# Strigolactones Are Involved in Root Response to Low Phosphate Conditions in Arabidopsis[W][OA]

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Strigolactones (SLs) are plant hormones that suppress lateral shoot branching, and act to regulate root hair elongation and lateral root formation. Here, we show that SLs are regulators of plant perception of or response to low inorganic phosphate (Pi) conditions. This regulation is mediated by MORE AXILLARY GROWTH2 (MAX2) and correlated with transcriptional induction of the auxin receptor TRANSPORT INHIBITOR RESPONSE1 (TIR1). Mutants of SL signaling (*max2-1*) or biosynthesis (*max4-1*) showed reduced response to low Pi conditions relative to the wild type. In *max4-1*, but not *max2-1*, the reduction in response to low Pi was compensated by the application of a synthetic strigolactone GR24. Moreover, AbamineSG, which decreases SL levels in plants, reduced the response to low Pi in the wild type, but not in SL-signaling or biosynthesis mutants. In accordance with the reduced response of *max2-1* to low Pi relative to the wild type, several phosphate-starvation response and phosphate-transporter genes displayed reduced induction in *max2-1*, even though Pi content in *max2-1* and the wild type were similar. Auxin, but not ethylene, was sufficient to compensate for the reduced *max2-1* response to low Pi conditions. Moreover, the expression level of *TIR1* was induced under low Pi conditions in the wild type, but not in *max2-1*. Accordingly, the *tir1-1* mutant showed a transient reduction in root hair density in comparison with the wild type under low Pi conditions. Therefore, we suggest that the response of plants to low Pi is regulated by SLs; this regulation is transmitted via the MAX2 component of SL signaling and is correlated with transcriptional induction of the TIR1 auxin receptor.

Strigolactones (SLs) or their derivatives have been shown to act as long-distance hormones that suppress the growth of preformed axillary shoot buds (Gomez-Roldan et al., 2008; Umehara et al., 2008). SLs were identified in a number of plant species (Xie et al., 2010) as carotenoid-derived terpenoid lactones (Matusova et al., 2005) that are synthesized in roots and shoots (for review, see Dun et al., 2009). Both SL-biosynthesis and SL-response mutants have been identified. Among the SL-biosynthesis mutants in Arabidopsis (*Arabidopsis thaliana*) are *more axillary growth3* (*max3*) and *max4*, which are mutated in carotenoid cleavage dioxygenase7 (CCD7) and CCD8, respectively. These two enzymes are suggested to be involved in SL biosynthesis (Booker et al., 2004; Bainbridge et al., 2005; Gomez-Roldan et al., 2008; Umehara et al., 2008). Among the SL-response mutants in Arabidopsis is *max2*, mutated in the F-box protein MAX2 (Stirnberg et al., 2002, 2007; Umehara et al., 2008).

Additional roles for SLs have been found in plants, including in the regulation of secondary growth (Agusti et al., 2011) and adventitious root formation (Rasmussen et al., 2012). SLs also play a role in modulation of root development, particularly lateral root (LR) formation and root hair (RH) elongation (Kapulnik et al., 2011a; Ruyter-Spira et al., 2011). This role was shown to be conveyed via intimate cross talk of SLs with auxin and ethylene (Kapulnik et al., 2011b; Koltai, 2011), and to be mediated by the MAX2 SL-signaling component (Kapulnik et al., 2011a, 2011b; Ruyter-Spira et al., 2011).

Phosphorus (P) is an essential macronutrient required by plants, as it plays a vital role in major metabolic processes, and is a structural component of cellular macromolecules. P availability varies considerably in soils (Maathuis, 2009). However, the soil-solution concentration of inorganic phosphate (Pi), the form that is most readily accessible to plants, is often a growth limiting factor (Bieleski, 1973). Plants have evolved strategies to exploit localized sources of P to cope with low P conditions. Among the structural changes that plants under P deprivation display are alterations in root architecture and RH density and length (for review, see Péret et al., 2011).

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www.plantphysiol.org/cgi/doi/10.1104/pp.112.202358

Pi starvation-induced (PSI) genes are known to be associated with the response of plants to low Pi conditions and are commonly used as markers for low Pi response (Chiou and Lin, 2011; Supplemental Table S1). Of the PSI genes, *ACP5* encodes a type 5 acid phosphatase. It displays both phosphatase and peroxidation activity, and could be involved in phosphate mobilization and the metabolism of reactive oxygen species (del Pozo et al., 1999). Another PSI gene is *INDUCED BY PHOSPHATE STARVATION1* (*IPS1*), the RNA of which contains a short, nonconserved open reading frame and is specifically induced by Pi starvation. It is interesting to note that exogenous cytokinins repress the expression of *AtIPS1* in response to Pi deprivation (Martín et al., 2000). *PHO2* (for *PHOSPHATE2*) encodes a ubiquitin-conjugating E2 enzyme, and functions in xylem P loading and in Pi-associated signaling (Hamburger et al., 2002; Wang et al., 2004) to regulate P uptake and translocation in plants (Delhaize and Randall, 1995; Dong et al., 1998; for review, see Chiou and Lin, 2011). A family of genes involved in P uptake encode high-affinity phosphate transporters (PHTs), which substantially increase the capacity for Pi influx and partitioning in the plant (e.g. Lee, 1993; Mudge et al., 2002; Shin et al., 2004; Misson et al., 2005). In addition, it has been shown for many plant species that an important part of PHT gene regulation takes place at the transcriptional level (Muchhal et al., 1996; Misson et al., 2005).

Several plant hormones, such as auxin and ethylene, have been suggested to be involved in linking Pi signaling with plant growth responses; auxin signaling was shown to be associated with changes in root system architecture caused by Pi deprivation, whereas Pi-deprived plants were shown to be more sensitive to exogenous auxin than Pi-nourished plants with regard to the arrest of primary root (PR) growth and induced formation of LR (for review, see López-Bucio et al., 2002; Chiou and Lin, 2011). In addition, the gaseous plant hormone, ethylene, was implicated in Pi-starvation-mediated inhibition of PR growth, increased LR elongation, and RH formation (López-Bucio et al., 2002; Chiou and Lin, 2011).

SLs have been shown to be involved in shoot responses to low Pi: in both rice (*Oryza sativa*) and Arabidopsis, decreased Pi concentration in the media led to increased SL levels in the roots, and to inhibition of tiller or lateral bud outgrowth in the wild type, but not in SL-insensitive or deficient mutants (Umehara et al., 2010; Kohlen et al., 2011). Shoot responsiveness to exogenous SL was also observed to be slightly increased by Pi deficiency (Umehara et al., 2010). Moreover, SL regulation of LR formation was affected by Pi conditions: under high Pi conditions, SLs repressed LR formation (Kapulnik et al., 2011a), whereas under low Pi conditions, they induced it (Ruyter-Spira et al., 2011). Hence, SLs have been suggested to be regulators of plant architecture, particularly in response to Pi growth conditions. However, the pathways that are involved with SLs regulation of low Pi response are still unclear.

Here we show that SLs are regulators of either the plant's perception of or response to low Pi conditions, and that this regulation is transmitted via the MAX2 component of SL signaling and is correlated with increased *TRANSPORT INHIBITOR RESPONSE1* (*TIR1*) expression. This is based on a phenotypic and pharmacological study of SL- or auxin-related mutants using application of SL, indole-3-acetic acid (IAA), or AbamineSG (an SL reducing compound), in combination with the determination of the expression profile of PSI genes and the auxin receptor *TIR1* gene in the different genotypes and under the various treatments.

## RESULTS

### Strigolactones-Signaling and Biosynthesis Mutants Show Reduced Response to Low Pi Conditions in Terms of RH Density

SLs have been suggested to be regulators of shoot architecture in response to Pi growth conditions (Umehara et al., 2010; Kohlen et al., 2011). To better understand the pathways involved with SLs regulation of low Pi response, we sought to examine whether SLs are involved in the influence of Pi levels on RH density, as both SLs and Pi levels are known to affect RH development (Kapulnik et al., 2011a; Péret et al., 2011). For that purpose, RH density was measured in the SL signaling and biosynthesis mutants *max2-1* and *max4-1*, respectively. Compared with wild-type Columbia-0 (Col-0) seedlings grown on high Pi (2 mM) plates, wild-type seedlings on low Pi (1 $\mu$M) media displayed a characteristic higher density of RH, as determined by the number of RH per 500 $\mu$m of root length at 48 h post germination (hpg; Fig. 1). In contrast with the wild type, the SL mutants showed significantly less RH density under low Pi conditions (Fig. 1). The RH density of the SL-signaling mutant *max2-1* and the SL-biosynthesis mutant *max4-1*, was similar in both high and low Pi treatments and equal to the RH density of the wild type grown on high Pi plates at 48 hpg (Fig. 1, A and B). At 72 and 96 hpg, however, *max2-1* and *max4-1* RH density grown on low Pi plates was similar to that of the wild type under the same conditions (Fig. 1, A and B), suggesting that this genetic effect of diminished response to low Pi is apparently transient and ameliorated over time.

