Phosphate (Pi) limitation causes plants to modulate the architecture of their root systems to facilitate the acquisition of Pi. Previously, we reported that the Arabidopsis (Arabidopsis thaliana) SUMO E3 ligase SIZ1 regulates root architecture remodeling in response to Pi limitation; namely, the siz1 mutations cause the inhibition of primary root (PR) elongation and the promotion of lateral root (LR) formation. Here, we present evidence that SIZ1 is involved in the negative regulation of auxin patterning to modulate root system architecture in response to Pi starvation. The siz1 mutations caused greater PR growth inhibition and LR development of seedlings in response to Pi limitation. Similar root phenotypes occurred if Pi-deficient wild-type seedlings were supplemented with auxin. N-1-Naphthylphthalamic acid, an inhibitor of auxin efflux activity, reduced the Pi starvation-induced LR root formation of siz1 seedlings to a level equivalent to that seen in the wild type. Monitoring of the auxin-responsive reporter DR5:uidA indicated that auxin accumulates in PR tips at early stages of the Pi starvation response. Subsequently, DR5:uidA expression was observed in the LR primordia, which was associated with LR elongation. The time-sequential patterning of DR5:uidA expression occurred earlier in the roots of siz1 as compared with the wild type. In addition, microarray analysis revealed that several other auxin-responsive genes, including genes involved in cell wall loosening and biosynthesis, were up-regulated in siz1 relative to wild-type seedlings in response to Pi starvation. Together, these results suggest that SIZ1 negatively regulates Pi starvation-induced root architecture remodeling through the control of auxin patterning.

Phosphorus is an essential macronutrient of plants because it is an integral component of nucleic acids and phospholipids and is conjugated to proteins and smaller molecules (Cohen, 1989; Berndt and Kumar, 2007). However, phosphate (Pi), the metabolically relevant elemental form of phosphorus, is often limited in the soil stratum (Schachtman et al., 1998). Pi is most prevalent at the soil surface, where microbes recycle the molecule from the organic matter of decaying organic substances. However, Pi is not adsorbed effectively into soil micelles, which are negatively charged, and is readily leached from the rhizosphere. Pi starvation induces root architecture changes that facilitate the efficient mobilization and acquisition of Pi (Lynch, 1995; Forde, 2002). Enhanced root branching contributes to the spatial configuration of the root system in the soil, which promotes the capacity of the plant to locate and absorb water and nutrients (Malamy, 2005).

Plants sense and respond to Pi deficiency by initiating a number of processes that are presumed to facilitate the capacity for Pi acquisition from external sources, intraplant distribution, and remobilization from organic molecules and stores (Raghothama, 1999; Abel et al., 2002; López-Bucio et al., 2003; Raghothama...
and Karthikeyan, 2005). Generally, three fundamental mechanisms have been proposed to cope with Pi starvation: plants activate the uptake of Pi from external organic and inorganic sources (Baldwin et al., 2001; Karthikeyan et al., 2002); internal Pi is mobilized and plants optimize Pi use by a wide range of metabolic alterations (Vance et al., 2003; Cruz-Ramírez et al., 2006); and plants modulate their root system architecture in response to low external Pi availability to access nutrients in other regions of the soil and increase the absorption surface area (Bates and Lynch, 1996; López-Bucio et al., 2003; Sánchez-Calderón et al., 2005). Low Pi availability attenuates primary root (PR) growth, promotes lateral root (LR) initiation and development, and increases root hair production (López-Bucio et al., 2003). Reduced PR growth presumably diverts resource allocation to the LR, which enhances access to Pi nutrient-rich regions that are near the soil surface (Williamson et al., 2001; Neumann and Martinoia, 2002; López-Bucio et al., 2003). It is presumed that the PR tip senses low levels of Pi and initiates a signaling cascade that reduces the proliferative activity of the meristematic cells and PR growth (Sánchez-Calderón et al., 2006; Franco-Zorrilla et al., 2007). Reduction in PR growth is presumably connected to LR development. Although the determinants of low Pi sensing, signal transduction in the PR, and long-distance communication between the PR and LR have not yet been elucidated (Williamson et al., 2001), increasing evidence suggests that phytohormones, particularly auxin, are involved (López-Bucio et al., 2002; Al-Ghazi et al., 2003; Narcy et al., 2005; Jain et al., 2007).

Auxin gradients regulate the initiation and elongation of the PR and LR (Casimiro et al., 2001; Himanen et al., 2002; Vanneste and Friml, 2009). Auxin is accumulated to the pericycle founder cells and contributes to LR initiation and elongation (Dubrovsky et al., 2001; De Smet and Jürgens, 2007; Péret et al., 2009). Because low external Pi values mimic the effect of indole-3-acetic acid (IAA) on LR development (Nacry et al., 2005), it is hypothesized that Pi starvation may be a signal for auxin redistribution. LRPI (for low phosphate-resistant root), also known as BIG, is required for the root auxin transporter (Gil et al., 2001) and LR development (Ruegger et al., 1997). lpr1 reduces the LR development that occurs in response to Pi deprivation (López-Bucio et al., 2005). Both TIR1- and ARF19-dependent auxin signaling are implicated in having roles in the LR development that is caused by Pi deficiency (Pérez-Torres et al., 2008). Thus, LR development under low Pi seems to be regulated by auxin.

