

# Functional Analysis and Expressional Characterization of Rice Ankyrin Repeat-Containing Protein, *OsPIANK1*, in Basal Defense against *Magnaporthe oryzae* Attack

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## Abstract

The ankyrin repeat-containing protein gene *OsPIANK1* (AK068021) in rice (*Oryza sativa* L.) was previously shown to be upregulated following infection with the rice leaf blight pathogen *Xanthomonas oryzae* pv *oryzae* (*Xoo*). In this study, we further characterized the role of *OsPIANK1* in basal defense against *Magnaporthe oryzae* (*M.oryzae*) by 5' deletion analysis of its promoter and overexpression of the gene. The promoter of *OsPIANK1* with 1,985 bps in length was sufficient to induce the *OsPIANK1* response to inoculation with *M.oryzae* and to exogenous application of methyl jasmonate (MeJA) or salicylic acid (SA), but not to exogenous application of abscisic acid (ABA). A TCA-element present in the region between -563 bp and -249 bp may be responsible for the *OsPIANK1* response to both *M.oryzae* infection and exogenous SA application. The JERE box, CGTCA-box, and two MYB binding sites locating in the region between -1985 bp and -907 bp may be responsible for the response of *OsPIANK1* to exogenous MeJA. *OsPIANK1* expression was upregulated after inoculation with *M.oryzae* and after treatment with exogenous SA and MeJA. Overexpression of *OsPIANK1* enhanced resistance of rice to *M.oryzae*, although it did not confer complete resistance. The enhanced resistance to *M.oryzae* was accompanied by enhanced transcriptional expression of SA- and JA-dependent genes such as *NH1*, *WKRY13*, *PAL*, *AOS2*, *PR1b*, and *PR5*. This evidence suggests that *OsPIANK1* acted as a positive regulator in rice basal defense mediated by SA- and JA-signaling pathways.

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## Introduction

Plants naturally co-exist with numerous and varied microbial pathogens and the diseases that can result represent a heavy loss in crop productivity worldwide. In consequence, plants have evolved effective immune systems to defend themselves against the attack of microbial pathogens. Two interconnected modes of the innate immune system are used by plants to defend against phytopathogens. The first mode, pattern-triggered immunity (PTI), results from the activation of plant cell-surface pattern recognition receptors (PRRs) following the exposure of these receptors to molecular patterns common to many types of microbes (pathogen-associated molecular patterns [PAMPs]). Infection by a particular pathogen can be blocked by PTI. Pathogens have evolved effector molecules that are secreted into plant cells and suppress PTI, and, in turn, plants have evolved resistance (R) proteins that recognize the effectors and activate the second mode of the innate immune system, effector-triggered immunity (ETI), in a gene-to-gene way [1–4]. In addition to the protein-protein interactions mediated by PRRs, R proteins, PAMPs, and effectors, other proteins such as the leucine-rich repeat (LRR) domain-containing proteins [5–7]

and the ankyrin (ANK) domain-containing proteins [8,9] are important in plant defense.

The ANK domain is one of the most common protein motifs in eukaryotic proteins. Repeated ankyrin domains are ubiquitous, can function in protein-protein interactions, and may act as molecular chaperones for a class of membrane-bound proteins in plants [10,11]. In addition to the ANK-repeat domain, ANK proteins often contain several other functional domains, for examples, PEST, calmodulin binding motifs, and Ring Finger domain [12,13]. Mediation of protein-protein interactions by the ANK domain is involved in a number of physiological and developmental responses such as the cell cycle, cell differentiation [14–19], plastid differentiation [14], pollen germination and pollen tube growth [20], lateral root development [21], leaf morphogenesis [16], targeting of proteins to the plastid outer envelope [22,23], grana formation [24], anthocyanin biosynthesis [25], nitrogen-fixing symbiosis in root nodules of *Lotus japonicus* [26], plant microbe interaction [27,28], male-female gamete recognition [29], plant immunity [8,9,30–36], plant resistance to abiotic stress associated with Cd [37–39], regulation of a plant potassium channel [40], and ethylene signaling [41,42]. In higher plants,

**Table 1.** Primer information for real-time PCR.

Gene name	Forward primer sequence (5' -3')	Reverse primer sequence (5' -3')
<i>PIANK1</i>	gAgATgTgCCCgTCgCTgTAT;	CACTgTCTgTgCTgAATgATgCC
<i>PR1b</i>	ggCAACTTCgTCggACAgA;	CCgTggACCTgTTTACATTTTCA
<i>PR5</i>	CAACAgCAACTACCAAgTCgTCTT;	CAAggTgTCgTTTTATTTCATCAACTTT
<i>NH1</i>	CACgCCTAAgCCTCggATTA;	TCAgTgAgCAGCATCCTgACTAg
<i>WRKY13</i>	TCAgTggAgAAGCgggTggTg;	gggTggTTgTgCTCgAAggAg
<i>AOS2</i>	CAATACgTgTACTggTCgAATgg;	AAggTgTCgTACCgAggAA
<i>PAL</i>	AgCACATCTTggAgggAAgCT;	gCgCggATAACCTCAATTTg
<i>Actin</i>	TgTATgCCAgTggTCgTACCA;	CCAgCAAggTCgAgACgAA

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ANK proteins constitute a large multi-gene family. For example, 175 and 105 ANK repeat genes have been found in rice and the *Arabidopsis* genome, respectively [12,13]. However, the function and the underlying mechanism of most of the genes of the ANK family of proteins remains poorly understood.

Here, we report on a study of the ANK domain in the plant immune response, using rice as a model system. Rice is one of the most agriculturally important crops worldwide, but approximately a million tons are lost to disease each year. Better understanding of the genetic mechanisms of rice defense responses could lead to better agricultural management and reduced losses due to diseases.

