OsSIZ1, a SUMO E3 Ligase Gene, is Involved in the Regulation of the Responses to Phosphate and Nitrogen in Rice

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SIZ1-mediated SUMOylation regulates hormone signaling as well as abiotic and biotic stress responses in plants. Here, we investigated the expression profile of OsSIZ1 in rice using quantitative reverse transcription–PCR (qRT–PCR) and pOsSIZ1–GUS transgenic plants, and the function of OsSIZ1 in the responses to phosphate and nitrogen using a reverse genetics approach. OsSIZ1 is constitutively expressed throughout the vegetative and reproductive growth of rice, with stronger promoter activities in vascular bundles of culms. ossiz1 mutants had shorter primary roots and adventitious roots than wild-type plants, suggesting that OsSIZ1 is associated with the regulation of root system architecture. Total phosphorus (P) and phosphate (Pi) concentrations in both roots and shoots of ossiz1 mutants were significantly increased irrespective of Pi supply conditions compared with the wild type. Pi concentration in the xylem sap of ossiz1 mutants was significantly higher than that of the wild type under a Pi-sufficient growth regime. Total nitrogen (N) concentrations in the most detected tissues of ossiz1 mutants were significantly increased compared with the wild type. Analysis of mineral contents in ossiz1 mutants indicated that OsSIZ1 functions specifically in Pi and N responses, not those of other nutrients examined, in rice. Further, qRT–PCR analyses revealed that the expression of multiple genes involved in Pi starvation signaling and N transport and assimilation were altered in ossiz1 mutants. Together, these results suggested that OsSIZ1 may act as a regulator of the Pi (N)-dependent responses in rice.

Keywords: Nitrogen • OsSIZ1 • Phosphate • Regulation • Rice.

Abbreviations: AMT, ammonium transporter; GUS, β-glucuronidase; N, nitrogen; NRT, nitrate transporter; P, phosphorus; Pi, phosphate; PT, phosphate transporter; qRT–PCR, quantitative reverse transcription–PCR; SUMO, small ubiquitin-related modifier; WT, wild type.

Introduction

Phosphorus (P) and nitrogen (N) are essential for plant growth and development. In addition to being a component of nucleic acids and phospholipids, P plays important roles in signal transduction, energy transfer, photosynthesis and respiration (Plaxton and Carswell 1999). N is a primary constituent of the nucleic acids and proteins that are vital for life (Xu et al. 2012). Plant growth and development is a dynamic and complex process often subject to nutrient limitation. This is particularly true of P and N availability, which are the major growth-limiting nutrients in natural environments. On encountering phosphate (Pi) or N limitation, plants undergo an array of acclimation responses, e.g. the activation of numerous genes, leading to changes in many physiological and morphological processes in plants (Franco-Zorrilla et al. 2004, Yang and Finnegan, 2010, Xu et al. 2012).

With recent advances in genomics, numerous genes involved in these adaptive responses have been intensively studied (Schachtman and Shin 2007, Chio and Lin 2011, Secco et al. 2011, Xu et al. 2012, Rojas-Triana et al. 2013). PHR1 and PHL1 in Arabidopsis play central roles in the plant’s response to Pi starvation, as most of the transcriptional activation and repression responses to Pi starvation are affected in phr1 and phl1 mutants (Rubio et al. 2001, Bustos et al. 2010). OsPHR2 is an ortholog of AtPHR1 in rice, and is an important regulator of downstream genes involved in Pi starvation signaling, such as phosphate transporter (PT) genes, which are responsible for Pi uptake and translocation (Zhou et al. 2008, Liu et al. 2010). miR399 serves as a systemic Pi starvation signal that negatively regulates the transcription of a ubiquitin E2-conjugating enzyme gene, PHO2 (Bari et al. 2006, Chio and Lin 2006, Lin et al. 2009, Hu et al. 2011). IPS1 is specifically induced by Pi starvation and acts downstream of PHR1 to inhibit the miR399-induced cleavage of PHO2 transcripts (Franco-Zorrilla et al. 2007, Wu et al. 2013). Four and six SPX genes have been identified in Arabidopsis and rice, respectively, and are involved in Pi starvation signaling (Duan et al. 2008, Z. Wang et al. 2009). OsSPX1, which regulates the expression of OsSPX2, OsSPX3 and OsSPX5 depending on Pi supply conditions, is specifically induced by Pi starvation, and suppression of OsSPX1 resulted in overaccumulation of Pi (C. Wang et al. 2009, Z. Wang et al. 2009). PHO1 encodes an SPX domain-containing protein, and is the first isolated gene involved in Pi loading into the xylem of roots in Arabidopsis (Poirier et al. 1991, Hamburger et al. 2002). Three PHO1 homologs have been characterized in rice, and OsPHO1;2 is involved in the long-distance transport of Pi.
from roots to shoots (Secco et al. 2010). Recently, degradation of PHO1 was found to be PHO2 dependent in Arabidopsis (Liu et al. 2012), and translation of PHO1;2 was found to be promoted by cis-NATPHO1;2 (a cis-natural antisense transcript) in rice (Jabnoune et al. 2013).

In comparison, the most striking advances in understanding the regulation of N use in plants during the past decades have mainly been in identifying transporters for nitrate and ammonium along with the functions of plant-specific nitrate sensors and N-dependent transcription factors (Krouk et al. 2010, Alvarez et al. 2012, Wang et al. 2012, Xu et al. 2012). Some members of the nitrate and ammonium transporter families (NRT2, NPF and AMT) have been identified in Arabidopsis and rice (Hsu and Tsay 2013, Ranathunge et al. 2014, Xia et al. 2015). AtNPF6.3 (NRT1.1, CHL1) functions as a nitrate sensor responsible for the nitrate-regulated transcriptional response (Ho et al. 2009, Parker and Newstead 2014). In addition, it has been reported that the transcription factors NLP7 and LBD37/38/39 are positive and negative regulators, respectively, of nitrate-related genes, for example NRT and NIA (Castaings et al. 2009, Rubin et al. 2009).