We have previously reported that the siz1 mutation enhances root system architecture modifications that are presumed to be an adaptive response to Pi deficiency, including the inhibition of PR elongation and the development of LR (Miura et al., 2005). SIZ1 encodes a SUMO E3 ligase that is also involved in stress responses (Miura et al., 2007a, 2007b, 2009; Miura and Hasegawa, 2008, 2009, 2010). This study provides evidence for the involvement of SIZ1 in the negative regulation of auxin patterning for Pi starvation-induced root architecture remodeling in Arabidopsis (Arabidopsis thaliana). siz1 seedlings, relative to wild-type seedlings, exhibited an exaggeration of root architecture changes in response to Pi starvation. However, a similar pattern was observed for wild-type seedlings with the addition of IAA. N-1-Naphthylphthalamic acid (NPA), an inhibitor of auxin efflux carrier activity (Morris, 2000), suppressed the LR elongation of wild-type and siz1 seedlings to a similar extent. The auxin patterning monitored by DR5::uidA expression indicated that early in the Pi starvation response, auxin initially accumulated in the PR meristem and was correlated with the cessation of PR elongation. Coincidently, with the cessation of PR growth, DR5::uidA expression was observed in the LR primordia and was followed by LR elongation. Microarray and quantitative reverse transcription (RT)-PCR analyses determined that several auxin- and Pi starvation-inducible genes were up-regulated in siz1 relative to the response of wild-type seedlings to Pi starvation. Some of these genes are implicated in wall loosening and cell wall biosynthesis.

RESULTS

SIZ1 Facilitates the Auxin-Regulated Root Architecture Remodeling That Occurs in Response to Pi Deficiency

siz1 mutant seedlings exhibited a hypersensitive response to Pi starvation, including the inhibition of PR elongation and greater LR density and elongation (Fig. 1, A and B; Supplemental Fig. S1, A and B; Miura et al., 2005). PR growth was inhibited within 2 d after transfer to low-Pi medium (0.0125 mm KH2PO4), and the response of siz1 seedlings was more substantial than that in the wild type (Fig. 1, A and B). The average length of visible LR (more than 1 mm in length) was 2.6 ± 0.2, 4.4 ± 0.2, and 4.3 ± 0.3 mm for wild-type, siz1-2, and siz1-3 seedlings, respectively, when grown on low-Pi medium for 7 d. The LR length of siz1 mutants grown under low-Pi conditions was significantly longer than that of the wild type (t test; P < 0.01). The number of LRs increased in response to low Pi in both wild-type and siz1 seedlings, but the siz1 mutation enhanced LR production (Fig. 1B; Supplemental Fig. S1B).

To determine whether SIZ1 is involved in auxin-mediated root architecture remodeling that occurs in response to Pi starvation (López-Bucio et al., 2005; Narcy et al., 2005; Pérez-Torres et al., 2008), the effects of exogenous IAA on the root architecture of wild-type and siz1 seedlings were determined. IAA reduced PR elongation and increased LR density of wild-type and siz1 seedlings similarly under Pi-sufficient conditions (Figs. 1, C and D, and 2, A and B; Supplemental Fig. S1C). However, in the low-Pi conditions, IAA enhanced the cessation of PR elongation and substantially increased the LR density of siz1 relative to wild-type.
seedlings (Figs. 1, C and D, and 2, A and B; Supplemental Fig. S1D). At low Pi, exogenous IAA (0.05 μM) increased the LR length of siz1 and wild-type seedlings (average visible LR length was 4.0 ± 0.2, 4.3 ± 0.2, and 4.1 ± 0.2 mm for wild-type, siz1-2, and siz1-3 seedlings grown under the low-Pi condition, respectively). In addition, IAA eliminated the difference in the LR elongation between siz1 and wild-type seedlings that was observed at low Pi (Supplemental Fig. S1D). These results suggest that the SIZ1 regulation of Pi deficiency-induced root architecture remodeling involves auxin transport.

SIZ1 Regulates an Auxin Response That Is Linked with Root Architecture Remodeling at Low Pi

To further link SIZ1 with the regulation of auxin accumulation, siz1 and wild-type seedlings were treated with NPA, an inhibitor of auxin efflux carrier activity (Morris, 2000). NPA reduced the root gravitropism (Supplemental Fig. S1, E and F) and inhibited the growth of PRs; this was most evident under Pi-sufficient conditions (Fig. 2, C and D). At low Pi, NPA treatment substantially inhibited LR density and development (Fig. 2D; Supplemental Fig. S1F). Wild-type seedlings had a LR density of 2.1 ± 0.2 LR per cm of PR at 7 d after transfer to a medium with low Pi, whereas siz1-2 seedlings had 6.3 ± 0.5 LR per cm of PR (Fig. 2D; 0 μM NPA). NPA (2.5 μM) reduced the siz1 seedling LR density to 2.1 ± 0.2 (Fig. 2D; Supplemental Fig. S1F). These results suggest that the SIZ1 regulation of LR development in response to low Pi involves auxin transport.