The *OsANK* proteins in rice can be classified into 10 subfamilies. Expression profile analysis of *OsANK* proteins suggested that members of this gene family may play important roles in pollination, fertilization, and signaling [12]. Among all of the 175 known ANK-repeat genes in rice, only a few members have been functionally characterized. The ANK-repeat domain-containing XA21 binding protein 3 was required for full *Xa21*-mediated disease resistance [33], *OsCBT*, containing three ANK repeats, is a transcription activator modulated by CaM [43], *OsBIANK1*, encoding a plasma membrane-anchored ANK-repeat protein, was induced by treatment with either benzothiadiazole or by infection with *Magnaporthe grisea* [31]. *OsPIANK1*, an ankyrin protein in rice, was late up-regulated by infection of the rice leaf blight pathogen *Xoo* at both early and late infection stages, and had different expression patterns against the attack of *Xoo* [44]. Both PTI and ETI are accompanied by major transcriptional reprogramming, which is considered a key step of the plant defense program [45,46]. Up to 25% of all *Arabidopsis* genes respond to pathogen infection by altering their transcript levels [47,48]. Genetic evidence indicates that genes that are transcriptionally up-regulated during plant immune responses are important in disease resistance [49–51]. This reasoning suggests that *OsPIANK1* plays an important role in rice immunity, although there is no direct evidence for this role. In the present study, we performed functional identification of *OsPIANK1* by its overexpression in rice and expressional characterization by a 5' deletion assay of its promoter. The results suggest that *OsPIANK1* is a positive regulator in the rice basal defense mediated by the SA and JA signaling pathways.

## Materials and Methods

### Isolation of the Full Length cDNA and Promoter of *OsPIANK1*

The full length cDNA of *OsPIANK1* was amplified by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA

from leaves of Nipponbare (*Oryza sativa* L. japonica) was extracted using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Invitrogen) was used for cDNA synthesis according to the manufacturer's protocol. The newly synthesized cDNA was used as the template for PCR amplification with the *OsPIANK1*-specific forward primer (5'-TACGGCATgCATACTCCATCA-3') and reverse primer (5'-TACATGCATGCAAGCTGTCA-3'). Genomic DNA of Nipponbare was extracted from leaf tissues using the hexadecyltrimethylammonium bromide (CTAB) method. The *OsPIANK1* promoter region was amplified by PCR using the specific primer pair (5'-TTTGCACCTTTTACCCTg-3) and (5'-CGCTTATTAGAGATAAAGAGGA-3'). All of the PCR products was cloned into pMD18-T vector and sequenced. The *OsPIANK1* promoter sequences were analyzed by the PLACE Web Signal Scan program (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

### Vectors Construction and Rice Transformation

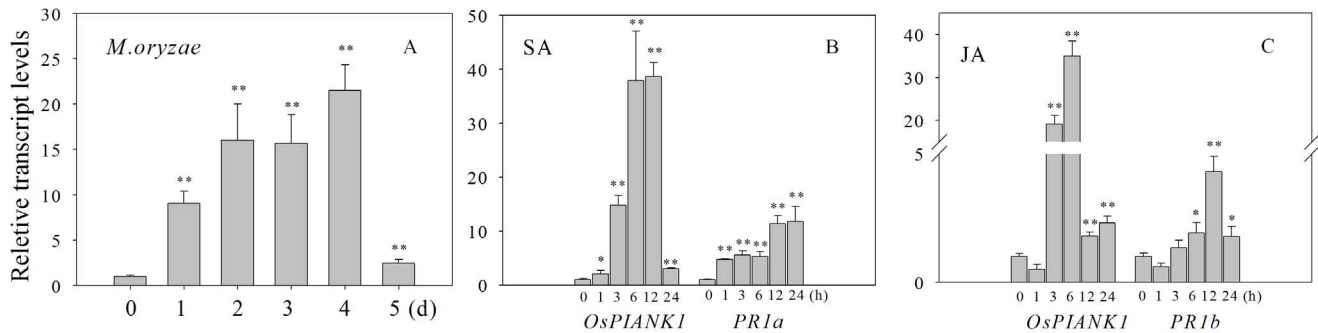
To construct the overexpression vector of *OsPIANK1*, the open reading frame of *OsPIANK1* was amplified by PCR with *Kpn*<sup>-</sup>-*SpeI*<sup>-</sup> (indicated by lowercase letters) linker primers (5'-GGgac-cATGCATACTCCATCAAT-3', 5'-GGgactagtTCAGGTCGT-CATCACAT-3') using pMD18T-*OsPIANK1* as the template, which was further cloned into the *Kpn* I and *SpeI* I sites of the modified binary expression vector pCambia1390 under the control of the maize ubiquitin promoter.

Serially 5'-deleted *OsPIANK1* promoters were created by PCR using the full-length promoter fragment as templates with four forward primers (-1101:5'-TATCTGGTACCTTCAGAG-3', -907:5'-TTCGGCCAATAGGATGTA-3', -563:5'-TGTTGGTCTAATCTTCGG-3', -249:5'-TTGCAATATTGACAGCAG-3') and the reverse primer (5'-CGCTTATTAGAGATAAAGAGGA-3'). The four promoter deletion-GUS fusion constructs were further cloned into the binary destination vector pMDC163 [52] using the Gateway cloning technique.

After verification by sequencing, all of the vectors were transferred into *Agrobacterium tumefaciens* strain EHA105, and then were transformed into Nipponbare rice. The *Agrobacterium*-mediated transformation was performed according to the method of Toki S [53] using vigorously growing callus derived from mature embryos. More than 10 independent transgenic lines were obtained for each vector.

### Pathogen and Exogenous Phytohormones Treatment

To analyze the response of *OsPIANK1* promoter to inoculation with the pathogen and exogenous application of phytohormones,



**Figure 1. Expression profiles of *OsPIANK1* in response to inoculation with *M.oryzae* or exogenous application of SA and MeJA.** Transcript levels of *OsPIANK1* were determined by quantitative real-time PCR. Total RNA was prepared from leaf tissues of 3-week-old seedlings at the times indicated. The expression level of untreated plants at each time was used as the control and assigned a value of 1. Relative expression levels were normalized using the expression of *actin*. Data represents the mean  $\pm$  SE of three independent biological replicates. (\*\*, \* indicate significant differences between the treatment and control at  $P < 0.01$  or  $P < 0.05$ , respectively, as determined by Student–Newman–Keuls (SNK) test). doi:10.1371/journal.pone.0059699.g001

T<sub>1</sub> transgenic rice plants with the *GUS* reporter gene controlled by the *OsPIANK1* promoter were developed. These plants may have had a single T-DNA insertion since the T<sub>1</sub> progeny from individual positive clones exhibited a nearly 3:1 ratio in resistance to hygromycin. The sterilized T<sub>1</sub> transgenic and wild-type rice seeds were germinated for 2 weeks on Murashige and Skoog agar medium with or without 50 mg L<sup>-1</sup> hygromycin, respectively. The resulting seedlings were transferred to soil in pots and grown in a greenhouse (25°C/27°C, solar radiation). Rice seedlings were treated at the four-leaf stage. For inoculation with the pathogen, leaves were sprayed with a suspension of conidia ( $1 \times 10^5$  mL<sup>-1</sup>) of *M.oryzae* strain guy11. For exogenous phytohormone treatment, 100  $\mu$ M ABA in sterile double-distilled H<sub>2</sub>O, SA (100  $\mu$ M in 10% ethanol), or 100  $\mu$ M MeJA (in 10% ethanol) was sprayed on the leaves and the plants were kept in a greenhouse (25°C/27°C, solar radiation) until the samples were harvested at the designated times.