SI1 is a SIZ/PIAS-type SUMO (small ubiquitin-related modifier) E3 ligase with pivotal roles in the plant’s response to environmental stresses, such as drought (Catala et al. 2007), cold (Miura et al. 2007b), salt (Miura et al. 2011b) and heat shock (Yoo et al. 2006). It acts both positively and negatively in controlling Pi starvation responses (Miura et al. 2005), and positively controls N assimilation by promoting SUMOylation of nitrate reductases in Arabidopsis (B.S. Park et al. 2011). In rice, both OsSIZ1 and OsSIZ2 were moderately expressed in different tissues (Chaikam and Karlson 2010). Mutation of OsSIZ1 caused serious growth and developmental defects in rice (Thangasamy et al. 2011, Wang et al. 2011). Heterologous expression of OsSIZ1 in creeping bentgrass enhanced the resistance to abiotic stresses (Li et al. 2013). These findings indicate that SIZ1 plays important roles in the plant’s response to environmental stresses and plant development.

In this study, we investigated the functions of OsSIZ1 in Pi and N homeostasis in rice. We found that OsSIZ1 was expressed throughout the plant’s life cycle and was not very responsive to either Pi or N starvation at the transcript level. Mutation of OsSIZ1 increased either the P or N concentration and changed the Pi distribution in various tissues of OsSIZ1;ossiz1-1 and OsSIZ1;ossiz1-2, each containing a T-DNA insertion within the 15th exon of OsSIZ1, and its WT (cv. Dongjin) were used in this work (Supplementary Fig. S1A, B). To detect the transcripts of OsSIZ1 in the homozygous mutant lines, the leaves of 5-week-old mutant and WT plants were harvested. RT–PCR analysis revealed the absence of OsSIZ1 transcript in ossiz1-1 and ossiz1-2, respectively (Supplementary Fig. S1C), confirming the knockout of this gene. The two lines were subsequently used for in-depth characterization.

WT plants and ossiz1-1 mutants were grown in hydroponic culture. The mutants showed shorter primary roots than the WT at 7 d (Fig. 2A) and 10 d (Fig. 2B), respectively. Time-course analysis showed that ossiz1-2 mutants began to show shorter primary roots 2 d after germination (Fig. 2C). After 7 d, the primary root length of ossiz1-1 mutants was approximately 29% shorter than that of the WT (Fig. 2A, C). Ten-day-old seedlings were subjected to different Pi supply for another 14 d. Significant reductions in the length were obtained in the five longest adventitious roots of ossiz1-1 compared with WT plants under both +P and –P conditions (Fig. 2D, E).

**Results**

**OsSIZ1 is constitutively expressed, and not very responsive to phosphate and nitrogen starvation in rice**

We determined the spatial expression pattern of OsSIZ1 in aerial parts of rice, and found that OsSIZ1 was expressed in all organs examined, with the expression being strongest in leaves and lowest in flowers (Fig. 1A). To investigate the expression of OsSIZ1 in response to Pi and N starvation, RNAs from roots and leaves of rice (Oryza sativa L. ssp. japonica cv. Nipponbare) subjected to different concentrations of Pi and N, respectively, were extracted for qRT–PCR analysis. The results showed that OsSIZ1 was not very responsive to Pi and N starvation at the transcript level in either leaves or roots (Fig. 1B).

To analyze the tissue-specific expression of OsSIZ1 in rice, a 2,318 bp fragment immediately upstream of the translation start site was fused to the β-glucuronidase (GUS) reporter gene, and transformed into rice (cv. Nipponbare). As shown in Fig. 1C, GUS expression driven by the OsSIZ1 promoter was constitutive in various tissues throughout the plants’ life cycle. GUS-stained organs and their cross-sections showed strong expression in the lateral root primordia, root epidermis, root cortex, root endodermis, root and shoot vascular bundles and leaf mesophyll cells (Fig. 1C–vi). In culms, GUS activity was the strongest in vascular bundles (Fig. 1Cvii, viii). In flowers, GUS activity was observed in the lemma, palea, anther and stigma (Fig. 1Cix, x). In seeds, GUS activity was detected in the embryo, aleurone layer, pericarp layer, and weakly in the endosperm (Fig. 1Cxi). In addition, strong GUS activity was detected in both the coleoptile and coleorhiza of germinating seeds (Fig. 1Cxii).

**OsSIZ1 is involved in the regulation of root architecture independently of Pi supply conditions**

To determine the function of OsSIZ1 in rice, two mutant lines, ossiz1-1 and ossiz1-2, each containing a T-DNA insertion within the 15th exon of OsSIZ1, and its WT (cv. Dongjin) were used in this work (Supplementary Fig. S1A, B). To detect the transcripts of OsSIZ1 in the homozygous mutant lines, the leaves of 5-week-old mutant and WT plants were harvested. RT–PCR analysis revealed the absence of OsSIZ1 transcript in ossiz1-1 and ossiz1-2, respectively (Supplementary Fig. S1C), confirming the knockout of this gene. The two lines were subsequently used for in-depth characterization.

