Strigolactones: mediators of osmotic stress responses with a potential for agrochemical manipulation of crop resilience

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1 **Strigolactones: mediators of osmotic stress responses with a potential for agrochemical**

2 **manipulation of crop resilience**

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17 **Running title:** Strigolactones in osmotic stress resistance and mitigation

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Highlight

We review the role and regulation of strigolactones during osmotic stress, namely on organ-specific dynamics of synthesis and interaction with abscisic acid and on their potential for crop protection.

29

Abstract

After quickly touching upon general aspects of strigolactones biology and functions, including structure, synthesis and perception, this review focuses on the role and regulation of the strigolactone pathway during osmotic stress, in light of the most recent research developments. We discuss available data on organ-specific dynamics of strigolactone synthesis and interaction with abscisic acid in the acclimatization response, with emphasis on the ecophysiological implications of the effects on the stomatal closure process. We highlight the importance to consider roots and shoots separately as well as combined *vs* individual stress treatments; and to perform reciprocal grafting experiments to work out organ contributions and long-distance signalling events and components under more realistic conditions. Finally, we elaborate on the question of if and how synthetic or natural strigolactones, alone or in combination with crop management strategies such as grafting, hold potential to maximise crop resilience to abiotic stresses.

42 **Key words**

43 Abscisic acid, Drought, Hormone cross-talk, Osmotic stress, Resilience, Root-shoot

44 communication, Stomata closure, Strigolactones

45

46

47 **Abbreviations**

48 ABA: Abscisic Acid

49 ABCG: ABC Transporter G Family Protein

50 ABI: ABA Insensitive

51 AM: Arbuscular Mycorrhizal

52 CCD: Carotenoid-Cleavage Dioxygenase

53 D: Dwarf

54 DAD: Decreased Apical Dominance

55 HAB: Hypersensitive to ABA

56 HTD: High Tillering and Dwarf

57 IPA: Ideal Plant Architecture

58 KAI: Karrikin Insensitive

59 KL: KAI2 Ligand

60 LBO: Lateral Branching Oxidoreductase

61 LGS: Low Germination Stimulant

62 MAX: More Axillary Growth

63 N: Nitrogen

64 NCED: Nine-*Cis*-Epoxycarotenoid Dioxygenase

65 ORA: Octadecanoid-Responsive AP2/ERF-domain transcription factor

66 P: Phosphate

67 PDR: Pleiotropic Drug Resistance

68 PIN: PIN-formed

69 PPP: Plant Protection Product

70 RMS: Ramosus

71 SL: Strigolactone(s)

72 SLAC: Slow Anion Channel-Associated

73 SMAX: Suppressor of MAX2

74 SMXL: SMAX-Like

75 TPL: Topless

76 TPR: TPL-related

77

1. Introduction

The quest for Strigolactones (SL) as endogenous regulators of plant development started when mutants affected in shoot development, displaying stunted and bushy phenotypes, were identified in a number of model species: *Oryza sativa*, rice (*d*, *dwarf*; or *htd*, *high tillering and dwarf* mutants), *Petunia hybrida*, petunia (*dad*, *decreased apical dominance*), *Arabidopsis thaliana*, Arabidopsis (*max*, *more axillary growth*), *Pisum sativum*, pea (*rms*, *ramosus*) (Waters *et al.*, 2017). These phenotypes were quickly shown not to be due to mutations in any known developmental pathway, and to be related to a novel kind of mobile signal molecules mainly but not exclusively produced in roots. From there, these compounds would be transported to the shoot to inhibit branching, contrasting cytokinin while reinforcing auxin activity on axillary buds. Such molecules were identified in 2008 as SL (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008), a family of lactone derivatives of carotenoids, produced in roots and exuded in soil, first detected in 1966 (Cook *et al.*, 1966) and identified a few years later (Cook *et al.*, 1972). Besides their endogenous role in the control of shoot branching, SL have several demonstrated functions in the rhizosphere, all favoured by the steep SL gradient around the root, which makes the presence of SL in soil a reliable indicator of proximity to a living plant root. Indeed, SL are rather labile molecules due to inherent instability of the enol-ether bond between ring C and D (Figure 1), whose integrity is essential for bioactivity (§ 2.1) (Al-Babili and Bouwmeester, 2015). Such exogenous signalling roles include stimulation of seed germination in parasitic plants belonging to the genera *Striga* and *Orobanche* (some former species of which now belong to the genus *Phelipanche*) – an obviously detrimental outcome for the producing plant. A second, indirect positive effect on plant mineral nutrition was proven in 2005, when SL exuded in soil were shown to trigger hyphal branching in arbuscular mycorrhizal (AM) fungi, thus increasing the chances of contact between the symbionts (Akiyama *et al.*, 2005). More recently, stimulating effects of SL on rhizobial swarming and on infection thread formation were also suggested to favour nodulation in legumes (Lopez-Raez *et al.*, 2017) (see (Lumba *et al.*, 2017b) for a graphical timeline of SL-related discoveries).

After the identification of the endogenous hormonal role of SL, further pervasive effects in the producing plant were assigned to this molecular family, comprising at present about 20 described molecular structures (Al-Babili and Bouwmeester, 2015). Reproduction (including flower and seed setting in several species), senescence, and secondary growth are all seemingly promoted by SL to various extents (especially based on the defects of SL-depleted or insensitive plants) (Brewer *et al.*, 2013). Also, their involvement in abiotic stress responses was highlighted by the initial observation of their inducibility by N and especially P deprivation; and later, by phenotypic comparison of mutant plants under nutritional stress. These studies proved that part of the molecular and morphological responses needed for acclimatization to a nutritionally poor environment are indeed

113 mediated by SL (Marzec *et al.*, 2013). More recently though, it has appeared that SL may also be
114 one of the endogenous molecular workings in acclimatization responses to water deprivation,
115 possibly the major environmental constraint to crop productivity. This fact, given also their strong
116 developmental effects, places SL in an optimal position to act as an integration hub between
117 environmental stimuli and endogenous cues, favouring proper resource allocation decisions by the
118 plant (Liu *et al.*, 2013).

119

120 The above-mentioned general aspects of SL biology and functions are covered in detail by other
121 reviews (Al-Babili and Bouwmeester, 2015; Lumba *et al.*, 2017a; Lumba *et al.*, 2017b; Makhzoum *et*
122 *al.*, 2017). In this review, we provide a quick overview on structure, synthesis, transport, and
123 perception of SL, and we focus thereafter on the role and regulation of the SL pathway during
124 osmotic stress. We discuss available data on organ-specific dynamics of SL synthesis and
125 interaction with abscisic acid (ABA) in the response process, highlighting the importance to
126 consider roots and shoots separately as well as to compare combined *vs* individual stress
127 treatments, to simulate more realistic conditions; and to perform reciprocal grafting experiments to
128 work out organ contributions and long-distance signalling events and components. Finally, we
129 discuss if and how synthetic or natural SL, alone or in combination with crop management
130 strategies such as grafting, may contribute to maximise crop resilience to abiotic stress.

