Osmotic stress represses Strigolactone biosynthesis in Lotus japonicus roots: exploring the interaction between Strigolactones and ABA under abiotic stress

Title

Osmotic stress represses Strigolactone biosynthesis in *Lotus japonicus* roots: exploring the interaction between Strigolactones and ABA under abiotic stress

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Main Conclusion

Strigolactone changes and cross-talk with ABA unveil a picture of root-specific hormonal dynamics under stress.

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Abstract

Strigolactones (SLs) are carotenoid-derived hormones influencing diverse aspects of development and communication with (micro)organisms, and proposed as mediators of environmental stimuli in resource allocation processes; to contribute to adaptive adjustments therefore, their pathway must be responsive to environmental cues. To investigate the relationship between SLs and abiotic stress in *Lotus japonicus*, we compared wild-type and SL-depleted plants, and studied SL metabolism in roots stressed osmotically and/or phosphate starved. SL-depleted plants showed increased stomatal conductance both under normal and stress conditions, and impaired resistance to drought associated with slower stomatal closure in response to Abscisic acid (ABA). This confirms that SLs contribute to drought resistance in species other than Arabidopsis. However, we also observed that osmotic stress rapidly and strongly decreases SL concentration in tissues and exudates of wild-type Lotus roots, by acting on the transcription of biosynthetic and transporter-encoding genes, and independently of phosphate abundance. Pre-treatment with exogenous SLs inhibited the osmotic stress-induced ABA increase in wild-type roots and down-regulated the transcription of the ABA biosynthetic gene *LjNCED2*. We propose that a transcriptionally regulated, early SL decrease under osmotic stress is needed (but not sufficient) to allow the physiological increase of ABA in roots. This work shows that SL metabolism and effects on ABA are seemingly opposite in roots and shoots under stress.

Keywords GR24, drought, phosphate starvation, stomatal conductance, *CCD7*, *CCD8*, *D27*, *MAX1*, *PDR1*, *NCED*, Strigolactone.

Footnotes

The online version of this article (doi:----) contains supplementary material, which is available to authorized users.

Introduction

Phytohormones are key effectors of the morphological plasticity of plants in response to changing environmental conditions. Abscisic acid (ABA), ethylene, jasmonic acid and salicylic acid are perhaps the best-studied hormones in the adaptive adjustments of plants to biotic and abiotic stress (Xiong et al. 2002). Strigolactones (SLs) were identified in 2008 as a new class of plant hormones modulating development (Cheng et al. 2013; Ruyter-Spira et al. 2013). SLs are also involved in the establishment of the symbiosis with nitrogen (N)-fixing bacteria (Foo and Davies 2011; Foo et al. 2013b; Liu et al. 2013b; Soto et al. 2010), and the interaction with pathogenic fungi (Torres-Vera et al. 2013). They are also key molecules in rhizosphere communication with arbuscular mycorrhizal fungi (AMF) and parasitic weeds (Akiyama et al. 2005; Foo et al. 2013b; Yoneyama et al. 2010)

Knowledge of the SL biosynthetic pathway is growing, with several key genetic determinants identified. Mutations affecting D27, a β-carotene isomerase (Alder et al. 2012; Lin et al. 2009); the carotenoid cleavage dioxygenase (CCD) enzymes MAX3/CCD7 and MAX4/CCD8 (Beveridge and Kyozuka 2009; Booker et al. 2004; Sorefan et al. 2003); and MAX1, a cytochrome P450 (Booker et al. 2005; Cardoso et al. 2014; Challis et al. 2013; Kohlen et al. 2011) have been associated with a hyper-branching phenotype and with reduced SL production and/or exudation in several species. The *in vitro* proof of the activity of D27, CCD7 and CCD8 was also provided, showing that these enzymes can produce carlactone – the precursor of SLs - from all-*trans*-β-carotene (Alder et al. 2012; Seto et al. 2014). Finally, first proof of the role of cytochrome P450 CYP711 subfamily members (MAX1 protein homologues) in carlactone B-C ring closure and subsequent structural diversification step(s) was recently obtained in rice (Zhang et al. 2014). MAX1 can also catalyze the conversion of carlactone to carlactonoic acid and its methyl ester; the latter substance was shown to be endowed with SL-like activity in Arabidopsis (Abe et al. 2014). Once produced, mainly in roots, SLs are transported acropetally to the shoot or are exuded into the rhizosphere by the ABC transporter PDR1, which was identified in *Petunia hybrida* (Kretzschmar et al. 2012). The F-box protein MAX2, and the α/β-fold hydrolase D14/DAD2 are the main candidate components of the SL perception complex in higher plants (Arite et

al. 2009; Gaiji et al. 2012; Hamiaux et al. 2012; Kagiyama et al. 2013; Stirnberg et al. 2007; Waters et al. 2012a; Zhao et al. 2013).

With their dual role as endogenous hormones and signalling molecules in the rhizosphere, SLs are well placed as regulators of the changes in development of plants in response to external cues. To do so, SL metabolism or signalling must be sensitive to environmental conditions. Several lines of evidence suggest a connection between SLs, or genes encoding their metabolism, and light - both as photoperiod, intensity and wave length [reviewed in (Liu et al. 2013a)]. A connection with nutrient availability has also been established: phosphate (P) and/or N starvation promote SL production and exudation in a number of species. The responses to P and N availability show species-specific diversity and were thus proposed to control nutrient-acquisition strategies exploiting AMF or Rhizobia symbionts [reviewed in (Xie and Yoneyama 2010)]. While this may be true in some plants, later physiological analyses indicated that SLs are not required to regulate these symbioses in response to nutrient deficiency in pea (Foo et al. 2013a; Foo et al. 2013b), and that the species-specific differences in SL production under P starvation rather correlate with the shoot P levels across species/families (Yoneyama et al. 2012). SLs were also demonstrated to be part of the plant perception/response system to low-P conditions, and to mediate, in a MAX2-dependent fashion, some of the morphological changes triggered by P starvation (Kohlen et al. 2011; Koltai 2013; Mayzlish-Gati et al. 2012).

Osmotic stress can be induced by drought, freezing, or salt, and represents a main limitation to plant growth and yield worldwide. At a metabolic level, plants under water stress rapidly accumulate ABA, which is essential for stomatal closure (Zhu 2002). The accumulation of ABA is largely due to increased activity of carotenoid-cleavage enzymes of the 9-*cis*-epoxycarotenoid dioxygenase family (NCED), which catalyse the rate-limiting step in ABA biosynthesis (Tan et al. 1997). Catabolism also contributes to the fine-tuning of ABA levels under stress conditions (Nambara and Marion-Poll 2005). Considering the shared carotenoid precursors and the involvement of carotenoid-cleaving enzymes, interactions between ABA and SL metabolism have been proposed (Tsuchiya and McCourt 2009). However so far, only a few experimental works have attempted to unravel such interactions,

131 with contradictory results to some extent. A positive correlation between ABA and SL
132 biosynthesis was demonstrated in the shoot with a set of tomato mutants blocked at different
133 steps in ABA biosynthesis, with the application of specific inhibitors for NCED enzymes,
134 and with ABA-insensitive mutants (López-Ráez et al. 2008; López-Ráez et al. 2010).
135 Accordingly, leaves of SL-deficient tomato plants were reported to contain less ABA than
136 WT (Torres-Vera et al. 2013). However, in Arabidopsis seeds, exogenous GR24 reduces
137 ABA levels, thereby alleviating dormancy induced by thermo-inhibition (Toh et al. 2012).
138 Also, SLs seem to promote the release of broomrape (*Phelipanche ramosa*) seeds from
139 dormancy by reducing ABA levels during conditioning (warm stratification) (Lechat et al.
140 2012). SL insensitivity does not change ABA levels, neither under normal or stress
141 conditions (Bu et al. 2014).
142 Two recent independent studies demonstrated that the F-box component of the putative SL
143 receptor, MAX2, plays a positive role in drought resistance at the shoot level, and that this
144 correlates to reduced response to exogenous ABA by guard cells (Bu et al. 2014; Ha et al.
145 2014). However, besides this shared conclusion, the two reports are contradictory at several
146 levels. While some inconsistencies could be explained with the slightly different
147 experimental set-up, it is puzzling that in one instance biosynthetic mutants were found not
148 to be compromised in drought tolerance (Bu et al., 2014), while in the other they were as
149 drought-sensitive as *max2* (Ha et al., 2014). Furthermore, exogenous SL treatment could
150 rescue the drought-sensitive phenotype of SL biosynthetic mutants and even increase
151 performances of drought-stressed WT plants in the latter report (Ha et al., 2014). This
152 implies that the SL metabolite would (Ha et al., 2014) be responsible for the observed
153 drought-hypersensitive phenotype, in contradiction with Bu and co-workers' findings (Bu et
154 al., 2014). Contradictions extend to the SL-ABA cross-talk, since *max2* mutants were found
155 hypo- (Ha et al., 2014) or hyper-sensitive (Bu et al., 2014) to ABA at germination and
156 post-germination developmental stages; and to the effect of SL insensitivity on stomatal
157 density, which was found increased (Ha et al., 2014) or unchanged in *max2* mutants
158 compared to the WT (Bu et al., 2014). Additionally, and in relation with SL role in both root
159 and shoot development, a few reports point to a direct influence of osmotic stress on the

emergence of lateral root primordia and on axillary bud outgrowth, but with divergent effects in different model systems or ecotypes (Deak and Malamy 2005; Kolbert et al. 2008; Osorio et al. 1998; Vandemoortele et al. 2001). Clearly, more work is needed to figure out the detailed contribution of SLs to osmotic stress responses, in Arabidopsis but also in other plants. Moreover, because of the partially contradictory data available, the interactions between SL and ABA metabolism also need further exploration in the context of abiotic stress, taking into account a possible diversity of responses in different plant species and in different organs or developmental processes for the same plant.

In the present study, we confirmed the positive role of SLs to drought resistance at the shoot level in the model legume *Lotus japonicus*, but most importantly investigated the response of SL synthesis and exudation to water stress and P deprivation in the roots, where we studied the organ-specific relationship between SLs and stress-induced ABA. The results suggest root-specific hormonal dynamics under stress.

Materials and methods

Plant material, growth conditions and treatments

Wild-type (WT) *L. japonicus* ecotype Gifu B-129 and the *LjCCD7*-silenced line P16 (*Ljccd7*) in the same background were used. *Ljccd7*, here at T1-T2 generation, shows an average reduction of 5-deoxystrigol in root tissues and exudates up to 80% (Liu et al. 2013b). Unless otherwise specified, in all experiments seeds were sterilized in 2.5% NaClO containing 0.02% (v/v) Tween 20 for 20 min, rinsed thoroughly with sterile water, stratified on Gamborg B5 medium for 2-3 d at 4°C and then moved for 2-3 d in a growth chamber (24°C, 16h light/8h dark). Germinating seedlings were then transferred in the greenhouse (20-21°C, 16h light/ 8h dark; 60% relative humidity) in a 2:1 mixture of perlite and Arabidopsis special soil (Horticoop) and watered with 'Hornum' solution twice a week (Handberg and Stougaard 1992).

For P starvation and osmotic stress treatments, fifteen plants per genotype and treatment were transplanted into an X-stream 20 aeroponic system (Nutriculture) running with 5 litres of modified half-strength Hoagland Solution (Hoagland and Arnon 1950; López-Ráez et al.

2008). Plants were cultivated another 4 weeks to ensure full root-system development and given fresh half-strength Hoagland solution (+P) twice a week. P starvation (-P) was accomplished by replacing the nutrient solution with half-strength Hoagland solution containing KCl instead of K_2HPO_4. Osmotic stress was imposed with 10% (w/v) Polyethylene glycol 6000 (PEG, Duchefa) completely dissolved in either P-rich (+P/+PEG) or P-deficient (-P/+PEG) half-strength Hoagland solution, corresponding to -1.61 MPa (http://www.plantstress.com/methods/peg.htm). For the early response of SL biosynthetic genes to osmotic stress, a short time-course was conducted with seedlings that after 3-week cultivation in the greenhouse as above had been transferred into 50 ml Falcon tubes refilled with fresh half-strength Hoagland solution every three days. Three plantlets were introduced in each tube through a hole in the lid; roots were kept in the dark. After another 2-week cultivation, osmotic stress was applied with 10% PEG, and samples were harvested at 0, 6, 12, 24 and 36 h after the beginning of stress. Each sample corresponded to three independent replicates, and each replicate was the pool of the three plantlets of a tube.

To compare the physiological behaviour of WT and *Ljccd7* plants under drought stress, 20 plants per genotype were cultivated in pots (Ø 10 cm) filled with the mixture of perlite and soil described above. After 9-week incubation in a growth chamber (20-22°C, 16h light/8h dark), plants were transferred to the greenhouse (22-24°C, natural daylight) for 2-3 days. Subsequently the plants were freshly watered one last time, carefully drained (time 0) and stressed by being kept unwatered for 9 additional days. Physiological measurements and sampling were performed at day 0, 2, 5 and 9. For the ABA treatment experiment (see following paragraph), plants were cultivated in a similar way and treated after about 8 weeks in the greenhouse.