Hyperaccumulation of Auxin Facilitates Low-Pi-Induced Root Architecture Remodeling

The flavin monooxygenases, YUCCA, play important roles in auxin biosynthesis (Zhao et al., 2001; Cheng et al., 2006; Kim et al., 2007). The overexpression of YUCCA1 or YUCCA6 under the control of the cauliflower mosaic virus 35S promoter results in the hyperaccumulation of auxin in plants (Zhao et al., 2001; Cheng et al., 2006; Kim et al., 2007). Interestingly, YUCCA1, but not YUCCA6, overexpression enhanced the root architecture remodeling in response to Pi deficiency in a manner similar to that of the siz1 mutation (Fig. 3). YUCCA1 overexpression in seedlings revealed a shorter PR under the low-Pi conditions (Supplemental Fig. S2A); however, the same effect was not observed with YUCCA6 overexpression (Supplemental Fig. S2A). Similarly, the LR density of the YUCCA1 overexpression line under low Pi was enhanced in a manner similar to that of siz1 (Supplemental Fig. S2B).
PR growth inhibition and LR development at low Pi. Results indicate that accumulation of auxin regulates grown on low-Pi medium for 7 d, respectively. These YUCCA1 and siz1-phenotype to Pi deficiency in the root system architecture. Wild-type to Pi Enhances/Alters Root Auxin Patterning in Response to Pi deficiency in seedlings (Fig. 4B). The maximal GUS expression was detected at 1 and 3 d after the transfer to low-Pi medium for siz1 and wild-type seedlings, respectively (Fig. 4B). Afterward, the GUS expression in the PR tips was decreased. Very little and no GUS expression was observed after 11 and 14 d, respectively, in siz1-2 seedling root tips (Fig. 4B).

When the seedlings were transferred to Pi-sufficient medium 9 d after the treatment with low Pi, the PR tips of the wild-type seedlings elongated again but those of the siz1-2 seedlings did not (Fig. 5A). The root growth of the LR was observed in both the wild-type and the siz1-2 seedlings (Fig. 5A). Nine days after the transfer to low Pi, the PR meristem of siz1-2 was disorganized, but the cell files were still intact for the wild-type seedlings (Fig. 5, B and C). Propidium iodide is excluded by intact cell membranes but can pass through damaged cell membranes and intercalate with DNA (Ma et al., 1997). Nine days after the transfer to low Pi, the root cells of siz1-2 seedlings took up propidium iodide, which was intercalated with DNA (Fig. 5C). The expression of pCycB1;1::uidA, which is a cell cycle marker (Colón-Carmona et al., 1999), was also lower in the PR tip cells of siz1-2 under Pi deficiency as compared with those of the wild type (Fig. 6A).

Interestingly, low-Pi induction of DR5::uidA expression was inversely correlated with SIZ1 expression (Figs. 4B and 6B). The peak of DR5::uidA expression in the PR tips occurred 3 d after the transfer to low-Pi medium (Fig. 4B) and was associated with reduced pSIZ1::uidA expression (Fig. 6B). The expression of pSIZ1::uidA was then increased 7 d after the transfer to low-Pi medium (Fig. 5B). These results link SIZ1 negatively with the auxin response of the root system architecture in low-Pi conditions, which likely involves auxin patterning.

In addition, the average LR length was 2.2 ± 0.3, 4.3 ± 0.4, 4.5 ± 0.4, and 2.7 ± 0.2 mm for the wild-type, siz1-2, YUCCA1, and YUCCA6 overexpression lines that were grown on low-Pi medium for 7 d, respectively. These results indicate that accumulation of auxin regulates PR growth inhibition and LR development at low Pi.

siz1 Enhances/Alters Root Auxin Patterning in Response to Pi

To characterize the auxin patterning in roots, DR5::uidA expression was monitored in wild-type and siz1-2 seedlings. This expression cassette is a fusion of the auxin-responsive DR5 promoter with uidA that encodes the GUS gene (Guilfoyle, 1999). GUS expression was similar in the PR tip of both the wild-type and the siz1-2 seedlings when Pi was sufficient (Fig. 4A). Pi deprivation caused a transient increase and then a decrease in GUS expression in the seedling PR tips; this occurred more rapidly in siz1 than in wild-type

Microarray Data Reveal That Several Auxin-Induced Genes Are Up-Regulated in siz1 in Response to Low Pi

A microarray analysis was conducted to assess the role of SIZ1 in Pi deficiency-regulated gene expression; the comparison was of wild-type and siz1 seedlings with and without sufficient Pi (Supplemental Tables...
Figure 4. DR5::uidA expression in PR tips and LR tips. A, No significant change was observed in high-Pi conditions. B, DR5::uidA expression in the PR tips of the wild type (WT) was increased after 3 d of growth under the low-Pi condition and was then decreased. In contrast, the expression in siz1 was increased 1 d after transfer onto Pi-deficient medium. C and D, DR5::uidA expression in the LR tips of wild-type and siz1-2 seedlings, respectively, after 1, 2, 3, and 5 d of treatment with Pi starvation. Three representative LR tips are shown. E and F, DR5-GUS accumulation in the LR tips of wild-type and siz1-2 seedlings, respectively, was observed at 7, 9, 11, and 14 d after the transfer to low-Pi medium. The top panels are two representative younger LR tips, and the bottom panels are older LR tips. Bars = 100 μm. [See online article for color version of this figure.]