131

132 **2. General structure, biosynthesis, transport and signal transduction of SL**
133 *2.1 Structure*
134 The term SL was proposed in 1995 to indicate a group of terpenoid derivatives sharing a conserved
135 lactone ring and able to induce seed germination in *Striga hermontica*, a holoparasitic plant that,
136 together with other *Orobanchaceae*, imposes huge yield losses in several crops worldwide
137 (Fernandez-Aparicio *et al.*, 2011). Most, though not all, SL analysed so far are characterized by a 4-
138 ring structure, in which the AB and C rings are condensed in a tricyclic lactone, while ring D is a
139 butenolide bound to ring C by an enol ether bridge (Al-Babili and Bouwmeester, 2015; Lumba *et al.*,
140 2017a) (Figure 1). Substitutions on ring A and stereochemistry of the B-C junction make up most of
141 the diversity within the family, with β- and α-oriented C rings being typical of strigol- and
142 orobanchol-like compounds, respectively; while both subgroups share the *R* orientation of C-2′
143 (Figure 1). Structure-activity relationship studies on natural and synthetic variants of SL indicate
144 that the bioactiphore includes the C and D rings and the connecting enol-ether bridge (Lumba *et al.*,
145 2017a), while the D ring alone is proposed to become part of the activated receptor complex (*vide*
146 *infra*, § 2.4). Racemic (*rac*) GR24, the most commonly used synthetic analogue of SL, is composed of

147 the equimolar mixture of the two enantiomers GR24^{5DS} (with the same stereochemistry as strigol)

148 and GR24$^{ent\text{-}5DS}$ (with stereochemistry at 2'S not occurring in natural SL; Figure 1).

149 While it the structural diversity of naturally occurring SL has been described at least in part, its

150 biological and ecological meaning is largely unexplained yet. In plant species that interact with AM

151 fungi or parasitic plants, co-evolution with the guest, be it friend or foe, might justify the drive to

152 diversification of molecular signals. However, there is no proof that such diversity is only targeted to

153 rhizosphere partners. Indeed, the possibility that multiple endogenous SL within a single species

154 may induce different responses due to specificities in perception or localization has not been

155 addressed experimentally yet. Future studies will test whether different SL regulate different

156 processes within a single species, but high quantities of natural SL are hard to obtain, given that the

157 daily production rate is very low (in the picomoles/plant/day range) (Yoneyama *et al.*, 2010).

158 *2.2 Biosynthesis*

159 A combination of pharmacological and forward genetic strategies reconstructed a basic SL-

160 biosynthetic module highly conserved across species, and composed of the plastid-localized, iron-

161 binding carotenoid isomerase named D27 in rice; of carotenoid cleavage dioxygenase 7 (CCD7)

162 (Arabidopsis MAX3, rice D17/HTD1, pea RMS5, and petunia DAD3); and of CCD8 (Arabidopsis

163 MAX4, rice D10, pea RMS1, and petunia DAD1) (Al-Babili and Bouwmeester, 2015). These three

164 enzymes act sequentially to produce carlactone, a compound sharing with SL the number of C

165 atoms and the presence of a butenolide ring (Figure 2). It is actually debated whether carlactone

166 should be considered a true ("canonical") SL or not, given the lack of B and C rings; nonetheless, its

167 identification as a product of the concerted action of D27, CCD7 and CCD8 solved the core SL-

168 synthesis pathway, providing the missing molecular link between linear carotenoids and tricyclic SL,

169 and pointing to CCD8 as an unusual CCD able to perform multiple operations on its substrate

170 (Bruno *et al.*, 2017).

171 The subsequent steps leading to the mature SL structures are less clearly defined, and might vary

172 substantially in different species. The cytochrome P450 MAX1 in Arabidopsis converts carlactone to

173 carlactonoic acid, which undergoes further methylation by an unknown methyltransferase (Abe *et*

174 *al.*, 2014; Seto *et al.*, 2014). The resulting methyl carlactonoate needs further oxygenation by an

175 oxidase such as LBO (Lateral Branching Oxidoreductase) to become bioactive (Brewer *et al.*, 2016).

176 In rice instead, one of the four functional MAX1 orthologues (Os900) acts as a carlactone oxidase,

177 catalysing the formation of the condensed B and C rings to give 4-deoxyorobanchol. Os1400,

178 another MAX1 paralogue, can then form orobanchol from 4-deoxyorobanchol (Zhang *et al.*, 2014).

179 In sorghum, functional loss of the putative sulfotransferase LOW GERMINATION STIMULANT1

180 (LGS1) converts the dominant SL in root exudates from 5-deoxystrigol to orobanchol, via an

181 unknown mechanism (Gobena *et al.*, 2017).

182 Therefore, our current understanding of the SL biosynthetic pathway indicates that the natural
183 diversity of SL, which is obvious among species but may be also represented in the same plant by a
184 blend of different SL, originates mainly from the action of modifying enzymes downstream of the
185 core set formed by D27, CCD7, CCD8 and MAX1. These late-acting enzymes are proving hard to
186 identify, possibly because their expression patterns do not necessarily overlap if intermediates are
187 mobile (*vide infra*), and/or because the corresponding mutants have weak phenotypes, and/or
188 because enzyme redundancy masks their molecular, physiological or morphological defects totally
189 or in part (Al-Babili and Bouwmeester, 2015).

190 In spite of the analytical difficulties due to the very low concentrations, evidence collected so far
191 indicates that SL synthesis is highest in roots, especially tips and vasculature (Al-Babili and
192 Bouwmeester, 2015). Grafting experiments and tracking of SL and of the SL analogue GR24 showed
193 that SL (or their precursors) move from the root to the shoot (Domagalska and Leyser, 2011; Kohlen
194 *et al.*, 2011; Sasse *et al.*, 2015; Xie *et al.*, 2015). However, SL may also be synthesized in stem nodes
195 as well as along the shoot vasculature (Lopez-Obando *et al.*, 2015). Local synthesis aboveground is
196 sufficient for SL-dependent shoot phenotypes, as shown by grafting experiments (Foo *et al.*, 2001;
197 Sorefan *et al.*, 2003; Visentin *et al.*, 2016). SL synthesis in shoots, possibly in leaves, was also
198 proposed to be important for the regulation of guard cell sensitivity to ABA and for proper response
199 to water deprivation (Visentin *et al.*, 2016) (see § 3). However, conclusive proof - beyond SL-
200 biosynthetic gene activation - that leaf tissues are, or not, a true SL source is still missing. Such
201 proof will likely not come until markers (transcriptional or FRET-based for example, as for ABA)
202 (Jones, 2016) are described, that could be used to localize SL synthesis/activity at or close to the
203 single-cell level; and/or until methods are developed to reliably quantify individual SL in small tissue
204 portions or individual cell types such as axillary buds or stomata.

