Differential Effects of Nitrogen Forms on Cell Wall Phosphorus Remobilization Are Mediated by Nitric Oxide, Pectin Content, and Phosphate Transporter Expression[1][OPEN]

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NH_4^+ is a major source of inorganic nitrogen for rice (*Oryza sativa*), and NH_4^+ is known to stimulate the uptake of phosphorus (P). However, it is unclear whether NH_4^+ can also stimulate P remobilization when rice is grown under P-deficient conditions. In this study, we use the two rice cultivars 'Nipponbare' and 'Kasalath' that differ in their cell wall P reutilization, to demonstrate that NH_4^+ positively regulates the pectin content and activity of pectin methylesterase in root cell walls under −P conditions, thereby remobilizing more P from the cell wall and increasing soluble P in roots and shoots. Interestingly, our results show that more NO (nitric oxide) was produced in the rice root when NH_4^+ was applied as the sole nitrogen source compared with the NO_3^-. The effect of NO on the reutilization of P from the cell walls was further demonstrated through the application of the NO donor SNP (sodium nitroprusside) and c-PTIO (NO scavenger 2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide). What's more, the P-transporter gene *OsPT2* is up-regulated under NH_4^+ supplementation and is therefore involved in the stimulated P remobilization. In conclusion, our data provide novel (to our knowledge) insight into the regulatory mechanism by which NH_4^+ stimulates Pi reutilization in cell walls of rice.

Phosphorous (P) is an important inorganic nutrient that plays pivotal roles in plant growth and development. It is part of cellular components such as membranes and nucleic acids and is vital for both vegetative and reproductive growth (Marschner, 1995). However, inorganic phosphorous (Pi) is the least available resource, as it is easily leached out from soil. Furthermore, the Pi retained in the soil could be bound with cations such as Fe^{3+} and Al^{3+}, or converted to organic matters through microorganisms, thereby becoming immobile and hardly to be utilized by plants (Marschner and Rimmington, 1988; Raghothama, 1999; Tiessen, 2008). For instance, 80% to 90% of applied P-fertilizer is fixed in soil particles (Gerke et al., 1994), decreasing the availability of P for plants and resulting in lower primary crop productivity. To ensure crop production, one of the most common agricultural practices is to apply chemical Pi-fertilizer at high concentrations. However, such measures are harmful to the environment and economic development (Bennett et al., 2001). Therefore, it is desirable to develop crops that are able to use P more efficiently under conditions of low P availability.

To maintain cellular Pi homeostasis under Pi-deficient conditions, plants have developed two main adaptive processes: one is to facilitate Pi acquisition from the external environment, while the other is to reutilize the Pi already inside the plant (Lin et al., 2009; Zhu et al., 2014). One of the most valuable mechanisms to improve P uptake is to remodel the architecture of the root system, such as to increase their root-to-shoot ratio, root branching, or root hair number (Wu et al., 2003; Misson et al., 2005; Lynch and Brown, 2008; Vance, 2008). In addition to changes in root architecture, roots can induce chemical and biological changes in the soil. For instance, under P-deficient conditions, roots will secrete protons (Hinsinger, 2001), organic acids (Noriharu et al., 1990), phosphatases (Vance et al., 2003; Vance, 2008), and other substances (Ae et al., 1996), thereby increasing the uptake of Pi from external environment.

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In addition, the content and activity of phosphohydrolases, acid phosphatase (APase), and RNase within the plant are increased under P-deficient conditions. These increases stimulate the reutilization of the internal P (Yun and Kaeppler, 2001; Gong et al., 2011). Kuga et al. (2008)speculated that the vacuole may play a role in storage of P in plant cells such that under P-sufficient conditions, vacuoles would store phosphate, and under P-deficient conditions, vacuoles would release Pi as needed (Kuga et al., 2008). However, other studies demonstrated that the Pi released from the vacuole under P starvation is insufficient (Pratt et al., 2009). Recently, Zhu et al. (2014) found that nearly 50% of total root P is stored in the cell walls of two rice (*Oryza sativa*) cultivars ('Nip' and 'Kas') and that cell wall pectin can facilitate the reutilization of the cell wall P due to its high affinity for Al^{3+}, Fe^{3+}, and Cd^{2+}, which firmly combine with Pi (Blamey et al., 1990; Chang et al., 1999; Zhu et al., 2012). However, whether other factors also affect the P remobilization capacity of pectin is still unclear.

Accumulating evidence has demonstrated that nitrogen (N) can induce the uptake of P by plants (Grunes, 1959; Miller, 1974), such as in the seedlings of maize (*Zea mays*; Smith and Jackson, 1987). Recently, Jin et al., (2014) demonstrated that nitrogen in the forms of urea and nitrate affect plant P uptake differently (Jin et al., 2014). NH_4^+ and NO_3- are the two major N sources that are taken up by plant roots (Marschner, 1995; Falkengren-Grerup et al., 2000). In general, with the absorption of NH_4^+ by plants, the related proton release decreases the pH of the rhizosphere (Wang et al., 1993; Mistrik and Ullrich, 1996; Schubert and Yan, 1997; Zhao et al., 2009), which leads to increased solubility and uptake of P by the plants (Sarkar and Jones, 1982; Hoffmann et al., 1994). The opposite appears to be true with the uptake of N in the form of NO_3^- (Smiley, 1974; Marschner and Römheld, 1983; Moorby et al., 1985; Watanabe et al., 1998). Furthermore, Zeng et al. (2012) demonstrated that in rice the increased P uptake in the presence of NH_4^+ instead of NO_3^- is due to increased activity of the plasma membrane H^+-ATPase. However, whether these two different forms of nitrogen under P-deprivation conditions affect the P remobilization in rice is still unclear.

Accumulating evidence has pointed to NO (nitric oxide) as a signaling molecule involved in physiological and developmental processes in higher plants, such as

