1 **Running Title:** SLs modulate Pi starvation signalling
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6 **Exogenous strigolactones impact metabolic profiles and phosphate starvation signalling**
7 **in roots**
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28 • Total word count: **5794**
29 • Summary: **192**
30 • Number of tables: **0**
31 • Number of figures: **7**
32 • Supplementary material: **4 Tables and 3 Figures**

SUMMARY

Strigolactones (SLs) are important *ex-planta* signalling molecules in the rhizosphere, promoting the association with beneficial microorganisms, but also affecting plant interactions with harmful organisms. They are also plant hormones *in-planta*, acting as modulators of plant responses under nutrient deficient conditions, mainly phosphate (Pi) starvation. In the present work, we investigate the potential role of SLs as regulators of early Pi starvation signalling in plants. A short-term pulse of the synthetic SL analogue 2'-*epi*-GR24 promoted SL accumulation and the expression of Pi starvation markers in tomato and wheat under Pi deprivation. 2'-*epi*-GR24 application also increased SL production and the expression of Pi starvation markers under normal Pi conditions, being its effect dependent on the endogenous SL levels. Remarkably, 2'-*epi*-GR24 also impacted the root metabolic profile under these conditions, promoting the levels of metabolites associated to plant responses to Pi limitation, thus partially mimicking the pattern observed under Pi deprivation. The results suggest an endogenous role for SLs as Pi starvation signals. In agreement with this idea, SL-deficient plants were less sensitive to this stress. Based on the results, we propose that SLs may act as early modulators of plant responses to P starvation.

KEYWORDS

2'-*epi*-GR24, metabolism, molecular markers, Pi starvation, signalling, plant responses, strigolactones

58 **INTRODUCTION**

59

60 Phosphorus (P) is an essential nutrient for plants, as it is a structural component of many
61 biomolecules, including nucleic acids, lipids and proteins, and it is involved in many cellular
62 processes such as primary metabolism, protein activation, energy transfer, and signal
63 transduction cascades (Ham et al., 2018; Scheible & Rojas-Triana, 2015). However, despite
64 its relevance, P is one of the less-abundant macronutrients present in soils. It is mainly
65 acquired by plants in the form of inorganic phosphate (Pi), which has high-affinity to mineral
66 particles and organic matter, thus reducing its bioavailability markedly, and limiting plant
67 growth and development (Lynch, 2011; Raghothama, 2000). Along evolution, plants have
68 developed a set of complex physiological, biochemical, metabolic and molecular
69 modifications to cope with Pi limitation in the soil, collectively known as Pi starvation
70 responses (PSRs) (Ham et al., 2018; Puga et al., 2017). PSRs include alterations in shoot and
71 root morphology, the regulation of high-affinity Pi transporters (PHT), modifications in the
72 primary and secondary metabolism, as well as the exudation into the rhizosphere of Pi-
73 releasing enzymes, organic acids and signalling molecules to associate with beneficial soil
74 microorganisms that can improve Pi uptake (Andreo-Jiménez et al., 2015; Campos et al.,
75 2018; Lambers et al., 2015; Puga et al., 2017). Overall, PSRs aim to improve P-use efficiency
76 by affecting both Pi acquisition and reallocation and remobilization of internal P.

77 Establishment and regulation of PSRs require a fine-tuned coordination and integration
78 of local and systemic signalling pathways, which are mediated by a number of genes and
79 signalling molecules (Ham et al., 2018; Lan et al., 2015; Puga et al., 2017; Scheible & Rojas-
80 Triana, 2015). It is well established that the transcriptional activator PHOSPHATE
81 STARVATION RESPONSE 1 (PHR1), and related transcription factors, play a central role
82 by regulating the expression of many Pi starvation-induced genes (Bustos et al., 2010; Rubio

et al., 2001; Zhou et al., 2008). PHR1 is constitutively expressed, but its activity is regulated by the plant Pi status. Indeed, PHR1 activity is negatively regulated by SYG1/Pho81/XPR1 (SPX)-domain proteins (Fig. 1), which sense inositol phosphates (InsP) as a Pi signal (Puga et al., 2017; Secco et al., 2012). Under Pi limitation, InsP concentration drops, making the complex SPX-PHR1 no longer stable releasing PHR1. Then, PHR1 induces the expression of certain high-affinity transporters of the PHT1 family (Fig. 1), facilitating Pi-acquisition and translocation *in-planta* (Huang et al., 2013; Liu et al., 2012; Puga et al., 2017). PHR1 also promotes the expression of the microRNA miR399, whose levels are highly induced soon upon Pi limitation (Pant et al., 2008). miR399 modulates the activity of *PHO2*, encoding an ubiquitin-conjugating E2 enzyme involved in protein degradation (Lin et al., 2008). Subsequently, down-regulation of *PHO2* prevents the degradation of PHO1, a Pi transporter involved in Pi loading into the xylem (Liu et al., 2012). In parallel to miR399, PHR1 also promotes the expression of the non-protein coding gene *IPS1* (Franco-Zorrilla et al., 2007). *IPS1* sequesters free miR399 through a target mimicry mechanism, preventing the interaction miR399-*PHO2* and the degradation of *PHO2* transcripts (Fig. 1) (Franco-Zorrilla et al., 2007). Thus, plant Pi acquisition and homeostasis is finely regulated mainly by the interaction of the triad *IPS1*-miR399-*PHO2*.

Plant responses to environmental challenges, including Pi starvation, are also mediated by phytohormones. Thus, it has been shown that Pi deficiency response is associated with downregulation of gibberellins and cytokinins, while other phytohormones such as auxin, ethylene, abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and strigolactones (SLs) are up-regulated (Chiou & Lin, 2011; Khan et al., 2016; López-Ráez et al., 2008; Pérez-Torres et al., 2008; Prerostova et al., 2018; Song et al., 2016). SLs are the latest plant hormones described, acting as modulators of plant responses under nutrient deficient conditions, mainly Pi starvation, and other abiotic stresses such as drought and salinity (Al-

108 Babili & Bouwmeester, 2015; Andreo-Jiménez et al., 2015). They are carotenoid-derived,
109 belonging to the apocarotenoid class, as ABA. They are produced by the action of a β-
110 carotene isomerase (D27) and sequential oxidative cleavage by two carotenoid cleavage
111 dioxygenases (CCD7 and CCD8), giving rise to carlactone, the precursor of all canonical SLs,
112 including strigol- and orobanchol-type (Al-Babili & Bouwmeester, 2015; Waters et al., 2017).
113 Under Pi limitation, they hinder shoot growth and promote root system development -
114 inhibiting primary root growth, promoting lateral root formation, and root hair number and
115 elongation -, thus increasing soil exploration capacity and improving minerals and/or water
116 acquisition under stress (Kapulnik et al., 2011; Ruyter-Spira et al., 2011). In addition to their
117 role as phytohormones, they are important *ex-planta* signalling molecules in the rhizosphere,
118 promoting the association with beneficial microorganisms, such as arbuscular mycorrhizal
119 fungi and rhizobia, also to improve nutrients (mainly Pi and nitrogen) and water acquisition
120 (López-Ráez et al., 2017). Despite the key role of SLs under Pi starvation, how they modulate
121 plant responses and whether they are also involved in P signalling remain unclear. We have
122 previously proposed that higher Pi acquisition efficiency in a commercial wheat cultivar
123 might be related to its improved SLs-P signalling, which modulates *PHO2* activity (Campos
124 et al., 2019). To further investigate the potential role of SLs as regulators of early Pi
125 starvation signalling, here we explore the transcriptional and metabolic responses of the plant
126 to a short-term pulse of the synthetic SL analogue 2'-*epi*-GR24, both under normal and Pi
127 limitation conditions. Moreover, the expression pattern of Pi starvation signalling maker
128 genes was assessed in the tomato SL-deficient line *SlCCD8*-RNAi L04. Our results suggest
129 that SLs can act as modulators of plant responses during Pi limitation. Improving our
130 understanding of Pi starvation signalling is essential to develop new agricultural strategies in
131 order to optimize plant Pi uptake and reduce the use of P fertilizers.
132

133 **MATERIALS AND METHODS**

134

135 ***Plant growth conditions and treatments***

136 Tomato (*Solanum lycopersicum* L. cv. MoneyMaker) and wheat seeds (*Triticum aestivum* cv.
137 Tukan) were surfaced-sterilized in 4% sodium hypochlorite containing 0.02% (v/v) Tween
138 20, rinsed thoroughly with sterile water and germinated for 2 d in a plate on moistened filter
139 paper at 25°C in darkness. Subsequently, seedlings were grown hydroponically in 3 L plastic
140 containers with modified Long Ashton nutrient solution (Hewitt, 1966) containing 800 µM of
141 Pi with constant aeration in a greenhouse for 4 weeks. After that, half of the plants were
142 transferred to a modified nutrient solution without Pi (-P) and were let to grow for another
143 week before applying 2'-*epi*-GR24 treatments. The other half was maintained under normal
144 Pi (+P) conditions. Nutrient solution was replaced twice in a week. The active diasteroisomer
145 2'-*epi*-GR24 (orobanchol-type) (Fig. S1) was applied to the nutrient solution (with and
146 without Pi) at 4 different concentrations (0, 10, 100 and 1000 nM) for 1 h. The SL analogue
147 2'-*epi*-GR24 was kindly provided by Dr. Xie (Utsunomiya University, Japan). To prepare 2'-
148 *epi*-GR24, 1 mg of the compound was dissolved in 330 µl of pure acetone to obtain a stock
149 solution of 1 M. The stock was serially diluted in sterile demiwater to obtain the desired final
150 concentrations. Then, the corresponding nutrient solution was replaced without 2'-*epi*-GR24,
151 and plants were grown for additional 24 h. Six seedlings per treatment were grown. Shoots
152 and roots were collected, weighted, frozen in liquid nitrogen and kept at -80ºC until use.