205 **2.3 Transport**
206 The ABCG protein Pleiotropic Drug Resistance1 (PDR1) of *Petunia hybrida* is the only *bona fide* SL
207 transporter characterized thus far (Figure 2). The defective mycorrhizal phenotype of *pdr1* mutants
208 (Kretzschmar *et al.*, 2012) compared to the faster mycorrhization in plants over-expressing the
209 PDR1 protein (Liu *et al.*, 2017), and the pattern of PDR1 localization (Sasse *et al.*, 2015) strongly
210 suggest that SL transport is important for SL effects on mycorrhiza establishment. On the other
211 hand, SL transport contributes to inhibition of lateral bud outgrowth and to resource allocation in
212 responses to environmental constraints, both at the root and shoot levels. This is suggested by 1)
213 the activity profile of the *PhPDR1* promoter (besides root cortex also in elongating root hairs, leaf
214 petioles and at the base of lateral axils) (Liu *et al.*, 2017); 2) the bushy shoots of *pdr1* mutants
215 (Kretzschmar *et al.*, 2012); 3) the fact that petunia plants over-expressing PDR1 show increased
216 lateral root formation and extended root hair elongation. There are also indications that mature

217 leaves may transport SL towards the stem and subtended axillary bud to join root-produced,
218 upstream-flowing SL (Liu *et al.*, 2017). This route seems to be relevant for leaf senescence
219 regulation, which is partly SL-dependent (Ueda and Kusaba, 2015) and is increased in PDR1-
220 overexpressing plants (Liu *et al.*, 2017). It is thus becoming increasingly clear that the SL source/sink
221 map may be more complicated than initially postulated (*i.e.* following a main root-to-shoot
222 concentration gradient), due to a new leaf-to-stem SL transport route that is important to regulate
223 SL levels in leaves and stems (Liu *et al.*, 2017). Indeed, the possibility that systemic and local
224 transport establish SL gradients both throughout the plant and/or between adjoining tissues is
225 certainly worth exploring. It is possible that local peaks of synthesis and distribution and the
226 resulting local gradient(s), rather than absolute hormone concentrations, are important
227 determinants of the physiological output of SL, as demonstrated for other phytohormones such as
228 auxin (Krupinski and Jonsson, 2010). It is worth noticing also that the expression profile of *D14* (the
229 gene encoding the SL receptor, see § 2.4) is poorly overlapping with that of the core biosynthetic
230 enzymes in Arabidopsis (Chevalier *et al.*, 2014); and that the D14 protein itself was recently proven
231 to act as an intercellular signal molecule, travelling in the phloem to fine-tune and specify the
232 location of SL perception (Kameoka *et al.*, 2016). Of course, the fact that both the SL signal and the
233 receptor are mobile complicates the interpretation of mutant phenotypes, and even more, the
234 deciphering of local *vs* systemic SL functions.

235 *2.4 Perception and transduction*

236 A remarkable amount of information has been gathered on the perception and early signal
237 transduction mechanisms in the SL pathway (Figure 2). The SL receptor proteins in vascular plants
238 are called D14-type receptors after the first characterized member of the clade, D14 in rice (Arite *et*
239 *al.*, 2009). These proteins are members of the α/β hydrolase-fold superfamily, and cleave the SL
240 molecule generating a tricyclic ABC and a D-ring moiety (Hamiaux *et al.*, 2012). At this point the D
241 ring, or a derivative thereof, is proposed to be trapped and covalently bound within the catalytic
242 pocket (de Saint Germain *et al.*, 2016; Yao *et al.*, 2016). Even though available crystallographic data
243 are not resolving nor decisive enough in this respect (Lombardi *et al.*, 2017), the hydrolysed SL
244 molecule should dock more favourably than the intact one in the active pocket (Gaiji *et al.*, 2012).
245 This peculiarity would explain the very low catalytic turnover of D14-type receptors (de Saint
246 Germain *et al.*, 2016; Hamiaux *et al.*, 2012; Nakamura *et al.*, 2013) and suggests that hydrolytic
247 activity is needed for signal transduction events and/or to de-sensitize the cell in subsequent SL
248 perception events, by lowering the number of available receptor pockets. As D14 itself is actively
249 degraded after physical interaction with SL (Chevalier *et al.*, 2014; Hu *et al.*, 2017), SL perception
250 indeed entails destruction both at the metabolite (Smith and Waters, 2012) and at the receptor
251 level.

252 Pervasive changes in the 3-D structure of D14 are triggered by the interaction with protein partners
253 (Nakamura *et al.*, 2013; Zhao *et al.*, 2013), prominently the F-box protein MAX2 (Bythell-Douglas *et*
254 *al.*, 2017). F-box proteins are a leitmotiv in phytohormone biology: as promiscuous adaptors
255 recruiting protein targets for ubiquitination and degradation by the proteasome, they suit perfectly
256 the function of specifically and quickly relieving constitutive response repression (Santner and
257 Estelle, 2010). The direct targets of MAX2 certainly include members of the SUPPRESSOR OF
258 MAX2 1 (SMAX1) and D53 protein families (Jiang *et al.*, 2013; Zhou *et al.*, 2013) (Figure 2). Genetic
259 and biochemical data support for these proteins a repressive role of MAX2 functions, though at
260 different developmental stages and in dependence of distinct receptor/ligand pairs (Waters *et al.*,
261 2012). Further work in Arabidopsis points to the combined action of SMAX1-LIKE (SMXL)
262 paralogues no. 6, 7 and 8 in branching promotion, *i.e.* as D53 orthologues (Soundappan *et al.*, 2015).
263 These proteins may act through interaction with TOPLESS (TPL)/TOPLESS-RELATED (TPR)
264 proteins, analogously to what observed in the auxin and jasmonate pathway. However, non-TPR-
265 dependent action mode(s) should not be excluded (Lumba et al., 2017b; Waters et al., 2017). Indeed
266 recently, IDEAL PLANT ARCHITECTURE1 (IPA1) has been shown to be one of the long-sought
267 transcription factors repressed by D53 in rice (Song *et al.*, 2017).
268 Much interesting research has been done on the molecular evolution of SL perception, both in the
269 producing and in the parasitic plant (Lumba *et al.*, 2017b). D14-type SL receptors seem to have
270 generated by gradual neo-functionalization of KARRIKIN INSENSITIVE2 (KAI2) paralogues in higher
271 plants (Bythell-Douglas *et al.*, 2017). KAI2, a close homologue of D14-type proteins, functions as a
272 receptor for karrikins (smoke-derived compounds that stimulate seed germination and share some
273 structural features with SL) (Smith and Li, 2014; Waters *et al.*, 2017). The primary function of KAI2
274 may be in the recognition of an uncharacterized, endogenous SL-like signal named KL (for KAI2-
275 Ligand), and in the transduction of the KL signal by interaction with MAX2 (Conn and Nelson, 2016)
276 (Figure 2). The D14 and KAI2-mediated pathways therefore converge on MAX2, a crucial issue for
277 researchers trying to disentangle the effects of SL and KL.
278
279 **3. Organ-specific dynamics of SL synthesis and cross-talk with ABA under single and combined**
280 **abiotic stress**
281 *3.1 Do SL contribute to shoot acclimatization under osmotic stress?*
282 Given their inducibility by nutrient deprivation, contribution to nutritional root symbioses, and
283 ability to shape plant morphology, SL were quickly proposed as a molecular interface between
284 phenotypic plasticity and a changing and often challenging environment (Liu *et al.*, 2013). Indeed,
285 SL contribute to root and shoot morphological and physiological responses to nutrient (N and
286 especially P) scarcity in soil. This concept was later tested also for other abiotic stresses. SL-

287   deficient or insensitive *Arabidopsis thaliana*, *Lotus japonicus* and *Solanum lycopersicum* are
288   hypersensitive to osmotic stress and respond less to endogenous and exogenous ABA, which
289   strongly suggests that SL synthesis and perception are important for acclimatization (Ha *et al.*,
290   2014; Li *et al.*, 2017; Liu *et al.*, 2015; Lv *et al.*, 2017; Visentin *et al.*, 2016). In these experiments,
291   survival and physiological performances of SL-related mutants were severely affected when either
292   progressively dehydrated (Ha *et al.*, 2014; Li *et al.*, 2017; Visentin *et al.*, 2016) or exposed to PEG at
293   the root level (Liu *et al.*, 2015).

