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

3 Ivan Visentin^a, Marco Vitali^a, Manuela Ferrero^a, Yanxia Zhang^{b, c}, Carolien Ruyter-Spira^b, Ondřej
4 Novák^d, Miroslav Strnad^d, Claudio Lovisolo^a, Andrea Schubert^a, Francesca Cardinale^{a1}

5
6 **AUTHOR AFFILIATIONS & INFO**

7 ^a Laboratory of Plant Physiology, DISAFA - Turin University, 10095 Grugliasco (TO) Italy

8 ^b Laboratory of Plant Physiology, Wageningen University, 6708 PB Wageningen, The Netherlands

9 ^c Laboratory of Growth Regulators, Institute of Experimental Botany ASCR & Palacky University
10 Olomouc, Czech Republic

11

12 ¹To whom correspondence should be addressed. E-mail: francesca.cardinale@unito.it; phone
13 +390116708875

14

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19 **SHORT TITLE:** Strigolactones in systemic drought signalling

20

21 **SUMMARY**

- 22 • Strigolactones (SL) contribute to drought acclimatization in shoots, since SL-depleted plants
23 are hypersensitive to drought due to stomatal hyposensitivity to abscisic acid (ABA).
24 However, under drought, SL biosynthesis is repressed in roots, suggesting organ specificity
25 in their metabolism and role. Since SL can be transported acropetally, such drop may also
26 affect shoots, as a systemic indication of stress.
- 27 • We investigated this hypothesis by analysing molecularly and physiologically WT tomato
28 scions grafted onto SL-depleted rootstocks, compared to self-grafted WT and SL-depleted
29 genotypes, during a drought time-course.
- 30 • Shoots receiving few SL from the roots behaved as under mild stress even if irrigated. Their
31 stomata were hypersensitive to ABA (likely via a localized enhancement of SL synthesis in
32 shoots). Exogenous SL also enhanced stomata sensitivity to ABA.
- 33 • As the partial shift of SL synthesis from roots to shoots mimics what happens under
34 drought, a reduction of root-produced SL might represent a systemic signal unlinked from
35 shootward ABA translocation and sufficient to prime the plant for better stress avoidance.

36

37 **KEYWORDS:** Abscisic acid, Drought, Strigolactones, Systemic signalling, Tomato

38

39

40 **INTRODUCTION**

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

57 Recently, the hormones strigolactones (SL) have been also proposed as signal mediators under
58 environmental stress. SL have pervasive roles in development, from germination and reproduction
59 to root and shoot architecture; at various levels, they also promote the interaction with beneficial
60 root symbionts as well as with detrimental (micro)organisms [reviewed by (Ruyter-Spira *et al.*,
61 2013)]. SL and ABA share their biosynthetic precursor, both being carotenoid-derived terpenoid
62 lactones (Matusova *et al.*, 2005). Several enzymes act sequentially in SL biosynthesis: DWARF 27
63 (D27) is a β -carotene isomerase, CCD7 and CCD8 are Carotenoid-Cleavage Dioxygenases (CCD) and
64 MORE AXILLARY GROWTH 1 (MAX1) is a class III cytochrome P450 that, with its orthologues and
65 paralogues and the recently characterized LATERAL BRANCHING OXIDOREDUCTASE (LBO) (Brewer
66 *et al.*, 2016), is thought to contribute to the oxidation of the SL precursor carlactone and to the
67 chemical diversification of SL family members [reviewed by (Al-Babili & Bouwmeester, 2015)]. The
68 core enzyme set is mostly active in roots; root-produced SL are then exported out of the producing
69 cell by ABC_G transporter protein(s) such as PhPDR1 (Kretzschmar *et al.*, 2012; Sasse *et al.*, 2015),
70 both to be exuded in soil and to travel shootward, as shown in *Arabidopsis* and tomato (Kohlen *et al.*,
71 *et al.*, 2011). Although transcripts of SL-related genes, and final metabolites, are mostly not or barely

72 detectable in shoots, biosynthesis in above-ground tissues is known to occur, possibly at specific
73 spots. In fact, wild-type (WT) shoots grafted onto SL-depleted rootstocks do not display the typical
74 morphological phenotype of SL-depleted plants (Foo *et al.*, 2001; Sorefan *et al.*, 2003).

75 Recently, SL metabolism and physiological effects in plants under osmotic stress conditions have
76 been analysed. SL-depleted *A. thaliana* and *Lotus japonicus* (Liu *et al.*, 2013) are hypersensitive to
77 drought at the shoot level, a feature linked to the hyposensitivity of their stomata to endogenous
78 and exogenous ABA. This finding supports a positive role for SL in the acclimatization to drought in
79 above-ground organs (Ha *et al.*, 2014; Liu *et al.*, 2015). Consistent with this idea, the transcript of
80 SL biosynthetic genes is increased by drought in Arabidopsis leaves (Ha *et al.*, 2014). However,
81 transcription of biosynthetic and SL transporter-encoding genes is repressed along with the
82 accumulation of SL in non-mycorrhizal *L. japonicus* and tomato roots under drought (Liu *et al.*,
83 2015; Ruiz-Lozano *et al.*, 2016). This is surprising *per se*, since roots are the main SL production site
84 under normal conditions; and suggests different dynamics for shoot- and root-derived SL. A
85 negative correlation between ABA and SL levels was observed in non-mycorrhizal, water-stressed
86 roots of *L. japonicus* and tomato (Liu *et al.*, 2015; Ruiz-Lozano *et al.*, 2016). Since drought stress-
87 triggered ABA accumulation is hampered by exogenous SL in *L. japonicus* roots, the drop in SL
88 biosynthesis in roots under drought might have the role to allow an increase of local ABA and
89 possibly, also of its levels in the xylem sap, leading to systemic responses to a dropping root water
90 potential in plants that rely also on ABA for chemical signalling of drought (Liu *et al.*, 2015).
91 However, the possibility exists that such drop has also a direct physiological effect on shoots,
92 namely as a systemic indication of stress at the root level, since root-produced SL can also be
93 transported to the whole plant (Kohlen *et al.*, 2011). This, and the fact that SL are needed locally in
94 stressed shoots for efficient control of water loss by transpiration (Ha *et al.*, 2014; Liu *et al.*, 2015),
95 led us to hypothesize that WT scions grafted onto SL-depleted rootstocks may behave as if
96 stressed even in the absence of stress, at least under some respects, and perform differently
97 under stress than if grafted onto WT rootstocks.

98 In this work, we investigated the possible systemic significance of the SL decrease in roots under
99 drought, by analysing molecularly and physiologically WT scions grafted over SL-depleted (*CCD7*-
100 silenced) tomato rootstocks, compared to self-grafted WT and SL-depleted genotypes, both under
101 normal and stress conditions. The results proved that indeed stomata of shoots receiving less SL
102 from the roots are hypersensitive to ABA also in the absence of stress, possibly through an
103 enhancement of local SL synthesis. This is likely to mimic what normally happens under drought,

104 and suggests that root-derived SL - or better, a reduction thereof - might be a component of the
105 systemic signal of stress in tomato.

106

107 **MATERIALS AND METHODS**

108 ***Plant material and growth conditions***

109 The tomato (*Solanum lycopersicum* L.) *SICCD7*-silenced line 6936, hereafter called SL-, and its WT
110 genotype M82 were a kind gift by Dr. H. J. Klee (University of Florida). Seeds were sterilized in 4%
111 (v:v) sodium hypochlorite containing 0.02% (v:v) Tween 20, rinsed thoroughly with sterile water,
112 and then germinated for 48 h on moisten filter paper at 25°C in darkness. Subsequently, seedlings
113 were grown in inert substrate (sand:vermiculite; 1:1, v:v) and the pots watered with Hoagland
114 solution twice per week. The three grafted lines were produced by the clamp grafting technique
115 on plants at the 2/4-leaf stage and with stem diameter of about 1.5-2 mm. Water stress was
116 applied to plants four weeks after grafting by withholding water starting at day zero (T0); shoots
117 and roots were collected 0, 1, 3 and 5 days after the beginning of the stress (T0 through T5,
118 respectively; 3 plants per line and sampling point) and stored to -80°C. At T5, 3 plants per line
119 were watered and collected after 2 additional days to give the rehydrated (recovery) samples. The
120 experiment was repeated twice. Supporting Information Fig. S1 shows how relative water content
121 and soil water potential were dropping during the course of one drought experiment. Relative soil
122 water content was gravimetrically determined by collecting daily ~10 ml of soil from three
123 different points and depths in each pot (at 5, 10 and 15-cm depth with 120° of angular separation
124 between each of the respective sample points). The soil was weighed, oven-dried at 100°C for 24 h
125 and then re-weighed to assess water content. At the same time, the soil water retention curve was
126 assessed with pressure plate measurements of the potting substrate according to (Tramontini *et*
127 *al.*, 2014).

128

129 ***Gene transcript quantification***

130 Total RNA from tomato roots and shoots was extracted as described (Gambino *et al.*, 2008) and
131 treated with DNase I (ThermoScientific) at 37°C for 30 min to remove residual genomic DNA. First-
132 strand cDNA was synthesized from 3 µg of purified total RNA using the High-Capacity cDNA
133 Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. For
134 transcript quantification of *SICCD7*, *SICCD8* and *SINCE1* by quantitative reverse-transcription PCR
135 (qRT-PCR), the StepOne system (Applied Biosystems) was used, with transcript of the Elongation

136 factor 1 α (*SIEF1 α* gene) as a reference; primers used are reported as Supporting information in
137 Supplementary Table S1. Three independent biological replicates were analysed and each qRT-PCR
138 reaction was run in technical triplicates. Transcripts of the target genes were quantified by the 2⁻
139 $\Delta\Delta C_t$ method.