### GUS Activity Assay

Histochemical staining for beta-glucuronidase (*GUS*) activity was performed as reported previously [54]. Leaves of *OsPIANK1*-pro-*GUS* transgenic plants were detached and incubated in *GUS* reaction buffer (0.2 M phosphate pH 7.0 containing 0.1 M K<sub>3</sub>[Fe(CN)<sub>6</sub>], 0.1 M K<sub>4</sub>[Fe(CN)<sub>6</sub>], 1.0 M EDTA-Na<sub>2</sub>, 0.1% 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide [X-Gluc]) at 37°C for 24 h. For better visualization of the stained tissue, leaves were rinsed at room temperature with an ethanol series for at least 1 h to remove chlorophyll.

Total protein was extracted from the leaf tissue of the *OsPIANK1*-pro-*GUS* plants. *GUS* activity was measured using a spectrophotometer to quantify the rate of release of *p*-nitrophenol ( $\lambda = 415$  nm) from *p*-nitrophenyl- $\beta$ -D-glucuronide (PNPG) [54]. *GUS* activity assays were repeated with at least two independent lines, each with three replicates. When similar results were obtained in repeated experiments, only one experiment has been presented.

### Subcellular Localization of *OsPIANK1*

For *OsPIANK1* localization, the full-length coding sequence of *OsPIANK1* was amplified and fused in frame to the N-terminus of the green fluorescent protein (*GFP*) gene to generate the CaMV35S::*OsPIANK1*-*GFP* construct by Gateway-mediated recombination into the vector pMDC83. The CaMV35S::*GFP* construct was used as the control. Both constructs were further transformed into *Agrobacterium tumefaciens* strain GV3101. The cells

of GV3101 harboring CaMV35S::*OsPIANK1*-*GFP* constructs was co-infiltrated into *N. benthamiana* leaves. One day after agro-infiltration, the leaves were visualized under a fluorescence microscope (Olympus DP72), with an excitation wave length of 488 nm and a 505–530 nm band-pass emission filter. 4',6-diamidino-2-phenylindole (DAPI) fluorescence was also imaged using an excitation wavelength of 405 nm and a 435–480 nm band-pass emission filter.

### Quantitative Real-time PCR

For quantitative real-time PCR analysis, total RNA from the leaves of the wild-type (Nipponbare) and transgenic plants were extracted. First-strand cDNA was generated by converting 500 ng total RNA using Primescript RT reagent (perfect real time, TaKaRa), and the cDNA was diluted to 100  $\mu$ L with water. Real-time PCR using Mastercycler ep *realplex* (Eppendorf, Hamburg, Germany) was performed with SYBR® Premix Ex Taq™ II (perfect real time, TaKaRa). Each reaction mix (25  $\mu$ L) contained 12.5  $\mu$ L SYBR Premix Ex Taq, 0.5  $\mu$ L PCR forward/reverse gene specific primers (10  $\mu$ M), 2.5  $\mu$ L diluted cDNA, and 9.5  $\mu$ L water. For each gene, three experimental replicates were obtained using different cDNAs synthesized from three biological replicates. The thermal cycle used was as follows: one cycle of 30 s at 95°C; 40 cycles of 5 s at 95°C, 34 s at 60°C; one cycle of 15 s at 95°C, 1 min at 60°C, 15 s at 95°C, 15 s at 60°C. Rice *actin* gene (X15865) was used for normalization. The gene-specific primer pairs are listed in Table 1. The relative expression levels were determined as described by Livak and Schmittgen [55].

### Quantification of *M.oryzae* DNA in Rice Leaves

Rice and fungal DNA were extracted from leaves inoculated with *M.oryzae* and quantified by real-time PCR in accordance with the method of Qi and Yang [56] using two specific primer pairs, which were designed based on the 3' noncoding region of a *MPGI* gene in *M.oryzae* (5'-GGGATGATGGTGGTGGAGGAC-3'; 5'-GCCAGGTGCTTAGGACGAAAC-3'). The data were normalized to the amount of DNA of a rice *actin* gene (AK060893), which was quantified using the forward primer (5'-GAGTATGATGAGTCCGGTCCAG-3') and reverse primer (5'-ACACCAA-CATCCCAAACAGAG-3').





**Figure 2. Nucleotide sequence of 5' flanking promoter regions and stress-related sequence motifs putatively acting as *cis*-elements of the *OsPIANK1* gene.** HSE: *cis*-acting element involved in heat stress responsiveness. CGTCA-box: *cis*-acting regulatory element involved in the MeJA-responsiveness. JERE: *cis*-acting regulatory element involved in the MeJA-responsiveness. ERE: ethylene-responsive element. DRE: *cis*-acting element involved in dehydration, low-temp, salt stresses. MBS: MYB binding site involved in drought-inducibility. GCC-box: ethylene-responsive element. ABRE: *cis*-acting element involved in the abscisic acid responsiveness. TCA-element: *cis*-acting element involved in salicylic acid responsiveness.

