The Function of LPR1 is Controlled by an Element in the Promoter and is Independent of SUMO E3 Ligase SIZ1 in Response to Low Pi Stress in Arabidopsis thaliana

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In Arabidopsis thaliana, there exist many typical responses to low phosphate (LP) stress, such as inhibition of primary root elongation, proliferation of lateral roots and accumulation of anthocyanin in leaves. The physiological, genetic and molecular mechanisms of these developmental responses remain undefined. We have isolated a phosphorus starvation-insensitive (psi) mutant. The mutant shows impaired inhibition of primary root growth, reduction of root hair growth and reduction of anthocyanin accumulation compared with the wild-type (WT) plants under an LP level. CycB1;1::GUS (cyclin B1;1::β-glucuronidase) staining suggests that the mutant has a higher ability to maintain cell elongation and cell division than the WT. The genetic analysis and gene cloning indicate that psi is a new allele of lpr1 and that an AC-repeat element in the promoter plays important roles in controlling the expression of LPR1. The psi mutant also shows less sensitivity to auxin treatment compared with the WT and the mutant has an enhanced higher ability to maintain the auxin response in the root tip under LP. However, enhancing the auxin response in the quiescent center cannot mimic the mutant phenotype. These observations suggest that LPR1 is involved in the regulation of the auxin response to Pi starvation and auxin is probably not the only factor affected for maintaining the long-root phenotype under LP stress. Our results also indicate that the function of LPR1 is probably independent of SUMO E3 ligase SIZ1 in response to Pi starvation. The insensitive response of the psi mutant to brefeldin A suggests that LPR1 and PDR2 (Pi Deficiency Response 2) function in opposite ways in regulating the root growth response to Pi starvation in the endoplasmic reticulum.

Keywords: Arabidopsis thaliana • LPR1 • Pi signaling • Root system architecture • SUMO E3 ligase SIZ1.

Abbreviations: BFA, brefeldin A; DAG, days after germination; EMS, ethyl methane sulfonate; ER, endoplasmic reticulum; GUS, β-glucuronidase; HP, high phosphate; IAM, indole-3-acetamide; LP, low phosphate; MCO, multicopper oxidase; NPA, N-1-naphthylphthalamic acid; PRL, primary root length; qRT–PCR, quantitative reverse transcription–PCR; RSA, root system architecture; WT, wild type.

Introduction

Phosphate (Pi) is an essential plant nutrient that plays important roles in plant growth and development. Because the bioavailability of Pi is extremely low due to mineralization and fixation processes in alkaline and acid soils (Bar-Yosef 1991), plants have developed a wide range of adaptive strategies to compensate for Pi deficiency and improve Pi mobilization and uptake efficiency from the soil (Raghothama 1999). Among these strategies, alteration in root architecture is an important mechanism that enables plants to explore and exploit the insoluble phosphorus in soils more efficiently. In Arabidopsis, these alterations are reflected in the reduction of primary root elongation, proliferation of lateral roots, enhancement of root hairs developing toward the root apical meristem, and accumulation of anthocyanin in the leaves (Williamson et al. 2001, Linkohr et al. 2002). The changes in root architecture induced by Pi starvation are the result of changes in meristem activity and cell elongation (Sánchez-Calderón et al. 2005, Reymond et al. 2006). A deeper understanding of the physiological, genetic and molecular mechanisms of the regulatory system, however, is still lacking.

In addition to the complexity of the changes in root development induced by Pi starvation, the signaling pathways triggering these modifications remain mostly obscure.
Increasing evidence suggests that hormones play a critical role in modulating the root system architecture (RSA) under Pi starvation. The distal auxin maximum correlates with root pattern formation and extension of cell division in the root meristem (Sabatini et al. 1999). Furthermore, the auxin distribution also regulates cell expansion in the primary root in Arabidopsis (Billou et al. 2005). The application of low concentrations of exogenous auxin could increase the density of lateral roots and root hairs. These observations indicate that auxin sensitivity may be involved in the root architecture in response to Pi starvation (López-Bucio et al. 2002, Pérez-Torres et al. 2008). It has been suggested that Pi deficiency leads to alteration of the local auxin concentration within the root system through changes in auxin transport (Nacry et al. 2005). Conversely, it has also been reported that primary root growth arrest in low Pi (LP) stress conditions is independent of auxin, while the stimulation of lateral root primordium emergence requires auxin transport, and the shoot Pi status influences the RSA (Williamson et al. 2001, López-Bucio et al. 2005).

Several genes have been identified as components of the Pi starvation signaling pathways in Arabidopsis. AtPHR1 encodes a MYB-like transcription factor with a homolog to PHOSPHORUS STARVATION RESPONSE 1 (PSR1) from Chlamydomonas reinhardtii (Wykoff et al. 1999) and regulates a subset of Pi starvation responses (Rubio et al. 2001). A small ubiquitin-like modifier (SUMO) E3 ligase (AtSIZ1) targets AtPHR1 (Miura et al. 2005). Loss of function of AtSIZ1 causes Arabidopsis plants to exhibit exaggerated prototypical Pi starvation responses, including cessation of primary root growth, extensive lateral root and root hair development, increase in root/shoot mass ratio and elevated anthocyanin accumulation in leaves. However, the phr1 mutant does not exhibit these RSA changes. These data suggest that the root architectural changes in response to Pi signaling are controlled by complicated systems and AtSIZ1 is one of the multiple target genes involved in Pi signaling pathways.

The Arabidopsis pho2 mutant overaccumulates Pi to toxic levels in the shoot (Dong et al. 1998). Recently, it was reported that PHO2 which encodes an unusual E2 conjugase gene, which was subsequently identified as UBC24, is modulated by the Pi-dependent miR399 (Bari et al. 2006, Aung et al. 2006). This research presents a working model for the mechanism of Pi sensing in higher plants. In this model, PHR1 plays a central role and the miR399–PHO2 pathway regulates the expression of a subset of the Pi starvation-induced genes (Bari et al. 2006).

