OsARF16, a transcription factor, is required for auxin and phosphate starvation response in rice (*Oryza sativa* L.)

CHENJIA SHEN1*, SUIKANG WANG1*, SAINA ZHANG1, YANXIA XU1, QIAN QIAN2, YANHUA QI1 & DE AN JIANG1

1State Key Laboratory of Plant Physiology and Biochemistry, College of Life Sciences, Zhejiang University, Hangzhou 310058 and 2State Key Laboratory of Rice Biology, China National Rice Research Institute, Chinese Academy of Agricultural Sciences, 359 Tiuyuchang Road, Hangzhou, China

**ABSTRACT**

Plant responses to auxin and phosphate (Pi) starvation are closely linked. However, the underlying mechanisms connecting auxin to phosphate starvation (−Pi) responses are largely unclear. Here, we show that OsARF16, an auxin response factor, functions in both auxin and −Pi responses in rice (*Oryza sativa* L.). The knockout of OsARF16 led to primary roots (PR), lateral roots (LR) and root hair losing sensitivity to auxin and −Pi response. OsARF16 expression and OsARF16::GUS staining in PR and LR of rice Nipponbare (NIP) were induced by indole acetic acid and −Pi treatments. In −Pi conditions, the shoot biomass of osarf16 was slightly reduced, and neither root growth nor iron content was induced, indicating that the knockout of OsARF16 led to loss of response to Pi deficiency in rice. Six phosphate starvation-induced genes (PSIs) were less induced by −Pi in osarf16 and these trends were similar to a knockdown mutant of OsPHR2 or AtPHR1, which was a key regulator under −Pi. These data first reveal the biological function of OsARF16, provide novel evidence of a linkage between auxin and −Pi responses and facilitate the development of new strategies for the efficient utilization of Pi in rice.

**Key-words:** auxin response factor.

**INTRODUCTION**

Phosphorus (P) deficiency leads to a serious obstruction of plant growth and development by affecting many physiological and biochemical reactions. Plants cope in a low P environment by altering their root system architecture (RSA; Bates & Lynch 1996; López-Bucio *et al*. 2002; Sánchez-Calderón *et al*. 2005). Plants grown under phosphate starvation conditions exhibit a reduction in primary root (PR) length, an abundance of root hairs (RH) and an increase in lateral root (LR) density (Dinkelaker, Hengeler & Marshner 1995; Bates & Lynch 1996; Borch *et al*. 1999; Williamson *et al*. 2001). These changes help to increase the root surface area and they allow plants to more effectively absorb P (Stitt & Rudiger-Scheible 1998). RSA alterations due to P deficiency can also be achieved by modulating the auxin sensitivity in roots (López-Bucio *et al*. 2002; Pérez-Torres *et al*. 2008). For example, P deficiency leads to an increase in the auxin content as well as its redistribution (Borch *et al*. 1999; Nacry *et al*. 2005). Auxin polar transport is essential for LR formation under adequate P conditions (Reed, Brady & Muday 1998), but an auxin transport-independent pathway is involved in phosphate stress-induced root architectural alterations in *Arabidopsis* (López-Bucio *et al*. 2005). A recent study showed that SUMO E3 ligase SIZ1 negatively regulates Pi starvation-induced root architecture remodelling through the control of auxin patterning (Miura *et al*. 2011).

Auxin signalling is involved in Pi starvation-induced root architecture remodelling. In *Arabidopsis*, Pi deficiency influences LR development that depends on TIR1 via ARF7/19, which are two of the many auxin response factors that act downstream of auxin (Ulmasov; Hagen & Guilfoyle 1997; Guilfoyle, Ulmasov & Hagen 1998, Guilfoyle & Hagen 2001, 2007). Auxin signalling via the TIR receptor triggers the degradation of AUX/indole acetic acid (IAA) proteins, which allows ARF7/19 to regulate the expression of a series of downstream genes controlling LR development (Pérez-Torres *et al*. 2008).

Cross-talk between auxin and −Pi response has been shown in *Arabidopsis* (Pérez-Torres *et al*. 2008; Miura *et al*. 2011), but it has only been implicated in rice (Inukai *et al*. 2005; Zhou *et al*. 2008; Attia *et al*. 2009). The underlying mechanism linking the two responses is unknown. Understanding the relationship between auxin and −Pi response will ultimately facilitate the development of new strategies for the efficient utilization of P in rice. Here, we showed that an osarf16 mutant was defective in both auxin response and −Pi signalling, and established OsARF16 as a critical link between auxin and −Pi response in rice.