doi:10.1371/journal.pone.0059699.g002

## Results

### Expression Pattern of *OsPIANK1* in Response to Pathogen Infection and Exogenous Application with Phytohormones

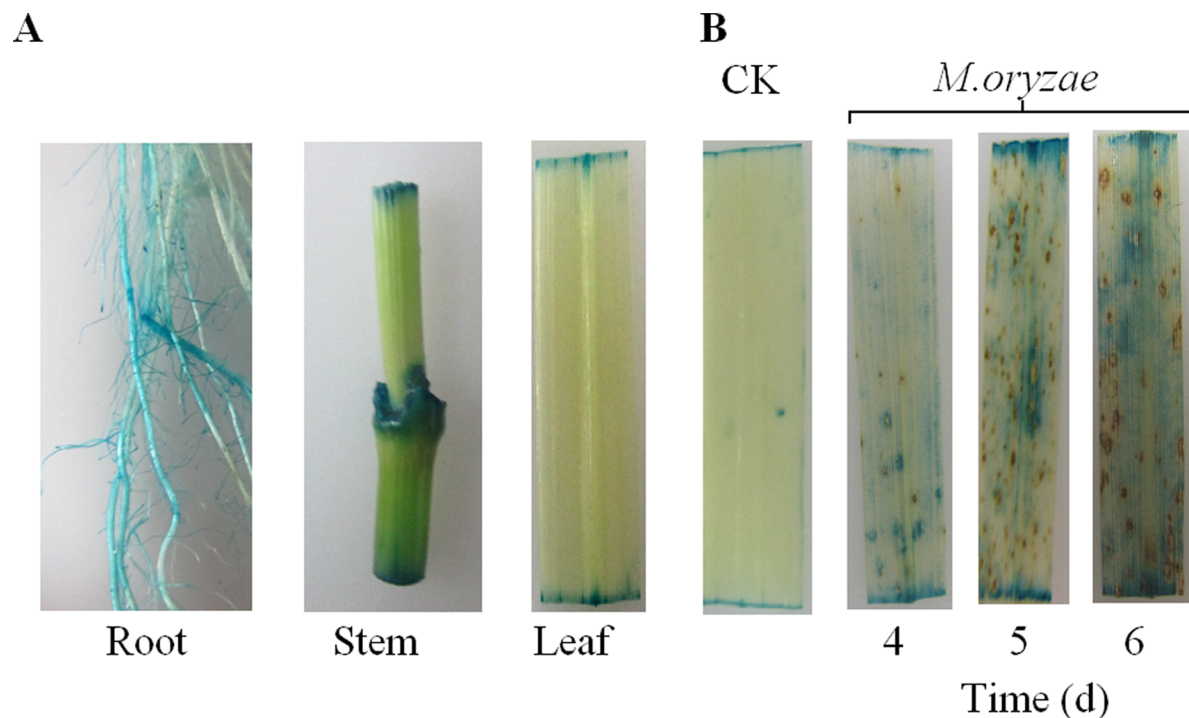
Expression of *OsPIANK1* in rice has been reported to be transcriptionally up-regulated after infection of the rice leaf blight pathogen *Xoo* [44]. We wished to evaluate the possibility that *OsPIANK1* is an important regulator for the rice response to other pathogens and to universal defense signaling molecules such as SA and JA. To this end, the transcriptional levels of *OsPIANK1* following infection with the *M.oryzae* strain guy11 or exogenous application of SA and MeJA were examined by real-time PCR. Transcripts of *OsPIANK1* increased approximately 10-fold 1 day after inoculation with *M.oryzae*, remained high for 2 to 4 days after inoculation, and decreased sharply 5 days after inoculation (Figure 1A). *OsPIANK1* transcripts increased markedly 3–12 h after treatment with SA and declined dramatically 24 h after treatment. SA treatment induced the rice *acidic pathogenesis-related (PR) protein1 (PR1a)* gene that was used as a comparable control (Figure 1B). In response to MeJA, the mRNA accumulation of *OsPIANK1* was increased remarkably for 3 h, reached a maximal level after 6 h, and decreased sharply 12 h after treatment. MeJA induced the rice *basic PR protein1 (PR1b)* gene that was used as a comparable control (Figure 1C). These results indicate that, in

addition to responding to infection by *Xoo*, *OsPIANK1* is involved in the response of rice to *M.oryzae* infection regulated by the signaling pathway mediated by SA and JA.

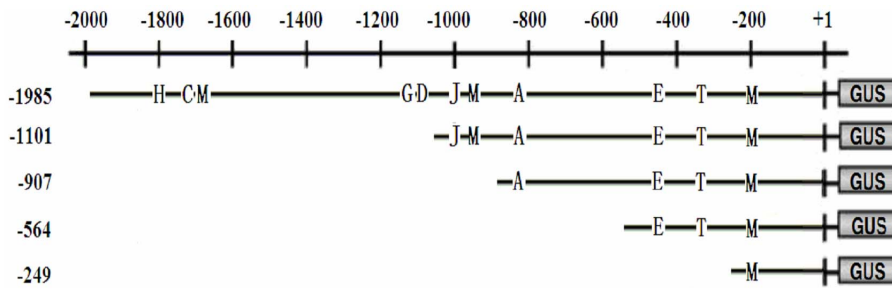
### Deletion Analysis of the *OsPIANK1* Promoter

Gene expression was largely controlled by its promoter. Therefore, deletion analysis of *OsPIANK1* promoter was carried out to dissect the possible molecular mechanism of *OsPIANK1* response to *M.oryzae*, SA, and JA. A 1985-bp upstream region of the *OsPIANK1* open reading frame was isolated from Nipponbare genomic DNA by PCR amplification and *cis*-acting elements in this region were analyzed (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). Several stress-related sequence motifs putatively acting as *cis*-elements were identified in the *OsPIANK1* promoter (Figure 2). These sequence motifs included two MYB binding sites (MBS) involved in response to drought; a *cis*-acting element involved in response to heat stress (HSE); a *cis*-acting element involved in abscisic acid responsiveness (ABRE); a *cis*-acting element involved in dehydration, low-temperature, and salt stresses (DRE); two ethylene-responsive elements (GCC box and ERE); two *cis*-acting regulatory element involved in the MeJA-responsiveness (JERE and the TCACG motif); and a *cis*-acting element (TCA-element) involved in SA responsiveness.

To analyze the activity of the *OsPIANK1* promoter in major organs of rice seedlings, the transgenic rice plants of *OsPIANK1* pro-