Sánchez-Calderó et al. (2006) have isolated several low phosphorus-insensitive (lpi) Arabidopsis mutants. The lpi mutants do not show the typical determinate developmental program induced by Pi stress in the primary root, while other root developmental aspects of the LP rescue system, including increased root hair elongation and anthocyanin accumulation, remained unaltered in lpi mutants. Some Pi starvation-inducible genes were repressed in lpi mutants. These Pi starvation-inducible genes were also down-regulated in the previously reported phr1 mutant (Rubio et al. 2001). Since lpi mutants are not impaired in anthocyanin accumulation and phr1 mutants are not affected by the RSA when subjected to Pi deprivation, it is proposed that the LPI genes function in a pathway different from PHR1.

Several genetic factors for root architectural change in response to LP stress have been reported (Reymond et al. 2006). Three quantitative trait loci (QTLs), LPR1, LPR2 and LPR3, were detected on chromosomes 1, 3 and 4, from a recombinant inbred line population derived from a cross between a Pi starvation-insensitive accession Bay-0 (Bayreuth, CS6608) and a Pi starvation-sensitive accession Sha (Shahdara, CS929). The genetic analysis indicates that LPR1 is a dominant locus and LPR2 is epistatic to LPR1. By using Bay-0 and Sha near isogenic lines (NILs) homologous for the Bay-0 allele and Sha allele, respectively, Reymond et al. (2006) found that LPR1 controls the cell length of primary root and root hair density. Their finding supports the earlier reports that root architectural change in response to LP stress is mediated by different physiological strategies for the different root components. Subsequently, Svisofoff et al. (2007) reported that LPR1 and LPR2 encode multicopper oxidases (MCOs). Physical contact of the primary root tip with LP medium is necessary and sufficient to arrest root growth. Loss of function of LPR1 and its close paralog LPR2 greatly reduces this inhibition, highlighting the essential role of MCOs for plant development.

Pi Deficiency Response 2 (PDR2) has been proposed to function at a Pi-sensitive checkpoint in root development. The pdr2 mutant displays hypersensitive responses to LP stress, showing shorter primary root and higher density of lateral root meristems under the stress (Ticconi et al. 2004). Most recently, it was reported that PDR2 encodes the P5-type ATPase, which is required for maintaining the steady-state level of SCR protein under LP stress. Furthermore, the LPR1 enzyme interacts genetically with the P5-type ATPase PDR2 in the endoplasmic reticulum (ER). PDR2 and LPR1 are presumptive components of an ER-resident pathway mediating root growth responses to external Pi (Ticconi et al. 2009).

In this work, we isolated a mutant from a Columbia (Col-0) accession mutant library mutagenized by ethyl methane sulfonate (EMS). The mutant showed Pi starvation insensitivity in terms of cell elongation and cell division activity of the primary root, root hair density and accumulation of anthocyanin in leaves. Therefore, it is designated as psi (Pi starvation insensitive). The decrease in auxin response in the root tip is impaired in the mutant compared with the wild type (WT), but the higher auxin concentration in the root tip of the WT is not sufficient to restore the primary root growth under LP stress. Genetic analysis revealed that a semi-dominant gene controls the long-root phenotype of the psi mutant under LP stress. The map-based cloning result showed that psi is a new allele to lpr1. An AC-repeat sequence in the promoter of LPR1 is a key element in controlling the function of this gene. Our results also indicate that LPR1 probably functions independently of SUMO E3 ligase SIZ1 and functions in an
opposite manner to PDR2 in regulating root growth response to Pi starvation in the ER.

Results

Isolation and characterization of the Pi starvation-insensitive mutant

EMS-generated M2 seeds (Col-0) were screened according to the root architecture of plants grown on the surface of Johnson agar plates with an LP level (15 µM Pi). One mutant was isolated which, in contrast to the WT, was able to sustain primary root length (PRL) under the LP level compared with that under the high Pi (HP, 1,000 µM Pi) level. No significant difference in PRL was observed between the WT and the mutant under nitrogen- (N), sulfur- (S) or iron- (Fe) deficient conditions, indicating that the maintenance of PRL in the mutant is specific to Pi deficiency (Fig. 1A). The PRL of WT seedlings was reduced by 73% under the LP level after growing for 8 days after germination (DAG), while the mutant was only reduced by approximately 23% (Figs. 1A, 2A). Under LP levels, the number and length of root hairs were reduced in the mutant compared with WT plants (Figs. 1B–E, 2B). A similar number of lateral roots was observed in the WT and mutant under LP levels (Fig. 2C). Under growth room conditions, the aerial part of the mutant plants was normal, presenting similar vegetative development, fruit development and seed production to the WT (data not shown). In Arabidopsis, one of the most conspicuous symptoms of LP stress is the accumulation of anthocyanin in leaves (Raghothama 1999). A remarkable accumulation of anthocyanin in the leaf tissue of the WT was observed under LP conditions, while in the mutant, the anthocyanin content was only slightly elevated (Fig. 2D). Taking these results together, we designated the mutant as Pi starvation insensitive (psi).

To determine whether the maintenance of PRL of the mutant at LP levels is caused by longer cell length and/or higher cell division rate, we measured the cell length in the mature zone adjacent to the elongation zone each day after germination, up to 8 DAG at HP and LP levels. The length of epidermal cells of the WT and the mutant was relatively constant under HP conditions. Under LP conditions, the epidermal cell length of the WT was dramatically reduced at 3 DAG, while the mutant showed only a slight reduction in cell length (Fig. 2E).

