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OsRPK1, a novel leucine-rich repeat receptor-like kinase, negatively regulates polar auxin transport and root development in rice

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ABSTRACT

Background: Leucine-rich-repeat receptor-like kinases (LRR-RLKs) represent the largest subfamily of putative RLKs in plants. Although several members in this subfamily have been identified, the studies about the relationships between LRR-RLKs and root development are still few. We previously identified a novel LRR-RLK in rice roots, and named it OsRPK1.
Methods: In this study, we first detected OsRPK1 kinase activity in vitro, and assessed its expression profile. We then investigated its biological function using transgenic rice plants over- and under-expressing *OsRPK1*.
Results: The *OsRPK1* gene, which encodes a Ca^{2+}-independent Ser/Thr kinase, was predominantly expressed in root tips, leaf blades, and undifferentiated suspension cells, and was markedly induced by treatment with auxin or ABA. Knockdown of *OsRPK1* promoted the growth of transgenic rice plants, and increased plant height and tiller numbers. In contrast, over-expressing plants showed undeveloped adventitious roots, lateral roots, and a reduced root apical meristem. *OsRPK1* over-expression also inhibited the expression of most auxin efflux carrier *OsPIN* genes, which was accompanied by changes in PAT and endogenous free IAA distribution in the leaves and roots.
Conclusions: The data indicated that OsRPK1, a novel leucine-rich-repeat receptor-like kinase, affects the root system architecture by negatively regulating polar auxin transport in rice.
General significance: This study demonstrated a common regulatory pathway of root system development in higher plants, which might be initiated by external stimuli via upstream receptor-like kinases and downstream carriers for polar auxin transport.

1. Introduction

The root systems of monocots and dicots have different architecture. In monocots like rice plants, there are embryonic and postembryonic root systems. The embryonic root system consists of a short-lived primary root (PR) and many shoot-borne adventitious roots (ARs), whereas the postembryonic system consists of ARs and lateral roots (LRs) [1]. During most of the growing period, ARs and LRs are the main parts of uptaking water and nutrient in rice.

Several studies suggested that the biosynthesis, transport, and signaling of auxin play pivotal roles in the development of the root system in both dicots and monocots [2]. In addition, efforts have been made to identify factors that regulate polar auxin transport (PAT) and root development. For example, Li and Jia [3] reported that enhanced levels of cGMP promoted the initiation of lateral root formation in *Arabidopsis* by regulating PAT and auxin gradients via modulation of the localization and expression of the auxin efflux carrier PINs (PIN FORMED). Wang et al. [4] found that *Arabidopsis* TWISTED DWARF1 interacts with the auxin efflux carrier ABCB1, and affects shootward root auxin reflux and gravitropism of the root. *Arabidopsis* SPIKE1 belongs to the conserved DHR2-Dock family of Rho guanine nucleotide exchange factors. Lin et al. [5] found that *SPIKE1* loss-of-function mutations increased lateral root density and retarded gravitropic responses by inducing PIN2 internalization in roots. Other regulators of PAT in *Arabidopsis* include phosphatidylinositol monophosphate 5-kinase (PIP5K) [6], ABA-regulated AP2 domain transcription factor ABI4 [7], RUS2/WXR1 [8], and blue-light photoreceptors CRY1 [9]. These factors all play roles in the regulation of lateral root formation and/or the root gravity response by regulating PAT by affecting the expression or cycling of PIN proteins. Liu et al. [10] identified OsGNOM1, a guanine nucleotide exchange factor for ADP-ribosylation factor, as a regulator of PAT in rice. Loss-of-function mutants of *OsGNOM1* were defective in AR formation, and exhibited reduced numbers of LRs and partial loss of gravitropism. The mutants displayed enhanced sensitivity to PAT inhibitors, and the expression of *OsPIN2*, *OsPIN5b*, and *OsPIN9* was also

Abbreviations: LRR, leucine rich repeat; RLK, receptor-like kinase; PAT, polar auxin transport; RSA, root system architecture; PR, primary root; LRs, lateral roots; ARs, adventitious roots; IAA, indole-3-acetic acid; GST, glutathione-S-transferase; PIN, PIN-FORMED; NPA, naphthyphthalamic acid

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altered in the mutants. Until now, most PAT regulators that affect root system architecture (RSA) were identified in dicots such as *Arabidopsis*, and only a small number of studies have focused on the fibrous root system in monocots. Thus, knowledge of PAT regulators in rice is still very limited.

Receptor-like protein kinases (RLKs) are a large gene family found in plants. The family includes over 600 members in *Arabidopsis thaliana*, and 1100 members in rice [11]. The best-studied RLKs are those containing extracellular leucine-rich repeat (LRR) domains. LRR-RLKs are the largest RLK class, with over 200 members in 13 subfamilies (LRR I to XIII) classified according to the organization of the LRRs in the extracellular domain [11,12]. Some of these LRR-RLKs have diverse roles in development. These include CLV1 and OsLRK1, the pathogen resistance kinases Xa21 and FLS2, the hormone perception kinase BRI1, and the abiotic stress tolerance kinases AtRPK1, SRLK, and OsSIK1 [13–21].

Very few studies have focused on auxin-related LRR-RLKs. Recent studies implicated plant Rho GTPase (RAC/ROPs) as a mediator of auxin-regulated gene expression, rapid cell surface-located auxin responses, and polar auxin transport (PAT) [22]. FERONIA is a ROPGEF-interacting receptor-like kinase at the plasma membrane that acts as an upstream regulator of RAC/ROPs [23]. Sakaguchi et al. [24] cloned the *COE1* gene, whose amino acid sequence is similar to brassinosteroid-insensitive 1-associated receptor kinase 1 (BAK1). Functional analysis suggested that COE1 might regulate commissural vein intervals downstream of auxin and brassinosteroid signals. Singla et al. [25] identified several LRR-RLKs belonging to the SERK/SERL gene family that regulate somatic embryogenesis. These LRR-RLKs are also homologs of BAK1, and some of them can be induced by auxin. Recently, a collection of homozygous T-DNA insertion lines of 69 root-expressed LRR-RLK genes was screened in *Arabidopsis* for root developmental defects and altered responses after exposure to environmental, hormonal/chemical, and abiotic stress [26]. Nineteen T-DNA insertion lines corresponding to 16 RLK genes showed increased resistance to indole-3-acetic acid (IAA), and two T-DNA insertion lines corresponding to *BAK1/SERK3* and *IRK* showed increased sensitivity to IAA. These results suggest that a number of LRR-RLKs are involved in auxin signaling and responses.

Previously, we identified Os05g0486100 (mRNA sequence: NM_001062412) as a putative 969-amino-acid LRR-RLK in rice, and named it OsRPK1 [27]. In this study, we describe its biological function in detail. Our results suggest that OsRPK1 is a Ca^{2+}-independent Ser/Thr kinase that is induced by auxin and abscisic acid (ABA). Overexpression and knockdown analyses suggested that OsRPK1 affects the root system by negatively regulating PAT and auxin accumulation in the roots.