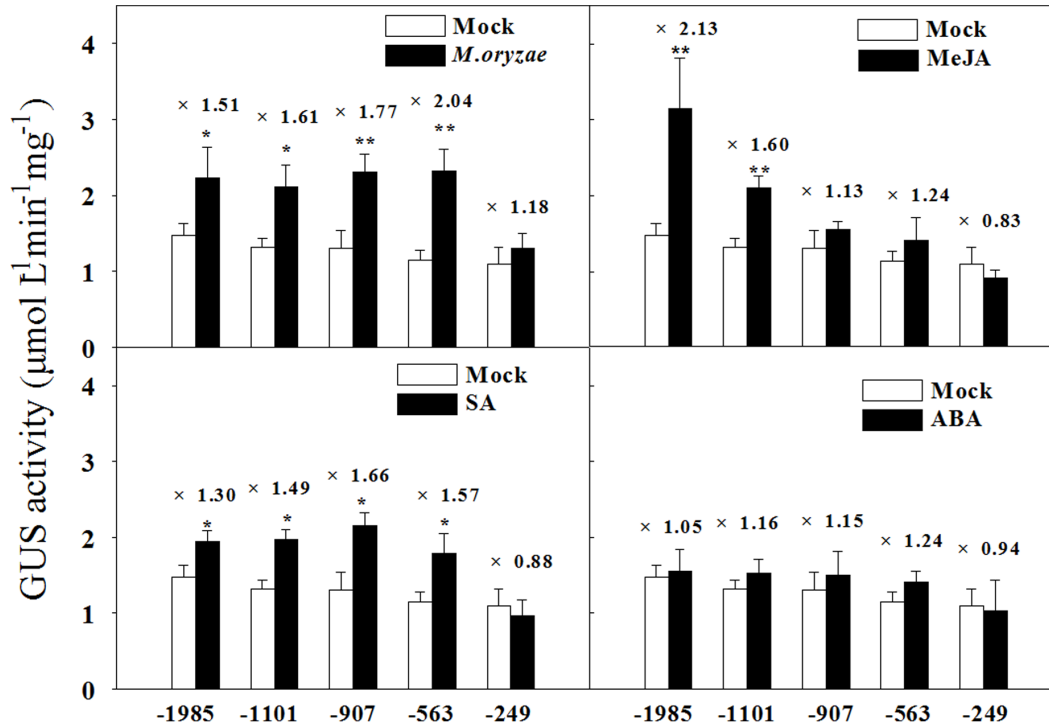
**Figure 3. The *OsPIANK1* promoter activities in different organs and its activation in response to *M.oryzae*.** (A) Histochemical analysis of GUS activity in different organs of *OsPIANK1*pro-*GUS* transgenic rice. (B) Histochemical analysis of GUS activity in *OsPIANK1*pro-*GUS* transgenic rice response to inoculation of *M.oryzae*. The leaves were harvested at 4, 5, and 6 days after inoculation. doi:10.1371/journal.pone.0059699.g003



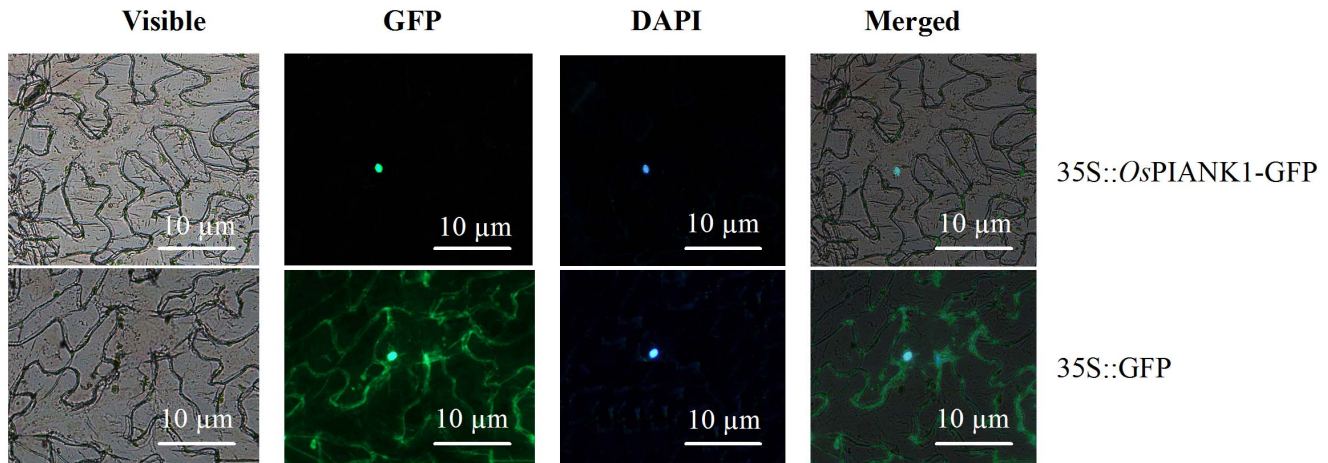
**Figure 4. Schematic representation of *OsPIANK1* promoter constructs for assaying GUS ( $\beta$ -glucuronidase) expression in transgenic rice leaves.** The serially 5' -deleted promoter constructs of the *OsPIANK1* gene were fused to the GUS reporter gene in the vector pCMDC163. H: HSE, *cis*-acting element involved in heat stress responsiveness. C: CGTCA-box, *cis*-acting regulatory element involved in the MeJA-responsiveness. J: JERE, *cis*-acting regulatory element involved in the MeJA-responsiveness. E: ERE, ethylene-responsive element. D: DRE, *cis*-acting element involved in dehydration, low-temp, salt stresses. M: MBS, MYB binding site involved in drought-inducibility. G: GCC-box, ethylene-responsive element. A: ABRE, *cis*-acting element involved in the abscisic acid responsiveness. T: TCA-element, *cis*-acting element involved in SA responsiveness.  
doi:10.1371/journal.pone.0059699.g004

$\beta$ -glucuronidase (*GUS*) and their corresponding T<sub>1</sub> lines were acquired. These lines were phenotypically normal and their growth was similar to wild-type plants. *GUS* expression was detected histochemically in leaves, stems, and roots of *OsPIANK1*-*pro-GUS* plants (Figure 3A). Next, the activation of the *OsPIANK1* promoter by pathogen infection was assessed. *OsPIANK1**pro-GUS* plants were inoculated with *M.oryzae* and the responses of *OsPIANK1* promoter were monitored by determining *GUS* activity. *GUS* staining was more intense in *M.oryzae*-inoculated leaves of *OsPIANK1**pro-GUS* rice plants 4 to 6 days after inoculation than in the uninoculated leaves (Figure 3B).