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different Pi or N supply. Compared with the WT, ossiz1 mutants exhibited a negative growth response under various treatments (Figs. 3A, B, Fig. 4A, B, Supplementary Fig. S2). The biomass of ossiz1-1 and ossiz1-2 was significantly lower than that of WT plants independently of Pi supply conditions (Supplementary Fig. S2A). The root/shoot ratios of ossiz1-1 and ossiz1-2 were similar to those of WT plants under +P conditions, and were significantly reduced compared with WT plants under –P conditions (Supplementary Fig. S2B). Irrespective of Pi supply, total P and Pi concentrations in roots and shoots were significantly higher in ossiz1-1 and ossiz1-2 than in WT plants, respectively. Under +P conditions, total P concentrations of ossiz1-1/ossiz1-2 were 51.8%/57.4% and 20.9%/29.0% higher in roots and shoots than those of WT plants, respectively (Fig. 3C). Under –P conditions, total P concentrations of ossiz1-1/ossiz1-2 were 54.0%/85.9% and 25.4%/39.5% higher in roots and shoots than those of WT plants, respectively (Fig. 3D). Similarly, the Pi concentrations in ossiz1 mutants were increased more in roots than in shoots compared with WT plants under both +P and –P conditions (Fig. 3E, F).

Similar to the finding in Pi supply treatments, the biomass of ossiz1-1 and ossiz1-2 was significantly lower than that of WT plants under both +N and –N conditions, and the root/shoot ratios of ossiz1-1 and ossiz1-2 were reduced compared with WT plants under –N conditions (Supplementary Fig. S2C, D). The total N concentration in roots of ossiz1 mutants was significantly increased compared with that of the WT under both +N and –N conditions (Fig. 4C, D). Under +N conditions, total
Fig. 2 Root architectural features of wild-type (WT) and ossiz1 mutants. (A, B) Primary root growth of 7- and 10-day-old seedlings of WT and ossiz1 mutants. (C) Primary root length of WT and ossiz1 mutant seedlings from germination to 7 d old. Data are means ± SE (n = 15). Significant differences between WT and ossiz1 plants at each time point are indicated with asterisks (P < 0.05, one-way ANOVA). (D) Root growth of 24-day-old WT and ossiz1 mutants grown under +P (200 μM Pi) and –P (10 μM Pi) conditions for 14 d. (E) Average length of the five longest adventitious roots of the WT and ossiz1 mutants grown under +P and –P conditions for 14 d. Data are means ± SE (n = 10). Different letters indicate significant differences according to two-way ANOVA (P < 0.05). Scale bars = 2 cm.
N concentrations in roots of *ossiz1-1*/*ossiz1-2* were 15.3%/17.8% higher than those of WT plants. Under –N conditions, total N concentrations in roots of *ossiz1* mutants grown under +P and –P conditions. Scale bars = 10 cm. (C, D) Total P concentration in shoots and roots of WT and *ossiz1* plants grown under +P and –P conditions. (E, F) Pi concentration in shoots and roots of WT and *ossiz1* plants grown under +P and –P conditions. Data are means ± SE (n = 5). Significant differences between the WT and *ossiz1* mutants in roots or shoots are indicated with asterisks (P < 0.05, one-way ANOVA).

To analyze the effects of *OsSIZ1* mutation on P (N) concentrations throughout the plant’s life cycle, plants were grown in a greenhouse in pots containing soil with different levels of available Pi or N. Mutation of *OsSIZ1* affected not only the vegetative growth, but also the development of reproductive organs. The height of plants, length of panicles and 1,000-grain weight of *ossiz1* plants were significantly lower than those of the WT (*Supplementary Fig. S3*). As shown in *Fig. 5A–F*, total P and total N concentrations in leaf blades, culms and grains of *ossiz1* mutants were significantly higher
than those of WT plants under different soil Pi and N supply treatments.

To confirm that OsSIZ1 may play a role in regulating Pi transport from roots to shoots, we measured the Pi concentration in the xylem sap of the culms of WT, ossiz1-1 and ossiz1-2 plants (Fig. 5G). The Pi concentration in the xylem sap of ossiz1-1 and ossiz1-2 was 980 and 790 μmol l⁻¹, respectively, which was 100% and 61% higher than that of the WT (480 μmol l⁻¹), indicating that mutation of OsSIZ1 enhanced Pi transportation to shoots. We also determined the P distribution among various tissues of shoots at the harvest stage. P contents in the vegetative tissues of ossiz1-1 and ossiz1-2 were 2.8- and 2.5-fold higher than those in WT plants, respectively, whereas in the reproductive tissues of ossiz1-1 and ossiz1-2, P contents were 43% and 32% of that in WT plants, respectively (Supplementary Fig. S4). These results indicated that mutation of OsSIZ1 altered P distribution in the different tissues of ossiz1 plants.

ossiz1 mutants displayed a seriously low seed-setting rate, and subsequently, a great reduction in grain yield per plant (Fig. 6A). To assess whether the large reduction in seed-setting rate led to P and N accumulation in various tissues of ossiz1 mutants, we mimicked the low seed-setting rate of ossiz1 mutants by either extracting the immature embryos or removing the florets from WT plants at the flowering stage (Fig. 6B). As shown in Fig. 6, artificial reduction of the seed-setting rate in the WT plants caused few effects on the increase of P and N concentrations, especially in the vegetative tissues, compared with that in ossiz1 plants. These results suggested that the increase of P and N concentrations in ossiz1 mutants are mainly due to defects in OsSIZ1, not to a reduction in seed-setting.

Mutation of OsSIZ1 specifically affected P and N accumulation, not that of other nutrients detected in rice

The results above showed an effect of OsSIZ1 on P and N accumulation in rice. To determine whether the concentrations of other nutrients were affected by the mutation of OsSIZ1, we performed analyses of eight nutrients, i.e. K, Ca, Mg, Mn, Zn, Fe, Cu and Si, in flag leaf blades, culms and grains of WT and...
ossiz1-1 plants, and P analysis was used as a control. As shown in Table 1, the P concentration of these tissues in ossiz1 mutants was significantly higher than that in the WT. However, there were no significant differences in the concentrations of other nutrients detected in flag leaf blades, culms and grains in ossiz1-1 compared with WT plants, except for the reduced Cu and Mg concentrations in flag leaf blades and culms of ossiz1-1, respectively, and the increased Zn concentration in culms of ossiz1-1 (Table 1). These results indicated that mutation of OsSIZ1 predominantly affects P and N homeostasis, not other nutrients detected in ossiz1 mutants.