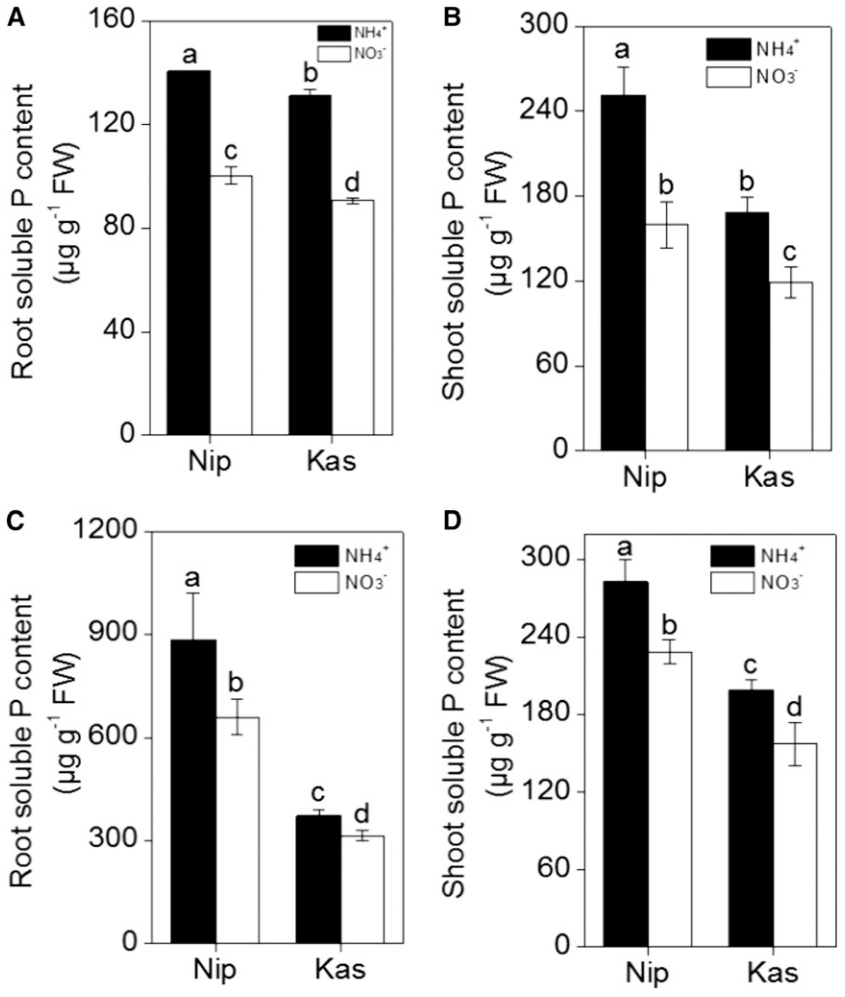

Figure 1. Soluble Pi in the two rice cultivars 'Nip' and 'Kas'. Seedlings were grown in P-deficient (A, B) or P-sufficient (C, D) solution containing different nitrogen forms (NH_4^+ or NO_3^-) for 1 week and the root (A, C)- and shoot (B, D)-soluble Pi contents were measured. Data are means ± SD ($n = 4$). Columns with different letters are significantly different at $P < 0.05$.

root growth (Pagnussat et al., 2002), leaf expansion (An et al., 2005), and the cytokinin signaling pathway (Stöhr and Stremlau, 2006), as well as in responses to stresses, including to drought in wheat (*Triticum* spp.; García-Mata and Lamattina, 2001), high temperature in Lucerne, Switzerland, low temperature in tomato (*Solanum lycopersicum*), wheat, and maize (Cueto et al., 1996); Al toxicity in rice bean (*Vigna umbellata*; Zhou et al., 2012); Fe deficiency in Arabidopsis (Chen et al., 2010b); and P deficiency in white lupin (*Lupinus albus*; Meng et al., 2012). NO improves the growth of white lupin under P deficiency through inducing cluster-root development and citrate exudation (Wang et al., 2010). Two pathways for NO production have be identified in plants: one is to reduce nitrite through the activity of NR (nitrate reductase), and the other is to oxidate Arg to form citrulline through NOS (nitric oxide synthase) activity (Wendehenne et al., 2001; Stöhr and Ullrich, 2002; García-Mata and Lamattina, 2003; Lamattina et al., 2003). However, the exact mechanism underlying NO accumulation in response to P deficiency in rice remains elusive.

Rice is one of the most important cereal crops and previous studies have demonstrated that different rice cultivars use P with different efficiencies. For example, due to the specific *PHOSPHORUS STARVATION TOLERANCE1* gene, the root system of Kas exhibits greater uptake of P in P-limited soils and is therefore more vigorous than that of Nip (Gamuyao et al., 2012). However, when grown in P-deficient solutions, Nip plants display increased P reutilization compared to Kas plants. This phenomenon can be explained by the higher pectin content in the cell walls of Nip plants compared with Kas plants (Zhu et al., 2014). In this study, we use these two rice cultivars with different P reutilization efficiencies to study the correlation between nitrogen forms and cell wall P reutilization. This correlation was then further verified by studying the pectin content as an indicator of the cell wall P reutilization efficiency. This study is the first, to our knowledge, to propose a mechanism for reutilization of cell wall P in the presence of different nitrogen forms and under P-deficient conditions.

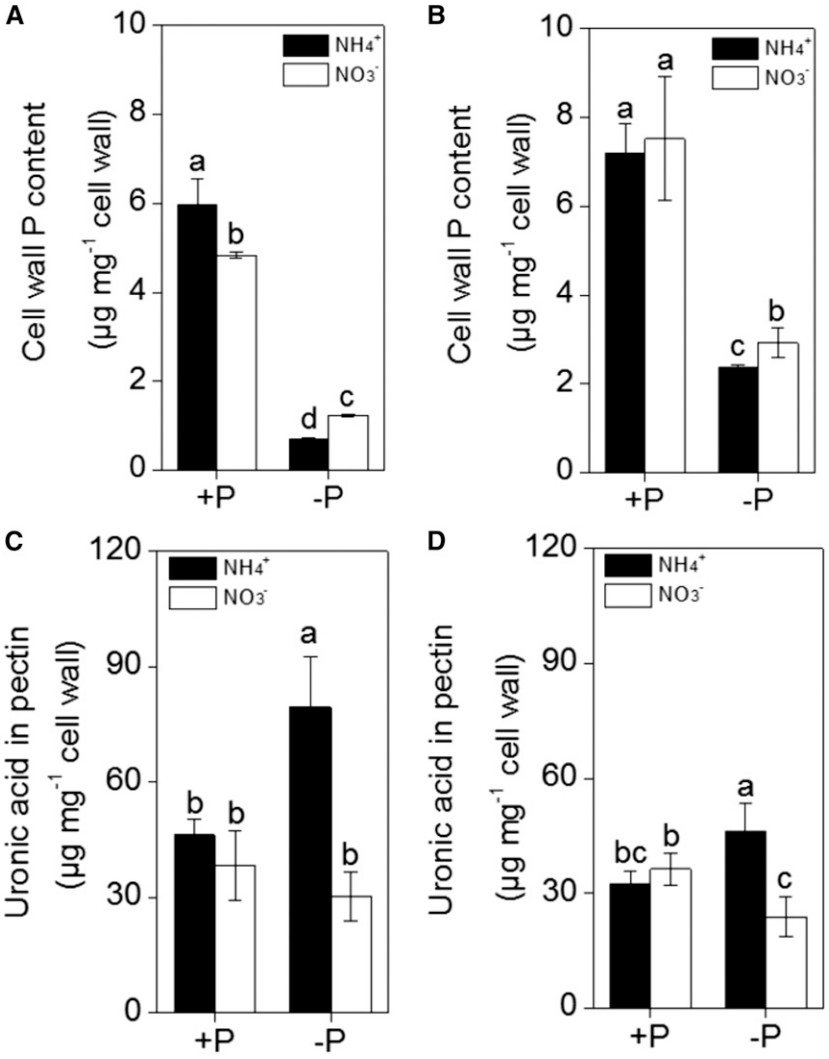

Figure 2. Effect of different nitrogen form (NH_4^+ or NO_3^-) on the retention of the P in the cell wall in 'Nip' (A) and 'Kas' (B) and the pectin content in 'Nip' (C) and 'Kas' (D). Seedlings were transferred to NH_4^+ or NO_3^- nutrient solution in the presence or absence of P for 1 week. Pectin content is reported by uronic acid content in the cell wall. Data are means ± SD ($n = 4$). Columns with different letters are significantly different at $P < 0.05$.