To perform 5' deletion analysis of the *OsPIANK1* promoter, serially 5'-deleted *OsPIANK1* promoters were created by PCR (Figure 4), cloned into the vector pMDC163 upstream of the *GUS* reporter gene, and transformed into rice via the *Agrobacterium*-mediated method. The transgenic rice plants and their corresponding T<sub>1</sub> lines were acquired and detected by PCR. A 5' deletion assay of the *OsPIANK1* promoter was performed with the transgenic plants. *GUS* expression in response to infection with *M.oryzae* or exogenous SA was similar for the following promoters: *OsPIANK1**pro*-1985 bp, *OsPIANK1**pro*-1101 bp, *OsPIANK1**pro*-907 bp, and *OsPIANK1**pro*-564 bp but different between these and *OsPIANK1**pro*-249 bp (Figure 5), suggesting that the *cis*-



**Figure 5. *OsPIANK1* promoter activation in response to inoculation with *M.oryzae* or exogenous application of MeJA, SA or ABA.** The leaves were harvested at 6 days after inoculation with *M.oryzae* and 12 h after treatment with the phytohormones. The numbers over the bars indicate the increase in induction of *GUS* activity compared to the mock. Data represent the means  $\pm$  SE from the leaf extracts collected from three experimental plant units. (\*\*, \*) indicate significant differences between the untreated (mock) and treated plants at  $P < 0.05$  and  $P < 0.01$ , respectively, as determined by the SNK test).  
doi:10.1371/journal.pone.0059699.g005



**Figure 6. Nuclear localization of *OsPIANK1* protein in *N. benthamiana* leaves.** *OsPIANK1*-GFP exclusively localized in the nucleus of cells in *N. benthamiana* leaves. Only the green fluorescent (GFP) localized throughout the whole cells. Cells were detected for GFP fluorescence by fluorescence microscopy 48 h after agroinfiltration.

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elements responsible for the response of *OsPIANK1* to *M.oryzae* infection and exogenous SA locate in the region between -564 bp and -249 bp. GUS expression in response to exogenous MeJA differed between the promoters *OsPIANK1pro*-1985 bp and *OsPIANK1pro*-1101 bp, as well as between *OsPIANK1pro*-1101 bp and *OsPIANK1pro*-907 bp (Figure 5). Thus, at least two *cis*-elements may be responsible for the response of *OsPIANK1* to exogenous MeJA. These may locate in the region between -1985 bp and -907 bp.

#### A Transiently Expressed *OsPIANK1*-GFP Fusion Protein is Localized in the Nucleus

The full-length cDNA of *OsPIANK1* was amplified with a specific primer pair of *OsPIANK1* by PCR with cDNA synthesized from the total RNA of rice leaves inoculated with *M.oryzae*. To determine the subcellular localization of *OsPIANK1*, the construct of a Cauliflower mosaic virus 35S-controlled *OsPIANK1*-green fluorescent protein chimera (CaMV35S::*OsPIANK1*-GFP) and the control vector (CaMV35S::GFP) were constructed, transformed into *Agrobacterium* strain GV3101, and infiltrated into *N. benthamiana* leaves. Localization of the *OsPIANK1*-GFP fusion protein was visualized exclusively in the nuclei (Figure 6).

#### *OsPIANK1* Overexpression Enhanced the Resistance of Rice to Infection by *M.oryzae*

We wished to characterize the function of *OsPIANK1* in rice against *M.oryzae* attack. First *OsPIANK1*-overexpression (OE) lines were generated. From these, three lines (lines 3#, 4#, and 6#) were chosen based on their high transcript levels of *OsPIANK1*. The function of *OsPIANK1* was studied in T<sub>2</sub> generations of those transgenic lines compared to WT rice plants. The expression of *OsPIANK1* in the three *OsPIANK1*-OE lines was higher than in WT plants (Figure 7A).

Three-week-old plants of *OsPIANK1*-OE transgenic and WT plants were inoculated with *M.oryzae* strain guy11 and evaluated for symptoms of rice blast 6 days later. The three *OsPIANK1*-OE lines had fewer and smaller expanding lesions (Figure 7B) with less fungus in the leaves (Figure 7C) than WT plants. *OsPIANK1*-OE and wild-type rice plants, both at the four-leaf stage, were inoculated with spores of *M.oryzae* strain guy11 and grown in a greenhouse. The *OsPIANK1*-OE and WT rice plants began to

wilt at 15 days after inoculation and some of the WT plants were dead at 30 days after inoculation. In contrast, all of *OsPIANK1*-OE rice plants survived with slight disease symptoms (Figure 7D). These results suggest that *OsPIANK1*-OE enhanced resistance to *M.oryzae*.

