

Short
Communication**Molecular Cloning and Characterization of Rice Pollen Profilin**

YE Qiu, XU Yin, YAN Fang, TANG Lin, CHEN Fang*

(The College of Life Science, Sichuan University, Chengdu 610064, China)

Abstract Profilin is a low molecular weight protein and is identified as an actin-binding protein in higher plants. Two pollen-specific cDNA clones of rice, *RproA* and *RproB*, were isolated from a cDNA library of rice mature pollen. The two cDNA clones was 821 bp and 805 bp long and contained an open reading frame of 131 amino acids, 5' and 3' untranslated regions and a long poly(A) tail. The comparison of deduced amino acid sequences from *RproA* and *RproB* cDNA and other plant profilin cDNA indicated that the two rice profilin are high homologous to each other, showed that 89% similarity to maize (*ZmPRO3*), 87% to *C. dactylon*, 83% to *H. brasiliensis* and 89% to *P. pratense*. Genomic southern blot analysis suggested the existence of two genes at least. Northern blot and reverse transcriptase polymerase chain reaction analysis demonstrated that the two rice profilin cDNA are anther or pollen specific.

Key words actin cytoskeleton; profilin; cloning and expression; pollen

Profilin is a low molecular weight protein that was originally identified as an actin-binding protein in calf spleen^[1]. Profilin is known to regulate the organization of the actin cytoskeleton in a complex fashion; It can promote or prevent actin nucleation or polymerization, depending on the profilin-to-actin ratio^[2-4]. Profilin forms a one to one complex with the actin monomer and can thereby affect the rate and extent of actin polymerization^[5]. It also binds to protein with proline-rich motifs and may be linked to signal transduction cascades through binding to polyphosphoinositides^[4-6]. Profilin has been found in organisms from all eukaryotic kingdoms and plays a significant role in regulating the dynamics of the actin cytoskeleton^[3,4,7].

In plants, profilin was originally discovered as a major birch pollen allergen^[8] and has subsequently been isolated or cloned from many plants^[4]. Such as maize^[9], timothy grass^[10], wheat^[11], tobacco^[12], common bean^[13], tomato^[14] and *Arabidopsis*^[15]. Plant profilin are members of large multigene families with unique expression patterns and can be grouped into two classes: those that are constitutively expressed in vegetative tissues and those expressed primarily in reproductive tissues. Multiple profilin isoforms can be expressed in individual tissues and cells, particularly in pollen^[15].

To investigate the function of profilin gene in rice, we have identified and characterized two members of the profilin multigene family from the plant rice (*Oryza sativa* L.c v. IR 36). Two cDNAs was isolated from a rice pollen library (designated *RproA* and *RproB*) and their transcripts were expressed in mature pollen and anther.

1 Materials and Methods

1.1 Materials

Rice plants (*Oryza sativa* L.c v.IR36) were grown under greenhouse conditions. Mature pollen grains and anthers were collected and immediately frozen in liquid nitrogen. Leaves, stems and roots were collected from two-week-old seedlings. Frozen tissues were stored at -70 °C until use.

1.2 Construction of rice pollen cDNA library

The poly(A) RNA was purified from mature pollen using poly Atract System 1000 kit (Promega) and the rice pollen cDNA library was constructed using SMART™ cDNA library construction kit(Clontech). The first-strand cDNA was primed with the CDSIII /3' primer and the SMART™III Oligo serves as a short, extended template at the 5' end of the mRNA. After first-strand cDNA was synthesized, the primer-extension step generates full-length ds cDNA. The ds cDNA was digested with *Sfi*I and size fractionation using CHROMA SPIN™ columns, 1.5 µl cDNA was ligated into the *Sfi*I-digested λTrip1Ex2™ vector and packaged using Gigapach Gold system(Stratagene).