RESULTS

Effect of Different N Sources on the Concentration of Soluble P in Rice

To investigate the effects of different nitrogen forms on the reutilization of P in rice roots, we tested the *japonica* variety 'Nipponbare' ('Nip') and the *indica* variety 'Kasalath' ('Kas'), which showed different P reutilization efficiencies in previous research (Zhu et al., 2014). To analyze the soluble Pi content, roots and shoots were collected separately after seven days of growth in nutrient solution with NH_4^+ or NO_3^- as the sole N source under P-sufficient (+P) or P-deficient (−P) conditions. The results clearly showed that there was more soluble P in 'Nip' roots and shoots compared with those of 'Kas' (irrespective of P status and nitrogen form; Fig. 1), which is in agreement with the previous study (Zhu et al., 2014). However, both 'Nip' and 'Kas' showed greater soluble P concentrations under −P conditions when grown with NH_4^+ as the N source, compared with NO_3^- as the N source (Fig. 1). These results imply that NH_4^+ as an N source may stimulate P reutilization in both rice cultivars.

Effect of Different N Sources on the Cell Wall Soluble P Content, Pectin Content, and Pectin Methylesterase Activity in Rice Roots

Because approximately 50% of the total P is accumulated in the cell walls of rice, and pectin contributes to the P remobilization differences in 'Kas' and 'Nip' (Zhu et al., 2014), we extracted root cell walls and measured the P retained in the cell walls. As shown in Figure 2, A and B, less P accumulated in the cell walls of both rice cultivars when NH_4^+ was applied as the sole N source when compared with NO_3^- as the sole N source under P-deficient conditions. In addition, under −P conditions, the P content in the Nip cultivar cell walls was lower than the P content in 'Kas' cultivar cell walls, which is consistent with previous results (Zhu et al., 2014).

Although the cell wall is composed of a polysaccharide matrix mainly composed of cellulose, hemicellulose, and pectin, only pectin contributes significantly to the release of P from cell walls (Zhu et al., 2014). Under P-deficient conditions, there was a significant increase in cell wall pectin content (as reported by uronic acid content) in both rice cultivars when NH_4^+ was the sole N source, while such an increase in pectin content was not observed with NO_3^- (Fig. 2, C and D). This difference indicates that the decrease of P in the NH_4^+-treated cell wall may be related to the increase of the cell wall pectin content.

We measured PME (pectin methylesterase) activity because the negative charges of the cell wall are caused by the demethylation of pectin, which is catalyzed by PME. As shown in Figure 3, the PME activity was significantly higher after NH_4^+ treatment than after NO_3^- treatment under different P concentrations in both rice cultivars. This finding indicates that NH_4^+ treatment may enhance negative charges in the cell wall.

Effect of Different N Sources on Nitric Oxide Accumulation in Rice Roots

Because the nitrogen form can affect the endogenous NO content (Chen et al., 2010a) and NO is involved in P deficiency (Wang et al., 2010), we hypothesized a direct relationship among nitrogen form (NH_4^+ or NO_3^-), P condition (+P or −P), and NO production. The NO content increased in both rice cultivars under P-deficient conditions, and treatment with NH_4^+ significantly increased the NO content compared with NO_3^- treatment, independent of P conditions (Fig. 4). To determine whether this increase of NO was involved in the NH_4^+-stimulated cell-wall P reutilization, a NO

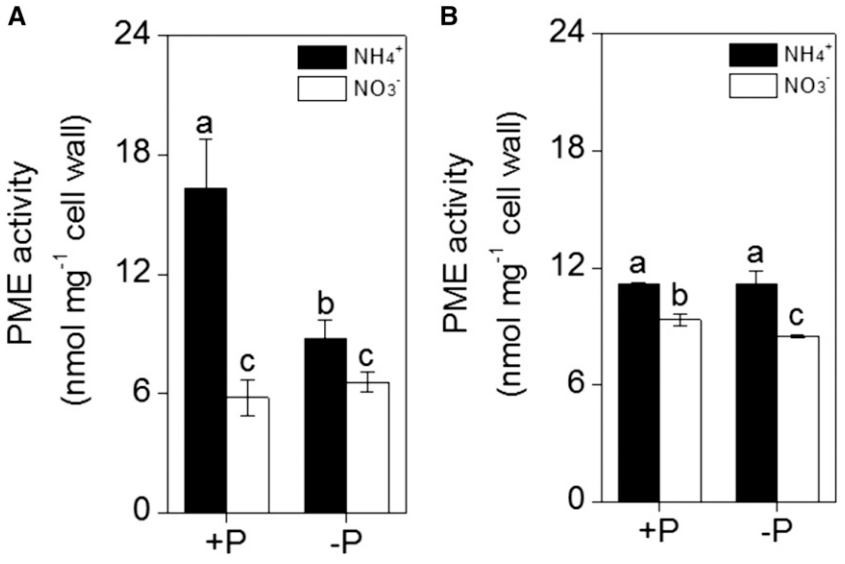

Figure 3. Effect of nitrogen form (NH_4^+ or NO_3^-) on the activity of PME in the cell wall of A, 'Nip' and B, 'Kas' roots. Seedlings were transferred to NH_4^+ or NO_3_ nutrient solution in the presence or absence of P for 1 week. Data are means ± SD ($n = 4$). Columns with different letters are significantly different at $P < 0.05$.

scavenger (c-PTIO) was added to the nutrient solution. As expected, the fluorescence associated with the presence of NO was decreased (Fig. 5). The presence of c-PTIO eliminated the NH_4^+-induced increase in soluble P concentration in the root and shoot in both rice cultivars (Fig. 6, A and B) and eliminated the difference of root cell-wall P content between NH_4^+ and NO_3^- treatment (Fig. 6C) under −P conditions. Then, a question arose whether there is a direct relationship between NO and cell wall P reutilization. Thus, we applied NO donor (SNP) exogenously, and found that with the increment of NO accumulation in rice root tip (Supplemental Figs. S1 and S2), the content of cell wall pectin and activity of cell wall PME were both increased whenever put under NH_4^+ or NO_3^- treatment, irrespective of P conditions (Supplemental Figs. S5 and S6). As a result, more root- and shoot-soluble P content was detected (Supplemental Figs. S3 and S4). This finding further indicates that NO plays an important role in the NH_4^+-regulated reutilization of cell-wall P in rice root.