2. Materials and methods

2.1. Plasmid construction and rice transformation

The full length *OsRPK1* cDNA was amplified from the first-strand cDNA of *Oryza sativa* L. *japonica* cv. Nipponbare cDNA (NM_001062412) using gene-specific primers 5′-*GGATCC*CTCGAGATGTGCACCAACGGCAGGG-3′ and 5′-*GGATCC*CTACTTGGGCT-TGACCTCAAAATAG-3′ (*Bam*H I site is shown in italics). The sequence-confirmed PCR products were single-digested with *Bam*H I, and sense or antisense orientation was inserted into the plant expression vector pCAMBIA 1304 (35S promoter) and then introduced respectively into *Agrobacterium tumefaciens* strain EHA105 by electroporation. *Agrobacterium*-mediated transformation of rice callus (*Oryza sativa* L. *japonica* cv. Nipponbare) was performed according to the protocol of Hiei et al. [28]. Transformed calli, selected by G418 resistance, were used to regenerate transgenic seedlings. Transgenic rice plants (T_0/T_1 re-generates) were selected on 1/2 MS media containing G418, transferred to soil and grown to obtain T_1/T_2 progeny seeds in the field under nature conditions.

2.2. Plant growth conditions and different treatments

Wild type and T_2 generation transgenic rice seeds were sterilized in a solution of 3% NaClO and 0.01% antifoam A (Sigma-Aldrich) for 15 min, and then imbibed in sterilized water for 2 days at 25 °C in the dark. The germinated seeds were transferred into a nutrient solution in a greenhouse under fluorescent light (600 μM m^{-2} s^{-1}, 14-h light/10-h dark) at 28 °C and 75% relative humidity. The composition of the nutrient solution was followed IRRI standard protocol. For rice agronomic characters analysis, the seedlings of the wild-type and transgenic plants were transplanted in pots and grown under nature conditions for 3 weeks. At maturity, the agronomic characteristics were measured. To evaluate the phenotypes of the transgenic rice plants, the seedlings of the wild-type and transgenic plants were germinated in 1/2 MS medium for 4 days, and the germinated seedlings were sown in the 96-well plates from which the bottoms were removed. The plate was floated and grown in nutrient solution. 4-week-old rice plants were measured. For NPA treatment assays, at 4 days following germination on 1/2 MS medium, the seedlings were transferred to 0.5 μM NPA 1/2 MS medium. After 5-days, the numbers of adventitious roots and the lateral root density were measured.

2.3. Expression and purification of the GST-KD fusion protein and phosphorylation assay

The cDNA fragment encoding the OsRPK1 protein kinase domain (from 1888 to 2803 bp) was amplified from the cDNA using gene-specific primers 5′-GCGGAATTCAACTTCTCTGACAACCACG-3′ and 5′-ATAGCGGCCGCGATCTCCTT-CACCACGGCG-3′, and then cloned into expression vector pGEX4T-1. The GST-KD fusion protein was expressed in *Escherichia coli* and then purified by GSH resin (Merck, Germany). The purified GST-KD was subjected to phosphorylation assay in a 25 μl reaction mixture containing 50 mM Tris–HCl (pH 7.6), 50 mM KCl, 2 mM DTT, 10% glycerol, and 5 mM $MnCl_2$ (or 5 mM $CaCl_2$, 5 mM $MgCl_2$). Phosphorylation was initiated after the addition of 1.6 μl ATP (0.2 μCi/μL [γ-^{32}P] ATP, 50 mM ATP). To assay the OsRPK1 kinase activity, GSH beads conjugated GST-KD and negative control protein GST alone were incubated for 30 min at 4 °C with total proteins that were prepared from the root tips of rice. The reactions were maintained at 30 °C for 1 h and were stopped by adding 5× SDS loading buffer, then boiled for 5 min. After electrophoresis on 12% SDS-PAGE, the radioactive gel was exposed to X-ray film. The protein samples were also resolved on SDS-PAGE to examine the loading after coomassie blue staining.

2.4. RNA isolation and real-time quantitative RT-PCR analysis

To detect the expression profile of *OsRPK1,* different tissues were harvested from wild-type seedlings. Total RNA was isolated using TRIzol reagent (Invitrogen, USA) and 1.0 μg RNA was used as templates for cDNA synthesis using the Reverse Transcription System kit with an oligo (dT) 18 primer. The *OsRPK1* gene was analyzed using the primers: 5′-AACTTCTCTGACAACCACG-3′ and 5′-GGAGGATGTTGGTGGACTTG-3′. The conditions for PCR were 95 °C for 5 min, and then 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s for 35 cycles. The *18S rRNA* gene was analyzed using the primers 5′-CCTATCAACTTTCGATGGTAGGATA-3′ and 5′-CGTTAAGGGATTTAGATTGTACTCATT-3′, as an internal positive control. The conditions for PCR were 95 °C for 5 min, and then 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 20 s for 30 cycles. To examine the effect of various hormones on *OsRPK1* expression, the 2-week-old wild-type seedlings were treated with solution of 100 μM 2,4-D,100 μM IAA, 100 μM ABA, 100 μM 6-BA, 100 μM GA3, 100 μM MeJA, and 1 mM ethephon, respectively, and sampled at 0 and 12 h. For analysis of auxin transport genes and auxin synthesis gene *OsYUCCA1*, the total RNA of 2-week-old transgenic and wild-type rice was isolated from the roots or leaves. PCR was performed using SYBR Premix

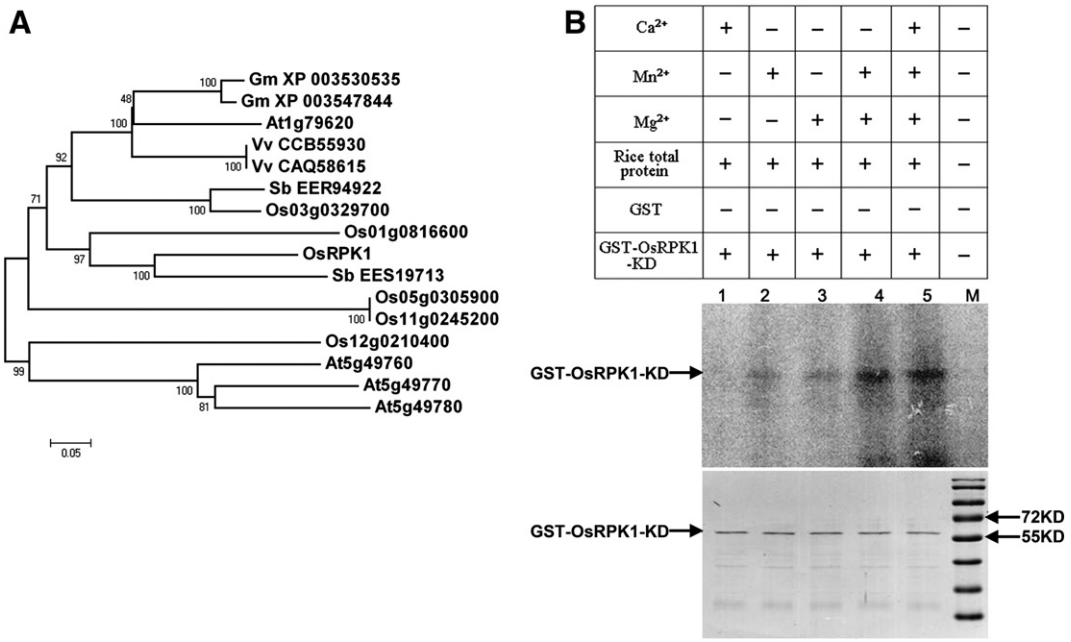

Fig. 1. Characterization of OsRPK1 protein. (A) Phylogenetic relationship among OsRPK1 homolog. A phylogenetic tree was constructed by neighbor-joining method. The bootstrapping value (out of 10,000 samples) for each node, obtained with the same software, was shown. Species abbreviations: At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Vv, *Vitis vinifera*; Sb, *Sorghum bicolor*; Gm, *Glycine max*. (B) In vitro phosphorylation activity of OsRPK1. Bacterially produced GST-OsRPK1-KD (OsRPK1 kinase domain) fusion proteins were incubated with [γ-^{32}P] ATP and separated by PAGE. The upper panel shows autoradiography and the lower panel shows coomassie blue staining.

