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Identification, Expression and Functional Analysis of a Receptor-like Cytoplasmic Kinase, OsRLCK1, in Rice

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Abstract

Pollination involves a series of complex cellular interactions and signal transduction events. Numerous reports have suggested a central role for protein kinases in pollen germination and pollen tube growth and a large number of receptor-like kinases have been detected exclusively in pollen in higher plants. However, few are well characterized, especially for the receptor-like cytoplasmic kinases. Here we report a receptor-like kinase gene, *OsRLCK1*, which belongs to the receptor-like cytoplasmic kinase VIII subfamily. Real-time quantitative polymerase chain reaction analysis and whole mount RNA *in situ* hybridization showed that *OsRLCK1* is a pollen-specific gene and expressed only in the mature pollen. When expressed in the onion epidermal cells, the OsRLCK1-GFP fusion protein was diffused throughout the cell, indicating its cytoplasmic and nuclear localization. The Maltose Binding Protein-OsRLCK1 recombinant protein was found to be capable of autophosphorylation on threonine residue, showing that it encodes a functional kinase. These results suggest that *OsRLCK1* is likely to play a role in a signaling pathway associated with pollen performance during pollination in rice.

Key words: pollination; receptor-like cytoplasmic kinase; rice (*Oryza sativa*).

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Protein phosphorylation is a key mechanism for intracellular signal transduction in both eukaryotic and prokaryotic cells. Because plants are sessile and cannot move to avoid biotic attack or abiotic stress, plant cells often use receptors at the cell surface to sense environmental changes, and then transduce this information via activated signaling pathways to trigger adequate responses (Morris and Walker 2003). Unlike only a limited number of this kinase family members contained in Plasmodium and animals, receptor-like kinases (RLKs, defined as plant receptor kinases resembling animal receptor tyrosine kinases) comprise a major gene family in plants and control a wide range of biological processes, including development, disease resistance, hormone perception and self-incompatibility. The *Arabidopsis* genome contains about 610 RLKs, representing nearly 2.5% of the annotated protein-coding genes in *Arabidopsis*; and more than 1 100 RLKs were annotated in rice genomes. RLK family members vary greatly in their domain organization and the sequence identity of their extracellular domains. Among them, the majority (75% in *Arabidopsis*) have a signal sequence (in amino-terminal extracellular domains), a transmembrane domain, and a carboxyl-terminal kinase domain; and the rest, defined as receptor-like cytoplasmic kinases (RLCKs), have a carboxyl-terminal kinase domain but no apparent signal sequence or transmembrane domain. Although a huge number of RLKs are contained in the plant genome, the function of most of the RLKs are still unknown, especially for the RLCKs (Shiu and Bleecker 2001a, 2001b, 2003; Shiu et al.

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2004; Morillo and Tax 2006). To date, only a few RLCKs have been characterized, for example, *Brassica* (*Brassica rapa*) M-locus protein kinase MLPK in self-incompatibility (Murase et al. 2004), tobacco (*Nicotiana tabacum*) NtPK1 and NtPK2 involving pollen germination and pollen tube elongation (Kumara et al. 2004), tomato (*Lycopersicon esculentum*) Pto and Pti1 in disease resistance (Zhou et al. 1995), soybean (*Glycine max* L.), GmPti1 in coordinating plant defense responses (Tian et al. 2004), *Arabidopsis* (*Arabidopsis thaliana*) CDG1 related to BR signaling (Muto et al. 2004), maize (*Zea mays*) ZmPti1a in pollen callose deposition, and ZmPti1b, a putative LePti1 ortholog, in pathogen defense (Herrmann et al. 2006). However, no RLCKs have been characterized in rice, the most important monocot model plant.

Pollination is one of the most important steps in the reproductive process in higher plants. Successful pollination is owing to a series of crucial events, such as pollen adhesion, hydration, pollen tube growth and guidance to the ovules; and this process involves a series of complex cellular interactions that culminates in the fusion between male and female gametes (Wheeler et al. 2001; Lord and Russell 2002). A large number of receptor-like kinases have been detected exclusively in pollen, and numerous reports have suggested a central role for protein kinases in pollen germination and pollination (Franklin-Tong and Franklin 2003; Honys and Twell 2003; Dai et al. 2006, 2007). However, our knowledge about signaling events during pollination is still fragmented, and almost non-existent in rice. We report here that *OsRLCK1* expressed exclusively in mature pollen encodes a novel functional receptor-like cytoplasmic serine/threonine and is likely to be involved in pollination in rice.

