SL− and WT/WT plants more quickly than in roots of WT/SL− plants, where ABA was significantly less concentrated than in the roots of the other grafts (Fig. S5a). Correlation curves to leaf water potential values were, however, substantially superimposable (Fig. 3a). Transcript quantification for *SlNCED1*, a key

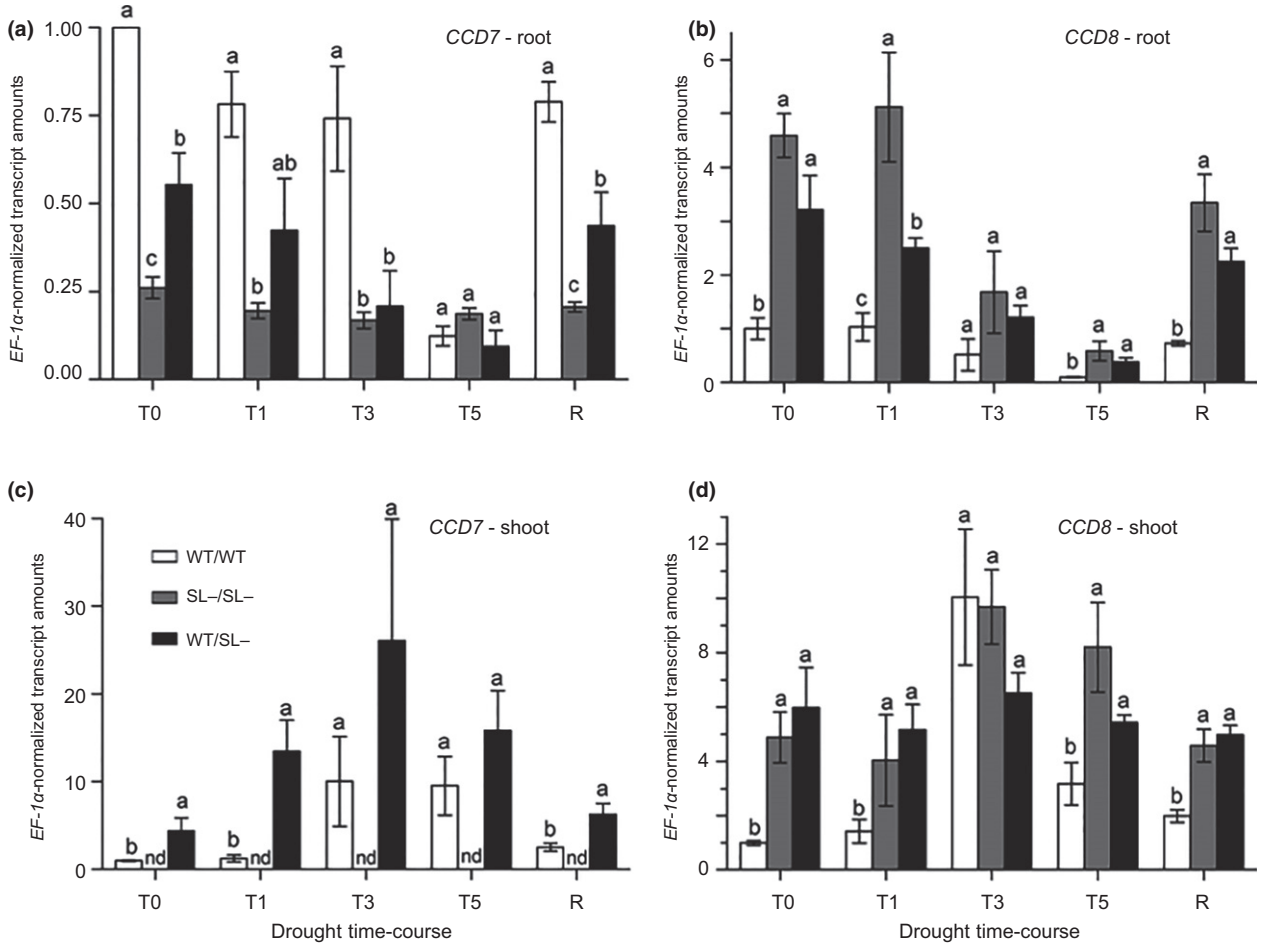

Fig. 2 Effect of drought on the transcript amounts of strigolactones (SL) biosynthetic genes (*SlCCD7* and *SlCCD8*) of (a, b) roots and (c, d) shoots of grafted tomato plants (WT/WT, SL−/SL− and WT/SL−) during a time-course (0, 1, 3 and 5 d from water withdrawal for T0 to T5). R indicates the rehydrated samples (recovery). Gene transcript abundance was normalized to endogenous *EF-1α* and presented as fold-change value over WT/WT at T0, which was set to 1. Data represent the mean and ± SE of $n = 6$ biological replicates from two independent experiments. Different letters indicate significant differences between plant lines for the same time-point, as determined by a two-way ANOVA test ($P < 0.05$). nd, not detectable. WT, wild-type; SL, strigolactones.

biosynthetic gene for stress-induced ABA in tomato (Munoz-Espinoza *et al.*, 2015), showed good correlation with free ABA content but for a few points and grafting combinations (Figs 3b, S5b). These discrepancies between *SlNCED1* transcript amounts and ABA concentration may be due to post-transcriptional regulation of biosynthetic enzymes, and/or to the activity of catabolic genes, for example, or to the release/sequestration of free ABA from/in conjugated forms (reviewed by Xiong & Zhu, 2003).