153 Plants of the SL-deficient tomato line *SlCCD8*-RNAi line L04 and its corresponding
154 wild-type cv. Craigella (LA3247) were grown in pots as described in Lopez-Raez et al 2008
155 (López-Ráez et al., 2008). Seeds were surfaced-sterilized and germinated as described above.
156 The seedlings were sown and grown in 0.5 L pots with sand/vermiculate (1:1) for 4 weeks in
157 a greenhouse at 21/18°C with 16/8 h photoperiod and 70% humidity. Plants were watered

158 twice a week with modified Long Ashton nutrient solution (Hewitt, 1966). Half of the plants
159 (Craigella and *SlCCD8*-RNAi) were watered with standard Pi levels (800 μM), whereas the
160 other half was watered with 25% Pi of the standard solution (200 μM), to subject the plants to
161 Pi limitation and induce the characteristic SL-deficient phenotype. Six seedlings per cultivar
162 and treatment were grown. Roots from each pot were collected separately, frozen in liquid
163 nitrogen and stored at -80°C until use.

164

165 ***Extraction and quantification of strigolactones from roots***

166 SL quantification was performed as described in Rial et al. 2018 (Rial et al., 2009). Fifty mg
167 of tomato root extracts from each treatment were ground in a mortar with liquid nitrogen.
168 Root material was extracted with 1 mL of ethyl acetate in an ultrasonic bath for 10 min,
169 centrifuged for 10 min at 5000 rpm, concentrated in a rotary evaporator, and stored at -80 °C.
170 Before the analysis, the extracts were dissolved with MeOH to achieve a ratio 1:1 $g \cdot L^{-1}$
171 (w/v). (±)-GR24 (*rac*GR24), used as internal standard, was dissolved in MeOH to 10 $mg \cdot L^{-1}$
172 and added to all samples at 10 $\mu g \cdot L^{-1}$. The samples were analysed on a Bruker EVOQ Triple
173 Quadrupole Mass Spectrometer (Bruker, Madrid, Spain), using as ionization source an
174 electrospray (ESI+). The samples were injected and separated using an ACE Excel 1.7 C18
175 (100 mm × 2.1 mm, 1.7 μm particle size) (Advanced Chromatography Technologies Ltd.,
176 Aberdeen, Scotland) maintained at 40°C. The mobile phases were solvent A (water, 0.1%
177 formic acid) and solvent B (MeOH, 0.1% formic acid), with a flow rate set to 0.3 mL/min.
178 The linear gradient was: 0-0.5 min, 50% B; 0.5-5 min, to 100% B; 5-7 min, 100% B; 7-7.5
179 min, to 50% B, and 7.5-10.5 min, 50% B. The injection volume was 5 μL. The instrument
180 parameters were: spray voltage +4500 V, cone temperature 300°C, cone gas flow 15 psi,
181 heated probe temperature 400°C, heated probe gas flow 15 psi, nebulizer gas flow 55 psi and
182 collision pressure 2.0 mTorr. The compound-dependent parameters for orobanchol, solanacol

183 and the IS, the parent or precursor ions, the fragments obtained by MRM analysis and the
184 collision energy to achieve each fragmentation are provided in Table S1. Orobanchol and
185 solanacol were kindly supplied by Professor Xiaonan Xie and Professor Koichi Yoneyama
186 (Weed Science Center, Utsunomiya University, Japan), and *rac*GR24 was provided by
187 Professor Binne Zwanenburg (Department of Organic Chemistry, Radboud University,
188 Nijmegen, Netherlands).
189

190 ***RNA isolation and gene expression analysis by quantitative real time RT-PCR (qPCR)***
191 Total RNA was extracted using TRIsure reagent (Bioline, Barcelona, Spain) according to the
192 manufacturer's instructions. Subsequently, the RNA was treated with RQ1 DNase (Promega,
193 Madrid, Spain) and purified through a silica column using the RNA Clean & Concentrator kit
194 (Zymo Research, Madrid, Spain). RNA was quantified using a Nanodrop (Thermo Fisher
195 Scientific, Madrid, Spain), and its integrity checked by gel electrophoresis before stored at -
196 80ºC. The first strand cDNA was synthesized with 1 µg of purified total RNA using the
197 PrimeScript RT Master Mix kit (Takara, Saint-Germain-en-Laye, France) according to the
198 manufacturer's instructions. Real time quantitative RT-PCR (qPCR) was performed in a
199 StepOnePlus real-time PCR system (Thermo Fisher Scientific, Madrid, Spain), using the TB
200 Green Premix *ExTaq* kit (Takara, Saint-Germain-en-Laye, France) and specific primers
201 (Table S2). Five independent biological replicates were analysed per treatment. Relative
202 quantification of specific mRNA levels was performed using the comparative $2^{-\Delta(\Delta Ct)}$ method
203 (Livak & Schmittgen, 2001). Expression values were normalized using the housekeeping
204 gene *SlActin* for tomato and *TahnRNPQ* (the heterogeneous nuclear ribonucleoprotein Q) for
205 wheat (Grün et al., 2018).
206

207 ***Metabolic analyses***

208 Untargeted metabolic profiles of tomato roots were performed by liquid chromatography and
209 electrospray ionization (LC-ESI) full scan mass spectrometry, as describe in Rivero et al.,
210 2008 (Rivero et al. 2018). Briefly, 50 mg of freeze-dried root material was extracted at 4°C
211 with 1 ml of MeOH:H_2O (10:90, v:v) containing 0.01% of HCOOH. After the centrifugation
212 at full speed at 4°C for 15 min, the supernatant was filtered through 0.2 µm cellulose filters
213 (Regenerated Cellulose Filter, 0.20 µm, 13 mm D. pk/100; Teknokroma, Barcelona, Spain).
214 Subsequently, 20 µl were injected into an Acquity UPLC system (Waters, Mildford, MA,
215 USA) interfaced with a hybrid quadrupole time-of-flight instrument (QTOF MS Premier).
216 Subsequently, a second fragmentation function was introduced into the TOF analyser to
217 identify the signals detected. This function was programmed in a t-wave ranging from 5 to 45
218 eV to obtain a fragmentation spectrum of each analyte (Gamir et al. 2012). Positive and
219 negative electrospray signals were analysed independently to obtain a global view of the data
220 conduct. To elute the analytes, a gradient of methanol and water containing 0.01% HCOOH
221 was used. Six independent biological samples were randomly injected. The LC separation was
222 performed using an UPLC Kinetex 2.6 µm particle size EVO C18 100 A, 50 x 2.1 mm
223 (Phenomenex, Madrid, Spain). Chromatographic conditions and solvent gradients and further
224 were established as described by Rivero et al. (2018).
225
226 ***Full scan data analysis***
227 Full scan data files were acquired with the Masslynx 4.1 software (Masslynx 4.1; Waters,
228 Barcelona, Spain) and were transformed from .raw format into .cdf with Databridge tool
229 provided by Masslynx software. The software R (http://www.r-project.org/) was used to
230 process chromatographic data file using the XCMS algorithm (www.bioconductor.org) to
231 obtain the peak peaking, grouping signals and signal corrections. Peak area was normalized
232 relative to the dry weight. To test the metabolic differences between treatments, a

