A substitution mutation in OsPELOTA confers bacterial blight resistance by activating the salicylic acid pathway[®]

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Research Article

Abstract We previously reported a spotted-leaf mutant *pelota* (originally termed HM47) in rice displaying arrested growth and enhanced resistance to multiple races of *Xanthomonas oryzae* pv. *oryzae*. Here, we report the mapbased cloning of the causal gene *OsPELOTA* (originally termed *spl*^{HM47}). We identified a single base substitution from T to A at position 556 in the coding sequence of *OsPELOTA*, effectively mutating phenylalanine to isoleucine at position 186 in the translated protein sequence. Both functional complementation and over-expression could rescue the spotted-leaf phenotype. *OsPELOTA*, a paralogue to eukaryotic release factor 1 (eRF1), shows high sequence similarity to *Drosophila* Pelota and also localizes to the endoplasmic reticulum and plasma membrane. *OsPELOTA* is constitutively expressed in roots, leaves, sheaths, stems, and panicles. Elevated levels of salicylic acid and decreased level of jasmonate were detected in the *pelota* mutant. RNA-seq analysis confirmed that genes responding to salicylic acid were upregulated in the mutant. Our results indicate that the rice PELOTA protein is involved in bacterial leaf blight resistance by activating the salicylic acid metabolic pathway.

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INTRODUCTION

The hypersensitive response (HR) is a highly effective mechanism by which plants protect themselves from different pathogenic attacks. Once plants recognize an invading pathogen, they induce rapid cell death in the attacked areas, restricting further pathogenic invasion or spread to adjacent cells. Lesion mimic mutants (LMMs) characteristically form necrotic lesions similar to HR lesions in the absence of pathogenic infection and can be used to determine the mechanisms involved in plant disease resistance. Over the last decade, many LMMs have been reported in a variety of plant species, including *Arabidopsis* (Lorrain et al. 2003), barley

(Buschges et al. 1997), groundnut (Badigannavar et al. 2002), and rice (Wu et al. 2008).

In rice (*Oryza sativa* L.), LMMs are specifically termed *spotted-leaf* (*spl*) mutants, and more than 11 *spl* genes have been isolated. Among them, *spl7*, the first isolated *spl* gene, encodes a predicted heat stress transcription factor, the expression of which is induced by light and temperature (Yamanouchi et al. 2002). Other *spl* genes have a wide range of biological functions, encoding a diversity of proteins/enzymes such as CC-NBS-LRR-type proteins (Tang et al. 2011), zinc finger proteins (Wang et al. 2005), E3 ubiquitin ligase (Zeng et al. 2004; Liu et al. 2017), membrane-associated proteins (Zhou et al. 2009; Zhou et al. 2010), and a eukaryotic translation elongation

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factor (Zhao et al. 2017), as well as enzymes involved in biochemical pathways responsible for a salicylic acid (SA)-dependent defense response (Chern et al. 2005) and chlorophyll biosynthesis (Sun et al. 2011). Such a diverse collection of functionally different genes involved in lesion production suggests that complicated mechanisms underlie the initiation of lesion mimics.

The fruitfly pelota mutant was first identified in a screen for male-sterile mutants in Drosophila melanogaster (Castrillon et al. 1993). Subsequently, the Pelota locus was shown to encode a protein homologous to yeast Dom34, with wide evolutionary conservation (Eberhart and Wasserman 1995). Both Pelota and Dom34 are involved in the cell division cycle by influencing protein quality control in eukaryotes. Drosophila pelota mutants demonstrate spermatogenic arrest, female sterility, and disturbances in eye patterning. During Drosophila pelota spermatogenesis, the germline mitotic divisions are normal, but meiosis is blocked (Eberhart and Wasserman 1995). In mice, genetic ablation of Pelota is embryo lethal at the early implantation stage by impairing the development of the extraembryonic endoderm (Nyamsuren et al. 2014; Raju et al. 2015). Human Pelota promotes the degradation of messenger RNA (mRNA) lacking a stop codon and poly(A) tail (Ikeuchi et al. 2016). In tomato, silencing Pelota confers high resistance to tomato yellow leaf curl virus (TYLCV) (Lapidot et al. 2015). However, the role of Pelota in plants is still largely unknown.