**Mutation of OsSIZ1 changed the expression of genes involved in Pi starvation signaling**

To gain insight into the molecular mechanisms underlying the enhanced total P and Pi concentrations in ossiz1 mutants under different Pi supply conditions, we investigated the expression of genes involved in Pi starvation signaling in the roots of ossiz1 mutants and WT plants. There are 13 putative genes encoding high-affinity PTs in rice (Paszkowski et al. 2002). Compared with the WT, the transcripts of most PT genes detected in this study were higher or lower in ossiz1 mutants under both +P and –P conditions (Fig. 7). The expression level of OsPT2 in ossiz1 mutants was about 60% of that in the WT under +P conditions and reduced to about 20% of that in the WT under –P conditions (Fig. 7). The expression level of OsPT6 in ossiz1 mutants was <40% of that in the WT under both +P and –P conditions (Fig. 7). The expression of OsPT3 and OsPT4 in ossiz1 mutants was greatly suppressed under –P conditions and there was no change under +P conditions (Fig. 7). In contrast, OsPT1 under –P conditions and OsPT8 under both +P and –P conditions were significantly up-regulated in ossiz1 mutants (Fig. 7), which probably resulted in the increase of total P and Pi concentrations in ossiz1 mutants (Figs. 3, 5).

We detected the expression levels of several Pi-regulated genes in ossiz1 mutants (Fig. 8). OsSIZ1 was down-regulated in ossiz1 mutants under both +P and –P conditions (Fig. 8). Compared with the WT, OsSIZ1 was constitutively suppressed in ossiz1 mutants (Fig. 8). In contrast, their expression levels were similar to that of WT plants under –P conditions (Fig. 8). OsSIZ1 was constitutively suppressed under both +P and –P conditions, and OsSIZ1 was suppressed under –P conditions (Fig. 8). We also evaluated the expression of two transcription factor genes, OsSIZ1 and OsSIZ2, which were differentially suppressed in ossiz1 mutants. OsSIZ1 and OsSIZ2 were constitutively suppressed under both +P and –P conditions, and OsSIZ1 was suppressed under –P conditions (Fig. 8). Together, these results indicated that OsSIZ1 is involved in the responses to Pi through regulating the expression of these regulatory genes in rice.

**Mutation of OsSIZ1 changed the expression of genes involved in nitrogen uptake and assimilation**

To investigate whether mutation of OsSIZ1 affected N uptake-related genes, we determined the expression of both nitrate and ammonium transporters in the WT and ossiz1 mutants under

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**Fig. 5** Total P and total N concentrations in the tissues and Pi concentrations in the xylem sap of wild-type (WT) and ossiz1 plants. (A, D) Leaf blades; (B, E) culms; and (C, F) grain. Seedlings were grown in a pot experiment containing varying concentrations of: phosphate, LP (0 mg fertilizer Pi kg–1 soil), MP (80 mg fertilizer Pi kg–1 soil), HP (160 mg fertilizer Pi kg–1 soil); and nitrogen, –N (100 mg fertilizer N kg–1 soil), +NH4+ (400 mg fertilizer NH4+ kg–1 soil), +NO3− (400 mg fertilizer NO3− kg–1 soil), +NH4+ + NO3− (400 mg fertilizer NH4+ kg–1 soil) and +N (400 mg fertilizer N kg–1 soil). Samples were collected and analyzed at the grain harvest stage. Data are means ± SE (n = 5). Different letters indicate significant differences according to two-way ANOVA (P < 0.05). (G) Pi concentrations in xylem sap of WT and ossiz1 plants. Xylem sap was collected at the grain-filling stage 20 d after pollination. Data are means ± SE (n = 5). Different letters indicate significant differences between WT and ossiz1 mutants (P < 0.05, one-way ANOVA).

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different N supply conditions. Two NRT1 genes (OsNRT1.1 and OsNRT1.2), two NRT2 genes (OsNRT2.1 and OsNRT2.2) and three AMT1 genes (OsAMT1.1, OsAMT1.2 and OsAMT1.3) were selected for the qRT–PCR analysis. As shown in Fig. 9, mutation of OsSIZ1 affected the expression of all of these genes, except for OsNRT1.1, in rice. The expression of OsNRT2.1 and OsNRT2.2 was constitutively suppressed in ossiz1 mutants independently of the N supply conditions (Fig. 9). The expression of OsNRT1.2, OsAMT1.2 and OsAMT1.3 was reduced under the +N condition in ossiz1 mutants compared with the WT (Fig. 9). In contrast, the expression of OsNRT1.2, OsAMT1.2 and OsAMT1.3 under −N conditions, and of OsAMT1.1 under both +N and −N conditions was up-regulated, with higher transcript abundance in ossiz1 mutants compared with the WT (Fig. 9), which probably resulted in the higher total N concentrations in ossiz1 mutants (Figs. 4, 5).

It has previously been reported that AtSIZ1 regulates N assimilation in Arabidopsis through its SUMO E3 ligase function (B.S. Park et al. 2011). Moreover, our results showed that mutation of OsSIZ1 increased N accumulation in ossiz1 mutants compared with WT plants at the harvest stage (Fig. 5D–F). To understand the possible function of OsSIZ1 in N assimilation in rice, we investigated the expression levels of N metabolism-related genes, such as nitrate reductase genes (OsNia1 and OsNia2) and glutamine synthase genes (OsGS1.1 and OsGS1.2) in leaves at the grain-filling stage. The results showed that the expression levels of the four genes were significantly up-regulated in leaves of ossiz1 mutants (Fig. 10). These results indicated that OsSIZ1 might regulate the expression of genes involved in N assimilation in rice.