Although in the absence of stress SL− shoots contained more ABA per gram fresh weight than WT ones, as stress proceeded and leaf water potential started becoming more negative, ABA levels increased faster in WT than in SL− scions; at the moment of maximum stress, ABA concentration was minimum in WT scions grafted onto SL− rootstocks and intermediate in SL− shoots (Fig. 3c, and T5 in Fig. S5c). The same trend is seen for transcripts of *SlNCED1*, which again showed a good correlation with free ABA content but for a few points and grafting combinations (Figs 3d, S5d). These results confirmed that especially under stress, SL depletion in the shoot partially compromises the ability to synthesize ABA. Furthermore, coupled to the physiological data in Fig. 1, they strongly suggested that the low gas exchange phenotype of hetero-grafted WT/SL− plants was not due to increased free ABA content, given the comparatively low ABA concentration in their tissues.

WT scions are hypersensitive to ABA if grafted onto SL-depleted rootstocks

In order to explore whether altered sensitivity to ABA might rather underlie the physiological and metabolic results described earlier, shoot sensitivity to exogenous ABA dependent on the rate of SL production in the roots was investigated. ABA at different concentrations was applied to and absorbed by excised petioles of composite leaves of the three grafted lines, while measuring the time required for the stomata to start closing. On the one hand, this assay confirmed in tomato what was already known in Arabidopsis and Lotus, that is, that SL-depleted scions are hyposensitive to ABA (at all three – but more convincingly at the lower –