294   It must be noted here that one controversial study in Arabidopsis (Bu *et al.*, 2014) reports that
295   signalling (*max2*) but not biosynthetic (*max1*, *max3* and *max4*) mutants are hypersensitive to stress.
296   This led these authors to absolve SL as culprit for the *max2* phenotype, in favour of other pathways
297   in which MAX2 would be involved. There are several apparent contrasting points between this
298   dataset and that of Ha *et al.* (2014), which call for careful reassessment of ABA-related phenotypes
299   especially at the early developmental stages for Arabidopsis SL mutants. The observed
300   discrepancies may derive from differences in the experimental design (see Table S1 for a detailed
301   comparison), and from the difficulty of pinpointing subtle phenotypes, in particular in SL-
302   biosynthetic mutants. This, in turn, might be due to leaking of the biosynthetic mutants, with
303   residual SL being produced at a sufficient level to confound results. Another possibility is that MAX2
304   might take part in additional pathways also contributing to drought resilience, making the *max2*
305   phenotype more severe than that of biosynthetic mutants: in this context, one rather obvious
306   possibility is that KL, the thus far unidentified endogenous KAI2 ligand, may contribute to the
307   observed phenotype (Li *et al.*, 2017), and do so to variable extents in different species. Given our
308   current understanding of signalling for SL-related molecules, one way to sort this point out would
309   be to test the effects of the pure GR24 enantiomers, to assess if the reported KAI2-dependent
310   activity of the 2'*S* enantiomer (GR24$^{ent\text{-}5DS}$) in Arabidopsis might possibly extend to other species
311   and conditions (Scaffidi *et al.*, 2014; Waters *et al.*, 2017), and how this would relate to drought
312   resilience. On this point, it must be noted that the stress-relieving effect of *rac*-GR24 treatment in
313   Ha *et al.* (2014) is consistent with a positive role of SL in stomatal closure as in Visentin *et al.* (2016)
314   and Lv *et al.* (2017), but all three these works cannot exclude a contribution by GR24$^{ent\text{-}5DS}$.
315   Additionally, *d14* and *kai2* mutants should be included in the panel of analysed lines - if available for
316   the species under study. In two very recent articles this was done for Arabidopsis, supporting a role
317   both for SL and KL in drought responses, including stomatal closure (Li *et al.*, 2017; Lv *et al.*, 2017).
318   So, both KAI2- and D14-dependent signalling pathways seem to contribute additively to
319   acclimatization, given the drought-sensitive phenotype of single and double *kai2/d14* mutants (Li *et*
320   *al.*, 2017). These data confirm that most likely, the relatively stronger drought-related phenotype in
321   SL-depleted *vs max2* mutants is due to the two pathways converging onto MAX2 – the D14- and

322 KAI2-dependent ones- being both involved. The time is ripe now to work out in detail the individual

323 contributions of the two pathways; the identification of KL would represent, in this sense among

324 many others, a major leap forward.

325 Notwithstanding these *caveats* and still open questions, the fact that guard cells in SL-depleted

326 plants are hypersensitive to stress and hyposensitive to ABA was confirmed in three different

327 eudicot species by independent groups with a combination of different eco-physiological

328 approaches, including the analyses of SL-depleted plants and now, also of the signaling mutant *d14*

329 (Ha *et al.*, 2014; Li *et al.*, 2017; Liu *et al.*, 2015; Lv *et al.*, 2017; Visentin *et al.*, 2016). Therefore, SL

330 contribution to proper guard cell functioning and acclimatization responses to water deprivation is

331 supported enough to be included among the effects of SL as phytohormones. Expression data for

332 SL-biosynthetic genes upon treatments such as drought, salinity and osmotic stress (Ha *et al.*, 2014;

333 Lv *et al.*, 2017; Visentin *et al.*, 2016), as well as transcript enrichment for *D14* and *MAX2* in the

334 stomatal cell lineage (Lv *et al.*, 2017) are also consistent with this picture (see § 3.3).

335 *3.2 Current understanding of SL mechanism of action in osmotic stress responses: cross-talk*

336 *between the SL and ABA pathways*

337 **3.2.1 At the biosynthesis level**

338 When it comes to the aetiology of such physiological effect, a modulation of free ABA

339 concentration seems not to be blamed in general terms, since free ABA content in Arabidopsis

340 leaves is comparable in WT and *max2* mutants (Bu *et al.*, 2014), even though stomata are

341 consistently more open in the latter genotype (Bu *et al.*, 2014; Ha *et al.*, 2014). Whole-leaf analyses

342 of course do not rule out that the modulation of ABA biosynthesis, catabolism, and transport could

343 lead to transient and/or very localized accumulation of ABA in a specific tissue, ultimately

344 contributing to the observed phenotypes. Invariant free ABA was observed also in WT *vs CCD7-*

345 silenced Lotus plants under no stress, or individual osmotic or nutritional stress (P deprivation);

346 however when both stresses were applied together, lower free ABA was recorded in leaves of SL-

347 depleted plants (Liu *et al.*, 2015). The situation in tomato is yet slightly different: quantification in

348 well-watered plants showed slightly more (Visentin *et al.*, 2016) or less (Torres-Vera *et al.*, 2013)

349 concentrated free ABA in leaves of SL-depleted plants than WT, likely depending on whether values

350 were expressed per fresh or dry tissue weight unit, respectively. These slight fluctuations are indeed

351 reasonably explained by the fact that SL-depleted and replete leaves have different relative water

352 content already in the absence of stress (Visentin *et al.*, 2016). In tomato suffering moderate and

353 severe drought though, free ABA was significantly less concentrated in *CCD7*-silenced plants than in

354 WT; these values were obtained per fresh weight unit and could not be underestimated in SL-

355 depleted plants, which are more dehydrated than corresponding WT controls. Less concentrated

356 ABA may of course contribute to the poor fitness of this line under water deprivation conditions
357 (Visentin *et al.*, 2016).

358 SL influence on ABA concentration under stress is far less documented at the root level. While no
359 data exist for Arabidopsis, the profile of free ABA concentrations in roots of SL-depleted tomato
360 and Lotus roughly reflects what happens in shoots (Liu *et al.*, 2015; Visentin *et al.*, 2016).
361 Additionally, roots of WT Lotus pre-treated with *rac*-GR24 are unable to increase free ABA
362 concentration in response to subsequent PEG-induced osmotic stress. This observation suggests
363 that - at least in Lotus - there might also be some root-specific negative effect of SL on ABA
364 synthesis under drought (Liu *et al.*, 2015); and/or that once again, the non-natural enantiomer in the
365 *rac*-GR24 used for treatment might be responsible for the effect. A very similar situation is observed
366 in seeds of parasitic plants, in which GR24 is thought to stimulate germination also by accelerating
367 ABA degradation via the ABA-8' hydroxylase *PrCYP707A1* (Lechat *et al.*, 2012). Analogously, SL may
368 relieve secondary dormancy, *i.e.* thermoinhibition of Arabidopsis seed germination, by lowering
369 ABA concentration (Toh *et al.*, 2012). These examples highlight once again how, depending on the
370 examined organ and conditions, the SL and ABA pathways might be wired differently. It might be
371 worth mentioning here that free ABA concentrations are higher in *kai2* mutants of Arabidopsis than
372 in the WT, both in the absence and presence of drought. This effect is likely due to compromised
373 activity of ABA-8'-hydroxylase enzymes (such as AtCYP707A3), given the lower transcript levels in
374 the *kai2* background (Li *et al.*, 2017). Therefore, also the endogenous KAI2 ligand might interfere
375 with ABA levels so once again, care should be taken in separating the effects of the two.