Results

Identification and structural feature of OsRLCK1 protein

Previously, we constructed a 10K cDNA microarray (http://plantbiol.genetics.ac.cn) that was used to monitor gene expression profiles during pollination in rice (Lan et al. 2004). An anther-specific cDNA *p768b08* (accession number: CR283882 and CR283761) encoding a putative receptor-like kinase was selected for detailed functional analyses. A homology search in GenBank (http://www.ncbi.nlm.nih.gov) revealed that the gene consists of eight exons and seven introns, the proposed translation start is located in exon 2 (Figure 1A) and the predicted open reading frame (ORF) encodes a 364 amino acid peptide with a calculated molecular mass of 39.7 kDa. SMART analysis showed that the putative protein harbors a cytoplasmic catalytic domain of RLKs, but lacks the extracellular receptor domains and the transmembrane domain, indicating that it may be a RLCK. Further BLAST comparison indicated that the putative protein has a higher homology to plant RLCKs including NtPK1 and NtPK2 (Kumara et al. 2004), GmPti1 (Tian et al. 2004), LePti1 (Zhou et al. 1995) and ZmPti kinases (Herrmann et al. 2006). For example, OsRLCK1 has the highest overall homology and identity with At2g41970 in *Arabidopsis* (Honys and Twell 2003), 82% and 70% respectively. And the overall homology (identity) is 81% (70%) with NtPK1 and 77% (65%) with ZmPti1a. Interestingly, based on the PlantsP Kinase classification (Shiu and Bleecker 2003), these proteins all belong to the receptor-like cytoplasmic kinase VIII (RLCK VIII) subfamily, which has a common monophyletic origin with receptor-like kinases but has no apparent signal sequence or transmembrane domain (http://plantsp.sdsc.edu/plantsp/family/class.html). In addition, the protein has a lower homology to plant RLCKs of the RLCK VII subfamily, such as Pto, MLPK and CDG1 (data not shown). So far, to our knowledge, there has been no report about functional studies on RLCKs in rice. So, we designated it OsRLCK1.

Furthermore, we conducted alignment of OsRLCK1 with the above-mentioned RLCKs of the RLCK VIII subfamily. The result showed that they have conserved kinase domains with all 11 subdomains of eukaryotic protein kinases and all the invariant amino acid residues are conserved in their proper positions (Hanks and Hunter 1995) (Figure 1B). Since it contains no membrane-spanning regions, extra membrane domains and other targeting signal-like myristoylation sites, OsRLCK1 is likely to be either a cytoplasmic and/or nuclear kinase.

Subcellular localization of OsRLCK1

To test the subcellular localization of OsRLCK1, we constructed a translation fusion between OsRLCK1 and a synthetic green fluorescent protein (sGFP). The *OsRLCK1-GFP* fusion and *GFP* alone, both driven by the 35S promoter, were introduced into onion epidermal cells by particle bombardment. The result showed that the OsRLCK1-GFP fusion protein was diffused throughout the cell, indicating its cytoplasmic and nuclear localization (Figure 2).

Temporal and spatial expression of *OsRLCK1*

To examine the expression profile of *OsRLCK1*, we carried out real-time polymerase chain reaction (PCR) analysis. The results showed that the *OsRLCK1* transcripts strongly accumulate in the anthers before anthesis (ABA), and could be detected in mature panicles at heading stage (S8P), and pistils of 3 h and 5 h after pollination (3hP and 5hP), but could not be detected in panicles at early developmental stages (S4P, S5P, S6P and S7P) and the unpollinated pistils (UP) (Figure 3A), indicating that *OsRLCK1* is specially expressed in the mature anther and is likely to exist exclusively in mature pollen but not