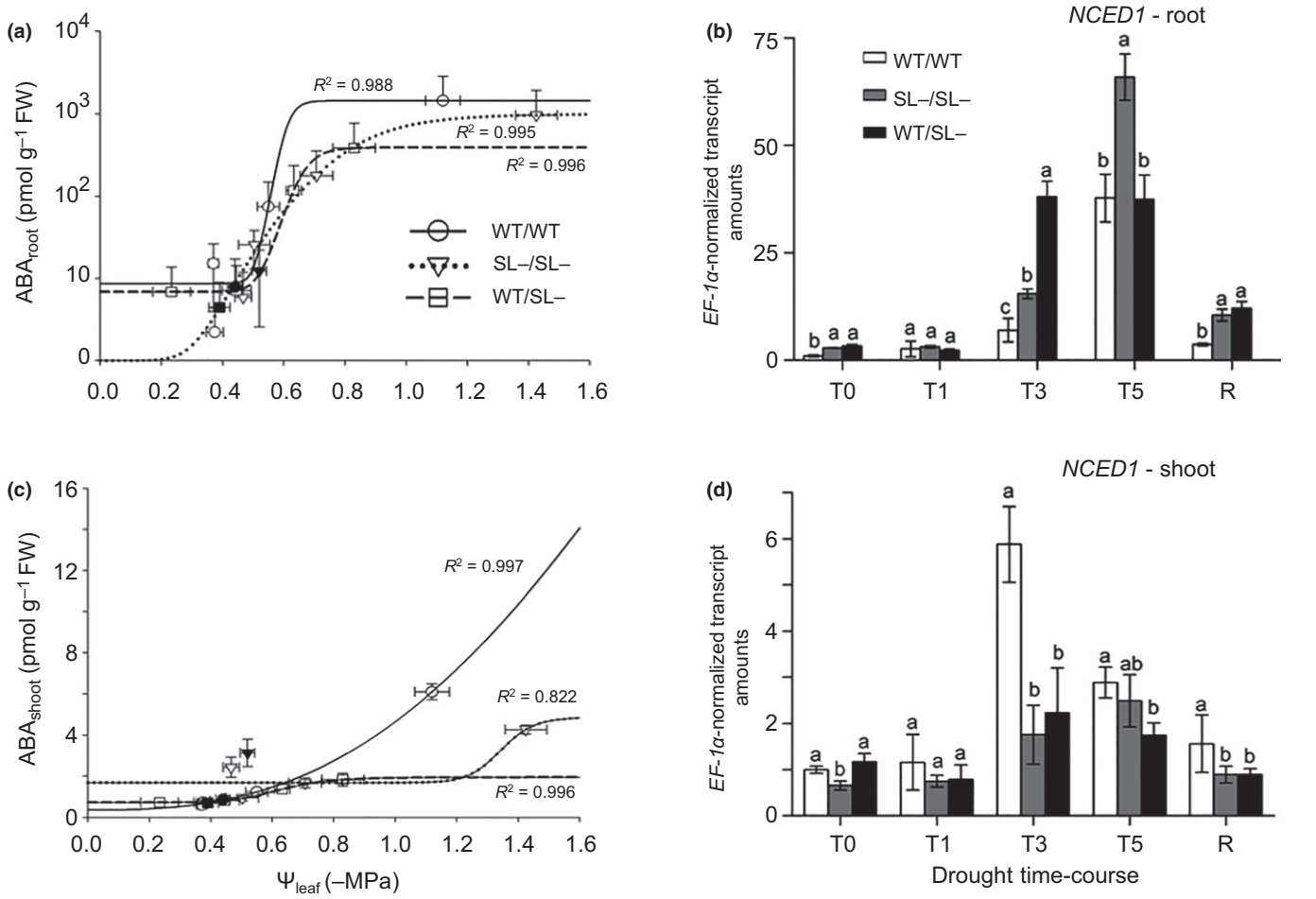

Fig. 3 Effect of drought on free abscisic acid (ABA) as a function on leaf water potential (Ψ_{leaf}) and on transcript amounts of the ABA biosynthetic gene *SlNCED1* in (a, b) roots and (c, d) shoots of grafted tomato plants (WT/WT, SL−/SL− and WT/SL−) during a time-course (0, 1, 3 and 5 d from water withdrawal for T0 to T5). (a, c) Closed symbols or (b, d) R indicate the rehydrated samples (recovery). Gene transcript abundance was normalized to endogenous *EF-1α* and presented as fold-change value over WT/WT at T0, which was set to 1. Data on ABA represent the mean and ± SE of $n = 4$ biological replicates (each replicate a pool of two plants) from two independent experiments. Data on *SlNCED1* represent the mean and ± SE of $n = 6$ biological replicates from two independent experiments. Different letters in (b) and (d) indicate significant differences between plant lines for the same time-point, as determined by a two-way ANOVA test ($P < 0.05$). WT, wild-type; SL, strigolactones.

concentrations tested), with respect to WT (SL−/SL− vs WT/WT; Fig. 4). On the other hand, the same analysis proved also that WT scions are indeed hypersensitive to ABA if grafted onto SL- instead of WT rootstocks (WT/SL− vs WT/WT, Fig. 4), as hypothesized on the basis of the stomatal conductance and shoot ABA quantification experiments reported earlier (Figs 1a, 3c vs Figs S3a, S5c). We also tested (at 5 μM ABA, the concentration for which differences among our lines were more evident) if a pretreatment with the synthetic SL analogue *rac*GR24 could by itself increase sensitivity to ABA, in a complementary way to SL depletion decreasing it. This was indeed the case (WT/WT plants, GR24-treated vs untreated, Fig. 4).

These data confirmed that the physiological phenotype displayed by the WT/SL− plants both under irrigated and drought conditions was more likely to be due to a higher sensitivity to endogenous ABA, rather than to its absolute levels. This effect could be linked to a local increase of SL synthesis, given the higher transcript concentration for SL biosynthetic genes under these conditions, and – as a more indirect indication – the fact that ABA sensitivity increased in stomata treated with exogenous SL.

Discussion

Low SL in the roots prime shoots for drought stress avoidance in tomato

In this study, we investigated in tomato the possible systemic implications of the drop in strigolactone (SL) synthesis happening in roots under osmotic stress. A parsimonious starting hypothesis was that SL depletion in roots could directly or indirectly act as a signal of stress for the shoots. On this basis, heterografted plants with wild-type (WT) scions and SL-depleted rootstocks were to behave as at least mildly stressed, even in the absence of stress. Our physiological data are in agreement with this theory: stomatal conductance values of WT shoots grafted