Ex Taq (Takara, Japan) and Mastercycler ep realplex 2 (Eppendorf, Germany) according to the manufacturer's protocols. The primers were as follows: *OsRPK1*-q-F: AAACGCAGCACTTCCATTTCAAC; *OsRPK1*-q-R: TTCCTATCCAATCGGAGAACTTCG; *OsPIN1a*-q-F: TGCACCCTAGCATTCTCAGCA; *OsPIN1a*-q-R: CCCTCCTCCCAAATTCTACTTC; *OsPIN1b*-q-F: CTCTCCAAGTCTCAACTCAAC; *OsPIN1b*-q-R: TGTCTATGTTCCTAATCTTCAATC; *OsPIN1c*-q-F: CGTCTTCGCCAAGGAGTACA; *OsPIN1c*-q-R: ACGACCCTCACCTGCAAA; *OsPIN1d*-q-F: CACAGCCGTGATATTCGGGAT; *OsPIN1d*-q-R: AGTTCTCGGATAATTGTCCACCT; *OsPIN2*-q-F: CAGGGCTAGGAATGGCTATGT; *OsPIN2*-q-F: GCAAACACAAACGGGACAA; *OsPIN3a*-q-F: CGCAACCCCAACACTTACTC; *OsPIN3a*-q-R: GACGACGGCGGCTGATTT; *OsPIN3b*-q-F: ACTGTTCAAGCCTTCAAGATTC; *OsPIN3b*-q-R: GTCCATCCACGGTTCTGC; *OsPIN5a*-q-F: CCCTACCTCAATCCATCACATC; *OsPIN5a*-q-R: CATTGGCTCTGCGTCTACC; *OsPIN5b*-q-F: ACCGGGGTTGGACTCTCCATGT; *OsPIN5b*-q-R: ATGATGGCAAGACGCAGGAGGT; *OsPIN5c*-q-F: TACCGATAACTCATCTGCTCAG; *OsPIN5c*-q-R: CATCCTTCTGTTGCGTCATTG; *OsPIN8*-q-F: CAATGACGGCTCGCTGTG; *OsPIN8*-q-R: GAAACGGTAAGAATTATGTATGGC; *OsPIN9*-q-F: ACTGAAGGATGACAACAAGGTG; *OsPIN9*-q-R: TCAACTGGTGGGCTGTAATAAG; *OsYUCCA1*-q-F: TCATCGGACGCCCTCAACGTCGC; *OsYUCAA1*-q-R: GGCAGAGCAAGATTATCAGTC; *18S-rRNA*-q-F: CCTATCAACTTTCGATGGTAGGATA; and *18S-rRNA*-q-R: CGTTAAGGGATTTAGATTGTACTCATT.

Relative quantification values for each target gene were calculated by the $2^{-\triangle\triangle CT}$ method [29], using *18S rRNA* as an internal reference gene for comparing data from different cDNA samples. All the experiments were repeated for three times, and the data were statistically analyzed and presented as means plus standard deviation (SD). Bars with different letters indicate significant differences at $P < 0.05$ (Fisher's protected LSD test).

2.5. Quantification of endogenous IAA

The 4-week-old rice roots and leaves (500 mg) were homogenized with 3 mL of 80% chilled methanol and kept under dark condition at 4 °C for over 24 h. The homogenate was centrifuged at 14,000 *g* for 20 min at 4 °C. The supernatants were collected, and the pellets were re-extracted with 3 mL of 80% cold methanol as the first extraction method. The sample preparation and measurement of endogenous IAA by HPLC were performed according to the method described by Lu et al. [30] with some modifications: HPLC is Waters 600–2487: Hibar column RT 250 × 4.6 mm, Purospher STAR RP-18 (5 μm), and column temperature was 35 °C. Fluid phase was methanol: 1% acetic acid (40:60, v:v), isocratic elution; fluid rate was 1.0 mL min^{-1}; UV detector, $\lambda = 269$ nm; and injection volume was 20 μL. A 0.45 μm filter was used for filtration of both the buffer and the samples before HPLC

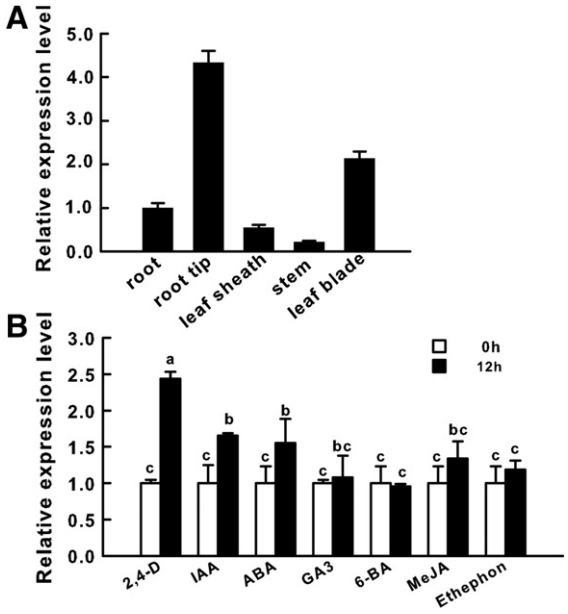

Fig. 2. Expression profiles of *OsRPK1* in wild type rice. (A) Tissue specific expression of *OsRPK1* in wild-type rice plants as assessed by real-time quantitative RT-PCR analysis. (B) Relative expression levels of *OsRPK1* were determined by real-time quantitative RT-PCR in the roots of 2-week old wild type seedlings with 2,4-D (100 μM), IAA (100 μM), ABA (100 μM), 6-BA (100 μM), GA3 (100 μM), MeJA (100 μM), and ethephon (1 mM) treatment for 0 h and 12 h, respectively. Data are means ± SD of three independent experiments. Bars with different letters indicate significant differences at $P < 0.05$.

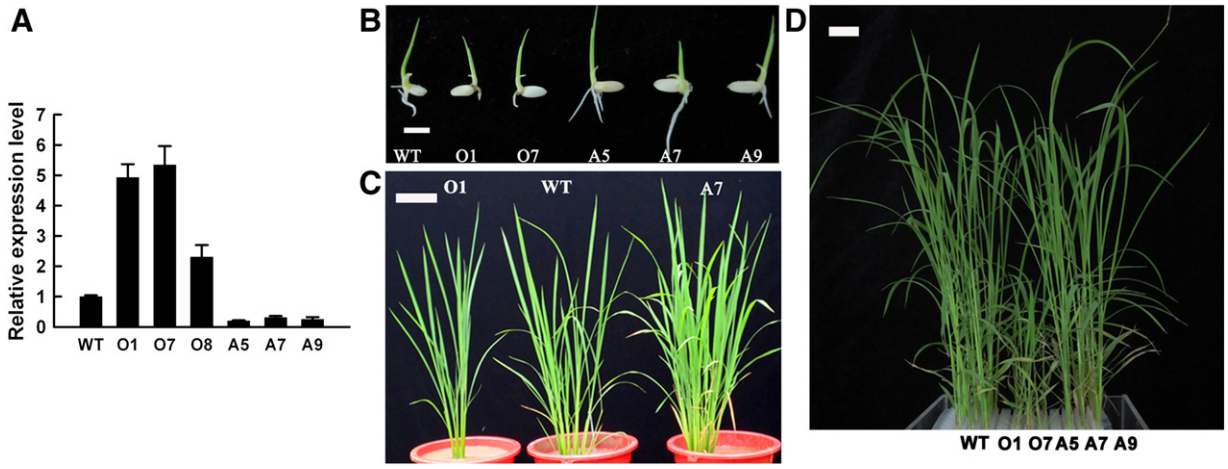

Fig. 3. Phenotypes of *OsRPK1* transgenic plants. (A) Real-time quantitative RT-PCR analysis for *OsRPK1* expression in the wild type (WT), three independent *OsRPK1* over-expressing lines (O1, O7, and O8), and three independent *OsRPK1* under-expressing lines (A5, A7, and A9). Total RNA was isolated from whole seedlings of WT and transgenic plants. (B) 1-week-old seedlings, white bar represents 0.4 cm. (C) 4-week-old seedlings, white bar represents 1.5 cm. (D) plants at tillering stage, and white bar represents 9.5 cm.