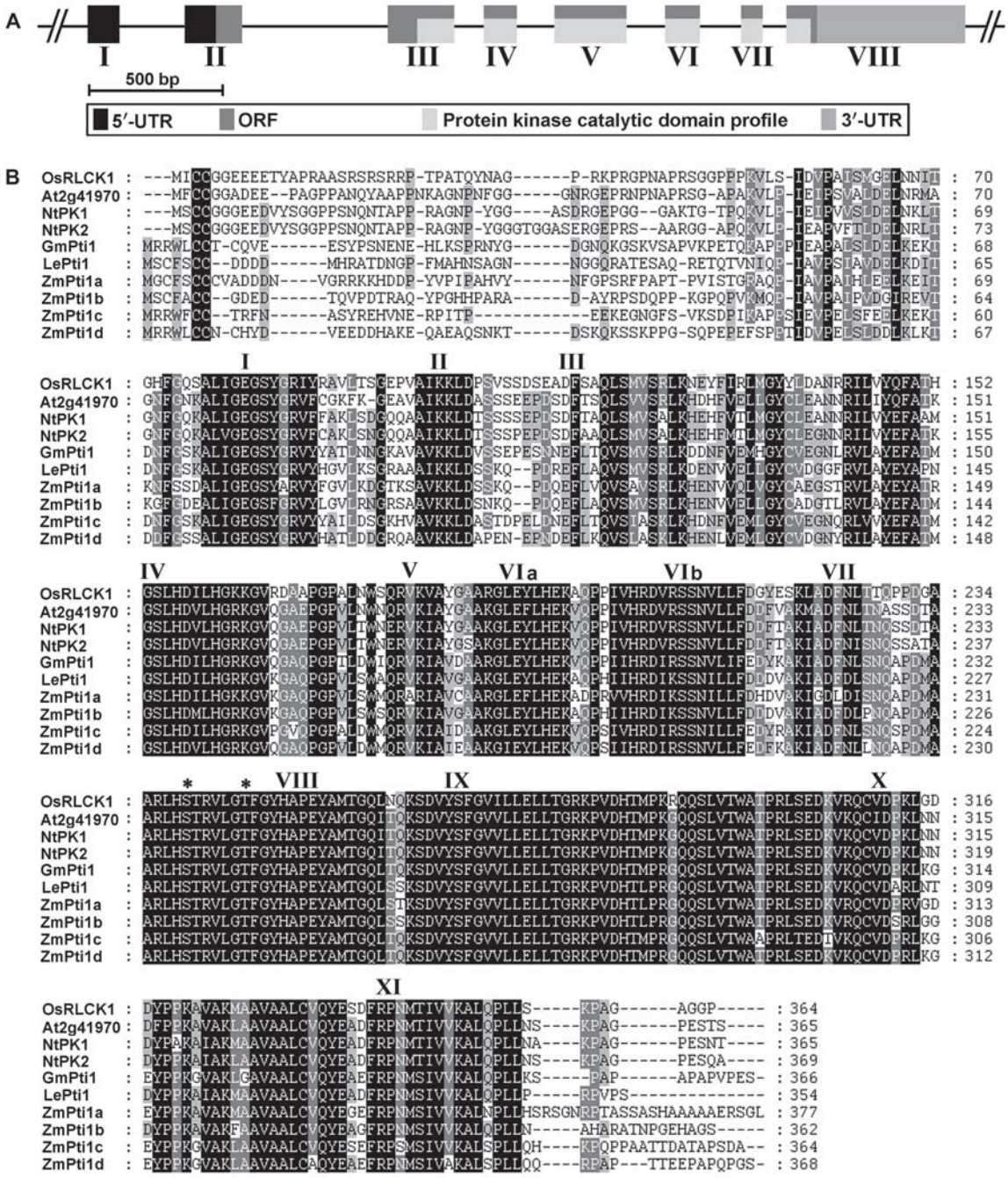

Figure 1. Structural features of *OsRLCK1* and its coding protein.

(A) Genomic structure of *OsRLCK1*. Exons are indicated as boxes with Roman numerals. The 5'-untranslated region (UTR), the open reading frame (ORF), protein kinase domain and the 3'-UTR are indicated in dark, dark gray, pale and gray, respectively. The scale bar indicates 500 base pairs (bp).

(B) Alignment of amino acid sequences of OsRLCK1, NtPK1 (AJ608156), NtPK2 (AJ608157), LePti1 (AAC61805), GmPti1 (AAO92595), ZmPti1a (AAT57906), ZmPti1b (ABG36850), ZmPti1c (ABG36851) and ZmPti1d (ABG36852). The alignment was made with CLUSTAL W (http://www.ddbj.nig.ac.jp/E-mail/ clustalw-j.html). A dash indicates a gap inserted to maximize the alignment. The locations of the 11 protein kinase subdomains are indicated by roman numerals, and invariant or highly conserved amino acids in the subdomains are in bold. Asterisks indicate Ser and Thr residues of putative phosphorylation site (S/T-X(5)-S/T) in MAPKKs (Morris et al. 1997; Ichimura et al. 1998).

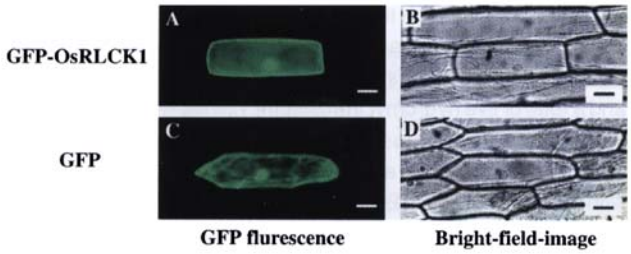

Figure 2. Subcellular localization of OsRLCK1 protein.

Subcellular localization of OsRLCK1. Fluorescent microscopy of transiently transformed epidermal onion cells expressing a 35S promoter driven OsRLCK1-green fluorescent protein (GFP) fusion protein **(A)** and the 35S promoter-driven GFP control **(C)**; and their bright-field images, **(B)** and **(D)**, respectively. Bars, 20 μm.

in immature pollen. To further confirm the expression pattern of *OsRLCK1*, we conducted a whole mount RNA *in situ* hybridization analysis with mature pollen just before anthesis, and a positive signal was detected in the mature pollen (Figure 3B). Taken together, these results indicate that *OsRLCK1* is specifically expressed in mature pollen, suggesting that it is likely to be involved in the pollination in rice.

OsRLCK1 encodes a functional protein kinase

To express OsRLCK1 protein for further analysis, we cloned the coding sequence CDS of *OsRLCK1* into the pET-30a vector and expressed the HIS-OsRLCK1 recombinant proteins in *Escherichia coli* BL21 and formed inclusion bodies. Then the inclusion bodies were denatured and refolded by dialysis and

