SIZ1 Regulation of Phosphate Starvation-Induced Root Architecture Remodeling Involves the Control of Auxin Accumulation[1][C][W][OA]

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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.

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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 organisms. 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; Nacry 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. *LPR1* (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 KH_2PO_4), 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 $\pm$ 0.2, 4.4 $\pm$ 0.2, and 4.3 $\pm$ 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; Nacry 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

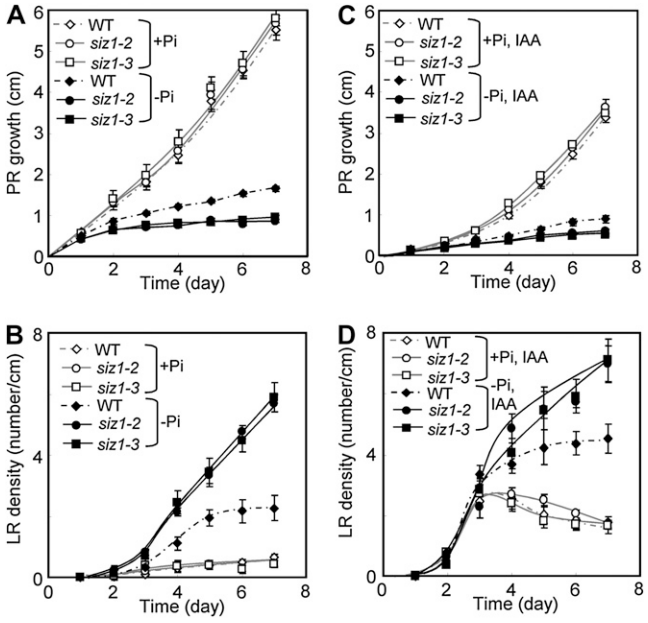

Figure 1. Effects of Pi availability and the application of 0.05 μM IAA on the root system architecture of wild-type (WT) and *siz1* seedlings. The seedlings grown on basal medium for 3.5 d were transferred onto medium containing high Pi (1.25 mM KH_2PO_4; +Pi) or low Pi (0.0125 mM KH_2PO_4; −Pi), and the growth of PR (A) and density of LR (B) were scored each day. The LR density was calculated by visible LR (more than 1 mm length) divided by the PR length. To monitor the effect of IAA on the root system architecture under high or low Pi availability, the seedlings were transferred onto medium containing 0.05 μM IAA (C and D). The growth of PR after the transfer and the density of the LR were scored. All values shown represent averages of 12 seedlings ± SE.

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 accumulation.

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).

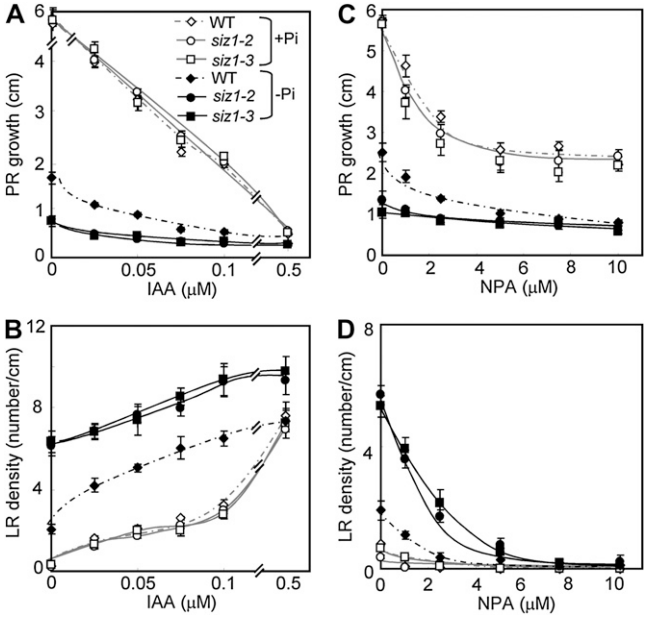

Figure 2. Application of IAA reduces PR growth and increases LR density, whereas NPA treatment decreases LR density in wild-type (WT) and *siz1* seedlings. Seedlings were grown on basal medium containing Pi for 3.5 d before being transferred onto medium containing high Pi (1.25 mM KH_2PO_4; +Pi) or low Pi (0.0125 mM KH_2PO_4; −Pi) and grown for 7 d. PR growth and LR density of wild-type and *siz1* seedlings that were grown on various concentrations of IAA (A and B) or NPA (C and D) were scored. All values represent means of 12 or more seedlings ± SE.

