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miR444a has multiple functions in the rice nitrate-signaling pathway

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SUMMARY

Nitrate (NO_3^-) is a key signaling molecule in plant metabolism and development, in addition to its role as a nutrient. It has been shown previously in Arabidopsis that ANR1, a MADS-box transcription factor, is a major component in the NO_3^--signaling pathway that triggers lateral root growth and that miR444, which is specific to monocots, targets four genes that are homologous to *ANR1* in rice. Here, we show that miR444a plays multiple roles in the rice NO_3^--signaling pathway – not only in root development, but also involving nitrate accumulation and even P_i-starvation responses. miR444a overexpression resulted in reduced rice lateral root elongation, but promoted rice primary and adventitious root growth, in a nitrate-dependent manner. In addition, overexpression of miR444a improved nitrate accumulation and expression of nitrate transporter genes under high nitrate concentration conditions, but reduced the remobilization of nitrate from old leaves to young leaves thus affecting the plant's ability to adapt to nitrogen-limitating conditions. Intriguingly, we found that P_i starvation strongly induced miR444 accumulation in rice roots and that overexpression of miR444a altered P_i-starvation-induced root architecture and enhanced P_i accumulation and expression of three P_i transporter genes. We further provide evidence that miR444a is involved in the interaction between the NO_3^--signaling and P_i-signaling pathways in rice. Taken together, our observations demonstrated that miR444a plays multiple roles in the rice NO_3^--signaling pathway in nitrate-dependent root growth, nitrate accumulation and phosphate-starvation responses.

Keywords: miR444a, nitrate signal, root, P_i-starvation response, rice.

INTRODUCTION

Nitrogen (N) is an essential macronutrient for the growth and development of plants. Plants obtain N from the soil mainly in the form of nitrate (NO_3^-) via high- or low-affinity NO_3^- transporters. In addition to being a nutrient source, NO_3^- also acts as a signaling molecule to regulate gene expression (Ho and Tsay, 2010; Krouk *et al.*, 2010). Several molecular components of the NO_3^- perception and signal transduction pathways have been identified in Arabidopsis. NRT1.1 (CHL1), a dual-affinity transporter, has been shown to be a NO_3^- sensor that regulates the primary NO_3^- response by changing the phosphorylation status of its threonine 101 residue (Ho *et al.*, 2009). The NO_3^- inducible protein kinase CIPK23 is a negative regulator of the high affinity response (Ho *et al.*, 2009). Another NO_3^- inducible protein kinase, CIPK8, is a positive regulator of the low-affinity response (Hu *et al.*, 2009). Transcription factors NLP7 and LBD37/38/39 are positive and negative regulators, respectively, of NO_3^--regulated genes (Castaings *et al.*, 2009; Rubin *et al.*, 2009). In addition, the MADS-box transcription factor ANR1, which acts downstream of CHL1, and the putative auxin receptor AFB3 both play important roles in NO_3^--stimulated lateral root proliferation (Zhang and Forde, 1998; Vidal *et al.*, 2010).

Phosphorus (P) is also an essential macronutrient for plant growth, development, and reproduction and is acquired mainly in the form of inorganic phosphate (P_i) by P_i transporters in plants (Chiou and Lin, 2011; Kuo and Chiou, 2011). The available P_i in the soil is usually limiting. To circumvent limited P_i availability, plants have evolved a repertoire of adaptive strategies including morphological, physiological, and biochemical responses to increase P_i acquisition and utilization (Chiou and Lin, 2011; Kuo and Chiou, 2011). Several components of the P_i signal transduction pathways have been identified and characterized.

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The transcription factor PHR1, the SUMO E3 ligase SIZ1, the SPX domain proteins SPX1 and SPX3, miR399 and its target PHO2, a ubiquitin-conjugating E2 enzyme (UBC24), are key players that regulate P_i homeostasis by modulating P_i acquisition, root architecture and metabolism (Rubio *et al.*, 2001; Miura *et al.*, 2005; Bari *et al.*, 2006; Chiou *et al.*, 2006; Liu *et al.*, 2010). There is increasing evidence to suggest that the P_i-starvation signal transduction pathway is highly conserved between rice and Arabidopsis. Several conserved regulators of P_i signaling have been characterized in rice (Hu and Chu, 2011). P_i homeostasis appears to be very important in rice reproduction, for example over-accumulation of P_i in the *Ospho2* mutant or the *OsPHR2*-overexpressing rice plant resulted in severely defective grain filling (Zhou *et al.*, 2008; Hu *et al.*, 2011).

Interaction between the NO_3^--signaling and P_i-signaling pathways has been observed. For example, P_i deprivation of bean (*Phaseolus vulgaris* L.) plants resulted in increased nitrate accumulation in roots and decreased nitrate levels in shoots (Gniazdowska and Rychter, 2000). However, Kant *et al.* (2011) have reported that low P_i slightly increased nitrate accumulation in Arabidopsis shoots. Low nitrate levels seem to improve P_i accumulation. It has been shown that decreased N availability in maize leaves resulted in P_i accumulation and in the down-regulation of genes involved in the P_i-starvation response (Schlüter *et al.*, 2012).

MicroRNAs (miRNAs) are small regulatory RNAs that can direct target mRNA degradation or translation repression (Voinnet, 2009). Plant miRNAs are not only essential for normal plant growth and development, but also play important regulatory roles in plant adaptive responses to various biotic and abiotic stresses (Jones-Rhoades *et al.*, 2006; Sunkar and Zhu, 2007; Ruiz-Ferrer and Voinnet, 2009). Recently, miRNAs have been shown to play vital regulatory functions in the uptake, assimilation and translocation of nutrients in plants (Kuo and Chiou, 2011). Two well studied examples, miR395 and miR399, have been reported to regulate the distribution and homeostasis of sulfur and phosphate, respectively, in Arabidopsis (Chiou *et al.*, 2006; Pant *et al.*, 2008; Kawashima *et al.*, 2009, 2011; Liang *et al.*, 2010). Recent studies have also revealed that some miRNAs are differentially expressed in response to altered N concentrations and are associated with N-regulated effects (Pant *et al.*, 2009). For instance, miR169 is involved in adaptation to N-starvation stress (Zhao *et al.*, 2011), and miR167 and miR393 participate in the regulation of NO_3^--signaling in lateral root growth (Gifford *et al.*, 2008; Vidal *et al.*, 2010). In addition, miRNAs may have several roles in the interaction between the NO_3^--signaling and P_i-signaling pathways, because some miRNAs are differentially expressed under both N- and P_i-deficient conditions (Pant *et al.*, 2009). It has been reported recently that miR827 regulates nitrate-dependent P_i accumulation by targeting *NLA* in Arabidopsis (Kant *et al.*, 2011). In rice, the use of small RNA sequence analysis has also shown that the accumulation of some miRNAs was altered by N or P starvation (Jeong *et al.*, 2011). However, the regulatory mechanisms of these N- or P-related miRNAs remain to be explored.

miR444 is specific to monocots and has been confirmed to target four MIKC-type MADS-box genes in rice (*OsMADS23*, *OsMADS27a*, *OsMADS27b*, and *OsMADS57*; Sunkar *et al.*, 2005; Lu *et al.*, 2008; Wu *et al.*, 2009; Li *et al.*, 2010). Phylogenetic analysis has shown that the miR444 targets are grouped into a clade with Arabidopsis ANR1 (Lee *et al.*, 2003; Arora *et al.*, 2007), which is a key regulator involved in the NO_3^--signaling pathway in lateral root growth (Zhang and Forde, 1998). Here, we report that miR444a, a member of the miR444 family, regulates NO_3^--signaling in rice root growth and nitrate accumulation in a way that is different from that of ANR1 in Arabidopsis. Interestingly, we also demonstrate that miR444a regulates multiple P_i-starvation responses in rice.