In our previous study, a rice spotted-leaf mutant obtained from ethyl methanesulfonate (EMS) mutagenesis was characterized and the causal gene locus was narrowed down to a 74 kb region in chromosome 4 by map-based cloning (Feng et al. 2013). Here, we show that the rice PELOTA protein (OsPELOTA), encoded by *LOC_Oso4g56480*, controls the spotted-leaf phenotype. High-throughput mRNA sequencing (RNA-seq), plant hormone level determination, and gene functional analysis suggest that OsPELOTA mediates disease resistance to *Xanthomonas oryzae* pv. *oryzae* in rice by activating the SA metabolic pathway.

RESULTS

OsPELOTA complements the spotted-leaf phenotype

The OsPELOTA (originally spl^{HM47}) locus was previously mapped to a 74 kb region of rice chromosome 4 (Feng et al. 2013). To fine-map the gene, an additional 1,754 F₂ individuals with the spotted-leaf phenotype were genotyped and the OsPELOTA locus was further refined to a 20 kb region containing three open reading frames (ORFs) (Figure 1A). After sequencing all three ORFs in this region, it was found that only one nonsynonymous mutation (T556A, F186I) was detected in the *pelota LOC_Oso4g56480*. The other two ORFs (*LO-C_Oso4g56470* and *LOC_Oso4g56490*) did not have any mutations compared to their respective wild-type loci (Figure 1B). The 7,427 bp *LOC_Oso4g56480* contains 15 exons, 14 introns, and was considered the most likely candidate gene responsible for the *pelota* spotted-leaf phenotype based on these results.

To validate the candidate gene, the complementary vector pC1300-C was introduced into embryogenic calli of pelota mutants, via Agrobacterium tumefaciensmediated transformation. A total of 14 positive transformants were obtained and all exhibited a normal green leaf color without lesions throughout their entire growth period. These results indicate that LO-C Oso4g56480 could rescue the spotted-leaf phenotype and was indeed the target gene (Figure 2A-C). Furthermore, *pelota* shows a high level of H_2O_2 accumulation at/around leaf lesions with high malonaldehyde (MDA) content and low catalase (CAT) activity compared to the wild-type IR64 (Feng et al. 2013). We measured the content of MDA and the activity of CAT in the complemented plants to assess whether they were restored to wild-type levels. The MDA content in both the complementation lines and the wild-type IR64 plants were similar and significantly lower than the MDA content in pelota plants. While the CAT activity in complementation lines was significantly higher than that of pelota, they still did not fully recover to the level of the wild-type IR64 (Figure 2D). Taken together, these results demonstrate that LOC Os04g56480 is the target gene that controls the spotted-leaf phenotype for OSPELOTA.

OsPELOTA has three different transcripts

When we tried to isolate the coding sequence of *OsPELOTA*, we identified three alternative splicing isoforms, which we termed transcripts 1, 2, and 3 (Figure 3). Transcript 1 was the full-length coding sequence (CDS) in *pelota* and the mutation occurred at position 556 (T/A) in exon 8 (Figure 3). For transcript 2, both the seventh and eighth introns were not spliced.

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Figure 1. Map-based cloning of OsPELOTA

(A) OsPELOTA locus is further refined to a 20 kb region between InDel marker YY84 and YY91. (B) Single base substitution at position 556 (T556A, F186I).

Instead, intron 7 was fully translated as indicated by a red box in transcript 2 (Figure 3). Similarly, the eighth intron in transcript 3 was not excised and a part of intron 8 was translated into the protein. To test the function of these isoforms, all three transcripts were over-expressed using the maize ubiquitin promoter in the mutant pelota, via Agrobacterium tumefaciensmediated transformation. Only the transgenic plants carrying transcript 1 restored the wild-type phenotype, although the expression levels of transcript 1 were different among various independent transgenic lines (Figure 4). Transformants carrying the other two transcripts exhibited the spotted-leaf phenotype similar to the pelota mutant (data not shown). These results indicate that only transcript 1 could rescue the mutant phenotype.