Effect of Different N Sources on the Expression of P-Transporter Genes in Rice Roots

To determine whether the different N forms influence the translocation of P from roots to shoots under P-deficient conditions, the expression of genes that are typically induced in response to P deficiency and that are involved in P translocation from roots to shoots was analyzed by quantitative RT-PCR. Roots from both cultivars were grown in normal or P-deficient medium, supplemented with NH_4^+ or NO_3^- as the sole nitrogen source. Under P-sufficient conditions, there were no significant differences between NH_4^+ and NO_3^- treatment, except for *OsPT2* in the 'Nip' cultivar, which showed higher expression in NH_4^+ treatment than NO_3^- treatment (Fig. 7). Interestingly, under −P conditions, NH_4^+ as a nitrogen source strongly induced *OsPT2* expression in both 'Nip' and 'Kas' when compared with NO_3^- as a nitrogen source (Fig. 7, A and D). This result is in agreement with the increased shoot-soluble P content, indicating that *OsPT2* may be involved in the NH_4^+ alleviated P deficiency.

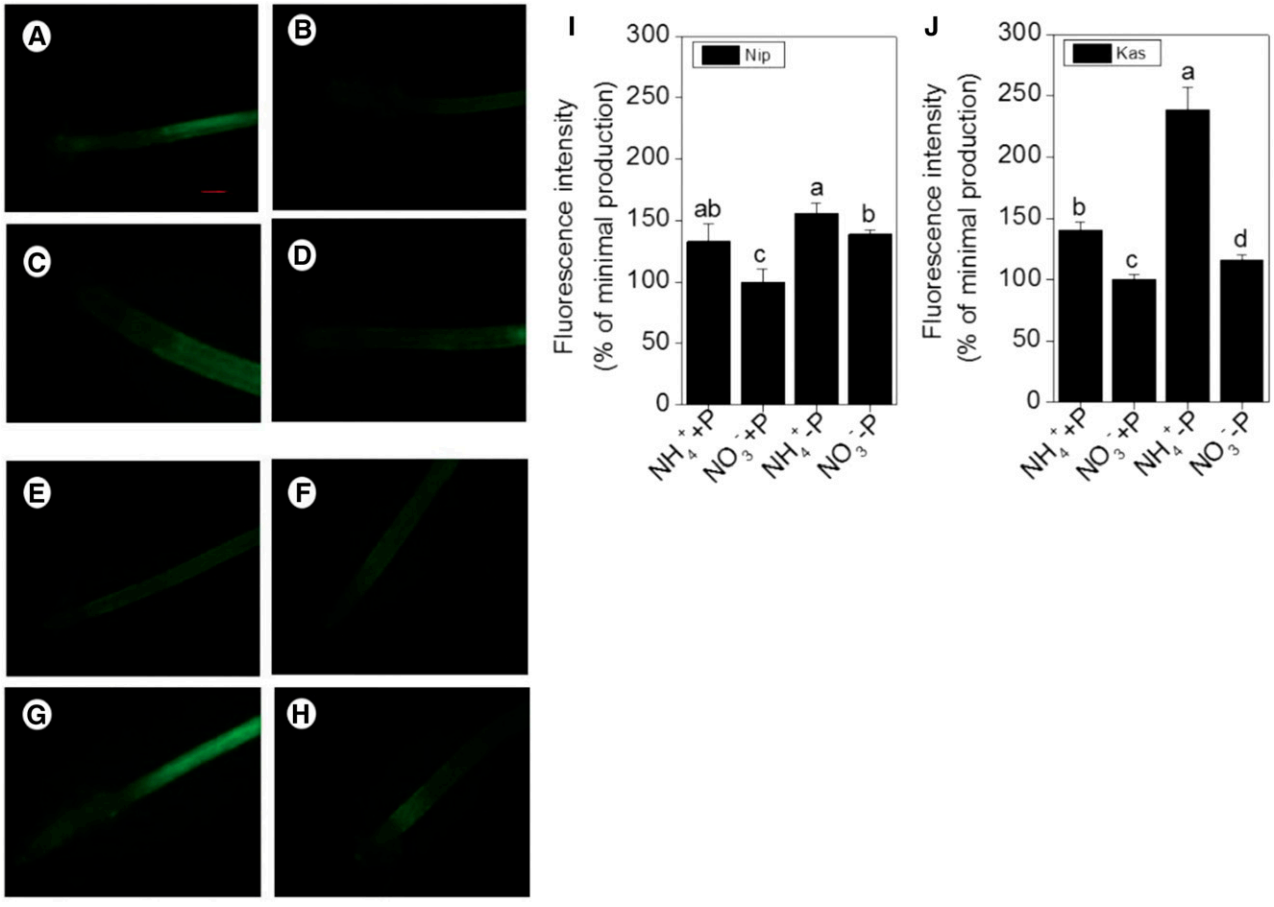

Figure 4. Effect of nitrogen form (NH_4^+ or NO_3^-) on NO production, as indicated by green fluorescence. A, B, Representative roots of 'Nip' cultivar in the full nutrition solution. C, D, 'Nip' cultivar in P-deficiency solution. E, F, 'Kas' cultivar in the full nutrition solution. G, H, 'Kas' cultivar in the P-deficiency solution. (A, C, E, G) Culture in the NH_4^+ solution. (B, D, F, H) Culture in the NO_3^- solution. I, J, NO production expressed as relative fluorescence intensity (% of minimal production). Data are means ± SD (n = 10). Scale bar = 1 mm.