1.3 Isolation and characterization of two cDNA from rice pollen cDNA library

Prior to the screening, an approximately 400 bp cDNA fragment was obtained using RT-PCR with a pair of primers, P₁(5'-GTNGACGAGCACCTNAT-3') and P₂(5'-RTARTCNCCNAGNCGYTC-3') which were synthesized according to the homogeneity. This amplified product was subcloned into the PMD18-T vector (TaKaRa). The nucleotide sequence of this fragment was determined and it was recognized as a partial fragment of profilin cDNA by the comparison with other known plant profilin cDNA. Using this PCR fragment, we screened a cDNA library, which was made from pollen of rice by standard method^[16].

1.4 Southern blot analysis

Total rice DNA was isolated from two-week-old seedlings of rice (*Oryza sativa*.L.c.v.IR36) according to the method^[17]. For analysis of the profilin gene copy number in rice, genomic DNA was completely digested with *Eco*RI, *Bam*HI, *Hind*III, *Pst*I and *Xba*I and then separated in a 1% agarose gel. DNA was transferred onto Hybond N⁺ filter (Biohringer Mannheim) The *Eco*RI/*Xba*I *RproA* insert labelled with [³²P]dCTP by the random priming technique was used as a probe. Blots were prehybridized at 65 °C in prehybridization solution[6; ÁSSC (1; ÁSSC is 0.15 mol/L NaCl and 0.015 mol/L sodium citrate), 0.5% SDS, 10% denatured salmon sperm DNA and 5; ÁDenhardt's solution [1; ÁDenhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA)] for 3 h and hybridized at 65 °C in prehybridization solution containing [³²P]-labeled probes using primer-a-Gene Labelling System(Promega). Blot were then washed under low stringency conditions (5 min in 2; ÁSSC and 0.1% SDS at room temperature and 10 min in 1; ÁSSC and 0.1% SDS at room temperature) and then exposed on Fuji X-ray film at -70 °C overnight.

1.5 Northern blot analysis

Total RNA from pollen, anther, shoots, leaves and roots was extracted using a guanadine thiocyanate extraction method^[18]. Fifteen µg of RNA per lane was separated in an agarose gel

containing 10% formaldehyde and blotted onto a Hybond N⁺ membrane in 10×SSC (1×SSC is 0.15 mol/L NaCl and 0.015 mol/L sodium citrate). The labelled *EcoRI/XbaI RproA* insert was used as a probe. After baking for 2 h at 80 °C, the membrane was hybridized to the profilin cDNA fragment, which has been labeled with [³²P]dCTP using primer-a-Gene Labelling System. The blot was washed at high stringency (5 min in 2×SSC, 0.01% SDS at 65 °C, 10 min in 1×SSC and 0.1% SDS at 65 °C) before X-ray film was exposed to them.

1.6 Nucleotide sequencing analysis

DNA sequencing was performed by the dideoxy chain-termination method using Bca BEST dideoxy sequencing kit (TaKaRa).

2 Results and Discussion

2.1 A rice pollen-specific cDNA library

To isolate and characterize the profilin gene in the development of pollen germination and tube growth, a rice pollen cDNA library was constructed in the expression vector λTriPEx2TM (Clontech) using cDNA synthesized from polyadenylated mRNA isolated from rice mature pollen. The vector λTriPEx2TM, used in this system allowed conversion from phage λ to plasmid clone by using the cre-lox site-specific recombination system. The library contained 2×10⁶ independent clones. The efficiency of recombinant clones was about 97%. It was conform to objective of the cDNA library constructed.

2.2 Isolation and sequence of cDN

A clonesA rice cDNA library derived from rice pollen mRNA was screened using a 366 bp nucleotide PCR fragment as a probe(see material and method). Several positive recombinant phages were isolated after screening 2×10⁵ plaques. These positive phages were then submitted to a second screen. Thirty-seven positive clones were isolated from a screen of 2×10⁶ clones. Based on the sequence information, we have obtained two full-length cDNA clones(*RproA* and *RproB*). The nucleotide sequence of *RproA* and *RproB* cDNA is 821 bp and 805 bp repectively and exhibits an open reading frame corresponding to a 333 bp amino acid polypeptide (Fig.1). The presence of a stop codon (TGA) two codon upstream of the first ATG suggests that this ATG is the initiation codon and thus that the coding sequence is complete. The cDNA presents 77 bp of 5'- and 319 bp of 3'-untranslated sequences and a long poly(A) tail.