OsPELOTA is highly conserved in eukaryotic organisms All eukaryotes contain a eukaryotic release factor 1 (eRF1) paralog, which is termed Pelota in *Drosophila* and Dom34 in yeast. Sequence analysis (http://www.ncbi. nlm.nih.gov) indicated that *OsPELOTA* encodes a paralogue of eRF1 consisting of three domains: the N domain that recognizes stop codons at the A site of the ribosome (Ma and Nussinov 2004), the M domain that mimics the acceptor stem of transfer RNA (tRNA) and contains a conserved GGQ motif essential for peptidyltRNA hydrolysis at the peptidyl transferase center (Bulygin et al. 2010), and the C domain that recognizes tRNA T Ψ C stems. We found OsPELOTA to be structurally composed of three domains, which we named eRF1_1, eRF1_2, and eRF1-3. The eRF1_2 and eRF1_3 domains were structurally analogous to the M and C domains, except that the eRF1_2 domain did not contain the critical GGQ motif. However, the eRF1_1 domain was different from the N domain and lacked the RNAbinding Sm fold (Lee et al. 2007; Graille et al. 2008).

According to our sequence alignment results, OsPELOTA was most comparable to monocot homologues such as ObPELOTA (90%) and ZmPelota1 (87%). OsPELOTA also had a high level of similarity to the dicot AtPELOTA1 (74%), and lesser similarity to the insect DmPelota (54%) and the yeast Dom34 (31%) (Figure 5). Notably, the amino acid mutation (F1861) in rice *pelota* was within domain eRF1_2, and the corresponding site was conserved in different species, especially in the plant kingdom (F186 in plants and Y186 in animals and yeast). These results indicate that OsPELOTA is

OsPELOTA is involved in rice disease resistance





(A) Complementation construct pC1300-C. (B) Phenotype of IR64, *pelota* and complementation transgenic line (Com-*PELOTA*). (C) Sequencing analysis of complementation transgenic lines. Arrowhead indicates the mutation site. (D) The contents of malonaldehyde (MDA) and activities of catalase (CAT) in the wild-type, *pelota* and complementation lines.



Figure 3. Transcripts of OsPELOTA (LOC_Os04g56480)

Grey, white, and red boxes indicate functional coding sequence, untranslated region (UTR), and shift open reading frames (ORF), respectively. The arrow shows the position at 556 bp where the point mutation occurs.





Figure 4. Over-expression of the wild-type OsPELOTA transcript 1 in *pelota*

(A) Wild-type. (B) *pelota*. (C–G) Different overexpression lines of *OsPELOTA* transcript 1. (H) *OsPELOTA* expression levels of wild-type and over-expression lines. OV-1, OV-2, OV-3, OV-4 and OV-5 represent five independent *OsPELOTA* transcript 1 over-expression transgenic lines in the *pelota* mutant background. Rice ubiquitin gene was amplified as internal standard.

structurally conserved and that the F186I mutation is likely critical to PELOTA function.

OsPELOTA is expressed constitutively and OsPELOTA-GFP localizes to both the endoplasmic reticulum and plasma membrane

To clarify the expression pattern of *OsPELOTA*, we performed quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis on the total RNA of roots, stems, nodes, internodes, leaves, leaf sheaths, panicles, and filling grains from the wild-type IR64. *OsPELOTA* was expressed in all the organs tested. The highest expression level occurred in the filling grains, followed by flag leaves, roots, and panicles at the grain filling stage. In other organs, it was expressed at a lower level, but these results indicate *OsPELOTA* is a constitutively expressed gene (Figure 6).

Having determined the tissue expression pattern of *OsPELOTA*, we carried out polyethylene glycol (PEG)mediated transient transformation of rice protoplasts to clarify the distribution of OsPELOTA-GFP (green fluorescence protein) in rice cells. When pAN580 and pOsPELOTA-GFP were introduced into rice protoplasts, the distribution of OsPELOTA-GFP was more pronounced in the plasma membrane and endoplasmic reticulum (ER) compared to cytosol-localized GFP (Figure 7). As OsRacGEF1-CFP predominantly localizes to the ER and plasma membrane (Akamatsu et al. 2013), we confirmed the subcellular location of OsPELOTA-GFP by expressing it with pGEF-RFP (Red fluorescence protein fused to the C-terminal of OsRacGEF1), finding that their subcellular localization patterns are similar. Thus, we concluded that OsPELOTA primarily localizes to the ER and plasma membrane.