**Discussion**

P and N are two major nutrients required for plant growth and development (Forde and Lorenzo 2001, López-Bucio et al. 2003, Raghothama and Karthikeyan 2005). Understanding the mechanisms of P and N use is an important step to improving growth and productivity of plants, and preventing negative effects on the environment. Previous studies demonstrated that the SUMO E3 ligase, AtSIZ1, controls Pi deficiency responses (Miura et al. 2005) and N assimilation (B.S. Park et al. 2011). Two homologous genes in rice (O. sativa), OsSIZ1 and OsSIZ2, were subsequently isolated (Park et al. 2010, Wang et al. 2011). OsSIZ1 is localized in the nucleus, and mediates SUMOylation in rice, tobacco and creeping bentgrass (Park et al. 2010, Thangasamy et al. 2011, Li et al. 2013). To determine the function of OsSIZ1 in P and N homeostasis in rice, we generated transgenic plants of OsSIZ1 promoter-driven GUS reporter expression as well as two independent OsSIZ1-knockout lines, and then investigated the roles of OsSIZ1 in response to different Pi and N supply conditions by characterizing these transgenic plants.

As shown previously in Arabidopsis (Miura et al. 2005), transcription of OsSIZ1 was not responsive to different Pi supply conditions in rice (Fig. 1B). This may be due to the fact that SUMOylation responds to Pi starvation through modification of proteins that function downstream of SIZ1 (Miura et al. 2007a, B.S. Park et al. 2011). We performed a histochemical analysis using transgenic plants harboring the GUS reporter gene driven by the OsSIZ1 promoter. The results revealed that OsSIZ1 is constitutively expressed throughout the
growth and development of rice (Fig. 1C), suggesting that OsSIZ1 performs important functions throughout the plant’s life. OsSIZ1 is strongly expressed in the root system, including lateral roots, lateral root primordia and root tips (Fig. 1Ci–iii), and acts on root development (Fig. 2), indicating that OsSIZ1 functions in controlling aspects of root development, which is consistent with the findings in Arabidopsis (Catala et al. 2007). Miura et al (2011a) reported that auxin is involved in the AtSIZ1 function. 

Table 1 Mineral concentrations in flag leaf blades, culms and grains of wild-type (WT) and ossiz1 plants

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<tr>
<th></th>
<th>P concentration (mg/g DW)</th>
<th>K concentration (mg/g DW)</th>
<th>Ca concentration (mg/g DW)</th>
<th>Mg concentration (mg/g DW)</th>
<th>Mn concentration (mg/kg DW)</th>
<th>Si concentration (mg/kg DW)</th>
<th>Fe concentration (mg/kg DW)</th>
<th>Zn concentration (mg/kg DW)</th>
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<td>WT</td>
<td>1.82 ± 0.11b</td>
<td>13.35 ± 1.02a</td>
<td>10.46 ± 0.85b</td>
<td>1.97 ± 0.39b</td>
<td>3.13 ± 0.34a</td>
<td>645.5 ± 41.0a</td>
<td>397.1 ± 37.8a</td>
<td>10.00 ± 1.04a</td>
<td>3.38 ± 0.44a</td>
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<td>ossiz1-1</td>
<td>2.35 ± 0.15a</td>
<td>12.27 ± 1.01a</td>
<td>11.34 ± 1.32b</td>
<td>1.90 ± 0.14a</td>
<td>3.27 ± 0.90a</td>
<td>546.6 ± 153.1a</td>
<td>445.0 ± 67.6a</td>
<td>9.40 ± 1.72b</td>
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<td>WT</td>
<td>1.13 ± 0.18b</td>
<td>25.91 ± 2.32a</td>
<td>0.92 ± 0.14a</td>
<td>1.91 ± 0.09a</td>
<td>0.47 ± 0.10a</td>
<td>159.2 ± 51.62a</td>
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<td>0.94 ± 0.25a</td>
<td>1.55 ± 0.10b</td>
<td>0.45 ± 0.07a</td>
<td>121.4 ± 18.24a</td>
<td>186.7 ± 72.1a</td>
<td>113.7 ± 18.76a</td>
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<tr>
<td>WT</td>
<td>3.59 ± 0.13b</td>
<td>2.96 ± 0.06a</td>
<td>0.24 ± 0.027a</td>
<td>1.53 ± 0.05a</td>
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<td>ossiz1-1</td>
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</table>

Data are means ± SE (n = 5). Significant differences between the WT and ossiz1 mutants are indicated with different letters (P < 0.05, one-way ANOVA).

Fig. 7 Expression of Pi transporter genes in the wild type (WT) and ossiz1 mutants. RNAs were extracted from the roots of seedlings grown in standard solution for 10 d, followed by treatments with either +P (200 μM Pi) or –P (10 μM Pi) supply for 14 d. OsActin1 was used as the reference gene. Data are means ± SE (n = 3). Significant differences between the WT and ossiz1 mutants under +P or –P conditions are indicated with asterisks, and ns indicates no significant differences (P < 0.05, one-way ANOVA).
Fig. 8 Expression of Pi regulatory genes in the wild type (WT) and ossiz1 mutants. RNAs were extracted from the roots of seedlings grown in standard solution for 10 d, followed by treatment with either +P (200 μM Pi) or –P (10 μM Pi) for 14 d. OsActin1 was used as the reference gene. Data are means ± SE (n = 3). Significant differences between the WT and ossiz1 mutants under +P or –P conditions are indicated with asterisks, and ns indicates no significant differences (P < 0.05, one-way ANOVA).