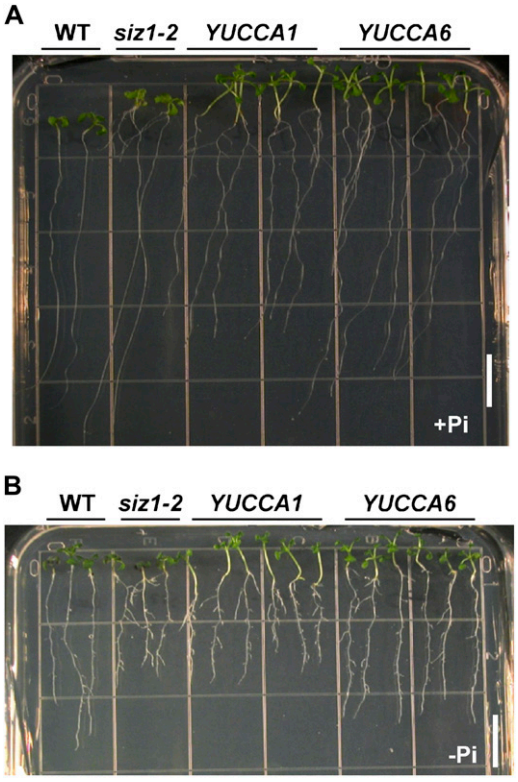

Figure 3. Seedlings overexpressing *YUCCA1* show a hyperresponse phenotype to Pi deficiency in the root system architecture. Wild-type (WT) and *siz1*-2 seedlings, as well as seedlings overexpressing *YUCCA1* and *YUCCA6*, were incubated on Pi-sufficient (A) or Pi-deficient (B) medium. The flavin monooxygenases gene, *YUCCA*, plays an important role in auxin biosynthesis (Zhao et al., 2001; Cheng et al., 2006; Kim et al., 2007). Seedlings overexpressing *YUCCA1*, but not seedlings overexpressing *YUCCA6*, exhibited a shorter PR and longer LR. The experimental procedure is the same as that described in Figure 1. Bars = 10 mm. Quantitative data of PR growth and LR density are shown in Supplemental Figure S2. [See online article for color version of this figure.]

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 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.

DR5::*uidA* expression was detected in the pericycle and LR primordia immediately after the transfer to low-Pi medium (1 d in Fig. 4, C and D). Coincident with earlier LR development (Fig. 1, A and B; Supplemental Fig. S1B), GUS expression was evident sooner after the transfer to low-Pi medium in *siz1* relative to wild-type seedlings (Fig. 4, C and D). After 7 d or more of treatment under Pi deficiency, GUS expression in older LR tips was decreased in comparison with younger LR tips (Fig. 4, E and F). At 9, 11, and 14 d, the older LR tips of *siz1-2* did not show any GUS activity (Fig. 4F) but the LR tips of wild-type seedlings still exhibited low levels of GUS activity (Fig. 4E). These data suggest that the LR development and meristem senescence of *siz1* seedlings occurred more rapidly after the transfer to low Pi than they did for wild-type seedlings.