### *max2-1* and *max4-1* Display Reduced Levels of PSI Gene Expression

To examine whether *max2-1* shows a reduced response to low Pi at the gene expression level, we examined the expression of six PSI genes (Chiou and Lin, 2011; Supplemental Table S1) at 48 hpg under low Pi, in comparison with high Pi conditions (Fig. 2; Supplemental Table S2). Most of the examined PSI genes (*ACP5*, *IPS1*, *PHT1;2*, *PHT1;4*, and *PHT1;5*, but not *PHT1;1*) showed lower levels of induction under low Pi

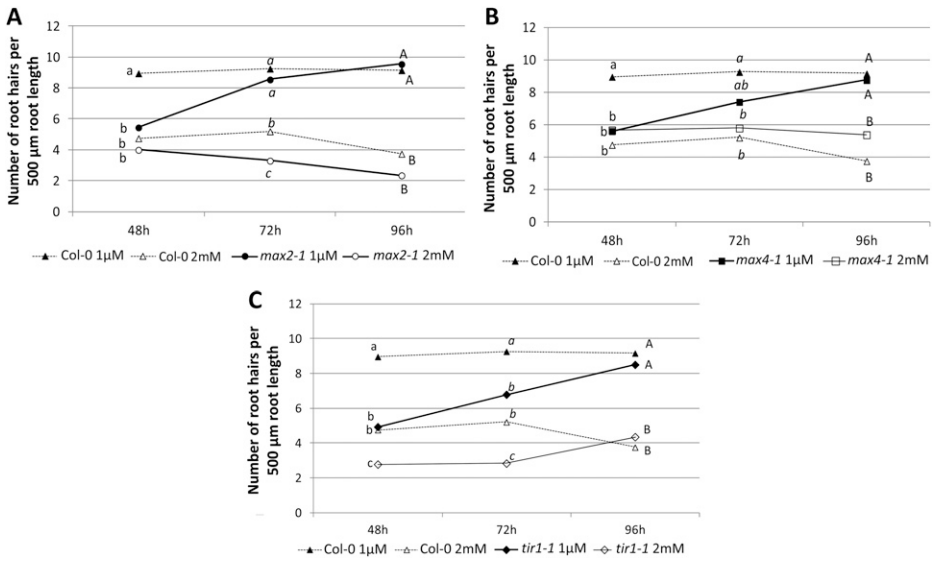


**Figure 1.** Effect of high and low Pi conditions on RH density of the wild type (Col-0; dotted line with triangles), *max2-1* (A; solid line with circles), *max4-1* (B; solid line with squares), and *tir1-1* (C; solid line with rhombuses) genotypes. A to C, RH densities are presented as the mean of the number of RHs per 500 $\mu$m of root length, under low (1 $\mu$M; closed symbols) and high (2 mM; open symbols) Pi conditions for a period of 48 to 96 hpg. Experiments were repeated three times; each treatment within each experiment included two replicates, with 10 germinated seedlings per replicate. Different letters indicate statistically significant differences between means at a particular time point by one-way ANOVA with Tukey-Kramer multiple comparison test ($P \leq 0.01$).

conditions in *max2-1* relative to the wild type (Fig. 2A; Supplemental Table S2). Also, the expression of *PHO2*, a repressor of Pi over accumulation (Delhaize and Randall, 1995; Dong et al., 1998), was reduced in the wild type under low Pi conditions, but increased in *max2-1* under these conditions (Fig. 2). Four of the genes that were differentially expressed between the wild type and *max2-1* were taken for further analysis. Reduced induction for two of these, *ACP5* and *PHT1;4*, was also evident for *max4-1* compared with the wild type (Fig. 2B; Supplemental Table S2). These results further suggest that seedlings of *max2-1* and *max4-1* have a reduced ability to respond to low Pi conditions in comparison with the wild type. Thus, SLs may play a role in the expression of PSI genes in response to Pi deprivation during early seedling development.

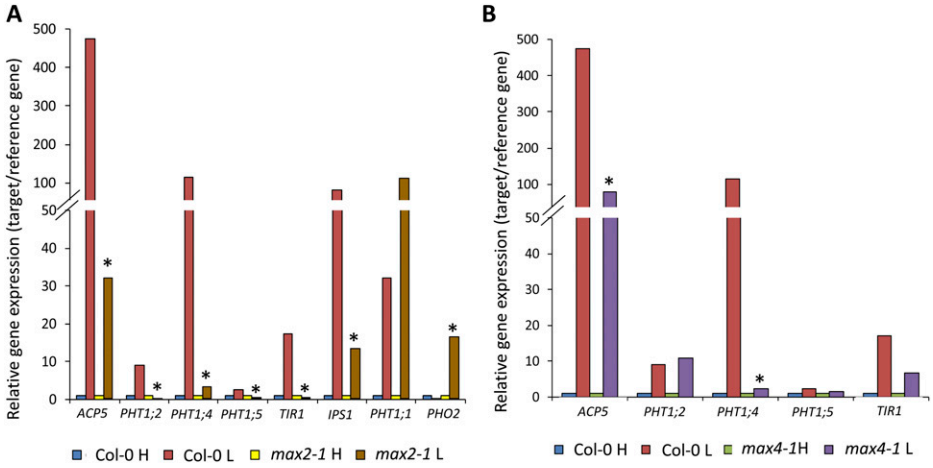


**Figure 2.** PSI gene expression (mean) at 48 hpg under low (1 $\mu$M) and high (2 mM) phosphate conditions in seedlings of the wild type (Col-0), *max2-1* (A), and *max4-1* (B) mutants. For comparison, in all graphs, the wild type is shown (Col-0 H and Col-0 L). Values of the steady-state level of gene transcripts were determined as a ratio between low or high Pi versus high Pi condition, using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001; Arocho et al., 2006). Values above or below 1.5 represent increase or decrease, respectively, in the steady-state level of gene transcripts for the examined conditions (i.e. that of the numerator versus that of the denominator) as described in the "Materials and Methods." RNA was extracted from seedlings of the wild type, *max2-1*, and *max4-1* mutants, from control treatments. The experiment was performed in five biological replicates; each replicate included 120 plants, on which three technical repeats were performed. Means and SE were determined from all biological replicates. Examined gene designation is indicated under each of the graphs. *Means are statistically different from that of the wild type in the control treatment, determined by ANOVA pairwise Student's *t* tests ($P \leq 0.05$) using the JMP statistical package. H, high (2 mM) phosphate conditions; L, low (1 $\mu$M) phosphate conditions.

### Exogenous Application of Synthetic Strigolactones (GR24) Compensates for the Reduced Response of *max4-1* to Low Phosphate Conditions

As our results suggested that there is a reduction in the response of *max2-1* and *max4-1* to low Pi conditions, we examined whether SLs are involved in the low-Pi response. We supplemented each of the seedlings grown on plates under low Pi conditions with GR24, a synthetic SL (Johnson et al., 1981), and examined their response to low Pi in terms of RH density at 48 hpg in comparison with treatment without GR24 supplied (Fig. 3). Because of the low amount of supplemented liquid (i.e. 20 $\mu$L for each of the seedlings), relatively high concentrations of GR24 were used (15–50 $\mu$M) because we assumed the supplied solutions would diffuse into the surrounding agar. These concentrations did not prevent plant growth under the examined conditions. As expected, under the low Pi control conditions, *max2-1* and *max4-1* had a reduced RH density compared with the wild type. Under these low Pi conditions, GR24 addition increased RH density in *max4-1* to the wild-type level, but it did not change RH density significantly in *max2-1* or the wild type, at all examined GR24 concentrations (Fig. 3). The response of *max4-1* was significant at 15 $\mu$M GR24 and restored to the wild type at 50 $\mu$M (Fig. 3).