233 nonparametric Kruskal-Wallis test ($P < 0.05$) was performed. Partial least square discriminant
234 analysis and heat map analysis were performed with the metaboAnalyst 4.0 (Chong & Xia,
235 2018). Adduct and isotope correction, filtering, clustering, exact mass mapping and metabolic
236 pathway exploration was carried out with the packages MarVis filter, MarVis cluster and
237 MarVis pathway that are integrated in the Marvis suit 2.0 (Kaever et al., 2015). Metabolite
238 identification was carried out based on exact mass accuracy and fragmentation spectra
239 matching with different online database. The database kegg (https://www.genome.jp/kegg/)
240 was used for exact mass identity and for fragmentation spectrum analysis, the Massbank and
241 the Metlin databases were used (www.massbank.jp; www.masspec.scripps.edu).
242
243 ***Statistical analyses***
244 Data were subjected to one-way analysis of variance (ANOVA) using the software SPSS
245 Statistics v. 20 for Windows (SPSS Inc., Chicago, IL, USA). Duncan's multiple range test
246 was applied when suited. Full scan data was subjected to Kruskal-Wallis test and signals with
247 P-value ≤ 0.05 between treatments were used for identification.
248
249 **RESULTS**
250
251 **Low doses of 2'-*epi*-GR24 stimulate strigolactone biosynthesis in roots under normal**
252 **and low Pi conditions**
253
254 It is well known that SL biosynthesis is promoted under Pi deficiency (López-Ráez et al.,
255 2008; Yoneyama et al., 2012). Here, we explore the potential feedback in SL biosynthesis
256 under both optimal and deficient Pi conditions. Tomato plants were grown hydroponically
257 under normal Pi conditions (+Pi) or subjected to Pi limitation for a week (-Pi). Then, half of

258 the plants of each condition were given a 1h-pulse with different concentrations (0, 10, 100 or
259 1000 nM) of 2'-*epi*-GR24. The SL analogue *rac*GR24 is a racemic mixture of four
260 diastereoisomers, where some of the enantiomers do not present SL activity (Scaffidi et al.,
261 2014). Here, to avoid side-effects of the non-active molecules, the active diasteroisomer 2'-
262 *epi*-GR24 (orobanchol-type) (Fig. S1) was applied to the nutrient solution. Upon the 1h-pulse,
263 plants grew for additional 24 h with the corresponding nutrient solution (with or without Pi)
264 without 2'-*epi*-GR24 to evaluate the response to the treatment.

265 As expected, the levels of the characterized tomato SLs orobanchol and solanacol (Fig.
266 S1) (López-Ráez et al., 2008) were promoted after one week Pi starvation (Figs. 2a and b). In
267 addition, the levels of these two SLs were further enhanced by the exogenous application of
268 2'-*epi*-GR24. Remarkably, this effect was dose-dependent, being most pronounced at the
269 lowest dose (10 nM) and disappearing at higher doses (100 and 1000 nM) (Figs. 2a and b).
270 The same pattern as for the analytical quantification was observed by qPCR when using
271 molecular markers for the SL biosynthesis pathway. The two genes studied were *SlD27*,
272 encoding for a β-carotenoid isomerase which converts all-*trans*-β-carotene to 9-*cis*-β-carotene
273 and *SlCCD8*, which encodes a carotenoid cleavage enzyme catalysing the production of
274 carlactone, the precursor of all canonical SLs (Al-Babili & Bouwmeester, 2015; Waters et al.,
275 2017). The expression of both genes was induced about 3 times under Pi starvation, and was
276 further promoted up to 8 and 6 times, respectively, by 10 nM of 2'-*epi*-GR24 (Figs. 2c and d).

277 Orobanchol and solanacol levels also increased by 2'-*epi*-GR24 in plants grown under
278 normal Pi conditions, somehow resembling those observed in Pi limitation. Here, the effect
279 was also dose-dependent, but the highest levels were observed at higher concentrations of 2'-
280 *epi*-GR24 (Figs. 2a and b). A very similar pattern was found regarding the expression of
281 *SlD27* and *SlCCD8*. They were also induced under these conditions (Figs. 2c and d). The
282 expression of these two SL biosynthesis genes was analysed in another important agricultural

283 crop as wheat. Interestingly, a similar trend was observed in the expression levels of the
284 wheat *TaD27* and *TaCCD8* genes, being both induced by Pi deprivation and by 10 nM 2-*epi*-
285 GR24 under normal Pi conditions (Fig. S2).
286
287 **Short-term application of strigolactones enhance plant Pi starvation signalling**
288
289 The influence of SLs on P-related signalling was assessed by analysing the expression of key
290 genes involved in plant P responses, such as the triad *IPS1*-miR399-*PHO2*, and the high-
291 affinity Pi transporter *LePT2*. *LePT2* belongs to the PHT1 family, and its expression is
292 strongly dependent on the plant Pi status (Franco-Zorrilla et al., 2007; Lin et al., 2008; Nagy
293 et al., 2005; Pant et al., 2008). The expression of *LePT2* was induced in the roots more than 2
294 times under Pi limitation. Interestingly, its expression was further increased up to 5 times
295 when 10 nM 2'-*epi*-GR24 was applied, while higher concentrations had no effect in its
296 expression under this Pi limiting conditions (Fig. 3a). The application of 2'-*epi*-GR24 under
297 normal Pi conditions induced an increase in the expression of *LePT2*, reaching at all SL
298 concentrations similar expression levels to those observed for Pi starvation (Fig. 3a). A very
299 similar expression profile was observed for the genes of triad *IPS1*-miR399-*PHO2*. Transcript
300 levels of *LeTPSI1*, the tomato homolog to *IPS1* (Liu et al., 1997), and *SlmiR399* were
301 promoted by Pi deprivation, and this induction potentiated by SL addition (Figs. 3b and c).
302 Moreover, under normal Pi conditions gene expression was enhanced by all 2'-*epi*-GR24
303 treatments (Figs. 3b and c). A different behaviour was detected for the other key gene in Pi
304 response. Transcript levels of *PHO2* were almost 2 times down-regulated by Pi starvation,
305 levels that were recovered upon application of 2'-*epi*-GR24 (Fig. 3d). Nevertheless, the
306 application of 10 nM 2'-*epi*-GR24 under normal Pi conditions repressed the expression of
307 *PHO2* 1.5 times (Fig. 3d), resembling the effect of Pi starvation.

308 The expression pattern of *IPS1*, miR399 and *PHO2* and of a high-affinity Pi transporter
309 (*TaPht2*) was also analysed in wheat. As for tomato, transcript levels of *TaPht2*, *taemiR399*
310 and *TaIPS1* were clearly induced by Pi starvation and by 10 nM 2'-*epi*-GR24 application
311 under normal Pi conditions (Fig. S3). In the case of *TaPHO2*, Pi starvation did not induce any
312 significant change. However, 2'-*epi*-GR24 increased its transcript levels only under Pi
313 limiting conditions (Fig. S3d).

314

315 **SL-deficient plants are less sensitive to Pi starvation**

316

317 We previously generated and characterized knock-down lines for the SL biosynthesis gene
318 *SlCCD8* in tomato (Kohlen et al., 2012). One of these transgenic lines - *SlCCD8-RNAi* L04 -
319 presented a 92% reduction on SLs levels. Here, we analysed the response of this SL-deficient
320 line to Pi starvation by checking the expression of Pi marker genes. Basal expression of the Pi
321 transporter *LePT2* was about 2-fold lower in the *SlCCD8-RNAi* line compared to the
322 corresponding wild-type under normal Pi conditions. An increase in *LePT2* transcript levels
323 was observed under Pi limitation both in the wild-type and the transgenic line. However, the
324 final value reached in *SlCCD8-RNAi* was lower because of their reduced basal levels (Fig.
325 4a). The same behaviour was observed for *SlmiR399* and *LeTPSI1*, showing an induction by
326 Pi starvation both in the wild-type and the transgenic line (Figs. 4b and c). As for *LePT2*,
327 basal transcriptional levels of these genes were also lower in *SlCCD8-RNAi*, therefore
328 reaching lower final values. In the case of *SlPHO2*, under normal Pi conditions the basal
329 levels in the *SlCCD8-RNAi* were higher than in the wild-type, in contrast to the pattern
330 observed for *SlmiR399* and *LeTPSI1* (Fig. 4d). On the other hand, an induction for *SlPHO2*
331 was detected in the wild-type in Pi starvation compared to normal Pi conditions, while in
332 *SlCCD8-RNAi* a slight reduction was observed (Fig. 4d). Thus, Pi starvation had opposite