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

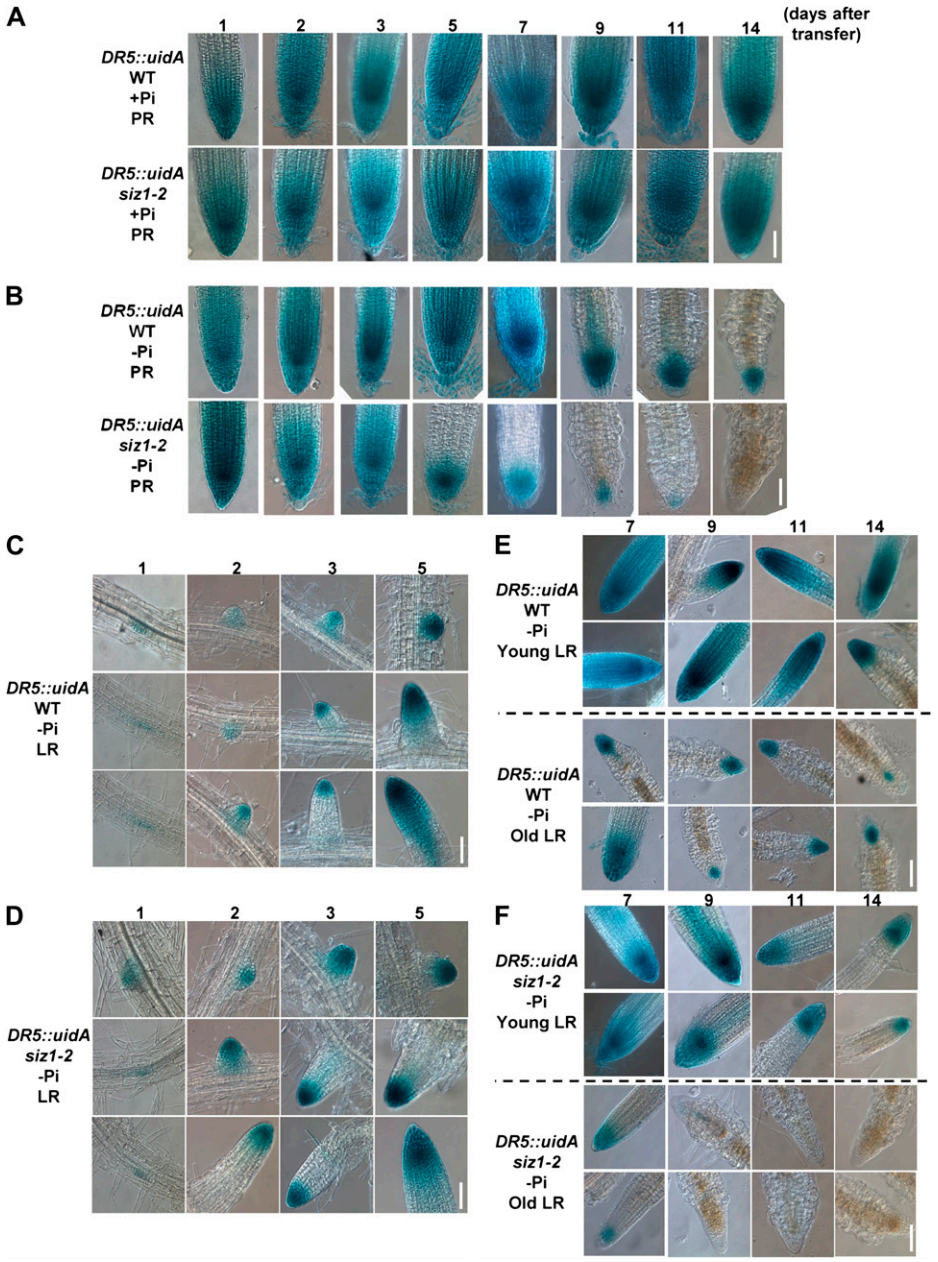

Figure 4. *DR5*::*uidA* expression in PR tips and LR tips. A, No significant change was observed in high-Pi conditions. B, *DR5*::*uidA* expression in the PR tips of the wild type (WT) was increased after 3 d of growth under the low-Pi condition and was then decreased. In contrast, the expression in *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