To investigate the cell division further, we crossed the mutant with a transgenic plant harboring a β-glucuronidase (GUS) reporter gene under the control of the cyclin B1;1 promoter, which allows the GUS staining to be examined in the dividing cells at the G2/M transition of the cell cycle (Colón-Carmona et al. 1999). The GUS staining was investigated in the primary root tips of WT and psi mutant plants under different Pi concentrations. The psi mutant showed a higher ability to sustain cell division in the root meristem than the WT at LP levels up to 100 µM Pi (Fig. 3A). Vertical sections of

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**Fig. 1** Phenotypic characterization of the psi mutant. (A) 8 DAG seedlings of the psi mutant and the WT on growth medium with a high Pi level (1,000 µM Pi) (HP) and a low Pi level (15 µM Pi) (LP), and deficiency of nitrogen (N), sulfate (S) and iron (Fe). Left, WT; right, psi. (B) Root tip of the WT on medium with HP. (C) Root tip of the psi mutant on medium with HP. (D) Root tip of the WT on medium with LP. (E) Root tip of the psi mutant on medium with LP. Scale bar = 1 cm (A), 1 mm (B–E).
the root meristem showed that a distortion of the root meristem region in the WT occurred at LP levels, while the \( \psi^i \) mutant sustained a relatively normal meristem (Fig. 3C). These results indicate that under LP stress, the mutant has a higher ability to maintain cell elongation and cell division than WT plants.

Given that the primary root of \( \psi^i \) is longer than that of the WT under LP stress, we predicted that \( \psi^i \) might have an increased ability to maintain cell elongation and cell division than WT plants. In summary, we isolated a Pi starvation-insensitive mutant, \( \psi^i \), which has a higher ability to maintain cell elongation, cell division and phosphate uptake than the WT under LP stress.

**Genetic characterization and cloning of \( \psi^i \)**

To determine the genetic basis of the mutant phenotype, the mutant was backcrossed to the WT (Col-0) that showed a short-root phenotype under LP conditions (Chevalier et al. 2003). At LP levels, the \( F_1 \) progeny from the backcross showed a PRL which was an average of that of the parents. The segregation of PRL among the backcross \( F_2 \) population (200 \( F_2 \) plants) showed a ratio of 1:2:1 of WT phenotype: intermediate phenotype: mutant phenotype (\( \chi^2 = 0.86 \)), which indicates that the long-root phenotype of the \( \psi^i \) mutant is controlled by a single semi-dominant Mendelian factor.

To determine the mutation locus, primary mapping was conducted using 100 long-root \( F_2 \) lines (PRL > 4 m at LP levels) selected from the \( F_2 \) population derived from the cross between

![Fig. 2 Root growth characterization and anthocyanin concentration of 8 DAG seedlings of the \( \psi^i \) mutant (black bars) and the WT (white bars) under HP and LP levels. (A) PRL; (B) root hair density (number of hairs in a 1 mm segment of the root from first root hair); (C) lateral root number; (D) anthocyanin content; (E) cell length of the root elongation zone within 8 DAG. The error bar represents the SD (anthocyanin content, \( n = 6 \) pools of approximately 20 seedlings; all other measurements, \( n = 15 \) seedlings). Different letters are used to indicate means that differ significantly (lower case letters, \( P < 0.05 \); upper case letters, \( P < 0.01 \)).](image-url)
Fig. 3 (A) Histochemical analysis of CycB1;1::GUS expression in primary root meristem of the WT and psi mutant. Seedlings were grown on the surface of agar plates containing Johnson medium with the indicated concentration of Pi. (B) Histochemical analysis of DR5::GUS expression in primary roots of the WT and psi mutant. (C) Longitudinal sections of the WT root tips grown on media with HP (a) and LP (b), and psi mutant root tips grown on media with HP (c) and LP (d). Scale bar = 100 µm.

Table 1 Inorganic P (Pi) concentration (µg/g FW) and total P concentration (mg/g DW) in root and shoot tissues of 8 DAG seedlings of Col wild-type (WT) and psi mutant (M) seedlings under HP (1,000 µM Pi) and LP (15 µM Pi) conditions ($n=6$)

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<td>WT</td>
<td>199.96 ± 13.36</td>
<td>399.55 ± 36.46</td>
<td>25.59 ± 2.46</td>
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<td>psi</td>
<td>206.19 ± 7.65</td>
<td>390.98 ± 35.27</td>
<td>25.04 ± 1.96</td>
<td>25.21 ± 0.99*</td>
<td>7.32 ± 0.72</td>
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*The concentration of the psi mutant is significantly higher than that of the WT at the 0.01 level.
the mutant and Sha which is sensitive to LP stress. The molecular marker MSAT1.10 linked with LPR1 (Reymond et al. 2006) showed close linkage with the mutant phenotype. Recently, it was reported that the LPR1 gene causes a long-root phenotype under LP conditions (Svistoonoff et al. 2007). Therefore, we developed a series of molecular markers for fine mapping around LPR1. By using 1,000 F2 mutants, the gene was fine mapped to a 5 kb region flanked by fssr1 and fssr2 (see Materials and Methods, Fig. 4A). We sequenced psi in this region and an 8 bp AC-repeat deletion in the promoter of LPR1 from −46 to −53 was found compared with the WT (Fig. 4A).

To check whether the long-root phenotype of psi is due to the mutation in the promoter of AtLPR1, we expressed the LPR1 coding sequence under the control of the WT LPR1 promoter in psi. Given that the long-root phenotype of the psi mutant is controlled by a single semi-dominant Mendelian factor, we would expect that the resultant transgenic lines would perform as psi heterozygous plants, if the mutant resulted from the mutation in the promoter of AtLPR1. As expected, we found that the long-root phenotype of psi under LP conditions was partially suppressed in the resultant transgenic lines (Fig. 4B).

Reymond et al. (2006) had reported that LPR1 did not regulate the anthocyanin content response. As we had found that the content of anthocyanins in the psi mutant grown under LP condition was lower than in the WT, we also measured the anthocyanin content of WT, psi, lpr1-1 and

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**Fig. 4** Map-based identification of PSI. (A) The results of primary mapping using 1000 F2 plants with the long-root phenotype as the psi mutant selected from the F2 populations derived from the crosses between the psi mutant and Sha accessions and the positions of deletion in different genotypes and the psi mutant. The dots represent the missing base. (B) Seedlings at 8 DAG of the WT, lpr1, and two lpr1/WT promoter-AtLPR1 transgenic lines grown on the basic medium. (C) qRT–PCR analysis of LPR1 expression in the WT and psi mutant under HP and LP levels. Scale bar = 1 cm.
lpr1-1/lpr2-1 under our HP and LP conditions. The results showed that the anthocyanin contents of the three mutants were all significantly lower than that of the WT under Pi starvation, although the anthocyanin contents in lpr1-1 and lpr1-1/lpr2-1 were relatively higher than that of psi (Supplementary Fig. S1), indicating that LPR1 also regulated the anthocyanin response under our conditions.