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GACCACCGCGACGAACACCCGACGAGGAGAAAGAAACCACCTGATCGAGCCCGTATCTTT 60
GCAGCCCGCAGGAGAGGAGGACGAGTCTGTTCTGTTGTCAGCAGGAGGCCGCCCGAAGATG 120
M
TCGTGGCAGACGTACGTCCGACGACACCTGATGTCCGAGATCGAGGGCCACCACCTCACC 180
S W Q T Y V D E H L M C E I E G H H L T
TCCGCCGCCATCGTCCGCCACGACGGCACCGTCTGGGCACAGAGCGCCGCCCTTCCCGCAG 240
S A A I V G H D G T V W A Q S A A F P Q
TTCAAACCGGAGGAGATGACCAACATCATGAAGGACTTCGACGAGCCCGGGTCTCTCGCC 300
F K P E E M T N I M K D F D E P G F L A
CCGACCGGACTGTTTTCTTGGACCAACCAAGTACATGGTCATCCAAGGTGAACCTGGCCCT 360
P T G L F L G P T K Y M V I Q G E P G A
GTCATTCTGTGCAAGAAGGGATCAGGAGGCATCACCGTGAAGAAGACCGGGCAAGGCCCTG 420
V I R G K K G S G G I T V K K T G Q A L
GTGGTCCGCATCTACGACGAGCCGATGACCCCAAGGCGAGTGCACATGCTGTGTCGAGAGG 480
V V G I Y D E P M T P G Q C N M V V E R
CTCCGCCACTACCTCGTAGAGCAGGGCCTGTAGATCACCGTCGCCCATCATCGTCTGCTT 540
L G D Y L V E Q G L
GCCACAGTTCAATTCTGTCAGCAACAATTAAACCGGAGACGAATGAGAGACACACACAGCA 600
CATAGTAGATCTCTATGCTATGCAATTTTTCTAGCCACCGTGCATGCATGCATGGGATTG 660
TGTTTTAATTTTCTCTCTCTCTTCTCTGTCACCTCCGCAAGCATGTTCTGGATCACCT 720
CTTGATCGTTCTGCCATATTTTGTGTGATCCCATTTCTTCATGCCGTTTTTGGCTAATAA 780
CTTTTTGTATTNAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

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Fig.1 Nucleotide and deduced amino acid sequences of the *Rpro* cDNA

DNA sequence corresponding to the *Rpro* transcript is shown, with the predicted translation product shown below. The nucleotide numbering is listed at the right side.

Comparison of the nucleotide sequences of *RproA* and *RproB* cDNA showed that the two sequences were homologous (89% identity) but not identical(Fig.2). Some differences localized in the 3'-untranslated sequence, suggested that the two cDNAs could derive from two different genes. Nucleotide sequence homology search in GenBank revealed that the cDNA fragment has a similarity of 89% to maize *ZmPRO3*, 87% to *C.dactylon*, 83% to *H.brasiliensis* and 89% to *P.pratense*(Fig.3). A G-actin-binding region, the VERLGDYL is located at 119;^a126 amino acid and a phosphatidylinositol 4,5-bisphosphate(PIP₂) region IRGKKGSGGITVKKT is located at 82;ⁱ97 amino acid, respectively. These results show that the deduced amino acid sequence of profilin from pollen is high conservation and the profilin is an actin-binding-protein. It could influence the pollen germination, subsequent tube growth, and movement of germinal cells down the tube by affecting the actin cytoskeleton. For example, profilin could both inhibit and promote actin polymerization because of its role in sequestering actin monomers and in catalyzing nucleotide exchange associate with monomer actin^[19]. In addition, profilin interacted with phospholipids and probably linked the remodeling of the actin cytoskeleton to the signal transduction pathway in response to extracellular stimuli^[6,20,21].