Activation of the SA response causes disease resistance to bacterial leaf blight pathogen in *pelota*

Our previous studies have shown that rice *pelota* is broadly resistant to *Xanthomonas oryzae* pv. *oryzae* (Xoo), the causal pathogen of bacterial leaf blight in rice (Feng et al. 2013). Although resistance to Xoo is highly associated with the spotted-leaf phenotype and a couple of defense genes were found to be upregulated in *pelota* (Feng et al. 2013), the resistance mechanism was still unclear.

To further explore the potential resistance mechanism, we carried out high-throughput RNA sequencing (RNA-seq) analysis. The complementary DNA (cDNA) libraries were prepared from the leaves of 23-day-old BC_1F_2 *pelota* mutant and wild-type IR64 plants. A total of 1,460 differentially expressed genes (DEGs) were detected between *pelota* and IR64. Among these, 801 genes were up-regulated and 659 genes were down-regulated in the *pelota* mutant. To verify the reliability of the sequencing results, 10 genes were randomly selected and analyzed Α



Figure 5. Sequence alignment and phylogenetic analysis of OsPELOTA with its homologues

(A) OsPELOTA shares high identity with its homologues, shown by the amino acids highlighted in black. (B) Phylogenetic analysis of PELOTA proteins from different organisms. Phylogenetic tree was constructed by neighbor-joining method using the MEGA program. Bootstrap values from 100 replicates are indicated at each node.

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Figure 6. Quantitative polymerase chain reaction (qPCR) analysis of wild-type OsPELOTA messenger RNA expression

Relative expression levels of OsPELOTA in various rice tissues at different growth stages. Means with different letters are significantly different at $P \le 0.01$ using Duncan's test.

using qRT-PCR. The expression levels of nine out of these 10 genes were the same in both methods, validating the RNA-seq data (Figure S1 and Table S2).

Gene Ontology (GO) analysis was used to classify the functions of the DEGs identified in *pelota*. From the GO term enrichment results, there were a total of 20 GO terms in biological processes with $P \le 0.01$ (Table S3). Among them, "response to chitin", "defense response to fungus" and "response to salicylic acid" were the most highly enriched terms with *P*-values of 1.18E-09, 4.98E-05, and 1.28E-04, respectively. More importantly, a total of 19 genes were enriched in the GO term "response to salicylic acid", with 13 genes up-regulated and six genes down-regulated (Table S4). Among the 13 up-regulated genes, there were eight lectin-like receptor kinase genes and 1 receptor like kinase. Three of the

six downregulated genes were WRKY genes (WRKY28, WRKY71, and WRKY76) (Table S4). These results indicate that the salicylic acid-mediated defense pathway was constitutively activated in *pelota*.

To elucidate the changes in endogenous plant hormones, including SA, leaf samples from the *pelota* mutant and the wild-type IR64 were tested for their hormone levels. For the sake of comparison, we set the content of various endogenous hormones in the wildtype IR64 to 1, providing a relative measurement for endogenous hormone in the *pelota* mutants (Figure 8). The results show the SA content in *pelota* increased by 68.7% ($P \le 0.01$), whereas the levels of abscisic acid (ABA) and jasmonate (MeJA) decreased by 38.3% and 47.1%, respectively. This indicates that the broadspectrum resistance of *pelota* was probably generated

Bar represents the number of amino acid differences per site. All PELOTA proteins used and their GenBank accessions are as follows: Oryza sativa OsPELOTA (XP_015637164.1), Oryza brachyantha ObPELOTA (XP_006652952.1), Sorghum bicolor SbPELOTA (XP_021319384.1), Zea mays ZmPELOTA (NP_001338038.1), Arabidopsis thaliana AtPELOTA (NP_194495.3), Medicago truncatula MtPELOTA (XP_013468922.1), Drosophila melanogaster DmPELOTA (NP_476982.1), Danio rerio DrPELOTA (NP_957430.1), Homo sapiens HsPELOTA (NP 057030.3) and Saccharomyces cerevisiae Dom34 (NP 014397.1).



Figure 7. Subcellular location of OsPELOTA-GFP in rice protoplasts

Upper panel: OsPELOTA-GFP (green fluorescence protein) and wild-type GFP are separately transformed into rice protoplasts; Lower panel: OsPELOTA-GFP and GEF-RFP are co-localized at endoplasmic reticulum (ER) and plasma membrane.

from the activation of the SA-mediated defense pathway.