333 effects in the wild-type and in the SL-deficient line in the expression of *SlPHO2*, supporting
334 the regulatory role of SLs in Pi responses.
335
336 **Low doses of 2-*epi*-GR24 impact metabolic profiles in the roots, resembling those of Pi**
337 **starvation**
338
339 The previous data evidence a parallelism between the plant response to Pi starvation and to
340 low doses of SLs (2'-*epi*-GR24). In addition, altered basal levels of Pi marker genes and
341 response to Pi limitation were observed in the SL-deficient line *SlCCD8-RNAi*. To investigate
342 a potential direct relationship between SLs and Pi signalling, the reprogramming of tomato
343 root metabolism associated to responses to Pi starvation and exogenous application of 2'-*epi*-
344 GR24 were compared. Since major effects at transcriptional and SL levels were observed at
345 low doses of 2'-*epi*-GR24 (10 nM), root metabolic profiles upon application of this
346 concentration of 2'-*epi*-GR24 under normal and limited Pi conditions were analysed.
347 Untargeted metabolomics analyses of extracts via HPLC coupled with a quadrupole time-of-
348 flight mass spectrometer were performed. Following the chromatographic analyses, a
349 bioinformatics processing of the detected signals was performed using the MarVis Suit 2.0
350 software tool for clustering and visualization of the metabolic markers (Kaever et al., 2015).
351 Clustering and functional pathway (KEGG *Solanum lycopersicum* pathway Database)
352 analyses were further performed to obtain potential biological information of the metabolic
353 reprogramming.
354 Metabolic analysis yielded a total of 1180 signals, 298 in ESI- mode (Table S3) and 882
355 in ESI+ mode (Table S4). A combined principal component analysis (PCA) ($P < 0.05$) of the
356 signals obtained from the ESI+ and ESI- modes showed that the principal source of variation
357 resulted from Pi starvation [Control -P (C-P) and GR24-treated -P (GR-P)]. Plant samples

358 subjected to one week of Pi deprivation grouped together in the PCA, and clearly separated
359 from those of plants grown under normal Pi conditions [Control +P (CP)], explaining 2.6% of
360 the variation (component 2) (Fig. 5a). Under Pi starvation, exogenous application of 2'-*epi*-
361 GR24 did not induce significant changes in the plant (GR-P *vs* C-P), showing a priority effect
362 of Pi starvation in the plant metabolome. However, under normal Pi conditions, 2'-*epi*-GR24
363 application [GR24-treated +P (GRP) *vs* Control +P (CP)] induced plant metabolic responses,
364 revealing some SL-derived metabolic responses leading to profiles closer to those observed in
365 plants grown under Pi limitation (Fig. 5a). Hierarchical cluster analysis of the different groups
366 confirmed the observations of the PCA analysis, supporting that the main source of variability
367 is the plant Pi status. Remarkably, the heatmap analysis showed that rather than inducing, Pi
368 starvation repressed the biosynthesis of most secondary metabolites detected (Fig. 5b). The
369 Kruskal-Wallis test revealed 408 significant ($P < 0.05$) features, of which 166 showed
370 differential signals when comparing control plants (CP) with 2-*epi*-GR24 treated plants (GRP
371 and GR-P) (Fig. 5c). Out of the 166 features, 40 signals were increased by 2-*epi*-GR24 (Fig.
372 5d).

373 The major impact took place at the primary metabolism, including signals associated to
374 carboxylic acids, fatty acids and purine metabolism, but also at the secondary metabolism,
375 mainly associated to phenylpropanoids (Fig. 6), changes already reported to be associated to
376 Pi starvation responses (Pant et al., 2015; Ziegler et al., 2016). Among the identified
377 compounds, we found the carboxylic acids malic and citric acids, whose levels were increased
378 by Pi starvation and by 2-*epi*-GR24 in normal Pi conditions. The same pattern was observed
379 for the fatty acids decanoic and azelaic acids, allantoic acid (purine metabolism), 3''-
380 Hydroxy-geranylhydroquinone (ubiquinone and other terpenoid-quinone biosynthesis),
381 isophenoxazine (tryptophan metabolism), and the flavonoid luteolin, all of them induced by Pi
382 starvation, but also by 2-*epi*-GR24 under normal Pi conditions. Among the compounds that

383 showed reduced accumulation under Pi starvation, several also showed a reduction with the
384 application of 2-*epi*-GR24 under normal Pi conditions, as is the case of some fatty acids,
385 especially compounds associated to the linoleic and alpha-linolenic acids metabolism,
386 including 9-Oxooctadeca-10, 12-Oxo-9(z)-dodecenoic acid and 9,10-Epoxyoctadecatrienoic
387 acid (Fig. 6).
388
389 **DISCUSION**
390
391 P is one of the less-abundant macronutrients in soils, which negatively impacts plant growth
392 and development, and therefore agricultural production. In intensive agriculture, the abuse of
393 P-fertilizers originates considerable costs and environmental damage, as soil and groundwater
394 contamination. Therefore, understanding how plants sense, signal and respond to low Pi
395 availability is essential to optimize the use of these fertilizers, alleviating agricultural costs
396 and the excessive consumption of this non-renewable resource.
397 SLs are key modulators of plant responses to Pi limitation in the soil, significantly
398 altering plant physiology and development to optimize Pi uptake and use (reviewed in Waters
399 et al., 2017). Indeed, their biosynthesis is highly promoted under Pi limiting conditions
400 (López-Ráez et al., 2008; Yoneyama et al., 2012). They have the capacity of inhibiting bud
401 outgrowth under Pi shortage in order to reduce shoot biomass and minimize Pi demand.
402 Actually, SL-deficient plants show a typical dwarf and bushy phenotype in different species,
403 which is restored upon exogenous application of *rac*GR24 (Gomez-Roldan et al., 2008;
404 Umehara et al., 2008). In the aerial part, they also promote internode elongation, secondary
405 growth of the stem and early leaf senescence to facilitate Pi reallocation, and inhibit
406 adventitious rooting. In the roots, where they are mainly produced and accumulated, SLs
407 repress the growth of the primary root, while stimulate the outgrowth of lateral roots, root hair

408 number and elongation (Kapulnik et al., 2011; Ruyter-Spira et al., 2011). All these
409 morphological modifications are well documented to be associated to plant responses to Pi
410 starvation, and they are oriented to increase the root surface area to facilitate Pi uptake in the
411 soil (Lynch, 2011; Raghothama, 2000). More recently, it has been reported that exogenous
412 *rac*GR24 application promoted anthocyanin accumulation and the activation of acid
413 phosphatases, typical early Pi starvation responses in plants (Ito et al., 2015). However, how
414 they modulate plant Pi responses and whether they are involved in P signalling is not clear.
415 We show here that a 1h-pulse of the active SL analogue 2'-*epi*-GR24 was able to further
416 promote the biosynthesis of endogenous SLs, already induced by Pi limitation, both in tomato
417 and wheat (Fig. 2), supporting a positive feedback loop in SL biosynthesis. Interestingly, 2'-
418 *epi*-GR24 application increased SL levels also under optimal Pi conditions, where SL levels
419 are usually low and even undetectable (López-Ráez et al., 2008; Yoneyama et al., 2012),
420 suggesting that SLs could act as signals triggering plant responses to Pi deficiency. In
421 agreement with their potential regulatory role in P signalling, the positive effect of GR24 was
422 mainly observed at low doses (10 nM) in plants grown under Pi starvation, with higher basal
423 levels of endogenous SLs, while the main effect under optimal Pi conditions (with low
424 endogenous SL levels) was detected at higher doses of GR24 (100 and 1000 mM). Other
425 dose-dependent effects for SL action have been previously reported. We previously showed
426 that *rac*GR24 application under normal Pi conditions supressed lateral root formation, while it
427 was promoted at Pi limitation. Therefore, it was proposed that endogenous SLs are important
428 for the final output in lateral root development (Ruyter-Spira et al., 2011). Similarly, De
429 Cuyper and co-workers showed that in the interaction *Sinorhizobium meliloti-Medicago*
430 *truncatula* *rac*GR24 treatment differentially affected nodulation, depending on its
431 concentration. The authors showed that low doses were able to promote the number of
432 nodules, whereas high doses reduced it (De Cuyper et al., 2015). Therefore, it seems that the