**MATERIALS AND METHODS**

**Plant material and growth condition**

Rice (*Oryza sativa* L.) Nipponbare (NIP), osarf16 mutant and OsARF16 overexpression lines were grown in normal
culture solution in a greenhouse with a light/dark cycle of 12/12 h at 30/24 °C. Under –Pi treatment, rice seedlings were grown in normal culture solution without NaH2PO4 for 7 d Phytomoroph hormone treatment was performed with 1 μM of 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthylacetic acid, 3-indolylbutyric acid (IBA) and 1 μM of IAA for 7 d, respectively. Treatments with polar auxin transport inhibitors (PATIs) were carried out with 1 μM of 1-naphthylphthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA), and 10 μM 1-naphthoxyacetic acid (NOA) for 7 d, respectively.

Identification of mutant osarf16

Identification of TOS17 insertion sites in the mutant osarf16 (RGRC-NC6645) and the expression levels of OsARF16 were determined according to http://www.rgrc.dna.afrc.go.jp. Primer TOS17-tail16 was used to confirm the integration of TOS17 in osarf16, and the gene-specific primers ARF16U/L were used to identify wild-type (WT)-bound OsARF16. The PCR insertion products were ligated with pUCm-T vector (Sangon, Shanghai, China) and transformed into Escherichia coli DH5α, and the flanking sequences of the TOS17 insertion site were sequenced by Invitrogen (Shanghai, China). To confirm the transcription level of the OsARF16 gene in the WT and osarf16 mutant, RT-PCR was performed using the primers RTARF16U/L. The methods of total RNA extraction, reverse transcription, and RT-PCR were as given in a previous report (Wang et al. 2010a). Primer sequences for the PCR and RT-PCR are listed in Supporting Information Table S1.

Overexpression of OsARF16 in the mutant osarf16 and NIP

The open reading frame (ORF) of OsARF16 (LOC_Os06g09660) was directly amplified from the full-length cDNA (AK103327) using the primers OVARF16U/L shown in Supporting Information Table S1, before being cloned into the binary vector pCAMBIA 1300 containing a CaMV 35S promoter to create an OsARF16 overexpression construct. The 35S:OsARF16 was introduced into Agrobacterium tumefaciens strain EHA105 using electroporation and infiltrated into the mutant osarf16 and NIP (Hiei et al. 1994). Overexpression analysis of OsARF16 genes was monitored by RT-PCR using the primers RTARF16U/L are listed in Supporting Information Table S1.

β-glucuronidase (GUS) staining

The construction of OsARF16 promoter–GUS was performed according to a published method (Cheng, Dai & Zhao 2007). Primers ProARF16U/L (listed in Supporting Information Table S1) were used for amplification of the promoter region. OsARF16::GUS was introduced into the Agrobacterium tumefaciens strain EHA105 and transformed into the rice WT, NIP. The DR5::GUS auxin reporter described by Ulmasov et al. (1997) was transformed into WT and osarf16 for detecting auxin distribution. GUS staining of seedlings used 100 mM sodium phosphate buffer (pH 7.0) containing 0.1% v/v Triton X-100 and 2 mM X-Gluc (Sangon, Shanghai, China), which was incubated at 37 °C overnight. Stained tissues were observed using a Carl Zeiss laser scanning system LSM510 (http://www.zeiss.com/) and a Leica MZ95 stereomicroscope (Leica Instrument, Nusslosh, Germany).

IAA measurement

The IAA concentrations of PR in the WT and osarf16 were measured by gas chromatography-selected reaction monitoring mass spectrometry, as described by Ljung et al. (2005). The germinated seeds of WT and osarf16 were grown in normal culture solution for 7 d (as a control, CK), or in normal culture solution without NaH2PO4 for 7 d (as –Pi treatment), or were treated with 1 μM of IAA for 3 h after being grown in normal culture solution for 7 d (as IAA treatment). The PRs after –Pi or IAA treatment were washed by deionized water five times to clear the PR surface. Five independent biological replicates of each 20 mg sample were purified after addition of 250 pg of 13C6-IAA internal standard using ProElu C18 (http://www.dikma.com.cn), and auxin contents were measured with FOCUS GC-DSQII (Thermo Fisher Scientific Inc., Austin, TX, USA).

qRT-PCR analysis

Total RNA was isolated from leaves or roots of 7-day-old seedlings. The methods for RNA extraction, reverse transcription and qRT-PCR were as given in a previous report (Wang et al. 2010a). The sequences of the corresponding primers for qRT-PCR are listed in Supporting Information Tables S2–S4.