For treatment with the synthetic SL analogue GR24 (purchased from StrigoLab S.r.l. or kind gift of B. Zwanenburg, NL), seedlings of WT plants (3 plants per replicate, in triplicate) were obtained as above, but kept in the greenhouse for three weeks instead of two, till root weight was ≥ 0.5 g. Plants were then rinsed well and kept for 2 days in 300 ml flasks covered with aluminium foil and filled with P-deficient half-strength Hoagland solution (-P), before being delivered exogenous SLs (-P/+SL). To do so, the flasks were filled with 300 ml

of -P solution containing 5.0 µM GR24 (a SL analogue; stock at 10 mM in acetone) or an equivalent volume of acetone (controls, -P/-SL) for 48 h. Plants were treated with GR24 in the absence of PEG 6000 in order to maximize GR24 uptake. At the end of the GR24 treatment, the solution was replaced with 300 ml of -P solution plus 10% PEG (-P/+PEG); samples were harvested 1 and 2 d after. Three plants from each biological triplicate were pooled as one analytical sample; roots were harvested and directly stored in liquid N_2 for further analyses.

Physiological measurements

For the drought experiments, soil or shoot water potential and stomatal conductance were measured daily between 10:00 and 12:00 am on five plants each of the WT (as a control) and *Ljccd7* genotype. Stomatal conductance was measured with a portable Gas exchange Fluorescence System (GFS-3000, Heinz Walz GmbH, Effeltrich, Germany) by clamping the most apical leaves of a shoot in the leaf chamber, where photosynthetically active radiation (PAR, 1000 µmol photons m^{-2} s^{-1}), air flow (700 µmol m^{-2} 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-day experiments. A calibration for CO_2 was set at the beginning of each day of measurement. Leaf area inside the chamber was set to 6.7 cm^2, value obtained after measurement with a portable area meter (LI 3000, Li-cor, Lincoln, NE, USA) of the apical part of 20 shoots (SE ± 0.15). Shoot water potential was measured with a pressure chamber (Scholander et al. 1965) on one shoot per plant, immediately after its gas exchange quantification. Soil water potential was calculated from the potential/moisture (assessed gravimetrically) curve previously obtained for the pot substrate (Tramontini et al. 2013). For a quantification of stomatal responses to saturating ABA in WT and *Ljccd7* plants, stomatal conductance was measured as above at 90 s intervals before and during ABA treatment. This was accomplished by cutting a leafy twig under filtered water, letting stomatal conductance stabilise with the twig dipped in water and then adding ABA to 5, 20 or 50 µM final concentration, while keeping recording every 30 seconds both stomatal conductance and transpiration rates as detailed above.

247

248 *Exudate collection, SL and ABA extraction and quantification*

249 Twenty-four hours before exudate collection, the roots of aeroponically-cultivated
250 plants were thoroughly rinsed with deionised water to remove all accumulated SLs and the
251 nutrient solution was refreshed. Root exudates were collected as previously reported (Liu et
252 al. 2011; López-Ráez et al. 2008) with minor changes (Liu et al. 2013b). Five litres of
253 exudates per sample were separately loaded onto pre-equilibrated C18 columns (GracePure
254 C18-Fast, 5000 mg/20 ml). Subsequently, each column was washed with 50 ml of deionised
255 water and 50 ml of 30% acetone in water. SLs were immediately eluted with 50 ml of 60%
256 acetone in water and stored at -20°C for later use. Exudates were consecutively collected
257 four times at two-day intervals (time-points 0, 2, 4 and 6 d after the beginning of treatments)
258 for SL quantification. At the end of the experiment, five plants from each replicate (three
259 replicates for controls, two for *Ljccd7* plants) were pooled together as one analytical sample;
260 roots and shoots were separately harvested and stored at -80°C until needed for gene
261 transcript, SL and/or ABA quantification.
262 The only detectable SL produced by Lotus, 5-deoxystrigol (Liu et al. 2013b; Sugimoto and
263 Ueyama 2008), was quantified in the shoots and in the root exudates and tissues, while ABA
264 was quantified in roots, and shoots (leaves included) when needed. Extractions were
265 performed as previously reported (Liu et al. 2013b; López-Ráez et al. 2010) with minor
266 modifications. For SL extraction from exudates, 2 ml of the elution fractions from C18
267 columns (see above) were mixed with 200 µl of internal standard solution (0.1 nmol ml^{-1}
268 $[^2H]_6$-5-deoxystrigol in acetone). The mixture was dried under speed vacuum, and residues
269 dissolved in 50 µl of ethyl acetate and then mixed with 4 ml of hexane. Samples were
270 loaded onto pre-conditioned Silica columns (GracePure 200 mg/3 ml). SLs were eluted
271 using 2 ml of hexane:ethyl acetate solvent mixtures (20, 40, 60, 80 and 100% of ethyl
272 acetate). The fractions of 40% or 60% ethyl acetate in hexane containing 5-deoxystrigol
273 (Liu et al. 2013b) were combined, dried under speed vacuum and dissolved in 200 µl of
274 acetonitrile:water:formic acid (25:75:0.1, v/v/v) for ultra-performance liquid
275 chromatography-tandem spectrometry (UPLC-MS/MS) quantification.

For SL and ABA extractions from tissues, 0.5 g of root and 0.3-0.5 g (for ABA) or 1.0 g (for 5-deoxystrigol) of shoot tissue were manually ground in liquid nitrogen. Samples were extracted with 2 ml of cold ethyl acetate containing $[^2H]_6$-ABA and $[^2H]_6$-5-deoxystrigol as internal standards (0.05 nmol ml^{-1} for both ABA and SL from roots, and for ABA from shoots; 0.025 nmol ml^{-1} for SL extraction from shoots) in a 10 ml glass vial. The vials were vortexed and sonicated for 10 min in a Branson 3510 ultrasonic bath (Branson Ultrasonics, Danbury, CT, USA). Samples were centrifuged for 10 min at 2500 *g*, the organic phase transferred to a 4 ml glass vial and the pellets re-extracted with another 2 ml of ethyl acetate. Samples for SL analysis were dried and the following steps were performed as described above for SL quantification in root exudates. Two-ml fractions of 100% ethyl acetate or 10% methanol in ethyl acetate were combined for ABA analysis.

Analyses of root exudates and tissue extracts were conducted by comparing retention times and mass transitions with those of available SL and ABA standards using a Waters Xevo tandem mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) source and coupled to an Acquity UPLC system (Waters, USA) as described previously (Jamil et al. 2011; Kohlen et al. 2011). The amount of GR24 taken up by plants was quantified by using the three transitions 299>97, 299>157, and 299>185 by external calibration curve. All datasets were generated and analysed with MassLynx 4.1 (Waters).

Gene expression by quantitative reverse-transcription PCR (qRT-PCR)

Total RNA from Lotus roots was extracted using Tri-Pure reagent (Roche) according to the manufacturer's protocol, sequentially digested on-column with DNaseI for 30 min and further purified by RNeasy Plant Mini Kit (both Qiagen) according to the manufacturer's instructions. RNA quality and integrity were checked by NanoDrop ND-2000 and standard gel electrophoresis. One µg of total RNA was reverse transcribed to cDNA with the iScript cDNA synthesis kit (Bio-Rad) as specified by the supplier's manual. qRT-PCR reactions were set up in 20 µl volumes using the iQ SYBR Green Supermix on the iQ5 Real-Time PCR detecting system (Bio-Rad). The target genes were retrieved from the Lotus EST

library available from Kazusa Institute (http://www.kazusa.or.jp/lotus/) by BlastP with the corresponding known orthologues as queries (Table S1). Gene-specific primers were designed with the Primer-3-Plus online programme (Table S1), and their specificity checked by blasting them against the available Lotus genomic sequences in NCBI. Ubiquitin (*UBI*) was used as internal standard (Yokota et al. 2009). Primers targeted to putative *LjPDR1s* could discriminate among the three paralogues, but not between the two splicing variants of *LjPDR1-226* and *-345*. On the contrary, the transcript of *LjPDR1-295a* and *b* were quantified separately. Three independent biological replicates per sample were analyzed and each PCR reaction had three analytical triplicates. Transcript quantification followed the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data were subjected to the analysis of variance (ANOVA) to determine treatment effects by SPSS 21.0 (IBM SPSS Inc., Cary, NC, USA). T-test was used for significance analysis of all gene expression data, while a Tukey's F-test was used for all other datasets, with $P < 0.05$.

Results

SL depletion reduces total shoot ABA content under combined P and osmotic stress, and dampens the response of plants to drought and exogenous ABA at the leaf level

ABA accumulates rapidly in response to various abiotic stresses, contributing to mediate plant acclimation to unfavourable environmental conditions. SL-related mutants were recently shown to be altered in ABA production/sensitivity, but evidence was partly contradictory (Bu et al. 2014; Ha et al. 2014; Torres-Vera et al. 2013) and needed confirmation in more plant species. Hereto, we first quantified ABA in tissues of WT plants either grown under normal aeroponic conditions, or P-starved and/or PEG-treated for 6 days. The P deprivation condition was added in the experimental set-up as a positive control, given that SLs are known to respond negatively to P availability [reviewed in (Xie and Yoneyama 2010)]; also, an earlier report suggests that low P enhances foliar ABA levels

following drought stress (Radin 1984), providing a hint that this interaction may be important at some level. In WT plants, osmotic stress significantly induced ABA accumulation both in roots and shoots (Fig. 1). P availability alone did not influence ABA levels, in agreement with previous studies (Jeschke et al. 1997). However, the increase in ABA due to PEG treatment was significantly enhanced under -P conditions, both in roots and shoots, with 1.75 and 1.27 times higher levels in -P/+PEG *vs.* +P/+PEG samples, respectively (Fig. 1).

In an attempt to understand if compromised SL biosynthesis would modify ABA content upon stress, SL-depleted *Ljccd7* plants (Liu et al. 2013b) were compared with WT plants in the presence or absence of osmotic stress. The plants were also P-starved (-P and -P/+PEG samples for each genotype), because these are the conditions under which these plants will presumably differ most from the WT in SL levels, given the strong induction observed in the WT by P starvation (see below, Fig. 3). *Ljccd7* plants did not display significant differences in ABA levels compared with the WT genotype in roots (Fig. 1, left-end bars), while in shoots a reduction of ABA concentration was observed upon combined P starvation and osmotic stress, compared to P starvation alone (Fig. 1, right-end bars). Under +P conditions, both roots and shoots of *Ljccd7* plants contained levels of ABA comparable to the WT.

Preliminary observations indicated that *Ljccd7* plants were more severely and precociously wilting than the WT plants and had lower leaf water potential 4 days after PEG treatment (Fig. 2), in spite of having the same stomatal density (Fig. S1b-d). The decreased tolerance to osmotic stress turned out to be in agreement with the phenotype of SL-related mutants in Arabidopsis (Bu et al. 2014; Ha et al. 2014). To confirm this phenotype and understand if it was due to reduced ABA levels and/or altered stomatal sensitivity to ABA, as in Arabidopsis, we then compared SL-depleted and WT plants as for ABA content and physiological performance upon drought stress in soil. The relationship between ABA and soil water potential was identical for the two genotypes (Fig. 3a). However, stomatal conductance (and transpiration accordingly, not shown) was higher in *Ljccd7* than in WT plants in irrigated and in drought stressed plants, at any root ABA content (Fig. 3b) or shoot

363 water potential (Fig. 3c), suggesting that the SL-depleted plants rather have a lower stomatal
364 sensitivity to ABA. When leafy twigs were fed ABA in the 5-50 µM range through the
365 petiole, there were differences in the sensitivity of stomata of SL-depleted plants compared
366 to WT, especially at low ABA concentrations. In fact SL-depleted leaves started closing
367 their stomata significantly later than WT controls for the 5 and 20 µM treatment (Fig. 3d), in
368 spite of the higher transpirational flux; while the trend was conserved, such difference was
369 less and less significant for higher ABA concentrations (Fig. 3d). SL-depleted leaves
370 reached final stomatal conductance values comparable to the WT, showing that guard cells
371 may not be compromised in their potential to fully close in response to ABA (Fig. S1a).
372 These results prove that chronic, whole-plant SL depletion in SL-depleted plants confers a
373 drought-sensitive phenotype in *L. japonicus*. This is most likely due to ABA hyposensitivity
374 at the guard cell level, which results in higher transpiration both under normal and stress
375 conditions.
376
377 *Osmotic stress represses accumulation and excretion of SLs in roots, independently of P*
378 *status*
379 Since SL depletion clearly affects osmotic stress resistance in Lotus at the shoot level
380 (as it does in Arabidopsis), we set to assess the effect of the same stress on SL
381 concentrations and tested if PEG-induced osmotic stress would influence SL concentration
382 in and exudation from the roots, under P-limiting as well as non-limiting conditions. P
383 availability can profoundly affect SL production and exudation (Xie and Yoneyama 2010;
384 Yoneyama et al. 2012). In Lotus, it was previously reported that 5-deoxystrigol is
385 significantly induced by P deficit in hydroponically cultivated plants (Akiyama et al. 2005;
386 Sugimoto and Ueyama 2008). Indeed, there is induction of 5-deoxystrigol accumulation in
387 root exudates of P-starved Lotus plants grown under aeroponic conditions, with an increase
388 over time starting to be significant four days after the beginning of P starvation (Fig. 4a).
389 This confirms that our experimental model reacts in a predictable way to abiotic stress at the
390 root level.