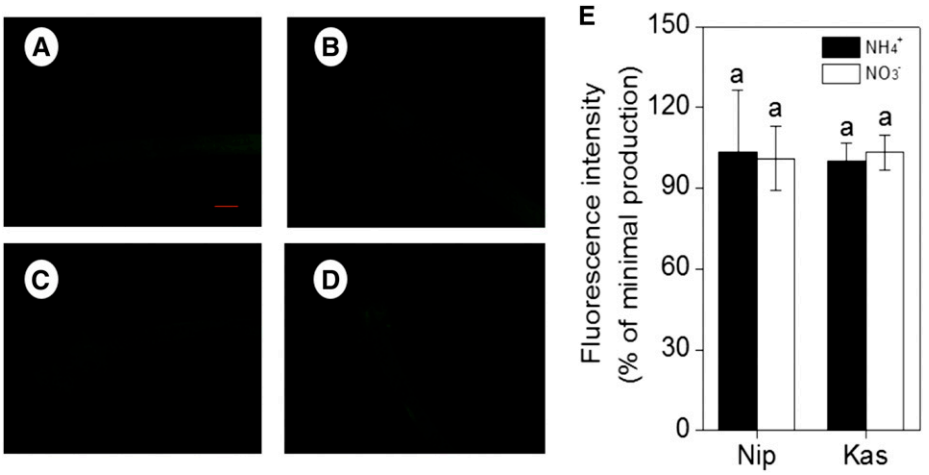

Figure 5. Effect of NO scavenger c-PTIO on NO accumulation, as indicated by green fluorescence. Representative roots are shown. A, B, 'Nip' and C, D, 'Kas' cultivar in P-deficiency solution. (A, C) Culture in the NH_4^+ solution. (B, D) Culture in the NO_3^- nutrition solution. (E) NO production expressed as relative fluorescence intensity (% of minimal production). Data are means ± SD (n = 10). Scale bar = 1 mm.

Effect of Different N Sources on Exudates from Rice Roots

It has previously been reported that the secretion of organic acids may affect the capacity of the cell wall to bind cations such as Al (Li et al., 2009). However, we found no significant difference in either the pH (because the nutrient solution was buffered by 5 mM MES, pH 5.5) or organic acid (malate, citrate, and oxalate) secretion between 'Nip' and 'Kas' (independent of P and nitrogen form status; Fig. 8). This suggests that organic acid efflux and acidification are unlikely to promote root P mobilization during P starvation in this study.

DISCUSSION

Nitrogen, specifically NH_4^+, has a stimulating effect on P uptake by plants. In this study, we found that NH_4^+ also stimulates the reutilization of P from the cell wall. NH_4^+ likely promotes the uptake of P from the soil via changes in the acidity and chemical composition of the rhizosphere (Blair et al., 1971; Riley and Barber, 1971; Jing et al., 2010). When plants absorb NH_4^+, their roots secrete protons, thereby acidifying their rhizosphere and causing a release of P from the soil. However, in this study, we buffered the nutrient solution with 5 mM MES at pH 5.5, and at a stable pH, the hydrolytic activity or pumping activity of the H^+-ATPase should be the same under both NH_4^+ and NO_3^- treatment (Schubert and Yan, 1997; Zhu et al., 2009). Furthermore, because of the absence of both soluble and insoluble P in the −P hydroponic solution, the involvement of the pH is excluded. In addition, secretion of organic acids was not involved in the NH_4^+-specific stimulation of P reutilization in the two rice cultivars (Fig. 8).

Because nearly half of the total P content is present in the cell walls of rice (Zhu et al., 2014), we speculate that both rice cultivars contain more soluble P when grown in NH_4^+ nutrient solution under P-deprivation conditions and the differences between cultivars may therefore result from differences in reutilization of P in the cell walls. The cell wall is composed of a matrix of polysaccharides such as cellulose, hemicellulose, and pectin. Pectin is a main source of negative charges in the cell wall that facilitate the binding of cations, such as Al (Eticha et al., 2005; Yang et al., 2008), Fe (Chang et al., 1999), and Cd (Zhu et al., 2012). In addition, previous studies have demonstrated that pectin in the root cell walls is involved in reutilization of insoluble P during P starvation (Nagarajah et al., 1970; Ae et al., 1996; Gessa et al., 1997; Zhu et al., 2014). What's more, it has been

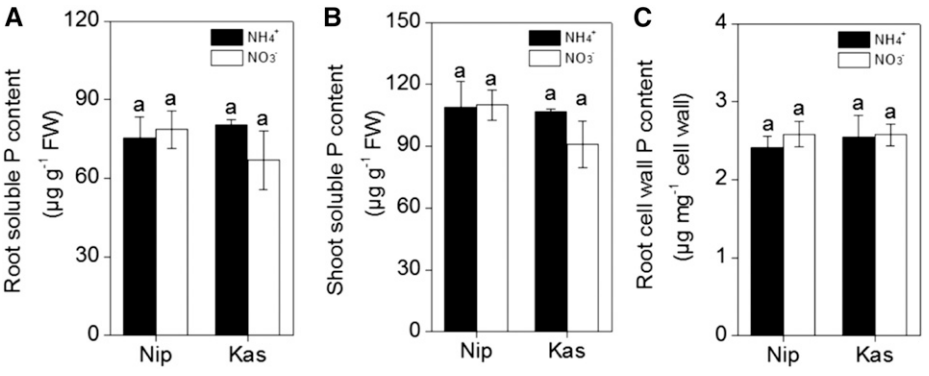

Figure 6. Effect of NO scavenger c-PTIO on (A) root- and (B) shoot-soluble Pi and (C) root cell wall P content in the two rice cultivars 'Nip' and 'Kas' under P-deficient conditions. Seedlings were subjected to P-deficient conditions in the presence of 10 μM c-PTIO with different nitrogen forms (NH_4^+ or NO_3^-) for 1 week and the root- and shoot-soluble Pi content was measured. Data are means ± SD (n = 4). Columns with different letters are significantly different at $P < 0.05$.