Measurement of metal ion contents

Ten-day-old rice WT and osarf16 mutants were analysed to determine their P and iron contents. P measurements were performed using Flow Analyser SAN++ (Skalar Analytical B.V., Breda, the Netherlands), while iron was determined using inductively coupled plasma mass spectrometry (Agilent 7500ce, Agilent Technologies, Palo Alto, CA, USA), according to published methods (Jia et al. 2011b). Five biological replicates were performed for each sample in all experiments.

Microarray data analysis

Microarray data published by Jain & Khurana (2009) and Zheng et al. (2009) were analysed using TreeView 1.60 and Cluster X 2.20 (Stanford University, Stanford, CA, USA).
RESULTS

OsARF16 is required for auxin responses in roots

Rice endogenous retrotransposon (TOS17) was integrated into the seventh exon of OsARF16 genes using analysis of the Rice Genome Resource Center (RGRC) database (http://www.rgrc.dna.affrc.go.jp) and sequencing (Fig. 1a). PCR analysis confirmed that the TOS17 fragment had been inserted into OsARF16 genes and the homozygous line was harvested (Fig. 1b). RT-PCR result demonstrated that OsARF16 was expressed in NIP and overexpressed in Ov16 and Ov16/mutant (Ov16/MT), but not the mutant osarf16 (Fig. 1c). The phenotypes of NIP, osarf16, Ov16 and Ov16/MT were approximately the same under control (CK) conditions (Fig. 1d). However, the mutant osarf16 showed longer PR than the other three lines under IAA treatments, indicating it was insensitive to auxin (Fig. 1e). These results confirmed that OsARF16 was knocked out in osarf16, and that it rescued the function of OsARF16 in Ov16/MT. Exogenous auxin can decrease PR length (Woodward & Bartel 2005) and induce LR formation (Dong et al. 2006) and RH elongation (Lee & Cho 2006; Duan et al. 2010). However, under IAA treatment, the PR length with 2,4-D and IBA treatments in Supporting Information Fig. S1 and the LR number in osarf16 were greater than NIP and Ov16, whereas the RH length in osarf16 was lower compared with both lines (Fig. 1f–h & Supporting Information Figs S1–S4). In Supporting Information Figs S2 and S3, Ov16 was more sensitive to IAA or NPA than NIP in terms of lateral root number. In addition, Ov16 was also more sensitive to IAA than NIP in terms of RH length, although the RH length in Ov16 under NPA treatment was similar to that of NIP. These results further confirmed that osarf16 is actually insensitive to auxin. Under IAA treatment, there was no difference in the adventitious root (AR) number between osarf16 and NIP, or Ov16, although more AR was produced in osarf16 than the other lines with an auxin influx transport inhibitor, that is, NOA treatment (Fig. 1i & Supporting Information Fig. S4). To know the phenotype of osarf16 when blocking auxin transport, the PR length was measured under PATIs. The PR length in osarf16 was longer than NIP and Ov16 with TIBA, NPA and 1-NOA treatments (Supporting Information Fig. S1), which indicated

Figure 1. Identification of mutant osarf16-Tos17 (osarf16) and phenotypic analysis. (a) TOS17 insertion site in osarf16. The black boxes represent exons and the black lines represent introns. The triangle shows the insertion site (the seventh exon). (b) PCR analysis to confirm the integration of TOS17 in OsARF16, where the upper bound indicates the OsARF16 gene fragment and the lower bound indicates the TOS17 insertion fragment. (c) RT-PCR analysis to confirm the knockout status of osarf16. The upper bounds show OsARF16 expression (30 cycles) in the same lines, respectively. OsACTIN (26 cycles) is shown at the bottom as a control. (d) Phenotypic characterization: left to right, shows the 7-day-old seedlings of Nipponbare (NIP), osarf16-Tos17 (osarf16), overexpression of OsARF16 in NIP (Ov16) and overexpression of OsARF16 in mutant (MT) osarf16 (Ov16/MT), bar = 2 cm. (e) Morphology of the same four lines under treatment with 1 μM of indole acetic acid (IAA) for 7 d after germination (DAG), bar = 2 cm. (f) PR length under various concentrations of IAA treatments at 7 DAG. (g) Lateral roots (LR) number under various concentrations of IAA treatments at 7 DAG. (h) RH length under 1 μM of IAA treatment for 24 h (3-day-old seedlings). (i) AR number under 1 μM of IAA treatment and 100 μM of 1-naphthoxyacetic acid (NOA) treatment at 7 DAG.