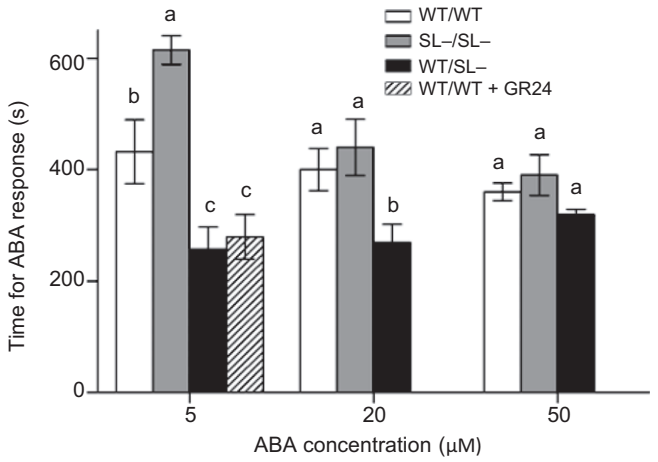

Fig. 4 Dose-response of leaves to treatment with exogenous abscisic acid (ABA) at different concentrations. Stomatal conductance was measured at 30-s intervals before and during ABA treatments performed on detached composite leaves from grafted tomato plants (WT/WT, SL−/SL−, WT/SL−). WT/WT plant pretreated with 5 μM *rac*GR24 were analysed only for the 5 μM ABA treatment (black bars). Values represent the mean and ± SE of at least $n = 6$ biological replicates from two independent experiments, and refer to the time (s) needed for the decrease of stomatal conductance to start, from the time of ABA addition to the dipping solution. Different letters indicate significant differences between plant lines for the same treatment, as determined by a two-way ANOVA test ($P < 0.05$). WT, wild-type; SL, strigolactones.

onto SL-depleted rootstocks are significantly lower than those of WT shoots self-grafted onto WT rootstocks in irrigated conditions, and are accompanied by less negative leaf water potential values and, as expected, higher intrinsic water use efficiency (defined as the ratio between net carbon assimilation and stomatal conductance; Fig. S3d). These data support the idea that SL depletion in root tissues affects (directly or indirectly) the physiological response in the shoot and leading to better acclimatization to drought. The ability of shoots to produce SL is needed for this to happen, because stomatal conductance is increased instead when the whole plant (and not only the roots) are *CCD7*-silenced; indeed, this latter condition rather leads to drought hypersensitivity, as shown in SL-depleted Arabidopsis, *Lotus* and, now, tomato plants (Ha *et al.*, 2014; Liu *et al.*, 2015; present study).

Low SL in the roots and (high) SL in the shoot render stomata hypersensitive to ABA

In order o determine whether the effects of root SL depletion on WT shoots may be due to altered abscisic acid (ABA) levels, this hormone was quantified in roots and shoots of plants in the three grafted sets. SL-depleted roots and especially shoots contain significantly more ABA per gram fresh weight than WT equivalents in the absence of stress. Our results in unstressed shoots are in apparent contradiction to the ones reported on *CCD8*-silenced tomato plants, where shoots of SL-depleted lines had lower ABA content (Torres-Vera *et al.*, 2014); the most likely explanation is that our data were normalized over fresh and not dry weight as in Torres-Vera *et al.* In any case during severe stress, free ABA increases less in tissues of self-grafted SL− than WT plants, a trend already observed in *Lotus* (Liu *et al.*, 2015); such a situation, coupled to the hyposensitivity to the hormone, will certainly exacerbate the drought sensitivity of SL-depleted shoots. Instead, the slower and less pronounced ABA increase in roots and shoots of WT/SL− plants compared with the other lines is in agreement with the physiological conditions of these plants (which being primed for better stress resilience, perform better and thus need less ABA). It is of course possible that ABA levels in guard cells may not be reflected by the total levels of free ABA in the whole leaf tissue, given the strong compartmentalization of the hormone in different cell types and compartments (Hartung & Slovik, 1991), and, thus, that WT/SL− plants had lower g_s because of locally enhanced ABA accumulation. However, the results of the ABA-feeding experiment rather supported the hypothesis that such a phenotype was (at least partly) due to stomatal hypersensitivity to the hormone. Finally, the same experiments also highlighted that SL in the shoot are not only necessary, but also sufficient to increase stomatal sensitivity to ABA.

Hormonal cross-talk and systemic signalling under drought: fitting SL in the picture

Because our experimental set-up mimics what normally happens during drought, we propose that these findings are relevant to stress resistance, at least in plants such as *Lotus* and tomato, for which a drop in SL synthesis is recorded in roots experiencing osmotic stress or drought. Such a drop might promote a pre-alerted (primed) status in the shoots, which become more sensitive to ABA at the guard cell level. This message may be conveyed directly (see later) or indirectly, that is, through a second messenger that ought to be, at least in tomato, different than ABA. It is to be noted here that SL were proven to cross-talk with other hormones, such as auxins, cytokinins, brassinosteroids and ethylene, in processes different than drought responses and stomatal closure (Cheng *et al.*, 2013); and that each of these hormones was shown to affect stomatal aperture locally (Daszkowska-Golec & Szarejko, 2013). Root-synthesized cytokinins were even proposed to act as a systemic signal promoting stomatal opening, in a similar way to SL (Davies & Zhang, 1991); however, SL− mutants display reduced cytokinin levels in the shoot, which is the opposite of what one would expect from a mediator of SL effect (because cytokinins promote stomata aperture and SL− shoots transpire more than WT) (Foo *et al.*, 2007). Additionally, shoots were proven to possess powerful homeostatic mechanisms for the regulation of cytokinin levels, that are largely unlinked from their concentration in xylem sap (Foo *et al.*, 2007). Resuming, we cannot exclude that the effect of SL on stomatal closure may be at least partly indirect – that is, mediated by any of these hormones or by yet other signals (and, indeed, sensitivity to ABA does play a role). It would be interesting to quantify other hormones in leaves of our lines, or even better to visualize their activity in guard cells; and to measure whether, for example, the xylem sap pH in hetero-grafted plants is different than in self-grafted