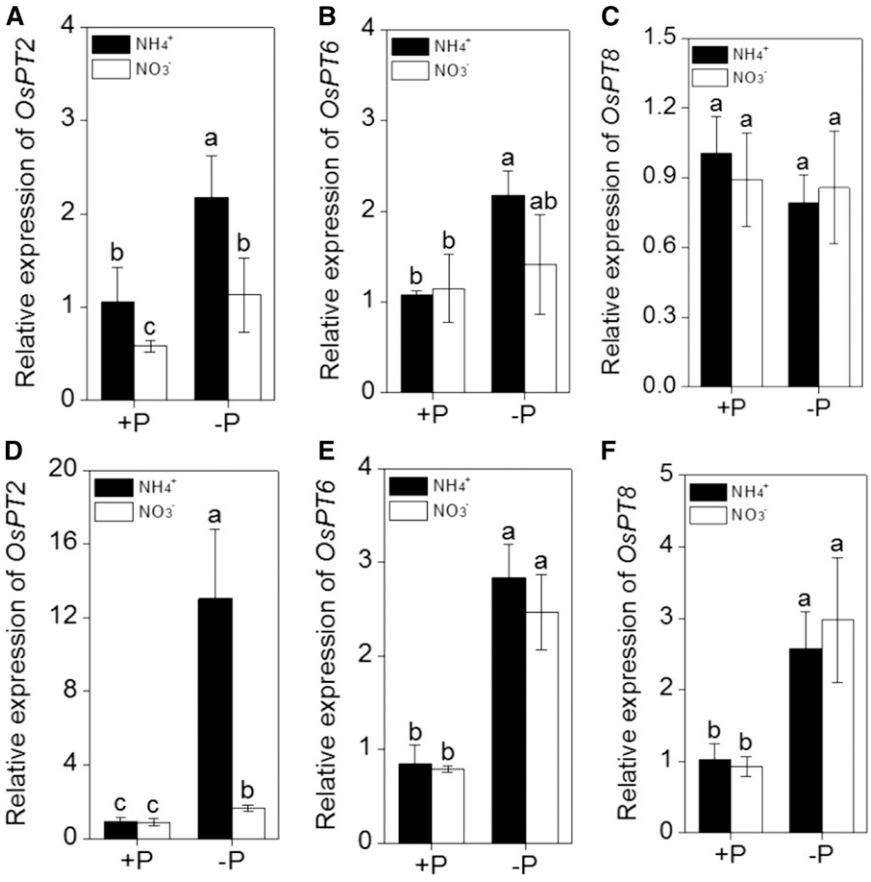

Figure 7. Effect of different nitrogen form (NH_4^+ or NO_3^-) on the expression of Pi translocation-related genes. A, B and C, 'Nip' cultivar; D, E and F, 'Kas' cultivar. Seedlings were transferred to P-deficient (−P) or P-sufficient (+P) nutrient solution in the presence of NH_4^+ or NO_3^- for 1 week. After treatment, total root RNA was extracted and used to synthesize cDNA. Quantitative RT-PCR was performed using gene-specific primers. Data are means ± SD ($n = 4$). Columns with different letters are significantly different at $P < 0.05$.

demonstrated that exposure of Arabidopsis and rice ('Kas') to a P deficiency condition led to a decrement of pectin content while this effect was diminished on another rice cultivar ('Nip'), and pectin contributed to the cell wall P reutilization in Arabidopsis and rice when suffering from P deficiency, which means, the more pectin content, the more cell wall P reutilization (Zhu et al., 2014).

Previous studies have demonstrated that compared with NO_3^-, there is lower root cell-wall pectin content under NH_4^+ treatment when two rice cultivars (YD6 and WYJ7) grow in the $CaCl_2$ solution without control pH (Wang et al., 2015), however, the change in pectin content might depend on plant cultivars, culture conditions, and physiological stresses. In this study, both rice cultivars showed an increase in cell wall pectin content when grown in the NH_4^+ nutrient solution under −P conditions, which means that they had an increased ability to reutilize the cell wall P. This is in agreement with the decrease in cell-wall P content

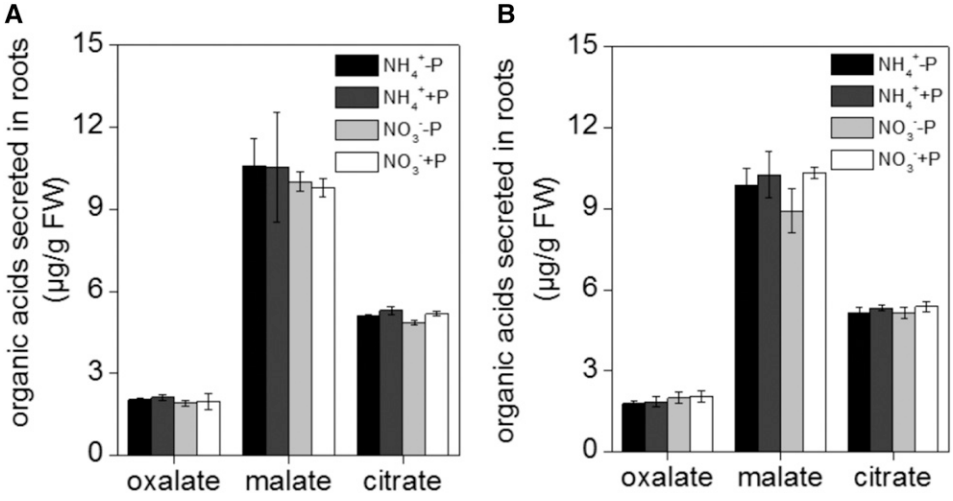

Figure 8. Effect of different nitrogen form (NH_4^+ or NO_3^-) on the secretion of organic acids. A, 'Nip' cultivar; B, 'Kas' cultivar. Seedlings were transferred to P-deficient (−P) or P-sufficient (+P) nutrient solution in the presence of NH_4^+ or NO_3^- for 1 week. After treatment, total root exudates were collected and measured by HPLC. Data are means ± SD ($n = 4$). Columns with different letters are significantly different at $P < 0.05$.

found in plants grown in NH_4^+ nutrient solution. What is more, pectin is synthesized and methylesterified in the Golgi apparatus and secreted into the cell wall in a highly methylesterified state (Micheli, 2001) that can only weakly to absorb cations. To increase its binding capacity for cations, free carboxylic groups of pectin must be exposed through demethylation. This process is catalyzed by the PME enzyme and renders pectin as the main binding site for cations in the cell wall. Once the amount of carboxylate group (–COO–) in root cell wall pectin is increased, the pectin will have higher capacity to bind Fe, thus facilitating the release of cell wall P. Under NH_4^+ nutrition solution, although there is no difference of the pectin content when compared with NO_3^- treatment under P sufficient condition, the PME activity is significantly higher. However, when under a P-deficient condition, the pectin content and the PME activity are both higher in the NH_4^+ treatment than in the NO_3^- treatment. So there may be higher cell-wall negative charges under NH_4^+ nutrition than NO_3^- nutrition, independent of P status. Therefore, the decreased retention of P in the cell wall and the increase in root-soluble P may further be due to the higher demethylation state of pectin under $-P+NH_4^+$ treatment (Fig. 7). However, it is strange that under NH_4^+ and $-P$ condition, PME activity in 'Kas' was higher than that in 'Nip' (Fig. 3), while the soluble P content in Nip was higher than that in Kas (Fig. 1). This is mainly because, in addition to PME activity, the content of pectin (which is the substrate catalyzed by PME enzymes) is another important factor that attributes to the cell wall P release. Maybe there is a threshold of the PME activity under P-deficient condition, and this needs our further study.