A	TACFACCGGACGAACACCCGACGAGGAGAAAGAAACAC	40
B	G-----	1
A	CTGATCGAGCCCGTATCTTTGCAGCCGAGGAGAGGAGGA	80
B	-----GAGC-----	5

A	CGAGTCGTTGGTTGTGTCAGCAGGAGGCGGCGGCGAAGATG	120
B	-----AGGCGA--GCGAAGATG	20
A	TCGTGGCAGACGTACGTGACGAGCACCTGATGTGCGAGA	160
B	TCGTGGCAGACGTACGTGACGAGCACCTGATGTGCGAGA	60
A	TCGAGGGGCCACCACCTCACCTCCGCCGCCATCGTCGGCCA	200
B	TCGAGGGGCCACCACCTCACCTCCGCCGCCATCGTCGGCCA	100
A	CGACGGCACCGTCTGGGCACAGAGCGCCGCCCTTCCC	240
B	CGACGGCACCGTCTGGGCACAGAGCGCCGCCCTTCCC	140
A	TTCAAACCGGAGGAGATGACCAACATCATGAAGGACTTCG	280
B	TTCAAACCGGAGGAGATGACCAACATCATGAAGGACTTCG	180
A	ACGAGCCGGGGTTCTCGCCCCGACCGGACTGTTTCTTGG	320
B	ACGAGCCGGGGTTCTCGCCCCGACCGGACTGTTTCTTGG	220
A	ACCAACCAAGTACATGGTTCATCCAAGGTGAACCTGGCGCT	360
B	ACCAACCAAGTACATGGTTCATCCAAGGTGAACCTGGCGCT	260
A	GTCATTTCGTGGCAAGAAGGGATCAGGAGGCATCACGGTGA	400
B	GTCATTTCGTGGCAAGAAGGGATCAGGAGGCATCACAGTGA	300
A	AGAAGACCGGGCAGGCGCTGGTGGTCCGGCATCTACGACGA	440
B	AGAAGACCGGGCAGGCGCTGGTGGTCCGGCATCTACGACGA	340
A	GCCGATGACCCCAGGGCAGTGCAACATGGTGGT	480
B	GCCCATGACCCCAGGGCAGTGCAACATGGTGGTTGAGAGG	380

A	CTCGGCCTGACTACCTCGTAGAGCAGGGCCTGTAGATC	519
B	CTCGGTGACTACCTCGTAGAGCAGGGCCTGTAGATCGATC	420
A	G-TCGC---CATCATCGT-CGTCGTTGCCACAGTTCA---	551
B	GATGACATCCATTGTCGCGCACCGTTCCCAATTTCACCA	460
A	---ATTCTG-TCAGC---AACAATTAAACGG---AGACAA	582
B	ACAAATTAACTCAGCCAAACGAGTCAGATTATAGATCA	500
A	TGAGAGACA-----AC-ACAGCACATA-GTAGAGC	612
B	TATGCAATTTTTCTAGCCACCACCGCGCATGCATGGGATT	540
A	--CTATGCT---ATGCAATTTTTCTAG---CCACCCTGCA	643
B	AATTGTGGTTTAATTTTTTTTTCTCACTTCCCTGTCA	580
A	TGC-ATGCATGG-GGAT-----TGTGGTTTAATT-FTC	673
B	CACCAACACATTGACAGATCACTTCCTGCAGTGCATAATC	620
A	CTCTCCTCTTTCTTTGTCCACCT--CGCAA--GCATGT	708
B	CTCTCGTTTCTTCGTGTCGTTTCTGCTAAAAATTGTGTGT	660
A	TCGCGATCACCCTCTTGTACGTTCTGCCATATT-----TTG	743
B	TTTTTTTTCTTTTTTGC TGGTTTGGTCATTTGGGGTGCCG	700
A	TTTGA-----TCC-----CATTTCTTCA--TGCCG	766
B	TTTGAGTTGTGAATCTGGAACAACATATATGTGATTGCCA	740
A	TT-ITTGGC--AATA-----ACTTTTGTATTAAAAA	797
B	TTGTTTCAITGCAATACAAAGAAGCCAGTATATCTAAAAA	780
A	AAAAAAAAAAAAAAAAAAAAAAAAAAAA	821
B	AAAAAAAAAAAAAAAAAAAAAAAAAAAA	805