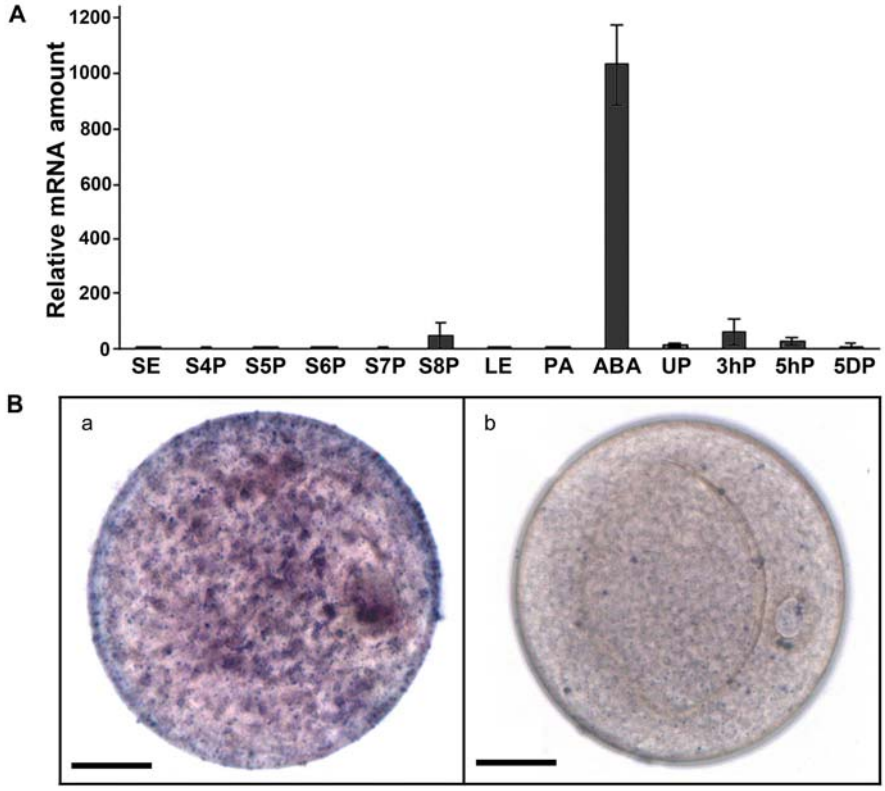

Figure 3. Expression pattern of *OsRLCK1*.

(A) *OsRLCK1* expression in different tissues at different growth stages. Panicles were from Stages 4 (immature panicle at stamen and pistil primordium differentiating stage, S4P), 5 (immature panicle at pollen mother cell formation stage, S5P), 6 (immature panicle at pollen mother cell meiotic division stage, S6P), 7 (immature panicle at pollen filling stage, S7P) and 8 (mature panicle at heading stage, S8P). Lemma (LA), palea (PA), anthers before anthesis (ABA) and unpollinated pistils (UP) were dissected from mature florets at the heading stage. 3hP and 5hP represent the pollinated pistils 3 h and 5 h after pollination, respectively. 5DP represents immature seeds after pollination for 5 days. SE represents seedlings at the three-leaf stage. Primers specific for *OsRLCK1* were used in real-time polymerase chain reaction (PCR) analysis; the rice *18S rRNA* was used as an internal control.

(B) Whole mount RNA *in situ* hybridization analysis of *OsRLCK1*. Mature pollen before anthesis was hybridized with digoxigenin-labeled antisense *OsRLCK1* RNA probes **(a)** and the sense *OsRLCK1* RNA probe as a negative control **(b)**, respectively. Bars, 10 μm.

purified by electroeluting, finally used to generate a polyclonal antibody by immunizing rabbits for subsequent Western analysis (Figure 5D).

To obtain the soluble fusion protein for further activity assay, we cloned the CDS of *OsRLCK1* into the pMAL-c2 vector and expressed the maltose binding protein MBP-OsRLCK1 recombinant protein in *E. coli* BL21. We successfully obtained soluble MBP-OsRLCK1 fusion protein, and then the MBP-OsRLCK1 was purified by amylose resin for further kinase assay.

To examine whether *OsRLCK1* encodes a functional protein kinase, the purified MBP-OsRLCK1 protein was used to carry out an *in vitro* autophosphorylation assay, purified MBP was used as a negative control. After incubation in a kinase reaction mixture at 30 °C for 30 min, phosphorylation was detected by immunoblotting with two commercially available sets of antibodies separately. The autophosphorylation of MBP-OsRLCK1 could be detected with the antiphosphothreonine-specific antibody, but could not with the antiphosphoserine-specific antibody (Figure 4). The results indicated that *OsRLCK1* indeed encodes a functional receptor-like cytoplasmic kinase.

Characterization on *OsRLCK1* RNAi lines indicated a functional redundancy in RLCKs in rice

To gain insight into *in vivo* function of *OsRLCK1*, we used RNAi interference to silence the expression of *OsRLCK1*, an RNAi construct driven by a maize ubiquitin promoter (Figure 5A) was introduced into *Oryza sativa* ssp. *japonica* var. Nipponbare, and a total of five hygromycin-resistant T_0 lines were generated. Two of the five T_0 lines were selected for further detailed analysis. Southern blot analysis of leaf genomic DNA digested with *Hin*dIII or *Eco*RI was carried out on these two plants using *HPT II* cDNA as a probe, the results showed that line 1 had two copies and line 2 contained three copies of the transgene insertion, showing that these two plants were independently transformed (Figure 5B).

To examine the expression of *OsRLCK1*, real-time PCR analysis was conducted using total RNA from mature pollen from the two transgenic lines and the wild-type plants. The results showed that transcription of *OsRLCK1* was reduced in the pollen of the two transgenic lines (line 1, 0.51 ± 0.15; line 2, 0.36 ± 0.09; WT, 1.00 ± 0.23) (Figure 5C). To further confirm the reduction of *OsRLCK1* expression, total protein extracts from mature pollen of the two transgenic lines and the wild-type plants were subjected to Western blot analysis using the anti-OsRLCK1 antibody. The result showed that its protein expression level was also reduced in the pollen of the transgenic lines compared to the wild-type plant (Figure 5D). Together, these results indicate that the expression of *OsRLCK1* was knocked down at both the transcriptional level and the translational level, further confirming the RNAi effect on *OsRLCK1* expression.

To decipher the biological function of *OsRLCK1* during pollination, pollen germination assay of the RNAi lines and the wild-type plants was carried out. However, the result did not show a statistically significant difference between the RNAi pollen and the wild-type (data not shown). Together, the results suggest that there is functional redundancy among rice RLCKs, and that OsRLCK1 is likely to play a role in the pollination signaling pathway synergistically with other RLCKs in rice.