(possibly, more basic as in droughted tomato plants; Wilkinson *et al.*, 1998). It remains clear that plant hormones, if capable of travelling over long distances, have a slow propagation velocity in comparison with hydraulic and/or electrical signals. However, the fact alone that in our model, stomatal closure is rather induced by the lack of an inhibitor in the shootward flow is attractive, because its decrease might be perceived faster than flow speed would predict for a positive modulator. In fact, the flow is slowed down by drought, thus adding to the decrease of the inhibitor itself; additionally, given that SL are degraded upon perception (Hamiaux *et al.*, 2012), they should be quickly depleted locally unless *de novo* synthesis or translocation occurs. Finally, expression pattern and intracellular location of the SL transporter(s) might add another regulation level, for mobility through living tissues.

As regards the activity of SL biosynthetic genes, shoots of irrigated, hetero-grafted WT/SL− plants behave as if under drought – that is, they show increased transcripts of *CCD7* and *CCD8*. These increases in gene activity might be due to the relief of direct repression of SL synthesis in the shoots by translocated, root-synthesized SL; a known pattern (e.g. Johnson *et al.*, 2006; Liu *et al.*, 2013) which might itself trigger SL accumulation at specific spots in the shoot (undetectable in whole-tissue analyses). Even if it is at present impossible to overcome the technical limitations that make the quantification of SL unfeasible in shoots, we propose that hypersensitivity to ABA in stomata of WT/SL− plants might be causally linked to higher production of SL in (limited tissue zones of) the shoot, because transcription of SL− biosynthetic genes is activated in WT shoots during stress, but also under nonstressful conditions if WT shoots are grafted onto SL− rootstocks. Sensitivity to ABA converts from higher to lower than normal, if not only roots but also shoots are SL-depleted, proving that SL synthesis in the shoots is needed for the effects on ABA sensitivity; exogenous GR24 treatment is sufficient to induce stomatal hypersensitivity to ABA. This latter effect is opposite to the one caused by SL depletion, and would explain the ability of GR24 to confer drought resistance in WT Arabidopsis (Ha *et al.*, 2014). The importance of SL produced in the shoot has been proposed also in branching, because micrografting of WT Arabidopsis scions on SL-defective rootstocks does not lead to an increased branching phenotype, as expected if SL synthesis is compromised in the whole plant (Foo *et al.*, 2001; Sorefan *et al.*, 2003). Whether osmotic/drought stress in the absence of such a decrease in root-synthesized SL is able to stimulate a similar shoot response, is still to be determined. A schematic drawing of our model is represented in Fig. 5. This model obviously implies that the shoot is able to discriminate between root- and shoot-produced SL; this ability needs to be proven experimentally, but could rely on differential loading in the upstream flow, and/or organ-specific production of the structurally different SL molecules, which make up species-specific SL blends and whose ecological and physiological meanings remain largely unexplored (Kohlen *et al.*, 2011, 2012; Bharti *et al.*, 2015; Brewer *et al.*, 2016). Alternatively, or in parallel, the uneven/nonoverlapping distribution of the receptor protein D14 and/or of SL transporter(s) in the plant might account for discrimination between locally and distally produced SL (Chevalier *et al.*, 2014; Sasse *et al.*, 2015).