Fig.2 Nucleotide sequences of two rice profilin cDNAs(*RproA* and *RproB*), isolated from a rice pollen library

The *RproA* is the longest cDNA.

<i>O. sativa</i>	MSWQTYVDEHLMCEIEG---HHLTSAAIVGHDGTVWAQSAAFPQFKPEEM	47
<i>Z. mays</i>	MSWQTYVDEHLMCEIEG---HHLSSAAIVGHDGAVWAQSTAFPQFKPEEM	47
<i>C. dactylon</i>	MSWQAYVDHLMCEIEG---HHLTSAAIIGHDGTVWAQSAAFPQFKPEEM	47
<i>P. pratense</i>	MSWQTYVDEHLMCEIEG---HHLASAAIIGHDGTVWAQSAAFPQFKPEEI	47
<i>H. brasiliensis</i>	MSWQTYVDEHLMCDIDG---HHLTAAAIIGHDGSVWAQSSSFQFKPEEV	47
<i>N. tabacum</i>	MSWQTYVDEHLMADIEGQOQHHLAAAIIGHDGSVWAQSSAFPQFKPEEI	50
<i>O. sativa</i>	TNIMKDFDEPGFLAPTGLFLGPTKYMVIQGEPGA VIRGKKGSGGITVKKT	97
<i>Z. mays</i>	TNIIGKDFDEPGFLAPIGLFLGPTKYMVIQGEPGA VIRGKKGSGGITVKKT	97
<i>C. dactylon</i>	ANIMKDFDEPGFLAPTGLFLGPTKYMVIQGEPGA VIRGKKGSGGVTVKKT	97
<i>P. pratense</i>	IGIMKDFDEPGH LAPTGMFVAGAKYMVIQGEPGA VIRGKKGAGGITIKKT	97
<i>H. brasiliensis</i>	AAIMKDFDEPGSLAPTGLIILGGTKYMVIQGEPGA VIRGKKGSGGITVKKT	97
<i>N. tabacum</i>	TNIMKDFDEPGH LAPTGLFLGGA KYMVIQGEPGA VIRGKKGSGGITIKKT	100
<i>O. sativa</i>	GQALVIGIYDEPMTPGQCNMVVERLGDYLVEQGL	131
<i>Z. mays</i>	GQALVIGIYDEPMTPGQCNMVVERLGDYLVEQGL	131
<i>C. dactylon</i>	GQALVIGIYDEPMTPGQCNMVIEKLGDYLI EQGM	131
<i>P. pratense</i>	GQALVIGIYDEPMTPGQCNMVVERLGDYLVEQGM	131
<i>H. brasiliensis</i>	GQALIIGIYDEPLTPGQCNMIVERLGDYLI EQGM	131
<i>N. tabacum</i>	NQALIFGIYEPEVTPGQCNMVVEKI GDYLVDOGY	134

Fig.3 Comparison of the rice pollen profilin amino acid sequences with other plant profilin sequences

The deduced amino acid sequence of rice profilin was compared with profilin sequences from *Z.mays*, *C.Dactylon*, *P.pratense*, *H.Brasiliensis*, *N.Tabacum*. Sequences were aligned using the DNASTar program. Identical amino acids are highlighted on a black background.