There may be another signal that acts downstream of this NH_4^+-mediated increased pectin content under P-deficient conditions. Increased NO production in various plant species has been widely observed in response to nutrient deficiency in general (Chen et al., 2010b) and P deficiency in particular (Wang et al., 2010). Interestingly, in this study, we found that NO is involved in the P deficiency response in rice. An increase in NO production under NH_4^+ combined with $-P$ treatment was associated with increased reutilization of cell wall P. Together, this increased root- and shoot-soluble P content (Fig. 1) and decreased the P retention in the cell wall (Fig. 2, A and B), indicating that NO may indeed be involved in the cell wall P reutilization process. This was further demonstrated by effect of the NO scavenger c-PTIO and the NO donor SNP. After the addition of c-PTIO, the stimulating effects of NH_4^+ on cell wall P reutilization under P deprivation were inhibited: no difference in root- and shoot-soluble P content and root cell-wall P content between NH_4^+ and NO_3^- treatment could be observed (Fig. 6). However, after being treated with SNP for 24 h, it was found that the concentration of rice root- and shoot-soluble P (Supplemental Figs. S3 and S4), the content of cell wall pectin, and the activity of cell wall PME were all increased (Supplemental Figs. S5 and S6), in company with the increment of NO content (Supplemental Figs. S1 and S2). It is noteworthy that the content of signal molecular NO was increased under NH_4^+ nutrition, which can stimulate the production of pectin and the increment of PME activity (Supplemental Figs. S5 and S6), thus more carboxylate group (–COO–) in pectin was produced (Supplemental Figs. S2 and S3). All the above results emphasize that NO plays an important role in rice cell wall P reutilization in response to different nitrogen forms.

In addition, up-regulation of the expression of multiple genes that mediate Pi translocation would be another effective way for plants to cope with P deficiency. As expected, NH_4^+ treatment in the absence of P significantly enhanced the expression of *OsPT2* (Fig. 8), which expressed mainly in the stele of primary and lateral roots under Pi-deficient conditions (Ai et al., 2009), indicating NH_4^+ may be involved in the transportation of P from root to shoot by regulating the expression of *OsPT2*. P deficiency also induced the expression of *OsPT6* (expressed in the epidermis, cortex, and stelar tissue under Pi-deficient conditions) in both rice cultivars and of *OsPT8* (expressed constitutively and functioning in P hemeostasis) in the 'Kas' cultivar (Fig. 7); however, there were no differences in responses to NH_4^+ and NO_3^- treatments, thus ruling out the possibility that *OsPT6* and *OsPT8* are transcriptionally regulated by NH_4^+ under P-deficient conditions.

In conclusion, we identified a novel (to our knowledge) physiological and molecular pathway of NH_4^+-induced cell wall P remobilization under P-deficient conditions. In the presence of NH_4^+, increased production of NO causes an increase of pectin and PME activity in the cell wall, which results in increased release of soluble P, and the concomitant up-regulation of *OsPT2* facilitates the translocation of P to the shoot.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Rice (*Oryza sativa*) spp. *japonica* 'Nipponbare' ('Nip') and *indica* 'Kasalath' ('Kas') were used in this study. Seeds were surface-sterilized with 1% NaClO for 10 min, washed with deionized water three times, and allowed to germinate on filter paper with deionized water for 24 h. Subsequently, seedlings were cultivated in 0.5 mM $CaCl_2$ (pH 5.5) solution for 2 d, and then transferred to full-strength nutrient solution containing 0.5 mM NH_4NO_3, 0.18 mM $NaH_2PO_4 \cdot 2H_2O$, 0.18 mM KCl, 0.36 mM $CaCl_2$, 0.6 mM $MgSO_4 \cdot 7H_2O$, 9 μM $MnCl_2 \cdot 4H_2O$, 0.1 μM $Na_2MoO_4 \cdot 4H_2O$, 10 μM H_3BO_3, 0.7 μM $ZnSO_4 \cdot 7H_2O$, 0.3 μM $CuSO_4$ and 20 μM $FeSO_4 \cdot 7H_2O$-EDTA. All experiments were conducted in a growth chamber with a 14-h/26°C day and a 10-h/23°C night regime, a light intensity of 400 μmol $m^{-2} S^{-1}$, and a relative humidity of 60%.

After 7 d, uniform seedlings were planted in 1.5-L pots (10 seedlings per pot) with the following treatments: $+P+NH_4^+$, $-P+NH_4^+$, $+P+NO_3^-$, $-P+NO_3-$, $-P+NH_4^+$+c-PTIO, $-P+NO_3^-$, and +c-PTIO. For NH_4^+ and NO_3^- treatments, 1 mM NH_4Cl and 1 mM $NaNO_3$ were applied, respectively. The final concentration of c-PTIO was 10 μM. The pH was adjusted to 5.5 with 5 mM MES. The solution was renewed every 3 d.

For the NO (nitric oxide) donor SNP application experiment, eight treatments, named $+P+NH_4^+$, $-P+NH_4^+$, $+P+NO_3-$, $-P+NO_3^-$, $+P+NH_4^+$+SNP, $+P+NO_3^-$+SNP, $-P+NH_4^+$+SNP, and $-P+NO_3^-$+SNP were performed. Seedlings with unanimous growth were treated with or without 2.5 μM SNP for 24 h under four respective treatments ($+P+NH_4^+$, $-P+NH_4^+$, $+P+NO_3^-$, and $-P+NO_3^-$). Afterward, the nutrient solution was totally renewed and the treated seedlings were still grown in P-deficient or P-sufficient solution containing different

nitrogen forms (NH_4^+ or NO_3^-) for another 6 d. The pH was adjusted to 5.5 with 5 mM MES. The solution was renewed every 3 d.

Determination of Soluble Inorganic Phosphorous Concentrations

The soluble inorganic phosphorous (Pi) concentration was determined according to Zhu et al. (2014). Briefly, after washing three times with deionized water, roots, and shoots were weighed and homogenized in 5-M sulfuric acid. After centrifugation at 4000 rpm for 5 min, 400 μL supernatant was transferred to an 1.5-mL Eppendorf tube, and 200 μL ammonium molybdate containing 15% (w/v) fresh ascorbic acid (pH 5.0) was added. The mixture was incubated at 37°C for 30 min and the absorbance was determined at 650 nm. And the Pi concentration was calculated by normalization of the fresh weight (Zheng et al., 2009).

Cell Wall Extraction and Fractionation

The extraction of cell wall materials were carried out according to Zhong and Läuchli (1993). Briefly, roots were homogenized in 8 mL 75% ethanol, incubated on ice for 20 min, and centrifuged at 4000 rpm for 10 min. Then, the pellets were homogenized in 8 mL acetone, a 1:1 ratio of methanol/chloroform, and methanol, respectively, for 20 min each. These steps were carried out at 4°C. The remaining pellets were freeze-dried and stored at 4°C until use.

The extraction of pectin was carried out by washing approximately 2 mg of isolated cell walls three times with 1 mL water at 100°C for 1 h. The supernatants containing pectin were collected in a 5-mL tube after centrifugation at 13,200 rpm for 10 min (Zhong and Läuchli, 1993; Yang et al., 2011).