140

141 ***Physiological measurements***

142 Leaf water potential, stomatal conductance and net carbon assimilation were measured daily
143 between 10:00 and 12:00 am on at least three plants per grafted line and independent
144 experiment, as reported by Liu *et al.* (2015). Briefly, stomatal conductance and net carbon
145 assimilation rate were measured with a portable gas exchange system (GFS-3000, Walz GmbH,
146 Effeltrich, Germany) by clamping the most apical leaves of a shoot in the leaf chamber, where
147 photosynthetically active radiation (1200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), air flow (750 $\mu\text{mol s}^{-1}$) and
148 temperature (25°C) were kept constant. Environmental conditions of CO₂ (450 ppm) and vapour
149 pressure deficit (2.3 kPa) were stable during the 10-day experiments. Leaf water potential was
150 measured with a pressure chamber (Scholander *et al.*, 1965) on one leaf per plant, immediately
151 after gas exchange quantification. For the quantification of responses to ABA, stomatal
152 conductance was measured as above at 30-s intervals before and during ABA treatment. This was
153 accomplished by cutting leafy twigs while submerged in filtered water (one leaf each, from three
154 plants per grafted line, treatment and experiment), by letting stomatal conductance stabilize with
155 the twig dipped in water and then by adding ABA to 5, 20 or 50 μM final concentration, while
156 continuously recording every 30 s both stomatal conductance and transpiration rates as detailed
157 above. For treatment with exogenous SL, WT plants were sprayed with a 5 μM solution of *racGR24*
158 (StrigoLab SrL, Turin, IT) 24 h before treatment with ABA 5 μM and stomatal conductance
159 recording as above.

160

161 ***Extraction and quantification of SL and ABA***

162 Solanacol, orobanchol and didehydro-orobanchol were quantified in the roots of the three grafted
163 lines, while ABA was quantified in both roots and shoots. For SL extractions, 3 plants per line and
164 time-point were pooled, while two independent biological assays were run. For SL quantification,
165 samples (0.5 g each) were manually ground in liquid nitrogen and extracted with 2 ml of cold ethyl
166 acetate containing D6-*epi*-5 deoxystrigol as internal standard (0.05 nmol ml⁻¹) in 10-ml glass vials.
167 Standards for didehydro-orobanchol isomers were not available, so quantities for this SL were

168 expressed as percentage ratio with respect to WT root tissues in the absence of stress (T0); the
169 isomer reported in Fig. S2C is the one with retention time of 4' 6'' in our conditions. The extraction
170 and quantification procedures for SL were performed as previously reported (Lopez-Raez *et al.*,
171 2010). For ABA extraction, 2 biological replicates of 2 pooled plants each were sampled per line
172 and time-point, while two independent biological assays were run. For ABA quantification, labelled
173 internal standard was added ($[^2\text{H}]_6\text{-ABA}$, 20 pmol) to each sample (20–25 mg homogenized in 1 ml
174 of cold 10% MeOH in H₂O, v/v) and subsequently extracted and analysed as detailed (Flokova *et*
175 *al.*, 2014).

176

177 **RESULTS**

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

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

191 Measuring stomatal conductance and leaf water potential confirmed that in tomato, as in
192 Arabidopsis and Lotus, whole-plant SL depletion increases stomatal conductance and decreases
193 leaf water potential in the absence of stress; under the same conditions, WT/SL- plants showed
194 instead significantly lower stomatal conductance than WT/WT (Fig. 1a and T0 in Supporting
195 Information Fig. S3a). Accordingly, leaf water potential values were significantly less negative in
196 WT leaves grafted onto SL- than WT roots (Supporting Information Fig. S3b). Photosynthesis of WT
197 scions grafted over SL- rootstocks was only slightly and non-significantly affected by the reduced
198 gas exchange of hetero-grafts compared to self-grafted WT plants, while both displayed
199 significantly lower values than SL- shoots (Fig. 1b and Supporting Information Fig. S3c).

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

210

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

213 To assess whether the change in metabolite abundance is regulated at the gene transcription
214 level, two SL biosynthetic genes (*SICCD7* and *SICCD8*) were profiled by qRT-PCR in roots and shoots
215 of the three grafted lines under irrigated and drought stress conditions, in the same plant material
216 used for SL quantification.

217 The analysis confirmed that in roots, transcript amount of both genes inversely correlated with
218 stress severity for all grafted lines (Fig. 2a, b and Supporting Information Fig. S4a, b). In the shoots
219 of the same sets of plants however, transcripts of both biosynthetic genes followed an opposite
220 trend compared to roots and accumulated under drought, as reported previously in *Arabidopsis*
221 and postulated in *Lotus* (Ha *et al.*, 2014; Liu *et al.*, 2015) (Fig. 2c, d and Supporting Information Fig.
222 S4d, e). It must be noted however that in terms of relative transcript abundance, values in shoots
223 remained much lower (about one hundredth; not obvious in the normalized data of Fig. 2) of root
224 values at T0, even in samples collected under very severe stress at T5. This justifies the fact that
225 we were unable to detect the final metabolites in these shoot samples (data not shown).
226 Relevantly here, expression of both biosynthetic genes in WT shoots was significantly higher when
227 the mutant was used as rootstock (WT/WT vs WT/SL-, Fig. 2c, d and Supporting Information Fig.
228 S4c, d). This is a known pattern (Johnson *et al.*, 2006) consistent with the idea of a general
229 negative feedback by the final metabolites on the SL biosynthetic pathway and supported by the
230 repressive effect of exogenous SL on the same genes [see for example (Liu *et al.*, 2015)]. Overall,
231 data on transcript of SL-biosynthetic genes indicated that the response of shoots to SL deficiency

232 in roots overlaps with the response to osmotic stress. In fact, both drought stress and depletion of
233 SL in the roots in the absence of stress induced transcript accumulation of SL biosynthetic genes in
234 tomato shoots.

235 As an additional observation, *SICCD7* transcripts in unstressed SL- (*CCD7*-silenced) rootstocks were
236 more abundant in grafts bearing a WT instead of a SL- shoot (WT/SL- vs SL-/SL-; T0 of Fig. 2a). This
237 correlated with a very slight increase of SL metabolites, especially orobanchol (see T0, Fig. S2a-c)
238 and suggested that a SL-dependent, shoot-to-root signal feeding back on the
239 transcription/transcript stability of this gene exists in tomato as in Arabidopsis and pea (Foo *et al.*,
240 2005; Johnson *et al.*, 2006), where it was shown to depend on the *RMS2* locus. Also, *SICCD8*
241 transcripts were more abundant in SL- than WT roots (as expected, given the already mentioned
242 negative feedback of SL on the transcription of their biosynthetic genes; Fig. 2b); and in SL- roots,
243 *SICCD8* transcripts were more concentrated in the presence of a SL- than of a WT scion (Fig. 2b). In
244 this sense, expression of *SICCD7* and *SICCD8* in the root seemed influenced oppositely by the
245 ability of the shoot to produce SL. We may hypothesize that not only locally-produced, but also
246 shoot-synthesized SL may participate (directly or indirectly) in the negative feedback on *SICCD8*
247 expression in the root, and thus that in SL- roots, the presence of a WT scion may lead to less
248 pronounced overexpression of *SICCD8* than in the presence of a SL- scion. Finally, it is noteworthy
249 that the concentration of *SICCD8* transcript in WT shoots grafted onto SL- roots was as high as in
250 SL- shoots in the absence of stress (T0, Fig. 2d) but remained stable along the time-course in the
251 former while it was further induced in the latter (Supporting Information Fig. S4d). We have no
252 easy explanation for this pattern, which might however be due to the fact that leaves of WT/SL-
253 plants dehydrate less and produce less ABA (see further on) along the time-course, than either
254 self-grafted control line.

255

256 ***The low-transpiration phenotype of hetero-grafted, WT/SL- plants is not due to increased total*** 257 ***free ABA***

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

264 Results showed that under normal conditions, WT roots contain less free ABA than SL- ones
265 (WT/WT vs SL-/SL- and WT/SL- plants, T0 in Supporting Information Fig. S5a) per gram of fresh
266 tissue weight. As stress increased, ABA started accumulating in roots of SL-/SL- and WT/WT plants
267 more quickly than in roots of WT/SL- plants, where ABA was significantly less concentrated than in
268 the roots of the other grafts (Supporting Information Fig. S5a). Correlation curves to leaf water
269 potential values were however substantially superimposable (Fig. 3a). Transcript quantification for
270 *SINCED1*, a key biosynthetic gene for stress-induced ABA in tomato (Munoz-Espinoza *et al.*, 2015),
271 showed good correlation with free ABA content but for a few points and grafting combinations
272 (Fig. 3b and Supporting Information Fig. S5b). These discrepancies between *SINCED1* transcript
273 amounts and ABA concentration may be due to post-transcriptional regulation of biosynthetic
274 enzymes, and/or to the activity of catabolic genes, for example, or to the release/sequestration of
275 free ABA from/in conjugated forms [reviewed by (Xiong & Zhu, 2003)].

276 While in the absence of stress SL- shoots contained more ABA per gram of fresh weight than WT
277 ones, as stress proceeded and leaf water potential started becoming more negative ABA levels
278 increased faster in WT than in SL- scions; at the moment of maximum stress, ABA concentration
279 was minimum in WT scions grafted onto SL- rootstocks, and intermediate in SL- shoots (Fig. 3c and
280 T5 in Supporting Information S5c). The same trend is seen for transcripts of *SINCED1*, which again
281 showed a good correlation with free ABA content but for a few points and grafting combinations
282 (Fig. 3d and Supporting Information Fig. S5d). These results confirmed that especially under stress,
283 SL depletion in the shoot partially compromises the ability to synthesize ABA. Furthermore,
284 coupled to the physiological data in Fig. 1, they strongly suggested that the low-gas exchange
285 phenotype of hetero-grafted WT/SL- plants was not due to increased free ABA content, given the
286 comparatively low ABA concentration in their tissues.

287

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

289 To explore whether altered sensitivity to ABA might rather underlie the physiological and
290 metabolic results described above, shoot sensitivity to exogenous ABA in dependence of the rate
291 of SL production in the roots was investigated. ABA at different concentrations was applied to and
292 absorbed by excised petioles of composite leaves of the three grafted lines, while measuring the
293 time required for the stomata to start closing. This assay on the one hand confirmed in tomato
294 what was already known in *Arabidopsis* and *Lotus*, i.e. that SL-depleted scions are hyposensitive to
295 ABA (at all three - but more convincingly at the lower - concentrations tested), with respect to WT

296 (SL-/SL- vs WT/WT; Fig. 4). On the other hand, the same analysis proved also that WT scions are
297 indeed hypersensitive to ABA if grafted onto SL- instead of WT rootstocks (WT/SL- vs WT/WT, Fig.
298 4), as hypothesized on the basis of the stomatal conductance and shoot ABA quantification
299 experiments reported above (Fig. 1a and 3c vs Supporting Information S3a and S5c). We also
300 tested (at 5 μ M ABA, the concentration for which differences among our lines were more evident)
301 if a pre-treatment with the synthetic SL analogue *racGR24* could by itself increase sensitivity to
302 ABA, in a complementary way to SL depletion decreasing it. This was indeed the case (WT/WT
303 plants, GR24-treated vs untreated, Fig. 4).