These results are consistent with SL regulation of RH density in these young seedlings, because the SL deficient mutant, *max4-1*, responded to GR24 whereas the *max2-1* signaling mutant did not (Fig. 3). At the gene expression level of the four PSI genes examined, the addition of GR24 to *max4-1* resulted in an increase in the expression of two PSI genes, *PHT1;4* and *PHT1;5*, but resulted in a reduction in the expression of *ACP5* (Figs. 2B and 4A; Supplemental Table S2).

### Exogenous Application of AbamineSG Reduces the Wild-Type Response to Low Phosphate Conditions

To further demonstrate the involvement of SL in the seedling response to low Pi conditions, we examined the effect of AbamineSG, which causes a reduction in SL levels (Kitahata et al., 2011), on the response to low Pi. Under low phosphate conditions, 20 $\mu$L of 1 mM AbamineSG was added to each of the seedlings grown on plates under low Pi conditions, and their response to low Pi in terms of RH density was examined at 48 hpg. The addition of AbamineSG reduced the RH density of wild-type plants to levels that were similar to those found in nontreated SL mutants (Fig. 5). Moreover, following application of AbamineSG, the induction of expression of three examined PSI genes (*ACP5*, *PHT1;4*, and *PHT1;5*) was reduced in the wild type (Fig. 4B; Supplemental Table S2) to at least that of the SL deficient mutant, *max4-1*. AbamineSG did not significantly affect the RH density of *max2-1* or *max4-1*, which are deficient in SL response and biosynthesis, respectively (Fig. 5).

Taken together, the results suggest that SLs are required in the response to low Pi during early seedling development, and that a deficiency in SL response or biosynthesis is the cause of the reduced response to low Pi in *max2-1* and *max4-1* mutants, respectively.

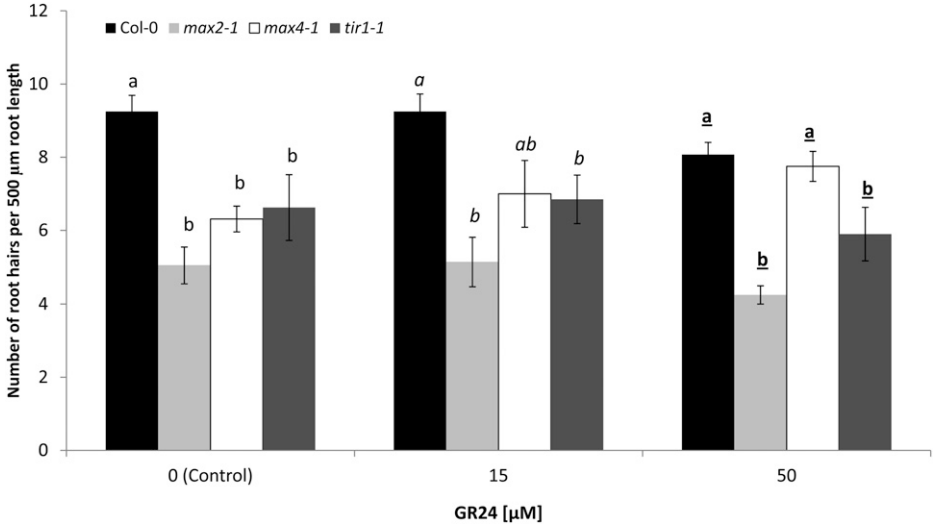


**Figure 3.** Effect of low Pi conditions and exogenous supplementation of GR24 on RH density of the wild type and *max2-1*, *max4-1*, and *tir1-1* mutants. RH densities at 48 hpg are presented as mean ± SE of the number of RHs per 500 $\mu$m of root length, following exogenous supplementation of 20-$\mu$L GR24 to each of the seedlings at concentrations of 0 (control), 15, and 50 $\mu$M. Experiments were repeated three times; each treatment within each experiment included two replicates, with 10 germinated seedlings per replicate. Different letters indicate statistically significant differences between means for particular GR24 treatment by one-way ANOVA with Tukey-Kramer multiple comparison test ($P \leq 0.01$). Two-way ANOVA analysis revealed lack of significance between genotypes and GR24 treatments ($P = 0.22$).

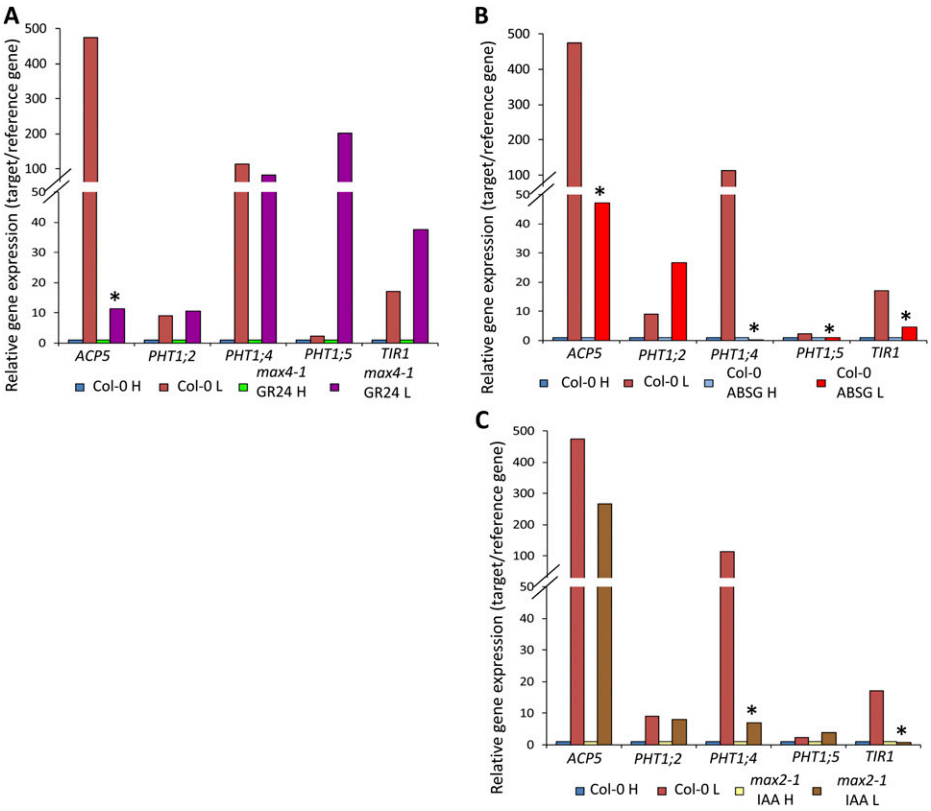


**Figure 4.** PSI gene expression (mean) at 48 hpg under low (1 $\mu$M) and high (2 mM) phosphate conditions in seedlings of *max4-1* treated with 15$\mu$M GR24 (A), of the wild type (Col-0) treated with 1 mM AbamineSG (B), and of *max2-1* treated with $1 \times 10^{-6}$ M IAA (C). For comparison, in all graphs, the wild type nontreated control is shown (Col-0 H and Col-0 L). Values of the steady-state level of gene transcripts were determined as a ratio between low or high Pi versus high Pi condition, using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001; Arocho et al., 2006). Value above or below 1.5 represents increase or decrease, respectively, in the steady-state level of gene transcripts for the examined conditions (i.e. that of the numerator versus that of the denominator) as described in the "Materials and Methods." RNA was extracted from seedlings of the wild type, *max2-1*, and *max4-1* mutants, from GR24, AbamineSG, and IAA treatments of the genotypes that responded to the corresponding treatments in terms of RH density under low Pi conditions (i.e. *max4-1* to GR24, the wild type to AbamineSG, and *max2-1* to IAA). The experiment was performed in five biological replicates. Each replicate included 120 plants, on which three technical repeats were performed. For each replicate of the experiment, controls were conducted, in which the exact concentration of the appropriate solvent was used to treat the seedlings (water for IAA, acetone for GR24 or AbamineSG). Means and SE were determined from all biological replicates. Examined gene designation is indicated under each of the graphs. *Means are statistically different from that of the wild type in the control treatment, determined by ANOVA pairwise Student's *t* tests ($P \leq 0.05$) using the JMP statistical package. H, high (2 mM) phosphate conditions; L, low (1 $\mu$M) phosphate conditions. GR24, GR24 treatment; ABSG, AbamineSG treatment; IAA, IAA treatment.