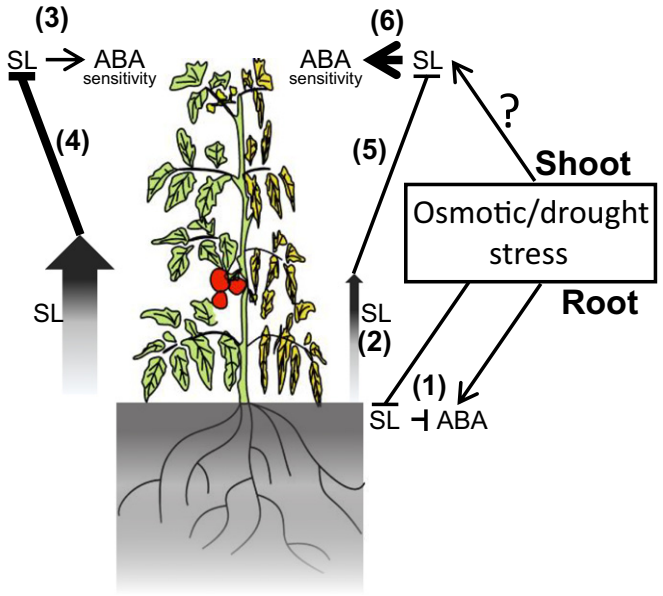

Fig. 5 Schematic drawing of the main connections between strigolactones (SL) and abscisic acid (ABA) in roots and shoots of tomato under drought stress. In the model, the effects of SL on ABA levels may be negative in the roots, as proven by *rac*GR24 treatment in *Lotus japonicus* (Liu *et al.*, 2015). Thereby, the drop in SL synthesis in this organ under osmotic (PEG-infused) stress may be needed but not necessarily sufficient to let ABA levels rise (results untested in other plant species so far; 1). SL synthesis is inhibited in roots under osmotic/drought stress, so shootward SL flow decreases (2); in tomato, root-produced ABA is neither translocated nor needed for appropriate shoot responses to stress (Holbrook *et al.*, 2002). The effects of shoot-produced or exogenous SL on ABA sensitivity of stomata are in turn positive (3) (Ha *et al.*, 2014; Liu *et al.*, 2015; this work). SL flowing shootward inhibit the transcription of SL biosynthetic genes (thicker line, 4), because reduced quantities in the upstream flow (or, possibly, a second messenger – different than ABA – produced in the roots in response to low SL) are sufficient to let transcripts of SL biosynthetic genes increase (thinner line; (5)) and as a likely consequence, also sensitivity to ABA (6). It is not known whether osmotic/drought stress can increase SL gene transcription and ABA sensitivity in the shoots, even when SL synthesis in the root is not decreased (question mark). Although SL remain undetectable in whole-shoot analyses of stressed tomato, localized accumulation may occur, as proposed by Liu *et al.* (2015) and suggested by transcript quantification of biosynthetic genes (Ha *et al.*, 2014; this work). Alternatively, steady-state SL levels may be necessary and sufficient to ensure wild-type sensitivity to ABA in stressed shoot tissues; or other, yet unidentified, SL(-like) molecules may be induced.

From a practical point of view, it remains to be assessed how such graft combinations will perform under other or combined stress. It is important to note in this regard that they will undoubtedly be advantageous in soil infested by parasitic weeds, that not all SL-depleted genotypes are also significantly compromised in mycorrhization (a possible detrimental side effect), and that with respect to SL synthesis, drought overrules P deficiency under combined stress (Kohlen *et al.*, 2012; Liu *et al.*, 2015). Nonetheless, our results highlight once more the importance of rootstocks in influencing shoot traits, and how they could be exploited to improve crop performances under stress (Albacete *et al.*, 2015; Cantero-Navarro *et al.*, 2016).

Acknowledgements

Research was supported by the SLEPS Project (Compagnia di S. Paolo and University of Turin, call 2012) to F.C., A.S., C.L., I.V. and M.F., and by the Czech National Program for Sustainability (LO1204) to O.N. and M.S. This article is based upon work from COST Action FA1206 STREAM, supported by COST (European Cooperation in Science and Technology).

Author contributions

F.C. conceived of the work and designed research supported by C.L. and A.S.; I.V. performed research helped by M.V. and M.F.; Y.Z. and O.N. analysed data; C.R-S. and M.S. provided logistic support to metabolite analyses; I.V. and F.C. wrote the paper. All authors read and helped to polish the final manuscript.

References

Al-Babili S, Bouwmeester HJ. 2015. Strigolactones, a novel carotenoid-derived plant hormone. *Annual Review of Plant Biology* **66**: 161–186.

Albacete A, Martinez-Andujar C, Martinez-Perez A, Thompson AJ, Dodd IC, Perez-Alfocea F. 2015. Unravelling rootstock × scion interactions to improve food security. *Journal of Experimental Botany* **66**: 2211–2226.

Bharti N, Tripathi S, Bhatla SC. 2015. Photomodulation of strigolactone biosynthesis and accumulation during sunflower seedling growth. *Plant Signaling and Behavior* **10**: e1049792.

Brewer PB, Yoneyama K, Filardo F, Meyers E, Scaffidi A, Frickey F, Akiyama K, Seto Y, Dun EA, Cremer JE *et al.* 2016. LATERAL BRANCHING OXIDOREDUCTASE acts in the final stages of strigolactone biosynthesis in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **113**: 6301–6306.

Cantero-Navarro E, Romero-Aranda R, Fernández-Muñoz R, Martínez-Andújar C, Pérez-Alfocea F, Albacete A. 2016. Improving agronomic water use efficiency in tomato by rootstock-mediated hormonal regulation of leaf biomass. *Plant Science* **251**: 90–100.

Cheng X, Ruyter-Spira C, Bouwmeester H. 2013. The interaction between strigolactones and other plant hormones in the regulation of plant development. *Frontiers in Plant Science* **4**: 199.