analysis. The content of samples was quantified by peak area with external standard. The IAA levels in each of the five plants were measured three times, and the standard deviations were calculated. Standard IAA was the product from Sigma-Aldrich.

2.6. Polar auxin transport assay

Polar auxin transport was measured according to Kant et al. [31]. The volume of the loading buffer (5 mM MES, 1% w/v Sucrose, 800 nM IAA, and 600 nM [^{3}H] IAA, pH 5.5) with or without 100 μM NPA was 50 μL, and the specific activity of [^{3}H] IAA was 15–30 Ci/mmol. After incubation at 25 °C for 3 h in the dark, stem segments were dissected into 1-cm-long sections, and the amounts of radioactivity in the sections were measured in a liquid scintillation counter.

2.7. Internal structure analysis

FM4-64 (Invitrogen, USA) staining of root tips was described by Wang et al. [32]. The 0.5 cm segment of the apical part of primary roots was cut from 5-day-old transgenic and wild-type seedlings, and stained immediately with 5 μM FM4-64 for 10 min on ice, followed by three rinses with water. Red fluorescence was observed with TCS SP2 confocal laser scanning microscope (Leica, Germany). FM4-64 fluorescence was excited with a 514-nm argon ion laser and a 600-nm long-pass emission filter. Images within a single experiment were captured with the same imaging system parameters.

For hematoxylin staining, longitudinal sections of root tips of 5-day-old transgenic and wild-type seedlings were used. The procedures of staining, dehydration, clearing, infiltration, and embedding were performed according to Liu et al. [33], and the microtome sections (8 mm) were mounted on glass slides for imaging.

2.8. Phylogenetic analysis

Phylogenetic analysis of OsRPK1 and its homologs was carried out using MEGA 4.0. The phylogenetic tree was performed by using the ClustalX 1.83 and DNAman software.

3. Results

3.1. OsRPK1 encodes a Ca^{2+}-independent LRR-RLK

To understand the relationship between OsRPK1 and LRR-RLK members from other plant species, phylogenetic analysis of OsRPK1 was performed on the full-length amino acid sequences from various plants using ClustalW and MEGA 4. A phylogenetic tree was constructed using the neighbor-joining method. The bootstrapping value (out of 10,000 samples) for each node, obtained with the same software, is shown in Fig. 1A. Data revealed that OsRPK1, which was designated Osi000900.2 and belongs to the LRR-VIII subfamily of RLK/Pelle in rice [50], had no close homologues in any other plant genome (Fig. 1A). For example, OsRPK1 shows 68 and 58% identity with EES19713 from *Sorghum bicolor* and CCB55930 proteins from *Vitis vinifera*, respectively, and 56% identity with XP_003547844 from *Glycine max*. In the rice genome, Os03g0329700 and Os01g0816600 proteins share 58 and 56% identity with OsRPK1, respectively. In addition, the results demonstrated that OsRPK1 is a novel leucine-rich-repeat receptor-like kinase (LRR-RLK), and no function had been assigned. OsRPK1 contains six periplasmic LRR motifs, one transmembrane domain, and one conserved

Table 1
Agronomic traits and yields of wild type and *OsRPK1* transgenic rice.

Genotype	WT	O1	O7	A5	A7	A9
Plant height (cm)	87.8 ± 2.2^a	74.5 ± 1.9^b	73.8 ± 1.2^b	87.7 ± 2.1^a	88.6 ± 2.5^a	89.6 ± 3.0^a
Total tiller number per plant	12.33 ± 0.82^{ab}	9.0 ± 0.89^b	8.7 ± 0.61^b	19.7 ± 1.15^a	19.0 ± 1.14^a	18.4 ± 1.23^a
Effective tiller number per plant	10.83 ± 0.41^{ab}	8.17 ± 0.75^b	7.70 ± 0.57^b	14.74 ± 1.76^a	14.2 ± 1.10^a	15.29 ± 1.38^a
Panicle length (cm)	20.62 ± 0.82^a	20.37 ± 1.25^a	20.50 ± 1.57^a	20.06 ± 2.24^a	20.48 ± 1.15^a	19.69 ± 1.81^a
Seed setting rate (%)	80.04 ± 4.38^a	82.83 ± 3.35^a	85.72 ± 1.92^a	73.61 ± 3.82^b	72.84 ± 2.72^b	75.15 ± 1.73^b
Grain number per panicle	88.39 ± 3.59^a	89.57 ± 6.36^a	91.22 ± 7.73^a	72.95 ± 4.62^b	71.35 ± 5.75^b	73.00 ± 7.21^b
Grain length (mm)	7.31 ± 0.27^a	7.40 ± 0.30^a	7.35 ± 0.27^a	7.22 ± 0.32^a	7.28 ± 0.27^a	7.50 ± 0.73^a
Grain breadth (mm)	3.40 ± 0.16^a	3.39 ± 0.16^a	3.52 ± 0.23^a	3.38 ± 0.27^a	3.33 ± 0.17^a	3.21 ± 0.23^a
1000-grain weight (g)	25.61 ± 2.09^a	24.80 ± 1.62^a	24.88 ± 1.80^a	24.54 ± 0.77^a	25.08 ± 1.00^a	25.47 ± 0.88^a
Yield (g/plant)	24.42 ± 0.93^a	17.72 ± 0.94^b	17.53 ± 1.38^b	24.37 ± 1.85^a	24.94 ± 1.85^a	24.57 ± 1.58^a

Numbers are presented as mean ± SD. The number of observations in each mean is 10. Means in the same column followed by the same letter are not significantly different ($P < 0.05$, LSD test).