Uronic Acid Measurement

The uronic acid concentration was used as an indicator for the pectin concentration and assayed according to Blumenkrantz and Asboe-Hansen (1973). Briefly, 200 μL pectin that was extracted as described above was incubated with 1 mL 98% H_2SO_4 combined with 0.0125 M $Na_2B_4O_7 \cdot 10H_2O$ at 100°C for 5 min, and chilled immediately. Subsequently, 20 μL 0.15% M-hydro-diphenyl was applied to the solution and incubated at 25°C for 20 min. Finally, the absorbance was measured spectrophotometrically at 520 nm, using GalUA (Sigma-Aldrich, St. Louis, MO) as a standard (Blumenkrantz and Asboe-Hansen, 1973).

P Retention in Cell Wall Materials

The cell wall P content was extracted by shaking approximately 2 mg of isolated cell walls with 1 mL HCl (2 M) in 1.5-mL Eppendorf tube. After the solution was shaken on a rotary shaker for at least 24 h, samples were centrifuged at 13,200 rpm at room temperature for 5 min to collect the supernatant. The P retained in the cell wall was determined by inductively coupled plasma atomic emission spectroscopy (Fisons ARL Accuris; Écublens, Vaud, Switzerland; Zhu et al., 2014).

Pectin Methylesterase Extraction and Activity Assay

For the extraction of crude PME (pectin methylesterase), 5 mg cell wall material was suspended in 1 M NaCl solution (pH 6.0) at 4°C for 1 h with repeated vortexing (for 20 s every 10 min). After centrifugation at 15,000 rpm for 10 min, the supernatant was retained as the PME sample.

The reaction solution used to analyze the PME activity consisted of 100 μL 200 mM PBS (containing 0.64 mg mL^{-1} pectin), 10 μL AO (alcohol oxidase), and 50 μL PME sample. After incubation at 30°C for 10 min, 200 μL NaOH (0.5 N) containing 5 mg mL^{-1} Purpald (Sigma-Aldrich) was added and the sample was incubated at 30°C for 30 min. Subsequently, 550 μL water was added to a final volume of 1.0 mL and that was used to measure the A_{550} spectrophotometrically (Zhu et al., 2012).

Determination of NO Content in Roots

The probe DAF-FM DA (4-amino-5-methylamino-2,7-difluorofluorescein diacetate) was used to determine the accumulation of endogenous NO in rice roots. The root tips were incubated with 10 μM DAF-FM DA in the dark for 30 min after being washed with HEPES-KOH (pH 7.4) for 15 min. After the incubation, the root tips were washed three times with HEPES-KOH (pH 7.4) to remove excess fluorescence. The epifluorescence images were captured by an Eclipse 80i, EX 460-500, DM 505, and BA 510-560 (Nikon, Melville, NY). The fluorescence intensity was measured using Photoshop 7.0 (Adobe Systems, Mountain View, CA) according to Besson-Bard et al. (2009).

Measurement of Organic Acid Efflux

To collect root exudates, plants were placed in 1.25 L 0.5 mM $CaCl_2$ (pH 5.5) for 12 h after treatment for 6 d. Subsequently, the root exudates were passed through a cation exchange column (16 mm × 14 cm) filled with 5-g Amerlite IR-120B resin (H^+ form; Muromachi Chemical, Tokyo, Japan) and an anion-exchange column filled with 1.5 g Dowex 1 × 8 resin (100–200 mesh, formate form; Dow Chemical, Midland, MI) successively. Then, 15 mL, 1 M HCl was used to elute the organic acid anions that were retained in the anion-exchange resin and the eluent was concentrated using a rotary evaporator at 40°C. The residue was redissolved in 1 mL H_2O and filtered (0.2 μm) before analysis. Organic acid anions were detected by ion chromatography (ICS 3000; Dionex, Sunnyvale, CA) equipped with an AS11 anion-exchange analytical column (4 × 250 mm) and a guard column (4 × 50 mm; Dionex). The mobile phase was 1200 mg L^{-1} NaOH at a flow rate of 0.6 mL min^{-1} (Zhu et al., 2014).

Gene Expression Analysis

To determine gene expression, roots were harvested after 7 d treatment and immediately frozen and ground in liquid nitrogen. Total RNA was isolated with TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and the RNA integrity and quality was confirmed by 1% agarose gel electrophoresis and spectroscopy. A PrimeScript RT reagent kit (Takara Bio, Kyoto, Japan) was used to reverse transcribe 1 μg total RNA into cDNA. A 10-μL real-time PCR mixture contained the following: 1 μL 10-fold-dilution of cDNA, 0.6 μL forward and reverse primers, 5 μL SYBR Premix ExTaq (Takara Bio), and 2.8 μL sterile distilled water. The sequences of the gene-specific primers are as follows: *OsPT2* (forward: 5′-GACGAGACCGCCCAAGAAG-3′; reverse: 5′-TTTTCAGTCACTCACGTCGAGAC-3′); *OsPT6* (forward: 5′-TATAACTGATCGATCGAGACCAGAG-3′; reverse: 5′-TGGATAGCCAGGCCAGTTATATATC-3′); *OsPT8* (forward: 5′-AGAAGGCAAAAGAAATGTGTGTTAAAT-3′; reverse: 5′-AAAATGTATTCGTGCCAAATTGCT-3′). Each cDNA sample was run in triplicate. Expression data were normalized to the expression level of the *actin* gene (forward: 5′-AAGTTCTGGGAAGTGGTT-3′; reverse: 5′-CTCCCAATGAGTGACAAA-3′; Jia et al., 2011; Wu et al., 2011).

Statistical Analysis

Each experiment was repeated at least three times, and one set of data are shown in the Results. Data were analyzed by one-way ANOVA and the mean values were compared by Duncan's multiple range test. Different letters indicate that the mean values were statistically different at the $P < 0.05$ level.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers *OsPT2* (AF536962), *OsPT6* (AF536966), *OsPT8* (AF536968), *actin* (AB047313).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Effect of Exogenous NO Addition on NO Accumulation in 'Nip' Root

Supplemental Figure S2. Effect of Exogenous NO Addition on NO Accumulation in 'Kas' Root

Supplemental Figure S3. Soluble Pi in the Rice Cultivar 'Nip'

Supplemental Figure S4. Soluble Pi in the Rice Cultivar 'Kas'

Supplemental Figure S5. Effect of Exogenous NO Addition on the Pectin Content in 'Nip' (A, B) and 'Kas' (C, D) Roots under P-Sufficient (A, C) or P-Deficient (B, D) Conditions

Supplemental Figure S6. Effect of Exogenous NO Addition on the Activity of PME in the Cell Wall of 'Nip' (A, B) and 'Kas' (C, D) Roots under P-Sufficient (A, C) or P-Deficient (B, D) Conditions

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