Characterization of low phosphorus insensitive Mutants Reveals a Crosstalk between Low Phosphorus-Induced Determinate Root Development and the Activation of Genes Involved in the Adaptation of Arabidopsis to Phosphorus Deficiency

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Low phosphorus (P) availability is one of the most limiting factors for plant productivity in many natural and agricultural ecosystems. Plants display a wide range of adaptive responses to cope with low P stress, which generally serve to enhance P availability in the soil and to increase its uptake by roots. In Arabidopsis (Arabidopsis thaliana), primary root growth inhibition and increased lateral root formation have been reported to occur in response to P limitation. To gain knowledge of the genetic mechanisms that regulate root architectural responses to P availability, we designed a screen for identifying Arabidopsis mutants that fail to arrest primary root growth when grown under low P conditions. Eleven low phosphorus insensitive (lpi) mutants that define at least four different complementation groups involved in primary root growth responses to P availability were identified. The lpi mutants do not show the typical determinate developmental program induced by P stress in the primary root. Other root developmental aspects of the low P rescue system, including increased root hair elongation and anthocyanin accumulation, remained unaltered in lpi mutants. In addition to the insensitivity of primary root growth inhibition, when subjected to P deprivation, lpi mutants show a reduced induction in the expression of several genes involved in the P starvation rescue system (PHOSPHATE TRANSPORTER 1 and 2, PURPLE ACID PHOSPHATASE 1, ACID PHOSPHATASE 5, and INDUCED BY PHOSPHATE STARVATION 1). Our results provide genetic support for the role of P as an important signal for postembryonic root development and root meristem maintenance and show a crosstalk in developmental and biochemical responses to P deprivation.

Phosphorus (P) is one of the most important nutrients for plant growth and development. P plays a myriad of essential biological functions as a structural element in phospholipids and nucleic acids, in energy metabolism, in the regulation of enzymatic activities, and in signal transduction cascades (Raghothama, 1999; Rausch and Bucher, 2002). Although the total content of P in the soil may be high, its bioavailability is generally very low due to mineralization and fixation processes (Hinsinger, 2001). Thus, low P availability is one of the prime limiting factors for plant growth and development in many ecosystems and a major constraint for agricultural productivity in alkaline and acid soils (López-Bucio et al., 2000).

Plants have evolved a wide range of adaptive strategies to adapt to P deficiency and improve P mobilization and uptake from the soil (Raghothama, 1999), including an increase in the synthesis and secretion of organic acids, which enhance the solubilization of P from insoluble inorganic compounds (Jones, 1998); an increase in the production of enzymes such as acid phosphatases and nucleases that release P from soil organic sources (Nürnberger et al., 1990; Löffler et al., 1992; Duff et al., 1994; Chen et al., 2000); an enhanced expression of high-affinity P transporters to optimize P uptake from the rhizosphere; and a differential distribution of photosynthates between shoots and roots, which results in increased root-shoot ratios caused by prolific growth of lateral roots (Muchhal et al., 1996; Raghothama, 1999; Karthikeyan et al., 2002). These developmental changes are believed to enhance the total exploratory capacity of roots in search of nutrient-rich patches present in the soil (Lynch, 1995; Lynch and Brown, 2001).

Arabidopsis (Arabidopsis thaliana) represents an excellent model for investigating the molecular mechanisms...
involves in the responses of plants to low nutrient availability. Most common plant responses to P starvation have been described to occur in Arabidopsis. For instance, the expression of several members of the purple acid phosphatase, S-like ribonuclease, and high-affinity phosphate transporter gene families are transcriptionally activated under low P conditions (Taylor et al., 1993; Bariola et al., 1994; Haran et al., 2000; Li et al., 2002; Mudge et al., 2002).

Arabidopsis also exhibits remarkable root architectural changes in response to low P availability. These include an enhanced growth and density of root hairs (Bates and Lynch, 1996), a reduction in primary root length, and an increased density of lateral roots (Williamson et al., 2001; López-Bucio et al., 2002). Recently, we reported that the reduction in primary root growth is due to a determinate low P-induced root developmental program that inhibits cell division in the primary root meristem and promotes differentiation processes within the root tip (Sánchez-Calderón et al., 2005). Adjustment of root system architecture via changes in meristem activity is integral to the low P adaptation process; however, the mechanisms by which plants monitor external P abundance and translate the nutritional signal into developmental processes remain to be elucidated. To study P starvation-signaling pathways in Arabidopsis, Ticconi et al. (2004) isolated the inorganic phosphate (Pi) deficiency response (pdr2) mutant, which is defective in local P sensing. The sensitivity and amplitude of metabolic P starvation responses, such as P-responsive gene expression or accumulation of anthocyanins and starch, are enhanced in pdr2 seedlings. Moreover, the most conspicuous alteration of pdr2 is the enhanced primary root growth inhibition in low P, caused by inhibited cell division in the root meristem. The characteristics of pdr2 suggest a P-sensitive checkpoint in root development and shows that root developmental responses to P deprivation can be dissected by a mutational strategy.