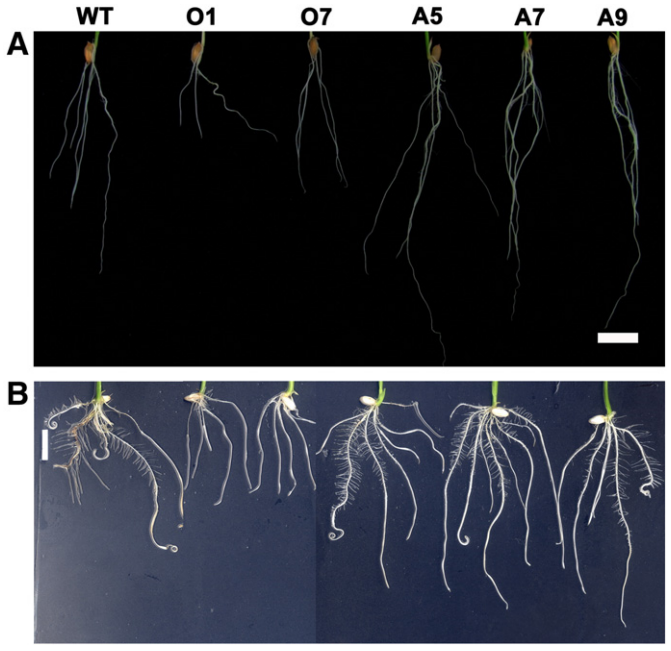

Fig. 4. Morphological characterization of root systems in wild type and transgenic rice plants. (A) Roots of 10-day-old WT, *OsRPK1* over-expressing (O1 and O7) and under-expressing (A5, A7, and A9) lines in rice nutrient solution. White bar represents 0.5 cm. (B) Roots of 4-week-old WT, *OsRPK1* over-expressing (O1) and under-expressing (A7) lines in rice nutrient solution. White bar represents 1.0 cm.

cytoplasmic kinase domain [27]. The kinase domain contained all 12 conserved sub-domains of the eukaryotic protein kinase, HRDIKSTN in the VIb sub-domain, and GTLGYLDPEY in the VIII sub-domain, indicating that OsRPK1 is a serine/threonine kinase (Fig. S1). To determine the phosphorylation activity of OsRPK1, the cytoplasmic kinase domain (OsRPK1-KD) was fused with a glutathione-S-transferase (GST) tag at the N-terminus (GST-OsRPK1-KD), and expressed in *E. coli* BL21. GST-OsRPK1-KD was purified using GSH resin and incubated with total protein extracted from rice root tips. As shown in Fig. 1B, the phosphorylation activity of GST-OsRPK1-KD was detected in the presence of Mn^{2+} and/or Mg^{2+}, but not in the presence of Ca^{2+}. This suggests that OsRPK1 is a Mn^{2+}/Mg^{2+}-dependent serine/threonine kinase.

3.2. Expression pattern of OsRPK1

The expression of *OsRPK1* in different tissues was analyzed by real-time quantitative PCR. The results showed that *OsRPK1* had a higher expression in root tips and leaf blades. Compared with the roots, the expression of *OsRPK1* was reduced 5-fold in the stem and 2-fold in the leaf sheath (Fig. 2A). Because the previous studies revealed that *OsRPK1* was induced by ABA [27], we assessed the effects of different plant hormones on its expression. Real-time PCR analyses (Fig. 2B) showed that the expression of *OsRPK1* was induced mainly by exogenous auxin (2,4-D and IAA) and ABA within 12 h of treatment.

3.3. Phenotypes of OsRPK1 transgenic rice

To assess the function of OsRPK1 in rice, transgenic plants over- and under-expressing *OsRPK1* were produced using 35S-driven *OsRPK1* sense and anti-sense constructs (Materials and methods). Three over-

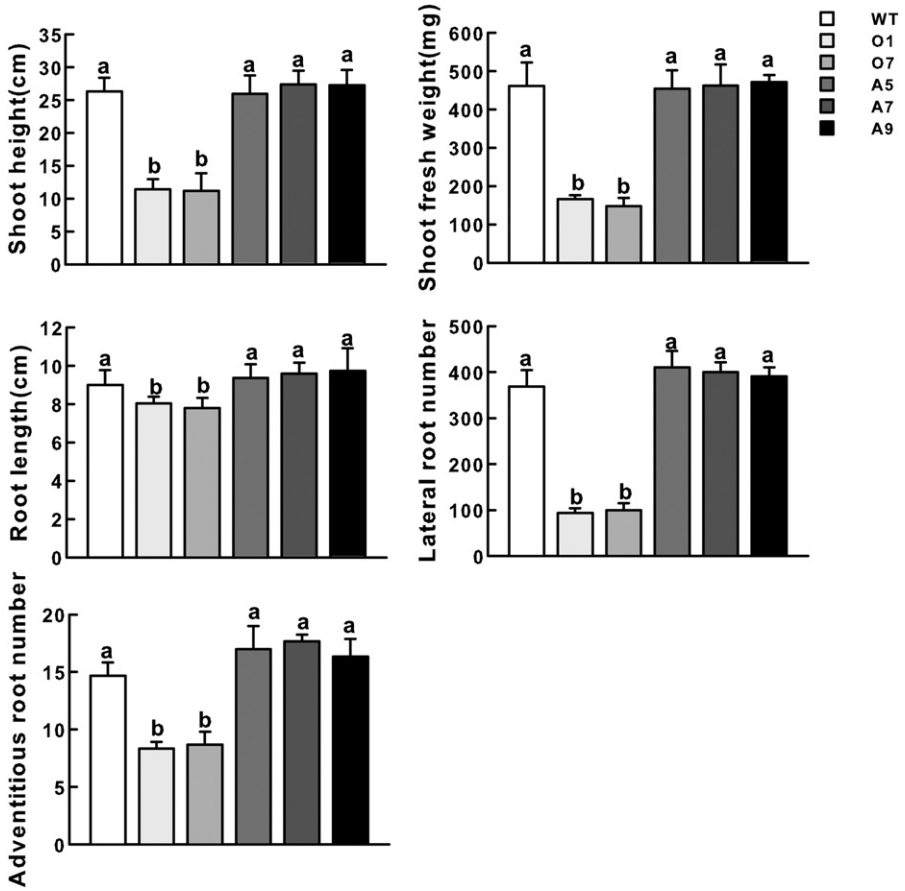

Fig. 5. Quantitative analysis of phenotypes in Figs. 3 and 4. Data are means ± SD of three independent experiments. The same letter indicated no significant difference, and different letters indicated significant differences at $P < 0.05$.