391 Conversely, PEG treatment consistently, time-dependently and significantly lowered
392 5-deoxystrigol concentration in Lotus exudates, irrespectively of P availability (Fig. 4a).
393 5-Deoxystrigol was also quantified within root tissues under -P and/or PEG-induced
394 osmotic stress, and showed a very similar trend to that of exudates after 6 days of treatment:
395 induction by P starvation and inhibition under osmotic stress (Fig. 4b, left-hand bars).
396 5-Deoxystrigol levels in shoots were just above the detection limit, but seemed to be slightly
397 induced as well by P deprivation. They remained stable under osmotic stress (Fig. 4b,
398 right-hand bars).
399 These results show that osmotic stress greatly reduces SL accumulation and exudation at the
400 root level, but not (or not detectably) in the shoot tissues of *L. japonicus*.
401
402 *Identification of SL-related genes in Lotus*
403 Since when this work was started no SL-related genes had been characterized in Lotus
404 except *LjCCD7* (Liu et al. 2013b), we used the protein sequences of the known orthologues
405 of biosynthetic and transporter-encoding genes in other species (Table S1) as BlastP queries
406 against the EST library and genomic sequence database at the Kazusa DNA Research
407 Institute. Maximum likelihood phylogenetic trees were constructed for the candidate *LjD27*
408 and *LjCCD8* genes, to support their identification as *bona fide* orthologues of the ones
409 characterized in other plant species (Fig. S2). *MAX1* is presumed to act on a later
410 biosynthetic step, which has been studied so far in Arabidopsis, petunia and rice (Booker et
411 al. 2005; Cardoso et al. 2014; Drummond et al. 2012; Kohlen et al. 2011; Abe et al. 2014;
412 Zhang et al. 2014). Functional redundancy of *MAX1* has been reported in rice, pea, sorghum
413 and several other species (Challis et al. 2013; Gomez-Roldan et al. 2008; Umehara et al.
414 2010), while only one EST from Lotus stands out for its similarity scores to *MAX1* queries
415 (Fig. S2). Recently, the genes we identified as putative orthologues of *D27*, *CCD8* and
416 *MAX1* were confirmed as such in an *in silico* analysis including the *L. japonicus* genome
417 (Challis et al. 2013). Finally, using the SL transporter PhPDR1 and its closest homologues
418 AtPDR12, GmPDR12 and NpPDR1 - in *Arabidopsis thaliana, Glycine max* and *Nicotiana*
419 *plumbaginifolia*, respectively - as BlastP queries, three putative homologues (*LjPDR1-226*,

LjPDR1-295 and *LjPDR1-345*), each with two splicing variants, were identified and confirmed by phylogenetic analysis (Fig. S3).

These *in silico* results provided a basis for wet analysis of SL-related gene expression in our experimental system.

Abiotic stress represses the transcription of SL-related genes in roots

To understand whether the observed changes in 5-deoxystrigol levels under stress are a result of differential gene regulation, we quantified the transcript levels of putative SL biosynthetic and transporter-encoding genes by qRT-PCR. Transcription of *LjD27*, *LjCCD7* and especially *LjCCD8* was induced in roots upon P starvation (-P *vs*. +P samples), and down-regulated under osmotic stress (+PEG *vs*. –PEG samples, both under P-sufficient or P-limiting conditions) (Fig. 5a), with an overall pattern roughly mirroring that of the 5-deoxystrigol metabolite in root tissues and exudates. Only transcription of the putative *LjMAX1* followed a different pattern than that of the other three biosynthetic genes, except for the low but significant reduction under combined stress (-P/+PEG *vs.* +P samples, Fig. 5a). qRT-PCR results showed that two of the three putative *PDR1* paralogues, *LjPDR1-226* and *LjPDR1-345*, were up-regulated upon P starvation and down-regulated by PEG treatment irrespectively of P availability (Fig. 4b; splicing variants were not discriminated for these paralogues). These values are in line with the observed changes in 5-deoxystrigol content of the root exudates under both single and combined stress. Both splicing variants *LjPDR1-295a* (Fig. 5b) and *b* (data not shown) instead displayed an opposite pattern under P deficiency than the other two putative paralogues, suggesting that SL changes in root exudates under P starvation are not due to differential transcription of *LjPDR1-295.*

Since the decrease in transcript abundance for the biosynthetic genes *LjD27* and *LjCCD7* was not significant in –P+PEG samples in comparison with +P samples at the 6-day time-point (Fig. 5a), while the decrease in 5-deoxystrigol levels was already evident around 2 days after the beginning of osmotic stress treatment between these two sample sets (Fig. 4a), we attempted to get a clearer picture of the transcriptional modulation preceding this time-point. To this purpose, we investigated *via* qRT-PCR the SL-biosynthetic genes *LjD27*,

449 *LjCCD7*, *LjCCD8* and *LjMAX1*, along with the putative transporter-encoding *LjPDR1-226*,
450 *-345* and *-295*, in a time-course within 36 h from the beginning of treatment. Transcription
451 of the first three putative biosynthetic genes and of *LjPDR1-345* was significantly
452 down-regulated by osmotic stress within a few hours (Fig. 5c). *LjPDR1-226* and *-295a*
453 showed slower down-regulation kinetics, being repressed after 6 days but not repressed or
454 even slightly induced after 36 h of PEG treatment (see Fig. 5b *vs.* d). The transcript of all
455 genes, except for *LjCCD8*, was significantly more abundant at 12 h than at the previous and
456 following time points (but still lower than untreated controls for *LjD27* and *LjCCD7*). This
457 could be a photoperiodic effect, as this time point coincides with the end of the light period
458 (i.e. plants had been exposed to light for already 12 - 13 h at this time of harvest).
459 Taken together, transcript quantification results suggest that the changes in 5-deoxystrigol
460 levels in root exudates and tissues under abiotic stress are at least partially due to a
461 transcriptional modulation of the three genes building the core SL biosynthetic pathway
462 (*LjD27*, *LjCCD7* and *LjCCD8*) and of the putative SL transporter-encoding gene *LjPDR1*.
463
464 *GR24 treatment prevents osmotic stress-induced ABA surge and transcriptional activation*
465 *of the biosynthetic gene* LjNCED2 *in roots*
466 To understand the physiological meaning of the sudden decrease in root SLs shortly
467 after initiation of osmotic stress treatment, we assessed whether an artificially high SL
468 concentration in the early phases of stress may affect the subsequent ABA increase in WT
469 roots. We measured root ABA levels 0, 1 and 2 days after the beginning of combined stress
470 (-P/+PEG samples) in plants that had been pre-treated or not for 2 days with the synthetic
471 SL analogue GR24 (+SL and -SL samples, respectively). To confirm that roots had taken
472 the GR24 up, we quantified its amounts within the root. GR24 was present at high
473 concentrations in the pre-treated plants but decreased with time after the end of the 2 days of
474 pre-treatment, at time 0 (Fig. S4a). This decrease could be due to non-specific catabolism, to
475 active exudation, and/or to the destruction-dependent perception of SLs, i.e. to its
476 degradation by D14 (Scaffidi et al. 2012; Smith and Waters 2012). The efficacy of GR24
477 treatment was demonstrated by the repression of the biosynthetic genes *LjD27*, *LjCCD7*,

LjCCD8 and *LjMAX1* and of *LjPDR1-226* by GR24 (Fig. S4b-c). This pattern is in agreement with the negative feedback regulation of SL biosynthesis demonstrated in Arabidopsis (Mashiguchi et al. 2009), although in Mashiguchi's work, *AtMAX1* was slightly induced by short-time GR24 treatment. Conversely, transcript of *LjPDR1-295a* and *-345* followed a different trend, being unresponsive to GR24 at later time-points but slightly up-regulated at time 0 (Fig. S4c), when GR24 levels are still high (Fig. S4a). The petunia SL transporter *PhPDR1* is also slightly induced by GR24 (Kretzschmar et al. 2012).

One and two days after the beginning of osmotic stress, plants that had not been pre-treated with GR24 showed a significant increase in ABA relative to time zero, as expected (Fig. 6a). In contrast, in roots pre-treated with GR24, ABA levels remained low in spite of the presence of PEG and the absence of P, with a significant decrease compared with the corresponding samples that had not been exposed to GR24 (Fig. 6a). In order to see whether this was due to a down-regulation of ABA production at the transcriptional level, we quantified transcripts for the putative ABA biosynthetic genes *NCEDs*. *NCED1*, *2* and *3* were obvious candidates, since they are known to play a major role in osmotic stress-induced ABA accumulation (Iuchi et al. 2001; Qin and Zeevaart 2002; Xiao et al. 2009). We identified and numbered them in Lotus on the basis of sequence similarity to known orthologues in pea (Fig. S5). Under our conditions, *LjNCED2* expression increased over time during PEG treatment (Fig. 6b), while the other NCED genes tested (*NCED1* and *3*) were unaffected (data not shown). GR24 treatment prevented this increase in transcription, suggesting that GR24 represses PEG-induced ABA increase through *LjNCED2*, at least for the 2 d time-point. We also tested other ABA-related genes, and among the putative orthologues of ABA catabolic genes and of other biosynthetic genes, we quantified the transcripts of *LjCYP707A1*, and *LjAAO3*, *LjABA2* and *LjABA3* (Nambara and Marion-Poll 2005). The phylogenetic analysis of the selected orthologues is shown in Fig. S6. The transcription of all genes was increased by PEG treatment. However, for none of them significant differences were detected between +SL vs. –SL samples, even if a trend for down-regulation by GR24 was apparent for the *LjAAO3* biosynthetic gene (Fig. S7).

As a whole, these results show that if SL levels are kept artificially high in roots under stress, ABA accumulation is inhibited. This may happen through transcriptional repression of the biosynthetic gene *LjNCED2*, even though under our conditions, a significant increase of the transcript for this gene was only seen at 2 days. Other, untested ABA-related genes must therefore contribute to the rise of ABA levels at earlier time-points.

Discussion

The ABA-SL relationship in L. japonicus *under abiotic stress*

Given the common metabolic precursor shared by ABA and SLs, cross-talk between the two pathways was investigated in tomato and, very recently, in Arabidopsis (Bu et al. 2014; Ha et al. 2014; López-Ráez et al. 2010; Torres-Vera et al. 2013). These reports point to a positive correlation between the levels of the two hormones in the shoot, although with some contradictions (see below). Since the main role of ABA is to orchestrate drought responses, the involvement of SLs in drought resistance was also investigated in SL-deficient Arabidopsis by two independent groups, but not observed by both (Bu et al. 2014; Ha et al. 2014). The picture is obviously far from complete, also because SLs were not quantified under stress in either work. Additionally, ABA was quantified only in one of them and only in whole seedlings of the SL-insensitive *max2* mutants (Bu et al. 2014).

In our system, ABA was significantly induced in roots and shoots of WT *L. japonicus* under osmotic stress but not under P starvation alone, as expected. Interestingly, we did observe a synergistic effect of the two stresses on ABA levels, both in roots and shoots. This is reminiscent of an earlier report demonstrating that low P enhances foliar ABA accumulation following drought stress (Radin 1984); and suggests that P deficiency enhances the effects of osmotic stress and *vice versa*. Also, since P starvation strongly enhances SL production, this synergism may have been indicative of a positive effect of SLs on ABA production under combined stress. To test this hypothesis, we compared the ABA levels of the genetically SL-depleted plants to the WT, and observed that less ABA accumulates in transgenic shoots under combined nutritional and osmotic stress, but not in control conditions. On the contrary, chronic SL depletion seems not to interfere with ABA

accumulation in roots, neither under normal growth conditions, nor under combined nutritional and osmotic stress. These findings imply that the synergistic effect of P deprivation and PEG on ABA synthesis is not mediated by SLs in the roots, while it could be in the shoots. The whole picture is in partial agreement with a recent communication, reporting that leaves of SL-deficient tomato plants contain moderately yet significantly less ABA than their WT counterpart in the absence of stress (Torres-Vera et al. 2013). The amount of ABA is reported similar between the WT and SL-insensitive mutants [*max2* in Arabidopsis, both irrigated and drought stressed (Bu et al. 2014), and *rms4* of pea (Dodd et al. 2008)]. However, SL depletion in our *Ljccd7* plants is not complete (about 80%), as it is not in the corresponding Arabidopsis mutants; therefore, residual SLs could mask more subtle SL-ABA correlations, calling for a confirmation in genotypes completely devoid of SLs and in other plant species. Also, our dataset does not allow us to conclude on the effects of SL depletion on ABA levels under osmotic stress alone; however, it is unlikely that such effects may be more intense than under –P conditions, the only situation where a difference in SLs could be detected in the shoots (see Fig. 4b, right-end bars). Conversely, it is not known yet what the effect of GR24 treatment is on ABA levels in shoots; in roots, it blocks stress-triggered ABA increase (see below). Finally, our data exclude that stress-induced ABA is sufficient to increase SL levels (at least in whole-organ analyses of Lotus, and with the difficulties in accurate quantification of low-abundance metabolites). In fact, SLs are seemingly as abundant in unstressed Lotus shoots as they are in drought-stressed shoots - when ABA concentration is higher; and are very low in roots under the same conditions, when again ABA levels are rising.