### Response in PR Length, LR Number, and LR Density of *max2-1* under Low Phosphate Conditions

Plants also respond to low Pi levels by modifying their root architecture resulting in a decrease in PR length and an increase in LR density (López-Bucio et al., 2003). As *max2-1* has the greatest reduction in response to low Pi in terms of RH density (at 48 hpg; Fig. 1A), we chose to investigate the response of *max2-1* mutants to low Pi level in terms of PR length and LR development. The wild type and *max2-1* mutants were grown under low and high Pi conditions and for a period of 7 to 11 d post germination (dpg), and PR length and the LR number were scored. As shown in Figure 6, when grown under low Pi, both the wild type and *max2-1* displayed a shorter PR and a lower number of LRs, resulting in a higher LR density, compared with high Pi conditions. Nevertheless, *max2-1* mutants did not respond to low Pi concentrations to the same extent as the wild type: *max2-1* mutants showed a lower reduction over time in both PR length and the number of LRs ($P < 0.001$ and 0.01, respectively; Fig. 6, A and B). However, these differences did not result in any significant difference in the increase of LR density between the wild type and *max2-1* mutants grown under low Pi conditions ($P = 0.117$; Fig. 6C).

### P Content in the Wild Type and *max2-1* Is Similar

Several Pi transporters are represented among the PSI genes with different levels of expression in

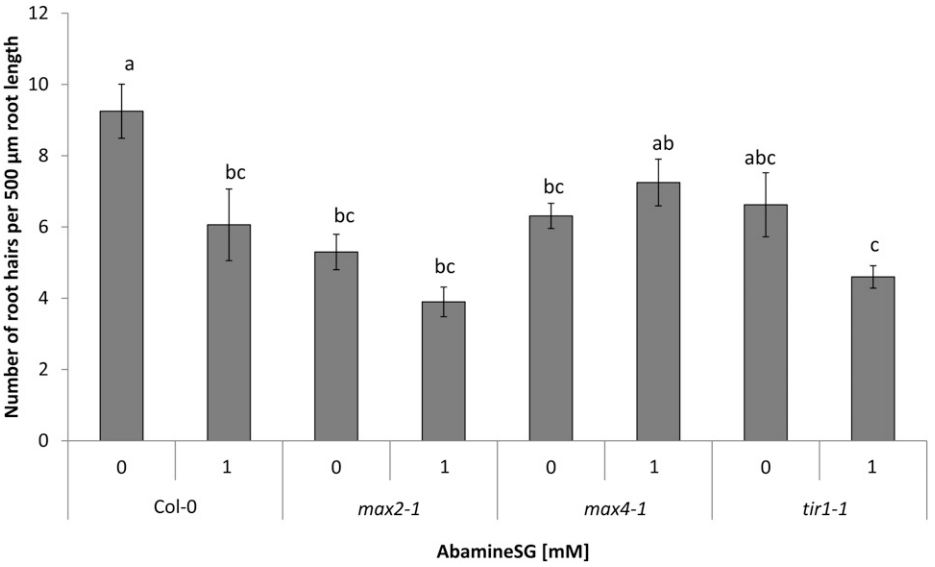


**Figure 5.** Effect of exogenous supplementation of AbamineSG on RH density of the wild type, *max2-1*, *max4-1*, and *tir1-1* mutants under low Pi conditions. RH densities at 48 hpg are presented as mean ± SE of the number of RHs per 500 $\mu$m of root length, following exogenous supplementation of 20-$\mu$L AbamineSG to each of the seedlings at concentrations of 0 and 1 mM. Experiments were repeated three times. Each treatment within each experiment included two replicates, with 10 germinated seedlings per replicate. Different letters indicate statistically significant differences between means by one-way ANOVA with Tukey-Kramer multiple comparison test ($P \leq 0.01$).

*max2-1* seedlings compared with the wild type under low Pi conditions (*PHT1;1* [PT1], *PHT1;2*, *PHT1;4* [PT2], and *PHT1;5*; Supplemental Table S1). These are necessary for increased Pi uptake (for review, see Lin et al., 2009). Hence, the deficiency in the transcriptional induction of particular Pi transporters in *max2-1* may suggest that in the first few hours of seedling growth under low Pi conditions, *max2-1* is unable to increase its P content. Therefore, we determined the concentration of P in seedlings of the wild type and *max2-1* during early seedling (48 hpg) growth on plates containing a range of Pi concentrations (0–1000 $\mu$M). As shown in Supplemental Figure S1, P concentration in wild-type and *max2-1* seedlings, relative to dry weight, varied

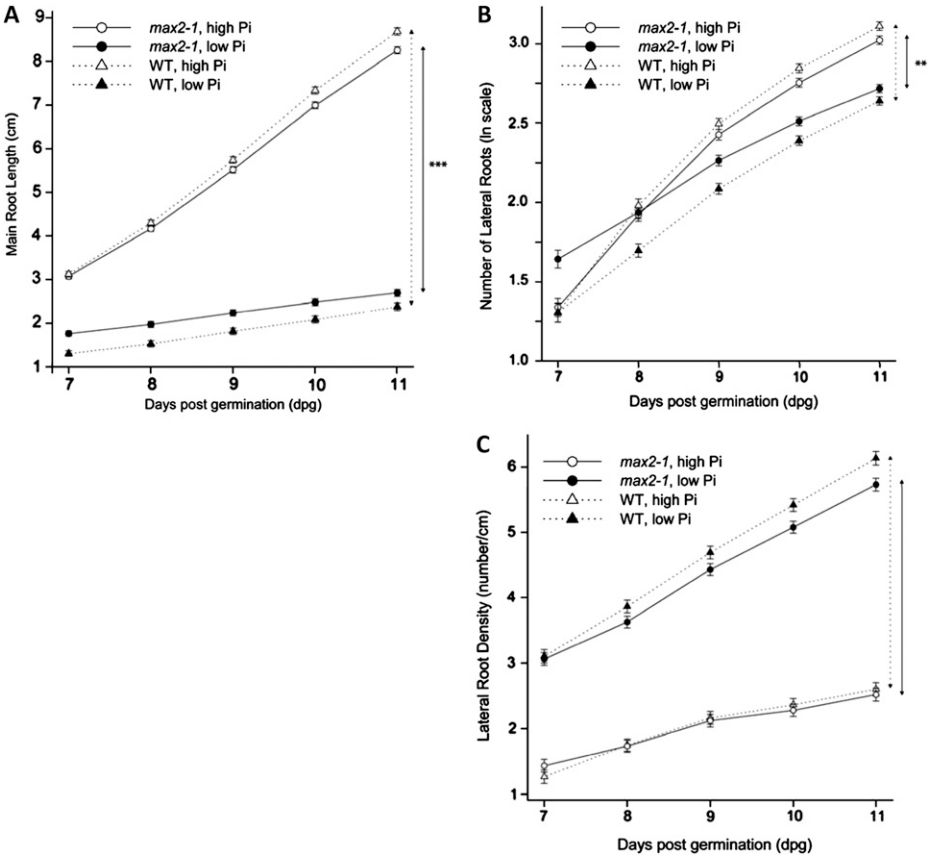


**Figure 6.** Effect of high and low Pi conditions on PR length, LR number, and density of the wild type (WT; Col-0) and *max2-1* mutants. Main PR length (A), LR number (B), and LR density (C) of the wild type (dotted line with triangles) and *max2-1* mutants (solid line with circles) are presented as mean ± SE under low (closed symbols) and high (open symbols) Pi conditions for a period of 7 to 11 dpg. Experiments were repeated twice; each treatment within each experiment included 25 germinated seedlings per replicate. Data were analyzed as repeated measurements, using the REML procedure. Significance of the genotype, treatment, and interaction effects (over the whole kinetics) was assessed by an *F*-test (**$P < 0.01$; ***$P < 0.001$).

across the treatments and significantly increased for both genotypes at 1000 $\mu$M. Moreover, the wild type and *max2-1* had similar concentrations of P. This lack of a significant difference between the wild type and *max2-1* was repeated across all examined Pi growth conditions (Supplemental Fig. S1).