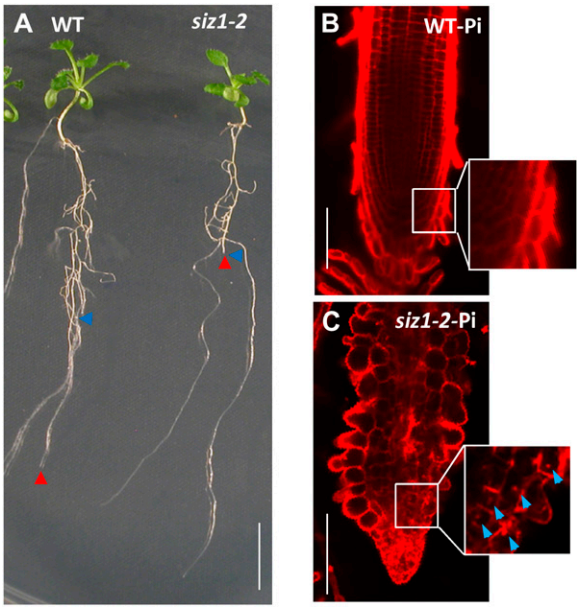

Figure 5. Recovery of PR. A, Nine days after the Pi starvation treatment, the seedlings were transferred onto the medium that was supplemented with 1.25 mM KH_2PO_4 and incubated for 5 d. The blue and red arrowheads indicate the start (just after transfer onto Pi-supplied medium) and end (5 d after the transfer) points, respectively. The root growth of the LR was observed in both wild-type (WT) and *siz1-2* seedlings. B and C, Nine days after the treatment of the Pi-deficient (−Pi) condition, the PR of wild-type and *siz1-2* seedlings was stained with propidium iodide, as described previously (Miura et al., 2010). B, In wild-type seedlings, only the cell membranes were stained because intact cell membranes block the penetration of propidium iodide. C, In *siz1-2* seedlings, the cells were disordered, and propidium iodide can pass through damaged cell membranes and intercalate with DNA. The arrowheads indicate nuclei intercalated with the propidium iodide. Bars = 10 mm (A) and 100 μm (B and C). [See online article for color version of this figure.]

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*, *GLH*, 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* seed-

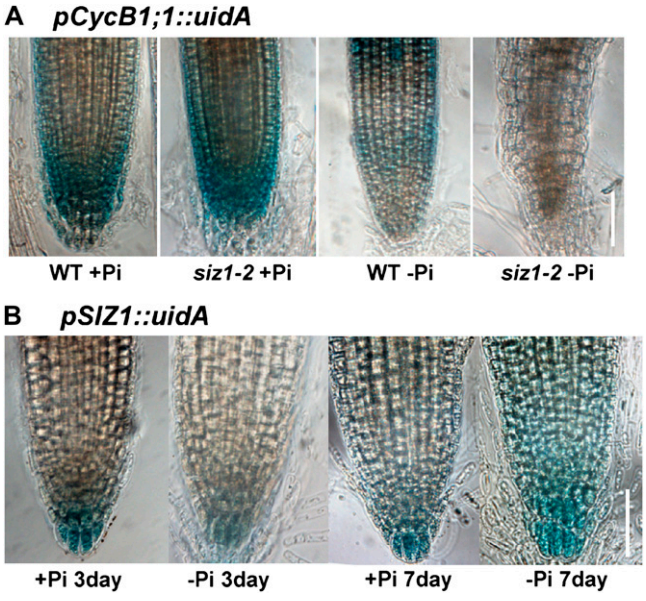

Figure 6. A, *pCycB1;1*::*uidA* expression in PR tips 7 d after the transfer into high- and low-Pi conditions. The *pCycB1;1*::*uidA* expression in the PR tips of *siz1-2* seedlings grown under Pi starvation was substantially decreased. B, *pSIZ1*::*uidA* expression in PR tips at 3 and 7 d after the transfer onto Pi-sufficient and starvation media, respectively. The expression of *pSIZ1*::*uidA* at 3 d after the transfer was substantially decreased. WT, Wild type. Bars = 100 μm. [See online article for color version of this figure.]