376 A positive influence of SL on ABA synthesis in shoots is therefore documented, especially but not
377 limited to shoots under drought, although there seem to be species-specific differences in
378 amplitude. The overall prevailing trend in leaves is for lower ABA concentration in SL-depleted
379 plants; indeed, transcripts of some ABA biosynthetic genes are less concentrated in leaf tissues of
380 Arabidopsis *max2* than WT under drought (Ha *et al.*, 2014). Additionally, *Nine-Cis-Epoxycarotenoid*
381 *Dioxygenase3* (*NCED3*), *Cytochrome P450 707A3*, *ABCG22*, *ABA Insensitive1* (*ABI1*), and
382 *Hypersensitive to ABA1* (*HAB1*) are all less transcribed in response to drought when *MAX2* is
383 mutated (Bu *et al.*, 2014). This picture is unsupportive of the initial hypothesis that SL and ABA
384 might be influencing each other's levels by merely competing for the same precursor substrate (i.e.
385 carotenoids). It is still not known whether excess SL, obtained for example by treatment with GR24,
386 modulates free ABA content in shoot tissues. On the other hand, the reverse effect - i.e. of
387 genetically reduced ABA content on endogenous SL concentration - was explored in tomato,
388 leading to the conclusion that the overall trend was for a positive correlation between ABA levels
389 and SL synthesis in the roots; correlations were not explored in the shoot, in which both the SL-
390 biosynthetic gene transcripts and final metabolites are undetectable under normal conditions

391 (López-Ráez *et al.*, 2010). However, ABA treatment induces *MAX3* and *MAX4* transcript

392 accumulation in Arabidopsis leaves (Ha *et al.*, 2014). One potential candidate regulator of both ABA

393 and SL levels in Arabidopsis is ORA47 (Octadecanoid-Responsive AP2/ERF-domain transcription

394 factor47) (Chen *et al.*, 2016), a transcriptional regulator involved in the cross-talk and integration of

395 several phytohormones, prominently of jasmonic acid and ABA. Its chromatin occupancy profile

396 includes, among others, the promoters of biosynthetic and signalling genes in the ABA pathway,

397 and of *MAX3* and *MAX4*. Occupancy is higher-than-background only under normal but not drought

398 conditions in leaves (Chen *et al.*, 2016), when transcripts of these genes accumulate (see § 3.3). This

399 suggests that beyond the most characterized role at the cross-road of ABA and jasmonic acid,

400 ORA47 may act as a transcriptional repressor and integration hub for the SL and ABA pathways as

401 well. This hypothesis is worth investigating and if indeed demonstrated, may define ORA47 as the

402 first molecular link in the SL-ABA crosstalk, namely under drought.

403 **3.2.2. At the ABA-sensitivity level**

404 Beyond the above observations, which suggest that the influence of ABA and SL on their mutual

405 concentrations may be more or less intimate in different species and organs, a combination of eco-

406 physiological measurements (including leaf temperature, stomatal conductance and water

407 potential) all pointed to increased stomatal conductance as a primary reason for higher sensitivity

408 to water deprivation in SL-biosynthetic or signalling mutants. Lower guard cell sensitivity to

409 endogenous and exogenous ABA is identified as another contributing factor to this phenotype.

410 Indeed, SL-depleted and insensitive plants have higher-than-WT stomatal aperture and

411 conductance in the absence and presence of stress, and slower closure in response to exogenous

412 ABA treatment (Ha *et al.*, 2014; Li *et al.*, 2017; Liu *et al.*, 2015; Lv *et al.*, 2017; Visentin *et al.*, 2016).

413 As expected for positive regulators of acclimatization responses, ABA, drought and/or osmotic

414 stress enhance transcript accumulation for SL biosynthetic genes in leaves (Ha *et al.*, 2014; Lv *et al.*,

415 2017; Visentin *et al.*, 2016). However, and unexpectedly perhaps, SL-related gene expression and

416 metabolite levels drop in the roots of non-mycorrhizal Lotus (Liu *et al.*, 2015), lettuce and tomato

417 (Ruiz-Lozano *et al.*, 2016; Visentin *et al.*, 2016) undergoing drought. It must be noted that in Lotus,

418 the drought-induced SL repression is independent of nutrient availability, *i.e.* if osmotic stress and P

419 scarcity are applied together, the drought response profile will prevail, and SL synthesis will be

420 inhibited (Liu *et al.*, 2015). These results indicate that the dynamics of SL synthesis are different in

421 different organs, which reinforces the need to separate above- and below-ground organs when

422 addressing issues related to systemic signalling under stress; and that the outcome of combined

423 stresses might not be easily predictable based on single-stress effects. These observations might

424 also explain why roots of SL-depleted and insensitive Arabidopsis plants grow comparably to the

425 WT, in the presence of high mannitol and NaCl (Ha *et al.*, 2014). In fact, if osmotic stress represses

426 SL synthesis in Arabidopsis roots (which is still to be demonstrated) as it does in lettuce, Lotus and

427 tomato, any genetic defect in SL metabolism or signalling will be less likely to cause a detectable

428 root-related phenotype under these conditions.

429 *3.3 Local and systemic effects of SL and SL-like molecules on stomatal conductance: a*

430 *parsimonious, preliminary model*

431 The inhibition of SL synthesis and possibly transport in dicot roots under osmotic stress is unlikely

432 to be due to mere metabolic suffering; in fact, gene transcript and metabolite concentrations are

433 quickly reduced, when local water potential has not dropped yet as a consequence of low water

434 availability (Liu *et al.*, 2015; Visentin *et al.*, 2016). Rather, a local consequence of this drop may be

435 the de-repression of ABA synthesis, as mentioned in § 3.2.1. This possibility however is so far

436 suggested only by a pharmacological approach in Lotus, and awaits confirmation in other species

437 and by using the SL enantiomer GR24^{5DS} before it can be generalized to any extent. Whatever the

438 local effect, SL and/or SL precursors travel shootward (Akiyama *et al.*, 2010; Domagalska and

439 Leyser, 2011; Kohlen *et al.*, 2011; Sasse *et al.*, 2015). Therefore, the possibility that a drastically

440 diminished flow of SL or SL-like molecules from the roots may carry precise information to the

441 shoots, could not be excluded. A reductionist approach (mimicking in the absence of stress the SL

442 gradient observed under drought) was taken to disentangle the inherent complexity of the

443 hypothesized interactions *in situ*. SL-replete (WT) tomato scions grafted to SL-depleted rootstocks

444 displayed more concentrated transcript of SL-biosynthetic genes, and higher sensitivity to

445 endogenous and exogenous ABA not only compared to shoots of SL-depleted plants, but also to

446 WT scions grafted onto WT rootstocks (Visentin *et al.*, 2016). The fact that root-produced SL

447 negatively feed back on the SL biosynthetic pathway in above-ground organs had been already

448 proposed in other species, based on similarly hetero-grafted plants (Johnson *et al.*, 2006). Although

449 SL remain stably under the analytical detection threshold in these leaf tissues, as they do under

450 drought (Visentin *et al.*, 2016) and osmotic/salt stress (Lv *et al.*, 2017); and in lack of detailed