### ACC Is Not Sufficient to Compensate for Deficiency in *max2-1* Response to Low Phosphate Conditions

Ethylene has been shown to be involved in the response to Pi deficiency: ethylene is important for inhibition of PR growth, for promotion of LR elongation, and RH formation (for review, see Chiou and Lin, 2011). On the other hand, under high Pi growth conditions, SLs have been shown to induce ethylene biosynthesis (Kapulnik et al., 2011b). We therefore examined whether 1-aminocyclopropane-1-carboxylic acid (ACC) can compensate for the deficiency in the response of *max2-1* to low Pi conditions at 48 hpg. 20 $\mu$L of ACC, at concentrations of $1 \times 10^{-12}$, $1 \times 10^{-13}$, and $1\times10^{-14}$ M, was added to each of the seedlings grown on plates under low Pi conditions, and their response to low Pi in terms of RH density was examined (Supplemental Fig. S2). Under our low Pi conditions, and regardless of concentration, ACC did not enhance *max2-1* RH density. The highest concentration of ACC ($1 \times 10^{-11}$ M) used did, however, lead to an increase in RH density in the wild type under these conditions (Supplemental Fig. S2). This is despite the fact that *max2-1* has been shown to be as sensitive as the wild type to ACC under high Pi conditions (Kapulnik et al., 2011b; data not shown). These results suggest that ethylene may not compensate for the deficiency in the response of *max2-1* to low Pi.

### IAA Is Sufficient to Compensate for the Deficiency in the *max2-1* and *max4-1* Response to Low Phosphate Conditions

Auxin has been shown to be involved in the response to low Pi through modification of the root system architecture (López-Bucio et al., 2002; for review, see Chiou and Lin, 2011), whereas SLs have been suggested to be either modulators of auxin transport or secondary messengers of auxin (e.g. Dun et al., 2009; Leyser, 2010). Therefore, we examined whether IAA can compensate for the reduction in the response of *max2-1* and *max4-1* to low Pi conditions at 48 hpg, in terms of RH density. 20 $\mu$L of IAA, at concentrations of $1\times10^{-8}$, $1\times10^{-7}$, and $1\times10^{-6}$ M, was added to each of the seedlings grown on plates under low Pi conditions, and their response to low Pi in terms of RH density was examined (Fig. 7). Different genotypes were found to react differently to the supplied IAA treatments ($P \leq 0.03$). Under our low Pi conditions, exogenous IAA led to an increase in RH density in both *max2-1* and *max4-1* roots. Under these conditions, no significant increase in RH density or changes in the PR length was observed for the wild type (Fig. 7; data not shown). The response of both *max2-1* and *max4-1* to IAA was dose dependent, whereas *max2-1* showed the highest sensitivity to IAA in comparison with *max4-1* and already reached a level insignificant from that of the wild type at IAA concentration of $1 \times 10^{-7}$. Treatment of *max2-1* or *max4-1* with the highest concentration of IAA led to a significant shortening of the PR (not shown) and, accordingly, an increase in RH density (Fig. 7). These results suggest that IAA may compensate for the deficiency in the response of *max2-1* and *max4-1* to low Pi growth conditions.

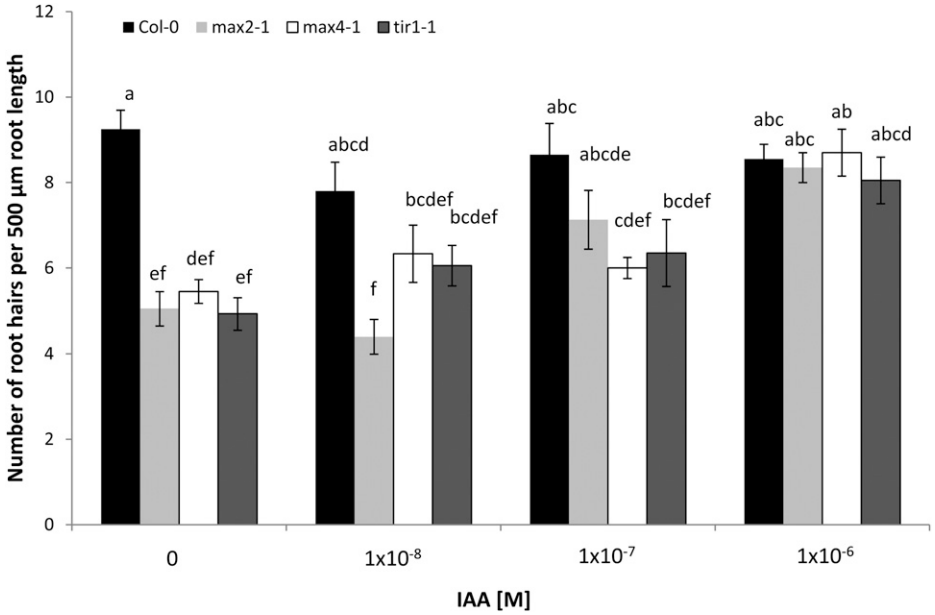


**Figure 7.** Effect of exogenous supplementation of IAA on RH density of the wild type and *max2-1*, *max4-1*, and *tir1-1* mutants under low Pi conditions. RH densities at 48 hpg are presented as mean ± SE of the number of RHs per 500 $\mu$m of root length, following exogenous supplementation of 20-$\mu$L IAA to each of the seedlings at concentrations of 0 (control), $1 \times 10^{-8}$, $1 \times 10^{-7}$, and $1 \times 10^{-6}$ M. Experiments were repeated three times; each treatment within each experiment included two replicates, with 10 germinated seedlings per replicate. Different letters indicate statistically significant differences between means for particular IAA treatment by one-way ANOVA with Tukey-Kramer multiple comparison test ($P \leq 0.01$). Two-way ANOVA analysis revealed significance between genotypes and IAA treatments ($P = 0.028$).

In agreement, the addition of IAA to *max2-1* resulted in an increase in expression of the 5 of the 6 examined PSI genes (except for *PHT1;4*) under low phosphate conditions compared with the treatment without IAA supplied (Figs. 2A and 4C; Supplemental Table S2), further suggesting that IAA is able to increase the response of *max2-1* to low Pi conditions.

### *TIR1* Expression Is Induced to a Lesser Extent in *max2-1* and *max4-1* Relative to the Wild Type under Low Phosphate Conditions

Our results showed that exogenous IAA may compensate for the deficiency in the response of *max2-1* and *max4-1* to low Pi growth conditions, thereby raising the possibility that IAA mediates the SL-regulated response to low Pi conditions. It has been suggested that low Pi response in roots is mediated via induction of *TIR1* expression (Pérez-Torres et al., 2008). To further examine the involvement of auxin and that of TIR1 in the SL-mediated response to low Pi conditions during early seedling growth, we measured *TIR1* expression levels in *max2-1*, *max4-1*, and the wild type under low Pi conditions, in comparison with the levels under high Pi conditions. In the wild type, *TIR1* expression was enhanced under low Pi conditions (17.23 ± 3.51-fold; Figure 2; Supplemental Table S2; Pérez-Torres et al., 2008), whereas in *max2-1*, *TIR1* expression was not induced but actually reduced under low Pi conditions, in comparison with its expression levels under high Pi conditions (0.27 ± 0.09-fold; Figure 2A; Supplemental Table S2). In *max4-1*, *TIR1* expression was induced to a lesser (but insignificant) extent than in the wild type (6.78 ± 2.09-fold; Figure 2B; Supplemental Table S2). These results indicate that the reduced ability of *max2-1* and *max4-1* to respond to low Pi conditions may be associated with their reduced ability to elevate *TIR1* expression.

### Hormonal Regulation of TIR1 Expression

We then examined the level of *TIR1* expression in the different genotypes following the treatments that altered their response to low Pi. In *max4-1*, exogenous addition of GR24 increased RH density in response to low Pi (Fig. 3). Concomitantly, this treatment to *max4-1* increased *TIR1* expression (37.48 ± 13.02-fold; Figure 4A; Supplemental Table S2) under low Pi conditions, in comparison with the untreated control (6.78 ± 2.09-fold; Figure 2B; Supplemental Table S2). In the wild type, exogenous addition of AbamineSG, which presumably reduces SL content, reduced the response to low Pi (Fig. 5). Accordingly, this treatment significantly reduced *TIR1* expression (4.56 ± 1.17-fold; Figure 4B; Supplemental Table S2) under low Pi conditions, in comparison with the untreated control (17.23 ± 3.51-fold; Figure 2; Supplemental Table S2). In *max2-1*, exogenous addition of IAA increased its response to low Pi (Fig. 7), and only slightly (but significantly) increased its *TIR1* expression (0.77 ± 0.19-fold; Figure 4C; Supplemental Table S2) in comparison with the control (0.27 ± 0.09-fold; Figure 2A; Supplemental Table S2). These results support the suggestion that the SL-mediated response to low Pi conditions is associated with the ability to elevate *TIR1* expression.