#### Overexpression of *OsPIANK1* Upregulated the Expression of Defense Marker Genes

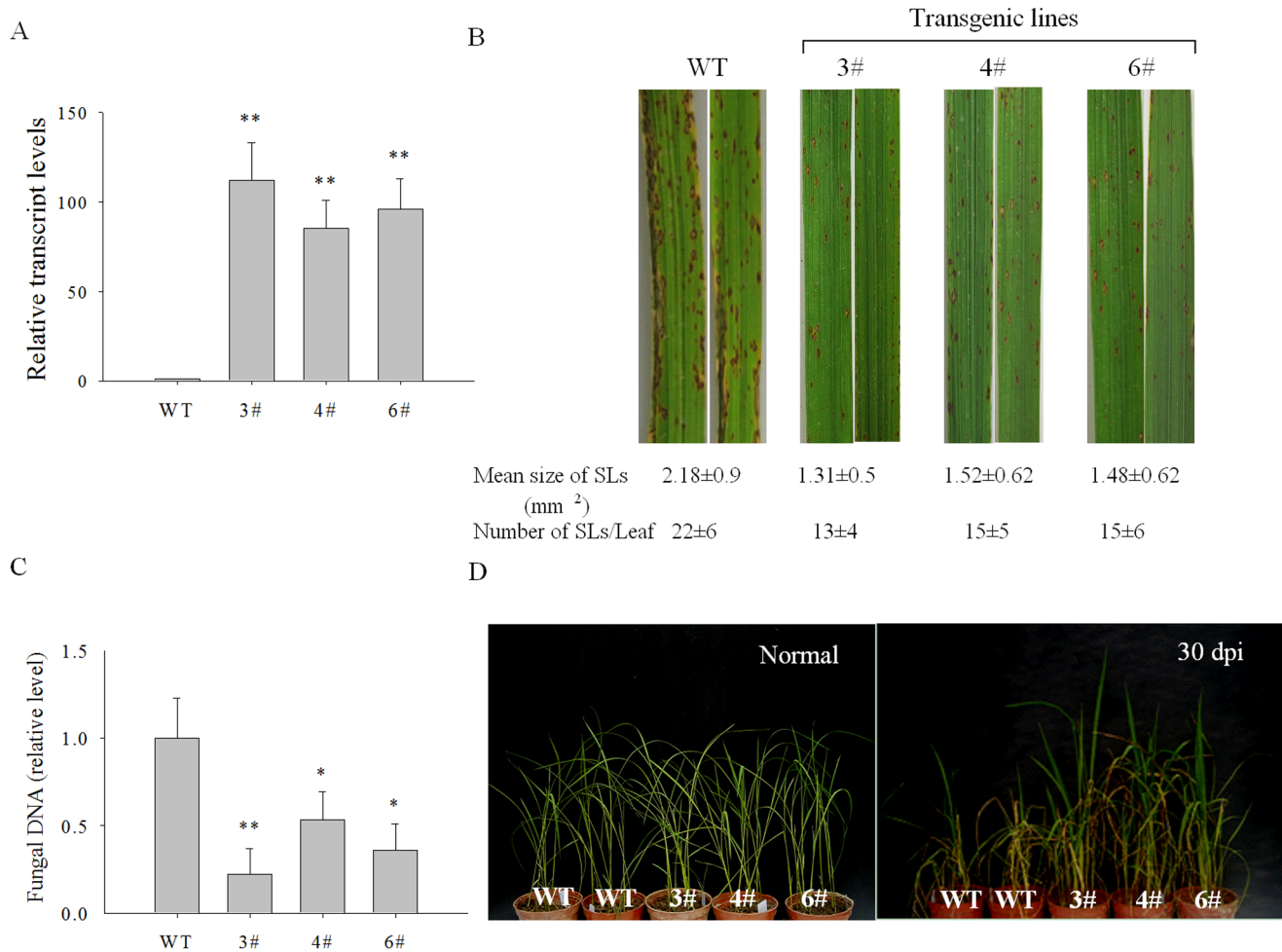
The mode of action of *OsPIANK1* in rice was studied by comparing the transcriptional expression of a set of pathogen-induced genes involved in defense in *OsPIANK1*-OE rice seedlings inoculated with *M.oryzae* strain guy11 and in un-inoculated controls. The genes included phenylalanine ammonia lyase (*PAL*; X87946), *Arabidopsis NPR1* homolog 1 (*NHI*; AY9123983), thaumatin-like protein (*PR5*; X68197), basic PR protein 1 (*PR1b*; U89895), *OsWRKY13* (EF143611), and allene oxide synthase 2 (*AOS2*; AY062258). The transcriptional expression of *PAL*, *NHI*, *PR1b*, and *AOS2* was enhanced by overexpression of *OsPIANK1* in both inoculated and control seedlings. Expression of *PR5* was transcriptionally enhanced only in the control seedlings while *WRKY13* was enhanced only in the inoculated seedlings (Figure 8).

#### Discussion

One hundred seventy-five *OsANK* proteins have been identified in rice, which have been classified into 10 subfamilies based on their domain compositions. Seventy-three (41.7%) belong to subfamily ANK-M, a classification based on the presence of the ANK domain in the absence of other known functional domains. Members of the other subfamilies contain not only the ANK domain but several other known functional domains [12]. The only distinguishing feature of the *OsPIANK1* protein is its three ANK domains; therefore, it may belong to the ANK-M subfamily. Little is known about the ANK-M rice proteins, and this report may be the first characterization of a protein in this subfamily.

Plant immune responses are accompanied by major transcriptional reprogramming and the genes involved appear to have important roles in the immune response [45–50,57]. *OsPIANK1* was upregulated following infection by the rice leaf blight pathogen *Xoo* [44], making it a likely player in the immune response of rice to pathogens. In the present study, *OsPIANK1* also





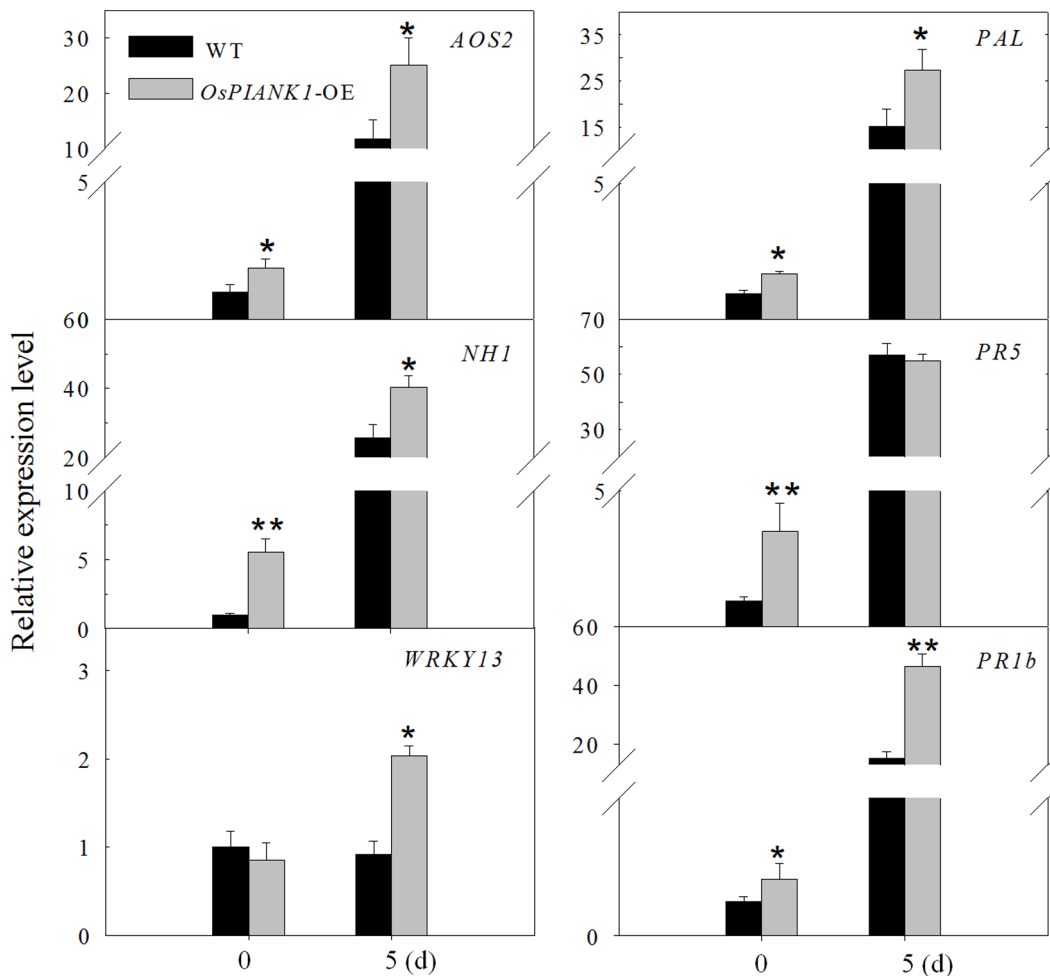
**Figure 7. Analysis of resistance to blast fungus in *OsPIANK1*-overexpressing (OE) rice plants.** (A) Quantitative PCR analysis of *OsPIANK1* expression in WT and OE plants (3#, 4#, and 6#). Data represent means  $\pm$  SE of three independent experiments. (B) Lesions in leaves at 6 days after inoculation. The number of expanding lesions (ELs) with an area greater than 0.5 mm<sup>2</sup> per leaf and their mean areas were determined using 10 leaves for WT (Nipponbare) and *OsPIANK1*-OE plants. Values represent means  $\pm$  SE. (C) The amount of *M. oryzae* DNA in the WT and *OsPIANK1*-OE rice leaves. The leaves were harvested at 6 days after inoculation. Values represent the mean  $\pm$  SE of three independent experiments. (D) Symptoms of rice blast in *OsPIANK1*-OE and WT rice plants grown in the greenhouse at 30 days after inoculation with spores of *M. oryzae* strain guy11. (\*\*,\* indicate significant differences between WT and *OsPIANK1*-OE plants at  $P < 0.01$  or  $P < 0.05$ , respectively, as determined by the SNK test.) doi:10.1371/journal.pone.0059699.g007