SL depletion induces ABA hyposensitivity in Lotus shoots

Ljccd7 plants showed an obvious drought-hypersensitive, wilting phenotype reminiscent of the *max* mutants of Arabidopsis (Bu et al. 2014; Ha et al. 2014), suggesting an influence of SLs on the control of leaf transpiration in Lotus. In this respect, our results are in contrast with those obtained by Bu et al. on *max3* and *max4* biosynthetic mutants. In this work in fact, Bu and co-workers excluded an effect of the SL metabolite on the

564 drought-sensitive phenotype, which would then be limited to *max2* mutants. Given the
565 involvement of this F-box in other pathways (Shen et al. 2012; Waters et al. 2012b), the
566 authors speculate that the effects of the *max2* mutation may be linked to other signalling
567 paths. Our dataset rather confirms and complements results by Ha et al., who came to the
568 opposite conclusion working on the same mutant lines.
569 Arabidopsis *max* mutants are shown (at least in one report) to have a modified leaf
570 morphology, with thinner cuticle and denser stomata (Ha et al. 2014). On the contrary, the
571 two Lotus genotypes studied here had comparable stomatal density, so the higher
572 transpiration rates in *Ljccd7* leaves are due to increased stomatal conductance. Such effect
573 on transpiration is not, or not only, exerted through altered ABA synthesis in Lotus, as ABA
574 concentrations were comparable in WT and transgenic plants under most tested conditions,
575 while transpiration rates were always different. Our results in Lotus can more easily be
576 explained by reduced stomatal sensitivity to ABA likely due to reduced capacity to transport
577 ABA to the guard cells. A deficit in the transporters of ABA to guard cells will appear as
578 hyposensitivity during ABA-treatment tests, but might not be associated to a true defect in
579 ABA perception and signal transduction. It must be noted in this respect that genes
580 *ABCG22/AT5G06530* and *ABCG40/AT1G15520* encoding ABA importer proteins in
581 well-watered and dehydrated leaves are down-regulated in *max2* leaves of Arabidopsis, both
582 under normal and stress conditions (Ha et al. 2014). The same guard-cell hyposensitivity to
583 exogenous ABA was recently documented in Arabidopsis *max* mutants (Bu et al. 2014; Ha
584 et al. 2014) and, along with the results of gene expression analyses and of treating with
585 exogenous SLs during stress (Ha et al. 2014), suggests that SL levels may increase (likely
586 locally) in shoots under drought for helping the plant to modulate transpiration appropriately
587 (Ha et al. 2014). It is noteworthy that in contrast to what shown previously (Bu et al., 2014;
588 Ha et al., 2014), the stomatal conductance values end up to be comparable for our WT and
589 SL-depleted genotypes of Lotus, if given enough time (see Fig. S1a). This apparent
590 discrepancy could be easily explained by the different plant species and especially by our
591 -less artificial- experimental set-up (whole leaves *vs*. epidermal peels). Indeed,
592 dysfunctional stomatal responses in isolated epidermis compared to intact leaves are

593 reported (Mott et al. 2008). Whatever the molecular defect underlying the SL-depleted
594 phenotype at the guard cell level, higher transpiration rates coupled to the about 2.5-fold
595 higher total biomass of *Ljccd7* plants (Liu et al. 2013b) will make SL-depleted plants wilt
596 faster than WT when water becomes limiting.
597
598 *SL synthesis and functions under drought may be organ-specific*
599 Ha et al. argue that SLs are important for drought resistance in Arabidopsis, but that
600 this effect does not apply to roots because the stress-sensitive phenotype of *max* plants does
601 not extend to root growth or development (Ha et al. 2014). Given their leading hypothesis
602 (i.e. that SLs are needed for full resistance, and probably increase under drought stress), and
603 being roots considered the main site of SL production, this observation is puzzling.
604 However the organ-specificity issue was not investigated further by these authors, or the
605 hypothesis confirmed at the metabolic level.
606 In this study we quantified SLs in exudates and extracts of roots under osmotic stress,
607 showing that they significantly decrease regardless of P availability. This correlates with a
608 fast transcriptional down-regulation (within a few hours from the beginning of stress) of the
609 genes encoding biosynthetic enzymes and putative transporters. The data suggests that the
610 shutdown of SL metabolism is actively controlled by the plant at least in the early stress
611 phases, and could be part of the adaptive adjustment process upon stress, rather than being
612 the result of a general metabolic impairment due to – for example - inefficient nutrient
613 absorption. The above result fits to the finding that soil salinity moderately reduces
614 weed-germination stimulating activity of lettuce roots (Aroca et al. 2013). It also explains
615 the observation by Ha and co-workers that the drought-sensitive phenotype of SL mutants is
616 not obvious at the root level: in fact, both WT and mutant plants will have similar, very low
617 SL levels under these conditions. Finally, even if – unexpectedly, given the physiological
618 data - whole shoots of stressed Lotus plants did not seem to contain more SLs than
619 unstressed ones, it is still possible that specific tissues or cell types support very localized
620 SL synthesis under stress. The drought-triggered, localized boost in SL production in the
621 shoot, if true, may be transcriptionally regulated, as it appears from Arabidopsis microarray

622 and qRT-PCR data; *MAX3* and *MAX4* transcripts accumulate in dehydrated WT leaves (Ha
623 et al. 2014). This, in turn, may be needed for full drought resistance, along with the
624 long-term effects (full ABA sensitivity and correct leaf morphology *in primis*) of
625 appropriate steady-state, systemic levels of SLs during development.
626 Even if other mechanistic explanations cannot be excluded, and notwithstanding the fact
627 that SLs in shoots were at the limit of detection, available data suggest as worth testing, e.g.
628 by grafting WT scions over SL-depleted rootstocks, the hypothesis that the main site of SL
629 production may be shifted under stress (from roots to shoots), and that SL contribution to
630 ABA modulation and osmotic stress resistance might also be organ-specific. The most
631 parsimonious hypothesis on the biological meaning of this shift, if true, is that the osmotic
632 stress-triggered drop of root-borne SLs flowing acropetally may be a direct and/or indirect
633 (through ABA) systemic stress signal, which is perceived by the shoot and contributes to a
634 timely closure of stomata. Whether a drought-induced rise of SL levels in specific tissues or
635 cell types of the shoot mediates the effects of such circuit-breaking signal on stomata, and if
636 these effects are indeed mediated by specific ABA importer proteins, is an hypothesis to be
637 directly addressed in a targeted work. Alternatively, the barely detectable SL production
638 may remain stable in all cell types of shoots under drought, in spite of the accumulating
639 gene transcripts, and be sufficient for normal ABA sensitivity under stress; or, the
640 biosynthesis of other SLs or SL-like molecules yet unidentified in Lotus may be induced.
641 Fig. 7 outlines the main demonstrated or plausible avenues of interaction between SLs and
642 ABA during abiotic stress in shoots and roots.
643
644 *The purpose of SL shutdown in roots may be to de-repress local ABA synthesis under stress*
645 In an attempt to understand more on the biological meaning of SL shutdown in roots
646 under osmotic stress, we studied the effect of keeping SLs artificially high in roots of
647 stressed WT plants. We observed that a pre-treatment of WT plants with the synthetic SL
648 analogue GR24 blocked the increase in root ABA content upon subsequent PEG treatment,
649 thus demonstrating a novel effect that adds to the many actions of SLs on plant metabolism.
650 This result suggests that the decrease in 5-deoxystrigol quickly triggered by osmotic stress

651 may be needed in *L. japonicus* to allow the accumulation of ABA induced by the same
652 stress, and is reminiscent of the negative effect of SLs on the thermo-induced ABA
653 accumulation in Arabidopsis seeds (Toh et al. 2012). In that system, the reduction in ABA
654 levels occurred through the down-regulation of the biosynthetic gene *NCED9*. Under our
655 conditions, transcript abundance for one of the putative *LjNCED* orthologues examined
656 (*LjNCED2*) was augmented by PEG treatment but not in the presence of exogenous SLs.
657 This suggests that in Lotus, SLs may inhibit ABA accumulation under stress by interfering
658 with the transcriptional activation of ABA biosynthetic genes; however other gene products
659 than LjNCED2 must be involved, to explain the increase of ABA levels after 1 d of PEG
660 treatment. On the other hand, low SL levels in the roots may be needed (as shown by the
661 GR24-treatment experiment) but certainly do not suffice to induce local ABA accumulation
662 (as shown by the lack of differences in root ABA levels between WT and *Ljccd7* plants,
663 under all tested conditions).
664 To conclude, we confirmed in *L. japonicus* the involvement of SLs in drought resistance
665 reported once in Arabidopsis (Ha et al. 2014) but that others did not observe (Bu et al. 2014).
666 Their role in the phenomenon is multifaceted. On the one hand, chronic SL depletion has a
667 rather complex effect on water balance and physiological responses to water stress at the
668 shoot level. On the other hand, we demonstrated for the first time that a strong
669 down-regulation of SL metabolism in the roots, early during osmotic stress, might be
670 needed (but not sufficient) to allow local ABA production. Since SLs were demonstrated to
671 regulate root architecture in response to P starvation (Koltai 2013; Mayzlish-Gati et al. 2012;
672 Ruyter-Spira et al. 2011), the independency of SL inhibition from P availability makes it
673 worth testing the hypothesis that water deficit may interfere - via SLs - with such responses
674 to P starvation. As a final consideration, the available data suggest that SL synthesis and
675 effects are not only developmentally and environmentally regulated, but also fine-tuned in
676 an organ- and possibly tissue- or cell-specific fashion.

677

678 **Authors' contributions**

679 JL, CL, FC designed the experiments. AS, HJB provided logistic support to the
680 experimental work. JL performed most experiments with occasional help by HH, while MV
681 and IV worked together at the physiological data collection. TC helped with the
682 UPLC-MS/MS assays and IH with the aeroponic cultivation system. FC conceived of the
683 study, supervised it, and analysed the data together with JL, MV, IV and CL. JL and FC
684 wrote the manuscript. IV, AS, CRS, CL and HJB critically revised the manuscript and
685 completed it. All authors read and approved the final manuscript.

686

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699

700 **Conflict of interest**

701 FC, IV and AS hold a share of StrigoLab S.r.l., which provided part of the GR24 used in the
702 study.

703

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944 **Figure legends**

945 **Fig. 1** ABA concentration in root and shoot tissues of WT and SL-depleted (*Ljccd7*) plants
946 under normal or stress conditions. Samples were collected 6 days after the beginning of
947 PEG treatment values are displayed as means ± SD of *n* biological replicates ($n = 3$ for WT,
948 and $n = 4$ for *Ljccd7* plants; each sample is the pool of five individual plants). Different
949 letters on top of bars indicate significantly different means for $P < 0.05$.
950 **Fig. 2** *Ljccd7* plants cope less well than WT with osmotic stress. Representative individuals
951 of the WT (left) or *Ljccd7* (right) populations are pictured after 4 days of PEG-infused
952 osmotic stress. Corresponding leaf water potentials are reported below. Data are the mean of
953 two measurements on three individual plants per genotype, ± SE.
954 **Fig. 3** Physiological responses of WT and SL-depleted (*Ljccd7*) plants to drought stress or
955 exogenous ABA treatment. **a** Root ABA concentration *vs*. water potential in soil. **b** Stomatal
956 conductance *vs*. ABA concentration in roots. **c** Stomatal conductance *vs*. water potential in

957 shoots. Data were obtained on pools of five plants per genotype and point, grown in soil and
958 undergoing progressive dehydration under greenhouse conditions, and are expressed as
959 means ± SE. **d** Average time of stomatal response to treatment with ABA at different
960 concentrations in WT and *Ljccd7* plants. Detached leafy twigs were treated by letting them
961 absorb ABA from the dipping solution through the petiole starting from time 0. The time of
962 response is defined as the time corresponding to first of a continuously dropping series of
963 stomatal conductance values in each treated sample. Data were collected on $n = 3$ WT and n
964 = 3 individual *Ljccd7* plants ± SE. Asterisks indicate significant differences between
965 corresponding time-points in the two genotypes, with ** = $P < 0.01$ and * = $P < 0.05$.
966 **Fig. 4** 5-Deoxystrigol quantification in root exudates and extracts of WT plants under
967 various stress conditions. **a** Time-course of 5-deoxystrigol accumulation as determined by
968 UPLC-MS/MS in root exudates of plants that had been P-starved and/or subjected to
969 PEG-induced osmotic stress for 0, 2, 4 or 6 days.. **b** Quantification of 5-deoxystrigol
970 concentration in root (left-hand bars) and shoot tissues (right-hand bars) under P deficit
971 and/or osmotic stress 6 days after the beginning of treatment. In all panels data are
972 represented as means ± SD of $n = 3$ independent replicates (each replicate is the pool of 5
973 plants). Different letters on top of bars (in **a**) or clustered bars (in **b**) indicate significantly
974 different means for $P < 0.05$.
975 **Fig. 5** qRT-PCR analysis of putative SL biosynthetic genes and transporter in WT Lotus
976 plants under different stresses. **a** Transcript abundance of putative biosynthetic and **b**
977 transporter-encoding genes in roots that had been P-starved and/or subjected to
978 PEG-induced osmotic stress for 6 days. Values are the average of three biological and three
979 analytical replicates ± SE, normalized to *LjUBI* transcript levels. Each biological replicate
980 was the pool of five individual plants. **c** Short-term time-course of transcript abundance for
981 the core SL biosynthetic genes *LjD27*, *LjCCD7* and *LjCCD8* and **d** of the three putative
982 *LjPDR1* paralogues in response to osmotic stress. Values are the average of three biological
983 and two technical replicates ± SE, normalized to *LjUBI* transcript levels. Each biological
984 replicate was the pool of three individual plants. In panels, different letters on top of
985 clustered bars indicate significantly different means for $P < 0.05$.