RESULTS

Expression pattern of miR444

To investigate whether miR444 expression responds to NO_3^- signals, we first analyzed the accumulation of miR444 and its target genes by treating N-starved roots with 5 mM KNO_3. Small RNA gel blot and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis showed that miR444 accumulation and the mRNA levels of the miR444 target genes were not altered to any obvious degree following 0.5, 1 or 2 h of KNO_3 stimulation. We also investigated the accumulation of miR444 under N, P and K starvation conditions. The results showed that, under N-starvation conditions, miR444 accumulation increased slightly. Interestingly, P-starvation induced an approximately two-fold increase in miR444 accumulation, whereas K starvation slightly reduced miR444 accumulation (Figure 1a). Accordingly, we determined the mRNA level of miR444 targets under N, P or K starvation conditions. The results showed that the mRNA levels of three of the four miR444 targets (*MADS-23, MADS-27a, and MADS-57*) decreased significantly under N-starvation conditions; P starvation reduced the mRNA levels of three miR444 targets (*MADS-27a/b, MADS-57*), however only one target, *MADS-23*, had significantly increased transcript levels under K starvation conditions (Figure 1b).

miR444a regulates the NO_3^--signaling pathway in rice root growth

To further explore the function of miR444, miR444a was overexpressed in rice. A 441-bp miR444a precursor cDNA sequence was transformed into *Oryza sativa* spp. *japonica* cv. Nipponbare under the control of the CaMV 35S promoter. Small RNA gel blot analysis showed that

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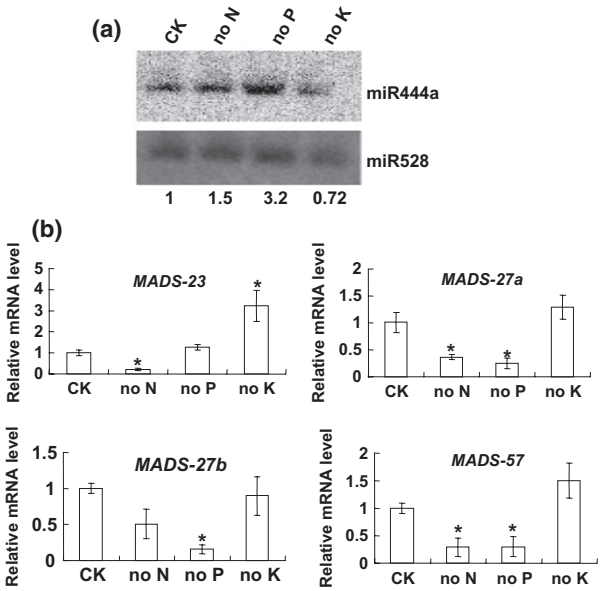

Figure 1. Expression patterns of miR444 and its targets under N, P, or K starvation conditions.
(a) Analysis of miR444 accumulation in rice roots under N, P, or K starvation conditions. After germination in water, wild type plants were transferred to half-strength Murashige and Skoog (½MS) medium (CK), N-starvation (no N), P starvation (no P), or K starvation (no K) medium and grown for 10 days. Then, root samples were collected and total RNA extracted. miR444 accumulation was detected by small RNA gel blot hybridization. The hybridization signal for miR528 on the same membrane formed after stripping off miR444 probes served as a small RNA loading control. Normalized intensities of miR444 signals are indicated under the blot panels.
(b) Analysis of the relative mRNA levels of four miR444 targets under N, P, or K starvation conditions in wild type rice roots. After reverse transcription from the total RNA samples described in (a), the relative mRNA levels of the four miR444 targets were analyzed by qRT-PCR. Results are the mean ± standard error (SE) for three replicates. Asterisks indicate significant differences between CK and no N, P or K conditions (Student's *t*-test analysis, $*P < 0.05$).

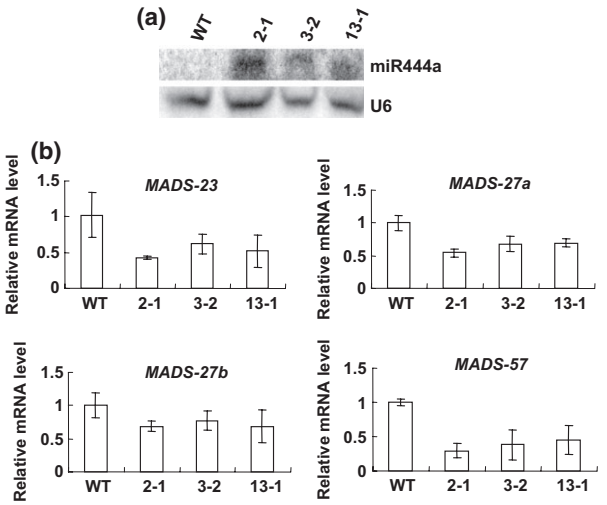

Figure 2. Expression of miR444 and its targets in miR444a-overexpressing rice.
(a) miR444 accumulation was increased in miR444a-overexpressing lines. After 10 days of growth in half-strength Murashige and Skoog (½MS) hydroponic culture, the shoots of three miR444a-overexpressing rice lines were collected and total RNA was extracted. miR444 accumulation was detected by small RNA gel blot hybridization. The hybridized signals for U6 small nuclear RNA (U6 snRNA) are shown as a loading control.
(b) Analysis of the relative mRNA levels of four miR444 targets in three miR444a-overexpressing rice lines. After reverse transcription from the total RNA samples described in (a), the relative mRNA levels of the four miR444 targets were analyzed by qRT-PCR. Error bars indicate standard error (SE; $n = 3$).

miR444 accumulation was increased in the transgenic rice lines (Figure 2a). Consistent with this finding, expression of the miR444 targets was decreased in these transgenic rice lines (Figure 2b). To investigate whether overexpression of miR444a affected the localized stimulation of rice lateral root growth in NO_3^--rich patches, adventitious roots of wild type plants and miR444a-overexpressing plants were grown in NO_3^--rich (5 mM KNO_3) or control (5 mM KCl) medium. In wild type plants, lateral root elongation was stimulated strongly in NO_3^--rich medium compared with the KCl-treated control (Figures 3a,b and S1). This stimulation was not observed when wild type plants were supplied with the same concentration of NH_4^+ (Figure S2), confirming the signaling properties of the NO_3^- ion itself in lateral root proliferation (Zhang and Forde, 1998). In contrast, NO_3^--rich treatment had no significant stimulatory effects on lateral root growth in the miR444a-overexpressing lines (Figures 3a,b and S1), much like *ANR1*-underexpressing Arabidopsis plants (Zhang and Forde, 1998). This situation indicated that overexpression of miR444a reduces the localized stimulatory effects of NO_3^- on rice lateral root proliferation.