Chevalier F, Nieminen K, Sanchez-Ferrero JC, Rodriguez ML, Chagoyen M, Hardtke CS, Cubas P. 2014. Strigolactone promotes degradation of DWARF14, an alpha/beta hydrolase essential for strigolactone signaling in Arabidopsis. *Plant Cell* **26**: 1134–1150.

Christmann A, Moes D, Himmelbach A, Yang Y, Tang Y, Grill E. 2006. Integration of abscisic acid signalling into plant responses. *Plant Biology* **8**: 314–325.

Christmann A, Weiler EW, Steudle E, Grill E. 2007. A hydraulic signal in root-to-shoot signalling of water shortage. *Plant Journal* **52**: 167–174.

Comstock JP. 2002. Hydraulic and chemical signalling in the control of stomatal conductance and transpiration. *Journal of Experimental Botany* **53**: 195–200.

Daszkowska-Golec A, Szarejko I. 2013. Open or close the gate – stomata action under the control of phytohormones in drought stress conditions. *Frontiers in Plant Science* **4**: 138.

Davies WJ, Zhang J. 1991. Root signals and the regulation of growth and development of plants in drying soil. *Annual Review of Plant Physiology and Plant Molecular Biology* **42**: 55–76.

Flokova K, Tarkowska D, Miersch O, Strnad M, Wasternack C, Novak O. 2014. UHPLC-MS/MS based target profiling of stress-induced phytohormones. *Phytochemistry* **105**: 147–157.

Foo E, Bullier E, Goussot M, Foucher F, Rameau C, Beveridge CA. 2005. The branching gene *RAMOSUS1* mediates interactions among two novel signals and auxin in pea. *Plant Cell* **17**: 464–474.

Foo E, Morris SE, Parmenter K, Young N, Wang H, Jones A, Rameau C, Turnbull CG, Beveridge CA. 2007. Feedback regulation of xylem cytokinin content is conserved in pea and Arabidopsis. *Plant Physiology* **143**: 1418–1428.

Foo E, Turnbull CG, Beveridge CA. 2001. Long-distance signaling and the control of branching in the *rms1* mutant of pea. *Plant Physiology* **126**: 203–209.

Gambino G, Perrone I, Gribaudo I. 2008. A rapid and effective method for RNA extraction from different tissues of grapevine and other woody plants. *Phytochemical Analysis* **19**: 520–525.

Ha CV, Leyva-Gonzalez MA, Osakabe Y, Tran UT, Nishiyama R, Watanabe Y, Tanaka M, Seki M, Yamaguchi S, Dong NV *et al.* 2014. Positive regulatory role of strigolactone in plant responses to drought and salt stress. *Proceedings of the National Academy of Sciences, USA* **111**: 851–856.

Hamiaux C, Drummond RS, Janssen BJ, Ledger SE, Cooney JM, Newcomb RD, Snowden KC. 2012. DAD2 is an alpha/beta hydrolase likely to be involved in the perception of the plant branching hormone, strigolactone. *Current Biology* **22**: 2032–2036.

Hartung W, Slovik S. 1991. Physicochemical properties of plant growth regulators and plant tissues determine their distribution and redistribution: stomatal regulation by abscisic acid in leaves. *New Phytologist* **119**: 361–382.

Holbrook NM, Shashidhar VR, James RA, Munns R. 2002. Stomatal control in tomato with ABA-deficient roots: response of grafted plants to soil drying. *Journal of Experimental Botany* **53**: 1503–1514.

Huber AE, Bauerle TL. 2016. Long-distance plant signaling pathways in response to multiple stressors: the gap in knowledge. *Journal of Experimental Botany* **67**: 2063–2079.

Johnson X, Brcich T, Dun EA, Goussot M, Haurogne K, Beveridge CA, Rameau C. 2006. Branching genes are conserved across species. Genes controlling a novel signal in pea are coregulated by other long-distance signals. *Plant Physiology* **142**: 1014–1026.

Kohlen W, Charnikhova T, Lammers M, Pollina T, Toth P, Haider I, Pozo MJ, de Maagd RA, Ruyter-Spira C, Bouwmeester HJ *et al.* 2012. The tomato CAROTENOID CLEAVAGE DIOXYGENASE8 (SlCCD8) regulates rhizosphere signaling, plant architecture and affects reproductive development through strigolactone biosynthesis. *New Phytologist* **196**: 535–547.

Kohlen W, Charnikhova T, Liu Q, Bours R, Domagalska MA, Beguerie S, Verstappen F, Leyser O, Bouwmeester H, Ruyter-Spira C. 2011. Strigolactones are transported through the xylem and play a key role in shoot architectural response to phosphate deficiency in nonarbuscular mycorrhizal host Arabidopsis. *Plant Physiology* **155**: 974–987.

Kretzschmar T, Kohlen W, Sasse J, Borghi L, Schlegel M, Bachelier JB, Reinhardt D, Bours R, Bouwmeester HJ, Martinoia E. 2012. A petunia ABC protein controls strigolactone-dependent symbiotic signalling and branching. *Nature* **483**: 341–344.