DISCUSSION

In the present study, we demonstrate that the spottedleaf phenotype of rice *pelota* is caused by a single nucleotide replacement (T556A) in the *OsPELOTA* gene (accession number MF457479). Both complementary assays and over-expression analysis clearly show that the expression of the *OsPELOTA* gene in *pelota* could restore a wild-type phenotype.

The amino acid sequence alignment reveals Pelota proteins are conserved in eukaryotes (Figure 5), but their functions may differ. In *Saccharomyces cerevisiae*, there are no obvious differences in the growth rates between the dom 34Δ strain and its wild-type counterpart under regular growth temperatures. However, the growth of the dom 34Δ strain is particularly slow at low temperatures, due to a reduced ribosome supply during

the transition out of stationary phase (Davis and Engebrecht 1998). Unlike Dom34 in yeast, mutations in Pelota proteins often lead to severe problems in animals, such as male infertility, female sterility, spermatogenic arrest in Drosophila (Eberhart and Wasserman 1995), and cell cycle arrest at the G2/M transition and embryonic death in mice (Nyamsuren et al. 2014; Raju et al. 2015). Currently, knowledge of Pelota's role in plants is still very limited. Only one report indicated that silencing Pelota in Solanum lycopersicum could lead to high TYLCV-resistance and significant reduction in fruit size (Lapidot et al. 2015). We have previously shown that rice pelota exhibits poor performance in agronomic traits, including plant height, number of tillers, panicle length, seed-setting, and 1,000-grain weight (Feng et al. 2013). Here, we reveal that a single nucleotide substitution mutation in OsPELOTA causes the spotted-leaf phenotype and enhances broad-spectrum disease resistance to Xoo. Our research shows that a loss of function in plant PELOTA protein can cause multiple effects, including



Figure 8. Hormone levels in rice leaves of *pelota* and the wild-type IR64

IAA, indole acetic acid; GA3, gibberellin A3; ABA, abscisic acid; MeJA, jasmonate; SA, salicylic acid. Single asterisk denotes $P \le 0.05$, and double asterisks denote *P*-value ≤ 0.01 (Student's *t*-test).

growth arrest, spotted-leaf phenotype, and enhanced resistance to bacterial leaf blight pathogens.

The nuclear localization signal sequence at the animal Pelota N-terminus is perfectly conserved from Drosophila to human beings, raising an interesting possibility that Pelota is a nucleus-localized protein. However, further research shows that Drosophila Pelota localizes to the cytoplasm (Xi et al. 2005) and human Pelota localizes to the cytoskeleton, at certain regions of plasma membranes, and at the site close to nuclear membranes (presumably to the endoplasmic reticulum) (Burnicka-Turek et al. 2010). However, murine Pelota is localized in the cytoskeleton and membrane-fractions, but not in nuclear and cytoplasmic fractions (Buyandelger and Mongolei 2007). Until now, the subcellular location of plant PELOTA has been unclear. Here, we show that OsPELOTA-GFP mainly localizes to the endoplasmic reticulum and plasma membrane in rice (Figure 7). In general, animal and plant PELOTA proteins share the same subcellular localization pattern, although the animal Pelota contains an additional nuclear-localized sequence at its N terminus.

A higher level of endogenous SA likely partially accounts for the broad-spectrum disease resistance in *pelota* plants. After pathogen infection, endogenous levels of SA increase significantly, preceding the induction of pathogenesis-related genes and the onset of disease resistance (Prithiviraj et al. 2005; An and Mou 2011; Yang et al. 2016). Similarly, overproduction of endogenous SA in transgenic tobacco and Arabidopsis enhances disease resistance (Verberne et al. 2000). By contrast, SA-deficient rice exhibits increased susceptibility to avirulent *Magnaporthe oryzae* (Yang et al. 2004).