### RH Density of *tir1-1* under Low Phosphate Conditions Was Increased by IAA But Not by GR24 or AbamineSG Application

Because this study suggests that *TIR1* expression is involved in SL regulation of the early response of seedlings to low Pi, we determined the RH density of *tir1-1* under low Pi conditions at 48 hpg on plates under low Pi conditions. Each seedling was untreated or treated by application of 20 $\mu$L of GR24, AbamineSG, or IAA, at the examined concentrations (as described above). During early seedling growth at 48 and at 72 hpg, *tir1-1* had a lower RH density than wild-type seedlings grown under comparable conditions, either high or low Pi (Fig. 1C). Both genotypes had a higher RH density under low P compared with high P at 48 hpg (Fig. 1C). Only at 96 hpg, *tir1-1* RH density grown on low Pi plates was similar to that of the wild type under the same low Pi conditions (Fig. 1C). Moreover, RH density of *tir1-1* under low Pi conditions was only moderately (and insignificantly) increased by GR24 application (Fig. 3). It was only moderately (and insignificantly) affected by application of AbamineSG (Fig. 5). *tir1-1* displayed a reduced sensitivity to IAA compared with *max2-1*, whereas only an IAA application of $1 \times 10^{-6}$ M was able to restore the response of *tir1-1* up to wild-type level (Fig. 7).

## DISCUSSION

Here we show that the SL pathway influences either root sensing of or response to low Pi, and it requires the activity of MAX2 and SL. Moreover, we show that the SL-regulated root response to low Pi conditions correlates with transcriptional induction of the auxin receptor TIR1.

Several lines of evidence suggest the SL pathway is involved in the very early events of the low-Pi response. First, *max2-1*seedlings have a weaker response to low Pi conditions compared with the wild type. This was evident by an absence in the increase of RH density (at 48 hpg but not at later developmental stages). Second, the *max4-1* mutant, deficient in SL biosynthesis, also showed a reduced response to low Pi conditions in comparison with the wild type, as determined by a reduction in the increase of RH density.

The third line of evidence suggesting involvement of the SL pathway in the very early events of the low-Pi response was the decreased induction level of expression of most of the examined PSI genes. PSI genes are transcriptionally induced upon Pi starvation (for review, see Chiou and Lin, 2011). However, PSI expression failed to be regulated by Pi in the SLs

mutants. Moreover, the expression of *PHO2* was reduced in the wild type under low Pi conditions, in line with the findings in other studies (e.g. Bari et al., 2006). However it was induced in *max2-1* under low Pi conditions. Together, these results support the suggestion of an averted response of the SLs mutants to low Pi conditions compared with the wild type. This might also suggest that SLs may be involved in the regulation of *PHO2* expression.

Fourth, the recovery of the low Pi responses by GR24 in *max4-1*, but not in *max2-1*, suggests that the deficiency in SL biosynthesis in *max4-1* is the cause of this reduced response to low Pi. The fifth line of evidence was that treatment of seedlings with AbamineSG, which reduces SL levels (Kitahata et al., 2011), led to a marked decrease in RH density in the wild type under low Pi conditions. These results suggest that a reduction in SL levels inhibits the wild-type response to low Pi conditions. In line with these findings are the results suggesting that AbamineSG has no such effect in *max4-1*, which is deficient in SL biosynthesis, or in *max2-1*, which is deficient in SL response. It should be noted, however, that AbamineSG is considered to be an abscisic acid biosynthesis inhibitor and target 9-cis-epoxycarotenoid dioxygenase (NCED; Kitahata et al., 2011), a close relative to CCD7 and CCD8. Therefore, it may affect additional pathways to that of SLs. As a result, it cannot be excluded that the effect of AbamineSG on the low Pi response of the wild type derived from different or additional pathways to that of SLs.

Together, the results demonstrate that SLs are associated with the ability of the root to respond to low Pi conditions, with respect to RH density. In previous studies, exogenous supplementation of SLs has been shown to have different effects on LR formation under low and high Pi conditions (Kapulnik et al., 2011a; Ruyter Spira et al., 2011). In the shoot, SLs have been suggested to be involved in the response to Pi growth conditions, based on differences in lateral bud outgrowth or tillering in response to low Pi conditions between the wild type and SL-insensitive or -deficient genotypes (Umehara et al., 2010; Kohlen et al., 2011). Hence, SLs and their signaling pathways may be important regulators of both root and shoot responses to Pi levels.

It is interesting to note that the response of *max2-1* and *max4-1* to low Pi increased with time, such that by 72 and 96 hpg they reached wild-type levels of RH density. This may be explained by the suggestion that there are factors acting in parallel with MAX2 in the SL signaling pathway under low Pi response over time. It might be that in *max2-1*, at 72 and 96 hpg under low Pi conditions, these other factors lead to the low Pi response. Similarly, at 72 and 96 hpg under low Pi conditions, in the SL-biosynthesis mutant *max4-1*, it is possible that SLs may be synthesized by other pathways, or *max4-1* may still have residual SLs content (Kohlen et al., 2011), or that even in the absence of SLs, the MAX2 F-box protein or its equivalent might still interact with its substrate, resulting in a more moderate phenotype (Hu et al., 2010). This can also indicate that SLs play a role in the response of root to low Pi conditions mostly at early stages in development and that other factor(s), such as other hormonal pathways or MAX2-independent IAA responses, dominate later stages. In agreement, the differences between *max2-1* and the wild type in terms of root architecture (LR number and PR length), examined 7 to 11 dpg, are moderate and did not result in a significant difference in LR density between *max2-1* and wild-type plants under low Pi conditions.

The reduced transcriptional induction of many of the PSI genes under low Pi conditions in *max2-1* seedlings may be a result of reduced sensitivity or response to Pi. It is interesting to note that because cytokinins were suggested to repress the expression of *IPS1* in response to Pi deprivation (Martín et al., 2000), the reduced level of *IPS1* transcription in *max2-1* occurs despite lower levels of xylem-sap cytokinins in these mutants (Foo et al., 2007). This may suggest that regardless of the cytokinin hormonal balance in this mutant, it is nevertheless unable to respond to low Pi conditions.

Alternatively, the reduction in low Pi response in *max2-1*could result from differences in the internal Pi content in the SL-insensitive mutant compared with the wild type. Of specific significance in this regard was the inability of *max2-1* to normally regulate expression of *PHO2*, a regulator of Pi uptake (for review, see Chiou and Lin, 2011). Also, the inability of *max2-1* to normally regulate expression of some of the examined Pi transporters might alter its ability to acquire sufficient Pi. In our experiments, under low Pi conditions at 48 hpg, the expression in seedlings of *PHT1;2*, *PHT1;4*, and *PHT1;5* Pi transporters was induced in the wild type but to a lesser extent in *max2-1*, whereas *PHT1;1* displayed increased expression in *max2-1* relative to the wild type.

Despite the differences in PHT and *PHO2* gene expression, the seedling concentrations of P in *max2-1* were similar to those in the wild type under low Pi conditions. First, the wild type-like P concentration in *max2-1* might be a result of the increased expression in *max2-1* of *PHT1;1* and other Pi transporters that were not examined here; this is despite the induction of *PHO2* expression in *max2-1* that might suggest down-regulation of Pi uptake. This contradicting evidence may hint at the existence of other pathways, besides SLs, that regulate low Pi responses. Second, these results show that under low Pi conditions, the relatively low concentration of P in the wild type that induce a low-Pi response, including PSI gene expression, is also present in *max2-1* tissues. However, these internal, low Pi concentrations in *max2-1* do not induce a normal low-Pi response. Although these results may suggest that the physiological importance of SL responses under a wide range of Pi concentrations might be compromised by other components of low Pi response, it can be concluded that *max2-1* mutants are deficient in their sensing of, or response to, internal P content, rather than in their P content itself, in comparison with the wild type.