To further identify the genetic components responsible for the Arabidopsis root architectural responses to low P availability, we performed a visual screening for Arabidopsis mutants that under low P conditions do not manifest primary root growth reduction. We identified a group of 11 low phosphorus insensitive (lpi) mutants. The primary roots of these mutants in P-deprived medium have a primary root growth quite similar to that observed for the primary root of wild-type plants growing in high P conditions. The mutant phenotypes are caused by single nuclear mutations in four different genes (lpi1–lpi4). Detailed cellular and development studies of two of these mutants indicate that lpi mutants do not show the typical determinate postembryonic developmental program induced by P stress in wild-type plants (Sánchez-Calderón et al., 2005). Our results also suggest that lpi mutants define crucial components of regulation of meristematic activity in the primary roots and of a subset of biochemical and genetic responses to low P availability.

RESULTS

Isolation of Arabidopsis Mutants with Altered Primary Root Growth Response to Low P Availability

One of the most conspicuous root developmental changes that occur in low P conditions is the reduction of primary root growth (Williamson et al., 2001; López-Bucio et al., 2002). To investigate the molecular basis of this response, we screened ethyl methane sulfonate (EMS)-induced mutant populations by observing the root architecture of plants growing over the surface of 0.1 M Murashige and Skoog agar plates with low (1 μM) P content. We isolated mutants that, in contrast to the wild type, were able to sustain normal primary roots under low P conditions (Fig. 1A, arrow). The mutants were backcrossed to wild-type plants four times prior to detailed phenotypical analysis. This screen yielded 11 lpi mutants whose insensitivity to low P stress in terms of primary root growth inhibition was confirmed in their progeny (data not shown). To further study the developmental alterations induced by low P in the wild type and lpi mutants, we grew ecotype Columbia (Col-0) and lpi plants side by side in

Figure 1. Genetic screen and phenotypic characterization of lpi mutants. A, Photograph of an agar plate with low P medium showing a putative lpi mutant (arrow). B, Five 14-d-old wild-type (Col-0) and lpi seedlings growing side by side on media containing high (left plate) or low (right plate) P. C, Col-0 (WT), lpi1, and lpi2 plants grown in soil.
vertically oriented agar plates with high (1 mM) or low (1 μM) P content. Wild-type plants grown in high P show the typical taproot system produced by indeterminate growth, in which the primary roots reached a greater length than lateral roots (Fig. 1B). In low P, wild-type plants have primary roots with limited growth that correlate with an increase in lateral root formation. Multiple branches developed, giving rise to a root system with different architecture than that observed in high P. In medium with high P, the lpi mutants showed a root system similar to that observed in wild-type plants, characterized by long primary roots with few lateral roots forming close to the root/shoot junction (Fig. 1B, left plate). In contrast to that observed in wild type, lpi mutants were insensitive to the primary root growth inhibition caused by P deprivation (Fig. 1B, right plate).

Under greenhouse conditions and a normal fertilization regime, the aerial part of lpi mutants is phenotypically normal, presenting vegetative development, fruit development, and seed production similar to the wild type. Under these conditions, the only observed difference is the lateral root density in the aerial part of mutants in comparison to wild-type plants. These observations indicate that the genetic defect present in the lpi mutants specifically impact the root developmental changes induced by low P availability.

Genetic Characterization of lpi Lines

To determine the number of genes represented in the lpi lines, we carried out complementation tests crossing all the lpi mutants to each other. These studies revealed four complementation groups among lpi lines, suggesting that four independent genes (lpi1–lpi4) were represented in the mutant collection (data not shown). To determine the genetic basis of the lpi phenotype, homozygous lpi1 to lpi4 plants were backcrossed to wild type. In F2 progeny from these crosses, three lines (lpi1, lpi3, and lpi4) segregated the mutant phenotype in a 1:3 ratio, indicating that each resulted from a recessive mutation. lpi2 segregated the mutant phenotype in a 3:1 ratio, indicating that it resulted from a dominant mutation (Table I). Two of these mutants, one recessive (lpi1) and one dominant (lpi2), were chosen for further analysis.