Fig. 9 Expression of nitrate and ammonium transporter genes in the wild type (WT) and ossiz1 mutants. Seedlings of WT and ossiz1 plants grown in standard solution for 20 d, followed by deprivation of N for 3 d, and supplied with nutrient solution containing high nitrogen (+N, 2.5 mM NH4NO3–) or low nitrogen (–N, 0.1 mM NH4NO3–) for 24 h. RNAs were extracted from roots. OsActin1 was used as the reference gene. Data are means ± SE (n = 3). Significant differences between the WT and ossiz1 mutants under +N or –N conditions are indicated with asterisks, and ns indicates no significant differences (P < 0.05, one-way ANOVA).
OsSIZ1 acts both positively and negatively to regulate the genes in Pi starvation signaling in rice, which is consistent with the findings in Arabidopsis (Miura et al. 2005).

Pi from the soil is transported to the various tissues of the shoot via the xylem and is re-mobilized inside the plant via the phloem to support plant growth and reproductive development (Ribot et al. 2008). OsSIZ1 was mainly expressed in the vascular bundles in culms (Fig. 1Cvii, viii), and the Pi concentration in the xylem sap was significantly higher in ossiz1 mutants than in the WT (Fig. 5G). These results strongly suggested that OsSIZ1 is involved in Pi translocation or distribution in rice. In addition, the proportion distributed to the reproductive parts was greatly decreased in ossiz1-1 and ossiz1-2 compared with the WT (Supplementary Fig. S4B), which possibly resulted from the effect of a seriously low seed-setting rate (Fig. 6), and greatly reduced sink strength in ossiz1 mutants.

To explore the molecular mechanism by which OsSIZ1 regulated Pi translocation or distribution in rice, we investigated the expression levels of genes involved in long-distance transport of Pi in rice. PHO1 loads Pi into the xylem for the long-distance transport of Pi from roots to shoots in Arabidopsis (Poirier et al. 1991, Hamburger et al. 2002, Stefanovic et al. 2011). Tissue localization analysis showed that OsPHO1;2 was strongly expressed in the vascular bundles of rice (Jabnoune et al. 2013), and mutation of OsPHO1;2 caused defects in transferring Pi from the roots to shoots in rice (Secco et al. 2010). Our qRT–PCR analysis indicated that the transcript level of OsPHO1;2 was up-regulated in ossiz1 mutants compared with WT plants (Fig. 8). It has been reported that PHO2-dependent degradation of PHO1 modulates Pi homeostasis in Arabidopsis (Liu et al. 2012). In our analysis, the expression of OsPHO2 was up-regulated in ossiz1 mutants (Fig. 8). Combined with the observation that OsPHO2 is also strongly expressed in the vascular bundles of rice (Hu et al. 2011), we proposed that a similar mechanism exists for the degradation of PHO1 by PHO2 in rice. Taken together, it is possible that OsSIZ1 regulates the translocation and distribution of Pi via OsPHO1;2 in co-ordination with OsPHO2 in rice, which needs to be verified next.

AtSIZ1 acts upstream of PHR1, and mediates SUMOylation of AtPHR1 (Miura et al. 2005). However, AtSIZ1 affects several Pi starvation response genes that are not controlled by PHR1 (Miura et al. 2005), indicating that SUMOylation has broad effects on the control of adaptation to Pi deficiency. We hypothesized that other proteins are SUMOylated by SIZ1-dependent processes. Many SUMO-modified proteins reside in the nucleus, and most of the targets of AtSIZ1 are transcription factors (Miura and Hasegawa, 2010, H. J. Park et al. 2011). Using the SUMOplot™ prediction program (http://www.abgent.com/sumoplot), potential SUMOylation sites were identified in protein sequences of two transcription factors, OsPHR2 and OsMYB2P-1, and it was found that they contain two and three SUMOylation sites, respectively (Supplementary Table S5).

Furthermore, the mutation of OsSIZ1 changed the transcript abundance of OsPHR2 and OsMYB2P-1 in ossiz1 plants under +P conditions (Fig. 8). These genes encoding transcription factors may thus act downstream of OsSIZ1 as independent regulators of Pi starvation signaling in rice.

regulation of phosphate starvation-induced root architecture remodeling. Whether auxin participates in the OsSIZ1 regulation of root development needs further characterization.