Liu J, He H, Vitali M, Visentin I, Charnikhova T, Haider I, Schubert A, Ruyter-Spira C, Bouwmeester HJ, Lovisolo C *et al.* 2015. Osmotic stress represses strigolactone biosynthesis in *Lotus japonicus* roots: exploring the interaction between strigolactones and ABA under abiotic stress. *Planta* **241**: 1435–1451.

Liu J, Novero M, Charnikhova T, Ferrandino A, Schubert A, Ruyter-Spira C, Bonfante P, Lovisolo C, Bouwmeester HJ, Cardinale F. 2013. CAROTENOID CLEAVAGE DIOXYGENASE 7 modulates plant growth, reproduction, senescence, and determinate nodulation in the model legume *Lotus japonicus*. *Journal of Experimental Botany* **64**: 1967–1981.

Lopez-Raez JA, Kohlen W, Charnikhova T, Mulder P, Undas AK, Sergeant MJ, Verstappen F, Bugg TD, Thompson AJ, Ruyter-Spira C *et al.* 2010. Does abscisic acid affect strigolactone biosynthesis? *New Phytologist* **187**: 343–354.

Manzi M, Lado J, Rodrigo MJ, Zacarias L, Arbona V, Gomez-Cadenas A. 2015. Root ABA accumulation in long-term water-stressed plants is sustained by hormone transport from aerial organs. *Plant & Cell Physiology* **56**: 2457–2466.

Matusova R, Rani K, Verstappen FW, Franssen MC, 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.

Munoz-Espinoza VA, Lopez-Climent MF, Casaretto JA, Gomez-Cadenas A. 2015. Water stress responses of tomato mutants impaired in hormone biosynthesis reveal abscisic acid, jasmonic acid and salicylic acid interactions. *Frontiers in Plant Science* **6**: 997.

Ruiz-Lozano JM, Aroca R, Zamarreno AM, Molina S, Andreo-Jimenez B, Porcel R, Garcia-Mina JM, Ruyter-Spira C, Lopez-Raez JA. 2016. Arbuscular mycorrhizal symbiosis induces strigolactone biosynthesis under drought and improves drought tolerance in lettuce and tomato. *Plant, Cell & Environment* **39**: 441–452.

Ruyter-Spira C, Al-Babili S, van der Krol S, Bouwmeester H. 2013. The biology of strigolactones. *Trends in Plant Science* **18**: 72–83.

Sasse J, Simon S, Gubeli C, Liu GW, Cheng X, Friml J, Bouwmeester H, Martinoia E, Borghi L. 2015. Asymmetric localizations of the ABC transporter PaPDR1 trace paths of directional strigolactone transport. *Current Biology* **25**: 647–655.

Scholander PF, Bradstreet ED, Hemmingsen EA, Hammel HT. 1965. Sap pressure in vascular plants: negative hydrostatic pressure can be measured in plants. *Science* **148**: 339–346.

Sorefan K, Booker J, Haurogne K, Goussot M, Bainbridge K, Foo E, Chatfield S, Ward S, Beveridge C, Rameau C *et al.* 2003. *MAX4* and *RMS1* are orthologous dioxygenase-like genes that regulate shoot branching in Arabidopsis and pea. *Genes & Development* **17**: 1469–1474.

Torres-Vera R, Garcia JM, Pozo MJ, Lopez-Raez JA. 2014. Do strigolactones contribute to plant defence? *Molecular Plant Pathology* **15**: 211–216.

Tramontini S, Doering J, Vitali M, Ferrandino A, Stoll M, Lovisolo C. 2014. Soil water-holding capacity mediates hydraulic and hormonal signals of near-isohydric and near-anisohydric *Vitis* cultivars in potted grapevines. *Functional Plant Biology* **41**: 1119–1128.

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

Wilkinson S, Corlett JE, Oger L, Davies WJ. 1998. Effects of xylem pH on transpiration from wild-type and *flacca* tomato leaves. A vital role for abscisic acid in preventing excessive water loss even from well-watered plants. *Plant Physiology* **117**: 703–709.

Xiong L, Zhu JK. 2003. Regulation of abscisic acid biosynthesis. *Plant Physiology* **133**: 29–36.

Supporting Information

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

Fig. S1 Relative soil water content and water potential of soil during the course of a drought experiment.

Fig. S2 Effect of drought on SL amounts in tomato roots.

Fig. S3 Physiological performances of the grafted lines in the absence and presence of stress as a function of time.

Fig. S4 Transcript amounts of key SL biosynthetic genes as a function of leaf water potential.

Fig. S5 Effect of drought on free ABA as a function of time, and on transcript amounts of the ABA biosynthetic gene *SlNCED1* as a function on leaf water potential.

Table S1 List of primers

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.