Root Development Alterations in lpi Mutants Are Specific for P Starvation

To evaluate the specificity of the lpi mutations to P starvation, we assessed the effects of other nutritional deficiencies, including potassium (−K), nitrogen (−N), sulfur (−S), and iron (−Fe), on root architectural traits of wild type and lpi1 and lpi2 mutants. The primary root length of wild-type seedlings showed a drastic reduction in low P medium (70%) and a smaller reduction in medium devoid of K (30%) compared with those grown in control medium, while no statistically significant differences where observed in medium lacking N, S, or Fe (Fig. 2A). The primary root of both lpi1 and lpi2 plants exhibited primary root elongation similar to the wild type in the different nutrient deficiency treatments, except in low P conditions, in which they presented a primary root growth similar to that observed in high P medium (Fig. 2A). lpi2 showed a small but statistically significant difference in primary root growth in −K media when compared to the wild type (Fig. 2A).

As previously reported (Williamson et al., 2001; Lópe-Bucio et al., 2002; Kutz et al., 2002), P and S deprivation increase lateral root number in wild-type seedlings (120% and 60%, respectively), whereas no effect on lateral root number was observed in media lacking other nutrients (Fig. 2B). lpi1 plants showed a similar response to P and S deprivation as that observed in the wild type, whereas lpi2 failed to increase its lateral root number in −P media but sustained the normal lateral root response to S deprivation (Fig. 2B). From the different nutrient deficiency treatments, only low P elicited dramatic differences in lateral root density between wild type and lpi mutants (Fig. 2C). P starvation induced a 6-fold increase in lateral root density in the wild type that is drastically reduced in lpi1 and lpi2. Significant differences were also found between lpi1 and lpi2 plants in lateral root density under low P conditions (Fig. 2C). Lateral root density of lpi1 and lpi2 seedlings was similar to that of wild type on −K, −N, −S, and −Fe medium.

lpi Mutants Are Not P Hyperaccumulators

To determine whether the lpi phenotype is due to a higher P uptake or accumulation capacity of these mutants, the P content in the shoot and root of 18-d-old wild-type, lpi1, and lpi2 plants subjected to high and

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<tr>
<th>Cross</th>
<th>Phenotype of Progeny F1</th>
<th>Phenotype of Progeny F2</th>
<th>Ratio Obtained</th>
<th>Ratio Tested</th>
<th>χ²</th>
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<tr>
<td></td>
<td>Short Root (WT)</td>
<td>Long Root (Mutant)</td>
<td>Short Root (WT)</td>
<td>Long Root (Mutant)</td>
<td>WT:Mutant</td>
</tr>
<tr>
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<td>0</td>
<td>44</td>
<td>17</td>
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<tr>
<td>lpi2 × WT</td>
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<td>13</td>
<td>60</td>
<td>0.71:3.29</td>
</tr>
<tr>
<td>lpi3 × WT</td>
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<td>50</td>
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<td>2.86:1.14</td>
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<tr>
<td>lpi4 × WT</td>
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<td>0</td>
<td>86</td>
<td>20</td>
<td>3.24:0.76</td>
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*With one degree of freedom and a critical value of 5%, the hypothesis is accepted if the χ² is smaller than 3.841.
low P treatments was determined (Table II). As expected, the shoot and root P content of wild-type and lpi mutant plants was higher in high P media than in low P media. In high P media, the shoot P content of lpi1 and lpi2 mutants was 17% and 15% lower than the wild type, respectively, whereas a slight (5%) but statistically significant reduction in root P content was observed in lpi1 but not in lpi2. In low P media, the shoot P content of the lpi mutants was similar to that of the wild-type controls, but the root P content was reduced by 21% and 24% in lpi1 and lpi2, respectively. These results show that the long primary root phenotype in lpi mutants is not due to a general or local increase in P content in the root of these mutants with respect to the wild type.

**lpi Mutants Have Normal Root Cell Elongation and Cell Division in Low P Conditions**

The primary root growth reduction observed in wild-type seedlings growing under P starvation has been related to both a reduction in root cell elongation and cell division in the root meristem (Williamson et al., 2001; Ma et al., 2003; Ticconi et al., 2004; Sánchez-Calderón et al., 2005). To analyze the cellular responses of lpi mutants to contrasting P availability, wild-type, lpi1, and lpi2 seedlings were grown in low and high P media for 14 d and cell parameters measured in several regions of the primary root. As shown in Figure 3A, the root epidermal cells of wild-type seedlings grown in low P were 80% shorter than in high P medium. In lpi1 and lpi2, no statistically significant reduction in epidermal cell length was observed in low P media when compared to those grown under optimal conditions (Fig. 3A). However, epidermal cells were 15% and 30% shorter in lpi1 and lpi2, respectively, than the wild type under high P conditions.