Taken together, we have shown that the LP-insensitive phenotypes of psi are caused by the mutation in the promoter of AtLPR1.

Deletion of AC in the LPR1 promoter led to lower expression of LPR1 in psi

Svistoonoff et al. (2007) showed that MCO activity, most probably resulting from the LPR1, is required for LP-dependent growth inhibition. Therefore, we examined the LPR1 transcription level in the mutant and WT using quantitative reverse transcription–PCR (qRT–PCR). The results showed that the level of LPR1 transcripts in the root of psi was significantly lower than in the WT (Fig. 4C), indicating that the special insensitivity of the root growth inhibition to LP is most likely to be due to a lower expression of the LPR1 transcripts.

Natural variations in response to Pi starvation have been reported in different Arabidopsis ecotypes (Chevalier et al. 2003, Reymond et al. 2006). Bay-0 and Ler (Landsberg erecta) accessions are relatively insensitive to LP stress, and the Col-0 accession is more sensitive (Reymond et al. 2006). In this study, the performances of PRL of these ecotypes are consistent with the earlier report, and the psi mutant showed similar PRL to Bay-0 and Ler (Supplementary Fig. S2). Therefore, we sequenced the promoter of LPR1 in Ler. This showed that the LPR1 promoter sequence of psi is identical to that of Ler. Together with the sequence data from Bay-0 and Sha, we found that the same missing 8 bp occurred in the LP-insensitive ecotypes, Bay-0 and Ler (Fig. 4A). A missing 10 bp AC-repeat was also detected in the same region in the LP-sensitive ecotype Sha. This indicates that the AC-repeat in the promoter region is important in maintaining the expression levels of LPR1.

In summary, we found that psi is a new allele of lpr1 and the AC-repeats in the promoter region are important in maintaining high expression of LPR1.

psi shows less sensitivity to auxin and a greater ability to maintain the auxin response in the root tip under LP stress

The auxin response in the WT and psi was investigated by monitoring DR5::GUS expression. DR5 is a highly active synthetic auxin response element (AuxRE), and provides a useful reporter gene for studying auxin-responsive transcription in WT plants and mutants (Ulmasov et al. 1997). A DR5::GUS auxin response reporter marker can visualize the distal auxin maximum (Sabatini et al. 1999). GUS staining showed that the auxin response was dramatically reduced in the root tip of the WT seedlings under LP stress at 8 DAG, while the mutant sustained a distal maximum pattern of auxin response under LP stress (Fig. 3B). To determine whether the maintenance of this distal maximum pattern of auxin response under LP conditions in psi contributes to the long-root phenotype, we tested the response of the mutant to exogenous auxin. As shown in Fig. 5A, under HP conditions, the mutant and WT plant showed identical sensitivity to IAA at concentrations from 0.005 to 0.5 µM, and to the inhibitor of auxin polar transport, N-1-naphthylphthalamic acid (NPA) at concentrations from 0.2 to 10 µM. Under LP stress, the mutant exhibited less sensitivity to auxin relative to the WT. At LP levels, 0.005–0.05 µM IAA produced a 16–61% reduction in PRL in the WT, while in the mutant, the PRL was only reduced by 0–30%. Similar, but less remarkable, results to those observed for IAA were obtained using NPA (Fig. 5A). These results indicated that the mutant is less sensitive to exogenous auxin compared with the WT under Pi starvation.

Following on from these results, another experiment using a WOX5::IAA transgenic line was conducted to examine whether maintaining the auxin response in the root tip by increasing auxin biosynthesis in the root tip can mimic the psi mutant phenotype. The WOX5::IAA line could induce auxin biosynthesis in the quiescent center by exogenous addition of the precursor indole-3-acetamide (IAM) (Biliou et al. 2005). After growing on LP medium for 4 d, seedlings of the WT, psi mutant and WOX5::IAA transgenic lines were transferred to LP medium containing different concentrations of IAM (0.01, 0.05 and 0.1 µM). Although the auxin maximum in the root tip can be maintained in the WOX5::IAA transgenic lines, the long-root phenotype was only observed in the mutant at LP levels at all of the IAM concentrations (Fig. 5B, C).

These results indicate that the psi mutant could maintain auxin response at the root tip under LP stress and the mutation altered the sensitivity to auxin and influenced the root development response of the root system under the stress. Thus, maintaining the auxin response at root tip by synthesizing auxin in the quiescent center could not mimic the long-root phenotype of the mutant under LP stress. Therefore, LPR1 may also influence other factors to cause the long-root phenotype under LP stress.

LPR1 functions independently of SIZ1

In Arabidopsis, the SUMO E3 ligase SIZ1 was reported to be a key player controlling not only the primary root architecture, but also the Pi signaling pathway (Miura et al. 2005). To investigate the relationship between SIZ1 and LPR1, a psi/siz1 double mutant was constructed.

Multiple phenotypes, including PRL, root hair length, root hair density and anthocyanin content were identified. On HP medium, siz1, psi and psi/siz1 double mutants have similar PRL, root hair length, root hair density and anthocyanin content. However, on LP medium, the double mutants showed an additive phenotype (Fig. 6A, B). For example, the primary root was shorter than that of the psi mutant, but longer than that of the siz1 mutant. Furthermore, Pi starvation-inducible
genes, including a member of TPS11/Mt4 family genes, AtIPS1 (At1g12820), a member of the phosphate transporters family, AtPT2 (At2g38940), one PHO81-like gene (At2g45130), and a MYB transcription factor PAP2 (At1g66390) (production of anthocyanin pigment 2), were tested in roots or shoots (PAP2) of 8-day-old seedlings of the WT, psi mutant and WOX5::IAAH seedling, vertically grown for 4 DAG at the HP or LP level, and subsequently transferred to medium with an LP level containing different concentrations of IAM for an additional 4 d.