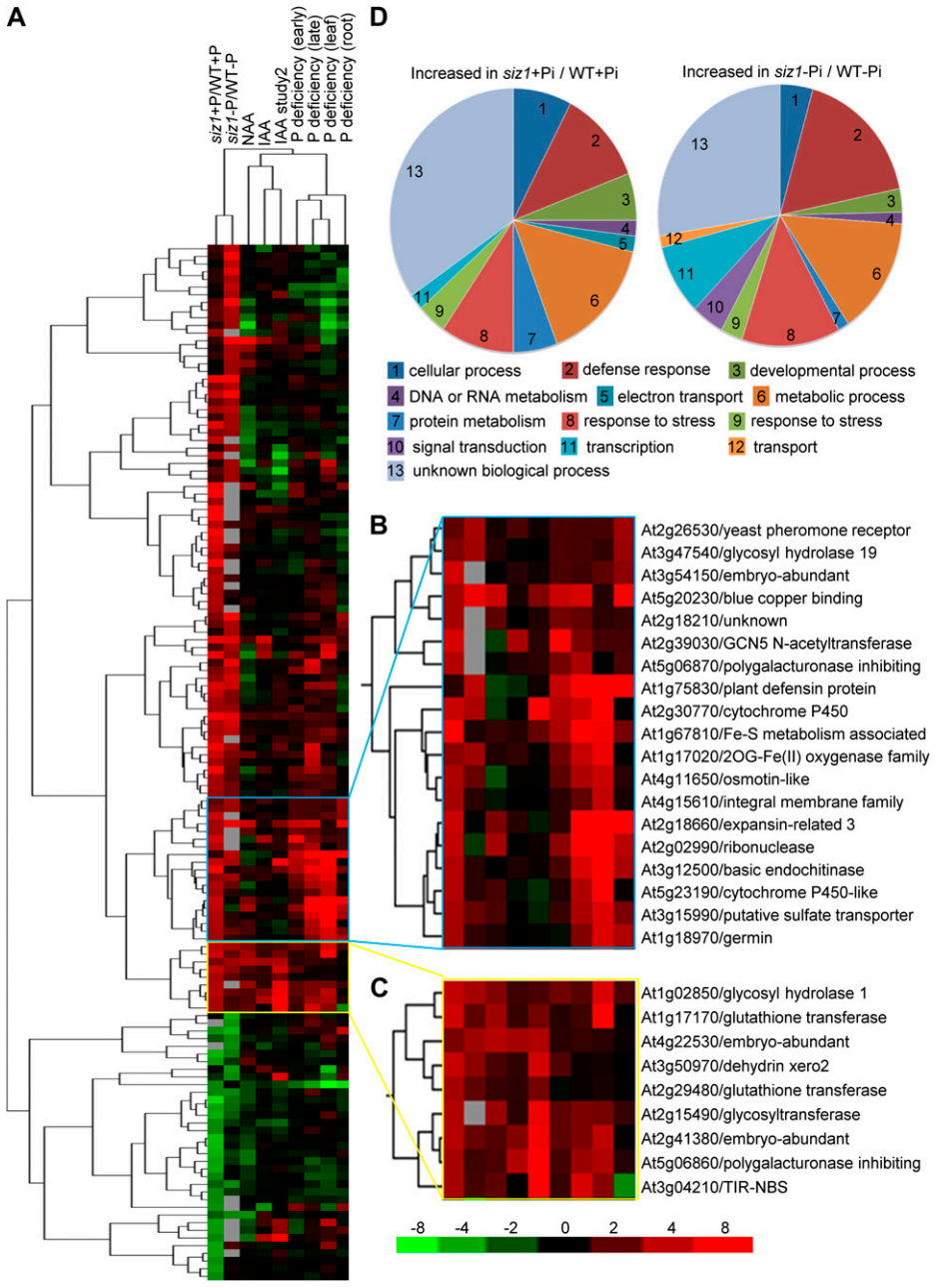

Figure 7. Functional categorization of *SIZ1*-regulated genes. A, Cluster analysis of transcripts, which were up- or down-regulated in *siz1-2*, before and after Pi starvation, with NAA, IAA, and Pi-deficient treatments. B and C, The expression data after NAA, IAA, and Pi-deficient treatments were obtained from Genevestigator. Two of the clusters were magnified. The genes, which were up-regulated in *siz1-2* and under Pi deficiency but were not significantly altered by auxin, were included in the cluster (B). In another cluster, the genes were up-regulated in *siz1-2* and by Pi starvation and auxin (C). The genes encoding glycosyl hydrolase and glycosyl transferase were included in this cluster (C). D, Functional classification of *SIZ1*-regulated genes. Genes whose expression levels were increased in *siz1* under the Pi-sufficient (left panel) and Pi-deficient (right panel) conditions were classified based on their GO biological processes. WT, Wild type. [See online article for color version of this figure.]