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

310

311 **DISCUSSION**

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

313 In this study, we investigated in tomato the possible systemic implications of the drop in SL
314 synthesis happening in roots under osmotic stress. A parsimonious starting hypothesis was that SL
315 depletion in roots could directly or indirectly act as a signal of stress for the shoots. On this basis,
316 hetero-grafted plants with WT scions and SL-depleted rootstocks were to behave as at least mildly
317 stressed, even in the absence of stress. Our physiological data are in agreement with this theory:
318 stomatal conductance values of WT shoots grafted onto SL-depleted rootstocks are significantly
319 lower than those of WT shoots self-grafted onto WT rootstocks in irrigated conditions, and are
320 accompanied by less negative leaf water potential values and, as expected, higher intrinsic water
321 use efficiency (defined as the ratio between net carbon assimilation and stomatal conductance;
322 Supporting Information Fig. S3d). These data support the idea that SL depletion in root tissues
323 affects (directly or indirectly) the physiological response in the shoot and leading to better
324 acclimatization to drought. The ability of shoots to produce SL is needed for this to happen, as
325 stomatal conductance is increased instead when the whole plant (and not only the roots) are
326 *CCD7*-silenced; indeed, this latter condition rather leads to drought hypersensitivity, as shown in

327 SL-depleted Arabidopsis, Lotus and now, tomato plants [(Ha *et al.*, 2014; Liu *et al.*, 2015); this
328 work].

329

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

331 To determine whether the effects of root SL depletion on WT shoots may be due to altered ABA
332 levels, this hormone was quantified in roots and shoots of plants in the three grafted sets. SL-
333 depleted roots and especially shoots contain significantly more ABA per gram of fresh weight than
334 the WT ones in the absence of stress. Our results in unstressed shoots are in apparent
335 contradiction to the ones reported on *CCD8*-silenced tomato plants, where shoots of SL-depleted
336 lines had lower ABA content (Torres-Vera *et al.*, 2014); the most likely explanation is that our data
337 were normalized over fresh and not dry weight as in Torres-Vera *et al.* In any case during severe
338 stress, free ABA increases less in tissues of self-grafted SL- than WT plants, a trend already
339 observed in Lotus (Liu *et al.*, 2015); such situation, coupled to the hyposensitivity to the hormone,
340 will certainly exacerbate the drought sensitivity of SL-depleted shoots. Instead, the slower and less
341 pronounced ABA increase in roots and shoots of WT/SL- plants compared to the other lines is in
342 agreement with the physiological conditions of these plants (which being primed for better stress
343 resilience, perform better thus needing less ABA). It is of course possible that ABA levels in guard
344 cells may not be reflected by the total levels of free ABA in the whole leaf tissue, given the strong
345 compartmentalization of the hormone in different cell types and compartments (Hartung & Slovik,
346 1991); and thus, that WT/SL- plants had lower g_s because of locally enhanced ABA accumulation.
347 However, the results of the ABA-feeding experiment rather supported the hypothesis that such
348 phenotype was (at least partly) due to stomatal hypersensitivity to the hormone. Finally, the same
349 experiments also highlighted that SL in the shoot are not only needed but also sufficient to
350 increase stomatal sensitivity to ABA.

351

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

353 Since our experimental set-up mimics what normally happens during drought, we propose that
354 these findings are relevant to stress resistance, at least in plants such as Lotus and tomato, for
355 which a drop in SL synthesis is recorded in roots experiencing osmotic stress or drought. Such drop
356 might promote a pre-alerted (primed) status in the shoots, which become more sensitive to ABA
357 at the guard cell level. This message may be conveyed directly (see below) or indirectly, i.e.
358 through a second messenger that ought to be, at least in tomato, different than ABA. It is to be

359 noted here that SL were proven to cross-talk with other hormones, such as auxins, cytokinins,
360 brassinosteroids and ethylene, in processes different than drought responses and stomatal closure
361 (Cheng *et al.*, 2013); and that each of these hormones was shown to affect stomatal aperture
362 locally (Daszkowska-Golec & Szarejko, 2013). Root-synthesized cytokinins were even proposed to
363 act as a systemic signal promoting stomatal opening, in a similar way to SL (Davies & Zhang, 1991);
364 however SL- mutants display reduced cytokinin levels in the shoot, which is the opposite of what
365 one would expect from a mediator of SL effect (because cytokinins promote stomata aperture,
366 and SL- shoots transpire more than WT) (Foo *et al.*, 2007). Additionally, shoots were proven to
367 possess powerful homeostatic mechanisms for the regulation of cytokinin levels, that are largely
368 unlinked from their concentration in xylem sap (Foo *et al.*, 2007). Resuming, we cannot exclude
369 that the effect of SL on stomatal closure may be at least partly indirect, i.e. mediated by any of
370 these hormones, or by other signals yet (and indeed, sensitivity to ABA does play a role). It would
371 be indeed interesting to quantify other hormones in leaves of our lines, or even better to visualize
372 their activity in guard cells; and to measure whether, for example, the xylem sap pH in hetero-
373 grafted plants is different than in self-grafted [possibly, more basic as in droughted tomato plants
374 (Wilkinson *et al.*, 1998)]. It remains clear that plant hormones, if capable of travelling over long
375 distances, have a slow propagation velocity in comparison with hydraulic and/or electrical signals.
376 However, the fact itself that in our model, stomatal closure is rather induced by the lack of an
377 inhibitor in the shootward flow is attracting, because its decrease might be perceived faster than
378 flow speed would predict for a positive modulator. In fact, the flow is slowed down by drought,
379 thus adding to the decrease of the inhibitor itself; additionally, given that SL are degraded upon
380 perception (Hamiaux *et al.*, 2012), they should be quickly depleted locally unless *de novo* synthesis
381 or translocation occurs. Finally, expression pattern and intracellular location of the SL
382 transporter(s) might add another regulation level, for mobility through living tissues.

383 As regards the activity of SL biosynthetic genes, shoots of irrigated, hetero-grafted WT/SL- plants
384 behave as if under drought, i.e. show increased transcripts of *CCD7* and *CCD8*. These increases in
385 gene activity might be due to the relief of direct repression of SL synthesis in the shoots by
386 translocated, root-synthesized SL; a known pattern [e.g. (Johnson *et al.*, 2006; Liu *et al.*, 2013)],
387 which might itself trigger SL accumulation at specific spots in the shoot (undetectable in whole-
388 tissue analyses). Even if it is at present impossible to overcome the technical limitations that make
389 the quantification of SL unfeasible in shoots, we propose that hypersensitivity to ABA in stomata
390 of WT/SL- plants might be causally linked to higher production of SL in (limited tissue zones of) the

391 shoot, since i) transcription of SL-biosynthetic genes is activated in WT shoots during stress, but
392 also under non-stressful conditions if WT shoots are grafted onto SL- rootstocks; ii); sensitivity to
393 ABA converts from higher to lower than normal, if not only roots but also shoots are SL-depleted,
394 proving that SL synthesis in the shoots is needed for the effects on ABA sensitivity; iii) exogenous
395 GR24 treatment is sufficient to induce stomatal hypersensitivity to ABA. This latter effect is
396 opposite to the one caused by genetically-due SL depletion, and would explain GR24 ability to
397 confer drought resistance in WT Arabidopsis (Ha *et al.*, 2014). The importance of SL produced in
398 the shoot has been proposed also in branching, because micro-grafting of WT Arabidopsis scions
399 on SL-defective rootstocks does not lead to an increased branching phenotype, as expected if SL
400 synthesis is compromised in the whole plant (Foo *et al.*, 2001; Sorefan *et al.*, 2003). Whether
401 osmotic/drought stress in the absence of such decrease in root-synthesized SL is able to stimulate
402 a similar shoot response, is still to be determined. A schematic drawing of our model is
403 represented in Fig. 5. This model obviously implies that the shoot is able to discriminate between
404 root- and shoot-produced SL; this ability needs to be proven experimentally, but could rely on
405 differential loading in the upstream flow, and/or organ-specific production of the structurally
406 different SL molecules, which make up species-specific SL blends and whose ecological and
407 physiological meanings remain largely unexplored (Kohlen *et al.*, 2011; Kohlen *et al.*, 2012; Bharti
408 *et al.*, 2015; Brewer *et al.*, 2016). Alternatively, or in parallel, the uneven/non-overlapping
409 distribution of the receptor protein D14 and/or of SL transporter(s) in the plant might account for
410 discrimination between locally and distally produced SL (Chevalier *et al.*, 2014; Sasse *et al.*, 2015).
411 From a practical point of view, it remains to be assessed how such graft combinations will perform
412 under other or combined stress. It is important to note on this regard that they will undoubtedly
413 be advantageous in soil infested by parasitic weeds; that not all SL-depleted genotypes are also
414 significantly compromised in mycorrhization (a possible detrimental side-effect); and that with
415 respect to SL synthesis, drought overrules P deficiency under combined stress (Kohlen *et al.*, 2012;
416 Liu *et al.*, 2015). Nonetheless, our results highlight once more the importance of rootstocks in
417 influencing shoot traits, and how they could be exploited to improve crop performances under
418 stress (Albacete *et al.*, 2015; Cantero-Navarro *et al.*, 2016).

419

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425

426 **AUTHOR CONTRIBUTION**

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

431

432

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561 **SUPPORTING INFORMATION**

562 **Figure S1. Relative soil water content (RWC) and water potential of soil (Ψ_{soil}) during the course**
563 **of a drought experiment**

564 **Figure S2. Effect of drought on SL amounts in tomato roots**

565 **Figure S3. Physiological performances of the grafted lines in the absence and presence of stress**
566 **as a function of time**

567 **Figure S4. Transcript amounts of key SL biosynthetic genes as a function of leaf water potential**

568 **Figure S5. Effect of drought on free ABA as a function of time, and on transcript amounts of the**
569 **ABA biosynthetic gene *SINCED1* as a function on leaf water potential**

570 **Table S1. List of primers**

571

572 **FIGURE LEGENDS**

573 **Figure 1. Physiological performances of the grafted lines in the absence and presence of stress.**

574 Stomatal conductance **(a)**, and mean carbon assimilation rate **(b)** as a function on leaf water
575 potential (Ψ_{leaf}) of grafted tomato plants (WT/WT, SL-/SL- and WT/SL-) along a water-deprivation
576 time-course. Full symbols in each series indicate rehydrated samples (recovery). Data represent
577 the mean and SEM of $n = 6$ biological replicates from 2 independent experiments.