Ethylene has been suggested in multiple studies to be involved in the response to low Pi (e.g. López-Bucio et al., 2002; Ma et al., 2003; Lei et al., 2011; Nagarajan et al., 2011). On the other hand, it has been shown that under high Pi conditions, SLs induce ethylene biosynthesis and require ethylene signaling for their effect (Kapulnik et al., 2011b). Hence, we examined the possibility that the deficient low-Pi response in *max2-1* may be a result of lower ethylene availability under low Pi conditions, as a result of SL insensitivity. However, addition of relatively high levels of ACC under low Pi conditions increased wild-type RH density but not that of *max2-1*. This suggests that ethylene does not compensate for the deficiency in the response of *max2-1* to low Pi and therefore, that the MAX2-regulated low-Pi response is downstream or independent of the ethylene pathway. Similar lack of ethylene involvement in the low-Pi response has been previously suggested for the wild type (e.g. Ma et al., 2001).

Conversely, addition of IAA to *max2-1* and *max4-1* roots led to complementation of the mutant phenotypes to that of the wild type, both in terms of RH density and, as examined for *max2-1*, at the level of expression of some of the PSI genes. However, the discrepancy between the complete restoration in *max2-1* of RH density and the partial restoration of PSI gene expression by IAA, as well as a wild type-like *PHT1;1* gene expression in *max2-1* under low Pi conditions, may suggest that there could also be a parallel pathway to TIR1 that involves IAA, in a MAX2-independent pathway. This pathway might be positively regulating PSI gene induction downstream to MAX2 activity.

These results suggest that IAA is part of the SL response pathway to low Pi growth conditions. The wild type did not respond to IAA under these conditions, possibly due to saturation in its IAA response, in agreement with published observations (Ma et al., 2001). These results for the wild type are in agreement with López-Bucio et al. (2003), who suggested that in the wild type, the auxin pathway plays a fundamental role in modifications of root architecture by P availability. Thus, the deficiency in the response of *max2-1* to low Pi might be associated with a reduced induction of the auxin pathway relative to the wild type, upon low Pi conditions.

Moreover, the auxin-mediated response to low Pi in the wild type was shown to be associated with transcriptional induction of *TIR1* (Pérez-Torres et al., 2008), encoding an auxin receptor (Dharmasiri et al., 2005). Therefore, we examined whether *TIR1* is differentially expressed under low Pi conditions (as compared with its expression under high Pi conditions) in each of the genotypes: the wild type, *max2-1*, and *max4-1*. Despite clear induction of *TIR1* expression in the wild type under low Pi conditions, results suggested that there is no such induction in *max2-1*, whereas in *max4-1*, induction of *TIR1* expression was reduced relative to the wild type. Hence, the reduced ability of *max2-1* and *max4-1* to respond to low Pi conditions may be a result of their reduced ability to induce *TIR1* expression. Furthermore, treatment of the wild type with AbamineSG, and of *max4-1* with GR24, reduced and increased *TIR1* expression, respectively, suggesting a correlation between *TIR1* expression and the SL content. However, IAA treatment of *max2-1* only slightly induced *TIR1* expression. Taken together, the results support a connection between the auxin pathway and the SL-mediated response to low Pi: either induction of *TIR1* expression under optimal levels of SLs (in the wild type or in *max4-1* following GR24 treatment), or the increase of IAA levels without induction of *TIR1* (in *max2-1* following IAA treatment), promote auxin signaling through TIR1, or the other IAA receptors (e.g. *AUXIN-SIGNALING F-BOX* (*AFB*) gene family; for review, see Parry et al., 2009) and induction of the low-Pi response.

Accordingly to the suggested connections between the auxin pathway and the SL-mediated response to low Pi, the *tir1-1* mutant showed a reduced RH density under low Pi in comparison with the wild type. Similar to the responses of *max2-1* and *max4-1* to low Pi, the *tir1-1* response increased with time, such that at 96 hpg, in terms of RH density, it reached wild-type levels. Hence, the suggested factors that act at later stages of seedling development in parallel to SLs signaling pathway and MAX2 in low Pi response, might also act in parallel to TIR1.

Moreover, the reduction in the response of *tir1-1* to low Pi could not be restored by GR24 application, and it was only slightly (and insignificantly) affected by

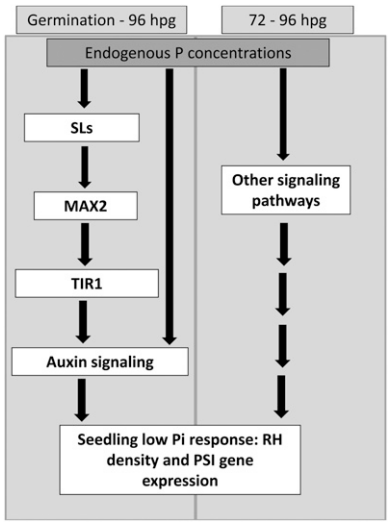


**Figure 8.** Schematic illustration of the hormonal pathways activated by SLs in response to low Pi growth conditions. Under conditions of Pi depletion, and in response to endogenous P concentrations, the SL pathway, through MAX2, is suggested to activate *TIR1* expression and the auxin signaling pathway, and thereby regulate root development to suit those growth conditions. Another possibility is that there could also be a parallel pathway to TIR1 that involves IAA, in a MAX2-independent pathway, which leads to partial low Pi response at early stages. However, evidence is found to the existence of other pathways that are MAX2 independent, which may dominate low Pi response during later stages of development.

application of AbamineSG. Hence, the deficiency in the response of *tir1-1* to low Pi might be downstream of the SL signaling. Exogenous supplementation of IAA at high concentration ($1 \times 10^{-6}$ M) was able to complement the response of *tir1-1* to low Pi, perhaps through other auxin receptors.

It is important to note that the stronger response of *max2-1* to auxin under low phosphate conditions, in terms of RH density and PR length, in comparison with the wild type and *tir1-1*, may suggest that under low Pi conditions *max2* is more sensitive to auxin than the wild type (or *tir1-1*), despite reduced levels of TIR1 induction. Such increased sensitivity may be one of the explanations for the increased DR5:GUS expression in the *max2-1* mutant root tip (Ruyter-Spira et al., 2011). Another possibility is that *max2-1* is saturated with auxin response, and therefore may not be able to respond to the low Pi conditions, in the same way as the wild type (Pérez-Torres et al., 2008), by elevation of *TIR1* expression. This assumption may be supported by the higher levels of auxin transport found in *max2-1* stem and the elevated levels of DR5:GUS expression found in *max2-1* roots in comparison with the wild type (Bennett et al., 2006; Ruyter-Spira et al., 2011). Rather, we found that low Pi conditions in *max2-1* lead to a reduction in *TIR1* expression. Thus, it might be that the reduction, rather than induction, of *TIR1* expression in *max2-1* under low Pi conditions is at least one of the factors that prevent *max2-1* responding to low Pi during the first few hpg.

## CONCLUSION

We show here that after germination the plant's response to Pi level and to endogenous P concentrations is regulated by SLs. We show that this regulation is mediated via the SL signaling component MAX2 (Fig. 8). Moreover, given the effect of IAA application on the wild type and SL- or auxin-related mutants, and the determination of *TIR1* expression in the different genotypes and under the various treatments, it is suggested that *TIR1* and the auxin signaling pathway are involved in this SL-regulated low Pi response (Fig. 8). Discrepancies between RH phenotype and PSI gene expression suggest that there could also be a parallel pathway to TIR1 that involves IAA, in a MAX2-independent pathway (Fig. 8). Furthermore, the only transient reduction in *max2-1*, *max4-1*, and *tir1-1* response to low Pi conditions point to the existence of other pathways, in addition to SLs and auxin, which may dominate low Pi response during later stages of seedling development (Fig. 8).

This work provides further evidence in support of the suggestion that the biological role of SLs is to act as mediators of plant responses to Pi conditions.

## MATERIALS AND METHODS

### Plant Growth

Seeds of Arabidopsis (*Arabidopsis thaliana*), wild-type Col-0, *max2-1*, *max3-11*, *max4-1*, *tir1-1* (all mutants are on the Col-0 background and were obtained from the Arabidopsis Biological Resource Center; http://abrc.osu.edu/) were surface-sterilized and germinated on one-half-strength Murashige and Skoog medium solidified with 0.5% (w/w) Gelzan (Sigma), supplemented with 1.5% (w/v) Suc and modified to contain 1 $\mu$M (for low level) or 2 mM (for high level) Pi in sterile culture plates. Plates were incubated vertically in the dark at 4°C for 2 d to synchronize germination. They were left unsealed to prevent accumulation of gases (e.g. ethylene). Plates were then positioned in an upright 45° position and incubated at 22°C with a light intensity of 50 to 60 mol photons $m^{-2}$ $s^{-1}$ provided by white fluorescent tubes under a photoperiod of 16 h of light/8 h of dark. Hormonal treatments including ACC ($1 \times 10^{-12}$, $1 \times 10^{-13}$, and $1 \times 10^{-14}$ M, water dissolved), IAA ($1\times10^{-8}$, $1\times10^{-7}$, $1\times10^{-6}$ M, water dissolved), GR24 (15 $\mu$M and 50 $\mu$M, acetone dissolved), and AbamineSG (1mM, acetone dissolved) were applied to the seedlings. Each seedling was treated with 20 $\mu$L of hormones (i.e. ACC, IAA, GR24, and AbamineSG) at 0 hpg. Controls were conducted for all experiments and all replicates. In each of the experiments, controls were the exact concentration of the appropriate solvent that was used to treat the seedlings. In all experiments, unless indicated otherwise, for each biological replicate, two plates were seeded with 10 plants per plate. Three biological replicates were performed for each experiment.