Fig. 6 Effect of exogenous SL treatment on ABA concentration and on the transcript of the ABA biosynthetic gene *LjNCED2* in roots. **a** Time-course of ABA accumulation in the roots of WT plants kept under -P/+PEG conditions for 0, 1 or 2 days after having been pre-treated or not (+SL or -SL samples, respectively) for 2 additional days with the synthetic SL analogue GR24 (5 µM). Data are displayed as means of three biological replicates ± SD (each replicate was the pool of three individual plants). Different letters on top of bars indicate significantly different means for $P < 0.05$. **b** qRT-PCR analysis of transcript abundance for the ABA biosynthetic gene *LjNCED2* under different conditions. qRT-PCR was performed using cDNA prepared from plants treated as in panel a. The transcript levels were normalized with *LjUBI*, and are displayed relative to the transcript level in mock samples at time 0 (which was therefore set to 1). Values are means of three biological and three analytical replicates ± SE (each biological replicate was the pool of three individual plants). Different letters indicate significantly different means for $P < 0.05$.

Fig. 7 Schematic drawing of the main connections between SLs and ABA in roots and shoots of *L. japonicus* under osmotic stress conditions. Grey lines indicate hypothetic links/events so far suggested by transcriptomic data published in Arabidopsis. In the model, the effects of SLs on ABA levels are negative in the roots, as proven by GR24 treatment; thereby, the shut-off of SL synthesis in this organ under stress should be needed (but not sufficient in the absence of stress) to let ABA levels rise locally. As a likely consequence, SLs flowing acropetally will decrease under stress, while ABA will increase. At the shoot level, the effects of SLs on ABA sensitivity are in turn positive, which explains the drought-sensitive phenotype of SL-depleted plants. Although whole-shoot analyses of SL levels in stressed Lotus do not show a detectable increase of SLs compared to unstressed tissues, localized synthesis may happen. Alternatively, steady-state SL levels are needed and sufficient to ensure wild-type sensitivity to ABA in stressed shoot tissues, or other, yet unidentified SL(-like) molecules may be induced.

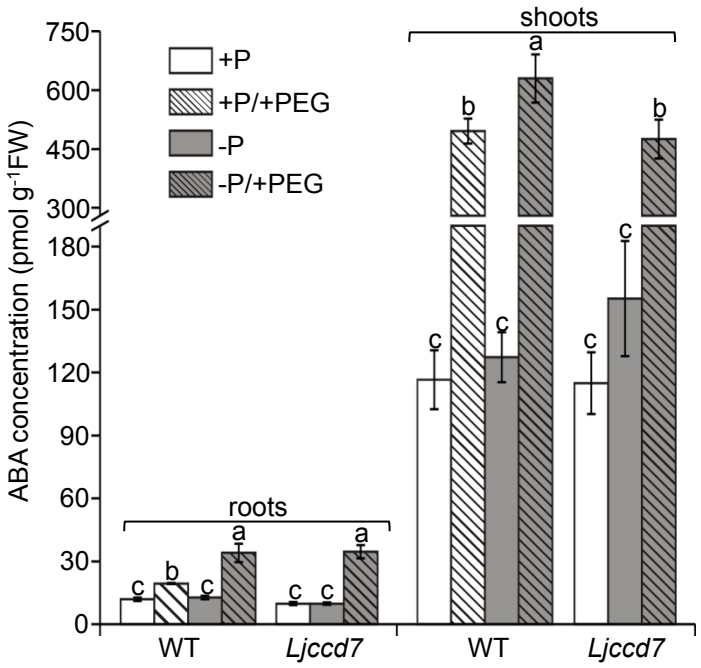

Figure 1
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Ψ	**WT**	***Ljccd7***
(MPa)	-0.75± 0.071	-0.84± 0.068

Figure 2
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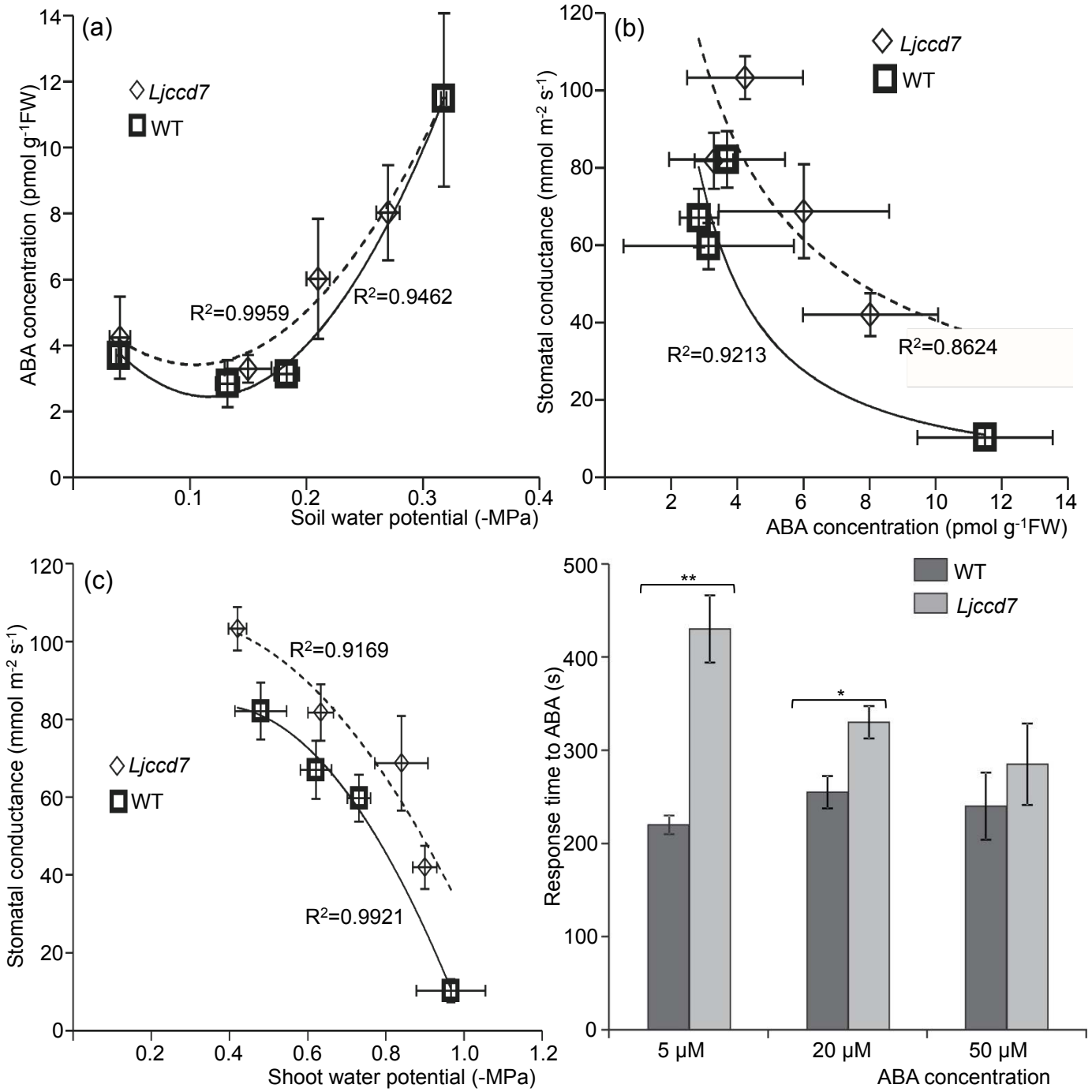

Figure 3
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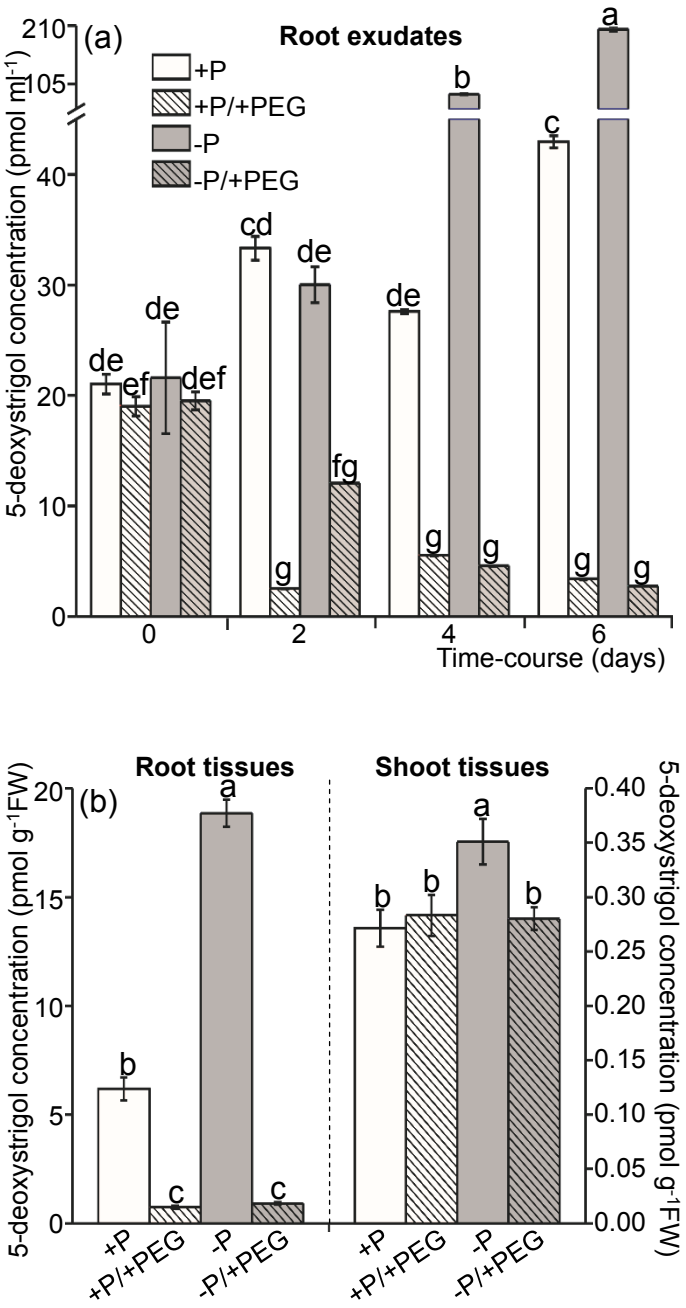

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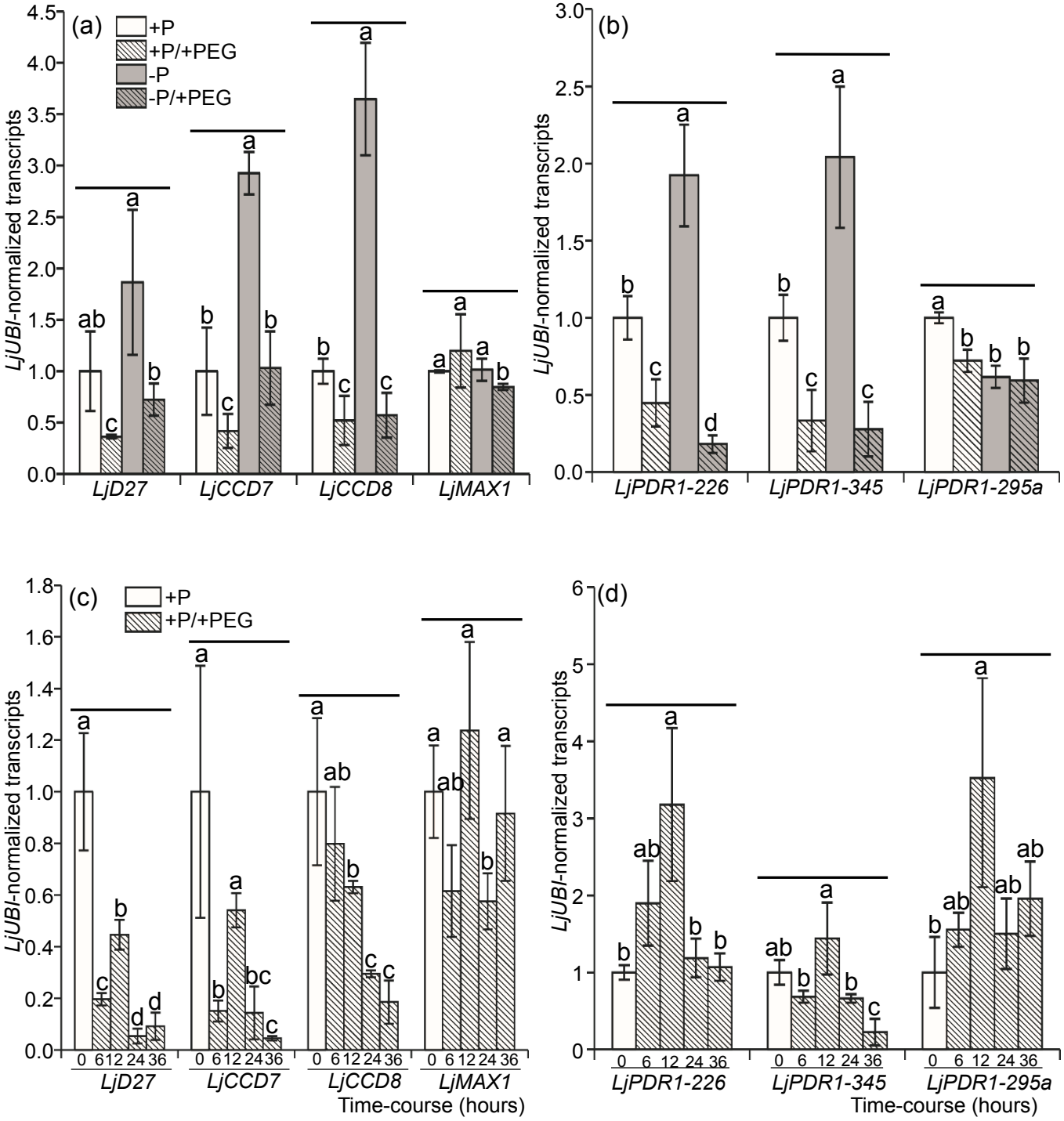