S2–S5). The data presented above suggest that the SIZ1 function in low-Pi root architecture remodeling involves auxin, and the analysis compared these results with those reported for 1-naphthaleneacetic acid (NAA)-inducible genes (Himanen et al., 2004; Vanneste et al., 2005). Fifteen genes, which were up-regulated in siz1-2 under Pi-deficient conditions, were also NAA-inducible genes (Supplemental Table S1), including the auxin-induced aldo/keto reductase (At1g60730). EXP17 (for expansin 17; At4g01630) belongs to a group of cell wall-loosening proteins (http://www.bio.psu.edu/expansins/; Li et al., 2002).

To further the analysis of our microarray data, expression profile data with NAA, IAA, or Pi deficiency were extracted from Genevestigator (https://www.genevestigator.com/; Zimmermann et al., 2004, 2005). By using these data and our microarray data (Supplemental Tables S2–S5), cluster analysis was performed (Fig. 7A). The genes that were up-regulated in siz1 and under Pi-deficient conditions but were slightly altered with auxin treatment were clustered into one clade (Fig. 7B): GLH19 (for glycosyl hydrolase 19), embryo-abundant, defense-related genes, and expansin-related 3 were included (Fig. 7B). In contrast, another cluster contained genes that were up-regulated in siz1 under Pi deficiency and auxin treatment: GLH1, glutathione transferase, embryo-abundant, and dehydrin xero2 were included (Fig. 7C). Because we assumed that genes up-regulated by the siz1 mutation, auxin, and Pi deficiency were candidates for the regulation of...
root architecture remodeling, the expression levels of EXP17 (At4g01630), GLH1 (At1g02850), and UGT73B4 (for UDP-glycosyltransferase 73B4; At2g15490) were confirmed by real-time RT-PCR (Fig. 8). These genes were also induced by Pi starvation in wild-type seedlings, and the level of these genes in siz1 was found to be higher than in the wild type (Fig. 8).

Because EXP17, GLH1, and UGT73B4 are also expressed in roots according to the microarray database Arabidopsis eFP Browser (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), it is possible that these genes are involved in the regulation of cell elongation in roots in response to Pi deficiency.

Based on Gene Ontology (GO) biological processes, the genes up-regulated in siz1 under high-Pi (Fig. 7D, left panel) and low-Pi (Fig. 7D, right panel) conditions were categorized. Under both conditions, genes involved in defense responses, metabolic processes, and response to stresses were major categories. Because the siz1 mutant accumulates SA and exhibited resistance to Pseudomonas syringae pv tomato DC3000 (Lee et al., 2007), many of the genes for defense responses were increased in the siz1 mutant (Fig. 7D). Genes such as GLH, UGT73B4, embryo-abundant protein, and cytochrome P450 were categorized as metabolic processes. These genes may be involved in the regulation of root architecture modification as described above.

Salicylic Acid Is Not Involved in Root Morphological Changes in Response to Pi Deficiency

siz1 mutations cause salicylic acid (SA) hyperaccumulation and constitutive pathogen resistance (Lee et al., 2007). The GO analysis indicated that about 15% of the genes that were up-regulated in siz1 relative to wild-type seedlings were categorized as being involved in the defensive response (Fig. 7D), including PATHOGENESIS-RELATED1 (Supplemental Table S2). The expression of the bacterial nahG gene in siz1 seedlings decreased SA accumulation (Yoo et al., 2006; Lee et al., 2007), because the encoded nahG, which has salicylate hydroxylase activity, catalyzes the conversion of SA to catechol (Yamamoto et al., 1965). PAD4 encodes a lipase-like protein that regulates SA accumulation for the control of defense responses in Arabidopsis (Zhou et al., 1998). At high Pi, the PR growth among all genotypes was similar (data not shown). At low Pi, nahG and pad4 seedlings had PR growth and morphology that were similar to the wild type, whereas the characteristics of nahG siz1-2 and pad4 siz1-2 seedlings resembled those of siz1-2 seedlings.
lings (Fig. 9). These results suggest that the low-Pi-induced root remodeling associated with siz1-2 is independent of SA levels.

DISCUSSION

Our data indicate that SIZ1 is a negative regulator of Pi deficiency-induced root architecture remodeling, including the inhibition of PR elongation and a greater LR density, that is presumed to enhance acquisition of Pi (Malamy, 2005). In addition, the role of SIZ1 in Pi deficiency-stimulated root morphological changes involves an auxin response. siz1 caused hypersensitivity to low Pi for root architecture remodeling that was associated with changes in auxin patterning (Figs. 1, 2, and 4). IAA exogenously supplied to seedlings under Pi sufficiency resulted in root responses that were similar to those caused by Pi deficiency and reduced the difference in Pi starvation sensitivity for PR growth inhibition between siz1 and wild-type seedlings (Fig. 2). Inhibition of the auxin efflux carrier activity by NPA abrogated PR elongation and LR development and differences in the root remodeling sensitivity between wild-type and siz1 seedlings caused by Pi starvation (Fig. 2). YUCCA1 overexpression in wild-type seedlings caused a similar hypersensitive response to low Pi as was found in the siz1 seedlings (Fig. 3; Supplemental Fig. S2). Monitoring auxin using
Controling the Accumulation of Auxin