Root performance of the miR444a-overexpressing lines and wild type plants grown under different nitrate concentration conditions was also investigated. T2 seeds of miR444a-overexpressing lines and wild type plants were germinated and grown for 14 days in high (10 mM KNO_3) or low (0.2 mM KNO_3) nitrate concentration conditions in agar tubes. After 14 days, the lateral roots of the miR444a-overexpressing plants were seen to be shorter than those of the wild type plants under both high nitrate and nitrate-limiting conditions (Figure 4a,b). Unlike the effects of *ANR1* down-regulation, which did not affect primary root growth, overexpression of miR444a significantly increased primary root elongation under 10 mM KNO_3 conditions and slightly increased primary root length under nitrate-limiting conditions. In addition, the adventitious roots of the miR444a-overexpressing plants were found to be longer than those of wild type plants under both high and low nitrate concentrations (Figure 4a,b). Moreover, the miR444a-overexpressing rice lines showed smaller root diameters compared with the wild type plants (Figure 4a, b). It is well known that carbon can affect the regulatory effects of N in plant growth and development. To test whether the miR444a-mediated effects of NO_3^--signaling in

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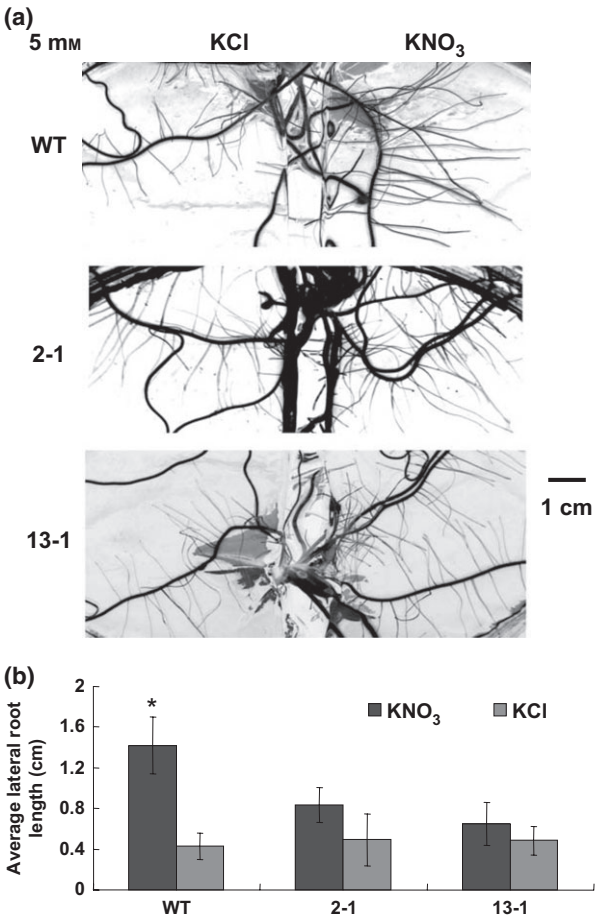

Figure 3. Lateral root performance of wild type and miR444a-overexpressing rice plants in NO_3^--rich patches.
(a) Overexpression of miR444a reduced lateral root stimulation in NO_3^--rich patches. Rice adventitious roots were divided into two parts and grown separately in medium that contained 5 mM KCl or 5 mM KNO_3. The roots were observed after 7 days of growth. Bar = 1 cm.
(b) Measurement of lateral root length under nitrate stimulation. Error bars indicate standard error (SE; $n = 10$). Asterisks indicate significant differences between wild type and miR444a-overexpressing plants (Student's *t*-test analysis, $*P < 0.05$).

root growth were changed by carbon, 2% sucrose was added to the high (10 mM) and low (0.2 mM) KNO_3 media and root growth was observed. After 10 days of germination and growth, the miR444a-overexpressing rice lines on combined nitrate/sucrose medium showed similar root phenotypes to those plants grown without sucrose, although the extent of phenotypic variation was slightly different (Figure S3). Therefore we concluded that miR444a participates mainly in NO_3^--affected root growth.

Taken together, our observations indicated that in rice miR444a negatively regulates the NO_3^--signaling pathway in lateral root growth by controlling the expression of *ANR1*-homologous genes. Moreover, in contrast with ANR1 in Arabidopsis, miR444a has additional functions in the regulation of growth of rice primary and adventitious roots.

Overexpression of miR444a improves nitrate accumulation and expression of nitrate transporters

To investigate whether miR444a-regulated NO_3^--dependent root development affected shoot growth and nitrate content, wild type and miR444a-overexpressing T2 seeds were germinated and grown in agar tubes on N-free medium that was supplemented with 0, 0.2 or 10 mM KNO_3. After 14 days of growth, the miR444a-overexpressing rice had shoots that were slightly shorter compared with wild type plants in 10 mM KNO_3 medium (Figure 5a,b). However, when the KNO_3 concentration was reduced to limiting conditions (0 or 0.2 mM), growth of the miR444a-overexpressing lines was inhibited more severely than that of the wild type plants (Figure 5a,b).

The nitrate content in the wild type plants and in the miR444a-overexpressing lines was measured and compared. miR444a-overexpressing rice shoots and roots contained more nitrate than those of the wild type plants for the same fresh weight when the plants were supplied with 10 mM KNO_3 (Figure 5c). There was no significant difference in nitrate levels between miR444a-overexpressing and wild type rice shoots under N-limiting conditions (0.2 mM KNO_3; Figure 5c).

Because of the increased nitrate accumulation in the miR444a-overexpressing rice plants, we examined the expression of four nitrate transporter genes to investigate whether the nitrate transport system was regulated by miR444a. The wild type plants and two miR444a-overexpressing lines were grown for 10 days in medium supplemented with 10 mM KNO_3. Shoot and root samples were subjected to quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. The results showed increased mRNA levels of the four nitrate transporter genes in the miR444a-overexpressing shoots and roots (Figure 5d).

Overexpression of miR444a disrupts remobilization of nitrate from old leaves to young leaves under N-starvation conditions

miR444a-overexpressing plants were more sensitive to N-starvation than wild type plants (Figures 5a,b), this finding suggested that up-regulation of miR444a reduced the adaptability of rice to N-limiting conditions. There was no significant difference in nitrate levels in rice shoots from miR444a-overexpressed or wild type plants under N-limiting conditions (0.2 mM KNO_3; Figure 5c); this result suggested that the reduced adaptability of miR444a-overexpressing plants to N-limiting conditions was not caused by a reduction in nitrate accumulation. To investigate whether the remobilization of nitrate from old organs to young organs was affected by overexpression of