© 2012 Blackwell Publishing Ltd, Plant, Cell and Environment, 36, 607–620
that osarf16 was also insensitive to PATIs. These results showed that OsARF16 was required for auxin responses in roots.

**OsARF16 expression pattern**

We evaluated the expression patterns of OsARF16 in various organs using the GUS reporter gene. The 2641 bp of the OsARF16 sequence upstream of its ATG (predicted by the annotated rice genome [http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/#search]) was fused to GUS and the transgene was introduced into rice NIP. Ten positive transgenic lines were obtained and three lines were used for further investigation. GUS staining was found to be prominent in the stele and root tip (Fig. 2a,b) of PR and AR (equal to PR, data not shown). In LR, OsARF16 was weakly expressed in the stele, root tip and primordia (Fig. 2c–e). OsARF16 was expressed at a lower level in the leaf relative to the leaf tip (Fig. 2f,g). It was not expressed in RH (Fig. 2h) and it was highly expressed in the vascular tissue of the stem (Fig. 2k,l). OsARF16 expression was also observed in the anther, the stigma of the flower (Fig. 2i,j,m) and the glume. Semi-quantitative RT-PCR (sqRT-PCR) further confirmed that OsARF16 was expressed at different levels in various tissues (Fig. 2n), consistent with the GUS staining results. Therefore, OsARF16 was expressed in

different organs and tissues, with the highest expression being in roots and vasculature.

OsARF16 expression in PR and LR was induced by IAA and –Pi treatments

We tested the effects of auxin and –Pi treatments on the expression of OsARF16 using the OsARF16::GUS reporter line. OsARF16 expression in the stele was completely inhibited (Fig. 3a,c) by IAA, whereas that in the PR tip was highly induced by about 10-fold (Fig. 3b,d,i). OsARF16 expression in the stele, epidermis and tip of PR was highly induced (about 12-fold; Fig. 3e,f,i) by –Pi treatment. Interestingly, under the –Pi/+IAA treatment, the OsARF16 expression was highly increased in tip of PR than with only –Pi or IAA treatment (about 15-fold; Fig. 3g,h,i) while it was impaired in epidermis. This data provide an evidence for that auxin controls OsARF16 expression at a higher degree than –Pi. qRT-PCR analysis of PR further confirmed these trends (Fig. 3i). We also compared the expression of OsARF16 in the initiation of LR under auxin and –Pi treatments. OsARF16::GUS staining was observed at the LR primordia (Fig. 3j–n), and induced by auxin and –Pi treatments (Fig. 3o–x). The above results suggest that the function of OsARF16 may be related to auxin and –Pi response.

Auxin content of PR in osarf16 was lower than in NIP under IAA and –Pi treatments

To understand the biological function of OsARF16 in auxin and –Pi responses, the auxin contents of PR in NIP and osarf16 were measured under CK, IAA and –Pi treatments (Fig. 4a). The PR auxin content in mutant osarf16 was lower than in NIP under IAA and –Pi treatments. The DR5::GUS staining in NIP and osarf16 under these conditions was observed since it reflects auxin distribution (Ulmasov et al. 1997). The results were consistent with the above measurements (Fig. 4b). Local auxin levels were determined by biosynthesis and intercellular transport in the Arabidopsis roots (Ding & Friml 2010), so we aimed to confirm what caused the alteration in the auxin content of osarf16, that is, auxin synthesis or transport. By recent report, IAA is produced from indole-3-pyruvate by the YUCCA family of flavin monooxygenases (Stepanova et al. 2011; Zhao 2012). We initially tested the expression of the OsYUCCA genes involved in auxin biosynthesis (Cheng, Dai & Zhao 2006; Cheng et al. 2007; Stepanova et al. 2008; Zhao 2008). The expressions of genes OsYUCCA1, 3, 4 and 5 in osarf16 were significantly lower than in NIP under IAA and –Pi treatments, respectively, suggesting decreased auxin synthesis in arf16 compared with NIP when response to IAA and –Pi (Fig. 4c,d). In addition, we also tested the expression of genes OsLAXs encoding the auxin influx transporters and OsPINs encoding auxin efflux transporters under CK, IAA and –Pi treatments. In osarf16, the expression of most OsLAXs and OsPINs was mainly induced by IAA and –Pi treatments, although to a lesser extent than in NIP (Fig. 4e,f). The weaker increases in the expression of auxin biosynthesis and auxin transporter genes in osarf16 compared with NIP may lead to lower auxin content in PR under IAA and –Pi treatments.
Auxin content of LR in osarf16 compared with NIP was less increased by –Pi, and P content in osarf16 compared with NIP was stable with various concentrations of auxin treatments.