The mutation of OsSIZ1 resulted in the significant increase of total P and Pi concentrations in roots and shoots under both +P and –P concentrations (Figs. 3, 5), which prompted us to evaluate the expression of genes involved in Pi response in rice (Figs. 7, 8). The rice genome contains 13 putative high-affinity PT genes belonging to the Ph1 family (Goff et al. 2002, Paszkowski et al. 2002). Changes in the expression of several OsPT genes have been reported to account for the variations in P concentration in rice (Ai et al. 2009, Jia et al. 2011, Sun et al. 2012, Wang et al. 2014, Zhang et al. 2015). Among 13 Ph1 members, OsPT8 and OsPT1 had higher expression and were not very responsive to Pi starvation in rice (Jia et al. 2011, Sun et al. 2012). Interestingly, mutation of OsSIZ1 increased their expression, especially under Pi starvation in this study (Fig. 7); thus it is possible that this causes the increased acquisition of Pi in ossiz1 mutants (Figs. 3, 5). Conversely, the expression of Pi starvation-induced PT genes, such as OsPT2 and OsPT6 (Ai et al. 2009), was significantly suppressed in ossiz1 mutants under –P conditions (Fig. 7). These results suggested that mutation of OsSIZ1 acts both positively and negatively on the expression of PT genes in rice. miR399 negatively regulates the expression of PHO2 in Arabidopsis (Bari et al. 2006, Chiou et al. 2006). Overexpression of two miR399 homologs in rice, OsmiR399f and OsmiR399j, suppressed the expression of OsPHO2 (Hu et al. 2011). We determined the expression of another rice miR399 homolog, OsmiR399a, and OsPHO2 in ossiz1 mutants and WT plants. OsmiR399a was suppressed, whereas OsPHO2 was induced significantly in ossiz1 mutants independently of Pi supply conditions (Fig. 8), which indicated that OsmiR399a may also negatively regulate the expression of OsPHO2 in rice. Taken together, these results suggest that
Similar to the response to Pi supply, OsSIZ1 transcription was not responsive to different N supply conditions (Fig. 1B), which is possibly due to the mechanism of SUMOylation mediated by SIZ1 functioning at the protein level (Miura et al. 2007a, B.S. Park et al. 2011). The mutation of OsSIZ1 resulted in the alteration of N concentrations in ossiz1 mutants, and meanwhile we noted that the total N concentration in the shoots of ossiz1 mutants did not change significantly at the seedling stage, and was subsequently increased in the shoot tissues of ossiz1 mutants at harvest stage compared with WT plants (Figs. 4, 5). This deviation is possibly related to the different rhizosphere environment, the different absorption rate of N in the different growth media and the different demands of N for metabolism at different developmental stages. To dissect the mechanism by which OsSIZ1 affected N accumulation in rice, the expression of nitrate and ammonium transporter genes and N assimilation-related genes was detected in ossiz1 mutants and WT plants. The expression of OsAMT1.7 was up-regulated under +N conditions, and the expression of OsNRT1.2 and three AMT1 genes (OsAMT1.1, OsAMT1.2 and OsAMT1.3) was up-regulated in ossiz1 mutants under –N conditions (Fig. 9). The induction of these genes may be associated with the N accumulation in ossiz1 mutants. Both nitrate reductase genes (OsNia1 and OsNia2) and glutamine synthase genes (OsGS1.1 and OsGS1.2) were up-regulated in ossiz1 mutants (Fig. 10), indicating that the mutation of OsSIZ1 influenced the N assimilation processes in ossiz1 mutants.

Taken together, OsSIZ1 is involved in the regulation of the responses to various Pi and N regimes in rice, which may be through mediating SUMOylation of the regulators and functional proteins in the Pi and N responses in rice.

Materials and Methods

Generation of the transgenic rice plants

For GUS expression analysis of the OsSIZ1 promoter, the 2318 bp sequence upstream of the coding region of OsSIZ1 was amplified from rice (O. sativa L. ssp. japonica cv. Nipponbare) genomic DNA using the primers listed in Supplementary Table S1. The HindIII and BamHI restriction sites were incorporated into the primers to facilitate cloning into the expression vector (pBPl21). PCR products were first cloned into the pMD19-T vector (TAKARA) for sequence confirmation. After digestion with HindIII and BamHI, the amplified fragments were purified and cloned into the linearized binary vector, pBPl21 (Jefferson et al. 1987), to replace the Cauliflower mosaic virus (CaMV) 35S promoter upstream of the virus gene. The expression vectors were transferred to Agrobacterium tumefaciens (TAKARA) for sequence confirmation. After digestion with (pBI121). PCR products were first cloned into the pMD19-T vector and meanwhile we noted that the total N concentration in the shoots of ossiz1 mutants did not change significantly at the seedling stage, and was subsequently increased in the shoot tissues of ossiz1 mutants at harvest stage compared with WT plants (Figs. 4, 5). This deviation is possibly related to the different rhizosphere environment, the different absorption rate of N in the different growth media and the different demands of N for metabolism at different developmental stages. To dissect the mechanism by which OsSIZ1 affected N accumulation in rice, the expression of nitrate and ammonium transporter genes and N assimilation-related genes was detected in ossiz1 mutants and WT plants. The expression of OsAMT1.7 was up-regulated under +N conditions, and the expression of OsNRT1.2 and three AMT1 genes (OsAMT1.1, OsAMT1.2 and OsAMT1.3) was up-regulated in ossiz1 mutants under –N conditions (Fig. 9). The induction of these genes may be associated with the N accumulation in ossiz1 mutants. Both nitrate reductase genes (OsNia1 and OsNia2) and glutamine synthase genes (OsGS1.1 and OsGS1.2) were up-regulated in ossiz1 mutants (Fig. 10), indicating that the mutation of OsSIZ1 influenced the N assimilation processes in ossiz1 mutants.

Taken together, OsSIZ1 is involved in the regulation of the responses to various Pi and N regimes in rice, which may be through mediating SUMOylation of the regulators and functional proteins in the Pi and N responses in rice.

Hydroponic, pot and field experiments

Rice seeds were surface sterilized for 1 min with 75% ethanol (v/v), and for 30 min with diluted (15 : v/v) NaClO (including 5.5% of efficient Cl), followed by thorough rinsing for 30 min with deionized water. Seeds were germinated in darkness at 25 °C for 3 d. The hydroponic experiments were carried out in a growth room with a 16 h light (30 °C)/8 h dark (22 °C) photoperiod, and the relative humidity was maintained at approximately 70%. Ten-day-old rice seedlings were transferred to complete nutrient solution containing 1.25 mM NH4NO3, 200 μM KH2PO4, 0.35 mM K2SO4, 1 mM CaCl2, 1 mM MgSO4, 0.5 mM Na2SiO3, 20 μM Fe-EDTA, 20 μM H3BO3, 9 μM MnCl2, 0.32 μM CuSO4, 0.77 μM ZnSO4 and 0.39 μM Na2MoO4. KH2PO4 (200 μM) and KH2PO4 (10 μM) were used as P-sufficient (+P) and P-deficient (−P) treatments, respectively, and 2.5 mM NH4NO3 and 0.1 mM NH4NO3 were used as N-sufficient (+N) and N-deficient (−N) treatments, respectively. The initial pH of the solution was adjusted to 5.5, and deionized water was used throughout the experiments. The nutrient solution was replaced every 3 d. After 10 d, plants were transferred from standard nutrient solution to either + P or – N solutions for 14 or 10 d, respectively. Root and leaf samples were collected for observation of phenotype, determination of total P, Pi and total N concentrations, and qRT–PCR analysis of genes involved in Pi starvation signaling. For qRT–PCR analysis of the nitrate and ammonium transporters, 20-day-old seedlings grown in N-sufficient condition were deprived of N for 3 d, and resupplied with nutrient solution containing 2.5 mM NH4NO3 (−N) or 0.1 mM NH4NO3 (–N) for 24 h. In experiments involving transgenic plants of OsSIZ1 promoter-driven GUS reporter expression, the seeds were germinated and screened in a solution containing 25 mg l−1 hygromycin for 7 d before being transferred to the hydroponics system.