578

579 **Figure 2. Effect of drought on the transcript amounts of SL biosynthetic genes (*SICCD7* and**

580 *SICCD8*) of roots **(a-b)** and shoots **(c-d)** of grafted tomato plants (WT/WT, SL-/SL- and WT/SL-)
581 during a time-course (0, 1, 3 and 5 days from water withdrawal for T0 through T5). R indicates the
582 rehydrated samples (recovery). Gene transcript abundance was normalized to endogenous *EF1 α*
583 and presented as fold-change value over WT/WT at T0, which was set to 1. Data represent the
584 mean and SEM of $n = 6$ biological replicates from 2 independent experiments. Different letters
585 indicate significant differences between plant lines for the same time point, as determined by a
586 two-way ANOVA test ($P < 0.05$). n.d. = not detectable.

587

588 **Figure 3. Effect of drought on free ABA as a function on leaf water potential (Ψ_{leaf}) and on**

589 **transcript amounts of the ABA biosynthetic gene *SINCED1* in roots (a-b) and shoots (c-d) of**
590 **grafted tomato plants (WT/WT, SL-/SL- and WT/SL-) during a time-course (0, 1, 3 and 5 days from**
591 **water withdrawal for T0 through T5). Full symbols (a, c) or R (b, d) indicate the rehydrated samples**
592 **(recovery). Gene transcript abundance was normalized to endogenous *EF1 α* and presented as**
593 **fold-change value over WT/WT at T0, which was set to 1. Data on ABA represent the mean and**
594 **SEM of $n = 4$ biological replicates (each replicate a pool of 2 plants) from 2 independent**
595 **experiments. Data on *SINCED1* represent the mean and SEM of $n = 6$ biological replicates from 2**

596 independent experiments. Different letters in **(b)** and **(d)** indicate significant differences between
597 plant lines for the same time point, as determined by a two-way ANOVA test ($P < 0.05$).

598

599 **Figure 4. Dose-response of leaves to treatment with exogenous ABA** at different concentrations.

600 Stomatal conductance was measured at 30-s intervals before and during ABA treatments
601 performed on detached composite leaves from grafted tomato plants (WT/WT, SL-/SL-, WT/SL-).

602 WT/WT plant pre-treated with 5 μ M *racGR24* were analysed only for the 5 μ M ABA treatment
603 (black bar). Values represent the mean and SEM of at least $n = 6$ biological replicates from 2

604 independent experiments, and refer to the time (seconds) needed for the decrease of stomatal
605 conductance to start, from the time of ABA addition to the dipping solution. Different letters

606 indicate significant differences between plant lines for the same treatment, as determined by a
607 two-way ANOVA test ($P < 0.05$).

608

609 **Figure 5. Schematic drawing of the main connections between SL and ABA in roots and shoots of**
610 **tomato under drought stress.** In the model, the effects of SL on ABA levels may be negative in the

611 roots, as proven by *racGR24* treatment in *L. japonicus* (Liu *et al.*, 2015). Thereby, the drop in SL
612 synthesis in this organ under osmotic (PEG-infused) stress may be needed but not necessarily

613 sufficient to let ABA levels rise (results untested in other plant species so far; **1**). SL synthesis is
614 inhibited in roots under osmotic/drought stress, so shootward SL flow decreases (**2**); in tomato,

615 root-produced ABA is not translocated nor needed for appropriate shoot responses to stress
616 (Holbrook *et al.*, 2002). The effects of shoot-produced or exogenous SL on ABA sensitivity of

617 stomata are in turn positive (**3**) [(Ha *et al.*, 2014; Liu *et al.*, 2015) and this work]. SL flowing
618 shootward inhibit the transcription of SL biosynthetic genes (thicker line, **4**), as reduced quantities

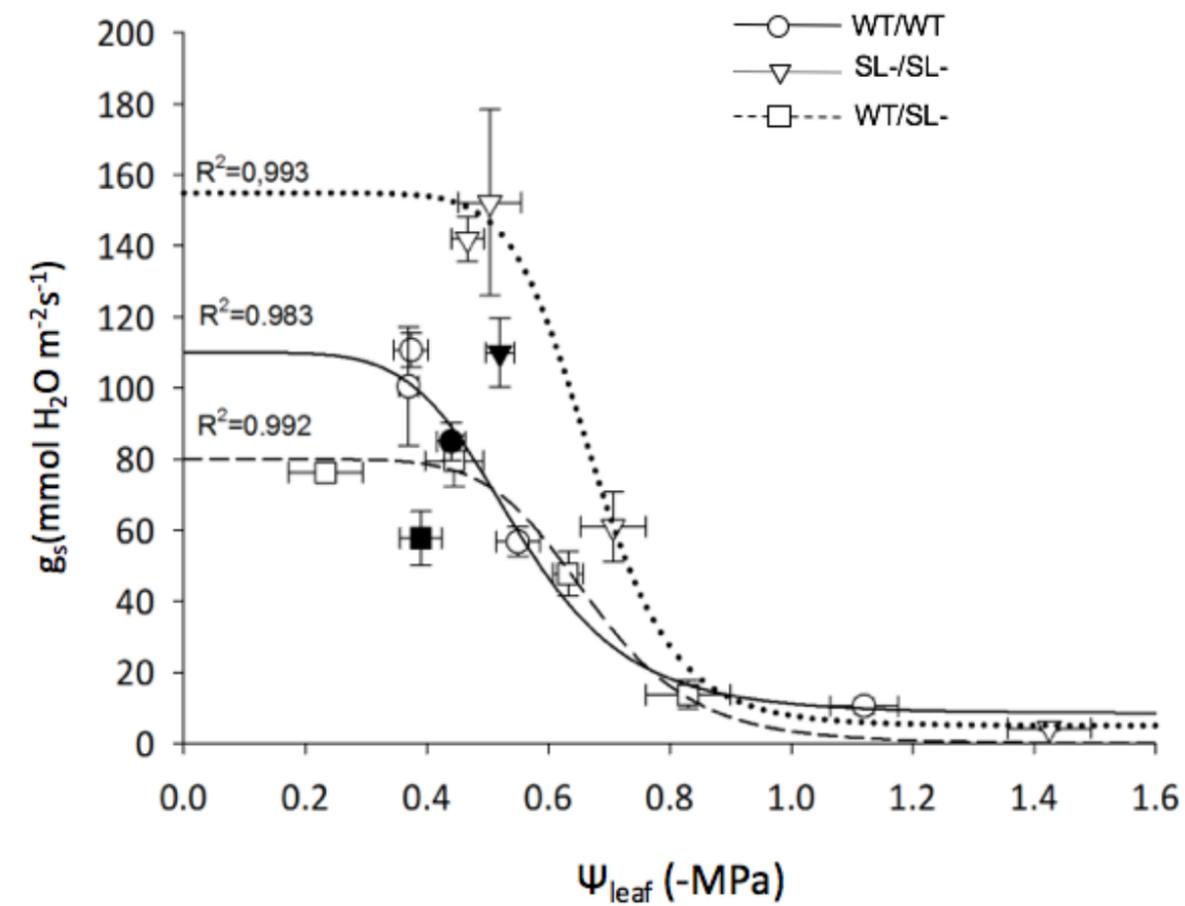
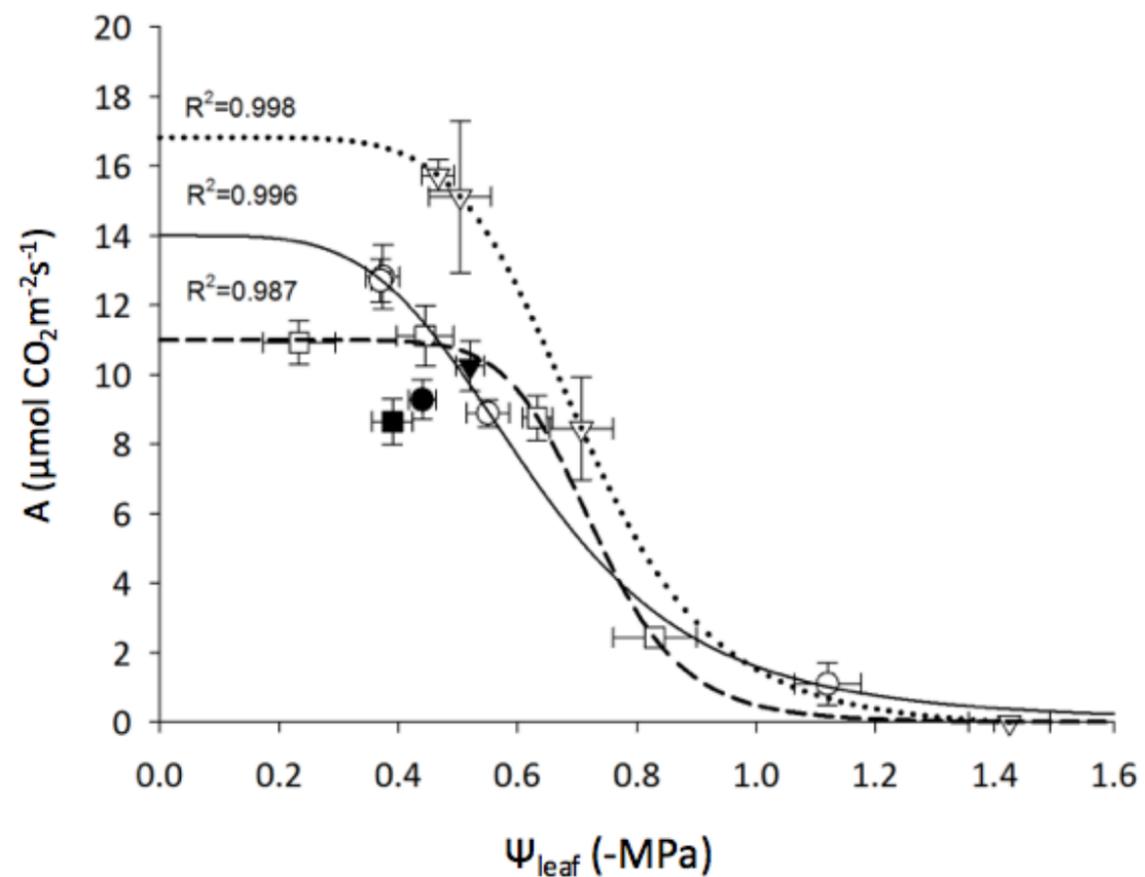
619 in the upstream flow (or possibly, a second messenger – different than ABA - produced in the
620 roots in response to low SL) are sufficient to let transcripts of SL biosynthetic genes increase