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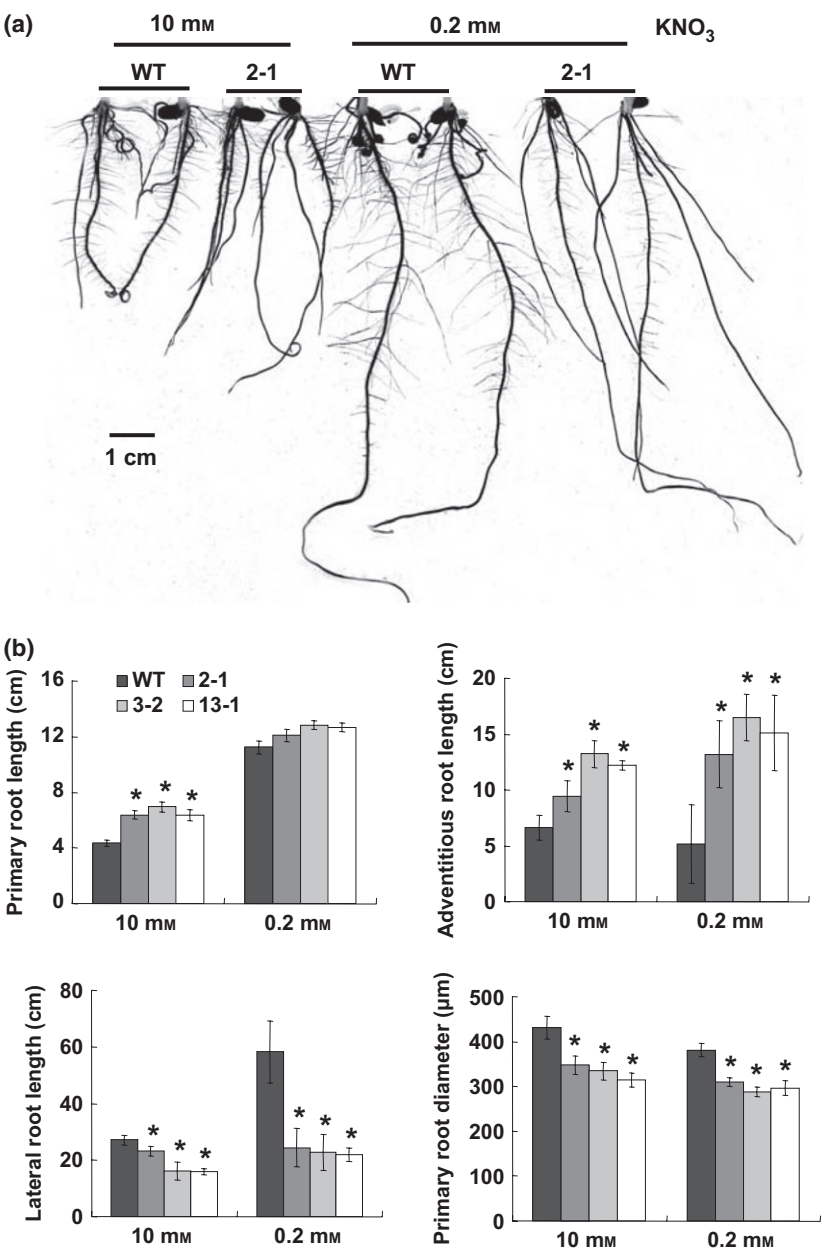

Figure 4. Root performance of wild type and miR444a-overexpressing rice under different nitrate concentration conditions.
(a) Roots of 14-day-old wild type and miR444a-overexpressing rice plant grown under high (10 mM KNO_3) or low (0.2 mM KNO_3) nitrate concentration conditions. Bar = 1 cm.
(b) Measurement of the length of primary roots, the three longest adventitious roots and lateral roots, and the primary root diameter. Error bars indicate standard error (SE; $n = 10$). Asterisks indicate significant differences between wild type and miR444a-overexpressing plants (Student's *t*-test analysis, $*P < 0.05$).

miR444a, wild type and miR444a-overexpressing plants were grown to the three-leaf stage under N-limiting conditions (0.2 mM KNO_3) and the nitrate contents of the second (old) and third (young) leaves were analyzed. The results showed that, compared with the wild type plants, the miR444a-overexpressing lines accumulated more nitrate in old leaves, but less nitrate in young leaves (Figure 6a). The total amino acid content of miR444a-overexpressing young leaves was also lower compared with that of their wild type counterparts (Figure 6b). Decreased accumulation of nitrate and amino acids in young leaves was not observed in plants that were grown under high nitrate conditions (10 mM KNO_3; Figure 6b,c). Taken together, the above results suggested that up-regulation of miR444a reduced the plant's ability to remobilize N from old leaves to young leaves, thus rendering the plant more sensitive to the N-limited conditions.

miR444a is involved in regulating root architectural changes in response to P_i starvation

Interestingly, miR444a accumulation was induced strongly by P_i starvation (Figure 1a), implying that miR444a is involved in the regulation of P_i-starvation responses. A typical morphological and developmental response to P_i starvation observed in many plant species is the reduced growth of the primary root and an increased length in

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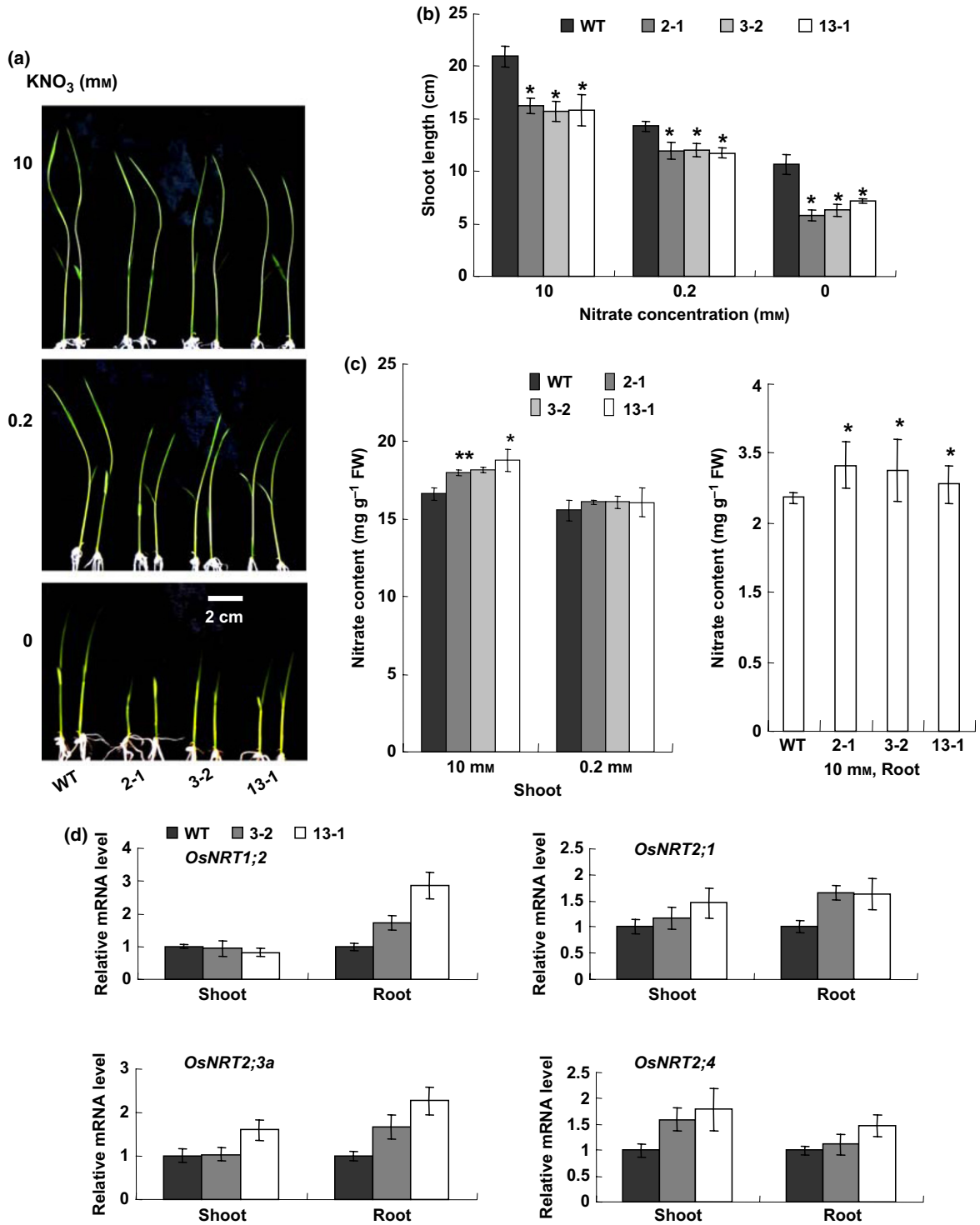