433 net effect of SLs depends on the P nutritional conditions and on their optimum endogenous
434 levels, as described for most plant hormones.
435 As mentioned, plant P responses are finely regulated by PHR1 and the triad *IPS1*-
436 miR399-*PHO2* (Fig. 1) (Franco-Zorrilla et al., 2007; Ham et al., 2018; Puga et al., 2017). We
437 have recently proposed that higher basal levels of SLs in a commercial wheat cultivar would
438 act as a priming signal to boost plant responses to Pi starvation, regulating the expression
439 levels of the three genes of the triad *IPS1*, miR399 and *PHO2* (Campos et al., 2019). The
440 fine-tuning modulation of PHO2 activity would reduce shoot Pi loading and favour the
441 development of the root system, thus improving Pi acquisition efficiency and use (Campos et
442 al., 2019). Here, we show that the short-term application of 2'-*epi*-GR24 also affected the
443 expression of *IPS1*-miR399-*PHO2* and that of the high affinity transporter *LePT2* genes, both
444 in tomato and wheat. Low doses of GR24 boosted the expression of these genes, already
445 promoted by Pi starvation, both in tomato and wheat. Also in the case of *PHO2*, which
446 expression was reduced under Pi limitation, it was promoted by 2'-*epi*-GR24. This fact could
447 be explained as an effect of timing and/or endogenous concentration of SLs. Indeed, a time-
448 dependent increase of *PHO2* transcripts has been shown in wheat, which would coincide with
449 a progressive increase in SL levels (Campos et al., 2019). 2'-*epi*-GR24 also induced the
450 expression of these Pi marker genes even at optimal Pi conditions, partially mimicking the
451 effect observed in Pi starvation. A promoter effect of *rac*GR24 in the expression of the high
452 affinity Pi transporter *Pht1;7* under Pi deprivation has also been observed in Arabidopsis
453 (Prerostova et al., 2018). Conversely, a down-regulation of Pi transporters from the *Pht1*
454 family in the SL-deficient mutant *max1-1* was shown (Ito et al., 2015). Altogether, the results
455 point to an involvement of SLs in regulating early P signalling in plants (Figure 7). In
456 agreement with this idea, the SL-deficient line *SlCCD8-RNAi* showed altered levels of these
457 key Pi response regulatory elements, being also less sensitive to Pi starvation. A similar effect

458 was observed in Arabidopsis, where the SL-deficient *max1-1* and the SL-signalling *max2-1*
459 mutants were less sensitive to Pi limitation, producing less root hairs and anthocyanins under
460 stress. This mutant showed reduced transcript levels of *IPS1*, whereas those of *PHO2* were
461 up-regulated (Ito et al., 2015). We report here the same pattern for tomato. Interestingly, the
462 application of *rac*GR24 partially rescued the phenotype in the SL-deficient mutant, but not in
463 the SL-signalling mutant (Ito et al., 2015; Kapulnik et al., 2011). Thus, our results support an
464 important role of SLs in the plant response to P levels. Further research is required to decipher
465 how plants perceive Pi stress and how the relationship SL-P signalling is regulated.

466 In addition to promoting SL biosynthesis and increasing the gene expression of P
467 signalling marker genes under low and optimal Pi conditions, low doses of 2'-*epi*-GR24
468 altered root metabolic profiles in plants grown under optimal Pi conditions, partially
469 resembling those observed under Pi starvation (Fig. 5a). Among the identified compounds, an
470 increase in malate and citrate was observed under both Pi starvation and 2'-*epi*-GR24
471 application. An accumulation and exudation into the rhizosphere of these carboxylic acids is
472 generally observed in plants exposed to Pi shortage (Pant et al., 2015). It is suggested that
473 they can improve Pi availability by mobilizing different P forms from the soil through the
474 chelation of metal ions such as Fe, Al or Ca. Moreover, a key role for malate in the
475 characteristic changes triggered by Pi starvation in root system architecture has been recently
476 described (Mora-Macías et al., 2017). Another important metabolite associated to plant
477 responses to Pi availability which levels were accumulated by Pi starvation and 2'-*epi*-GR24
478 under optimal Pi conditions was allantoic acid. The accumulation of nitrogen rich compounds,
479 including the ureides allantoin and its degradation product allantoate, is related to increased
480 nucleotide degradation and the consequent Pi mobilization under this stress condition, and the
481 crosstalk between P and nitrogen metabolism (Medici et al., 2019; Pant et al., 2015). Overall,

482 the accumulation of these compounds would help the plant to cope with low Pi availability by
483 optimizing P use and internal mobilization.

484 Another metabolite specifically accumulated under Pi limitation and 2'-*epi*-GR24
485 application was the saturated dicarboxylic acid azelaic acid. This compound has been
486 associated to priming of plant immunity (systemic acquired resistance, SAR), conferring local
487 and systemic resistance against bacterial pathogens by inducing the production of SA (Jung et
488 al., 2009). Increased levels of SA in roots subjected to Pi starvation have been shown
489 (Prerostova et al., 2018) (López-Ráez et al., unpublished data), whereas a reduced
490 accumulation of this hormone was observed in the tomato SL-deficient *SlCCD8-RNAi* line L9
491 (Torres-Vera et al., 2014). Interestingly, this SL-deficient line was more susceptible to the
492 fungal pathogen *Botrytis cinerea*. Similarly, Arabidopsis SL-deficient plants were
493 hypersensitive to the actinomycete *Rhodococcus fascians*, whereas the application of
494 *rac*GR24 to wild-type plants induced resistance against this pathogen (Stes et al., 2015). A
495 connection between Pi starvation and SAR through the stimulation of SA by the Pi transporter
496 *PHT4;1* was made in Arabidopsis (Wang et al., 2011), where the authors proposed a critical
497 role of this Pi transporter in regulating innate immunity in Arabidopsis. Parallelism between
498 Pi starvation and 2'-*epi*-GR24 application was also found in the reduction of compounds
499 associated to the linoleic and alpha-linolenic acids metabolism was observed (Fig. 6). These
500 pathways are related with the biosynthesis of essential fatty acids and oxylipins metabolism,
501 which includes the biosynthesis of JA and derivatives. The reduction of these intermediate
502 compounds could indicate an increase of the final products of these pathways such as JA,
503 among others. Indeed, a promotion of JA content by Pi starvation has been reported (Khan et
504 al., 2016; Prerostova et al., 2018). Interestingly, Khan and co-workers showed that the
505 accumulation of JA in Pi-starved plants was mediated by PHR1, and that it was associated
506 with resistance to insect herbivores (Khan et al., 2016). As for SA, reduced levels of JA were

507 observed in the SL-deficient *SlCCD8-RNAi* line L9 (Torres-Vera et al., 2014), pointing to a
508 role of SLs in defence responses. A cross-talk between Pi starvation and signalling pathways
509 regulating plant responses to other environmental stresses, including biotic stresses, has been
510 suggested, opening up a broad field of research. SLs, through its interaction with other
511 phytohormones, might be regulating these plant stress responses in a dose- and likely tissue-
512 dependent manner.

513 Summarizing, we provide experimental evidences supporting that SLs are early
514 modulators of plant responses to low Pi availability, promoting the expression of key
515 regulatory genes and that of high-affinity Pi transporters associated to this stress, and altering
516 metabolic profiles to cope with Pi limitation (Figure 7). A short-term pulse of low doses of
517 the SL analogue 2'-*epi*-GR24 at optimal Pi conditions was able to partially mimic the plant
518 response to Pi starvation, supporting the role of SLs in Pi-related signalling. The results
519 presented here could be extrapolated to crop varieties with higher endogenous SLs' levels or
520 with increased sensitivity to this plant hormone. This knowledge may help to develop new
521 strategies to optimize plant Pi acquisition efficiency and use, thus reducing the excessive use
522 of P fertilizers for a more sustainable agriculture.

523

524 **ACKNOWLEDGEMENTS**

525

526 This work was supported by grants RTI2018-094350-B-C31 and AGL2017-88-083-R from
527 the Spanish National R&D Plan of the Ministry of Science, Innovation and Universities
528 Economy and Competitiveness (MICIU) and the European Regional Development Fund
529 (ERDF), and 201640I040 from the Spanish National Research Council (CSIC). JG was
530 supported by two Postdoctoral fellowships: FJCI-2015-23575 from Ministry of Science,
531 Innovation and Universities, and CDEIGENT/2018/015 from Generalitat Valenciana. The

532 laboratory participates in the National Network of Carotenoids (CaRed) (BIO2017-90877-
533 REDT). We thank Dr Koichi Yoneyama and Dr Xiaoan Xie for kindly providing the natural
534 strigolactones used to set up their analytical quantification and Dr Binne Zwanenburg for
535 providing *rac*GR24. We also thank the SCIC of the University Jaume I for technical support
536 in the metabolomic analysis. We declare that we do not have any conflict of interest.