For further investigation, the expression patterns of PHO2 and miR399 precursors, which are regulated by the SIZ1/PHR1 function in different pathways for controlling root architecture and Pi signaling under LP stress.
**Fig. 6** The phenotypic characterization and the expression of Pi starvation-inducible genes in the psi/siz1 mutant. (A) Primary root phenotype of psi/siz1 on HP (left) and LP (right) medium. (B) Phenotypic characterization of psi/siz1. (a) Primary root length of the WT, psi, siz1 and psi/siz1; (b) root hair length; (c) root hair density (number of hairs in a 1 mm segment of root from the first root hair); (d) anthocyanin content. (C) qRT–PCR analysis of Pi starvation-inducible genes in the psi mutant, WT (Col-0) or psi/siz1 double mutant under HP and LP levels: AtIPS1 (At1g12820), AtPAP2 (At1g66390), AtPT2 (At2g38940), PHO81-like (At2g45130), PHO2 (At2g 33770) and miR399d (At2g34202). Values are the means of three replicates.
regulation system (Miura et al. 2005), were tested in the WT and \( \psi \) mutant. No significant differences in the expression levels of these genes were found between the WT and the \( \psi \) mutant (Fig. 6C, for the miRNA399 precursors, only miRNA399d was shown). These results further confirmed the independent function of \( \text{LPR1} \) and \( \text{SIZ1} \) for the regulation of root architecture and Pi signaling under LP stress.

Recent reports have shown that \( \text{PDR2} \) encodes the ER-localized single P5-type ATPase, which play a role in maintaining the steady-state level of the nuclear SCR protein when Pi is limiting. The participation of the ER in the root meristem response to LP stress was further supported by the observation that the \( \text{pdr2} \) mutant is hypersensitive to brefeldin A (BFA) treatment (BFA inhibits vesicle trafficking and induces redistribution of Golgi proteins into the ER) (Ticconi et al. 2009). Ticconi et al. also showed that the \( \text{lpr1}/\text{lpr2} \) mutant is epistatic to \( \text{pdr2} \) and LPR1 localizes to the ER, indicating that the LPR1 and PDR2 proteins interact genetically in the ER. This raised the question of whether the \( \psi \)/\( \text{lpr1} \) mutants are insensitive to BFA treatment.

Therefore, we also examined the response of \( \psi \) and \( \text{lpr1-1}/\text{lpr2-1} \) to BFA. Primary root growth of the WT was sharply attenuated under BFA treatment in a dosage-dependent manner. However, the primary root growth only showed a moderate decrease in \( \psi \) and a slight decrease in \( \text{lpr1-1}/\text{lpr2-1} \) under the treatments (Fig. 7). This result indicated that the \( \psi \) and \( \text{lpr1-1}/\text{lpr2-1} \) mutants were less sensitive to BFA.

Discussion

LPR1 is involved in LP stress-regulated primary root growth and signaling pathway

Analysis of the Pi starvation response indicated that the \( \psi \) mutation causes insensitivity to LP. The \( \psi \) mutant accumulates less starch and anthocyanin (data not shown and Fig. 2D) than the WT under Pi starvation. The mRNA levels of Pi starvation-inducible genes, such as \( \text{PAP2} \) (production of anthocyanin pigment 2), which is involved in anthocyanin synthesis, are also significantly decreased. This reduction was supported by the decreased accumulation of anthocyanin in the mutant. We have shown that the \( \psi \) mutant is insensitive to LP stress, but not to N, S or Fe deficiency. Furthermore, a reduced response of Pi starvation-inducible genes, including the high-affinity Pi transporter \( \text{AtPT2} \), in the \( \psi \) mutant was observed in shoots and roots (Fig. 6B). However, we also observed that Pi and P concentrations in the \( \psi \) mutant shoots were higher than those of the WT under LP. Since the PRL of the \( \psi \) mutant is 2.8 times as long as that of the WT under LP (Fig. 2A), we proposed that this enlarged root area could compensate for the reduction in \( \text{AtPT2} \) expression and therefore increase the Pi absorption and Pi content in shoots of \( \psi \) under LP. Also the higher Pi and P concentrations in the shoot of the \( \psi \) mutant further confirmed that the Pi signaling pathway is disordered in the mutant.

Taken together, we showed that LPR1 is involved in the LP stress-regulated primary root growth and signaling pathway.

A higher auxin concentration under LP stress is not the sole cause of the longer PRL phenotype in the \( \psi \) mutant

Despite numerous studies showing that LP availability strongly influences RSA, little is known about the physiological and molecular events responsible for sensing of Pi limitation signaling, and its affect on root system development. The role of hormone signaling in the adaptive response of RSA to Pi starvation is complex, and previous studies led to conflicting conclusions.

Concerning the primary root, it was first reported that root developmental responses under LP stress were auxin independent (Williamson et al. 2001, Linkohr et al. 2002).
Later, two research groups found that the PRL and elongation rate are significantly altered in both auxin-treated WT plants and mutants defective in auxin responses. These results demonstrated that auxin mediates the effect of Pi stress on regulating the PRL (López-Bucio et al. 2002, Al-Ghazi et al. 2003). Recently, López-Bucio et al. (2005) proposed that only some aspects of the RSA response to Pi availability are under auxin control, while inhibition of primary root growth is independent of auxin. In our case, the decrease of Pi concentration in the medium leads to a decrease of auxin response activity in the root tip of the WT (Fig. 3B), which also shows a tight linkage with shortening of PRL (data not shown). The psi mutant sustains a higher auxin response activity than the WT under 100 μM Pi conditions and also exhibits a longer PRL than the WT. Furthermore, our results showed that the psi mutant is less sensitive to exogenous auxin in terms of the PRL under Pi starvation compared with the WT. Changes in the auxin sensitivity of the root seem to be involved in the developmental response of the Arabidopsis root system to Pi starvation (López-Bucio et al. 2002. Pérez-Torres et al. 2008). Therefore, it is likely that LPR1 altered the Pi signaling, impaired the sensitivity to auxin and contributed to the long-root phenotype under Pi starvation. These results indicated that auxin may participate in the adaptation of primary root growth under LP stress. However, maintaining the auxin response activity in the root of WOX5:IAAH by external addition of the auxin precursor IAM cannot mimic the long-root phenotype of the mutant under LP stress. It implied that changes in auxin response activity in the root tip need to co-function with other factors regulated by LPR1 to affect primary root elongation under LP stress. Some other signaling pathways, including other phytohormones, might co-function with auxin in this process. Borch et al. (1999) previously found that ethylene restricts the primary root growth of beans with adequate phosphorus sufficiency but maintains root growth under phosphorus deficiency. Ma and his colleagues confirmed the effect of ethylene on primary root elongation under Pi starvation (Ma et al. 2003). Taken together, these results suggest that the control of primary root elongation by Pi availability relies on a complex cross-talk between auxin and other factors, but it remains to be clearly defined.