Figure 5. Overexpression of miR444a improves nitrate accumulation and expression of nitrate transporter genes.
(a) Shoot performance of wild type and miR444a-overexpressing rice under high (10 mM KNO_3) or low (0.2 or 0 mM KNO_3) nitrate concentration conditions after 14 days of growth. Bar = 2 cm.
(b) Measurement of the length of shoots described in (a). Error bars indicate standard error (SE; $n = 8$).
(c) Nitrate contents in shoots and roots of wild type and miR444a-overexpressing plants grown in high (10 mM KNO_3) or low (0.2 mM KNO_3) nitrate concentration conditions. FW, fresh weight.
(d) Analysis of the relative mRNA levels of four nitrate transporter genes. After reverse transcription from the total RNA extracted from shoots growing on 10 mM KNO_3, relative mRNA levels were analyzed by qRT-PCR. Asterisks indicate significant differences between wild type and miR444a-overexpressing plants (Student's *t*-test analysis, $*P < 0.05$).

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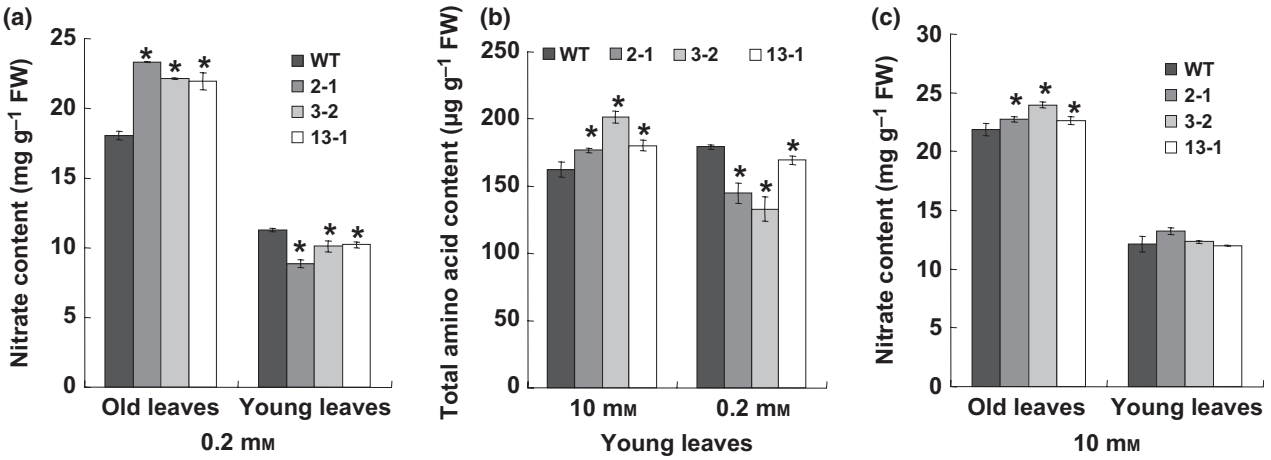

Figure 6. Overexpression of miR444a disrupts N remobilization from old leaves to young leaves.
Wild type and miR444a-overexpressing plants were grown to the three-leaf stage and the nitrate and total amino acid contents of the second leaf (old) and third leaf (young) were analyzed.
(a, c) Nitrate content in old leaves and young leaves under 0.2 mM (a), or 10 mM KNO_3 (c) conditions.
(b) Total amino content in young leaves under both 10 and 0.2 mM KNO_3 conditions. Error bars indicate standard error (SE; $n = 3$). FW, fresh weight. Asterisks indicate significant differences between wild type and miR444a-overexpressing plants (Student's *t*-test analysis, $*P < 0.05$).

lateral roots (López-Bucio *et al.*, 2003; Yuan and Liu, 2008). To investigate whether miR444a regulates P_i concentration-dependent root architecture, T2 seeds from the wild type and miR444a-overexpressing lines were germinated and grown for 10 days on solid medium under adequate P_i (0.3 mM Pi) or P_i-starvation (0 mM P_i) conditions. Both media contained 10 mM KNO_3 and 2% sucrose. In wild type plants, the growth of primary and adventitious roots was inhibited by P_i-starvation conditions (Figures 7a–c and S4). Lateral root elongation was induced in the wild type plant under P_i-starvation conditions, although the total lateral root length was shorter because of the shorter primary root (Figures 7a,d,e and S4). In contrast, the miR444a-overexpressing plants had longer primary and adventitious roots and shorter lateral roots than the wild type plants under both adequate P_i conditions and P_i-starvation conditions (Figures 7a–e and S4). These observations indicated that the root performances of miR444a-overexpressing rice plants under Pi starvation are similar to plants grown under high nitrate conditions (Figure 4) and that miR444a is involved in the regulation of root architectural changes in response to P_i starvation.

Overexpression of miR444a enhances P_i accumulation and expression of P_i transporters

To test whether miR444a regulation of root architecture affects rice P_i accumulation, T2 seeds of the wild type and two miR444a-overexpressing lines were germinated and grown for 10 days in solid medium supplemented with high concentrations of KNO_3 (10 mM) and 0, 0.2, 1 or 10 mM P_i. The P_i contents of shoots were then analyzed. P_i content was increased in the miR444a-overexpressing shoots under different P_i concentrations (Figure S5). It has been shown that P_i accumulation is affected by nitrate concentration and that low levels of nitrate promote P_i acquisition in Arabidopsis (Kant *et al.*, 2011). To test whether miR444a also plays a role in P_i accumulation in rice under low nitrate concentration conditions, we determined the P_i content of wild type and miR444a-overexpressing rice plants under different nitrate concentrations (10 or 0.2 mM NO_3^- with 0.3 mM P_i). Consistently, miR444a-overexpressing plants accumulated more P_i than wild type plants in shoots and roots under high nitrate concentrations (Figures S5 and 8a,b). More specifically, low nitrate concentrations promoted P_i accumulation in wild type rice shoots, as in Arabidopsis (Figure 8a,b). Comparatively, miR444a-overexpressing plants accumulated even more P_i than wild type plants in shoots under low nitrate concentrations (Figure 8a); this result suggested that miR444a improves Pi accumulation in shoots irrespective of the nitrate concentration. Low levels of nitrate had no apparent effect on P_i accumulation in wild type rice roots (Figure 8b).