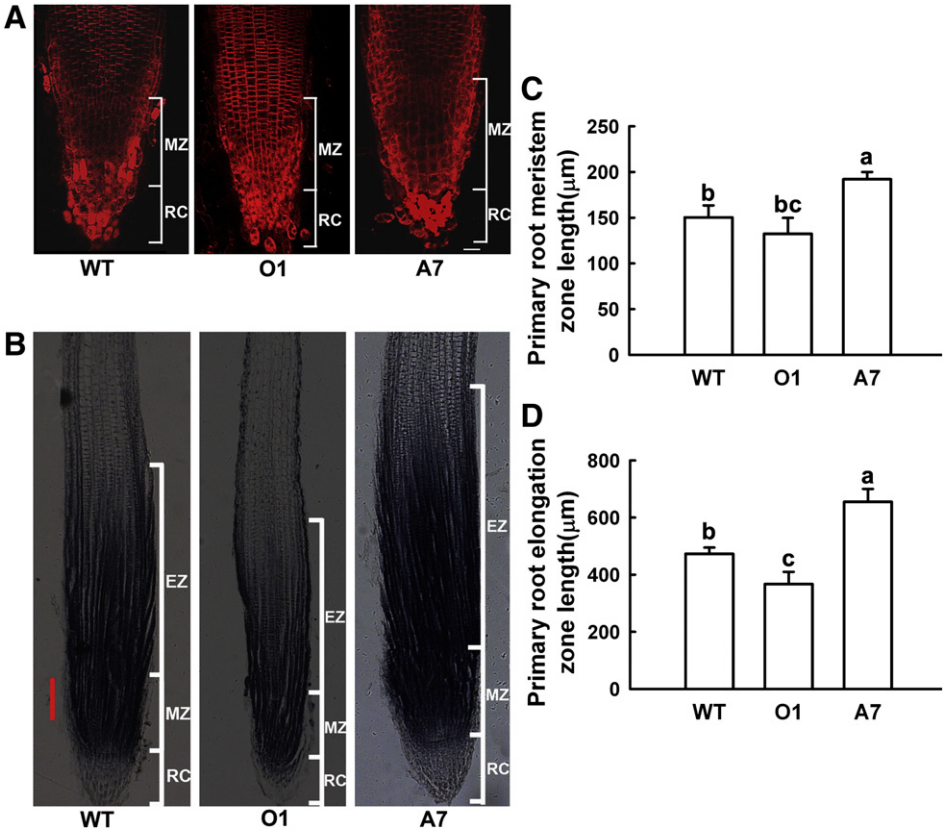

Fig. 6. Internal structure analysis of seminal roots of 5-day-old transgenic and wild-type seedlings. (A) Histology of root apex visualized on optical longitudinal sections of living embryonic primary roots from WT, O1, and A7 lines, which were stained with FM4-64 (in red). White bar represents 20 μm. (B) Longitudinal sections of primary root of WT and transgenic lines, which were stained with hematoxylin. The red bar represents 100 μm. RC, MZ, and EZ represent root cap, meristem zone, and elongation zone, respectively. (C) The quantification of meristem zone length of seminal root described in (A). (D) The quantification of elongation zone length of primary root described in (B). Data are means ± SD of three independent experiments. The same letter indicated no significant difference, and different letters indicated significant differences at $P < 0.05$.

expressing T_2 lines (O1, O7, O8) and three under-expressing T_2 lines (A5, A7, A9) were selected for analysis. Real-time quantitative PCR analysis showed that O1, O7, and O8 had higher levels of *OsRPK1* transcript than wild type (WT), whereas *OsRPK1* expression was suppressed in A5, A7, and A9 (Fig. 3A). One week after germination, the growth of the primary embryonic root was delayed in the over-expressing lines, whereas

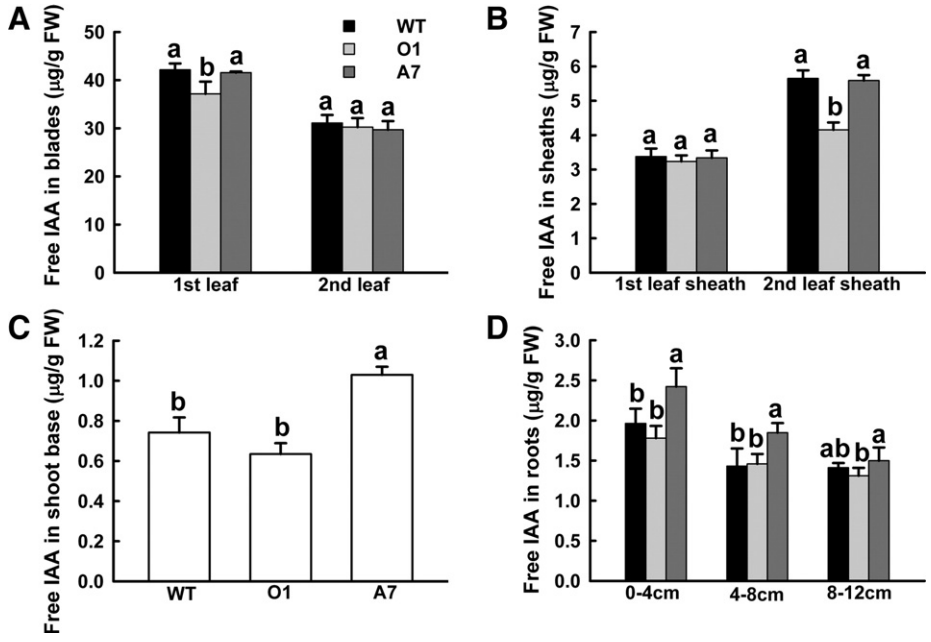

Fig. 7. HPLC analysis of endogenous free IAA concentrations in different tissues of 4-week-old wild-type and transgenic seedlings. (A) First and second leaf blades from the top. (B) First and second leaf sheaths from the top. (C) The root-shoot junctions. (D) Three different regions of roots: 0–4 cm, 4–8 cm and 8–12 cm from the root tip. Data are means ± SD of three independent experiments. The same letter indicated no significant difference; different letters indicated significant differences at $P < 0.05$.

Table 2
Phenotypes of wild type and *OsRPK1* transgenic seedlings with or without NPA treatment.

Treatments	Genotypes	ARN	LRD
−NPA	WT	7.67 ± 0.58[b]	23.4 ± 3.02[b]
	O1	6.0 ± 0.82[c]	13.1 ± 1.43[c]
	O7	6.17 ± 0.75[c]	15.3 ± 1.21[c]
	A5	12.7 ± 1.63[a]	30.3 ± 1.96[a]
	A7	11.7 ± 2.31[a]	31.8 ± 2.13[a]
	A9	11.3 ± 1.36[a]	34.0 ± 3.41[a]
+NPA	WT	3.75 ± 0.52[cd]	7.8 ± 0.50[d]
	O1	5.25 ± 0.45[d]	NA
	O7	5.50 ± 0.84[d]	NA
	A5	8.67 ± 0.82[b]	21.2 ± 3.12[b]
	A7	8.25 ± 0.64[b]	20.5 ± 4.33[b]
	A9	7.83 ± 0.75[b]	20.7 ± 2.13[b]

ARN, adventitious root number; LRD, lateral root density. Means in the same column followed by the same letter are not significantly different among the WT and the transgenic line with and without NPA treatment ($P < 0.05$, LSD test). Numbers are presented as mean ± SE. The number of observations in each mean is 5; 7-day-old seedlings were treated with 0.5 μM NPA for 5 days with five replicates.

the under-expressing lines had more adventitious root production compared with WT (Fig. 3B). At the seedling stage, over-expressing lines exhibited significant shorter shoot height and less fresh weight compared with the WT or under-expressing lines (Figs. 3C and 5). From the seedling to the tillering stage, under-expressing lines had an increased tiller number compared with the WT and over-expressing lines (Fig. 3C and D). At the ripening stage, there were significant differences between WT and the transgenic lines in the total and effective tiller number per plant. Compared with WT, over-expressing lines showed a 25% decrease in the effective tiller number, leading to a 27% reduction in grain yield per plant (Table 1). Although the effective tiller number in under-expressing lines was increased by 31% relative to WT, the seed setting rate and grain number per panicle were lower than those of WT, and the overall grain yield was similar (Table 1).

We further compared the root systems of the WT and transgenic plants. As shown in Fig. 4A, over-expressing lines had a smaller root system and fewer ARs. For example, compared with WT, over-expressing lines had 13% and 43% decreased seminal root length and AR number, respectively (Fig. 5). In addition, LR formation was severely defective in over-expressing lines (Figs. 4B and 5). The internal structure of the primary embryonic root tips also supported these observations. As shown in Fig. 6, under-expressing lines had well-developed meristems and elongation zones compared with WT, which were slightly defective in over-expressing plants.

3.4. OsRPK1 is a negative regulator of polar auxin transport

Because the expression of *OsRPK1* was related to tiller number, and LR and AR formation, we proposed that OsRPK1 could play a role in the physiological responses to auxin. To determine whether OsRPK1 affected auxin distribution, the levels of endogenous IAA in the leaves and roots of transgenic and WT plants were quantified using high performance liquid chromatography (HPLC; Fig. 7). Under-expressing lines had comparable levels of free IAA in the leaf and leaf sheath as WT, but higher levels of IAA in the shoot base and roots (increases of 39% in the shoot base, 23% in 0–4 cm roots, 29% in 4–8 cm roots, and 6% in 8–12 cm). In contrast, over-expressing lines had lower levels of IAA in the leaf blades, leaf sheath, shoot base, and root tips compared with WT. Since the younger leaves are the main tissues for auxin biosynthesis, these results suggest that OsRPK1 could affect PAT and/or auxin biosynthesis.