To determine the relationship between –Pi signalling and auxin distribution in osarf16, we further analysed the auxin content in LR under –Pi using a DR5::GUS reporter and the P content with various concentrations of auxin treatments. The auxin content from LR initiation to maturation in osarf16 under –Pi condition and under CK (Fig. 5g–i) were about equal, whereas that of NIP was greatly increased (Fig. 5a–f). In Arabidopsis, a strong GUS activity was also detected in the expression pattern of the DR5::GUS reporter gene under –Pi, which suggested that P starvation resulted in an auxin overaccumulation or that it drastically modified auxin sensitivity in the PR apex, initiated primordial and young LR (Nacry et al. 2005). The results in NIP were consistent with those in Arabidopsis mentioned above, and indicated a positive relationship between auxin distribution and –Pi response. Despite this, the P content in osarf16 remained at a stable level, whereas that in NIP was gradually enhanced along with an increase of auxin concentration to 0.01 μM (Fig. 5m). The results indicated that the loss of auxin sensitivity and the higher P levels in osarf16 compared to NIP control was independent of exogenous auxin at any concentration. It may be due to a higher number of lateral roots in the osarf16 mutant at any auxin concentration used.

osarf16 had a higher P content and reduced sensitivity to –Pi

LR development in response to Pi availability is mediated by changes in auxin sensitivity in Arabidopsis (Pérez-Torres et al. 2008). osarf16 exhibited an auxin-insensitive phenotype, so we further examined the P accumulation capacity of osarf16 under IAA and –Pi response. The P contents in leaves and roots of NIP were greatly enhanced by exogenous IAA treatment, while that in osarf16 was less enhanced (Fig. 6a,b and Supporting Information Table S6a). The P content in leaves of osarf16 under CK was 15% higher than that in NIP (Fig. 6a), even under –Pi; however, osarf16 still had higher P than NIP. The auxin content in osarf16 was higher than that in NIP and the P content in osarf16 was about equal to NIP under IAA treatment (Fig. 4a), that is, the P accumulation capacity of osarf16 was also stronger than that of NIP, suggesting that the P content increase in osarf16 may be closely correlated to endogenous...
High Pi content was also observed in the result in improved P absorption and an increased P content of rice (Zheng et al. 2009). To determine whether the Fe content was impacted by –Pi, the change of root-to-shoot ratio in osarf16 also differed with that in NIP (Fig. 6e,f, & Supporting Information Table S6c,d). Shoot fresh weight (FW) in osarf16 was greater than NIP, whereas root FW in osarf16 was less than NIP under –Pi (Fig. 6e). Thus, the root-to-shoot ratio was significantly amplified in NIP, but not in osarf16 with –Pi treatment (Fig. 6f). These results indicate that osarf16 attenuated or lost the functional response to –Pi, suggesting that OsARF16 was a requirement for –Pi response.

**RH length or LR number of osarf16 were not induced by –Pi, but were increased by Fe starvation (–Fe), while OsARF16 expression was up-regulated by –Pi and unchanged by –Fe**

LR development in response to Pi availability is mediated by changes in auxin sensitivity in Arabidopsis (Pérez-Torres et al. 2008). Our research showed that osarf16 had auxin insensitivity and the physiological characteristics of –Pi insensitivity (Figs 1 & 6), and the phenotype of osarf16 was also affected by –Pi. However, the Fe content in osarf16 under –Pi was not induced to the same degree as found in NIP (Fig. 6c,d, and Supporting Information Fig. S5), which led us to explore whether OsARF16 was also implicated in –Fe response. Thus, we examined the RH length and LR number in NIP and osarf16, and OsARF16::GUS staining under –Pi and –Fe conditions in detail. The RH length in osarf16 in NIP was not extended by –Pi, whereas they were all increased by –Fe (Fig. 7a–f,s). OsARF16::GUS staining was detected in RH with –Pi, but not with CK or –Fe (Fig. 7g–i). Similarly, the LR number in osarf16 in NIP was not greatly increased by –Pi (Fig. 7j–o,t), whereas they were all reduced by –Fe. The OsARF16::GUS staining was up-regulated in LR with –Pi but not with –Fe or CK (Fig. 7p–r). The experiments showed that RH and LR development in osarf16 were impaired by Pi deficiency. The visible phenotypes and physiological evidence (Figs 6 & 7) indicated that OsARF16 played an important role in the –Pi response because root morphology changed when it was absent. However, the RH length and LR number in osarf16 under –Fe was the same as that in NIP, suggesting that OsARF16 was not directly responsible for the –Fe response.