Pot experiments were performed with five replications in a greenhouse using soil collected from an experimental farm at Nanjing Agricultural University. The acidic soil (pH 5.0; soil : water = 1 : 1) contained 6.5 mg Pi kg−1 extracted by the Bray I method (Bray and Kurtz 1945). The Pi supply levels to the plants were 0 mg fertilizer Pi kg−1 soil (Pi = 6.5 mg kg−1 soil), 80 mg fertilizer Pi kg−1 soil (Pi = 22.5 mg kg−1 soil) and 160 mg fertilizer Pi kg−1 soil (Pi = 35.1 mg kg−1 soil). The N supply levels to the plants were 100 mg fertilizer N kg−1 soil [CO(NH2)2, + N], 400 mg of fertilizer N2O5 kg−1 soil [Ca(NO3)2, + NO3], 400 mg fertilizer NH4 “kg” soil [(NH4)2SO4 + NH4] and 400 mg fertilizer N kg−1 soil [CO(NH2)2, + N]. One WT plant, one ossiz1-1 plant and one ossiz1-2 plant were grown in each pot containing 15 kg of air-dried soil. Samples were collected at the grain harvest stage.

RT-PCR and quantitative real time PCR

Total RNAs were extracted from the roots and leaves of WT and ossiz1 mutants using Trizol reagent (Invitrogen; http://www.invitrogen.com), according to the manufacturer’s instructions. Semi-quantitative RT-PCR was performed for OsActin1 (LOC_Os03g50885) and OsSIZ1 (LOC_Os05g39330) using gene-specific primers (Supplementary Table S2). The PCR products were loaded onto 1% agarose gels and imaged with a CCD camera.

For quantitative real-time PCR, DNase I-treated total RNAs were used for reverse transcription using SuperScript II (Invitrogen). Triplicate quantitative assays were performed for each sample using SYBR Green Master Mix (SYBR Premix Ex Tag “II; TAKARA BIO INC.; http://www.takara-bio.com) with the StepOnePlus Real-time PCR System, according to the manufacturer’s instructions (Applied Biosystems). The relative quantification method was used to evaluate quantitative variation between the replicates, with amplification of OsActin1 (LOC_Os03g50885) being used as the reference gene to normalize all data. All primers used for quantitative real-time PCR are listed in Supplementary Table S3.

GUS histochemical analysis

Histochemical analysis of GUS staining was performed as described previously (Ai et al. 2009). Roots and leaves were collected 3 weeks after germination. Inflorescences were collected before flowering. Culms and seeds were collected 20 d after pollination. Samples were submerged in GUS reaction mix [0.05 mM sodium phosphate buffer, pH 7.0, 1 mM X-gluc and 0.1% (v/v) Triton X-100] with vacuum infiltration for 30 min, and incubated at 37 °C overnight. To analyze subcellular expression patterns, GUS-stained leaf blades and roots were embedded in Spurr’s resin and sectioned for optical microscopy. The stained and sectioned tissues were imaged using an Olympus MVX10 stereo-microscope coupled to a color CCD camera.

Identification of ossiz1 mutants

Two OsSIZ1 T-DNA insertion mutant lines (O. sativa L. ssp. japonica cv. Dongjin), PFG_3A-02154.L (ossiz1-1) and PFG_3A-02154.R (ossiz1-2) were obtained from RiceGE (http://signal.salk.edu/cgi-bin/RiceGE) in Korea. Both lines were inserted into the pGA2715 vector, and the T-DNA insertion sites were verified for the left and right borders (Supplementary Fig. S1). Primers used to identify the homozygous mutants and conformation of T-DNA insertion
sites are listed in Supplementary Table S4. RT-PCR was performed using gene-specific primers that flanking the T-DNA insertion sites (Supplementary Table S2) to determine the expression of OsSIZ1 in mutants. OsActin1 was used as the reference gene for the RT-PCR analysis.

Measurement of mineral concentration in plants

Total P concentration of plant samples was measured as described previously (Chen et al. 2007). Briefly, about 0.05 g of crushed dry sample was digested with H2SO4–H2O2 at 300 °C. After cooling, the digested samples were diluted to 100 ml in distilled water. P concentration was analyzed by the molybdenum blue method (Ames et al. 1966) and normalized by the dry weight of the sample. The concentration of P in plants was measured using a previously described method (Nanamori et al. 2004). The soil P concentration was determined using the Bray-I method (Bray and Kurtz 1945). Total N concentration in plants was determined by the Kjeldahl method (Tang et al. 2012) and normalized by the dry weight of the sample.

To measure the elemental composition of rice samples, about 0.2 g of fully crushed dry sample were completely digested in a total volume of 5 ml of extra pure grade HNO3 and HClO4 (871:3, v/v) at 180 °C. The concentrations of P, K, Ca, Mg, Mn, Zn, Fe, Cu and Si were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES; Perkin Elmer Optima 2000 DV) according to the manufacturer's instructions.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using the SPSS 20 program. Different letters or asterisks on the histograms between the mutants and the WT and/or different treatments indicate their statistically significant difference using Duncan test at \( P < 0.05 \).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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