The function of LPR1 may be controlled by the AC-repeat element in the promoter

Gene cloning and sequencing analysis revealed that an 8bp AC-repeat element was deleted in the promoter of LPR1 in the psi mutant and several LP-insensitive ecotypes. Interestingly, a 10bp AC-repeat deletion was also detected in the LP-sensitive ecotype Sha, which indicated that the different length of the AC-repeat had different regulatory effects on gene function. It has also been found in a human system that naturally occurring promoter region alleles, which possess a dinucleotide repeat polymorphism, could drive different levels of human NRAMP1 gene expression, and consequently different levels of disease susceptibility (Searle and Blackwell 1999). It has been demonstrated that it is the promoter, and not the gene coding region itself, that is the key regulator of function of the LPR1 gene (Svistoonoff et al. 2007). Our results provide further evidence for the functional effect of the LPR1 promoter and indicate that the AC-repeat element in this region has a subtle, but complicated function for gene regulation and the regulation of RSA under Pi starvation. However, how the different AC-repeats in the LPR1 promoter affect LPR1 function still needs to be investigated.

Our result regarding the regulatory role of LPR1 in anthocyanin accumulation is different from that of the previous report from Reymond et al. (2006). In their report, LPR1 did not regulate anthocyanin accumulation. These discrepancies may be caused by the different experimental conditions. We used Johnson medium for the seedling culture, while Reymond et al. adopted MS medium. Furthermore, the light intensity and culture temperature were different. In our conditions, we also found that the anthocyanin content in the psi mutant was significantly lower than in the other two mutants under LP stress (Supplementary Fig. S1), suggesting that psi was a stronger allele in regulating anthocyanin accumulation.

LPR1 may function independently from PHR1/SIZ1 regulation

PHR1, a MYB transcription factor, is an important regulator of Pi starvation-regulated genes (Rubio et al. 2001). It is a positive regulator of AtIPS1, and overexpression of OsPHR2 causes accumulation of excessive phosphate in the shoot (Nilsson et al. 2007, Zhou et al. 2008). Very recently, it was reported that the accumulation of AtPT2 mRNA was regulated by PHR1 (Karthikeyan et al. 2009). In the present case, AtIPS1 and AtPT2 were generally repressed in the psi mutant (Fig. 6C), while the expression of AtPHR1 showed no difference between the psi mutant and the WT at both HP and LP levels (data not shown). Furthermore, the expression levels of mir399 and PHO2, which are also downstream of PHR1, were also not changed in the psi mutant (Fig. 6C). Taken together with the fact that PHR1 is not involved in regulation of root architecture under LP stress, our data suggest that LPR1 may function independently from PHR1.

The Arabidopsis SUMO E3 ligase SIZ1 has been reported to be a key factor involved in the signaling pathway response to Pi starvation (Miura et al. 2005). Recent research reveals it may be a key regulator for environment stress responses (Catala et al. 2007, Miura et al. 2007). T-DNA insertionally mutated alleles of AtSIZ1 cause Arabidopsis to exhibit exaggerated prototypical Pi starvation responses, including cessation of primary root growth, extensive lateral root and root hair development, increase in root/shoot mass ratio and greater anthocyanin accumulation. The phenotype of the psi mutant is opposite to that of the Atsiz1 mutant in response to Pi starvation. The psi/siz1 double mutant exhibited an additive effect of the psi and siz1 mutant on PRL under LP stress (Fig. 6B). All of these results suggest that other regulation pathways exist. We propose that it is nearly impossible for LPR1 and SIZ1 to
function together by a direct interaction or regulation since the phenotype in either single loss-of-function mutant could not be completely shielded by a loss of function of the other gene. More probably, LPR1 and SIZ1 may function in two parallel signaling pathways for Pi starvation adaptation, and their function on the root phenotype in response to Pi starvation is antagonistic. Therefore, the root length of double mutants is probably the mean of that of the single mutants siz1 and psi in response to Pi starvation. Together with the phenotypic performance of the psi/siz1 double mutants, these results suggest that LPR1 can change the root phenotype under LP stress independently of SIZ1. The interactions between these two pathways remain to be clarified. Taken together, LPR1 may function independently of the PHR1/SIZ1 signaling pathway to regulate the RSA under Pi starvation in Arabidopsis.

**LPR1 and PDR2 have opposite functions in regulating root growth response to Pi starvation in the ER**

It has been proposed that PDR2 and LPR1 function together in an ER-resident pathway to adjust meristem activity to external Pi status (Ticconi et al. 2009). Furthermore, the PDR2-based function in maintaining root meristem activity can be supported by BFA treatment as the primary root growth of pdr2 is hypersensitive to BFA treatment. Here, we also showed that both of the psi and lpr1-1/lpr2-1 mutants are insensitive to BFA in primary root growth (Fig. 7). Therefore, it could be predicted that LPR1 and PDR2 had opposite functions in the regulation of ER function in the root growth response to LP stress. However, how the ER participates in the root meristem response to external Pi still needs to be addressed in the future.