Plants absorb P_i via P_i transporters (PT). To analyze whether increased expression of PT genes resulted in P_i over-accumulation in the miR444a-overexpressing plants, we examined the mRNA levels of 11 rice PT genes. The expression levels of three PT genes, *OsPT1*, *OsPT7* and *OsPT11*, was increased greatly in miR444a-overexpressing roots. The expression of *OsPT1* and *OsPT11* was also increased significantly in miR444a-overexpressing shoots (Figure 8c).

Phenotypes of miR444a-overexpressing rice in the field

The phenotypes of miR444a-overexpressing rice over the whole growth period were investigated in the paddy field under normal nutrition management. Changes in the

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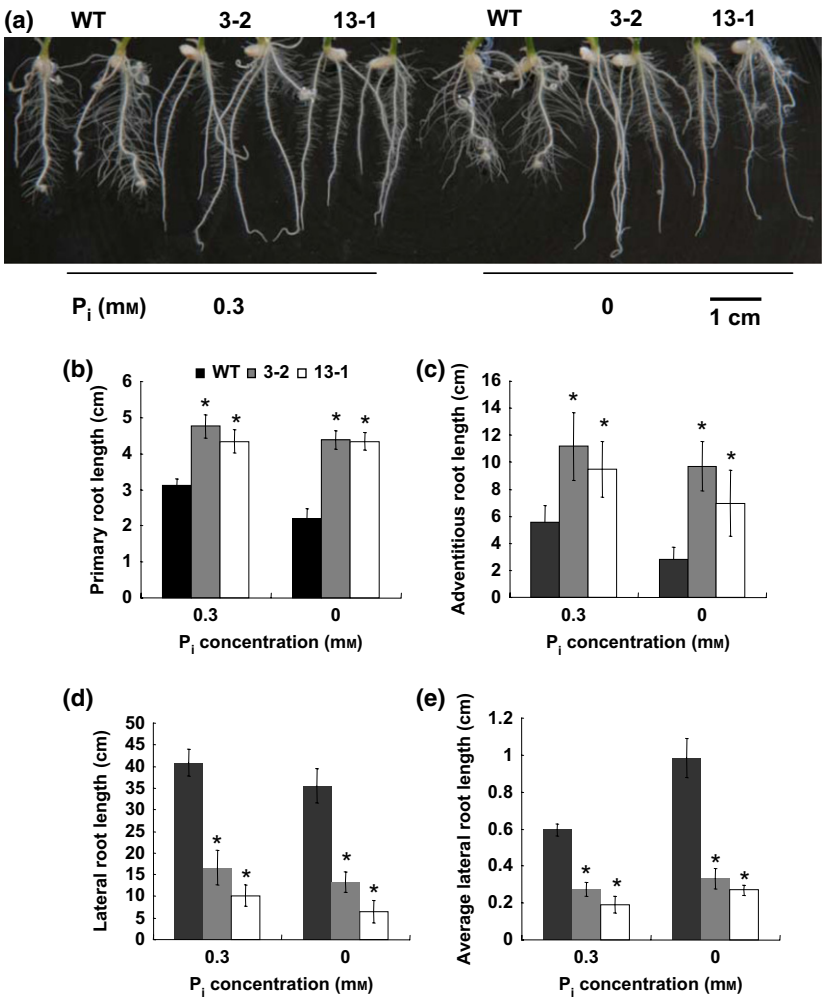

Figure 7. Overexpression of miR444a alters root architecture in response to P_i starvation.
(a) Root performance of wild type and miR444a-overexpressing rice under adequate P_i (0.3 mM) and P_i starvation (0 mM) conditions after 14 days of growth. Bar = 1 cm.
(b–e) Measurement of the length of primary roots (b), the three longest adventitious roots (c), and lateral roots (d, e). Error bars indicate standard error (SE; $n = 10$). Asterisks indicate significant differences between wild type and miR444a-overexpressing plants (Student's *t*-test analysis, $*P < 0.05$).

panicle compared with wild type rice plants were of particular interest because this organ is related to rice yield. Several panicle parameters were measured. miR444a-overexpressing plants had comparatively longer panicles (Figure 9a,b), however detailed analysis showed that although the panicle was longer, the number of primary branches in miR444a-overexpressing panicles did not increase; conversely, the number of second branches was decreased, which resulted in lower grain numbers per panicle in miR444a-overexpressing plants (Figure 9c–e). Furthermore, the panicle filling rate and 1000-grain weight were decreased (Figure 9f,g). These observations indicated that up-regulation of miR444a reduced rice grain number and filling.

DISCUSSION

miR444a regulates the NO_3^--signaling pathway in rice root and shoot development and nitrate accumulation

The targets of miR444, four MADS-box genes, are highly homologous to *ANR1* (Sunkar *et al.*, 2005; Wu *et al.*, 2009; Li *et al.*, 2010). In this work, we showed that overexpression of miR444a, which decreased the expression of the four MADS-box genes (Figure 2a,b), reduced nitrate-induced lateral root growth in rice (Figures 3, 4, S1 and S3), as in *ANR*-repressed Arabidopsis (Zhang and Forde, 1998). These observations indicated that miR444a participates in the NO_3^--signaling pathway to trigger plasticity of lateral root development by regulating *ANR1*-homologous genes in rice; this outcome suggests a conserved NO_3^--signaling regulatory mechanism in rice and Arabidopsis. Repression of *ANR1* in Arabidopsis did not affect primary root elongation (Zhang and Forde, 1998), whereas miR444a-overexpressing rice plants showed altered primary and adventitious root architecture in response to different nitrate concentrations (Figure 4). This difference in primary root performance may stem from the different root systems between dicots and monocots. Our observations suggested both conserved and divergent functions for the *ANR1* homolog(s) in the rice NO_3^--signaling pathway.

The functions of *ANR1* in shoot growth and nitrate accumulation remain to be explored in Arabidopsis. Recently,