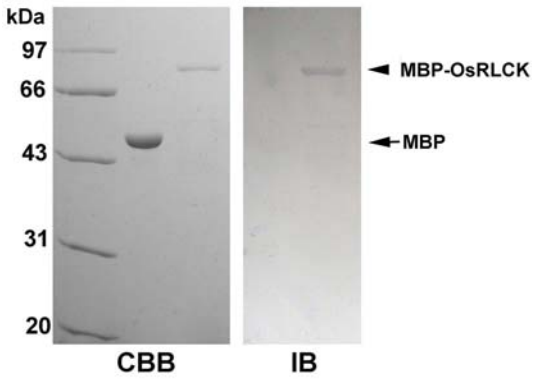

Figure 4. *In vitro* autophosphorylation assay of OsRLCK1.

In vitro kinase assay of OsRLCK1. Protein samples (MBP-OsRLCK fusion protein and MBP control) were incubated in a kinase reaction mixture. An aliquot of sample was separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblot analysis (IB) with either antiphosphoserine or antiphosphothreonine antibodies. Membranes were washed thoroughly with stripping solution and immunoblotted with anti-OsRLCK antibody confirming the protein signal (data not shown). One of the duplicate gels was stained with Coomassie Brilliant Blue R-250 (CBB) indicating the equal loading of proteins. Size markers are indicated on the left (kDa).

Discussion

OsRLCK1 encodes a novel functional receptor-like cytoplasmic kinase in rice

Receptor-like kinases comprise a major gene family in plants, but the functions of most RLKs are unclear, especially for the RLCKs (Shiu and Bleecker 2001b; Shiu et al. 2004). So far, only a limited number of the RLCKs have been characterized, such as NtPK1 and NtPK2 in tobacco (Kumara et al. 2004), Pto and Pti1 in tomato (Zhou et al. 1995), CDG1 in *Arabidopsis* (Muto et al. 2004), MLPK in brassica (Murase et al. 2004), GmPti1 in soybean (Tian et al. 2004) and ZmPti kinases in maize (Herrmann et al. 2006). To our knowledge, OsRLCK1 is the first RLCK to be identified and functionally characterized in rice. Blast comparison showed that OsRLCK1 is similar to NtPK1, NtPK2, LePti1,

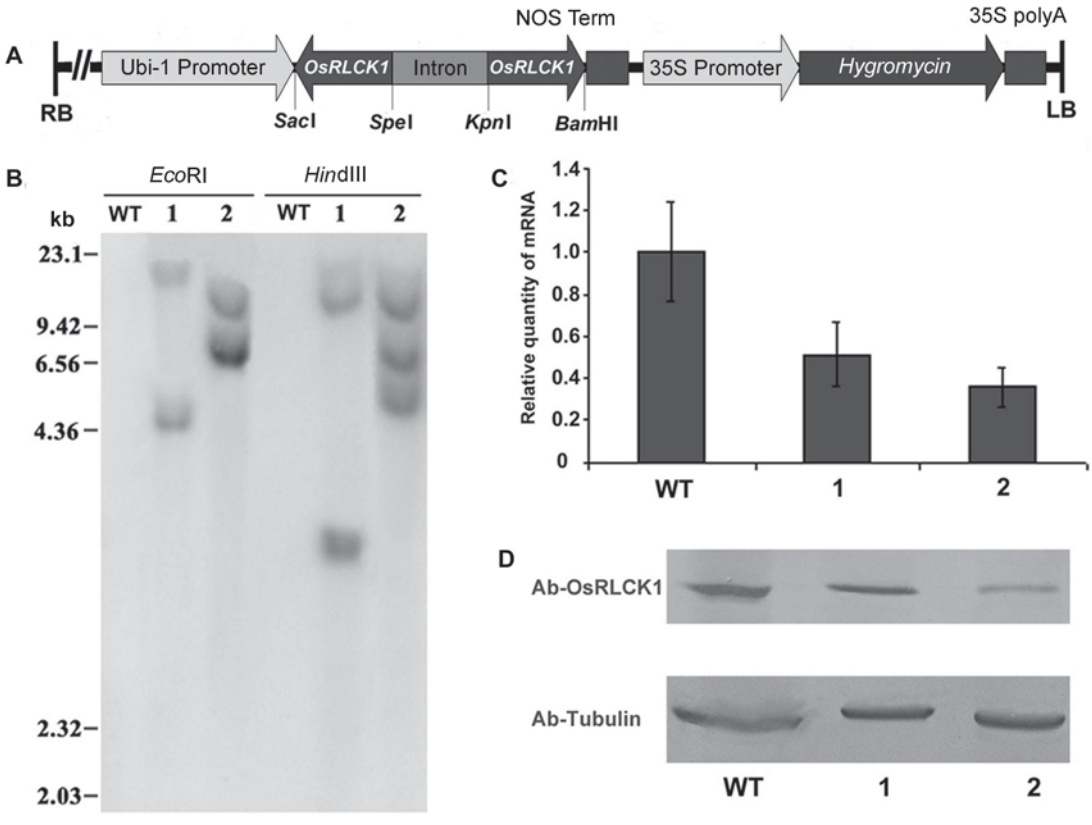

Figure 5. Generation of *OsRLCK1* RNAi lines.