We have previously reported that as a consequence of the determinate root developmental program induced by P deprivation, the number of cells in the elongation and meristematic regions is reduced due to premature cell differentiation in the elongation zone and the differentiation of cells present in the exhausted meristem (Sánchez-Calderón et al., 2005). To determine whether this low P-induced differentiation process also occurs in lpi1 and lpi2, cell counts in the elongation and meristematic regions of wild-type and lpi seedlings grown in high and low P conditions were carried out. It was observed that in low P media, the number of cells in the elongation and meristematic regions of wild-type roots decreased over 90% when compared with the same regions in high P plants. Interestingly, no significant differences were found in the number of cells in the cell elongation and meristematic zones of lpi1 and lpi2 in high and low P conditions (Fig. 3, B and C).

Reduction in the number of cells in the meristematic and elongation regions correlated with cell differentiation processes. For instance, in the roots of wild-type plants grown in low P, root hair formation was observed in the former meristematic zone (Fig. 4B, arrow), whereas in high P root hair formation took place 2 mm from the root tip. No root hair formation was observed near the root tip of lpi1 and lpi2 mutants when grown in low P media (Fig. 4, D and F).

To evaluate the contribution of cell division to the lpi phenotype, we crossed lpi1 and lpi2 with a transgenic line that expresses the Cyclin B1 promoter fused to the β-glucuronidase (GUS) reporter gene (CycB1;1:uidA).

<table>
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<tr>
<th>Lines</th>
<th>High P</th>
<th>Low P</th>
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<tr>
<td></td>
<td>Shoot</td>
<td>Root</td>
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<tr>
<td>Wild type</td>
<td>1.45</td>
<td>1.62</td>
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<tr>
<td>lpi1</td>
<td>1.26</td>
<td>1.46</td>
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<tr>
<td>lpi2</td>
<td>1.23</td>
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This marker is expressed only in dividing cells at the G2/M transition and is a good marker for proliferative activity (Colón-Carmona et al., 1999). In wild-type seedlings grown in high P media, a patchy pattern of single cells expressing \( \text{CycB1;1:uidA} \) was observed in the primary root meristems (Fig. 4A). Under low P conditions, GUS expression in the primary root tip decreased with time until it became undetectable in 14-d-old seedlings (Fig. 4B), suggesting that primary root meristems entered a determinate developmental program as previously reported (Sánchez-Calderón et al., 2005).

Interestingly, \( \text{CycB1;1:uidA} \) expression in the primary root apex of \( \text{lpi1} \) and \( \text{lpi2} \) seedlings was very similar in high and low P media (Fig. 4B). Furthermore, the primary root tip of \( \text{lpi} \) mutants grown in low P was anatomically very similar, including the presence of a well-defined quiescent center, as compared to that of the wild type grown in high P (Fig. 4, C–F). These results show that \( \text{lpi1} \) and \( \text{lpi2} \) maintain an indeterminate root development program under low P conditions.

**lpi Mutants Are Only Partially Affected in Low P Responses**

Among the most conspicuous responses of Arabidopsis to P deprivation are an increase in number and length of root hairs (Bates and Lynch, 1996), enhanced anthocyanin accumulation, and an increase in root/shoot ratio (Raghothama, 1999). To assess whether \( \text{lpi} \) mutants are affected in these typical responses to P deprivation, wild-type, \( \text{lpi1} \), and \( \text{lpi2} \) Arabidopsis seedlings were grown in high and low P conditions, and the length and density of root hairs, anthocyanin content, and root/shoot ratio were determined.

It was determined that root hairs of \( \text{lpi1} \) and \( \text{lpi2} \) were longer than those of the wild type in both high and low P conditions (Fig. 5A). Although root hairs of wild-type, \( \text{lpi1} \), and \( \text{lpi2} \) seedlings were longer in low than in high P media, the stimulation of root hair length by P deprivation was reduced in the two mutants. The root hair length in the wild type under low P conditions was on average 240% longer than those of the respective wild-type and mutant seedlings in high P media.
Figure 5. The effect of P availability on several rescue system responses. Wild-type and lpi seedlings were grown for 12 (A and B) and 18 (C and D) d on the surface of agar plates containing high or low P, and several rescue system responses were evaluated. Mean root hair length (A), root hair density indicating the number of root hairs per cm in the primary root (B), anthocyanin content (C), and root/shoot ratio (D) are shown. Values shown are the means ± SE of 20 seedlings (A and B), three repetitions (C), and five groups of 40 seedlings (D). Letters represent statistically different (P < 0.05) means.