**Alternating trends of gene expression related to –Pi response in osarf16 under –Pi conditions were similar to that in the mutant osphr2**

As described above, mutant osarf16 was insensitive to –Pi. To investigate the regulatory mechanism of OsARF16 by –Pi, we tested whether the phosphate starvation-induced genes (PSIs) were also affected by the knockout of OsARF16. The expression levels of six PSIs in osarf16, including OsIPS1/OsIPS2 (Hou et al. 2005), OsSQD2...
(Essigmann et al. 1998), OsPT2 (Ai et al. 2009), OsPT8 (Jia et al. 2011a) and OsSPX1 (Wang et al. 2009a) were much lower under –Pi compared with NIP (Fig. 8a–f), and similar trends were found in a OsPHR2 knockdown mutant, which is a key regulator of P signal transduction (Zhou et al. 2008), and in phr1 in Arabidopsis (Rubio et al. 2001). OsPHR2 was also not down-regulated in osarf16 under –Pi (Fig. 8g). Furthermore, there were two P1BS sequences (DNA element bound by OsPHR2) and one auxin responsive element (AuxRE) in the promoter region of OsARF16 by the sequences analysis (Supporting Information Fig. S6). However, the expression of OsARF16 was almost not altered in OsPHR2-RNAi and OsPHR2-Ov transgenic lines compared to NIP under IAA and –Pi condition (Supporting Information Fig. S6). These results suggested that the regulation relationship between OsARF16 and OsPHR2 might be complicated. In addition, a downstream gene OsPHO2 of OsPHR2 (Zhou et al. 2008) was less

Figure 7. The development of root hairs (RH) and LR (5-day-old) under CK, –Pi and –Fe conditions. Bar = 500 μm. (a–c) RH in Nipponbare (NIP) under CK (a), –Pi (b), and –Fe (c). (d–f) RH in osarf16 under CK (d), –Pi (e), and –Fe (f). (g–i) OsARF16:β-glucuronidase (GUS) in RH of NIP under CK (g), –Pi (h) and –Fe (i). (j–l) Lateral roots (LR) in NIP under CK (j), –Pi (k) and –Fe (l). (m–o) LR in osarf16 under CK (m), –Pi (n) and –Fe (o). (p–r) OsARF16::GUS in LR of NIP under CK (p), –Pi (q) and –Fe (r). (s) RH length under CK, –Pi and –Fe conditions. (t) Number of LR. The above data were based on analysis of 10 biological repeats.
inhibited by –Pi in osarf16 compared with NIP (Fig. 8h), further suggesting that OsARF16 was included in the –Pi response. It was interesting that the transport inhibitor response1 in rice (OsTIR1), which is a homologous gene of the auxin receptor implicated in the –Pi response in Arabidopsis (Pérez-Torres et al. 2008), which was also less up-regulated by –Pi in osarf16 compared with NIP (Fig. 8j). These results indicated that OsARF16 may be an essential regulator in the –Pi response at least at the transcription level.

Figure 8. Analysis of expression level for eight genes related to –Pi response in the roots of Nipponbare (NIP) and osarf16 under CK and –Pi conditions. (a) OsIPS1. (b) OsIPS2. (c) OsSQD2. (d) OsPT2. (e) OsPT8. (f) OsSPX1. (g) OsPHR2. (h) OsPHO2. (i) OsTIR1. qRT-PCR conditions were the same as those given in Fig. 3.
DISCUSSION