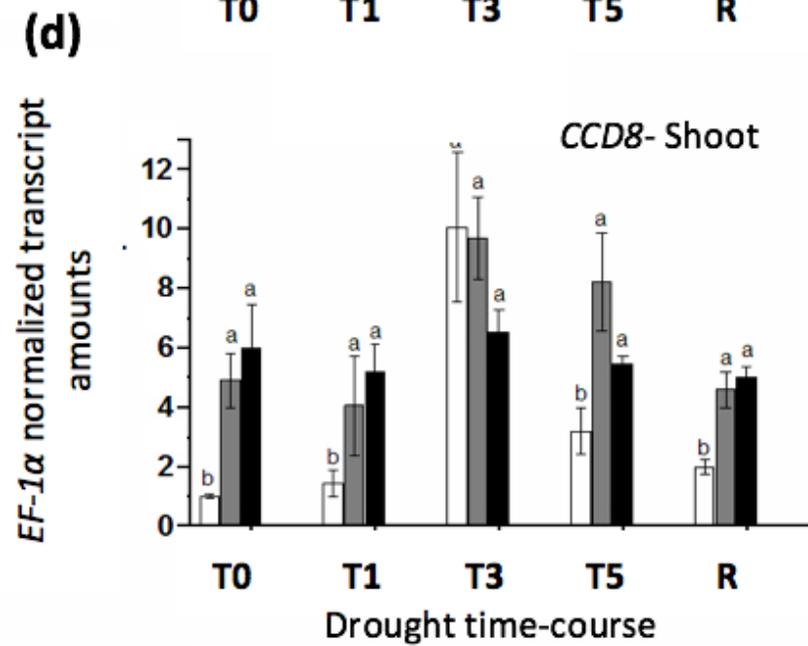
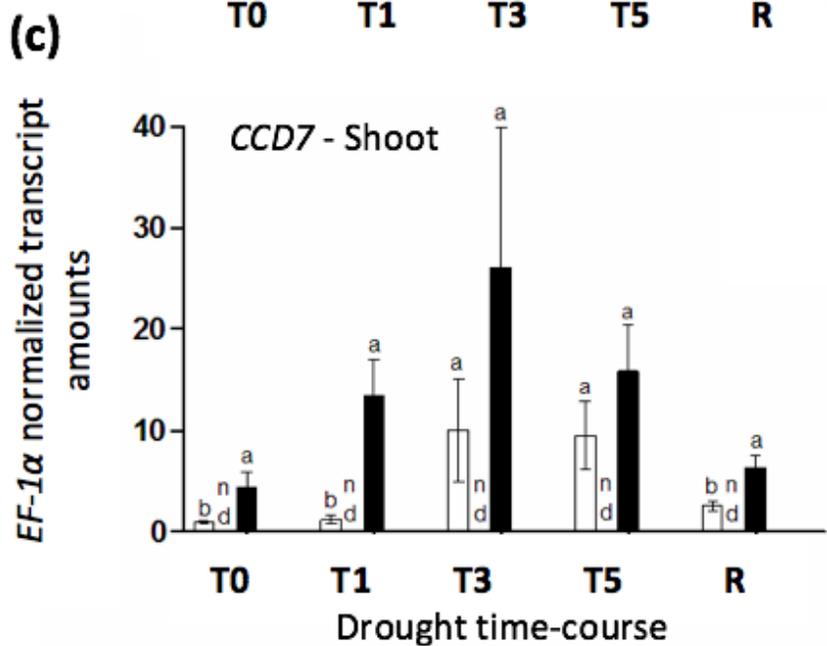
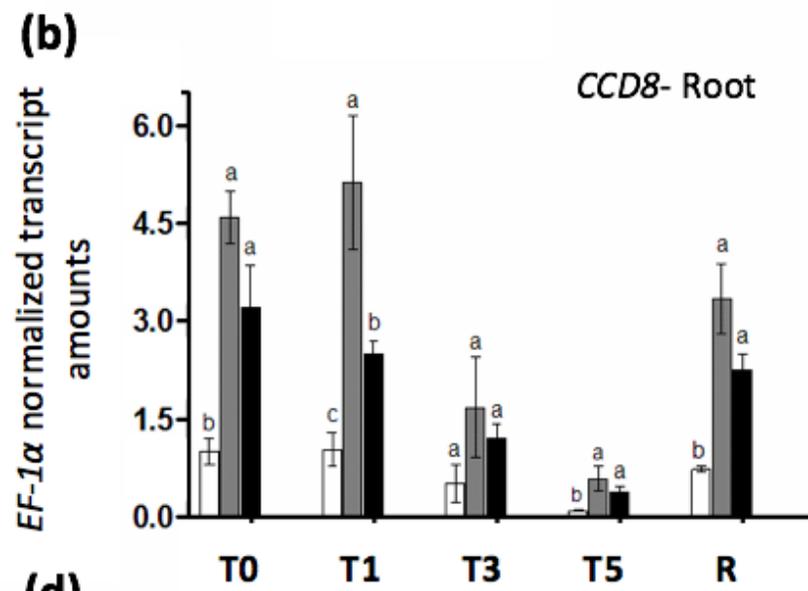
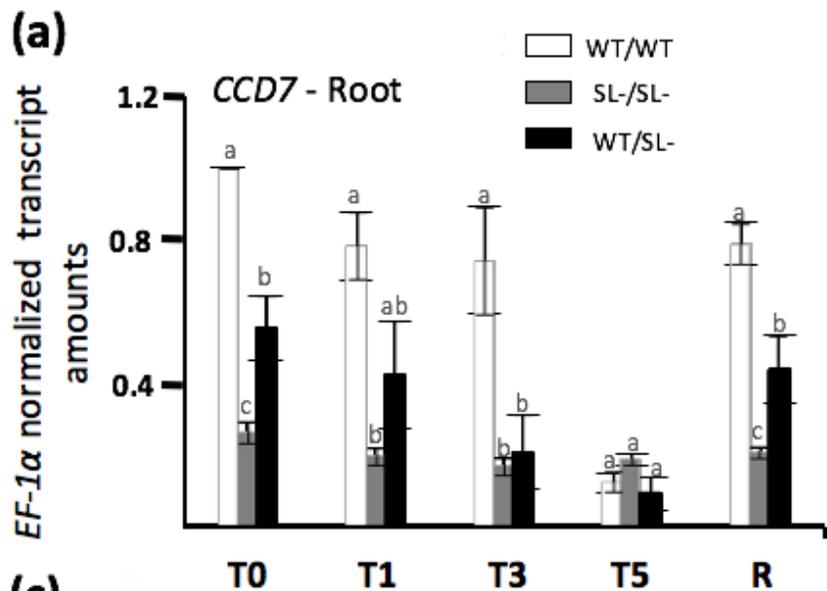
621 (thinner line; **5**) and as a likely consequence, also sensitivity to ABA (**6**). Whether osmotic/drought
622 stress can increase SL gene transcription and ABA sensitivity in the shoots even if SL synthesis in