In the present study, the endogenous SA level in pelota was 1.87-fold higher than that of wild-type plants. Furthermore, consistent with the higher endogenous SA content, multiple genes involved in response to SA were activated in the *pelota* mutant (Table S4), supporting the notion that resistance to Xoo is enhanced, via the SA-mediated defense pathway. Notably, a set of lectin receptor kinase genes are upregulated in pelota, and lectin receptor kinases have been shown to participate in plant innate immunity (Singh and Zimmerli 2013). For example, Pi-d2, a rice lectin receptor kinase, provides resistance against the fungal pathogen Magnaporthe oryzae (Chen et al. 2006b). In addition, the expression of WRKY28, WRKY71, and WRKY76 were all down-regulated in pelota. However, overexpression of OsWRKY28 and OsWRKY76 in rice plants results in drastically increased susceptibility to Magnaporthe oryzae (Chujo et al. 2013; Yokotani et al. 2013), while overexpression of OsWRKY71 enhances resistance to virulent Xoo strain 13751 (Liu et al. 2007). These results indicate that WRKY71 plays vital roles to modulate the innate immunity in rice against Xoo and highlight that further functional characterization of WRKY71 is needed. Nevertheless, the upregulation of the lectin receptor kinase genes and down-regulation of WRKY genes are consistent with the elevated resistance to Xoo in the mutant.

The interactions between SA and JA signaling appear to be complex, but their antagonistic relationship is well accepted (Kunkel and Brooks 2002; He et al. 2017). ABA treatment inhibits both upstream and downstream signaling in the SA-mediated defense pathway, independently of the jasmonic acid (JA)/ ethylene-mediated signaling pathway (Yasuda et al. 2008). Similarly, we detected a decreased level of endogenous MeJA and ABA in *pelota*. Considering the key role of SA in plant disease resistance, we conclude that the rice *pelota* mutant confers enhanced bacterial leaf blight resistance through the activation of the SA metabolic pathway.

Although the rice *pelota* exhibits a higher level of resistance to Xoo by activating the SA-mediated

defense pathway, the molecular mechanism remains unknown. We are currently carrying out yeast twohybrid screening to identify protein interactions with PELOTA. In any event, the present study provides a starting point to further elucidate OsPELOTA-mediated disease resistance in rice.

MATERIALS AND METHODS

Rice material

In this study, the spotted-leaf mutant *pelota* was obtained by EMS mutagenesis of *indica* rice (*Oryza sativa* L.) cultivar IR64. The rice *pelota* shows impaired growth, spotted-leaf, and broad-spectrum resistance to *Xanthomonas oryzae pv. oryzae* (Feng et al. 2013).

Seedlings of *pelota* and wild-type IR64 were planted in pots in a greenhouse at the China National Rice Research Institute. Twenty-three days after germination, rice leaves were collected for high-throughput RNA-Seq and determination of plant hormone levels.

Extraction and determination of hormone levels

The levels of MeJA, GA3, SA, IAA, and ABA were determined by Zoonbio Biotechnology Co., Ltd, Nanjing, China, and the methods were modified from those described by You et al. (2016). Approximately 1.0 g leaf samples were ground in a pre-cooled mortar that contained 10 mL extraction buffer (isopropanol:water: hydrochloric acid, 2:1:0.002 v/v). The extract was shaken at 4°C for 30 min. Then, 20 mL dichloromethane was added and the sample was shaken at 4°C for 30 min and then centrifuged at $16,020 \times g$ for 5 min. The organic phase was extracted and dried under liquid nitrogen. The pellets were dissolved in $150 \,\mu\text{L}$ methanol (0.1% methane acid) and filtered with a $0.22 \,\mu$ m filter membrane. The purified product was then subjected to high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis. The injection volume was 2 μ L. Mass spectometry conditions were as follows: the spray voltage was 4,500 V; the pressure of the air curtain, nebulizer, and aux gas were 15, 65, and 70 psi, respectively, and the atomizing temperature was 400°C.

Complementation test and over-expression analysis

To construct a plasmid for the complementation test, an 11.3 kb DNA fragment from the wild-type IR64 containing 2,200 bp upstream of the OsPELOTA transcription start site, the full-length OsPELOTA genomic DNA, and 1,500 bp downstream of the OsPELOTA termination site was amplified by LA taq polymerase (Takara *Inc.*) and cloned into the binary OsPELOTA vector pCAMBIA1300 to generate the construct pC1300-C. The primer set 47com was used to amplify the 11.3 kb OsPELOTA (Table S1).