(A) A schematic representation of the RNAi construct of *OsRLCK1* used for rice transformation. The hairpin structure consisting of an antisense *OsRLCK1* fragment, a rice intron and the sense *OsRLCK1* fragment was inserted between the maize ubiqutin1 promoter and the nopaline synthase terminator of the vector pTCK303.

(B) Southern blot analysis of the RNAi T_0 lines and the wild type (WT) control. The leaf genomic DNA was digested with *Eco*RI and *Hin*dIII, respectively, and was blotted and probed with a ~500-bp fragment of the hygromycin phosphotransferase (*HPT*) gene. Sizes of the markers are indicated in kilobase pairs.

(C) Reduced *OsRLCK1* expression in the RNAi T_0 lines. Mature pollen of the two independent RNAi T_0 plants and the WT control at the heading stage were used for real-time polymerase chain reaction (PCR) analysis.

(D) Reduced OsRLCK1 expression in the RNAi T_0 lines. Total proteins of mature pollen in the two independent RNAi T_0 plants and the WT control at the heading stage were used for Western blot analysis using anti-OsRLCK1 polyclonal antibody generated with HIS-OsRLCK1 fusion protein by immunizing rabbits. One of the duplicate gels was detected with anti-Tubulin antibody indicating an equal loading of the proteins.

GmPti1 and ZmPti kinases of the RLCK VIII subfamily, but different from Pto, MLPK and CDG1 of the RLCK VII subfamily, indicating that OsRLCK1 is a new member of RLCK VIII subfamily (Figure 1). Moreover, *in vitro* kinase assay indicated that OsRLCK1 is likely to be a functional protein kinase (Figure 4).

OsRLCK1 is a mature pollen-specific protein kinase that may facilitate the pollen performance during pollination in rice

The pollen-specific genes are differentially expressed and tightly regulated during the pollen developmental process, and they can be divided into two phases (McCormick 1993; Cheung and Wu 2001). The “early” pollen genes, which are expressed just after meiosis and are undetectable in mature pollen, are believed to secrete proteins necessary for cytoskeletal organization. The “late” pollen genes that are subsequently expressed are thought to be involved in pollen germination or

pollen tube growth (Mascarenhas 1990). In this study, *OsRLCK1* falls into the category of "late" pollen genes, and is specially expressed in the mature pollen in rice. We found that a strong *OsRLCK1* expression signal could be detected in mature anthers and a relative weak signal in mature panicles (Figure 3A), suggesting that *OsRLCK1* is specific to the mature pollen. Whole mount RNA *in situ* hybridization analysis on *OsRLCK1* confirmed its mature pollen-specific expression profile (Figure 3B). Interestingly, a similar mature pollen-specific expression has been shown for *OsRLCK1*'s homologs, such as *At2g41970*, *NtPK1*, *NtPK2*, *ZmPti1a* and *ZmPti1c*. Moreover, given that the *OsRLCK1* transcripts could be detected in the pistils 3 and 5 h after pollination (3hP and 5hP) but no *OsRLCK1* transcripts in the unpollinated pistils (UP) (Figure 3A), it seems that *OsRLCK1* is likely to play a role during pollen germination and pollen tube elongation. However, we could not detect any alteration with statistical significance during pollen germination and pollen tube elongation in *OsRLCK1* knock-down lines, which could be explained by a functional redundancy of RLCKVIII kinases in rice. Nevertheless, the fact that OsRLCK1 is a functional protein kinase suggests that *OsRLCK1* is likely to play an important role during pollination synergistically with other RLCKs in rice.

Pollination shares certain similarities with pathogen defense

Previously, we monitored gene expression profiles during pollination in rice by cDNA microarray analysis and found that large proportions of the differentially expressed genes during pollination and fertilization are involved or potentially involved in defense and/or stress response (Lan et al. 2004, 2005). The result indicated that an extensive overlap occurs between pollination/fertilization and defense- or stress-response pathways. Interestingly, in the present study, we have found that the pollen-specific genes (*OsRLCK1*, *At2g41970*, *NtPK1*, *NtPK2*, *ZmPti1a* and *ZmPti1c*) and defense-related genes (*LePti1*, *GmPti1*, *ZmPti1b*) show high sequence similarity and fall into one category (Figure 1); moreover, their gene organizations possess nearly identical exon/intron structures as compared to OsRLCK1, i.e. eight exons and a predicted translation start in exon 2 (data not shown). This finding further supports our previous speculation that the pollen-sporophyte interactions and pathogen defense share some common signaling pathways. It is possible that the pollen-sporophyte interaction during pollination and defense response are likely to share an evolutionary conserved signaling pathway(s), and that the underlying mechanism of the pollen recognition, germination and elongation in style (also considered as pollen tube-mediated pathogen ingress) shares a great similarity with that of the plant-pathogen recognition and hypersensitive response.

In conclusion, our results strongly suggest that OsRLCK1, a novel pollen-specific RLCK, acts as a functional protein kinase and plays a role during pollination. However, the underlying molecular mechanism is not entirely unclear due to possible functional redundancy of RLCKs. Further studies on OsRLCK1 and its homologs in rice, especially on the generation of the loss-of-function alleles, will shed light on the signaling transduction in pollination and the cross-talk between the pollination and pathogen defense.