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

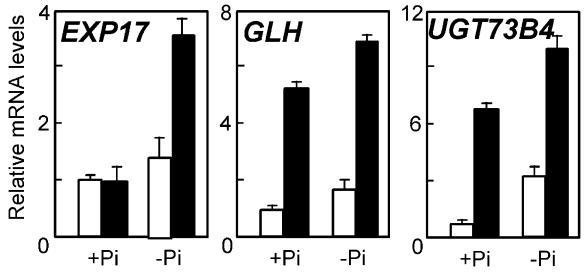

Figure 8. The expression levels of *EXP17*, *GLH*, and *UGT73B4* were up-regulated in *siz1* seedlings. Seedlings 3.5 d old were transferred onto medium that was supplemented with 1.25 mM (+Pi) or 0.0125 mM (−Pi) KH_2PO_4 and were incubated for 3 d. The relative mRNA levels were determined by quantitative RT-PCR analysis. White bars, wild type; black bars, *siz1* mutant. The data shown are means ± SD ($n = 3$).

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. 4B and 10, A–C), *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. 10D). 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

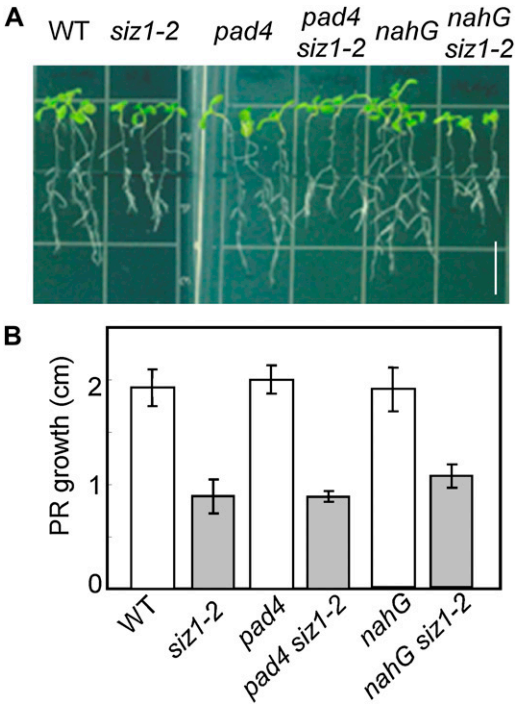

Figure 9. The response to Pi deficiency is independent of SA signaling. The *siz1-2* dwarf-like phenotype is suppressed by the expression of the *nahG* gene or the *pad4* mutation (Lee et al., 2007). Wild-type (WT), *siz1-2*, *pad4*, *nahG*, *siz1-2 pad4*, and *siz1-2 nahG* seedlings at 3.5 d old that were grown on high-Pi medium were transferred onto medium containing 0.0125 mM KH_2PO_4. A, This photograph was taken 7 d after the transfer. B, The growth of PR was scored. All values shown represent means of 12 seedlings ± SE. Bar = 10 mm. [See online article for color version of this figure.]