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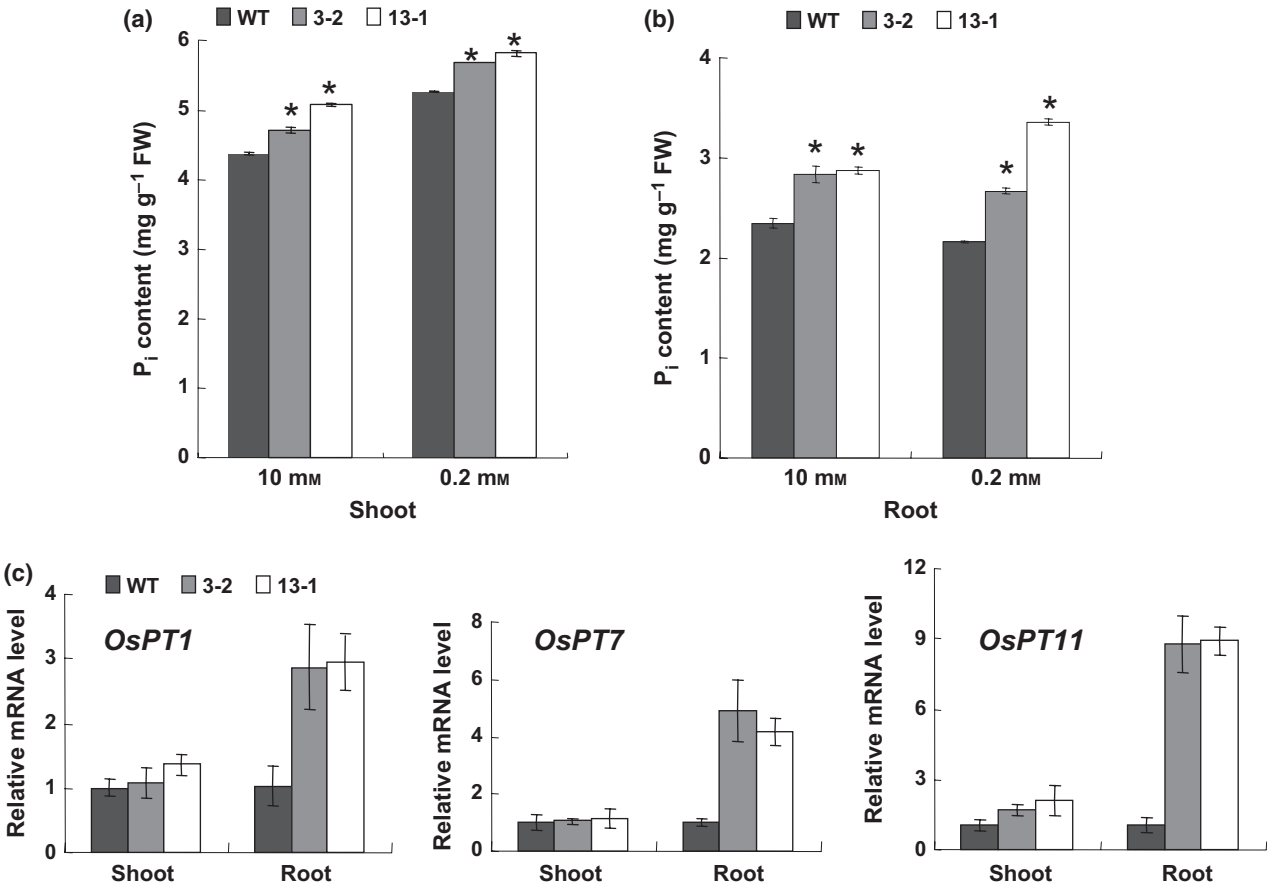

Figure 8. Overexpression of miR444a improves P_i accumulation and expression of P_i transporters.
(a, b) P_i content of shoots (a), and roots (b) under high (10 mM KNO_3) or low (0.2 mM KNO_3) nitrate concentration conditions with 0.3 mM P_i after 14 days of growth. Error bars indicate standard error (SE; $n = 3$).
(c) Analysis of the relative mRNA level of P_i transporter genes by qRT-PCR. Total RNA samples were the same as described in Figure 5(d). FW, fresh weight. Asterisks indicate significant differences between wild type and miR444a-overexpressing plants (Student's *t*-test analysis, $*P < 0.05$).

Gan *et al.* (2012) reported that overexpression of *ANR1* increased the length and number of lateral roots and the fresh weight of shoots. Our results showed that overexpression of miR444a slightly reduced shoot growth at the early seedling stage (Figure 5). It has been shown that the Arabidopsis nitrate transporter gene *NRT2.1* is down-regulated in the roots of an *ANR1*-knockout line, indicating that ANR1 is a positive regulator of *NRT2.1* expression (Gan *et al.*, 2005) and suggesting that ANR1 regulates nitrate acquisition. Consistent with this finding, it was also shown that overexpression of *ANR1* increased nitrate accumulation (Gan *et al.*, 2012). Our results showed that overexpression of miR444a increased nitrate accumulation in rice shoots and roots under adequate nitrate (10 mM) conditions (Figure 5). This result can be explained possibly by the increased length of primary and adventitious roots (Figure 4) and increased expression of nitrate transporter genes in miR444a-overexpressing rice (Figure 5d). Overexpression of miR444a also reduced nitrate remobilization from old leaves to young leaves under N-limiting conditions (Figure 6), and suggested that miR444a affects nitrate translocation in shoots under N-starvation conditions. Taken together, these observations indicated that miR444 plays important regulatory functions in the NO_3^--signaling pathway for root and shoot development and nitrate accumulation.

miR444a is involved in multiple P_i-starvation responses in rice

miR444a expression was induced by P_i starvation (Figure 1a), this finding suggests a role for miR444a in regulating P_i-starvation responses. Indeed, the following evidence demonstrates that miR444a regulates multiple P_i-starvation responses. First, overexpression of miR444a altered P_i-starvation-induced root architecture (Figure 7). Second, overexpression of miR444a increased the P_i content in roots and shoots and the expression of three PT genes (Figure 8), indicating that miR444a regulates P_i acquisition and accumulation. Third, to adapt to P_i starvation, plants also regulate the expression of genes that

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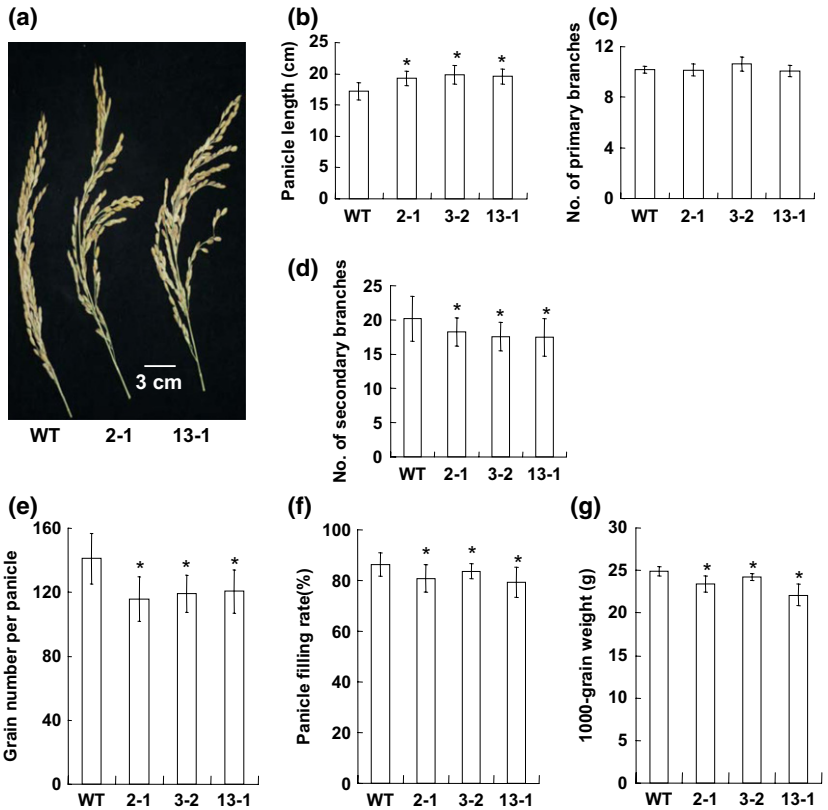

Figure 9. The panicle phenotypes of miR444a-overexpressing plants grown in the paddy field (Beijing, 40°06′N, 116°24′E).
(a) Panicle architecture.
(b–g) Measurements of panicle parameters including panicle length (b), number of primary branches per panicle (c), number of secondary branches per panicle (d), grain number per panicle(e), panicle filling rate (f) and 1000-grain weight (g). Error bars indicate standard error (SE); (b–f) $n = 10$ plants; (g) $n = 5$ replicates. Asterisks indicate significant differences between wild type and miR444a-overexpressing plants (Student's *t*-test analysis, $*P < 0.05$).