SIZ1

DR5::uidA expression revealed a spatial and temporal patterning (Fig. 4). Low Pi resulted in a more rapid transient induction of GUS expression in PR that was followed by a reduction in activity. Coincident with the decreased DR5::uidA expression reduction in the PR, the expression of DR5::uidA increased in the pericycle and the LR primordia and tips (Fig. 4, C and D). Pi starvation-induced auxin accumulation in the PRs and LRs occurred more rapidly in siz1 than in wild-type seedlings. Twenty-six NAA-inducible genes were up-regulated by siz1 (Supplemental Table S1), including those coding for expansin, glycosyl hydrolase, and glycosyl transferase (Fig. 8). Together, these results suggest a SIZ1 function in root remodeling that occurs in response to Pi deficiency by controlling auxin accumulation patterning.

SIZ1 Negatively Regulates Root System Architecture by Controlling the Accumulation of Auxin

Our results indicate that SIZ1 negatively regulates the low-Pi-inducible root system architecture, presumably by regulating the auxin transport system. Recent studies suggest that auxin accumulation is involved in the alteration of PR length and LR density and elongation (López-Bucio et al., 2002; Al-Ghazi et al., 2003; Nacry et al., 2005). Based on our study, during the first stage (days 1–3 in the wild type or day 1 in siz1; Figs. 3B and 4B), DR5::uidA expression in the PR tip increased to a very high level (Fig. 4B), which correlated with a decreased PR growth rate (Fig. 1A). SIZ1 is likely to repress high accumulation of auxin in the PR tips (Fig. 10C), because when the expression level of pSIZ1::uidA was decreased (Fig. 6B), DR5::uidA expression in the PR tips was highly promoted (Fig. 4B). At the second stage (days 4–7 in the wild type or days 2–5 in siz1; Fig. 4, B–D, and 10D), the DR5::uidA expression level was increased in the LR for elongation (Fig. 4, C and D), and the density of the LR was drastically increased (Fig. 1B). The DR5::uidA expression levels of the LR tips in siz1-2 (Fig. 4D) were higher than those in the wild type (Fig. 4C), suggesting that SIZ1 is involved in the negative regulation of auxin accumulation in LR tips (Fig. 1D). At the third stage (more than 8 d in the wild type or more than 6 d in siz1; Figs. 3, E and F, and 10, E and F), the younger LR tips accumulate auxin more than the older LR tips. This action is also likely to be regulated by SIZ1 (Fig. 10, E and F) because DR5::uidA expression in the older LR tips of siz1-2 was not observed after a 9-d treatment with Pi deficiency. However, expression in the older wild-type LR tips continued (Fig. 3, E and F). At the same stage, auxin accumulation in the PR tip was gradually decreased (Fig. 3B), the cells became swollen, and the meristem activity was lost (Fig. 10F). This event occurs earlier in siz1 than it does in the wild type (Fig. 3B); the PR roots of siz1-2 seedlings were not able to elongate, even though those of the wild-type seedlings were able to do so when the seedlings were transferred to the Pi-sufficient condition (Fig. 4). Finally, the plants began to die (Fig. 10G). Together, these data suggest that SIZ1 negatively regulates Pi starvation-induced root architecture modulation through the control of auxin accumulation patterning (Fig. 10).

Auxin patterning may play an important role in the modulation of root system architecture that is induced by low Pi. The transcription factors ARF7 and ARF19 (for auxin response factors 7 and 19), which activate...
auxin-responsive gene expression (Ulmasov et al., 1999; Tiwari et al., 2003), play a crucial role in LR development (Okushima et al., 2005, 2007). The LR formation pattern in response to Pi availability is mediated by changes in auxin sensitivity as controlled by TIR1 and ARF19 (Pérez-Torres et al., 2008). Our data demonstrated that SIZ1 plays a negative role in the regulation of LR formation (Fig. 1). Because SIZ1 is a SUMO E3 ligase (Miura et al., 2005), the sumoylation system may control TIR1 or ARF19 for the modification of root system architecture. SUMO conjugation occurs through covalent linkage to the Lys residue in the conserved sumoylation motif \( \Psi KXE/D \), where \( \Psi \) is a large hydrophobic amino acid and X is any amino acid (Geiss-Friedlander and Melchior, 2007). Neither TIR1 nor ARF19 has the conserved sumoylation motif, whereas ARF7 has one conserved sumoylation site. Thus, TIR1 or ARF19 may not be direct targets for sumoylation. In addition to the covalent attachment of SUMO to the Lys residues in the target proteins, the SUMO-interaction motifs (V/I-X-V/I-V/I and V/I-V/I-X-V/I/L) have been identified to mediate noncovalent interactions with SUMO (Song et al., 2004, 2005; Lin et al., 2006). This SUMO-interaction motif is important for interaction with sumoylated proteins. Both TIR1 and ARF19 have one SUMO-interaction motif (VEVI in TIR1, ILLV in ARF19). It is plausible that SIZ1 mediates sumoylation of another target, which promotes interaction with TIR1 or ARF19 to block LR formation.