537

538 **REFERENCES**

539

540 Al-Babili, S., & Bouwmeester, H. J. (2015) Strigolactones, a novel carotenoid-derived plant
541 hormone. In*: Vol. 66. Annual Review of Plant Biology* (pp. 161-186).
542 Andreo-Jiménez, B., Ruyter-Spira, C., Bouwmeester, H., & López-Ráez, J. A. (2015).
543 Ecological relevance of strigolactones in nutrient uptake and other abiotic stresses, and
544 in plant-microbe interactions below-ground. *Plant and Soil* 394, 1-19.
545 doi:doi:10.1007/s11104-015-2544-z
546 Bustos, R., Castrillo, G., Linhares, F., Puga, M. I., Rubio, V., Pérez-Pérez, J., . . . Paz-Ares, J.
547 (2010). A central regulatory system largely controls transcriptional activation and
548 repression responses to phosphate starvation in Arabidopsis. *PLoS Genetics* 6.
549 doi:10.1371/journal.pgen.1001102
550 Campos, P., Borie, F., Cornejo, P., López-Ráez, J. A., López-García, A., & Seguel, A. (2018).
551 Phosphorus acquisition efficiency related to root traits: Is mycorrhizal symbiosis a key
552 factor to wheat and barley cropping? *Frontiers in Plant Science* 9.
553 doi:10.3389/fpls.2018.00752
554 Campos, P. M., Cornejo, P., Rial, C., Borie, F., Varela, R. M., Seguel, A., & Lopez-Raez, J.
555 A. (2019). Phosphate acquisition efficiency in wheat is related to root:shoot ratio,
556 strigolactone levels, and Pho2 regulation. *Journal of Experimental Botany* 70, 5631-
557 5642. doi:10.1093/jxb/erz349
558 Chiou, T. J., & Lin, S. I. (2011) Signaling network in sensing phosphate availability in plants.
559 In*: Vol. 62. Annual Review of Plant Biology* (pp. 185-206).
560 Chong, J., & Xia, J. (2018). MetaboAnalystR: an R package for flexible and reproducible
561 analysis of metabolomics data. *Bioinformatics (Oxford, England)* 34, 4313-4314.
562 doi:10.1093/bioinformatics/bty528
563 De Cuyper, C., Fromentin, J., Yocgo, R. E., De Keyser, A., Guillotin, B., Kunert, K., . . .
564 Goormachtig, S. (2015). From lateral root density to nodule number, the strigolactone
565 analogue GR24 shapes the root architecture of *Medicago truncatula*. *Journal of*
566 *Experimental Botany* 66, 137-146. doi:10.1093/jxb/eru404
567 Franco-Zorrilla, J. M., Valli, A., Todesco, M., Mateos, I., Puga, M. I., Rubio-Somoza, I., . . .
568 Paz-Ares, J. (2007). Target mimicry provides a new mechanism for regulation of
569 microRNA activity. *Nature Genetics* 39, 1033-1037. doi:10.1038/ng2079

570 Gamir, J., Pastor, V., Cerezo, M., Flors, V. (2012). Identification of indole-3-carboxylic acid
571 as mediator of priming against Plectosphaerella cucumerina. Plant Physiology and
572 Biochemistry 61, 169-179. doi: 10.1016/j.plaphy.2012.10.004
573 Gómez-Roldán, V., Fermas, S., Brewer, P. B., Puech-Pagés, V., Dun, E. A., Pillot, J. P., . . .
574 Rochange, S. F. (2008). Strigolactone inhibition of shoot branching. *Nature* 455, 189-
575 194. doi: 10.1038/nature07271
576 Grün, A., Buchner, P., Broadley, M. R., & Hawkesford, M. J. (2018). Identification and
577 expression profiling of Pht1 phosphate transporters in wheat in controlled
578 environments and in the field. *Plant Biology, 20*(2), 374-389. doi:10.1111/plb.12668
579 Ham, B. K., Chen, J., Yan, Y., & Lucas, W. J. (2018). Insights into plant phosphate sensing
580 and signaling. *Current Opinion in Biotechnology,* 49 1-9.
581 doi:10.1016/j.copbio.2017.07.005
582 Hewitt, E. J. (1966). *Sand and water culture methods used in the study of plant nutrition*.
583 Paper presented at the Technical communication no. 22. Commonwealth Agriculture
584 Bureau, London, UK.
585 Huang, T. K., Han, C. L., Lin, S. I., Chen, Y. J., Tsai, Y. C., Chen, Y. R., . . . Chiou, T. J.
586 (2013). Identification of downstream components of ubiquitin-conjugating enzyme
587 PHOSPHATE2 by quantitative membrane proteomics in Arabidopsis roots. *Plant Cell*
588 25, 4044-4060. doi:10.1105/tpc.113.115998
589 Ito, S., Nozoye, T., Sasaki, E., Imai, M., Shiwa, Y., Shibata-Hatta, M., . . . Asami, T. (2015).
590 Strigolactone regulates anthocyanin accumulation, acid phosphatases production and
591 plant growth under low phosphate condition in Arabidopsis. *PLoS ONE* 10.
592 doi:10.1371/journal.pone.0119724
593 Jung, H. W., Tschaplinski, T. J., Wang, L., Glazebrook, J., & Greenberg, J. T. (2009).
594 Priming in systemic plant immunity. *Science* 324, 89-91.
595 doi:10.1126/science.1170025
596 Kaever, A., Landesfeind, M., Feussner, K., Mosblech, A., Heilmann, I., Morgenstern, B., . . .
597 Meinicke, P. (2015). MarVis-Pathway: integrative and exploratory pathway analysis
598 of non-targeted metabolomics data. *Metabolomics* 11, 764-777. doi:10.1007/s11306-
599 014-0734-y
600 Kapulnik, Y., Delaux, P. M., Resnick, N., Mayzlish-Gati, E., Wininger, S., Bhattacharya, C., .
601 . . Koltai, H. (2011). Strigolactones affect lateral root formation and root-hair
602 elongation in Arabidopsis. *Planta* 233, 209-216.
603 Khan, G. A., Vogiatzaki, E., Glauser, G., & Poirier, Y. (2016). Phosphate deficiency induces
604 the jasmonate pathway and enhances resistance to insect herbivory. *Plant Physiology*
605 171, 632-644. doi:10.1104/pp.16.00278
606 Kohlen, W., Charnikhova, T., Lammers, M., Pollina, T., Toth, P., Haider, I., . . . López-Ráez,
607 J. A. (2012). The tomato *CAROTENOID CLEAVAGE DIOXYGENASE8 (SlCCD8)*
608 regulates rhizosphere signaling, plant architecture and affects reproductive
609 development through strigolactone biosynthesis. *New Phytologist* 196, 535-547.
610 doi:10.1111/j.1469-8137.2012.04265.x
611 Lambers, H., Martinoia, E., & Renton, M. (2015). Plant adaptations to severely phosphorus-
612 impoverished soils. *Current Opinion in Plant Biology* 25, 23-31.
613 doi:10.1016/j.pbi.2015.04.002
614 Lan, P., Li, W., & Schmidt, W. (2015). 'Omics' approaches towards understanding plant
615 phosphorus acquisition and use. In W. C. Plaxton & H. Lambers (Eds.), *Phosphorus*
616 *Metabolism in Plants* (Vol. 48, pp. 65-98).