OsARF16 effects on the –Pi signalling were correlated with auxin distribution

To elucidate the function of ARF in rice, the structures of OsARF genes and OsARF12 features were investigated in our previous study (Shen et al. 2010a; Qi et al. 2012). Here, we further identified and characterized the biological function of OsARF16 with TOS17 insertion in greater detail in rice. osarf16 mutant or Os16 scarcely showed phenotypic differences in their roots, suggesting that OsARF16, one member of the ARF gene family might have functional redundancy with another member. However, the PR, LR, and RH of osarf16 showed auxin insensitivity (Fig. 1), suggesting that OsARF16 plays a role on auxin response in root development. OsARF16 was mainly expressed in the stele and root tip of PR (Fig. 2a,b), AR and LR (Fig. 2c,d), which further supported that it was implicated in root development. And, OsARF16 expression in PR and LR was induced by auxin and –Pi treatments (Fig. 3), and the expression of most OsLAX and OsPINs in osarf16 was markedly lower than in NIP under exogenous IAA treatment (Fig. 4e–f) – this demonstrated that absence of OsARF16 might affect auxin polar transport. The temporal and spatial distribution of auxin mainly depends on the dynamic expression and subcellular localization of auxin efflux proteins, PINs (Bureau et al. 2010). The genomic sequencing of these auxin transporter genes, OsLAX and OsPINs, in rice was recently published in our study where it was compared to Arabidopsis (Shen et al. 2010b), but their individual functions remain unknown. However, the expression pattern of OsARF16 in PR was the same as OsPIN1b, OsPIN4 (OsPIN1c in this paper) and OsPIN9 (Wang et al. 2009b), suggesting that OsARF16 may affect auxin transport mainly via these three genes. In addition, in osarf16 under –Pi, the expression of the four OsYUCAs was highly induced, and most OsLAXs and OsPINs showed a similar trend with IAA treatment, indicating that an auxin transporter was also involved in –Pi response. The results indicated that –Pi response may alter auxin distribution or auxin polar transport via the regulation of OsARF16. In NIP, the auxin content was increased by –Pi condition (Fig. 5a–f) while in osarf16 mutant, it was not affected (Fig. 5g–i). These results suggested that the impact of –Pi signalling on auxin distribution depends on OsARF16. On the other hand, in NIP applying exogenous auxin enhanced Pi absorption, but in the OsARF16 knockout mutant, the Pi content was not increased (Fig. 5m). Therefore, the improvement of Pi absorption caused by changes of auxin distribution also depends on OsARF16. Taken together, results in Figs 4 and 5 further confirmed that the effects of OsARF16 on –Pi signalling were correlated with auxin distribution.

Furthermore, using published microarray data (Jain & Khurana 2009; Zheng et al. 2009), we analysed 201 auxin early induction genes including classical OsIAAs, OsGH3, OsSAURs (Ulmasov et al. 1997) and some novel genes with unknown function in rice. We found that auxin signalling takes part in differences responses to Pi deficiency in the shoot and root. Most of the auxin-induced genes in the rice root were also up-regulated by Pi deficiency (Fig. 6 & Supporting Information Table S5). These data further confirmed that a number of genes co-participate in auxin and –Pi response, and not only OsARF16.

RH and LR development under P deficiency in rice depends on OsARF16-mediated –Pi signalling

The phenotype of a plant under auxin treatment is similar to that for Pi starvation (López-Bucio et al. 2003; Nacry et al. 2005; Vanneste & Friml 2009). Our study found that osarf16 had auxin insensitivity and was also insensitive to Pi deficiency (Figs 6 & 7), especially in terms of RH and LR development. In NIP, but not in osarf16, the RH length was extended by –Pi (Fig. 7e), and OsARF16::GUS staining was also induced in RH by –Pi (Fig. 7h). RH development in –Pi has been infrequently reported (Sánchez-Calderón et al. 2006; Bustos et al. 2010; Wang et al. 2010b). The present study was the first to demonstrate that OsARF16 was a key
regulator in RH expansion under –Pi in rice. Moreover, the LR number in osarf16 showed a small increase under –Pi, consistent with an arf19 mutant in Arabidopsis (Pérez-Torres et al. 2008). OsARF16 is highly homologous to ARF19, which is implicated in responses to Pi deficiency (Wang et al. 2007). The data indicated that OsARF16 also acted in LR development under –Pi as well as ARF19 (Fig. 7k–q,t). Plants respond to Pi deficiency by allocating more carbon to their roots, thereby increasing their root-to-shoot ratio (López-Bucio et al., 2003; Hermans et al. 2006). The root-to-shoot ratio in osarf16 was only slightly increased compared with NIP, which further suggested that osarf16 was insensitive to Pi deficiency. It is worth mentioning that Fe accumulation in osarf16 under –Pi was lower than in NIP (Fig. 6c,d). A previous report showed that –Pi induced Fe acquisition and increased the Fe content of rice (Zheng et al. 2009). Our results suggested that the knockout of OsARF16 may indirectly affect the Fe signal via the –Pi response.