To determine whether OsRPK1 is involved in PAT, WT and transgenic seedlings were treated with 0.5 μM NPA, an inhibitor of auxin transport (Table 2). As expected, exogenous NPA significantly decreased the adventitious root number (by 51%) and lateral root density (by 67%) in WT plants. In contrast, the under-expressing lines were less sensitive to NPA treatment, which resulted in only a 29% decrease in adventitious root number, and 35% reduction in lateral root density. Furthermore, we measured PAT directly in different stem segments from the base of 4-week-old transgenic and wild type plants. As shown in Table 3, four-stem segments in the over-expressing lines had significantly lower levels of [^{3}H]-IAA than those of WT and under-expressing lines. Treatment with exogenous NPA also reduced [^{3}H]-IAA movement in both WT and transgenic plants. Taken together, these data suggest that OsRPK1 is a negative regulator of PAT in rice.

Next, we detected the expression of *OsPIN* genes in transgenic plants. The data revealed that most *OsPINs* (except for *OsPIN3a*, *5b*, and *5c*) were suppressed significantly in over-expressing lines, and had slightly higher expression in under-expressing lines compared with WT (Fig. 8). This supports the hypothesis that PAT is affected in transgenic plants. To further study whether *OsRPK1* plays a role in the auxin biosynthesis pathway, we assessed the expression of *OsYUCAA1*, which is an important enzyme for IAA biosynthesis in rice [34]. As shown in Fig. 8, the expression of *OsYUCAA1* was similar

Table 3
Polar auxin transport in stems of wild type and *OsRPK1* transgenic seedlings.

Treatment and plant	[^{3}H]IAA in stem section			
	1	2	3	4
Cpm				
Basipetal (without NPA)				
WT	212,106 ± 18,022[a]	25,303 ± 3146[ab]	5096 ± 331[a]	1266 ± 89[ab]
O1	209,385 ± 14,198[b]	21,095 ± 2682[b]	4698 ± 461[b]	732 ± 34[b]
O7	189,453 ± 12,283[b]	20,398 ± 2456[b]	4409 ± 234[b]	765 ± 84[b]
A5	226,785 ± 15,849[a]	26,102 ± 3185[a]	5684 ± 472[a]	1506 ± 76[a]
A7	230,748 ± 12,521[a]	27,084 ± 3873[a]	5783 ± 343[a]	1458 ± 96[a]
A9	228,218 ± 14,586[a]	26,435 ± 3281[a]	5539 ± 459[a]	1447 ± 79[a]
Basipetal (with 100 μM NPA)				
WT	208,713 ± 21,671[a]	4778 ± 438[b]	812 ± 96[b]	309 ± 40[a]
O1	207,851 ± 18,845[b]	4009 ± 409[b]	723 ± 45[a]	214 ± 32[b]
O7	186,192 ± 18,290[b]	4134 ± 875[b]	765 ± 63[a]	228 ± 43[b]
A5	215,109 ± 16,853[a]	5098 ± 512[a]	1047 ± 86[a]	422 ± 65[a]
A7	221,983 ± 23,209[a]	4954 ± 209[a]	964 ± 76[a]	425 ± 73[a]
A9	220,754 ± 23,834[a]	4839 ± 321[a]	926 ± 82[a]	439 ± 80[a]

Four-centimeter stem segments of 4-week-old wild type and *OsRPK1* transgenic seedlings were incubated in [^{3}H] IAA solution for 3 h, and segments were further divided into four sections of 1 cm each. Section 1 was submerged in [^{3}H] IAA solution, and Section 4 indicates the farthest section. Numbers are presented as mean ± SE. The number of observations in each mean is 6. Means in the same column followed by the same letter are not significantly different ($P < 0.05$, LSD test).

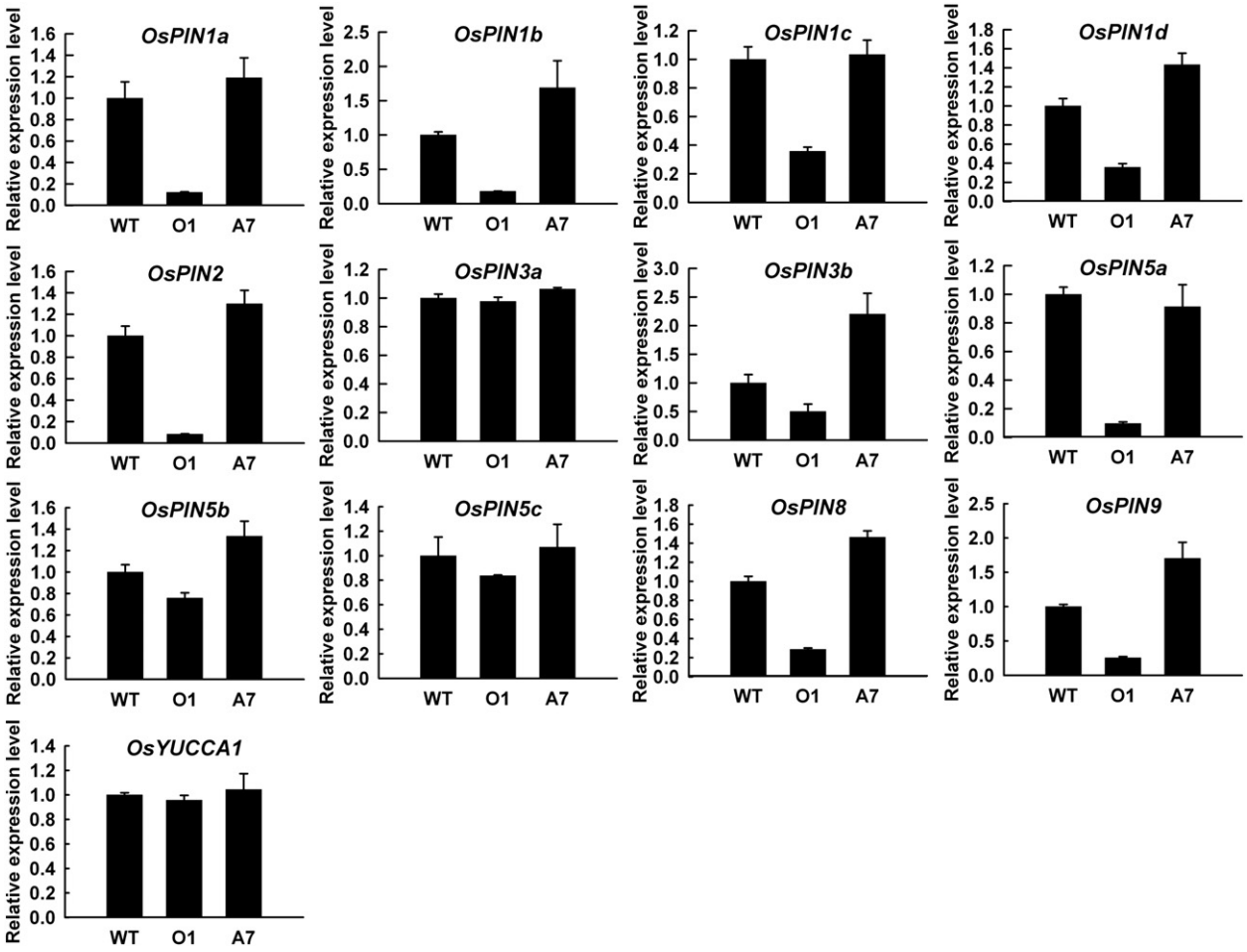

Fig. 8. Expression of auxin related genes in WT, *OsRPK1* under- and over-expressing plants. Relative expression levels were determined by real-time RT-PCR for Os*PINs* and Os*YUCCA1* genes in the roots of 2-week old seedlings of wild type (WT), over-expressing O1 line, and under-expressing A7 line. Data are means ± SD of three independent experiments.

in WT and transgenic plants. Since OsYUCCA1 functions in various organs, this suggests that OsRPK1 may have no effect on auxin biosynthesis.