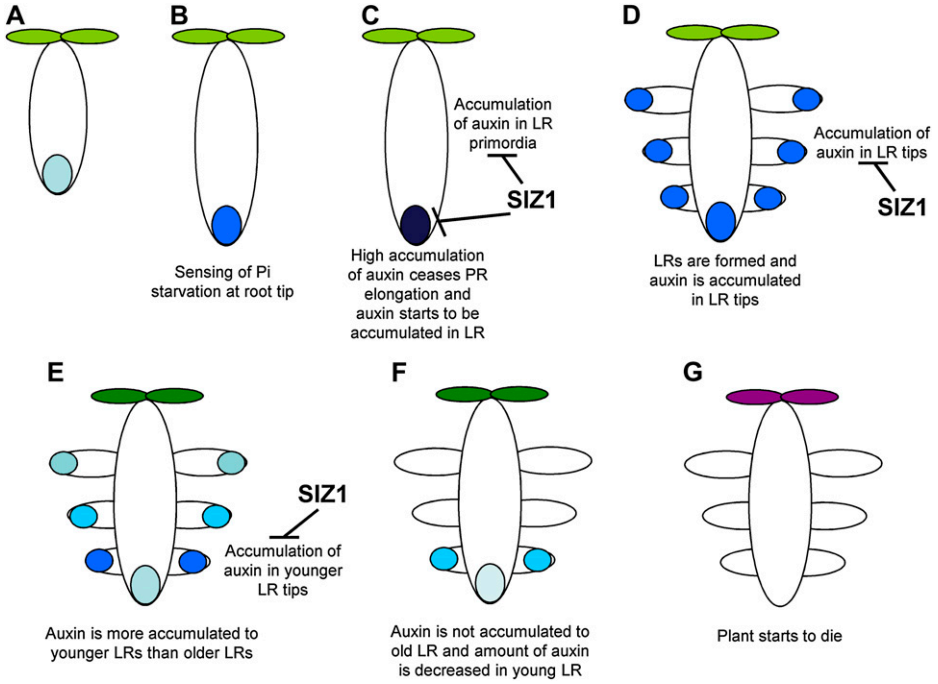

Figure 10. A model illustrating that *SIZ1* negatively regulates the Pi starvation-induced root system architecture modification through controlling the auxin accumulation. After Pi starvation (A), the plants somehow sense Pi starvation (B). Then, auxin is accumulated in the PR root tip, and PR elongation is inhibited (C). Auxin is then accumulated in the LR primordia and tips for elongation (D). Auxin accumulates more in younger LR tips than it does in older LR tips (E). After auxin accumulation in the LR tips cease (F), the plants are not able to survive (G). *SIZ1* is involved in the negative regulation of auxin accumulation in the PR and LR tips (C–E). [See online article for color version of this figure.]

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 ΨKXE/D, where Ψ 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; Marín-Rodríguez 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 PttCel9A 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 up-regulated 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 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 *AtPHT1;4* and *AtPS2* 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-PHO2 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-PHO2 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. CS6559 and CS6560, 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 KH_2PO_4 (Miura et al., 2005). The basal medium contained 1/10× MS macronutrients (2.05 mM NH_4NO_3, 1.8 mM KNO_3, 0.3 mM $CaCl_2$, and 0.156 mM $MgSO_4$) and 1× MS micronutrients (100 μM H_3BO_3, 100 μM $MnSO_4$, 30 μM $ZnSO_4$, 5 μM KI, 1 μM Na_2MoO_4, 0.1 μM $CuSO_4$, 0.1 μM $CoCl_2$, 0.1 mM $FeSO_4$, and 0.1 mM Na_2EDTA). 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$_{DR5::uidA}$ and Columbia$_{pCycB1;1::uidA}$ Arabidopsis plants were kindly provided by Dr. Angus Murphy (Purdue University). The homozygous progeny of genetic crosses with *DR5*::*uidA* or *CycB1;1*::*uidA* and *siz1-2* were used for the analysis of GUS expression. To make Columbia$_{pSIZ1::uidA}$, 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 KH_2PO_4) and high-Pi (1.25 mM KH_2PO_4) 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-glucuronide 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*::*uidA*, the GUS reaction buffer was supplemented with 3.5 mM each $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$. 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 KH_2PO_4 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://jtreeview.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 Genevestigator (http://www.genevestigator.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-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 mM Pi for the same time period (experiment AT-00122); Pi deficiency (shoot or root), Col-0 seeds 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 (+Pi) or 0.0125 mM (−Pi) KH_2PO_4. 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 Kits (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 Genome Initiative or GenBank/EMBL data libraries under the following accession numbers: CYCB1;1 (At4g37490), EXP17 (At4g01630), GLH1 (At1g02850), PAD4 (At3g52430), SIZ1 (At5g60410), UGT73B4 (At2g15490), YUCCA1 (At4g32540), YUCCA6 (At5g25620), and nahG (YP_534831).

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* 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 *siz1-2* 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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