**SIZ1 Regulates Root Initiation and Root Elongation Mechanisms**

According to our microarray and quantitative RT-PCR data (Figs. 7 and 8), several genes, including EXP17, GLH, and UGT73B4, were up-regulated in siz1 and induced by Pi starvation and/or auxin (Fig. 7, B and C). Some of these genes should be candidates for the regulation of root initiation and root elongation.

LR development and cell separation processes in adjacent root tissues are tightly coordinated to minimize tissue damage. Recent studies have identified cell wall remodeling enzymes, which are expressed in root cells next to new LR primordia, presumably to promote the emergence of LR primordia (Laskowski et al., 2006). Cell wall remodeling enzymes, such as xyloglucan:xyloglucosyl transferase 6, EXP17, and GLH17, are regulated by LAX3 (for like AUX1-3)-mediated auxin signaling (Henrissat, 1991; Cosgrove, 2000; Marin-Rodriguez et al., 2002; Vissenberg et al., 2005; Wen et al., 2006).

The plant cell wall has high tensile strength and must be loosened to enable the cell to grow (Cosgrove, 2005). Expansins are plant cell wall proteins that function in cell wall stress relaxation and irreversible wall extension, which are important processes for cell enlargement (Cosgrove, 2000). Thus, expansins are involved in plant cell growth, cell wall disassembly, fruit softening, and developmental processes. As the expression level of EXP17 was up-regulated by the siz1-2 mutation under the low-Pi conditions (Fig. 8), was regulated by LAX3 (Swarup et al., 2008), and was induced by Pi deficiency (Fig. 8), EXP17 may be involved in cell wall loosening for LR emergence.

Glycosyl hydrolases have large families and diverse biochemical functions, such as mannosidase, glucosidase, galactosidase, endoglucanase, and chitinase (Lopez-Casado et al., 2008). One of the glycosyl hydrolases, *Populus tremula × Populus tremuloides* glycosyl hydrolase family 9 isolog (PttCel9A), is up-
regulated during cell wall synthesis (Master et al., 2004), and both PitCelA9 and its homolog KORRIGAN1 decrease the cellulose crystallinity, which implicates them in cellulose biosynthesis (Takahashi et al., 2009). According to our data, GLH1 and GLH19 were upregulated in siz1 (Fig. 7, B and C). In Arabidopsis, 48 members are included in GLH1 and 14 members are included in GLH19. Family 19 has chitinase activity, but family 1 has several functions (Lopez-Casado et al., 2008). The precise biological function of GLH1 has not yet been identified. However, it is possible that At1g02850 is linked with cell wall synthesis. UDP-glycosyltransferase was also up-regulated in siz1 under IAA treatment and in the Pi starvation condition (Figs. 7C and 8). Because UGT73B4 is phylogenetically close to UGT73B5, which is induced by pathogens and (Figs. 7C and 8). Because UGT73B4 is phylogenetically close to UGT73B5, which is induced by pathogens and is important for resistance to P. syringae pv. tomato (Langlois-Meurinne et al., 2005), UGT73B4 may function in pathogenesis.

**SIZ1 Regulation of Root Architecture Remodeling Is Independent of Pi Signaling Pathways for Pi Uptake**

The root architecture of siz1 showed hyperresponses to low extracellular Pi (Fig. 1). However, the siz1 plant accumulates greater amounts of Pi in the shoot than the wild-type plant (Miura et al., 2005). The up-regulation of AIPH1;4 and AIP52 in the siz1 mutant (Miura et al., 2005) is likely to be correlated with the accumulation of Pi but not with the root system architecture modulation. The accumulation of Pi in the shoot and the root architecture modulation are not linked. The pho1 and pho2 mutants accumulate less and more Pi, respectively (Poirier et al., 1991; Delhaize and Randall, 1995). These mutants show a similar root morphology phenotype to wild-type seedlings (data not shown). The plants overexpressing miRNA399, which accumulate a larger amount of Pi, do not show a hyperresponse phenotype (Fujii et al., 2005). PHO2, encoding the ubiquitin E2 conjugate enzyme, is a target of miRNA399 (Aung et al., 2006; Bari et al., 2006). Taken together, these results suggest that it is more likely that the miRNA399-PH2O pathway preferentially regulates Pi uptake but does not regulate root architecture. PHR1 positively regulates the expression level of miRNA399 (Bari et al., 2006), and the phr1 mutant accumulates less Pi (Rubio et al., 2001) but shows no effect in the root system architecture (Sánchez-Calderón et al., 2006). It is plausible that the sumoylation of PHR1 regulates the Pi uptake that is mediated by the miRNA399-PH2O pathway but does not regulate the modulation of the root system architecture.

**Negative Regulators for Pi Starvation Responses**

To regulate Pi homeostasis, both positive and negative regulators are required. SIZ1 is involved in the negative regulation of Pi starvation-induced root architecture modification through auxin accumulation.