**Materials and Methods**

**Plant material and growth conditions**

*Arabidopsis thaliana* ecotype Col-0 was used for all experiments. Seeds were surface sterilized with 10% (v/v) bleach diluted with ethanol for 10 min. After several washes in sterile distilled water, seeds were germinated and grown on agar plates containing Johnson medium. The growth medium contained 3 mol m\(^{-3}\) KNO\(_3\), 2 mol m\(^{-3}\) Ca(NO\(_3\))\(_2\), 0.5 mol m\(^{-3}\) MgSO\(_4\), 25 mmol m\(^{-3}\) KCl, 12.5 mmol m\(^{-3}\) H\(_2\)BO\(_3\), 1 mmol m\(^{-3}\) MnSO\(_4\), 1 mmol m\(^{-3}\) ZnSO\(_4\), 0.25 mmol m\(^{-3}\) CuSO\(_4\), 0.25 mmol m\(^{-3}\) (NH\(_4\))\(_6\)Mo\(_7\)O\(_24\), 25 mmol m\(^{-3}\) Fe-EDTA, 0.55 mol m\(^{-3}\) myoinositol, 2.5 mmol m\(^{-3}\) MES, 29.2 kmol m\(^{-3}\) sucrose (Ma et al. 2001) and 7 g l\(^{-1}\) agar (Sigma-Aldrich Co., St Louis, MO, USA). The pH of the medium was adjusted to 5.7 with dilute KOH before the agar was added. For media with varied phosphorus concentrations, NH\(_4\)H\(_2\)PO\(_4\) was added to give the targeted phosphorus concentration. For low phosphorus (15 µmol m\(^{-3}\)) media, (NH\(_4\))\(_2\)SO\(_4\) was used to replace NH\(_4\)H\(_2\)PO\(_4\) with no additional source of phosphorus. The agar concentration used contains approximately 15 µmol m\(^{-3}\) of phosphorus in the final media as determined by the Malachite green method (Delhaize and Randall 1995).

To make the Fe-free medium, Fe-EDTA was omitted from the nutrient solution. The N-free medium was prepared by omitting KNO\(_3\) and substituting CaCl\(_2\) for Ca(NO\(_3\))\(_2\). For S-free medium, MnSO\(_4\), ZnSO\(_4\) and CuSO\(_4\) were replaced by their respective chloride salts. For BFA treatment, 5 mM BFA (Sigma-Aldrich Co.)/dimethylsulfoxide (DMSO) solution was added into the Johnson medium to give the working concentration. Plates were placed vertically to allow root growth along the agar surface. Plants were grown at 20–22°C in a plant growth cabinet (Percival Scientific, Perry, IA, USA), with a photoperiod of 14 h of light, 10 h of darkness, with a light intensity of 150 µmol m\(^{-2}\) s\(^{-1}\). Seeds of CycB1;1::uidA (Colón-Carmona et al. 1999), DR5::uidA (Ulmasov et al. 1997) and WOX5::IAAH (Biliou et al. 2005) transgenic Arabidopsis plants were provided by Dr. Peter Doerner, Dr. Tom J. Guilfoyle and Dr. Ben Scheres, respectively. The seeds of accessions of Col-0, Bay-0 and Ler were from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH, USA). For genetic analysis and gene mapping, we generated the F\(_1\) lines and F\(_2\) populations between the related accessions.

**Mutant isolation procedure**

EMS-mutagenized seeds (Col-0) were purchased from Lehle Seeds (Round Rock, TX, USA). Seeds were surface sterilized and plated on LP Johnson medium. A total of approximately 20,000 M\(_2\) seedlings descended from EMS-mutagenized seeds were screened for long primary roots by placing seeds on LP agar plates. The seeds were distributed in 2–3 rows on the agar surface at a density of 1 seed cm\(^{-1}\), stratified at 4°C for 48 h, and then incubated for 8 DAG. LP-grown plants have a short primary root and a large number of root hairs formed close to the root apex. Putative mutants with long primary roots were selected, transferred to soil and allowed to self-fertilize. Homozygous M\(_3\) seeds were screened for long primary roots in LP conditions. Root hairs were observed using a Leica MZ9\(_3\) stereomicroscope. Photographs were taken using a NIKON D70\(_5\) digital camera. To measure and count cells, images were taken from different root regions and processed with a ZEISS Axiovert 200 microscope and Axiovision 3.1 Software. For histochemical analysis of GUS activity, the seedlings were incubated overnight at 37°C in a GUS reaction buffer (0.5 mg ml\(^{-1}\) 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 100 mM sodium phosphate, pH 7.0), and the stained seedlings were cleared using the method described by Malamy and Benfey (1997). For each marker line and for each treatment, at least 15 transgenic plants were analyzed. A representative plant was chosen for each Pi treatment and photographed using the ZEISS Axiovert 200 microscope.

A psi/siz1 double mutant was constructed by crossing psi and siz1 mutants. After genotyping the T-DNA insertion site of SIZ1 and the 8bp element deletion in the promoter of LPR1, the double mutants were confirmed. For siz1, the genotyping was performed according to the SALK Institute’s protocol
mixed vigorously. After at least 30 min the 20 µl to 1.5 ml, depending on the Pi concentration, was analyzed (Whatman No. 4 filter paper), and a subsample, ranging from were added to the mixture. The resulting solution was filtered were ground in a hand-held device and then 3 ml of water 5 M H2SO4 was added per 20 mg of fresh sample. The samples roots were collected and weighed, and approximately 40 µl of sample. Pi content was determined by the Malachite green method (Delhaize and Randall 1995). A total of 20–40 shoots or sample. For all experiments, the overall data were statistically analyzed in the SPSS 10 program (SPSS, Chicago, IL, USA). Univariate and multivariate analyses with a Tukey's post hoc test were used for testing differences in each variable in Pi treatments of WT and psi mutant seedlings.