451 structural and biosynthetic information on other possibly concurring molecules, the most

452 parsimonious hypothesis at present is that stomata in such hetero-grafted plants display a ABA-

453 hypersensitive phenotype because synthesis of SL or SL-like molecules is enhanced in leaves (as

454 supported by gene expression data). Notably, *rac*-GR24 is sufficient to increase the speed of

455 stomatal closure in response to exogenous ABA in tomato (Visentin *et al.*, 2016), and to trigger

456 stomata closure in the absence of exogenous ABA in Arabidopsis (Lv *et al.*, 2017) just as it improves

457 survival rate under drought both in WT and SL-depleted, but not SL-insensitive *max2* Arabidopsis

458 (Ha *et al.*, 2014). Additionally, as *MAX2* and *D14* transcripts are more concentrated in the stomatal

459 lineage than in other leaf tissues, SL perception may be specifically enhanced in guard cells (Lv *et*

460 *al.*, 2017). In this context, low SL in roots may well be a component of the systemic drought stress

461 signal in tomato (Visentin *et al.*, 2016), in which (just as in Arabidopsis) ABA does not cover a long-
462 distance signalling function of drought stress (Christmann *et al.*, 2007; Holbrook *et al.*, 2002). Based
463 on the above data, obtained in herbaceous dicots, a mode of action in osmotic stress responses for
464 SL and/or SL-like molecules such as SL intermediates, or KL can be proposed (Figure 3). Such model
465 places a drop in SL synthesis at the root level above the dynamic concentration adjustment of SL
466 (and/or, of SL-like molecules) throughout the plant. As a direct or indirect (*i.e.* mediated by a second
467 messenger) consequence of such drop, synthesis of SL and/or SL-like molecules would be induced
468 in shoots, namely in leaves, to the immediate and positive purpose of making stomatal closure
469 more efficient. How this effect is achieved, and through which mediators, is not yet understood. As
470 an obvious path to beat, the possibility that the ABA transport, perception and/or signalling
471 machinery is primed by SL or SL-like molecules should be explored, with emphasis on the post-
472 transcriptional levels of regulation. However at least in Arabidopsis, all ABA signalling components
473 investigated were found not to be required for the effect of *rac*-GR24 on stomatal closure, which
474 was instead dependent on *MAX2, D14, SLOW ANION CHANNEL-ASSOCIATED1* (*SLAC1*) and an
475 ABA-independent H_2O_2/NO burst at the guard cell level (Lv *et al.*, 2017) (Figure 3). These results
476 unveil an interesting, completely novel link between SL or SL-like molecules and SLAC1 activity,
477 and open a new avenue of investigation in SL biology. However, they cannot explain why stomata
478 of SL-related mutants in Lotus, tomato and Arabidopsis are hyposensitive to exogenous ABA in
479 feeding experiments (Ha *et al.*, 2014; Liu *et al.*, 2015; Lv *et al.*, 2017; Visentin *et al.*, 2016). A possible
480 reconciliation key for these apparent discrepancies is that given the low background of stomata
481 reactivity they cause, mutations compromising endogenous SL synthesis or perception are able to
482 unveil a contribution of SL-dependent priming of ABA signalling/transport to stomata during ABA
483 feeding experiments. During *rac*-GR24 feeding experiments instead, the effects of ABA-
484 independent, direct SLAC1 stimulation by exogenous SL may be strong enough to mask milder
485 ABA-dependent ones. In other words, while the effect of ABA on stomatal closure is at least
486 partially dependent on endogenous SL, *rac*-GR24 effects on the same feature are largely ABA
487 independent. Clearly, this signalling module is not the only ABA-independent response to SL or SL-
488 like molecules: *max2* and *kai2* Arabidopsis mutants were reported to dismantle their photosynthetic
489 machinery more slowly, and switch on anthocyanin synthesis less efficiently than the WT, in an
490 ABA-independent way (Ha *et al.*, 2014; Li *et al.*, 2017) – two features that, once again, may worsen
491 performances under stress. It must be noted here that *rac*-GR24-triggered flavonoid synthesis was
492 shown to be dependent both on D14 and KAI2 in Arabidopsis roots (Walton *et al.*, 2016).
493
494 **4. Perspectives on abiotic stress relief and practical applications of SL in agriculture**

Modern agriculture requests continue, more and more specific interventions during the growth season in order to manage a wide range of biotic and abiotic challenges; and thus, innovative crop protection solutions must be continuously developed. In the last years, traditional breeding has been associated with the use of a new generation of agrochemical compounds. These give satisfying results in protection against biotic stresses such as bacterial or fungal diseases, and weed plant infestation. On the other hand, the same solutions cannot warrant adequate results against abiotic stresses such as water or nutrient deficiency. Generally, plants acclimate to adverse conditions by exploiting signal molecules that in turn, will modulate several genetic and metabolic pathways. Many among these signal molecules are already present as phytoregulators or biofertilisers in the catalogue of agrochemical companies, with a prominent role played by phytohormones (gibberellins to stimulate seed germination and fruit ripening, auxins to promote flower and fruit development etc.). SL as well could raise a similar interest by the agro-technical market thanks to their already characterized activity both as signal molecules in the rhizosphere and as endogenous hormones (Makhzoum *et al.*, 2017; Screpanti *et al.*, 2016a). The potential for application in the control of parasitic weeds has been the first to be investigated, both because of the huge market impact of these pathogens, and of the early discovery of SL as potent seed germination stimulants for *Striga*, *Phelipanche* and *Orobanche* seeds (Screpanti *et al.*, 2016b; Yoneyama *et al.*, 2010). Seed banks of parasitic species in these genera infest not only Asia and Africa but also the Mediterranean and Black Sea regions (Zwanenburg *et al.*, 2016), causing huge yield losses in commercial crops by hampering host growth and life-cycle completion through subtraction of water and nutrients from the phloem in colonized roots (Parker, 2009). The proposed SL-based control strategy is named "suicidal germination": SL are delivered to the parasitic seed-infested soils in the absence of a host crop, in order to lead germinated seeds to death. The strategy is covered in detail elsewhere (Fernandez-Aparicio *et al.*, 2011; Zwanenburg *et al.*, 2016). Similarly, as soon as SL were associated to the stimulation of hyphal branching in AM fungi, their soil application in combination with other compounds such as elicitors of defence responses or fungicides was promptly patented (Dahmen *et al.*, 2011; Suty-Heinze and Vors, 2008, 2009) as a mitigation strategy against combined stresses. Simplifying, marginal soils could be amended with exogenous SL and AM fungi (and/or Rhizobia where appropriate, given the effects on swarming discovered later), in order to increase the chances of successful host colonization and thus, of improving plant mineral nutrition. Analogously, plastic remodelling of root/shoot morphology and modulation of developmental progression (namely, of the juvenile to reproductive phase transition) are very interesting endogenous effects in a perspective of crop management practices, and could be possibly also achieved by targeted delivery to the site of action, in order to reduce the amount of active principle required. The latter strategy would of course be sustainable only in high-profitability

530 crops, and needs careful evaluation of goals and formulations on a case-by-case basis; for example,
531 mere spraying with exogenous SL is known, at least in certain model plants, not to inhibit shoot
532 branching (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008).