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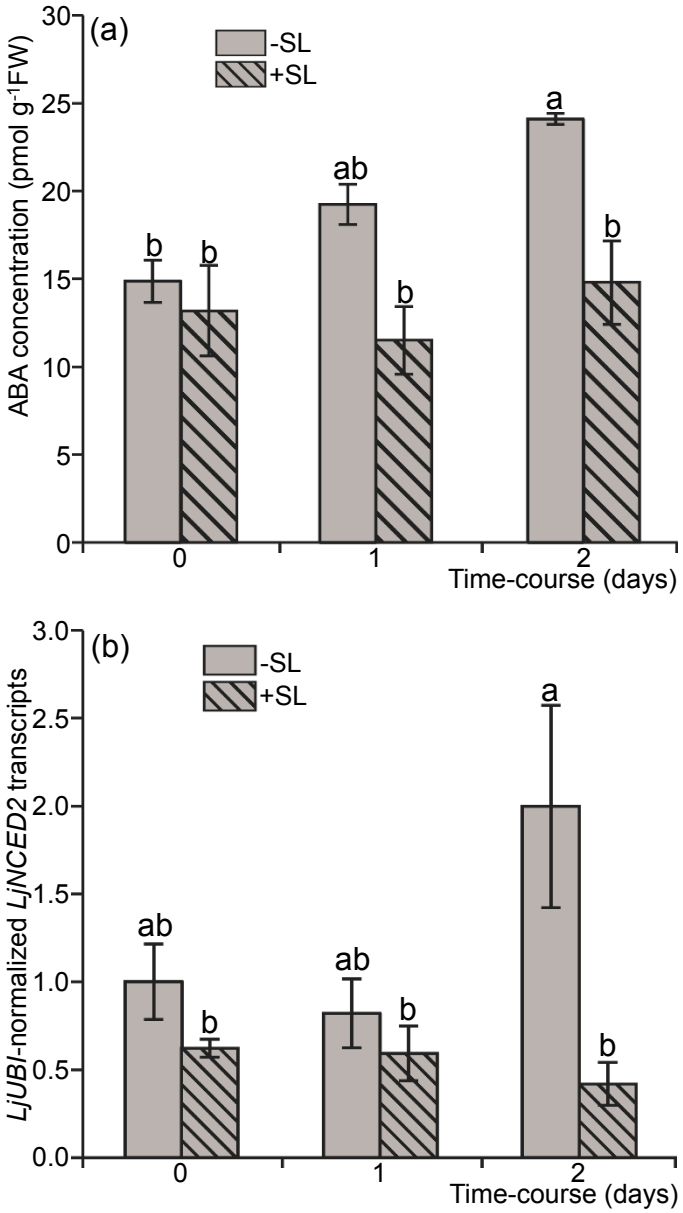

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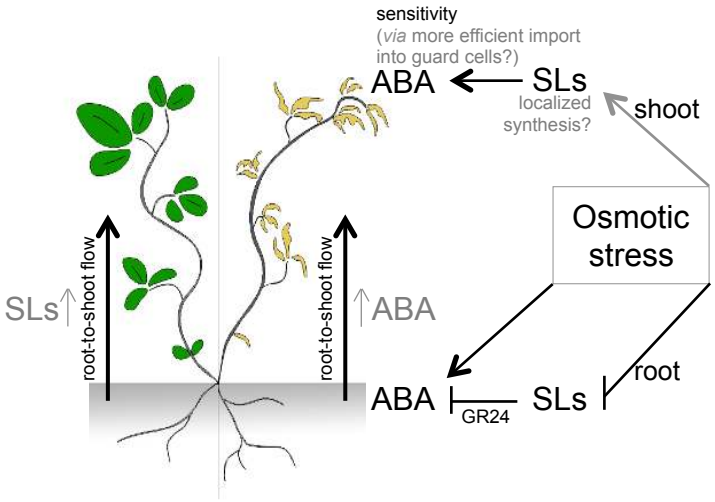

Figure 7
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Table S1. Primers used for gene expression analysis

Putative gene name	BlastP query used	Gene ID in Kasuza database	Primer name	Primer sequence (5'-3')
LjD27	MtD27/AtD27/OsD27	chr1.CM0375.30.r2.m	*qLjD27F*	TCTGCAAAATGCCATCTCAA
			qLjD27R	GCTCGGTCCATGCTGTTTAT
LjCCD7		LjSGA_131670.1	*qLjCCD7F*	GTATGGAGTGTTTAAGATGCCC
			qLjCCD7R	TAAAATGACTGCGTGGAAGC
LjCCD8	PsRMS1/PhDAD1/	chr1.LjB19M02.90.r2.m	*qLjCCD8F*	GTTCTGCCAGATGCTAAGGTTG
	AtCCD8		*qLjCCD8R*	GTTAGGGTTTATGCTGCACATGTC
LjMAX1	PhMAX1/AtMAX1	chr1.CM0133.560.r2.m	*qLjMAX1F*	TCTGGTTAGCGCTTGGAGTT
			qLjMAX1R	TGCAGGGAAAATTTCTGACC
LjPDR1	PhPDR1/AtPDR12/NpPDR1/ GmPDR12	chr3.CM0226.120.r2.m	*qLjPDR1-226F*	GTCCTGTGGCTTGGACTATCTAT
			qLjPDR1-226R	CAACAACCACAACTGCACAA
		chr5.CM0345.1620.r2.m	*LjPDR1-345F*	CATGATGGGATTTGATTGGTC
			LjPDR1-345R	ATATTGCGTAGAATGCCGATG
		chr1.CM0295.1210.r2.a	*LjPDR1-295aF*	ATGATTGGTTATGAATGGACTGTG
			LjPDR1-295aR	ATGAATCCTGAGAAGAGATTCCAC
		chr1.CM0295.1190.r2.a	*LjPDR1-295bF*	ATCACAGTCTCTGGGCATCC
			LjPDR1-295bR	ATGTCAGGGGACAATCGAAG
LjNCED1	PsNCED1	LjSGA_052946.1	*qLjNCED1F*	ATGATCTTGAGCATCAGTGGTTT
			qLjNCED1R	AGAAAATGGGTCAACTTTTGGAT
LjNCED2	PsNCED2	chr1.CM0794.180.r2.d	*qLjNCED2F*	ACAGGCGAGGTAAAGAAGTACC
			qLjNCED2R	ATGGTAGCCTCCAACTTCAAAT
LjNCED3	PsNCED3	LjSGA_102649.1	*qLjNCED3F*	TCCCTCAGTGTTCCAATTCC
			qLjNCED3R	CGAACATGTCTAGGGCCATT
LjABA2	AtABA2	LjSGA_036557.1	*qLjABA2F*	GTTTGGCCTTGGCTCATTT
			qLjABA2R	ACCTCGCATCATCACTTGC
LjABA3	AtABA3	chr3.CM0634.640.nc	*qLjABA3F*	ACATGCAGAGATGCAAGGAGT
			qLjABA3R	GTCTGCCTCCAAATACCACAA
LjAAO3	AtAAO3	chr2.CM0545.610.nd	*qLjAAO3F*	AGTGGGCATCACCAGCAT
			qLjAAO3R	TTTACCACCGGCATGGTT
LjCYP707A1	AtCYP707A3	chr2.CM0803.690.r2.m	*LjCYP707A1F*	GCACAGATCGTAGCCCAAAT
	PrCYP707A1		*LjCYP707A1R*	CACGTAAGCACACTGGCTGT
LjUBI			*qLjUBIF*	TTCACCTTGTGCTCCGTCTTC
			qLjUBIR	AACAACAGAACACACAGACAATCC

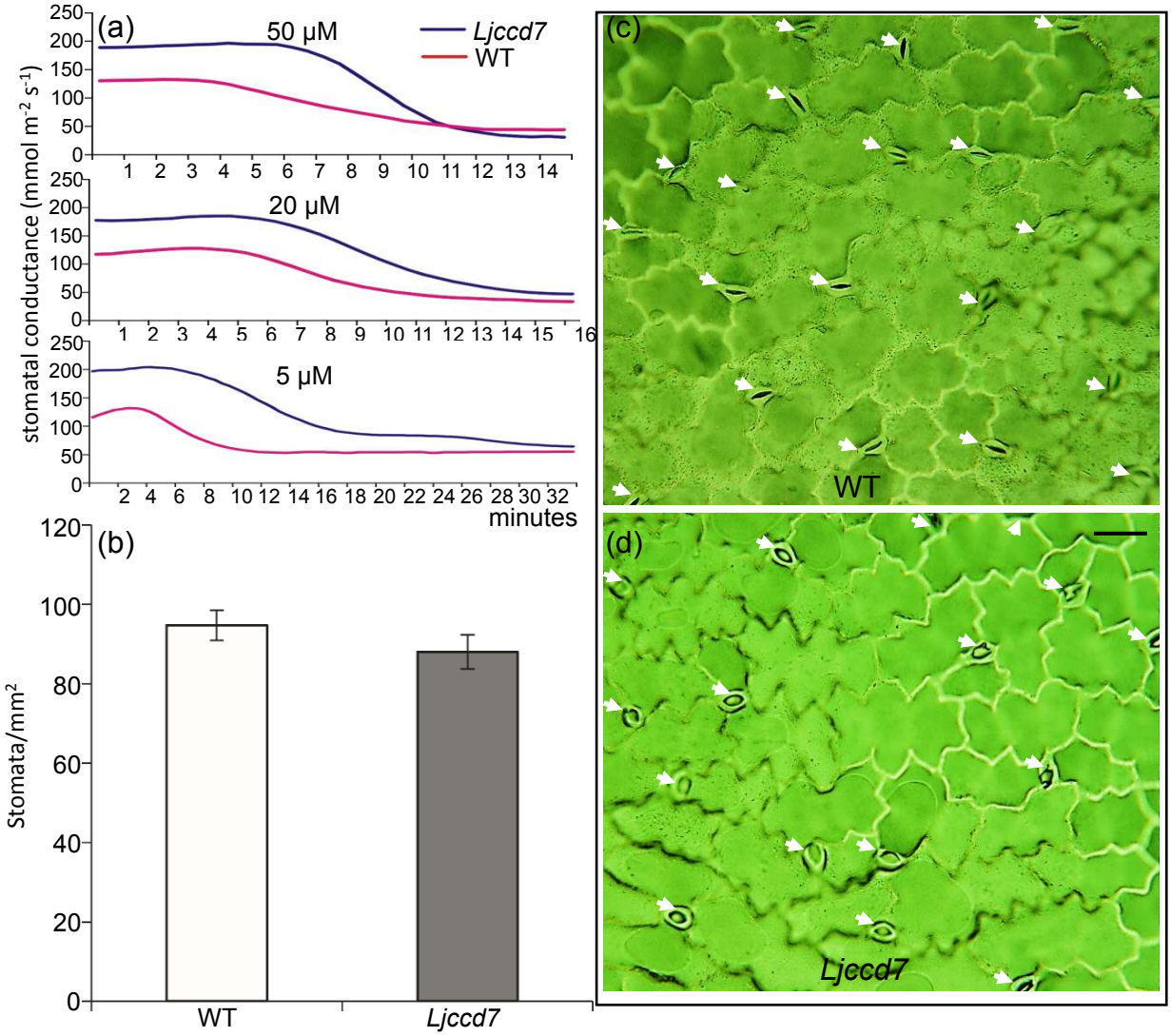

Fig. S1 Phenotype under osmotic stress and stomata density for WT and *Ljccd7* plants. **a** Representative curves of stomatal conductance values after treatment of detached leafy twigs with different ABA concentrations (5, 20, 50 µM). ABA was added to the dipping solution at time zero, after stomatal conductance had stabilized. Measurements were taken every 30 seconds on both WT and *Ljccd7* leaf tissues. **b** Stomatal density, expressed as number of stomata per surface unit, was analysed on the leaf abaxial epidermal layers from WT and *Ljccd7* plants. Leaves of similar physiological age and position in the plant were collected. Data are the mean of 2 counts on 15-20 individual plants per genotype. **c-d** Representative images of leaf abaxial epidermal layers from 8-week-old WT (**c**) and *Ljccd7* plants (**d**) aeroponically cultivated under 16-h diurnal photoperiod and in Hoagland solution. Stomata are pointed by red arrows. Bars = 50 µm.