Determination of anthocyanin and phosphorus content

Anthocyanin content was measured using a procedure based on the methods of Rabinow and Mancinelli (1986) and Feinbaum and Ausubel (1988). Seedlings were frozen in liquid nitrogen and ground. Tissue was gently shaken in 2.5 ml of 1% HCl/methanol for 2 h at room temperature, 2 ml of chloroform was added, the mixture was vortexed, 5 ml of water was added, and the vortex step was repeated. After separating the phases by centrifugation, 1 ml of the aqueous/methanol phase was assayed. A530 minus A657 was used to measure the anthocyanin content; values were normalized to the fresh weight of each sample. Pi content was determined by the Malachite green method (Delhaize and Randall 1995). A total of 20–40 shoots or roots were collected and weighed, and approximately 40 µl of 5 M H2SO4 was added per 20 mg of fresh sample. The samples were ground in a hand-held device and then 3 ml of water were added to the mixture. The resulting solution was filtered (Whatman No. 4 filter paper), and a subsample, ranging from 20 µl to 1.5 ml, depending on the Pi concentration, was analyzed for Pi content. The subsample was made up to 1.5 ml with water, added to 0.5 ml of Malachite green reagent, and then mixed vigorously. After at least 30 min the A650 of the solution was measured. The total phosphorus concentration was determined as follows: WT and psi shoots and roots of 8-day-old seedlings grown in HP and LP conditions were collected, weighed, and dried at 80°C for 24 h. Dried samples were digested with sulfuric acid and hydrogen peroxide (Mizuno and Minami 1990) and the concentration was measured by the Malachite green method.

Quantitative PCR analysis

For quantitative PCR analysis, WT, psi, siz1, or psi/siz1 seedlings were vertically grown for 8 DAG on HP and LP agar medium. Total RNA was prepared from shoots and roots. First-strand cDNA was synthesized from total RNA using a commercial cDNA synthesis kit (Promega, Sunnyvale, CA, USA) and stored at –20°C before use. The primers used in RT–PCR analyses were as follows: AtiPS1 (At1g12820) forward, 5’-TAAGAAAA GGTTTTATAAGATGG-3’; AtiPS1 reverse, 5’-TTCATCATAAGAAGATCAGAAGC-3’; AtiPAP2 (At1g66390) forward, 5’-CA TCAAGTTTCTTGGAGACG-3’; AtiPAP2 reverse, 5’-TTGGACC GGTGTTGTAGAGG-3’; AtiPT2 (At2g38940) forward, 5’-TGGG ATTCTTCATCTGCTTACG-3’; AtiPT2 reverse, 5’-CGAAGAA TACCCAGCGCCATGATC-3’; AtiPHO81-like (At2g45130) forward, 5’-GTGAATTGACCTTATAATTCGCG-3’; AtiPHO81-like reverse, 5’-CGAACATCTCTCCTGTATCCC-3’; AtiPHO2 (At3g33770) forward, 5’-GTTTAACAAAGCCACCAAGGCA-3’; AtiPHO2 reverse, 5’-TCACAGACATGTCCAAACCA-3’; AtmiR399d (At2g34202) forward, 5’-AAATACCTCTATGGCAGATTTT-3’; AtmiR399d reverse, 5’-TCTTGTGCAGAGAAGCCCTTACT-3’; AtmiTGA (At318780) forward, 5’-ACACCTGCTGGACG TGACCTTAC-3’; and AtmiTGA reverse, 5’-GATGACACCGCACA AAGAGAGAGC-3’. Quantitative real-time PCR was performed with an ABI 7000 sequence detection system according to the manufacturer’s protocol (Applied Biosystems, Foster, CA, USA). Triplicate quantitative assays were performed on each cDNA sample. The relative quantification method was used to evaluate quantitative variation between replicates examined. To account for differences in total RNA present in each sample, the amount of cDNA calculated was normalized using the amount of actin cDNA detected in the same sample. Data were then analyzed with ABI Prism 7000 SDS software version 1.1 (Applied Biosystems).

Genetic analysis and primary mapping

For genetic analysis of the performance of psi, the psi mutant was backcrossed to the WT (Col-0) to develop F2 populations. The PRL of 200 F2 plants (B DAG) under LP levels (15 µM Pi) was scored and the ratio of three PRL ranges—longer than the psi mutant, shorter than the WT (Col-0), and those in between—was counted for χ² analysis. For mapping, the psi mutant was crossed to the Sha accession to develop F2 populations. The genotypic patterns of 50 simple sequence repeat (SSR) markers distributed on five chromosomes were screened, and the new markers at the detected locus were developed for linkage analysis based on differences between Col-0 and Sha genomic sequences. Molecular markers closely linked with the PSI locus were investigated among 1000 long-root lines as the psi mutant selected from the F2 populations. Standard simple sequence length polymorphism and cleaved amplified polymorphic sequence PCR conditions were used. Marker information can be found at http://www.inra.fr/internet/Produits/vast/. For gene narrow down two primer pair were used (fssr1_forward: ATTTCACAGAACATTTTG, fssr1_reverse: TTGCATGTITTGTTCAT; fssr2_forward: GGGTGTGAACCAAG, fssr2_reverse: CGACACAAACAGATTC).

Complementation of psi

The expression construct was made by inserting a WT promoter + full-length LPR1 cDNA of psi into the binary plant vector pCAMBIA1300. The full-length promoter + LPR1 cDNA sequence can be found at http://signal.salk.edu/tdnaprimerst2.html. For psi, a primer pair was used to verify the 8 bp deletion (forward primer, TGAACTGAAAGACA; reverse primer, TTATCCTCTCCGACAC).
from psi was obtained by overlapping PCR. This vector was first digested by EcoRI then treated with T4 DNA polymerase to generate one blunt end, and then treated with CIAP (calf intestinal alkaline phosphatase) for dephosphorylation. The promoter + cDNA fragment was treated with DNA kinase and inserted into the modified plant vector pCAMBIA1301 by blunt-end ligation. Transformation was carried out using the floral dip method (Clough and Bent 1998). The transgenic plants were selected on hygromycin (20 mg l⁻¹) medium.

### Supplementary data

Supplementary data are available at PCP online.

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### References


Function of LPR1 in response to low Pi stress