In one case, the pho2 mutant exhibited an excessive amount of Pi (Delhaize and Randall, 1995), leading to growth retardation and necrosis in the mature leaves in both Arabidopsis (Aung et al., 2006) and rice (Oryza sativa; Wang et al., 2009). Several transcription factors have been isolated as potential negative regulators for Pi starvation signaling. The induction of the Pi transporter gene AtPht1;4 is negatively regulated (Mukatira et al., 2001). MYB-type transcription factors are repressed by Pi starvation (Wu et al., 2003). A WRKY transcription factor and two SPX-domain-containing proteins repress Pi starvation-induced genes (Devaiah et al., 2007; Duan et al., 2008).

To survive, plants rely on proper physiological and developmental adjustment. Auxin seems to be an essential integrator of root remodeling that is critical to Pi acquisition from soil.

Our study found that SIZ1 is involved in the control of auxin patterning to modulate Pi starvation-induced root system architecture, which is an important process for optimizing Pi starvation responses.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

Arabidopsis (Arabidopsis thaliana) ecotype Columbia (Col-0) and the mutants siz1-2 and siz1-3 (stock nos. C5659 and C5660, respectively, in the Arabidopsis Biological Resource Center at Ohio State University; Miura et al., 2005) were used. The seeds were surface sterilized with 70% (v/v) ethanol for 5 min and 10% (v/v) bleach for 15 min. After being washed three times with distilled water, the seeds were incubated at 4°C for 4 d. The seeds were then germinated on 1× Murashige and Skoog (MS) medium with a pH of 5.7, 3% (w/v) Suc, and 0.8% (w/v) agar. After incubation for 3.5 d, the seedlings were transferred to the basal medium containing B5 vitamin, 3% (w/v) Suc, 2.5 mM MES (pH 5.7), and 1.2% (w/v) agar with the indicated concentration of KH2PO4. The basal medium contained 1/10× MS macro-nutrients (2.05 mM NH4NO3, 1.8 mM KNO3, 0.3 mM CaCl2, and 0.156 mM MgSO4) and 1× MS micronutrients (100 mM H3BO3, 100 μM MnSO4, 30 μM ZnSO4, 5 μM KI, 1 μM Na2MoO4, 0.1 μM CuSO4, 0.1 μM CoCl2, 0.1 μM FeSO4, and 0.1 μM Na3EDTA). The seedlings were placed vertically, and the temperature was maintained at 23°C to allow root growth along the surface of the agar, with a photoperiod of 16 h of light and 8 h of dark.

Seeds of the transgenic ecotype Columbia_CaMV35S::SIZ1 and Columbia_CaMV35S::YUCCA1 or Columbia_CaMV35S::YUCCA6 by the Murashige and Skoog (MS) medium were transferred to the basal medium containing B5 vitamin, 3% (w/v) Suc, 2.5 mM MES (pH 5.7), and 1.2% (w/v) agar with the indicated concentration of KH2PO4. The basal medium contained 1/10× MS macro-nutrients (2.05 mM NH4NO3, 1.8 mM KNO3, 0.3 mM CaCl2, and 0.156 mM MgSO4) and 1× MS micronutrients (100 mM H3BO3, 100 μM MnSO4, 30 μM ZnSO4, 5 μM KI, 1 μM Na2MoO4, 0.1 μM CuSO4, 0.1 μM CoCl2, 0.1 μM FeSO4, and 0.1 μM Na3EDTA). The seedlings were placed vertically, and the temperature was maintained at 23°C to allow root growth along the surface of the agar, with a photoperiod of 16 h of light and 8 h of dark.

Arabidopsis plants were kindly provided by Dr. Angus Murphy (Purdue University). The homozygous progeny of genetic crosses with DR5::uidA or CaMV35S::uidA and siz1-2 were used for the analysis of GUS expression. To make Columbia_siz1::uidA, the promoter region of SIZ1 (− 2,035 to – 7 from ATG) was introduced into pCAMBIA1391Z, and the resulting construct was transformed into Arabidopsis. The expression pattern of pSIZ1::uidA in other organs (data not shown) was similar to that described previously (Catala et al., 2007). The seeds of the overexpression of YUCCA1 or YUCCA6 by the cauliflower mosaic virus 35S promoter were kindly provided by Dr. Yunde Zhao (University of California at San Diego; Cheng et al., 2006).

**Hormone Treatments**

To test the effects of IAA and NPA, low-Pi (0.0125 mM KH2PO4) and high-Pi (1.25 mM KH2PO4) nutrient media were supplemented with ethanol-dissolved IAA or NPA. These compounds were filter sterilized and added to the media at 60°C. IAA and NPA were purchased from Sigma Chemicals.