2.3 Southern blot analysis of the profilin gene family

Unlike most other organisms, in higher plants there are large multigene families for profilin and actin^[4,22]. Maize was predicted to have at least five profilin genes^[9] and six to eight actin isoforms^[23, 24]. In order to determine the size of the profilin multigene family in rice genomic DNA was digested with *EcoRI*, *BamHI*, *HindIII*, *PstI* and *XbaI* and probed with a cDNA fragment of the *RproA* and *RproB*, respectively (see materials and methods). Each probe hybridized to two or three bands. These results indicate that the profilin multigene family exist in the rice genome and have at least two or three profilin gene (Fig.4).

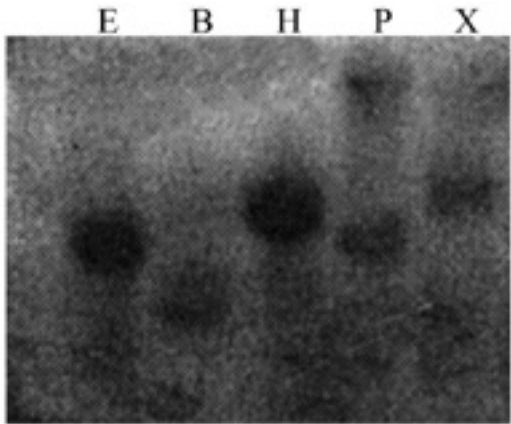


Fig.4 Southern blot analysis of rice genomic DNA digested with *EcoRI*, *BamHI*, *HindIII*, *PstI* and *XbaI*

Gene-specific probes made from digested *EcoRI/XbaI RproA* insert.

2.4 Analysis of the expression of the profilin gene in different organs of rice plants

The RNA gel blot analysis showed that a different expression pattern among various organs. The transcript was abundant in the pollen and anther, no transcripts present in roots, stems, leaves of rice plants. Identical results were also obtained through RT-PCR analysis. An abundance of *Rpro* transcript was only detected in pollen and anther, but not in other organs. These results indicated that the *Rpro* was a cDNA clone which was pollen specific(Fig.5). Identical results were also obtained in RT-PCR (Fig.6). An abundance of *Rpro* transcripts was only detected in pollen grains and anthers. Huang *et al* analyzed some plant profilin genes and had suggested two profilin gene classes(constitutive and pollen-specific) in plant. The pollen-specific gene in the gene class included profilin genes from the *Arabidopsis PRF4*, monocot maize (*Zma1-3*), wheat(*Tac*), and timothy grass(*Ppr*), and the dicots white birch(*Bve*) and tobacco(*Nta*). Similar results were also obtained in rice, in which genes could only be detected in mature and germinated pollen grains^[15]. Recent data indicated that profilin interacted with a soluble pollen component(s), resulting in dramatic alterations in the phosphorylation of a number of phosphoproteins. This implies a signaling role for profilin in angiosperm pollen^[25], suggests that profilin may act in a signaling capacity to regulate pollen tube growth through its modulation of protein kinase activity.