Materials and Methods

Plant materials and growth conditions

Rice (*Oryza Sativa* L. ssp. *japonica* var. Nipponbare) was used for various experiments in this study, including expression analysis, transformation. Transgenic plants and the untransformed wild-type control were grown in the field under natural conditions in the experimental farm of the Institute of Genetics and Developmental biology, Beijing.

Subcellular localization

The whole coding sequence of *OsRLCK1* was amplified using two primers (5'-CCGAGCTCATGATTTGCTGCGGCGGCGAG-3', *Sac* I site underlined) and (5'-CAGTCGACATAGGGCCCTCCTGCTCCGGCT-3', *Sal* I site underlined). The resulting PCR product was subcloned into a rebuilt vector pBI221 to generate *pBI221-35S:OsRLCK1-GFP* containing an *OsRLCK1-GFP* fusion construct under the control of the 35S promoter. The construct was sequenced to verify the in-frame fusion and no nucleotide mutations.

Then the *pBI221-35S:OsRLCK1-GFP* was used for transient transformation of onion epidermal cells using bombardment (Bio-Rad, Hercules, CA, USA), and the vector *pBI221-35S:GFP* was used as a positive control. Expression and localization of OsRLCK1-GFP and GFP control were observed under a fluorescent microscope (Leica DMR) and photographed using a Micro Color charge-coupled device (CCD) camera (Apogee Instruments, Roseville, CA, USA).

Real-time PCR

Total RNA preparation and real-time PCR were carried out as previously described (Lan et al. 2004). In brief, reverse transcription was performed using TaqMan Reverse Transcription Regents Kit (Applied Biosystems, CA, USA). The cDNA samples were diluted to 5.00 and 1.25 ng/μL. Triplicate quantitative assays were carried out on 1 μL of each cDNA dilution using the SYBR Green Master Mix (Applied Biosystems, PN 4309155) with an ABI 7900 sequence detection system according to the

manufacturer's protocol (Applied Biosystems). The gene-specific primers were designed by using PRIMEREXPRESS software (Applied Biosystems). The relative quantification method (DDCT) was revised and used to evaluate quantitative variation between replicates examined. The amplification of *18S rRNA* was used as an internal control to normalize all data. Gene-specific primers for *OsRLCK1* were 5'-GCATCAAACCAACTTAGCATCCA-3' and 5'-ATCACCAGCTGTTTAACAA GTCTCA-3', and for *18S rRNA* 5'-CGGCTACCACATCCAAGGAA-3' and 5'-TGTCACTACCTCCCCGTGTCA-3'.

Whole mount RNA *in situ* hybridization

RNA *in situ* hybridization was carried out as previously described (Xu et al. 1999). A fragment of 490-bp that was amplified from 3'-UTR of *OsRLCK1* with primers 5'-GCCCTAGCAGCACATGCATA-3' and 5'-TGCGAACAATCAAGCCACA-3' was cloned into pGEM-TEasy vector. Plasmid DNA was linearized by use of *Sal* I and *Nco* I, and then transcribed *in vitro* with T7 and SP6 RNA polymerase, respectively. The digoxigenin-labeled antisense and sense riboprobes were synthesized using a DIG Northern Starter Kit (Cat. No. 2039672, Roche, Basel, Switzerland) according to the manufacturer's instructions. These riboprobes were hydrolyzed into small fragments of about 150 nucleotides in length.

Images were observed under bright-field through a microscope (Leica DMR) and photographed using a Micro Color charge-coupled device (CCD) camera (Apogee Instruments).

Expression and purification of OsRLCK1 protein

Expression and purification of HIS-OsRLCK1 and antibody production

Previously, to express the OsRLCK1 for further analysis, the whole coding sequence of *OsRLCK1* was amplified using two primers (5'-CAGAATTCCATGATT TGCTGCGGCGGCGAG -3', *Eco*RI site underlined) and (5'- CAGTCGACCTAGGGCCCTCC-TGCTCCGGC -3', *Sal* I site underlined). The resulting PCR product was subcloned into the pET-30a vector. A single colony of *E. coli* strain BL21 transformed with the expression plasmid was grown overnight at 37 °C in Luria-Bertani (LB) medium containing 100 μg/mL ampicillin. An aliquot of 10 mL culture was inoculated into 990 mL LB medium and incubated at 37 °C to an OD600 of 0.5. Isopropyl-β-D-thiogalactoside (IPTG) was then added to a final concentration of 0.25 mmol/L and the cells were grown for a further 4 h at 37 °C. *E. coli* cells were harvested by centrifugation, resuspended in phosphate-buffered saline (PBS), and lysed by sonication for 30 min. However, the HIS-OsRLCK1 recombinant protein was expressed in inclusion bodies. The inclusion bodies were dissolved in 8 mol/L urea and refolded by dialysis and purified by electroeluting, and then the purified HIS-OsRLCK1 recombinant protein was used to immulizing rabbit to generate the anti-OsRLCK1 antibody.

Expression and purification of MBP-OsRLCK1

Subsequently, to express soluble OsRLCK1 for further kinase assay, the above-mentioned PCR product was subcloned into the pMAL-c2 vector and expressed the MBP-OsRLCK1 recombinant protein as the above-mentioned method. Fortunately, the MBP-OsRLCK1 fusion protein was expressed in a soluble form. Then the MBP-OsRLCK1 fusion protein was purified by amylose resin following the manufacturer's recommendation (New England Biolabs, Ipswich, MA, USA), and used for subsequent kinase assay.