under high P conditions, whereas that of lpi1 and lpi2 was 180% and 150%, respectively (Fig. 5A). In wild-type plants, root hair density was increased by 80% in low P conditions, whereas in lpi1 and lpi2 the increase was of 18% and 20%, respectively (Fig. 5B). No statistically significant differences in anthocyanin content were observed among mutant genotypes (C), and root/shoot ratio (D) are shown. Values shown are the means ± SE of 20 seedlings (A and B), three repetitions (C), and five groups of 40 seedlings (D). Letters represent statistically different (P < 0.05) means.

lpi Mutants Have Reduced Pi Starvation-Inducible Gene Expression

The indeterminate primary root growth of lpi seedlings in low P conditions suggests that lpi genes may act in a P-specific nutrient-sensing pathway that modulates mitotic activity and meristem maintenance in the root. To study whether the genetic lesions of lpi1 and lpi2 have an effect on the expression of genes induced by P deprivation, we analyzed the expression of the high-affinity P transporter genes under low P conditions in both wild-type and lpi seedlings. With this aim, we introduced gene constructs containing the promoters of the high-affinity P transporters AtPT1 and AtPT2 fused to the GUS reporter gene (pAtPT1:uidA and pAtPT2:uidA) into lpi1 and lpi2. These reporter gene constructs have been used to study the effect of low P on the expression of AtPT1 and AtPT2 (Karthikeyan et al., 2002). As previously reported for the wild-type seedlings grown in high P medium, pAtPT1:uidA had a low but detectable level of expression over the entire root system except for the nondifferentiated region of the root tip where GUS activity was undetectable (Fig. 6, A and B), whereas no pAtPT2:uidA expression was detected (Fig. 6, C and D). Under low P conditions, GUS expression in wild-type seedlings drastically increased for the two markers in the whole root system, but more markedly in the root tip of primary roots with exhausted meristem, including high levels of expression in root hairs (Fig. 6, E–H). Under high P conditions, the expression of pAtPT1:uidA and pAtPT2:uidA in the lpi1 and 2 genetic backgrounds was undistinguishable from that observed in the wild type (Fig. 6, I–L and Q–T). Although the expression of pAtPT1:uidA in lpi1 and 2 was induced in low P medium in most of the root, in contrast to that observed in the wild type, no GUS staining was detected in the primary root tips (Fig. 6, M, N, U, and V). In the case of pAtPT2:uidA, lower levels of induction were observed in lpi1 and lpi2 P-deprived seedlings as compared to the wild-type controls, and no expression was detected in their primary root tips (Fig. 6, O, P, W, and X).

To more closely investigate the role of the LPI genes on P starvation-inducible expression, we examined the effect of P deprivation on the steady-state mRNA level of Arabidopsis thaliana 4 (At4), Arabidopsis thaliana PURPLE ACID PHOSPHATASE 1 (AtPAP1), Arabidopsis thaliana ACID PHOSPHATASE 5 (AtACP5), AtPT1, Arabidopsis thaliana INDUCED BY PHOSPHATE STARvation 1 (AtIPS1), and AtPT2, all belonging to the P-responsive gene group (Muchhal et al., 1996; Burleigh and Harrison, 1999; del Pozo et al., 1999; Martin et al., 2000; Li et al., 2002). Northern-blot analysis of At4, AtPAP1, AtACP5, AtPT1, AtIPS1, and AtPT2 using total RNA extracted from wild type, lpi1, lpi2, and phr1,
a mutant affected in the induction of P-responsive genes, grown in high or low P media was carried out and the relative signal respect to tubulin mRNA, a P-nonresponsive gene, determined. As previously reported, the steady-state transcript levels of all the tested genes increased in wild-type plants when grown under P-limiting conditions, particularly in RNA extracted from roots (Fig. 7, A and B). This increase in the steady-state transcript levels was also observed in lpi1 and lpi2 mutants, however at a reduced level than that observed in wild-type plants. The induction ratio of AtPAP1, AtPT1, AtACP5, and AtPT2 transcripts in the root of lpi1 and 2 seedlings grown in low P media was reduced by 20% to 80% (Fig. 7, A and B). This reduction in the transcript levels upon P deprivation is similar to that observed for the phr1 mutant. The most drastic changes in the P-induced transcript levels were observed in lpi2 roots where no significant induction of AtIPS1 expression was detected. Interestingly, a strong At4 induction 2-fold higher than in the wild type was reproducing observed in lpi2 (Fig. 7). In shoots, no increase in AtPT1 transcripts was detected in phr1, lpi1, and lpi2, and the induction ratio of AtPAP1 and AtPT2 transcripts was reduced between 40% and 80%. In contrast, the AtACP5 transcript was found to be 6- and 3-fold higher in phr1 and lpi1 than in the wild type. lpi2 did not show At4 and AtIPS1 transcript induction. These results suggest that lpi mutations not only disrupt the root developmental response but also alter the response to P deprivation of several genes related to the adaptation of Arabidopsis plants to P deficiency.