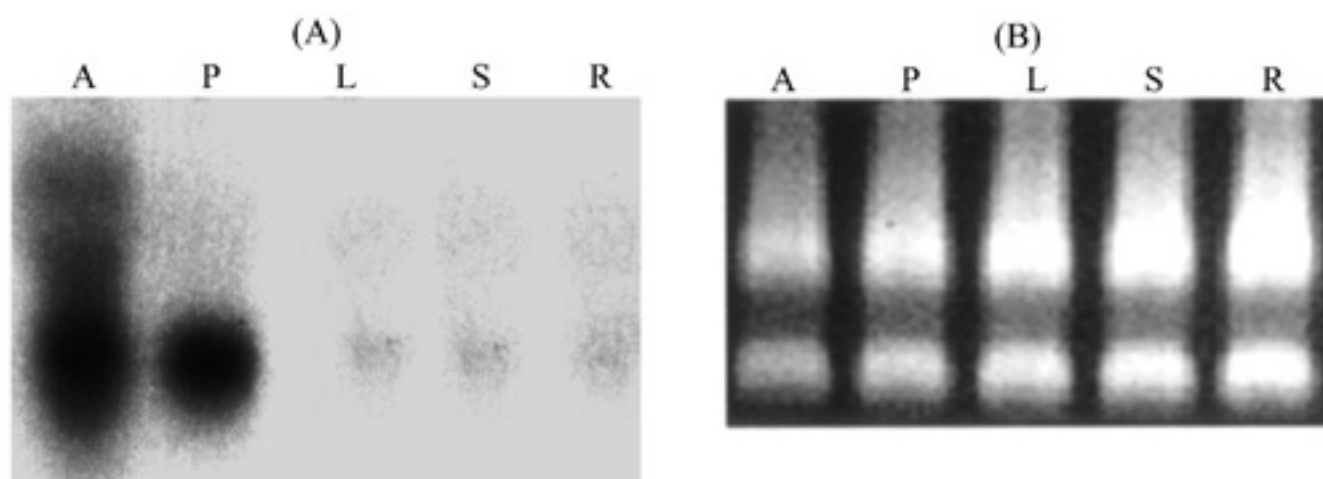


Fig.5 Northern blot analysis of profilin transcripts in various rice (IR 36) tissues and organs

(A) Northern blot analysis of total RNA (15 μ g in each lane) isolated from different tissue-specific expression of the profilin gene. Rice tissues: anthers(A), pollen grains(P), leaves(L), stems(S), root(R). The RNA was blotted onto a Hybond N⁺ membrane (Boehringer Mannheim) and probed with [³²P]-labeled *RproA* cDNA. A strong hybridization signal was detected in both pollen grains and anthers, but not in other organs. (B) Fifteen micrograms of total RNA were loaded in each lane and stained the gel with ethidium bromide.

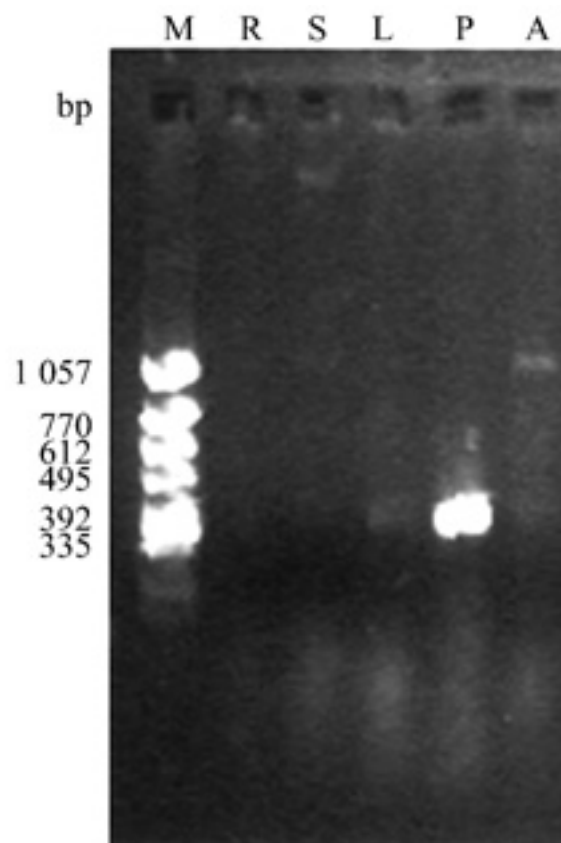


Fig.6 RT-PCR products amplified from rice RNA using a 366 bp long PCR fragment as primer
 0.5 µg RNA was used to synthesize the cDNA, the cDNA was amplified by PCR. The lanes are: Φ₁Á174-*Hinc*II digest DNA marker(M), root(R), stems(S), leaves(L), pollen grains(P), anthers(A). The RT-PCR products were shown in the pollen or anther.

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*Corresponding author: Tel, 86-28-5417281; Fax, 86-28-5417281; e-mail, chengfang@scu.edu.cn