To determine which transcript could restore the phenotype of the *pelota* mutant, 3 *OsPELOTA* transcripts with different ORF sizes were amplified and inserted into the rice overexpression plasmid pCAMBIA1300-Ubi-NOS (Huang et al. 2016) between the maize *ubiquitin* promoter and NOS terminator. The primers for each of the transcripts are listed in Table S1.

All the above constructs were used to transform embryogenic calli induced from *pelota* seeds using the *Agrobacterium tumefaciens*-mediated method (Hiei and Komari 2008).

Subcellular location and qPCR analysis

The full-length coding sequence (no stop codon) of OsPELOTA and OsRacGEF were amplified using the primers 47GFP F/R and OsRacGEF F/R (Table S1). The PCR product of OsPELOTA was fused to the GFP N-terminus and driven by the CaMV 35S promoter in the transient expression vector PAN580 to form a new construct: pOsPELOTA-GFP. The CDS of OsRacGEF was placed upstream of mRFP in the plasmid pB-mRFP (pBluescript II SK-35S-mRFP-NOS) to generate a new construct termed pGEF-RFP. Ten micrograms of pAN580, 10 µg of pOsPELOTA-GFP, or the mixture of 5 µg pGEF-RFP and 5 µg pOsPELOTA-GFP were introduced into the protoplasts generated from the IR64 seedlings according to the protocol described previously (Chen et al. 2006a). The GFP fluorescence was observed 48 h after transformation by a Zeiss Ism710 confocal laser scanning microscope (Carl Zeiss, Inc., Jena, Germany).

Total RNA was extracted from various plant tissues using TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The genomic DNA removal and first strand cDNA synthesis were carried out using the ReverTra Ace qPCR Master Mix kit (Toyobo, Tokyo, Japan). SYBR[®] Premix Ex TaqTM II (Tli RNaseH Plus) Kit was chosen for qRT-PCR analysis and performed on a Thermal Cycle Dice[®] Real Time System (TaKaRa, Dalian, China). A *ubiquitin* gene was used as an internal control. Primers used for qRT-PCR are listed in Table S1. All assays were repeated at least three times and the means were used for analysis.

High-throughput mRNA sequencing (RNA-seq) analysis

To eliminate other possible mutations, pelota was backcrossed to the wild-type IR64. The F₁ plants were self-pollinated to generate BC_1F_2 and BC_1F_3 . Three individual BC1F3 lines and three wild-type IR64 plants were chosen for isolation of total RNA. A total of six RNA samples were used for RNA-seq analyses. Library construction and approximately 6 Gb deep sequencing were carried out using the Illumina HiSeq 3000 platform (Illumina, 345 San Diego, CA, USA) following the manufacturer's instructions by Genergy 346 Biotechnology Co. Ltd. (Shanghai, China). The aligned read files were processed by Cufflinks. The unit of measurement is fragments per kilobase of exon per million fragments mapped (FPKM). DEGs between three biological replicates of either *pelota* and IR64 were identified using the Empirical Analysis of Digital Gene Expression data package in Cuffdiff (ver.2.1.1). An absolute fold change of >2 and a false discovery rate significance score of \leq 0.05 were used as thresholds to identify significant differences in gene expression. Each DEG was annotated, initially according to the top Oryza sativa annotation hit and then by GO enrichment analysis using GO Slim (http:// www.geneontology.org), which assigned each DEG to one of three principal GO categories: molecular function, cellular component, and biological process.

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AUTHOR CONTRIBUTIONS

X.Z. and J.W. conceived and designed the research, X.Z., B.F., H.W. and Y.H. performed the experiments, X.X., C.Z., A.P. and Y.S. carried out the data analysis, X.Z. and J.W. wrote and revised the manuscript. All authors read and approved the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article: http:// onlinelibrary.wiley.com/doi/10.1111/jipb.12613/suppinfo **Figure S1.** qRT-PCR validation of 10 genes expressed in IR64 and *pelota*

Actin1 was used as an internal control. One asterisk denotes p < 0.05, and double asterisks denote P-value < 0.01 (Student's t-test).

Table S1. The primer pairs used in this study

Table S2. The RNA-seq data of 10 randomly selectedgenes expressed in IR64 and *pelota*

Table S3. Enrichment of gene ontology in terms of biological processes with *P*-value \leq 0.01

Table S4. Different expression genes between IR64 and *pelota* plants in the Gene Ontology term: response to salicylic acid stimulus



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