DISCUSSION

Alterations in root system architecture are extremely important in plant productivity since they often determine the ability of plants to more efficiently explore and exploit the spatially heterogeneous soil environment. Because of its low mobility in the soil solution, P is one of the most limiting nutrients for plant growth and crop productivity. P availability is greater in the upper layers of soil, thus plant species may enhance P acquisition through increased root branching and root hair proliferation (Lynch, 1995). Although nutrients have been recognized as environmental signals that modulate root development, the mechanisms by which plants perceive nutrient availability and translate this signal(s) into particular developmental responses remain to be elucidated.

To dissect the physiological and genetic mechanisms of P sensing in angiosperms, several research groups have used Arabidopsis as a model system to study the root system developmental responses to low P availability. The most conspicuous root architectural alterations induced by P deprivation in Arabidopsis include an early arrest of primary root growth, increased growth and density of root hairs, and prolific growth of lateral roots (Bates and Lynch, 1996; Williamson et al., 2001; López-Bucio et al., 2002). Such adjustment of root system architecture by P deprivation has been suggested to occur via changes in meristem activity and maintenance (Sánchez-Calderón et al., 2005). This notion is supported by the finding that the pdr2 Arabidopsis mutant, originally isolated as a mutant incapable of utilizing RNA as a source of P (Chen et al., 2000), displays hypersensitive responses to P limitation, including greater primary root growth inhibition and early arrest of cell division in the root meristem (Ticconi et al., 2004).

LPI1 and LPI2 Are Required for the Root Architectural Changes Induced by Low P Conditions

Recent studies have shown that P deficiency induces a determinate growth program that includes early cellular differentiation, gradual reduction of cell elongation, loss of the root elongation zone, and meristematic activity (Sánchez-Calderón et al., 2005). Here we report on the isolation of lpi mutants that were

Figure 6. AtPT1:uidA and AtPT2:uidA expression in transgenic wild-type and lpi seedlings. Overnight GUS staining of AtPT1:uidA and AtPT2:uidA root system and its primary root tips in wild-type, lpi1, and lpi2 seedlings grown for 12 d in medium with high (A–D, I–L, and Q–T) or low (E–H, M–P, and U–X) P content. Photographs are representative individuals of at least 20 plants stained.
identified because of their indeterminate primary root growth under low P conditions, which leads to a long root phenotype under these experimental conditions (Fig. 1B). The finding that the genetic lesions affecting lpi1 and lpi2 affect the root architecture response only to P and not to low availability to other nutrients, such as S and Fe that are known to alter root architecture in Arabidopsis, suggests that nutrient sensing is mediated by specific signaling pathways (Fig. 2).

Further detailed cellular analysis of wild-type, lpi1, and lpi2 plants showed that lpi lines show neither a drastic reduction in cell length in the primary root nor decreased cell number in the root elongation zone (Fig. 3, A and B). Moreover, mutant plants sustained normal root meristematic activity as revealed by cell counts and CycB1;1:uidA expression in the meristematic region (Figs. 3C and 4). Interestingly, the typical increase in lateral root number under low P condition was observed in lpi1 mutants but not in lpi2 (Fig. 2B). Taking into account these observations, we conclude that LPI2 regulates both primary root growth and lateral root formation, whereas LPI1 plays an important role only in primary root growth but not in lateral root induction by P deprivation. Several types of experimental evidence suggested that conditions that reduce primary root meristematic activity, including destruction of meristematic activity by cell ablation or physical decapitation of the primary root meristem, elicit an increase in lateral root number (Torrey, 1950; Dubrovsky, 1997; Tsugeki and Fedoroff, 1999). Therefore, it has been suggested that the increase in lateral root number under P deprivation conditions could be a consequence of a reduced meristematic activity (Sánchez-Calderón et al., 2005). However, the finding that lpi1 is still able to respond in terms of lateral root formation to low P, although it sustains normal primary root growth, provides genetic evidence that lateral root formation is not a direct consequence of the observed meristem exhaustion in the low P response and could be mediated by an independent alteration of auxin sensitivity of pericycle cells during P deprivation (López-Bucio et al., 2005).
**LPI1 and LPI2 Have an Important Role in the Low P-Induced Root Growth Determinate Program**

The root architecture of higher plants is determined to a larger extent by the activities of the root meristems, in which most postembryonic cell division takes place. The primary root meristem contributes new cells to the primary root for continuous growth (Schiefelbein and Benfey, 1991). This indeterminate root growth program is changed to a determinate one under P deprivation conditions (Sánchez-Calderón et al., 2005). The isolation of lpi mutants provides strong evidence that root meristem exhaustion under low P availability is not a direct consequence of the lack of P, but rather is under control of a genetic program that controls root growth. The mechanisms by which P starvation regulates meristem activity remain unclear; nevertheless, the finding that lpi mutants do not enter a determinate root growth program under low P conditions suggests that LPI1 and 2 are negative regulators of meristem maintenance under this stress condition. Moreover, the finding that lpi mutants do not hyperaccumulate P (Table II) suggests that meristem maintenance under low P conditions is not regulated by the shoot P status but rather by local signals derived from the concentration of this nutrient in the root or by the sensing of P level in the rhizosphere as previously proposed by Ticconi et al. (2004).