OsARF16 is an essential regulator in –Pi response

Under –Pi conditions, a plant enhances P absorption efficiency by regulating the expression of genes induced by phosphate starvation (PSIs) to maintain normal growth and development (Martín et al. 2000; Schachtman & Shin 2007). In Arabidopsis, the complete regulatory network for the P signal is important for plant responses to –Pi. Thus, the absence of AtPHR1 located in the centre of the P signal network resulted in the expression of numerous downstream genes that were inhibited under –Pi, and with an impaired P signal (Rubio et al. 2001; Nilsson, Müller & Nielsen 2007; Panigrahy, Rao & Sarla 2009). In rice, the knockdown of OsPHR2 led to a series of PSIs genes that were not distinctly induced by –Pi (Zhou et al. 2008). The genetic effect of OsARF16 knockout was similar to the absence of AtPHR1 and OsPHR2. The knockout of OsARF16 greatly weakened the transmission of the P signal, leading PSIs genes to lose their correct response. The P deficiency response still was impaired in the osarf16 mutant, even if OsPHR2 was normally expressed (Fig. 8g). Thus, the P deficiency response via OsPHR2 was dependent on OsARF16-mediated –Pi signalling. The effect of the OsPHR2 function was based on the normal expression of the OsARF16 gene, which maintained P signal transmission and allowed rice to respond to P deficiency in time.

In Arabidopsis, the modulation of auxin sensitivity by Pi depends on the auxin receptor transport inhibitor response1 (TIR1) and ARF19. Auxin sensitivity is enhanced in Pi-deprived plants by an increased expression of TIR1, which accelerates the degradation of AUX/IAA proteins. This indicated that ARF transcription factors activate/repress genes that are related to auxin signalling (Pérez-Torres et al., 2008). In rice, OsTIR1 in osarf16 was also less up-regulated by –Pi compared with NIP (Fig. 8j). Taken together, the results indicate that OsARF16 may be an essential regulator in –Pi response.

In conclusion, mutant osarf16 was insensitive in terms of both the auxin and –Pi response, which indicated an important role for OsARF16 in regulating auxin and –Pi signalling. This is the first report to implicate the regulation of ARF in the –Pi response in rice.

ACKNOWLEDGMENTS

We would like to thank Professor Xuemei Chen from the University of California for her assistance in editing this manuscript. This research was supported by the National Natural Science Foundation of China (Grant no. 31071392, 30971703 and 31171462), the Natural Science Foundation of Zhejiang province, China (Grant no. Y3080111), the Genetically Modified Organisms Breeding Major Projects (2009ZX08009-123B) and China Transgenic Plant Research and Commercialization Project, 2009ZX08001-022B [Correction added on 14 January 2013, after first online publication: National Natural Science Foundation of China grant no. 3117462 has been amended to 31171462]. We gratefully acknowledge Professor Akio Miyao in the RGRC in Japan for providing the full-length cDNA clone of OsARF16 gene and osarf16-TOS17 mutant, and Professor Ping Wu in Zhejiang University for providing the PHR2-RNAi and OsPHR2-Ov transgenic line.

REFERENCES


© 2012 Blackwell Publishing Ltd, Plant, Cell and Environment, 36, 607–620


Received 15 March 2012; received in revised form 8 August 2012; accepted for publication 14 August 2012

SUPPORTING INFORMATION

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

Figure S1. PR length analysis of NIP and mutant osarf16 under auxin and PATIs.

Figure S2. The LR phenotypes of NIP, osarf16, and Ov16 under CK and IAA, 2,4-D and NPA treatments.

Figure S3. The AR phenotypes of NIP and osarf16 under CK and NOA treatment.

Figure S4. The RH phenotypes of NIP, osarf16, and Ov16 under CK, 2,4-D and NPA treatments.

Figure S5. Soluble Fe contents in leaves and roots of NIP and osarf16.

Figure S6. Analysis of OsARF16 promoter sequences and OsARF16 expression in NIP, OsPHR2-RNAi and OsPHR2-Ov transgenic lines.

Table S1. Primer sequences for the OsARF16 gene.

Table S2. Primers sequences for the auxin synthetase genes.

Table S3. Primer sequences for the auxin influx, OsAUX and efflux transporter, and OsPIN gene families.

Table S4. Primers sequences for the expression of genes related to the P signal.

Table S5. Probe set ID of 201 auxin upregulated genes.

Table S6. Response to–Pi in NIP and osarf16.