### RH Density

To examine RH density, roots were grown on plates as described above. After 48, 72, and 96 hpg, roots were examined on the plates using a stereomicroscope (Leica MZFLIII, Leica Microsystems GmbH) equipped with a Nikon DS-Fi1 camera. Pictures were taken of root segments that had grown on the plates every 24 h, with 10 separate roots per treatment. Measurements of RH density were performed on 10 pictures per treatment, for two segments of 500 $\mu$m each, using IMAGEJ (http://rsbweb.nih.gov/ij/). Experiments were repeated three times. Each treatment within each experiment included two replicates. Two-way ANOVA analysis was done to assess significance between genotypes and treatments. Different letters indicate statistically significant differences between means for particular treatment by one-way or two-way ANOVA with Tukey-Kramer multiple comparison test ($P \leq 0.01$) using the JMP statistical package (SAS Institute, Cary, NC).

### PR Length, LR Number, and LR Density

To examine PR length, LR number, and LR density, plants were grown as described above and at 7, 8, 9, 10, and 11 dpg, the PR and LRs were studied using a stereomicroscope (Leica S4E LED2500, Leica Microsystems GmbH). LRs were counted and plates were scanned to measure the PR length, using IMAGEJ (http://rsbweb.nih.gov/ij/). LR densities were calculated by dividing the number of LRs by the length of the PR. The experiment was repeated twice ($n = 25$). We used the residual maximum likelihood (REML) procedure as implemented in GenStat version 13 software (Payne, 2010) to perform the repeated measurements analysis. Repeated measurements data were analyzed by fitting the following linear mixed model $y_{ijkl} = \mu + genotype_i + treatment_j + time_k + genotype \times time_{ik} + treatment \times genotype_{jk} + genotype \times treatment_{ij} + genotype \times treatment \times time_{ijk} + e_{ijkl}$, where $y_{ijkl}$ is the phenotypic value of the $l$th individual of genotype $i$, treated with the $j$th treatment at time point $k$; $\mu$ is the mean term, and $e_{ijkl} \sim N(0, \sigma_e^2)$ is the residual effect, with $\sigma_e^2$ being the residual variance. Various ways of modeling the correlation structure (uniform, autoregressive order 1 [AR1] or AR2, and antedependence order 1 and 2) were compared in the REML framework. Selection of the best fit model was based on a likelihood ratio test statistic and/or the Aikake Information coefficient. When residuals from the analysis indicated increasing variance over time, this was modeled directly by specifying that heterogeneity is to be introduced into model. Significance of the fixed main and interaction effects (over the whole kinetics) was assessed by an $F$-test.

### Gene Transcription Levels in Seedlings

For each of the genotypes, gene transcription was examined under low and high Pi conditions, using quantitative PCR (qPCR). RNA was extracted as described by Mayzlish-Gati et al. (2010) from seedlings grown as described above for 48 hpg, 120 seedlings per sample. Treatments that were taken for RNA extraction included: control, $1 \times 10^{-6}$ M IAA, $1.5 \times 10^{-5}$ M GR24, and $1 \times 10^{-3}$ M AbamineSG. qPCR was performed by amplifying fragments of PSI genes (Supplemental Table S1). qPCR amplification was performed as described by Mayzlish-Gati et al. (2010). Briefly, qPCR was performed using components supplied in the KAPA SYBR FAST qPCR kits (Kapa Biosystems) and the gene-specific primers (Supplemental Table S1). The reaction mixture

consisted of the following components: 2× Master Mix with integrated antibody-mediated hot start, SYBR Green I fluorescent dye, $MgCl_2$, deoxyribonucleoside triphosphates, and stabilizers, 2 $\mu$L of the template, and PCR-grade water to a final volume of 10 $\mu$L. The reverse transcription qPCR analysis was carried out on a Rotor gene 6000 instrument (Corbett-Qiagen). The threshold cycle (Ct) was calculated by the Rotor gene 6000 instrument software. The level of expression of the target genes (genes of interest) was calculated relative to that of the reference mRNA. Arabidopsis 15S ribosomal RNA (GenBank accession no. AT1G04270.1) served as the reference internal control gene (Kapulnik et al., 2011b) for the amount of RNA, and was amplified using specific primers: 5′-CAAAGGAGTTGATCTCGATGCTCTT-3′ (forward) and 5′-GCCTCCCTTTTCGCTTTCC-3′ (reverse). Values of the steady-state level of gene transcripts were analyzed using the $2^{-\Delta\Delta C_T}$ method (e.g. Livak and Schmittgen 2001; Arocho et al., 2006; Schmittgen and Livak 2008; Postaire et al., 2010; Ralhan et al., 2012; Tsou et al., 2012), according to the below formula (adapted from Schmittgen and Livak 2008). Ct: number of PCR cycles, GI: gene of interest, IC: internal control, H: high Pi conditions, L: low Pi conditions.

$$\frac{\frac{Ct\ GI_H}{Ct\ IC_H}}{\frac{Ct\ GI_L}{Ct\ IC_L}} = \frac{2^{[Ct\ GL_H - Ct\ IC_H]}}{2^{[Ct\ GL_L - Ct\ IC_L]}} = 2^{[Ct\ GL_H\ - Ct\ IC_H]} - 2^{[Ct\ GL_L - Ct\ IC_L]} = 2^{\Delta\ Ct_H - \Delta\ Ct_L}$$

Values above or below 1.5 represents increase or decrease, respectively, in the steady-state level of gene transcripts for the examined conditions (i.e. that of the nominator versus that of the denominator). The experiment was performed in five biological replicates; each replicate included 120 plants, on which three technical repeats were performed. Means and SE were determined from all biological replicates. Statistical differences of means of genotypes and treatments from the wild-type control treatment were determined by ANOVA pairwise Student's $t$ tests ($P \leq 0.05$) using the JMP statistical package.

## P Concentrations in Seedlings

Seedlings grown as described above were taken at 48 hpg for determinations of total concentration of P. Per sample, 30 seedlings were collected and their dry weight was determined. P was determined after digestion with sulfuric acid and peroxide (Snell and Snell 1949): 0.2 mL of concentrate sulfuric acid was added to 10 to 20 mg of root and heated to 180°C. Hydrogen peroxide (0.1 mL) was added until clearance of the acid solution. Distilled water was then added to complete a volume of 20 mL. P concentration was determined using an Autoanalyzer (Lachat Instruments). Different letters indicate statistically significant differences between means determined under all examined Pi conditions by one-way ANOVA with Tukey-Kramer multiple comparison test ($P \leq 0.01$).

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Phosphorous concentrations (percentage of dry weight) in seedlings of the wild type and *max2-1* under different Pi growth conditions.

**Supplemental Figure S2**. Effect of exogenous supplementation of ACC on RH density of the wild type and *max2-1* mutant under low Pi conditions.

**Supplemental Table S1.** List of genes subjected to qPCR-based determination of steady-state transcription levels.

**Supplemental Table S2.** PSI gene expression (mean ± SE) at 48 hpg under low (1 $\mu$M) phosphate conditions calculated versus PSI gene expression under high (2 mM) phosphate conditions.

## ACKNOWLEDGMENTS

We would like to thank Dr. Mor Yaari, Bruria Ben-Dor, Rony Shema, Dikla Koren, and Danny Levi for their technical help. We are grateful to Dr. Koichi Yoneyama for the supplement of GR24 and Dr. Tadao Asami for the supplement of AbamineSG.

Received June 21, 2012; accepted September 10, 2012; published September 11, 2012.

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