533 Unfortunately, a key limit for the use of these potential biofertilisers in plant protection is the
534 chemical instability of natural SL in aqueous solution, which especially at alkaline pH, rather rapidly
535 hydrolyse by producing an ABC-formyl lactone and 5-hydroxybutenolide (Akiyama *et al.*, 2010). In
536 addition to this restriction, also the mass production of natural SL is at present technically and
537 economically challenging. In fact, about 20 different natural SL have been isolated and
538 characterized so far, but their concentration in plant-derived samples such as root exudates is very
539 low (Al-Babili and Bouwmeester, 2015). Complete chemical synthesis has been achieved, but
540 besides the low yield, it is labour- and time-consuming (Brooks *et al.*, 1985; Shoji *et al.*, 2009).
541 Therefore, the task of obtaining large quantities of natural SL from plants or through organic
542 synthesis is still daunting and/or not economically viable for the agrochemical market – certainly so
543 for commodity crops, on which mark-ups are generally low. For these reasons, synthetic molecules
544 with a simpler chemical structure than natural SL, yet showing comparable bioactivity to the
545 natural compounds were developed (Prandi and Cardinale, 2014). "Synthetic SL" can be classified
546 into two main categories: analogues, whose structure is very similar to natural SL though easier to
547 synthesize *in vitro*; and mimics, whose structure is much simpler. Both will retain all or a subset of
548 SL-like bioactivity features. With regard to the latter point, it must be noted that quite some effort
549 has been devoted by organic chemists, biochemists and modellers to design molecular structures
550 retaining SL-like bioactivity towards only a subset of target organisms or organs, if applicable
551 (Prandi and Cardinale, 2014). For example, the mimic molecule named 4-BD (4-Br debranone) is not
552 active as germination stimulant of parasitic seeds; thus, a 4-BD-based weed-avoidance strategy can
553 be envisaged, that couples SL-deficient plants (to prevent seed-bank stimulation by natural SL
554 exudation in the rhizosphere) and 4-BD (to compensate for possible unwanted phenotypic effects
555 of SL deficiency in the producing plant, without contributing to weed infestation) (Fukui *et al.*,
556 2013). A similar strategy was also proposed based on other analogues that retain their bioactivity on
557 plant morphology, but induce very little germination of parasitic weeds (Boyer *et al.*, 2014).

558 More recently, as described in § 3, treatment with exogenous *rac*-GR24 was shown to increase
559 stomata reactivity in tomato and Arabidopsis (Lv *et al.*, 2017; Visentin *et al.*, 2016) and
560 performances under drought in SL-depleted and WT, while not in SL-insensitive Arabidopsis (Ha *et
561 al.*, 2014). Notwithstanding the *caveats* on the use of racemic mixtures in proof-of-concept
562 experiments (see § 3.1), and taking into account that the non-natural enantiomer in the racemic
563 mixture likely contributes to the effect through KAI2, this ability of synthetic molecules to confer
564 drought resistance by foliar nebulization opens interesting scenarios. Synthetic SL derivatives were

565 indeed proven to relieve drought of maize under field conditions, and patented in this respect

566 (Davidson *et al.*, 2015; Lumbroso and De Mesmaeker, 2017); foliar application would bypass most

567 instability issues for molecules delivered in soil. This highlights how available SL analogues/mimics

568 and karrikins could serve as a blueprint for the development of future agrochemicals aimed at

569 controlling plant water use and improving yield under water stress conditions, just like ABA agonists

570 (Helander *et al.*, 2016). While it is clear indeed that ABA is a central regulator of plant water use, the

571 fact that *rac*-GR24 acts mostly ABA-independently on stomatal closure might allow for efficient

572 control of water losses, without stimulating the full array of ABA responses (Ha *et al.*, 2014; Lv *et al.*,

573 2017). On the other hand, different stresses may be associated to non-overlapping SL profiles in

574 different organs (see for example, osmotic stress and P deprivation); therefore, what outcome

575 combined stress might have in terms of metabolite profile, must be determined experimentally.

576 Only after such data are available might the effect of treatment with exogenous SL be foreseen. For

577 example, if SL are delivered to leaves of dicot plants under combined osmotic and nutritional stress

578 (by both of which SL may be induced in leaves), it is likely that the effects on stress resilience will be

579 positive; not necessarily so if treatments were targeted to the roots (in which, during combined

580 stress, the SL decrease triggered by osmotic stress will override the increase induced by P

581 deprivation) (see § 3). Additionally, since SL in soil may stimulate parasitic seed germination, foliar

582 application may be safer than soil delivery if the risk of weed infestation is not zero in any given

583 field. Wet testing is needed in this sense, but still missing for any realistic stress combinations.

584 It must be noted as well that a potentially exploitable effect on stomatal conductance could be

585 obtained in WT shoots of tomato plants grafted onto SL-depleted rootstocks (Visentin *et al.*, 2016).

586 This result, besides providing mechanistic insights in SL-dependent root-to-shoot communication,

587 opens the possibility to develop efficient drought resistance strategies for graftable plants, in which

588 SL dynamics under drought mirror what happens in tomato. The use of SL-depleted (possibly non-

589 transgenic) rootstocks for SL-replete scions leads to higher water use efficiency and better

590 performances under stress thanks to the demonstrated increase of ABA sensitivity in such scions

591 compared to WT shoots grafted onto WT roots (Visentin *et al.*, 2016); and this, without using any

592 natural or synthetic chemical endowed with SL-like activity. Additionally, the possibility cannot be

593 excluded that natural variants exist among tomato accessions and wild relatives, which are more

594 resilient than cultivated genotypes because they exploit more efficiently the SL- (or SL-like) related

595 toolbox. In this sense, collections could be screened looking for genotypes displaying the most

596 effective root/shoot activation profile of the SL or SL-like pathways, under normal and stress

597 conditions. It must be noted in this regard that rootstocks in which SL production is knocked down

598 (yet not completely out) may also induce less germination in seed banks of parasitic weeds, and yet

599 produce enough SL to allow for regular colonization by AM fungi (see for example (Vogel *et al.*,
600 2010), identifying a balance point between contrasting ecological needs.

601

602 Thus, the many features of SL bioactivity make them potentially interesting for agronomic
603 applications against abiotic stress: soil treatment to improve beneficial symbiosis with AM fungi
604 and Rhizobium, foliar nebulization and grafting contrasting genotypes for SL production to increase
605 drought resistance seem to be the most promising strategies at present. On the other hand, the
606 road to market uptake for any SL-based product is inevitably long: chemical instability in water
607 solution, difficulties in the isolation of such low-concentration natural metabolites, the economic
608 burden of productive scale-up and registration of synthetic molecules are the biggest challenges to
609 tackle. Nonetheless, if enrichment strategies and protocols can be optimized to allow for the
610 development of a natural SL-enriched biostimulant, a decrease of the industrial costs (due in
611 particular to the registration and certification load) could be achieved. A biostimulant can be
612 defined as a (mix of) substance(s) and/or microorganisms that, when applied to plants or the
613 rhizosphere, stimulates natural processes to enhance/benefit crop yield and quality, also by
614 enhancing resilience to and recovery from abiotic stress, drought included (Van Oosten *et al.*, 2017).
615 The positive influence of biostimulants is dependent on plant species, cultivars, climatic conditions,
616 dose, origin and time of application, but their use is fully compatible with both conventional and
617 organic agriculture. New, SL-enriched biostimulant formulations could be ideally developed and
618 tested for proof-of-concept, to the long-term goal of integrating them into the set of most effective
619 crop management practices and tools that prevent and mitigate the effect of abiotic stress. In
620 Europe, biostimulants can be currently placed on the market either under the national regulations
621 on fertilisers, or under the European pesticides law, which combines both supranational and
622 national provisions for introducing plant protection products (PPPs) on the market (EC regulation
623 No 1107/2009). However, a Fertiliser Proposal covering biostimulants as "fertilising products" (*i.e.*
624 distinct from fertilisers *sensu strictu,* but also from PPPs) is currently under discussion by the EC; its
625 goal is to amend the 2009 Regulation on PPPs, to explicitly exclude biostimulants. This currently
626 leaves biostimulants in a regulatory limbo, which is thought to be over shortly. Were biostimulants
627 to be registered for commercialization under less demanding regulations than PPPs, natural SL-
628 enriched versions might become as or more attractive than synthetic SL for certain applications.