encode acid phosphatases and nucleases and alter lipid metabolism (Chiou and Lin, 2011). Using qRT-PCR analysis, we showed that the expression of *acid phosphatase* (*OsPAP10*), two nuclease genes (*RNase PD2* and *S1/N1 nuclease*), *phospholipase C* (*PLC*) and *diacylglycerol galactosyltransferase* (*DGGT*) was altered in miR444a-overexpressing plants under P_i-sufficient conditions (Figure S6).

miR444a involves in the interaction between the NO_3^-- and P_i-signaling pathways

We have shown that miR444a is involved in P_i-starvation responses and also in the nitrate-signaling pathway, these findings imply that miR444a might play a role in the interaction between the NO_3^--signaling and P_i-signaling pathways. More interestingly, the roots of miR444a-overexpressing rice plants under P_i starvation showed similar phenotypes to those plants grown under high nitrate conditions (Figures 4 and 7). These findings suggested that miR444a regulates P_i-starvation-induced root architecture possibly via activation of the miR444a-regulated NO_3^--signaling pathway. Taken together, these observations suggested that miR444a regulation of P_i effects involves interaction between the NO_3^--signaling and P_i-signaling pathways in rice, although the detailed mechanism of this interaction needs to be investigated further.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Oryza sativa spp. *japonica* cv. Nipponbare was used as the wild type and the genetic background for transgenic plants. For phenotypic observations, plants were grown in the field or in a 25°C incubator with a 16-h light/8-h dark photoperiod and approximately 70% humidity. For different nutrient treatments, plants were grown in hydroponic cultures or on agar plates prepared with modified half-strength Murashige and Skoog (½MS) mixtures (PhytoTechnology Laboratories, Shawnee Mission, KS, USA, http://www.phytotechlab.com). To simulate different nitrate concentration cultures, a designated amount of KNO_3 was added to N-free medium (cat. no. M531). N-, P- and K-free medium (cat. no. M407) was used to prepare cultures of different P_i or K concentrations by adding the desired nutrients. Except for these modified nutrients, all nutrients were kept at the same concentration in all cultures. For NO_3^--rich treatment, 4-day-old primary roots were excised to promote the growth of adventitious roots. After 2 days of growth, adventitious roots were divided into two parts and grown separately on 5 mM KNO_3, and 5 mM NH_4Cl or 5 mM KCl agar plates.

Constructs and generation of transgenic rice plants

To generate a miR444a ectopic expression vector, a 441-bp miR444a precursor sequence was amplified and cloned into pCAMBIA1300 (Cambia, http://www.cambia.org). Then, the construct was electroporated into *Agrobacterium tumefaciens* EHA105 to transform rice using the method described by Hiei *et al.* (1994). The primers used for PCR are listed in Table S1.

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Small RNA gel blot analysis

Small RNA gel blot analysis was performed as described in Yan *et al.* (2011). Briefly, total RNA was extracted from rice samples with Trizol reagent (Life Technologies, http://www.lifetechnologies.com). Then, total RNA samples (10 and 30 μg from shoot and root materials, respectively) were separated on a denaturing 17% polyacrylamide gel and transferred electrophoretically to Hybond-N^+ membranes (GE Healthcare Life Sciences, http://www.gelifesciences.com). Hybridizations were performed at 38°C in PerfectHyb Plus buffer with DNA oligonucleotide probes labeled by T4 polynucleotide kinase (New England Biolabs, https://www.neb.com/). Hybridization signals were detected with a phosphorimager (GE Healthcare Life Sciences, http://www.gelifesciences.com). The sequences of the probes are given in Table S1.

qRT-PCR analysis

Total RNA (3 μg) treated with RNase-free DNase I was subjected to reverse transcription to produce cDNA products using SuperScript III reverse transcriptase (Invitrogen) following the supplier's protocol. qRT-PCR was performed by adding SYBR Green real-time PCR master mix (TOYOBO, http://www.toyobo-global.com/) to the reaction system and run on a DNA engine Opticon2 real-time PCR detection system (Bio-Rad, http://www.bio-rad.com) in accordance with the manufacturer's instructions. Three replicates were performed for each gene. Relative quantification of each sample was determined by normalization against the amount of glyceraldehyde 3-phosphate (GAPDH) cDNA detected in the same sample. Relative expression level was calculated by the formula $2^{-\Delta\Delta C_T}$. The sequences of the primers used for qRT-PCR are listed in Table S1.

Nitrate and total amino acid concentration measurement

Nitrate concentration was measured as described previously (Cataldo *et al.*, 1975). Fresh rice plant samples were collected and ground to a powder in liquid nitrogen. Then, the samples were suspended in 10 ml of deionized water and incubated at 45°C for 1 h. The suspension was centrifuged at 5000 ***g*** for 15 min. The supernatant and 5% (w/v) salicylic acid (1:4) were mixed in concentrated H_2SO_4. After 20 min at room temperature, 2 N NaOH was added slowly into the mixture to raise the pH to above 12. The samples were cooled to room temperature, and nitrate concentration was determined at 410 nm wavelength. Total amino acids were estimated according to the method of Moor and Stein (1957) using the same supernatant for measuring nitrate content. Briefly, the supernatant (100 μl) was mixed with 0.3% ninhydrin solution (300 μl, dissolved in 95% ethanol) and acetic acid (pH 5.4, 100 μl) in a 20 ml tube. Then, the tubes were heated for 20 min in a boiling water bath. After cooling by water, the total amino acid concentration was determined at 570 nm wavelength.

P_i concentration measurement

P_i content was measured following a previously described method with minor modifications (Zhou *et al.* 2008). Fresh rice plant samples were frozen with liquid nitrogen and homogenized in 1 ml of 10% (w/v) perchloric acid. The homogenate was then diluted 10 times with 5% (w/v) perchloric acid and placed on ice for 30 min. The mixture was centrifuged at 13 000 ***g*** for 5 min, and an aliquot of the supernatant was used in the P_i assay. The reaction mixture contained 700 μl of assay solution (0.35% NH_4MoO_4, 0.86 N H_2SO4, and 1.4% ascorbic acid) and 300 μl of sample and was incubated at 42°C for 30 min. P_i content was measured at 820 nm wavelength.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Lateral root performance of wild type and miR444a-overexpressing line (3-2) in NO_3^--rich patches.

Figure S2. Lateral root stimulation in NH_4^+-rich patches.

Figure S3. Root performance of wild type and miR444a-overexpressing rice plants under combined nitrate/sucrose conditions.

Figure S4. Root performance of wild type and miR444a-overexpressing line (2-1) under P starvation condition.

Figure S5. Overexpression of miR444a increases P_i accumulation.

Figure S6. qRT-PCR analysis of the expression of *acid phosphatase* (*OsPAP10*), two nuclease genes (*RNase PD2* and *S1/N1 nuclease*), *phospholipase C* (*PLC*) and *diacylglycerol galactosyltransferase* (*DGGT*) in miR444a-overexpressing and wild type rice plants under Pi-sufficient conditions.

Table S1. Primers for qRT-PCR; probes for small RNA hybridization.

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