**Histochemical Analysis**

For histochemical analysis of GUS activity in the Arabidopsis transgenic line, DR5::uidA and pSIZ1::uidA seedlings were incubated for 4 h at 37°C in a
GUS reaction buffer (0.522 mg mL⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-gluconoride and 0.3% [v/v] Triton X-100 in 100 mM sodium phosphate, pH 7.5), and the stained seedlings were washed with 70% (v/v) ethanol four times to stop the reaction and remove chlorophyll. For the histochemical analysis of GUS reporter enzyme activity in the Arabidopsis transgenic line pCycB1;1::GUS, the GUS reaction buffer was supplemented with 3.5 μM each K₃Fe(CN)₆, and K₅Fe (CN)₆. For each treatment, at least 10 seedlings were analyzed. Representative seedlings were photographed using the Nomarski optics on a Nikon E800 or a DM RXA-6 (Leica) microscope.

cDNA Microarray Analysis

One-week-old wild-type and siz1-2 seedlings grown in MS liquid medium were transferred to the MS liquid medium with 1.25 mM or 0.0125 mM KH₂PO₄ and were incubated for 3 d. The total RNA (70 μg) was isolated with the TRIzol reagent (Invitrogen; Miura et al., 2005). The cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). The cDNA was labeled with Cy3 or Cy5 by indirect labeling (Gong et al., 2005). Microarray slides harboring 25,425 probes, which were spotted as 70-mer oligonucleotides, were used (http://www.ag.arizona.edu/microarray; Gong et al., 2005). Three biological repeats were performed.

Evaluation and Statistical Analysis

The data evaluations and statistical analyses were carried out as described by Gong et al. (2005). Briefly, the signal intensities for each microarray element were evaluated using GenePix 4000B (Axon Instruments) and analyzed with GenePix Pro 4.0 (Axon Instruments). The spots with intensities lower than the background and aberrant spots were flagged by the GenePix software and checked manually. The resulting files were converted by ExpressConverter version 1.5 and analyzed using the TIGR-TM4 package (http://www.tm4.org; Saeed et al., 2003). A one-class t test with P = 0.01 was carried out to reveal the patterns of regulation (Gong et al., 2005; Lee et al., 2007).

Cluster analysis of the transcripts was performed with Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm), and Java Treeview (http://froEinview.sourceforge.net/) was used for visualization. The expression data after NAA, IAA, and Pi-deficient treatment with Col-0 were obtained from the microarray database Genevistator (http://www.genevistator.com; Zimmermann et al., 2004, 2005). The conditions were as follows. NAA, 5-week-old Col-0 leaf disc treated with 10 μM NAA for 1 h versus 0 h (experiment AT-00392); IAA, Col-0 seedlings treated with 1 μM IAA for 1 and 3 h versus seedlings with mock treatment for 1 and 3 h (experiment AT-00110); IAA study 2, 7- and 9-d-old Col-0 seedlings with 5 μM IAA for 2 h versus seedlings treated with 5 μM ethanol (experiment AT-00164); P deficiency (early or late), Col-0 seedlings treated with 5 μM Pi for 3, 6, and 12 h (early) or 1 or 2 d (late) versus seedlings treated with 1 μM Pi for the same time period (experiment AT-00122); Pi deficiency (shoot or root), Col-0 seedlings were sown onto low-Pi medium (5 μM) and high-Pi medium (500 μM) for 10 d (experiment AT-00122). The biological classifications of the genes were clustered according to the GO annotations described in The Arabidopsis Information Resource database (http://www.arabidopsis.org).

Quantitative RT-PCR

Three-and-a-half-day-old seedlings grown on MS medium were transferred onto the medium described above containing 1.25 mM (±P) or 0.0125 mM (–P) KH₂PO₄. Three days after incubation, the seedlings were harvested for RNA preparation (Miura et al., 2005, 2010). Total RNA was used for the first-strand cDNA synthesis, which was performed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed with the Thunderbird SYBR qPCR Mix (Toyobo) with gene-specific primers (Supplemental Table S6). The PCR products were detected using a Thermal Cycler Dice Real Time System (Takara BIO) as described previously (Miura and Ohta, 2010). The relative differences in expression were calculated as described previously (Miura et al., 2007b, 2009).

Sequence data from this article can be found in the Arabidopsis Initiative or GenBank/EMBL data libraries under the following accession numbers: CYCB1;1 (At4g37490), EXP17 (At1g01630), GLH1 (At1g02850), PAD4 (At1g52430), SIZ1 (At1g50410), UGT73B4 (At2g15490), YUCCA1 (At4g32540), YUCCA6 (At5g25620), and nahG (YP_334831).

Supplemental Data

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

Supplemental Figure S1. Wild-type seedlings treated with IAA in low Pi mimicked the siz1-2 phenotype in response to Pi deficiency, and treatment with NPA suppressed LR formation.

Supplemental Figure S2. Overexpression of YUCCA1 reduced PR elongation and enhanced LR density.

Supplemental Table S1. Genes with 3-fold or higher expression in siz1-2 that are also induced by NAA.

Supplemental Table S2. Genes 3.0-fold or higher up-regulated in siz1-2 plants under the low-Pi condition.

Supplemental Table S3. Genes 3.0-fold or higher up-regulated in siz1-2 plants under the high-Pi condition but less than 3.0-fold up-regulated in siz1-2 plants under the low-Pi condition.

Supplemental Table S4. Genes 3.0-fold or higher down-regulated in siz1-2 plants under the low-Pi condition.

Supplemental Table S5. Genes 3.0-fold or higher down-regulated in siz1-1 plants under the high-Pi condition but less than 3.0-fold down-regulated in siz1-2 plants under the low-Pi condition.

Supplemental Table S6. Primers used for quantitative RT-PCR analysis.

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