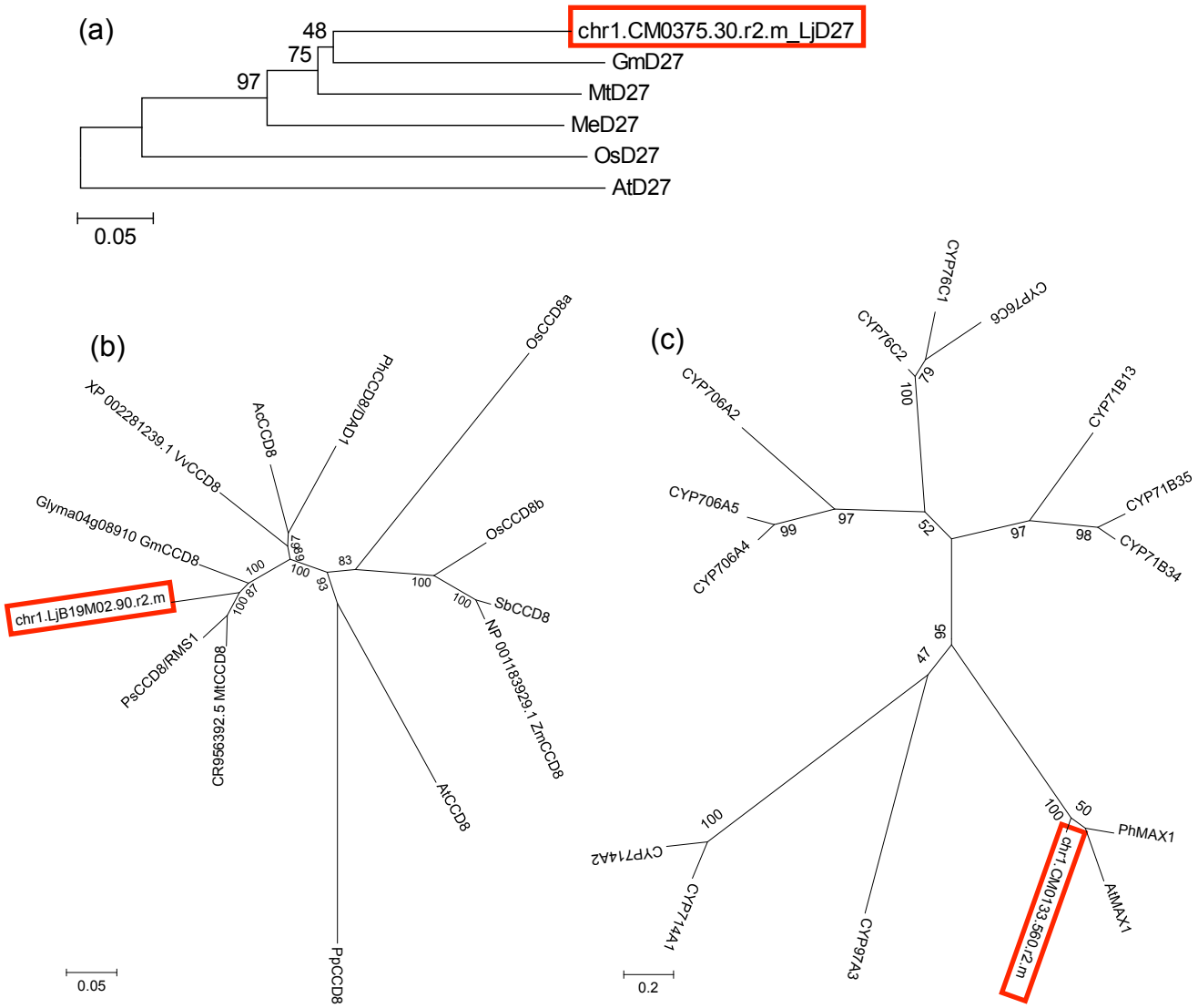

Fig. S2 Unrooted phylogenetic tree of the putative SL biosynthetic enzymes of Lotus LjD27, LjCCD8 and LjMAX1 (boxed in red). The predicted protein sequences were clustered using ClustalX 2.0 and MEGA 5.1. Phylogenetic relationships were calculated using the maximum-likelihood principle, and bootstrap values with 1000 replicates were determined. Scale bars give the number of substitutions per site. **a** LjD27 phylogenetic tree. Accession numbers used: AtD27 (NP_563673), GmD27 (BXP_003536919), MeD27 (TA7061_3983, from the TIGR plant transcript assemblies database), MtD27 (AEW07379.1), OsD27 (ABA94460). **b** Phylogenetic tree of LjCCD8. Accession numbers used: AcCCD8 (NP_001183929), AtCCD8 (AEE86121), GmCCD8 (NP_001242715), MtCCD8 (CR956392.5), OsCCD8a (AP003296 .3), OsCCD8b (AP003376 .3), PhCCD8/DAD1 (AY743219), PpCCD8 (ADK36681), PsCCD8/RMS1 (AY557341.1), SbCCD8 (XP_002458477.1), VvCCD8 (XP_002281239.1), ZmCCD8 (NP_001183929). **c** Phylogenetic tree of LjMAX1. Accession numbers used: AtMAX1 (AEC07803), CYP71B13 (AED93405.1), CYP71B34 (EFH53221.1), CYP71B35 (AEE77144.1), CYP76C1 (AEC10571.1), CYP76C2 (EFH58280.1), CYP76C6 (AEE31618.1), CYP97A3 (Q93VK5.1), CYP706A2 (AEE84642.1), CYP706A4 (EFH51020.1), CYP706A5 (AEE83113.1), CYP711A1 (At2g26170), CYP714A1 (AED93377.1), CYP714A2 (AED93376.1), PhMAX1 (AEB97383).

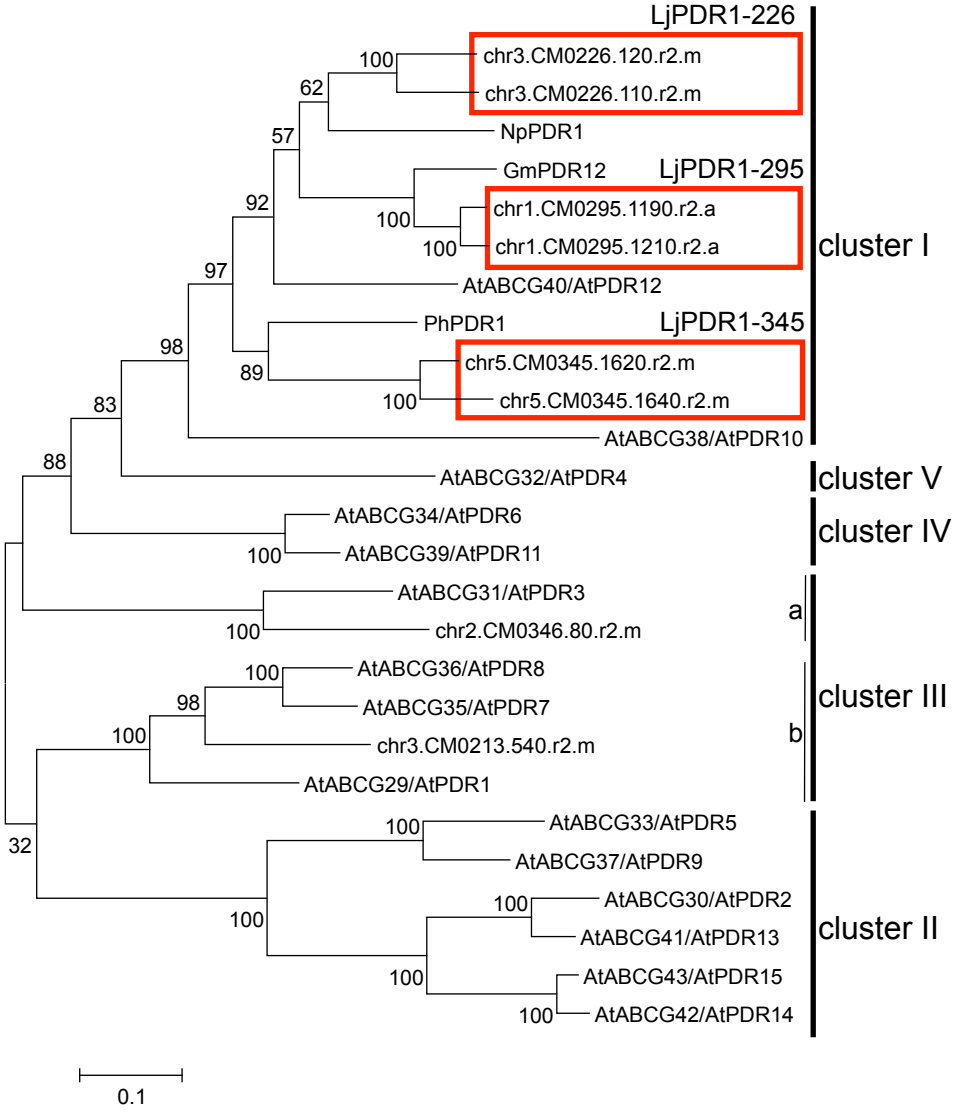

Fig. S3 Phylogenetic positioning of Lotus LjPDR1 in relation to GmPDR12, NpPDR1, PhPDR1, and the Arabidopsis full size ABCG/PDR subfamily of ABC transporters. The predicted protein sequences were clustered using ClustalX 2.0 and MEGA 5.1. Phylogenetic relationships were calculated using the maximum-likelihood principle, and bootstrap values with 1000 replicates were determined. Scale bar gives the number of substitutions per site. The putative Lotus homologues of PDR1 are boxed in red, and are the candidates matching best to PhPDR1 and AtPDR12. Accessions: AtPDR1 (BK001001), AtPDR2 (BK001000), AtPDR3 (BK001002), AtPDR4 (BK001003), AtPDR5 (BK001004), AtPDR6 (BK001005), AtPDR7 (BK001006), AtPDR8 (BK001007), AtPDR9 (BK001008), AtPDR10 (BK001009), AtPDR11 (BK001010), AtPDR12 (BK001011), AtPDR13 (BK001012), AtPDR14 (BK001013), AtPDR15 (BK001014), GmPDR12(NP_001237697), NpPDR1 (CAC40990), PhPDR1 (JQ292813). Clustering is adapted from Kretzschmar et al., 2012, Suppl. Fig. 3b.

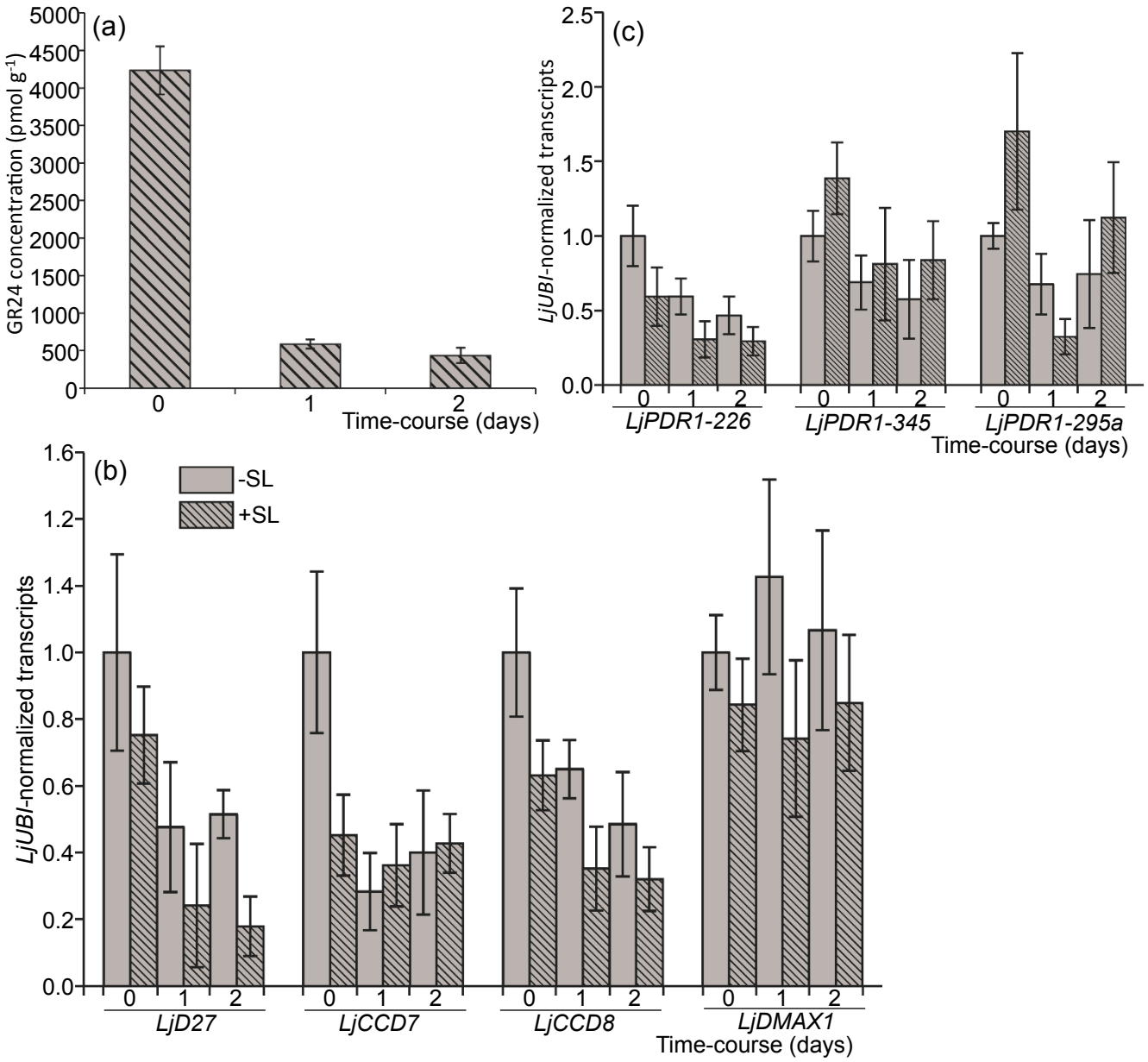

Fig. S4 Uptake control for GR24 in Lotus roots, and response of SL biosynthetic and transporter genes to GR24 application. **a** UPLC-MS/MS analysis of GR24 uptake and accumulation by Lotus roots 0, 1 or 2 days after the beginning of PEG treatment, which correspond also to the time elapsed after a 2-day pre-treatment with GR24. Data are displayed as means of three biological replicates ± SD (each replicate was the pool of three individual plants). **b** qRT-PCR analysis of the putative Lotus homologues of the SL biosynthetic genes *LjD27*, *LjCCD7* and *8*, *LjMAX1* and **c** PDR1-encoding *LjPDR1-226, -345* and *-295a* in the presence (+SL) or absence of GR24 (-SL) in the same experiment as in **a**. Data are expressed as the average of three biological and three analytical replicates ± SE, normalized to *LjUBI* transcript levels and to time 0 of –SL sample, which is therefore set to 1. Each biological replicate was the pool of three individual plants.