The phenotype of lpi mutants under low P conditions suggests that LPI1 and 2 are important components in the signaling pathway by which P deprivation triggers meristem exhaustion. Several genes required for meristem function and maintenance have been identified, including the putative AP2-class transcription factors PLETHORA (PLT1 and PLT2; Aida et al., 2004); the transcription factors of the GRAS family, SHORT-ROOT and SCARECROW (SCR); and AtCLE19, a member of the CLV3/ESR-related family (Casamitjana-Martínez et al., 2003). Our results open the possibility that the transcriptional or posttranscriptional regulation of PLT1, PLT2, SHORT-ROOT, SCARECROW, and/or AtCLE19 could be negatively regulated by LPI1 and LPI2 depending upon P availability. In particular, it is tempting to speculate that P availability could regulate the expression or activity of PLT1 and PLT2 because the root architecture of the double plt1/plt2 mutant, short primary root with an abundance of lateral roots, is quite similar to what it is observed for the wild type under P deprivation (Aida et al., 2004).

**LPI Genes Affect Only a Subset of P Deprivation Responses**

The low P rescue system includes morphological, physiological, biochemical, and molecular adaptive alterations, such as anthocyanin pigment accumulation, secretion of phosphatases and organic acids into the rhizosphere, and the increased expression of several genes (Raghothama, 1999). lpi mutants were affected not only in the morphological alterations in the root system, but also in the shoot/root ratio induced by low P, indicating that LPI genes participate in the differential distribution of photosynthates between shoots and roots that promotes the proliferation of the Arabidopsis root system under low P conditions. Nevertheless, our results show that several P deprivation responses are not affected in lpi1 and lpi2, such as anthocyanin accumulation and the stimulation of root hair elongation (Fig. 5), suggesting that low P-inducible responses in Arabidopsis are regulated by different signaling pathways, some of which are independent of LPI1 and 2.

In terms of P-regulated gene expression, it was found that lpi mutants were less responsive to low P in terms of the level of transcript accumulation of AtPAP1, AtACP5, AtPT1, AtIPS1, and AtPT2. The finding that mutants affected in root architecture responses are also altered in the regulation of P-responsive genes reveals a potential crosstalk between low P-induced determinate root development and the activation of genes involved in the adaptation of Arabidopsis to P deficiency.

**PHR1** encodes a MYB transcription factor required for the induction of P starvation-regulated genes and metabolic responses such as anthocyanin accumulation (Rubio et al., 2001). The observed changes in the P responsiveness of AtACP5, AtPT1, and AtIPS1 in lpi mutants were to some extent similar that those previously reported for phr1, an Arabidopsis mutant altered in P starvation gene regulation (Rubio et al., 2001), suggesting the possibility that LPI genes could alter the expression or function of PHR1. However, since lpi mutants are not impaired in anthocyanin accumulation and phr1 mutants are not affected the root system architecture changes when subjected to P deprivation, it is also possible that the LPI genes function in a different pathway than PHR1.

Isolation of genes affected in lpi mutants will provide further insights in the signaling pathways that regulate changes in root system architecture in response to low P availability.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