617 Lin, S. I., Chiang, S. F., Lin, W. Y., Chen, J. W., Tseng, C. Y., Wu, P. C., & Chiou, T. J.
618 (2008). Regulatory network of microRNA399 and PHO2 by systemic signaling. *Plant*
619 *Physiology* 147, 732-746. doi:10.1104/pp.108.116269
620 Liu, C. M., Muchhal, U. S., & Raghothama, K. G. (1997). Differential expression of *TPS11*, a
621 phosphate starvation-induced gene in tomato. *Plant Molecular Biology* 33, 867-874.
622 doi:10.1023/A:1005729309569
623 Liu, T. Y., Huang, T. K., Tseng, C. Y., Lai, Y. S., Lin, S. I., Lin, W. Y., . . . Chioua, T. J.
624 (2012). PHO2-dependent degradation of PHO1 modulates phosphate homeostasis in
625 Arabidopsis. *Plant Cell* 24, 2168-2183. doi:10.1105/tpc.112.096636
626 Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data
627 using real-time quantitative PCR and the 2_DDC$_t$ method. *Methods* 25, 402-408. doi:
628 10.1006/meth.2001.1262
629 López-Ráez, J. A., Charnikhova, T., Gómez-Roldán, V., Matusova, R., Kohlen, W., De Vos,
630 R., . . . Bouwmeester, H. (2008a). Tomato strigolactones are derived from carotenoids
631 and their biosynthesis is promoted by phosphate starvation. *New Phytologist* 178, 863-
632 874. doi:10.1111/j.1469-8137.2008.02406.x
633 López-Ráez, J. A., Charnikhova, T., Mulder, P., Kohlen, W., Bino, R., Levin, I., &
634 Bouwmeester, H. (2008b). Susceptibility of the tomato mutant *high pigment-2^{dg}* (*hp-*
635 *2^{dg}*) to *Orobanche* spp infection. *Journal of Agricultural and Food Chemistry* 56,
636 6326-6332.
637 López-Ráez, J. A., Shirasu, K., & Foo, E. (2017). Strigolactones in plant interactions with
638 beneficial and detrimental organisms: the yin and yang. *Trends in Plant Science* 22,
639 527-537. doi:10.1016/j.tplants.2017.03.011
640 Lynch, J. P. (2011). Root phenes for enhanced soil exploration and phosphorus acquisition:
641 Tools for future crops. *Plant Physiology* 156, 1041-1049. doi:10.1104/pp.111.175414
642 Medici, A., Szponarski, W., Dangeville, P., Safi, A., Dissanayake, I. M., Saenchai, C., . . .
643 Krouk, G. (2019). Identification of molecular integrators shows that nitrogen actively
644 controls the phosphate starvation response in plants. *Plant Cell* 31(5), 1171-1184.
645 doi:10.1105/tpc.18.00656
646 Mora-Macías, J., Ojeda-Rivera, J. O., Gutiérrez-Alanís, D., Yong-Villalobos, L., Oropeza-
647 Aburto, A., Raya-González, J., . . . Herrera-Estrell, L. (2017). Malate-dependent Fe
648 accumulation is a critical checkpoint in the root developmental response to low
649 phosphate. *Proceedings of the National Academy of Sciences of the United States of*
650 *America* 114, E3563-E3572. doi:10.1073/pnas.1701952114
651 Nagy, F., Karandashov, V., Chague, W., Kalinkevich, K., Tamasloukht, M., Xu, G. H., . . .
652 Bucher, M. (2005). The characterization of novel mycorrhiza-specific phosphate
653 transporters from *Lycopersicon esculentum* and *Solanum tuberosum* uncovers
654 functional redundancy in symbiotic phosphate transport in solanaceous species. *Plant*
655 *Journal* 42, 236-250. doi:10.1111/j.1365-313X.2005.02364.x
656 Pant, B. D., Buhtz, A., Kehr, J., & Scheible, W. R. (2008). MicroRNA399 is a long-distance
657 signal for the regulation of plant phosphate homeostasis. *Plant Journal* 53, 731-738.
658 doi:10.1111/j.1365-313X.2007.03363.x
659 Pant, B. D., Pant, P., Erban, A., Huhman, D., Kopka, J., & Scheible, W. R. (2015).
660 Identification of primary and secondary metabolites with phosphorus status-dependent
661 abundance in Arabidopsis, and of the transcription factor PHR1 as a major regulator of
662 metabolic changes during phosphorus limitation. *Plant Cell and Environment* 38, 172-
663 187. doi:10.1111/pce.12378
664 Pérez-Torres, C. A., López-Bucio, J., Cruz-Ramírez, A., Ibarra-Laclette, E., Dharmasiri, S.,
665 Estelle, M., & Herrera-Estrella, L. (2008). Phosphate availability alters lateral root

666 development in Arabidopsis by modulating auxin sensitivity via a mechanism
667 involving the TIR1 auxin receptor. *Plant Cell* 20, 3258-3272.
668 doi:10.1105/tpc.108.058719
669 Prerostova, S., Kramna, B., Dobrev, P. I., Gaudinova, A., Marsik, P., Fiala, R., . . . Vankova,
670 R. (2018). Organ–specific hormonal cross-talk in phosphate deficiency.
671 *Environmental and Experimental Botany* 153, 198-208.
672 doi:10.1016/j.envexpbot.2018.05.020
673 Puga, M. I., Rojas-Triana, M., de Lorenzo, L., Leyva, A., Rubio, V., & Paz-Ares, J. (2017).
674 Novel signals in the regulation of Pi starvation responses in plants: facts and promises.
675 *Current Opinion in Plant Biology* 39, 40-49. doi:10.1016/j.pbi.2017.05.007
676 Raghothama, K. G. (2000). Phosphate transport and signaling. *Current Opinion in Plant*
677 *Biology, 3*(3), 182-187. doi:10.1016/s1369-5266(00)80063-1
678 Rial, C., Varela, R. M., Molinillo, J. M. G., López Ráez, J. A., & Macías, F. A. (2009). A new
679 UHPLC-MS/MS method for the direct determination of strigolactones in root exudates
680 and extracts. *Phytochemical Analysis* 30, 110–116. doi:DOI: 10.1002/pca.2796
681 Rivero, J., Álvarez, D., Flors, V., Azcón-Aguilar, C., & Pozo, M. J. (2018). Root metabolic
682 plasticity underlies functional diversity in mycorrhiza-enhanced stress tolerance in
683 tomato. *New Phytologist* 220, 1322-1336. doi:10.1111/nph.15295
684 Rubio, V., Linhares, F., Solano, R., Martín, A. C., Iglesias, J., Leyva, A., & Paz-Ares, J.
685 (2001). A conserved MYB transcription factor involved in phosphate starvation
686 signaling both in vascular plants and in unicellular algae. *Genes & Development* 15,
687 2122-2133. doi:10.1101/gad.204401
688 Ruyter-Spira, C., Kohlen, W., Charnikhova, T., van Zeijl, A., van Bezouwen, L., de Ruijter,
689 N., . . . Bouwmeester, H. (2011). Physiological effects of the synthetic strigolactone
690 analog GR24 on root system architecture in Arabidopsis: another belowground role
691 for strigolactones? *Plant Physiology* 155, 721-734. doi:10.1104/pp.110.166645
692 Scaffidi, A., Waters, M. T., Sun, Y. K., Skelton, B. W., Dixon, K. W., Ghisalberti, E. L., . . .
693 Smith, S. M. (2014). Strigolactone hormones and their stereoisomers signal through
694 two related receptor proteins to induce different physiological responses in
695 Arabidopsis. *Plant Physiology* 165, 1221-1232. doi:10.1104/pp.114.240036
696 Scheible, W. R., & Rojas-Triana, M. (2015). Sensing, signalling, and control of phosphate
697 starvation in plants: molecular players and applications. In W. C. Plaxton & H.
698 Lambers (Eds.), *Phosphorus Metabolism in Plants* (Vol. 48, pp. 25-64).
699 Secco, D., Wang, C., Arpat, B. A., Wang, Z., Poirier, Y., Tyerman, S. D., Wu, P., Shou, H., &
700 Whelan, J. (2012). The emerging importance of the SPX domain-containing proteins
701 in phosphate homeostasis. *New Phytologist* 193, 842-851. doi:10.1111/j.1469-
702 8137.2011.04002
703 Song, L., Yu, H., Dong, J., Che, X., Jiao, Y., & Liu, D. (2016). The molecular mechanism of
704 ethylene-mediated root hair development induced by phosphate starvation. *PLoS*
705 *Genetics* 12. doi:10.1371/journal.pgen.1006194
706 Stes, E., Depuydt, S., De Keyser, A., Matthys, C., Audenaert, K., Yoneyama, K., . . .
707 Vereecke, D. (2015). Strigolactones as an auxiliary hormonal defence mechanism
708 against leafy gall syndrome in *Arabidopsis thaliana*. *Journal of Experimental Botany*
709 66, 5123-5134. doi:10.1093/jxb/erv309
710 Torres-Vera, R., García, J. M., Pozo, M. J., & López-Ráez, J. A. (2014). Do strigolactones
711 contribute to plant defence? *Molecular Plant Pathology* 15, 211-216.
712 doi:10.1111/mpp.12074

713 Umehara, M., Hanada, A., Yoshida, S., Akiyama, K., Arite, T., Takeda-Kamiya, N., . . .
714 Yamaguchi, S. (2008). Inhibition of shoot branching by new terpenoid plant
715 hormones. *Nature* 455, 195-200. doi: 10.1038/nature07272
716 Wang, G. Y., Shi, J. L., Ng, G., Battle, S. L., Zhang, C., & Lu, H. (2011). Circadian clock-
717 regulated phosphate transporter PHT4;1 plays an important role in Arabidopsis
718 defense. *Molecular Plant* 4, 516-526. doi:10.1093/mp/ssr016
719 Waters, M. T., Gutjahr, C., Bennett, T., & Nelson, D. C. (2017) Strigolactone Signaling and
720 Evolution. In*: Vol. 68. Annual Review of Plant Biology* (pp. 291-322).
721 Yoneyama, K., Xie, X., Kim, H. I., Kisugi, T., Nomura, T., Sekimoto, H., . . . Yoneyama, K.
722 (2012). How do nitrogen and phosphorus deficiencies affect strigolactone production
723 and exudation? *Planta* 235, 1197-1207. doi:10.1007/s00425-011-1568-8
724 Zhou, J., Jiao, F., Wu, Z., Li, Y., Wang, X., He, X., . . . Wu, P. (2008). OsPHR2 is involved in
725 phosphate-starvation signaling and excessive phosphate accumulation in shoots of
726 plants. *Plant Physiology* 146, 1673-1686. doi:10.1104/pp.107.111443
727 Ziegler, J., Schmidt, S., Chutia, R., Müller, J., Böttcher, C., Strehmel, N., . . . Abel, S. (2016).
728 Non-targeted profiling of semi-polar metabolites in Arabidopsis root exudates
729 uncovers a role for coumarin secretion and lignification during the local response to
730 phosphate limitation. *Journal of Experimental Botany* 67, 1421-1432.
731 doi:10.1093/jxb/erv539

732

733 **FIGURE LEGENDS**

734

735 **Figure 1.** Schematic model of core elements involved in the regulation of P signalling in
736 plants. Under Pi deficient conditions, the complex SPX-PHR1 is no longer stable. Once
737 released, PHR1 promotes the expression of high-affinity Pi transporters from the PHT1
738 family (e.g. LePT2 in tomato) in the roots, increasing Pi uptake. PHR1 also induces the
739 expression of the microRNA miR399 (SlmiR399 in tomato), which negatively regulates
740 *PHO2* (SlPHO2 in tomato) activity, and of the non-protein coding gene *IPS1* (TPSI1 in
741 tomato). *PHO2* down-regulation prevents the degradation of the Pi exporter PHO1, thus
742 allowing Pi xylem loading and the subsequent Pi transport into the shoots. On the other hand,
743 *IPS1* can interact and block miR399 transcripts, preventing miR399-*PHO2* binding and
744 degradation of *PHO2*. Adapted from Puga et al. 2017 (Puga et al., 2017).