responded to *M. oryzae*, exogenous SA, and exogenous MeJA, suggesting that *OsPIANK1* is a regulator in basal defense or PTI of rice against different pathogens. Transcriptional expression of genes is largely regulated by *cis*-elements in their promoters. Some stress-related *cis*-elements: the CGTCA-box and JERE, involved in the MeJA-responsiveness [58,59]; the ethylene-responsive element [60]; the GCC-box [61]; the TCA-element, involved in SA responsiveness [62]; MBS, involved in drought responsiveness [63]; and HSE, involved in heat stress responsiveness [64] were found in the promoter of *OsPIANK1*. GUS expression controlled by the full-length promoter was enhanced by inoculation of *M. oryzae*, a finding that confirmed our finding that overexpression of *OsPIANK1* enhanced the resistance of rice to *M. oryzae*. The region responsible for *M. oryzae* infection and response to exogenous SA was between -563 bp and -249 bp, where only one ERE and one TCA-element was found, suggesting that this TCA-element was responsible for the response of *OsPIANK1* to *M. oryzae* infection and that this TCA-element mediated SA-signaling pathway is sufficient for the response of *OsPIANK1* to *M. oryzae* infection. This result is consistent with previous studies that SA is

important in modulation of the redox balance and protection of rice plants from the oxidative stress caused by infection of *Magnaporthe grisea* [65] or *Xanthomonas oryzae* [66]. The regions responsible for the MeJA response in the promoter of *OsPIANK1* located between -1985 bp and -907 bp, where the CGTCA-box, JERE, the GCC-box, and MBS were located. Among these *cis*-elements, JERE and CGTCA-box *cis*-element may be responsible for the response of *OsPIANK1* to exogenous application of MeJA. In addition, MBS may play a role in the response of *OsPIANK1* to exogenous application of MeJA. In support of this, a myb gene that may bind to MBS was induced by jasmonic acid [67].

The data of the present study also suggested that the *OsPIANK1* protein targeted to the nuclei and acted as a positive regulator in basal defense in rice. The latter conclusion was supported by the findings that inoculation of the WT, Nipponbare, with spores of *M. oryzae* strain guy11 caused a compatible reaction. Resistance to *M. oryzae* attack was greater in *OsPIANK1*-OE rice plants than in WT, as manifested by fewer, smaller lesions, less *M. oryzae*, and greater vigor at 30 days after inoculation. These results indicated





**Figure 8. Overexpression of *OsPIANK1* influenced the expression of a set of genes functioning in SA- and JA-associated pathways during disease resistance against *M.oryzae* strain *guy11* analyzed by quantitative real time-PCR.** Bars represent mean  $\pm$  SE of three biological replicates. (\*\*, \*) indicate significant differences between the *OsPIANK1*-OE and wild-type plants at the same time at  $P < 0.01$  or  $P < 0.05$ , respectively, as determined by the SNK test. doi:10.1371/journal.pone.0059699.g008

that *OsPIANK1* was a regulator of basal defense or PTI, although overexpression did not confer complete resistance.

The transcriptional expression of *NH1*, *WRKY13*, *PAL*, *AOS2*, *PR1b*, and *PR5* were consistently enhanced by the overexpression of *OsPIANK1*. Of these genes, *NH1*, *WRKY13*, and *PR5* are either SA-dependent or involved in the regulation of SA [68–70], and *PAL* is associated with SA biosynthesis and induced by SA application [71]. *AOS2* is involved in JA synthesis [72–74] and *PR1b* has been reported to be associated with both JA and SA signaling pathways [69]. These results strongly suggest that *OsPIANK1* primes the expression of SA- and JA-responsive endogenous genes. Although the activated immune responses in ETI are more prolonged and robust than those in PTI [75], differences observed between compatible and incompatible interactions are temporal and quantitative, not qualitative [47,48]. Common defense signaling molecules such as SA and JA are often common to both PTI and ETI [75], but the way they use the common network is very different: synergistic relationships among the signaling sectors are evident in PTI, compensatory relationships among the sectors are dominant in ETI [2]. Thus, the

co-upregulation of JA- and SA-dependent genes by the overexpression of *OsPIANK1* is consistent with the role of *OsPIANK1* as a regulator in PTI or basal defense.

*OsPIANK1* was late up-regulated by infection of rice plants with the leaf blight pathogen (*Xoo*) and had different expression patterns against the attack of *Xoo* in the leaf-blight resistant cultivar IET8585 and the leaf-blight susceptible cultivar IR24 [44], as would be predicted. In the present study, *OsPIANK1* was cloned from Nipponbare, a japonica rice cultivar susceptible to *M.oryzae* and *Xoo*. Therefore, its expression patterns in response to pathogen attack and its function in disease resistance might be different from its analogues in resistant cultivars. Characterization of analogues of *OsPIANK1* should supply new insight into its function and underlying mechanism in rice immunity.

### Author Contributions

Conceived and designed the experiments: SM SH. Performed the experiments: SM ZL. Analyzed the data: ZL DG AQ YL. Contributed reagents/materials/analysis tools: SH. Wrote the paper: SM SH.

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