4. Discussion

In this study, we provided evidence that *OsRPK1*, a novel LRR-RLK, plays a role in root development in rice, and demonstrated that its over-expression resulted in reduced plant height, tiller number, and AR and LR numbers (Figs. 3, 4, and Table 1). IAA and ABA also induced the expression of OsRPK1 (Fig. 2). Although several LRR-RLKs were proposed to play a role in the auxin response in *Arabidopsis* [26], this is, to our knowledge, the first auxin-related LRR-RLK reported in rice.

The development of ARs and LRs and their relationship to the auxin response has been well studied in rice. Several studies suggested that auxin biosynthesis [34], transport [35], and signaling [36] are required for the initiation of ARs and LRs. For example, Inukai et al. [37] identified CRL1 as a target of auxin response factor (ARF), and demonstrated that its expression promoted the formation of ARs and LRs. Kitomi et al. [38] further identified CRL5, which promoted the initiation of ARs by suppressing cytokinin signaling via positively regulating OsRR1, a type-A response regulator. In these reports, transcription factors are components of auxin signaling or response pathways, and directly regulate RSA in rice. In contrast to these regulators, OsRPK1 was identified as a negative regulator of PAT. All the phenotypes observed in over-expressing plants, including auxin distribution (Fig. 7) and sensitivity to NPA (Table 2), could be explained by disrupted PAT (Table 3).

In this study, OsRPK1 exhibited wide-ranging regulation of the expression of PIN family members. Out of the 12 *OsPIN* genes in rice [39], nine genes (not *OsPIN3a*, *5b*, and *5c*) showed similar expression patterns in over- and under-expressing lines. Until now, only three *OsPINs* had been reported in rice. *OsPIN1a* played an important role in auxin-dependent adventitious root emergence and tillering in rice, and the significantly decreased AR numbers in *OsPIN1* RNAi plants were caused by the arrest of primordial emergence [35]. In the present study, OsPIN1a and three other members in OsPIN1 family were also inhibited significantly in the over-expressing lines. As a result, the numbers of ARs and LRs were decreased. Although OsPIN1a did not affect LR development [35], it is possible that other OsPIN1s may regulate this process, since AtPIN1 is essential for the initiation and development of LR primodia in *Arabidopsis* [40]. Recent studies implicated OsPIN2 as a homolog of AtPIN2. OsPIN2 played a role in the LAZY1-dependent gravity responses of rice shoots, and the over-expression of OsPIN2 in rice resulted in increased tiller angle, more tillers, and fewer ARs [41]. Os*PIN3* are monocot-specific PINs, with two members. *OsPIN3a* is also known as *OsPIN3t* [42] or *OsPIN10a* [43], and *OsPIN3b* is also called *OsPIN10b* [43]. The over-expression of *OsPIN3t* led to longer roots, more ARs, and improved drought tolerance [42]. Consistent with this, *OsPIN2* was suppressed, and *OsPIN3a* was induced slightly in over-expressing lines in our study. Although such changes should lead to increased AR numbers, it is likely that OsRPK1 regulates root development via PINs. It is also possible that different OsPIN proteins could mediate diverse functions in PAT and root development [42], and that OsPIN2 and OsPIN3t may play relatively small roles in AR and LR formation. This hypothesis is based on the several observations. First, the

suppression of *OsPIN2* was observed in AR-defective mutants. For example, *gnom1-1* mutants exhibited reduced LR and AR numbers. The expression of *OsPIN2*, *OsPIN5b*, and *OsPIN9* was altered in the mutants, which suggested that OsGNOM1 affects the formation of ARs by regulating PAT [10]. The inhibition of *OsPIN2* in *gnom1-1* mutant supports the hypothesis that this protein is not a key regulator of AR formation. Second, although over-expressing *OsPIN2* rice showed fewer ARs, it is still unknown whether *OsPIN2* under-expression leads to the formation of more ARs. Finally, the over-expression of *OsPIN3t* led to more ARs, and its knockdown resulted in only slightly shorter ARs. In WT, treatment with 20% PEG slightly induced OsPIN3t expression, but suppressed ARs significantly [42]. Because rice has several PIN family members and most of them are not functionally characterized, these data suggest that PAT and root development are regulated by a combination of different PINs, and that OsRPK1 could be a common switch of *OsPINs* under hyper-osmotic stress.

In this study, the expression of OsRPK1 was induced by auxin and ABA. Although the crosstalk between auxin and ABA remains unclear, many genes have been identified as auxin- and ABA-sensitive. For example, auxin responding factor-2 (ARF2) was also induced by ABA, and *arf2* mutants are extremely sensitive to ABA. Because PIN2 expression is suppressed by ABA during germination, an *arf2–pin2* double mutant has ABA sensitivity similar to wild type [44]. In fact, whole-genome analysis in rice showed that 154 auxin-induced and 50 auxin-repressed genes responded to abiotic stresses and ABA [45]. Among these multi-sensitive genes, several crosstalk points are now regarded as the nodes that link auxin/ABA/ROS signaling pathways. These include ROS-dependent MAPK cascades, Rho guanosine triphosphatases (GTPases), and calcium signaling [46–48]. We suggest that OsRPK1 is an auxin-related receptor-like kinase that is induced by auxin, ABA, and abiotic stresses, and may be a central point of crosstalk between auxins and ABA. Calcium signaling, G proteins, and MAPKs are the second messengers and downstream components of these pathways. As an upstream regulator at plasma membrane, OsRPK1 could affect the expression and activity of Os*PINs* (Fig. 8), possibly via the RAC/ROP pathway [49]. Since small GTPases and RAC/ROPs have been implicated as mediators of auxin-regulated gene expression, rapid cell surface-located auxin signaling, and directional auxin transport, it is possible that OsRPK1 and its phosphorylation/dephosphorylation play a role in RAC/ROP-mediated PIN expression and/or polar localization.

It should be noted that, in our previous study, OsRPK1 was identified as a salt stress-responding protein that was induced by treatment with 150 mM NaCl [27], and that the phenotypes of the over-expressing plants in Figs. 3 and 4 are very similar to that of the WT under high salt stress. Based on these observations, we propose that, in addition to regulating PAT, OsRPK1 might also play roles in the root system architecture under salt stress, and so the expression of OsRPK1 could remain low under normal growth conditions. Abiotic stresses such as high salt induce the expression of OsRPK1, resulting in altered expression of OsPINs, particularly OsPIN1 and OsPIN2. This leads to reduced PAT and auxin levels in roots, which inhibits the growth of ARs and LRs. However; additional studies are still needed to support this hypothesis, particularly the relationship between OsRPK1 activity and stress treatments, and the identification of OsRPK1-interacting proteins (ligands) and elucidation of its precise mechanism of action are important aims of future studies.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.01.003.

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