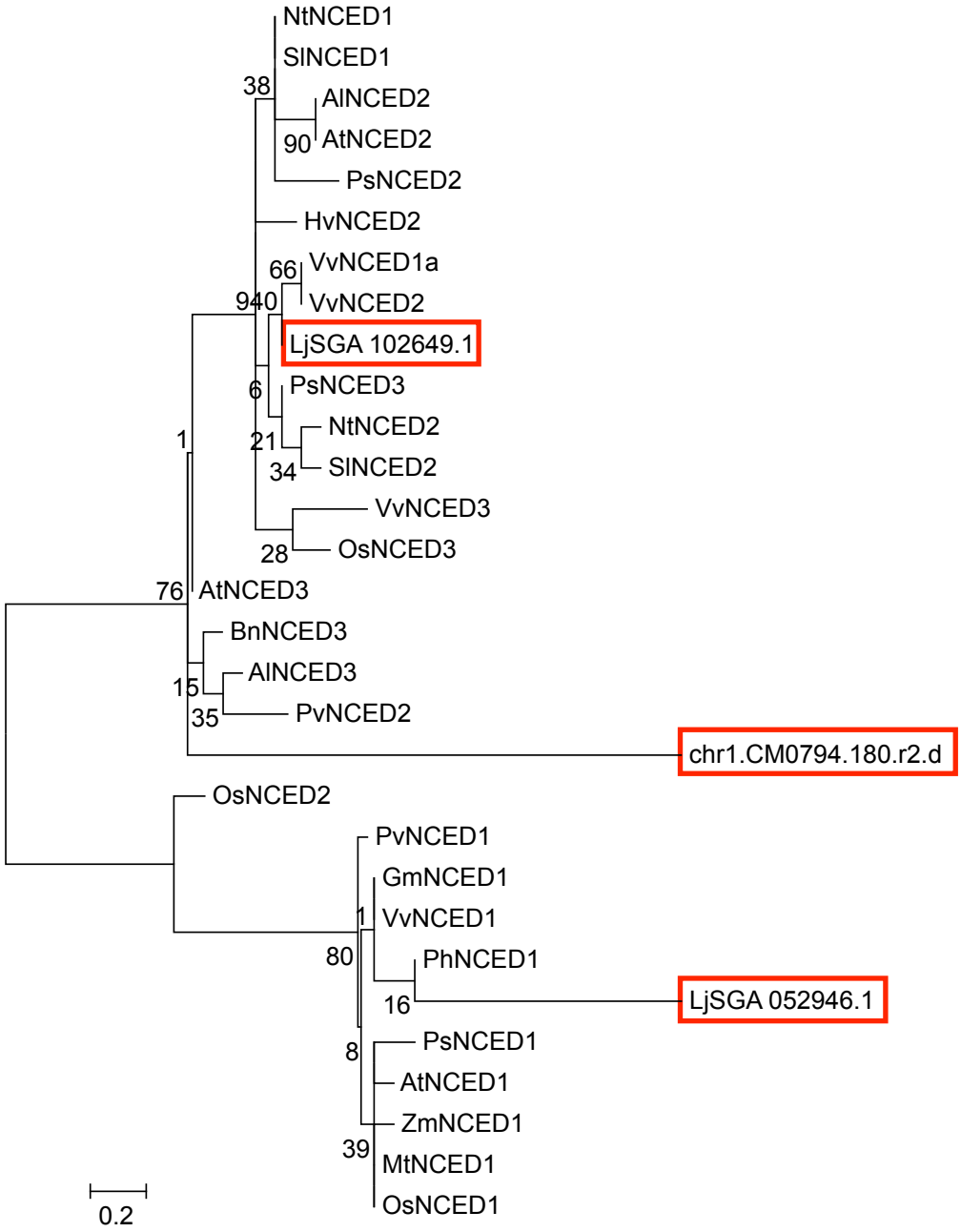

Fig. S5 Phylogenetic positioning of Lotus NCED1 (LjSGA_052946.1) NCED2 (chr1.CM0794.180.r2.d) and NCED3 (LjSGA_102649.1). The predicted protein sequences were clustered using ClustalX 2.0 and MEGA 5.1. Phylogenetic relationships were calculated using the maximum-liklihood principle, and bootstrap values with 1000 replicates were determined. Scale bar gives the number of substitutions per site. The putative Lotus homologues of NCEDs are boxed in red. Accessions used for NCED1: AtNCED1 (NP_191911.1), GmNCED1 (XP_003542847.1), MtNCED1 (CAR57918.1), NtNCED1 (AFP57677.1), OsNCED1 (ABA99623.2), PhNCED1 (AAT68189.1), PsNCED1 (Q8LP17.1), PvNCED1 (Q94IR2.1), SlNCED1 (CAD30202.1), VvNCED1 (XP_002278750.1), VvNCED1a (XP_003633030.1), ZmNCED1 (ABF85668.1); for NCED2: AlNCED2 (XP_002870052.1), AtNCED2 (O49505.1), HvNCED2 (ABB71584.1), NtNCED2 (AFP57678.1), OsNCED2 (AAW21318.1), SlNCED2 (ACL00683.2), PsNCED2 (BAC10550.1), PvNCED2 (AAY82457.1), VvNCED2 (AAR11194.1); for NCED3: AlNCED3 (EFH61304.1), AtNCED3 (Q9LRR7.1), BnNCED3 (AEN94304.1), OsNCED3 (AAW21319.1), PsNCED3 (BAC10551.1), VvNCED3 (AFP28804.1).

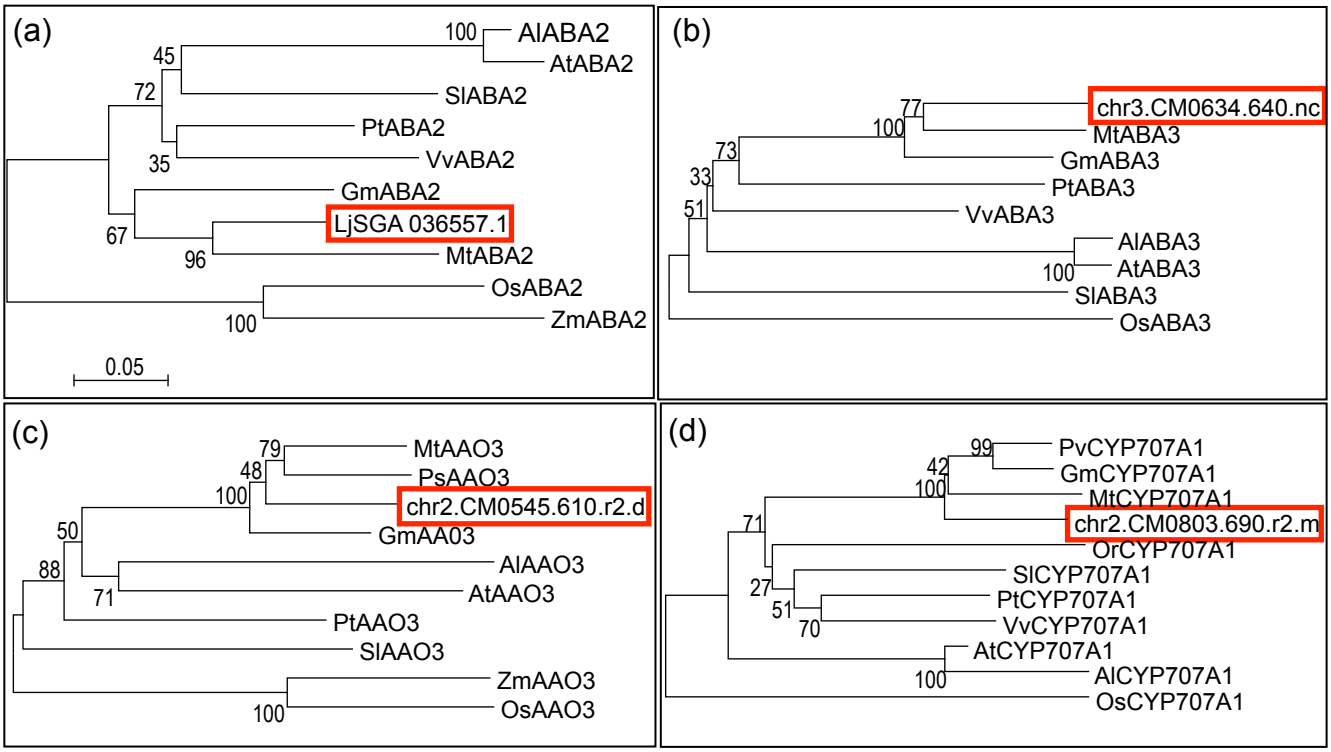

Fig. S6 Phylogenetic positioning of Lotus ABA2 (**a**), ABA3 (**b**), AAO3 (**c**) and CYP707A1 (**d**). The predicted protein sequences were clustered using ClustalX 2.0 and MEGA 5.1. Phylogenetic relationships were calculated using the neighbor-joining principle, and bootstrap values with 1000 replicates were determined. Scale bar gives the number of substitutions per site. The putative Lotus homologues are boxed in red, and are the candidates matching best to the corresponding leguminous orthologs. Accessions used for ABA2: AlABA2 (XP_002894375.1), AtABA2 (NP_175644.1), GmABA2 (XP_003539230.1), MtABA2 (XP_003598766.1), OsABA2(NP_001051666.1), PtABA2(ACE97459.1), SlABA2(XP_004237828.1), VvABA2(XP_002265724.1), ZmABA2(NP_001148513.1); for ABA3: AlABA3 (XP_002890171.1), AtABA3 (NP_564001.1), GmABA3 (XP_003534435.1), MtABA3 (XP_003605400.1), OsABA3 (Q655R6.2), PtABA3 (XP_002310102.1), SlABA3 (NP_001234144.1), VvABA3 (CBI21736.3); for AAO3: AlAAO3 (XP_002877283.1), AtAAO3 (NP_180283.1), GmAAO3 (XP_003519469.1), MtAAO3 (XP_003617051.1), OsAAO3 (NP_001105308.1), PsAAO3 (ABS32110.1), PtAAO3 (EEE71517.1), SlAAO3 (NP_001234456.1), ZmAAO3 (NP_001105308.1); for CYP707A1: AlCYP707A1 (XP_002869993.1), AtCYP707A1 (NP_567581.1), GmCYP707A1 (NP_001237490.1), MtCYP707A1 (XP_003612492.1), OsCYP707A1 (Q05JG2.1), OrCYP707A1 (AFP74114.1), PtCYP707A1 (XP_002328843.1), PvCYP707A1 (ABC86558.1), SlCYP707A1 (XP_004244436.1), VvCYP707A1 (XP_002269405.2).

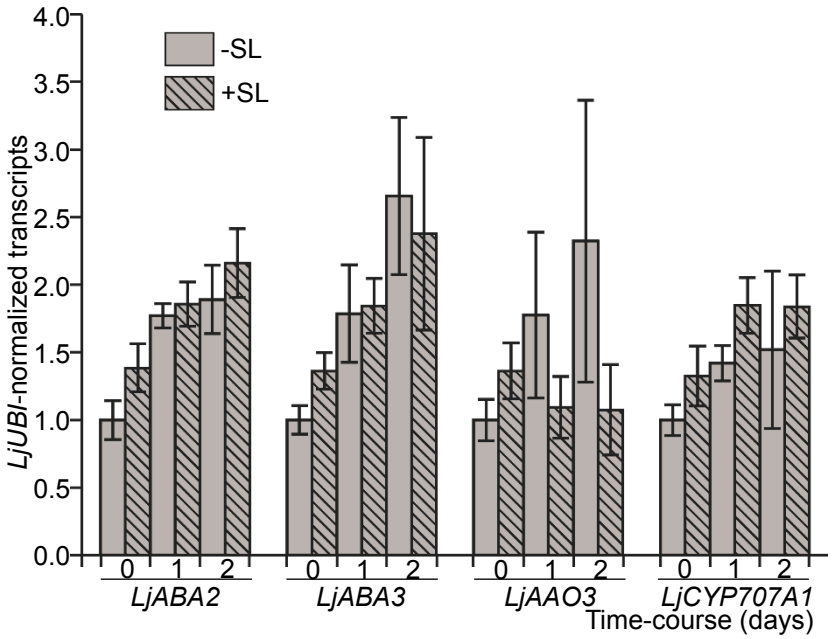

Fig. S7 qRT-PCR analysis of the putative Lotus homologues of the ABA biosynthetic genes *ABA2*, *ABA3* and *AAO3* and of the catabolic gene *CYP707A1* after 2 days exposure (+SL) or not (-SL) to GR24 under –P conditions, followed by PEG treatment starting at time 0. Data are expressed as the average of three biological and three analytical replicates ± SE, normalized to *LjUBI* transcript levels and to time 0 (-SL), which is therefore set to 1. Each biological replicate was the pool of five individual plants.