745

746 **Figure 2.** Effect of Pi starvation and 2'-*epi*-GR24 exogenous application on strigolactone
747 biosynthesis. Content of the SLs orobanchol (a) and solanacol (b) in tomato root extracts of 4-
748 week old plants from plants grown under normal (+P; light bars) or deficient (-P; dark bars)
749 phosphate conditions, and treated or not with different concentrations of the synthetic SL
750 analogue 2'-*epi*-GR24 [GR24]. Gene expression analysis (M value) of the SL biosynthesis
751 genes *SlD27* (c) and *SlCCD8* (d) in roots of 4-week old tomato plants. M value ($\log_2$ ratio) is
752 zero if there is no change; '+1' or '-1' indicate two-fold change induction or repression,
753 respectively. Bars presents the means of five independent replicates (±SE). Bars with different
754 letters mean significantly different ($P < 0.05$) according to the Duncan's multiple range test.
755
756 **Figure 3.** Expression analysis of genes associated to P signalling and homeostasis. Effect 2'-
757 *epi*-GR24 under normal (+P; grey bars) or deficient (-P; closed bars) phosphate conditions in
758 the expression (M value) of the gene encoding the Pi transporter *LePT2* (a), and the Pi
759 signalling genes *SlmiR399* (b), *LeTPSI1* (c) and *SlPHO2* (d) in tomato roots. Gene expression
760 values were normalized using the housekeeping gene *SlActin*. Bars presents the means of five
761 independent replicates (±SE). For statistics see legend in Fig. 2.
762
763 **Figure 4.** Gene expression analysis of tomato genes associated to P signalling and
764 homeostasis in the SL-deficient line *SlCCD8*-RNAi and its corresponding wild-type cv.
765 Craigella (WT). Plants were grown in pots under normal (+P; grey bars) or deficient (-P;
766 closed bars) phosphate conditions. The expression (M value) of the of the gene encoding the
767 Pi transporter *LePT2* (a), and the Pi signalling genes *SlmiR399* (b), *LeTPSI1* (c) and *SlPHO2*
768 (d) was analysed. Expression values were normalized using the housekeeping gene *SlActin*.
769 For statistics see legend in Fig. 2.
770

771 **Figure 5.** Overview of metabolite behaviour in roots of tomato plants grown under normal (P)
772 or deficient (-P) Pi conditions and treated (GR) or not (C) with 10 nM 2'-*epi*-GR24, using
773 principal component and heat map analyses. (a) Combined (ESI+ and ESI- modes) principal
774 component analysis (PCA) ($p < 0.05$) of the signals obtained from a non-targeted analysis by
775 HPLC-QTOF monitoring metabolic changes. (b) Heatmap of the metabolite profiling
776 generated with MarVis Filter and Cluster packages by combining ESI+ and ESI- modes. Each
777 colour band represents a single compound detected in CP, C-P, GRP and GR-P, whose
778 accumulation is indicated by the colour scale ranging from high (red) to low (blue). The
779 concentration of the metabolites was determined in all samples by normalizing the
780 chromatographic pick area for each compound with the dry weight of the corresponding
781 sample. (c) Non-parametric Kruskal-Wallis test to identify significant ($P < 0.05$) features
782 among the total number of signals. Red dots are features with significant differences and
783 green dots features without significant differences. The straight sets the threshold for the
784 statistical differences ($P < 0.05$). (d) Heatmap analysis of the 40 significantly signals obtained
785 from the Kruskal-Wallis test, whose levels were increased upon 2'-*epi*-GR24 treatment. Red
786 box indicates compounds up-regulated by Pi limitation and 2'-*epi*-GR24. Each colour band
787 represents a single compound detected in CP, C-P, GRP and GR-P, whose accumulation is
788 indicated by the colour scale ranging from high (red) to low (blue). Data points represent six
789 biological replicates injected randomly into the HPLC-QTOF MS. Values are relative to root
790 dry weight and normalized to the lowest amount.
791
792 **Figure 6.** Box plots of identified and selected metabolites from the untargeted metabolomics
793 analysis in tomato roots, showing similar accumulation patterns under Pi starvation and plants
794 treated with 2'-*epi*-GR24 under normal Pi. + and - indicate presence or absence, respectively,
795 of Pi and GR24. Compounds showing up-regulation by Pi limitation and 2'-*epi*-GR24: malic

796 acid, citric acid, decanoic acid, azelaic acid, allantoic acid, 3''-Hydroxy-
797 genarylhydroquinone, isophenoxazine and luteolin. Compounds showing down-regulation by
798 Pi limitation and 2'-*epi*-GR24: 9-Oxooctadeca-10, 12-dienoic acid, 12-Oxo-9(z)-dodecenoic
799 acid and 9,10-Epoxyoctadecatrienoic acid. Boxplot of the selected metabolomic features from
800 the user's uploaded data. Black dots represent the concentration of the selected features from
801 all samples. The notch indicates the 95% confidence interval around the median of each
802 group. The mean concentration of each group is indicated with yellow diamond. Data no
803 sharing a letter in common differ significantly according to the Fisher's least significant
804 difference test ($P < 0.05$). Six independent replicates were used.
805
806 **Figure 7.** Proposed model for the regulation of plant responses to Pi starvation. Phosphorus
807 deficiency induces SL biosynthesis, which would modulate the expression of the key Pi
808 signalling and regulatory genes, and that of Pi transporters. The regulation of the Pi response
809 modulators would promote plant responses, including changes in the metabolome, to cope to
810 the stress.
811
812 **SUPPLEMENTAL MATERIAL**
813
814 **Table S1.** Mass pair (*m/z*) and compound-dependent parameters of the standards used for
815 analytical quantification of SLs. *C.E.: Collision Energy
816
817 **Table S2.** Primer sequences used in the real time qRT-PCR analyses.
818
819 **Table S3.**
820 Dataset containing metabolic profiles from tomato roots in negative (ESI-) mode.

Table S4.

Dataset containing metabolic profiles from tomato roots in negative (ESI+) mode.

Figure S1. General structure of natural strigolactones and the active synthetic strigolactone analogue 2'-*epi*-GR24. Chemical structure of the tomato strigolactones quantified in this work: orobanchol and solanacol.

Figure S2. Effect of Pi starvation and 2'-*epi*-GR24 exogenous application on strigolactone biosynthesis in wheat. Gene expression analysis (M value) of the SL biosynthesis genes *TaD27* (a) and *TaCCD8* (b) in roots of 4-week old wheat plants grown under normal (+P; light bars) or deficient (-P; dark bars) Pi conditions, and treated or not with 10 nM 2'-*epi*-GR24 [GR24]. M value (log_2 ratio) is zero if there is no change; '+1' or '-1' indicate two-fold change induction or repression, respectively. Data presents the means of five independent replicates (±SE). Bars with different letters mean significantly different ($P < 0.05$) according to the Duncan's multiple range test.

Figure S3. Expression analysis of genes associated to P signalling and homeostasis in wheat. Effect of 10 nM 2'-*epi*-GR24 under normal (+P; grey bars) or deficient (-P; closed bars) phosphate conditions in the expression (M value) of the gene encoding the Pi transporter *TaPht2* (a), and the P signalling genes *taemiR399* (b), *TaIPS1* (c) and *TaPHO2* (d) in wheat roots. Gene expression values were normalized using the housekeeping gene *TahnRNPQ*. Data presents the means of five independent replicates (±SE). Bars with different letters mean significantly different ($P < 0.05$) according to the Duncan's multiple range test.