Arabidopsis (Arabidopsis thaliana) Col-0 was used for all experiments. Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min. After five washes in sterile distilled water, seeds were germinated and grown on agar plates containing 0.1 × Murashige and Skoog medium, pH 5.7, 0.5% (w/v) Suc, and 1% (w/v) agar (López-Bucio et al., 2002). The basic medium contained 2.0 mM NH4NO3, 1.9 mM KNO3, 0.3 mM CaCl2, 2H2O, 0.15 mM MgSO4, 7H2O, 5 mM KI, 25 mM H2O2, 0.1 mM MnSO4, 0.3 mM ZnSO4, 7H2O, 1 mM Na2MoO4·2H2O, 0.1 mM CuSO4·5H2O, 0.1 mM CoCl2·6H2O, 0.1 mM FeSO4·7H2O, 0.1 mM Na2EDTA·2H2O, inositol (10 mg L−1), and Glc (0.2 mg L−1). To make the Fe-free medium, FeSO4 and Na2EDTA were replaced by Na2SO4 in the nutrient solution. The N-free medium was prepared omitting NH4NO3 and KNO3 of the nutrient solution, and supplying the K source replaced by KCl. To make the K-free medium, KI was replaced by NaI. The Fe-free medium, Na2SO4, and Na2EDTA were replaced by their respective chloride salts, and FeSO4 and Na2EDTA were replaced by FeEDTA. The K-free medium was made by substituting KI by NaI and omitting KNO3.
Plates were placed at an angle of 65° to allow root growth along the agar surface and to allow unimpeeded hypocotyl growth into the air. Plants were grown at 22°C to 24°C in a plant growth cabinet (Percival Scientific), with a photoperiod of 16 h of light, 8 h of darkness, with a light intensity of 300 μmol m⁻² sec⁻¹. Seeds of transgenic CpcB1:uidA (Col-0; Carmona et al., 1999), AiptT1:uidA, and AiptT2:uidA (Karthikeyan et al., 2002) Arabidopsis plants were provided by Dr. Peter Doerner and Dr. Kaschandra Raghothama. Homozygous F₂ progeny of crosses with lpi₁ and lpi₂ were used for analysis of GUS expression.

Mutant Isolation Procedure

EMS-mutagenized seeds (Col-0) were purchased from Lehle Seeds. Seeds were surface sterilized and plated on low P (1 μM NaH₂PO₄) 0.1 × Murashige and Skoog medium. A total of approximately 25,000 M₀ seedlings descended from EMS-mutagenized seed were screened for long primary roots by placing seeds on low P nutrient agar plates. The seeds were distributed into two to three rows on the agar surface at a density of 1 seed/cm², stratified at 4°C for 48 h, and then incubated at 22°C. Fourteen days after germination, low P-grown plants have a short primary root and a large number of lateral roots formed close to the root apex. Putative mutants with long primary roots were selected, transferred to soil, and allowed to self fertilize. Homozygous M₁ seeds were screened for long primary roots in low P and backcrossed four times to wild type to remove unlinked mutations.

Histological Analysis

Wild-type and mutant plants were cleared using the method described by Malamy and Benrey (1997). All samples were observed using Nomarski optics on a Leica DMR microscope. Photographs were taken using a Leica DC180 digital camera and Leica IM50 4.6 software. To measure and count cells, images were taken from different root regions and processed with a Scion Image Software (Scion Corporation; www.scioncorp.com). For histochemical analysis of GUS activity, Arabidopsis seedlings were incubated overnight at 37°C in a GUS reaction buffer (0.5 mM MgCl₂, 5 mM b-mercaptoethanol, 100 μM sodium phosphate, pH 7.0), and the stained seedlings were cleared. For each marker and for each treatment, at least 10 transgenic plants were analyzed. A representative plant was chosen for each treatment and photographed using the Nomarski optics on a Leica DMR microscope.

Northern Analysis

Total RNA was extracted from roots and shoots using the TRIZOL Reagent (Invitrogen). Ten micrograms of total RNA were electrophoretically separated on a 1% (w/v) denaturing formaldehyde agarose gel and blotted onto Hybond-N+ (Amersham Biosciences). Both labeled probes for AipsP5 (Del Pozo et al., 1999), Aht (Burleigh and Harrison, 1999), AiptT1 and AiptT2 (Machut et al., 1996), AipsP1 (Martin et al., 2000), and AtIPAP1 (Li et al., 2002) were used to obtain a DNA-labeling kit (Ambion). RNA hybridization analysis was carried out as suggested by Sambrook et al. (1989).

P and Anthocyanin Determinations

Anthocyanin content was measured in 500 mg of 18-d-old seedlings grown on low and high P medium from fresh tissue of wild type and lpi mutants, as described previously (Giusti and Wrolstad, 2001). Wild-type and lpi shoots and roots of 18-d-old seedlings grown in high and low P treatments were collected, weighed, and dried at 70°C for 24 h. The total P content of 90 mg of dry tissue was evaluated by the vanadate-molybdate colorimetric method (Hesse, 1971).

Data Analysis

Arabidopsis root systems were viewed with an AFX-II-A stereomicroscope (Nikon). All lateral roots emerging from the primary one and observed under the x 8 objective were included in the lateral root number data. Primary root length was determined for each root using a ruler. For all experiments, the overall data was statistically analyzed in the SPSS 10 program (SPSS). Univariate and Multivariate analyses with a Tukey’s or Duncan Post Hoc test were used for testing differences in each variable in P treatments of wild-type and lpi seedlings. Different letters are used to indicate means that differ significantly (P < 0.05).

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LITERATURE CITED