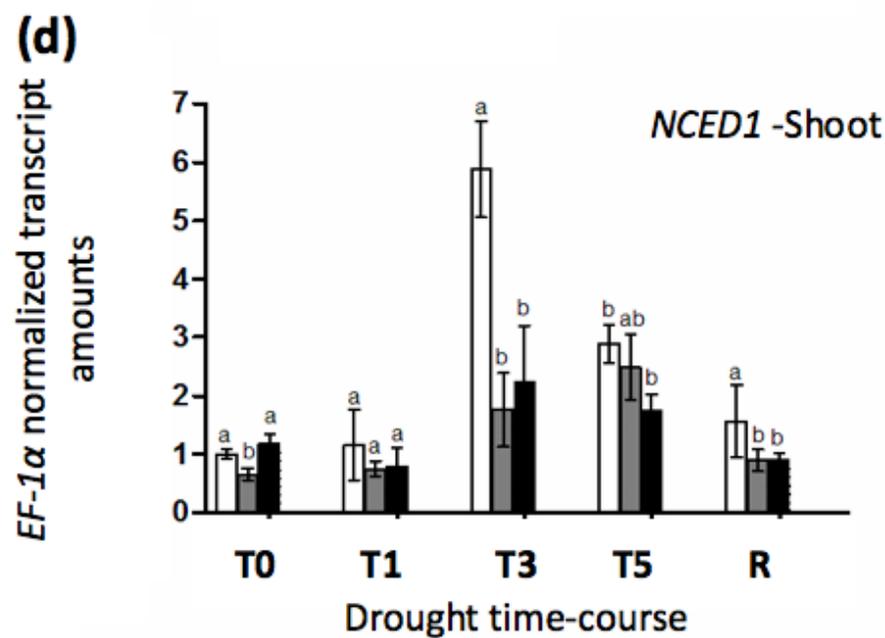
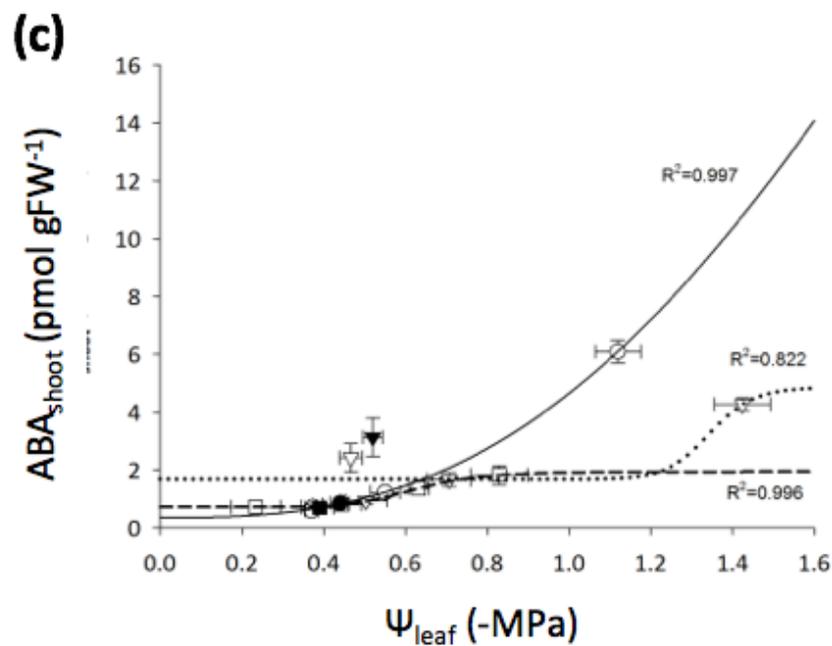
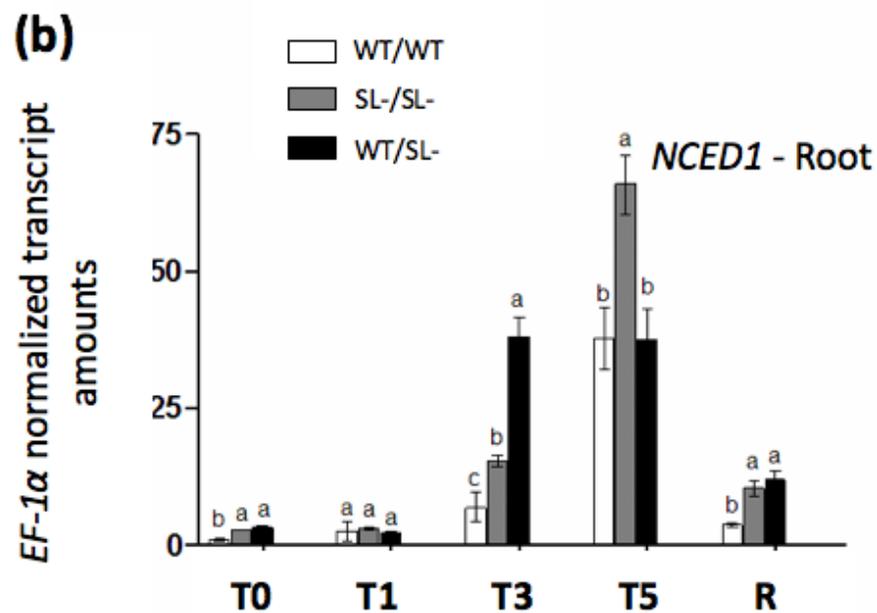
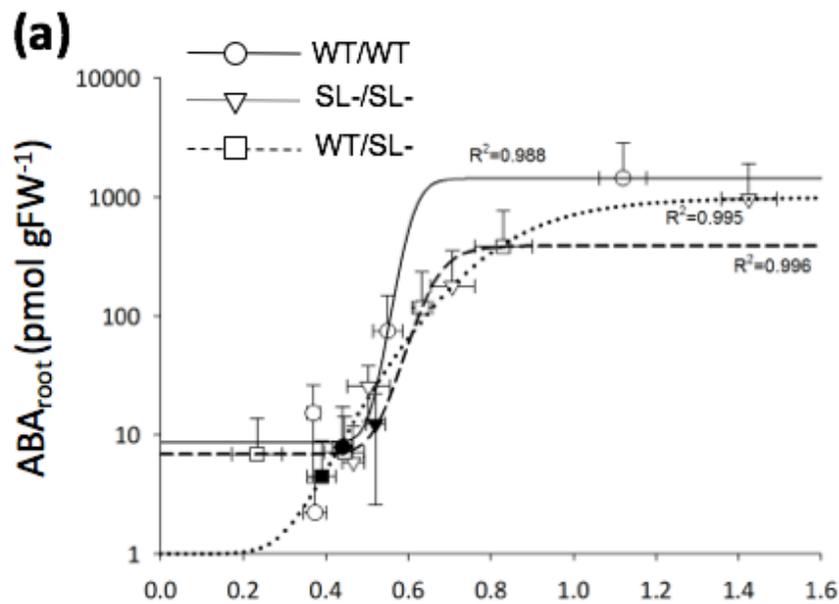
623 the root is not decreased is not known (question mark). Although SL remain undetectable in
624 whole-shoot analyses of stressed tomato, localized accumulation may occur, as proposed (Liu *et*

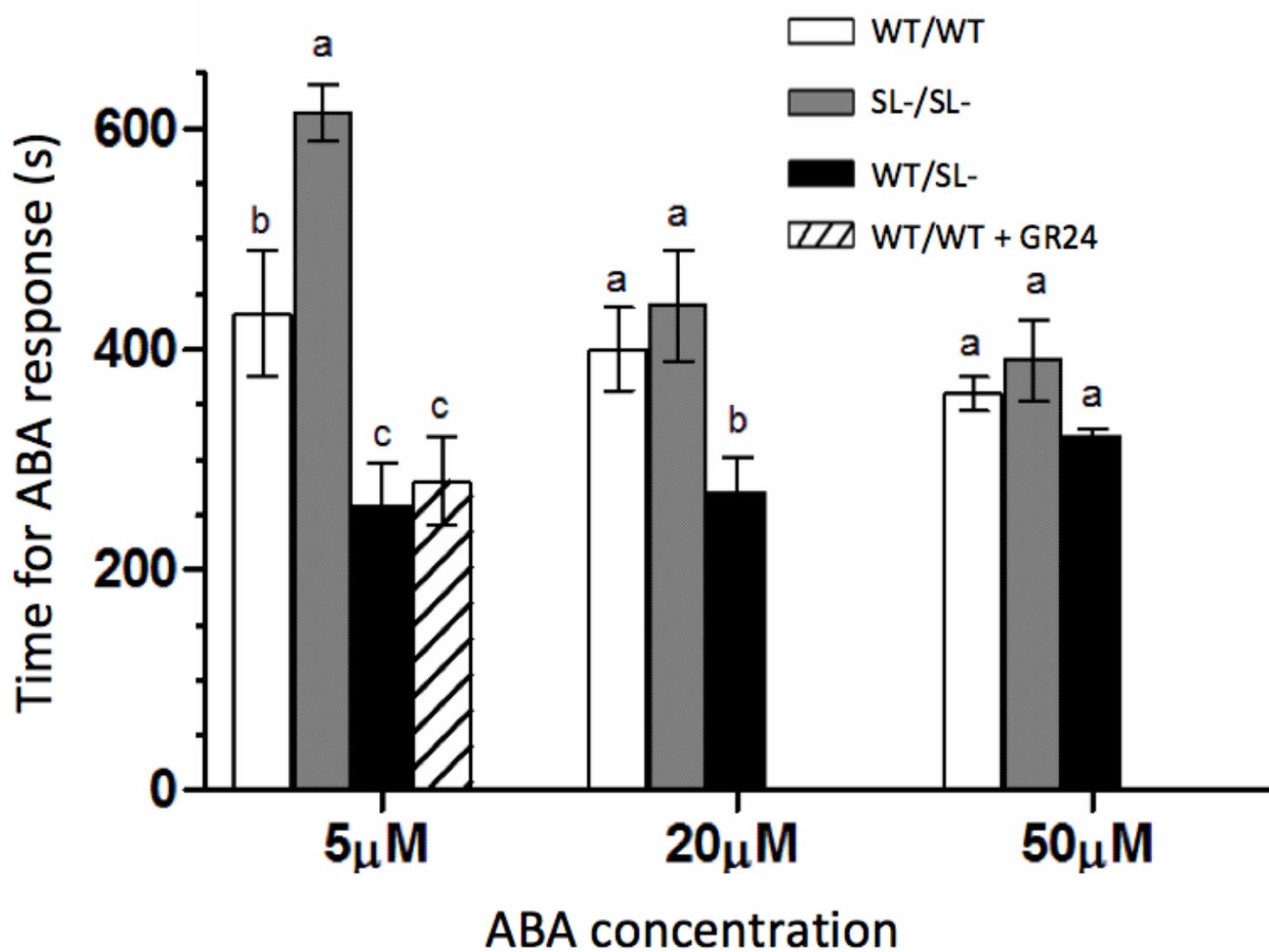
625 *al.*, 2015) and suggested by transcript quantification of biosynthetic genes (Ha *et al.*, 2015; this
626 work). Alternatively, steady-state SL levels may be needed and sufficient to ensure wild-type

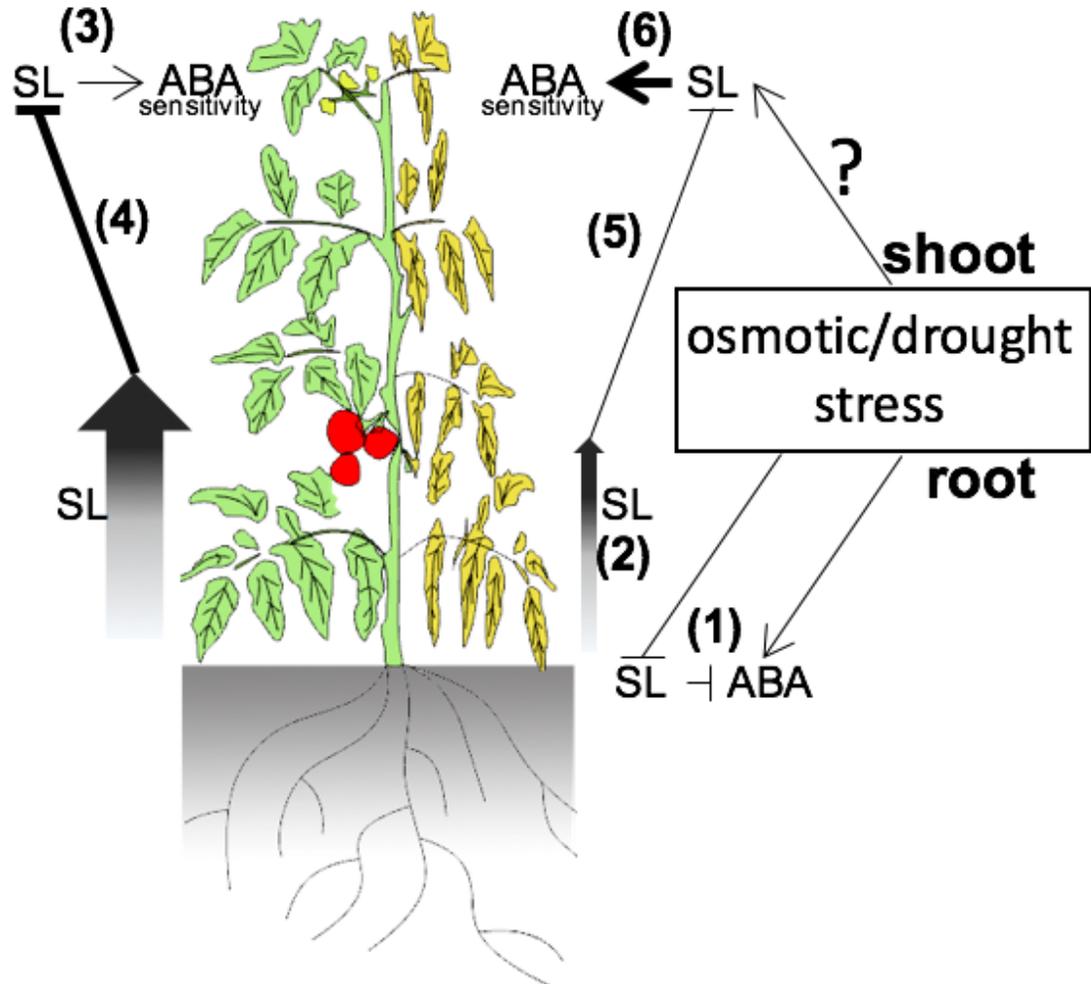
627 sensitivity to ABA in stressed shoot tissues; or other, yet unidentified SL(-like) molecules may be
628 induced.
629