In vitro kinase assay and autophosphorylation

For the *in vitro* kinase assay, 10 μg purified recombinant protein samples were incubated in 50 μL kinase reaction mixture (50 mmol/L Tris-HCl pH 7.5, 150 μmol/L ATP, 1 μmol/L dithiothreitol (DTT), 10 mmol/L $MgCl_2$ and 10 mmol/L $MnCl_2$) at 30 °C for 30 min. Kinase reaction mixture added with 10 μg purified MBP protein was used as a negative control. The reaction was stopped by adding 25 μL 2 × sodium dodecyl sulphate (SDS)-loading buffer (0.2 mol/L Tris-HCl, pH 6.8, 0.5 mol/L DTT, 4% SDS, and 25% glycerol), followed by boiling at 100 °C for 5 min. The labeled protein samples were resolved on 10% SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto Nitrobind (Micron Separations, Westborough, MA, USA) using a Bio-Rad Transblot SD wet electroblotting apparatus (Bio-Rad, Hercules, CA, USA). Blots were treated with either antiphosphoserine-specific mouse monoclonal antibody (1:10 000) or antiphosphothreonine-specific mouse monoclonal antibody (1:10 000) (4G10, Upstate Biotechnology, Lake Placid, NY, USA) at 37 °C for 1 h. After extensive washing of the membranes with TBS-T (Tris-Buffer Saline-Tween-20) buffer, the immune complexes were detected using alkaline phosphatase-conjugated secondary antibodies. Signals were detected with 0.33 mg/mL 4-nitro blue tetrazolium (NBT) and 0.165 mg/mL 5-bromo-4chloro-3indoyl phosphate (BCIP) in alkaline phosphate buffer (100 mmol/L Tris, pH 9.5, 100 mmol/L NaCl, and 5 mmol/L $MgCl_2$).

Plasmid constructs, rice transformation and generation of transgenic rice

A fragment of 490-bp was amplified from *OsRLCK1* with two primers 5'-GG GGTACC ACTAGT GCCCTAGCAGCACATGCATA -3' (*Kpn* I and *Spe* I sites underlined) and 5'-CG GGATCC GAGCTC TGCGAACAATCAAGCCACA -3' (*Bam*H I and *Sac* I sites underlined) containing two restriction enzymes at their 5' ends, respectively. The plasmid was constructed as previously described (Kong et al. 2006). The hairpin structure consisting of an antisense *OsRLCK1* fragment, a rice intron and

an *OsRLCK1* sense fragment was inserted between the maize ubiqutin1 promoter and the nopaline synthase terminator of the vector pTCK303 (Figure 4A). The construct was completely sequenced to ensure that it did not contain PCR or cloning errors.

Plant transformation was carried out as previously described (Ge et al. 2004). Briefly, rice embryonic calli were induced on scutella from germinated seeds and transformed with *Agrobacterium tumefaciens* strain EHA105 containing the desired binary vector. Transgenic plants were selected in half-strength MS medium containing 50 mg/L hygromycin (Roche, 843555). Hygromycin-resistant plants from calli, defined as transgenic plants of T_0 generation, were transplanted into the field. T_0 plants were further used for Southern blot analysis.

Southern blotting analysis

Genomic DNA isolation and Southern blotting analysis were carried out as described previously (Qiao et al. 2004b). DNA (5 μg) was digested, separated on 0.8% agarose gel, and transferred onto Hybond Nt (Amersham, Buckinghamshire, UK) membrane. Prehybridization, hybridization, and washing of the blot were carried out as recommended by the manufacturers. *HPT* probe was labeled with ^{32}P by random priming using the Prime-a-Gene labeling system (Promega, Madison, WI, USA). The *HPT* primers were 5'-GCAAGGAATCGGTCAATACAC-3' and 5'-TCCACTATCGGCGAGTACTTC-3'.

Protein extraction and Western blotting analysis

Protein extraction and Western blotting analyses were carried out as described previously (Qiao et al. 2004a). Fresh mature pollen were ground in liquid nitrogen and extracted in 50 mmol/L Tris buffer (pH 8.0) with 300 mmol/L NaCl, 10 mmol/L ethylenediaminetetraacetic acid (EDTA), 10 mmol/L DTT, and a protease inhibitor cocktail (Sigma, St Louis, MO, USA) at a dilution of 1:100. The extraction mixtures were kept on ice for 15 min and then centrifuged for 10 min at 10 000*g* to pellet cellular debris. The protein concentrations of the different fractions were estimated according to the method of Bradford.

Protein extracts were separated on 12% SDS-polyacrylamide gels and blotted to nitrocellulose (Pharmacia, Peapack, NJ, USA) using the Bio-Rad Mini-Protein II apparatus. After blotting, the filters were blocked with 15% skimmed milk power in TBS-T for at least 8 h at room temperature. Then, the membranes were incubated with the anti-OsRLCK1 polyclonal antibody (1:200) at 37 °C for 1 h. The pre-immune serum served as a control. The secondary antibodies were alkaline phosphatase-conjugated anti-rabbit secondary antibody (Sigma) diluted 1: 10 000 in TBS-T. Signals were detected with 0.33 mg/mL 4-NBT and 0.165 mg/mL BCIP in alkaline phosphate buffer (100 mmol/L Tris, pH 9.5, 100 mmol/L NaCl, and 5 mmol/L $MgCl_2$).

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