629

630 **5. Main open questions and conclusions**
631 Many open questions of course persist, both at the basic understanding level and on the feasibility
632 of practical applications of fundamental knowledge. Namely, main avenues of research will have to
633 give further details in the molecular underpinnings of SL effects on stomatal closure, explaining the

634 reasons for the ABA-dependent share of guard cell activity impairment in SL mutants. The fact that
635 SL accumulate in stressed *vs* unstressed leaves is still awaiting to be conclusively proven or
636 disproven; it is indeed possible that SL synthesis in droughted leaves is highly localized (for
637 example, in guard cells; and anyway enough to escape detection in whole-leaf analyses), and/or
638 that different metabolites than the known ones, such as KL, are co-responsible for the observed
639 phenotypes. To this goal, readouts of SL activity are needed, but yet to be developed, which are
640 both sensitive, quantitative and at high spatial resolution (ideally, at the single-cell level); and
641 knowledge on the elusive KL is to be acquired. Finally, the actual mitigation effects of SL-based
642 management strategies on abiotic stress consequences in realistic field (open or protected)
643 situations must be explored soon by the academic community, if we are to fully exploit the
644 theoretical potential of SL in modern agriculture.
645

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Fig. 1. Prototypal structures of natural SL and analogues. (A) General four-ring structure (ABCD) of SL, and relative C-atom numbering. **(B)** The racemic solution of GR24, the most commonly used synthetic analogue of SL, is composed of the equimolar mixture of the two enantiomers GR24^{5DS} (with the same stereochemistry as strigol) and GR24$^{ent\text{-}5DS}$. **(C)** Molecular structures of strigol and orobanchol, two naturally occurring SL characterized by β- and α-orientations of the C ring, respectively. They are representatives of the two main molecular types of natural SL; both share the *R* configuration at the C-2' of ring D.

Fig. 2. Main synthesis and perception avenues of SL. Left-hand panel: SL biosynthesis starts in plastids where three enzymes, D27, CCD7 and CCD8, act sequentially on carotenoids to produce carlactone, a precursor of SL. Carlactone is then transferred to the cytosol, where it is further processed in order to produce SL. SL and carlactone are then perceived in the same cell where they were produced (not shown) and/or transferred to other cells; while the first are probably transferred via the PDR1 protein, the transporter for carlactone is not identified yet (dotted arrow). It is also not known if some steps of the SL biosynthetic pathway are shared by other SL-like molecules. Right-hand panel: SL (or, other carlactone derivatives) activate MAX2-dependent signal transduction after physical binding with the receptor D14. Through this pathway, SL modulate transcription by destabilizing members of the SMXL family of transcriptional corepressors; induce stomatal closure by influencing the activity of the ion channel SLAC1; and influence auxin distribution by promoting the removal of PIN-FORMED (PIN) transporters. MAX2 is also a component of the KAI2-triggered transduction cascade. The ligands to this receptor are thought to be an endogenous, putative SL-like signal molecule (KL) and karrikins (which are also suspected to activate a MAX2-independent signalling pathway; dotted arrow).
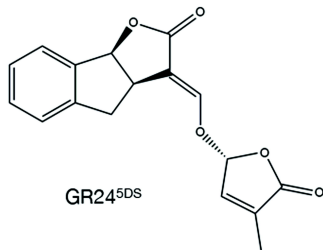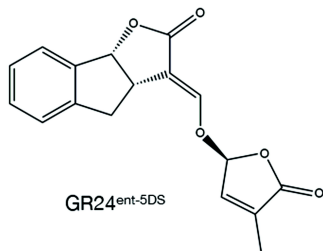
Fig. 3. Model for SL action in root-shoot communication and local signalling under drought. The main connections between SL (or SL-like signal molecules such as SL precursors, or KL) and ABA in roots and shoots under drought stress are highlighted. SL/SL-like molecules may have a negative effect on osmotic stress-induced ABA levels in roots, as indicated by *rac*-GR24 treatment in *Lotus japonicus*. This suggests that a drop in SL/SL-like synthesis in this organ under osmotic stress may be required (but not sufficient) to let ABA levels rise [1]. The shootward flow of SL/SL-like molecules represses by an unknown mechanism the transcription of SL/SL-like biosynthetic genes in shoots, especially under normal conditions when more SL are produced in the roots and likely translocated to the shoot [2] than under stress (*vide infra*). SL/SL-like synthesis is inhibited in roots under osmotic/drought stress and, as a positive consequence for acclimatization, shootward SL/SL-like flow is decreased [3]. The transcription of SL/SL-like biosynthetic genes is thus de-repressed in

shoots, likely increasing the metabolite levels [4] (dotted inhibition arrow indicates lower repression than in [2]). Shoot-produced SL/SL-like molecules may induce SLAC1-dependent stomatal closure directly, by triggering the production of H_2O_2 and NO in guard cells [5]; moreover, they could also impact stomatal closure more indirectly, by positively regulating ABA sensitivity in guard cells [6]. It is not known whether osmotic/drought stress can increase SL/SL-like biosynthetic genes transcription in shoots independently of SL-related signals from the roots [?]. Adapted from: Visentin *et al.* (2016) based on data by Liu *et al.* (2015); Li *et al.* (2017); Lv *et al.* (2017); Visentin *et al.* (2016).
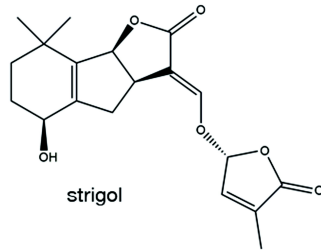
Supplementary Information

Table S1. Comparative table of main results in Ha *et al.* (2014) and Bu *et al.* (2014). "*Lower*", "*higher*" and "*equal*" are intended in comparison with the WT genotype; *n.a.*, not assessed.
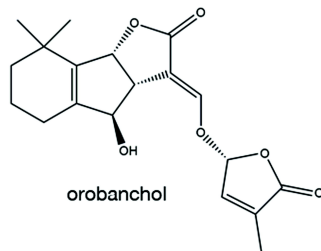
A
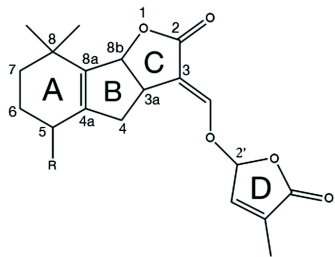
B *rac*-GR24 enantiomers

GR24⁵ᴰˢ

GR24ᵉⁿᵗ⁻⁵ᴰˢ

C

strigol

orobanchol