(a)**(b)**









New Phytologist Supporting Information

Article title: **Low levels of strigolactones in roots as a component of the systemic signal of drought stress in tomato**

Authors: Ivan Visentin, Marco Vitali, Manuela Ferrero, Yanxia Zhang, Carolien Ruyter-Spira, Ondřej Novák, Miroslav Strnad, Claudio Lovisolo, Andrea Schubert, Francesca Cardinale

Article acceptance date: 4 August 2016

The following Supporting Information is available for this article:

Fig. S1 Relative soil water content (RWC) and water potential of soil (Ψ_{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 *SINCED1* as a function on leaf water potential

Table S1 List of primers

Fig. S1 Relative water content (RWC) and water potential of soil (Ψ_{soil}) during the course of a drought experiment. Soil RWC was gravimetrically determined by collecting daily soil from three different points and depths in each pot, to assess water content after oven drying. At the same time, the soil water retention curve was assessed with pressure plate measurements of the potting substrate (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(10-11): 1119-1128). For both datasets values represent the mean and SEM of $n = 6$ samples from two independent experiments.

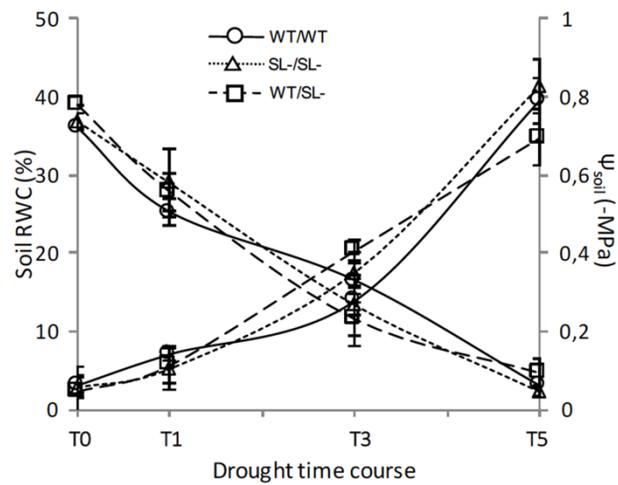


Fig. S2 Effect of drought on SL biosynthesis in tomato roots: solanacol (a), orobanchol (b) and the didehydro-orobanchol isomer with retention time 4'6'' (c) were quantified in roots of grafted plants (WT/WT, SL-/SL- and WT/SL-) along a time-course (0, 1, 3 and 5 days from the beginning of stress for T0 through T5). R indicates the rehydrated (Recovery) samples. Data represent the mean and SEM of $n = 2$ samples derived from the pool of 3 plants each, in two independent experiments. While solanacol and orobanchol are expressed as absolute amounts per g of fresh tissue weight, the didehydro isomer of orobanchol is expressed as a percentage ratio of MS/MS peak area normalized over values for WT tissues at T0, due to the lack of a standard.

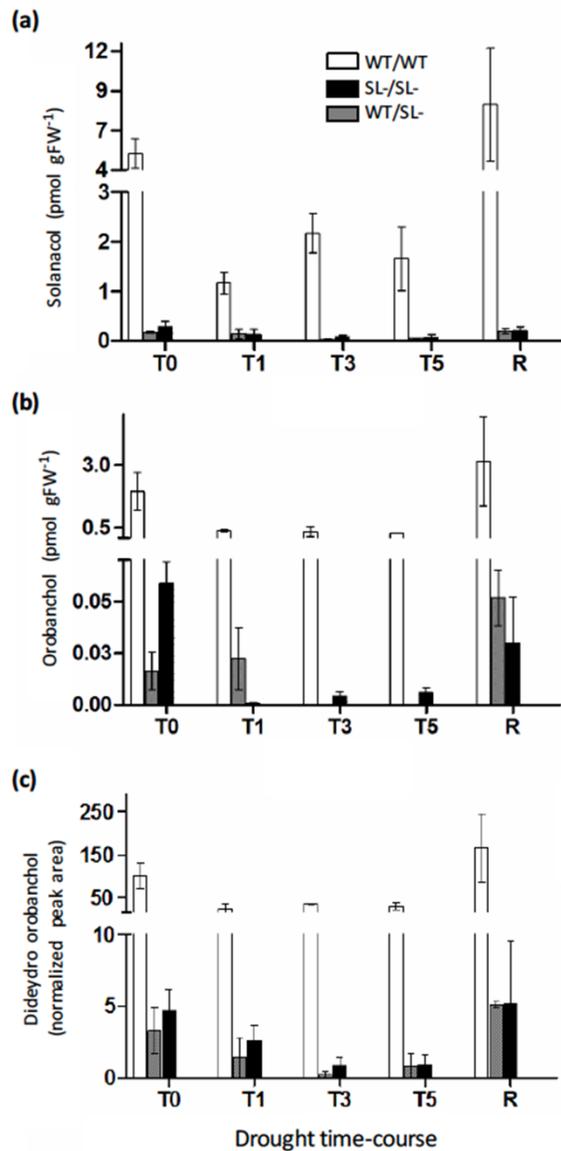


Fig. S3. Physiological performances of the grafted lines in the absence and presence of stress as a function of time. Stomatal conductance (a), leaf water potential (b) and mean carbon assimilation rate (c), and intrinsic water use efficiency (WUE) (d) of grafted tomato plants (WT/WT, SL-/SL- and WT/SL-) along a water-deprivation time-course (0, 1, 3 and 5 days from the beginning of stress for T0 through T5). R indicates rehydrated samples (recovery). Data represent the mean and SEM of $n = 6$ biological replicates from 2 independent experiments. Different letters indicate significant differences within the same time point as determined by a two-way ANOVA test ($P < 0.05$). n.d. = not detectable.

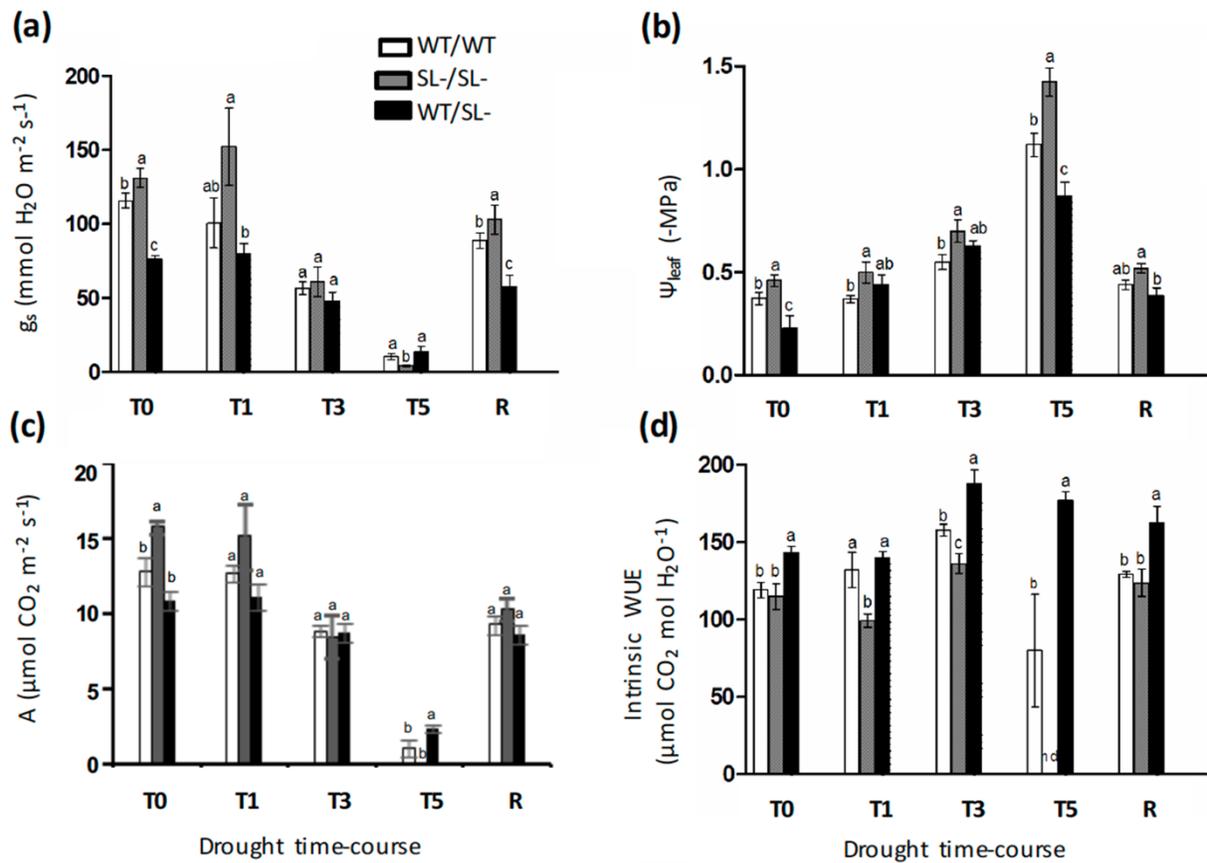


Fig. S4. Transcript amounts of key SL biosynthetic genes (*SICCD7* and *SICCD8*) in roots (a-b) and shoots (c-d) of grafted tomato plants (WT/WT, SL-/SL- and WT/SL-) as a function of leaf water potential during a drought time-course (0, 1, 3 and 5 days from water withdrawal). Full symbols in each series indicate the rehydrated samples (recovery). Gene transcript abundance was normalized to endogenous *EF1 α* and presented as fold-change value over WT/WT at T0, which was set to 1. Data represent the mean and SEM of $n = 6$ biological replicates from 2 independent experiments. *SICCD7* transcripts were undetectable in silenced (SL-) shoots.

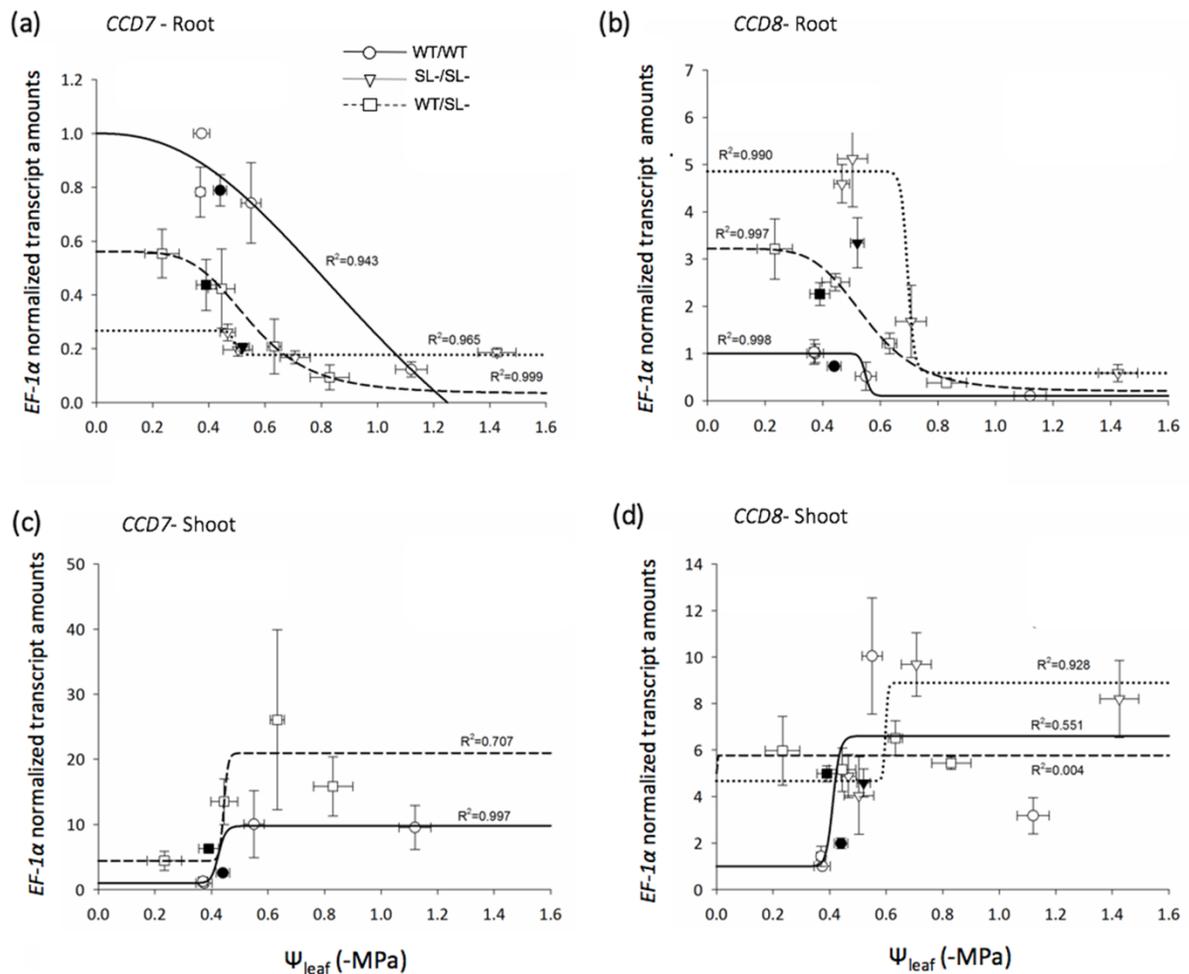


Fig. S5. Effect of drought on free ABA and on transcript amounts of the ABA biosynthetic gene *SINCED1* as a function on leaf water potential in roots (a-b) and shoots (c-d) of grafted tomato plants (WT/WT, SL-/SL- and WT/SL-) during a time-course (0, 1, 3 and 5 days from water withdrawal for T0 through T5). Full symbols (a, c) or R (b, d) indicate the rehydrated samples (recovery). Gene transcript abundance was normalized to endogenous *EF1 α* and presented as fold-change value over WT/WT at T0, which was set to 1. Data on ABA represent the mean and SEM of $n = 4$ biological replicates (each replicate a pool of 2 plants) from 2 independent experiments. Data on *SINCED1* represent the mean and SEM of $n = 6$ biological replicates from 2 independent experiments. Different letters in (a) and (c) indicate significant differences between plant lines for the same time point, as determined by a two-way ANOVA test ($P < 0.05$).

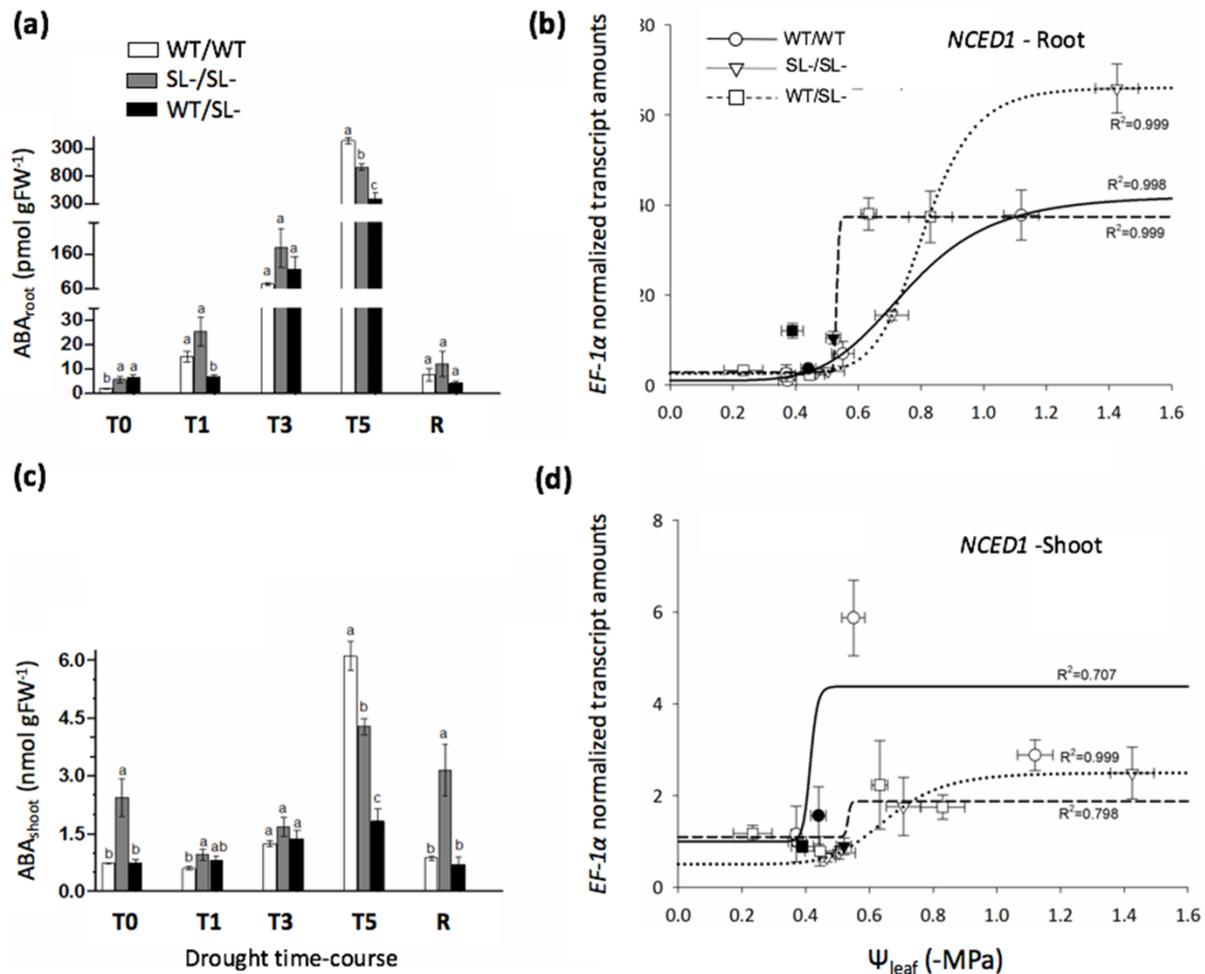


Table S1 Primer pairs for transcript quantification via qRT-PCR.

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 (SICCD8) regulates rhizosphere signaling, plant architecture and affects reproductive development through strigolactone biosynthesis. *New Phytologist* **196**(2): 535-547.

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**(2): 343-354.

Gene ID	Forward primer	Reverse primer	Reference
<i>SLCCD7</i>	GTTGCTCTTACCAATGGTTCAATTT	TACATTCATCATGGAAGGATCAAAGTT	(Kohlen <i>et al.</i> , 2012)
<i>SICCD8</i>	CCAATTGCCTGTAATAGTTCC	GCCTTCAACGACGAGTTCTC	(Kohlen <i>et al.</i> , 2012)
<i>SINCED1</i>	ACCCACGAGTCCAGATTTC	GGTTCAAAAAGAGGGTTAG	(Lopez-Raez <i>et al.</i> , 2010)
<i>SIEF1α</i>	GATTGGTGGTATTGGAAGTGC	AGCTTCGTGGTGCATCTC	(Kohlen <i>et al.</i> , 2012)

Supplementary Table S1

Gene ID	Forward primer	Reverse primer	Reference
<i>SLCCD7</i>	GTTGCTCTTACCAATGGTTCAATTT	TACATTCATCATGGAAGGATCAAAGTT	(Kohlen <i>et al.</i> , 2012)
<i>SICCD8</i>	CCAATTGCCTGTAATAGTTCC	GCCTTCAACGACGAGTTCTC	(Kohlen <i>et al.</i> , 2012)
<i>SINCE1</i>	ACCCACGAGTCCAGATTTC	GGTCAAAAAGAGGGTTAG	(Lopez-Raez <i>et al.</i> , 2010)
<i>SIEF1α</i>	GATTGGTGGTATTGGAAGTGC	AGCTTCGTGGTGCATCTC	(Kohlen <i>et al.</i> , 2012)

Primer pairs for transcript quantification via qRT-PCR