

Flower-preferential poly(A) binding (PAB) protein gene from rice

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Abstract

The Poly(A) binding (PAB) protein functions in posttranscriptional regulation by stabilizing mRNA and initiating translation. Using sequence homology to *Arabidopsis PAB* genes, we have identified seven members (*OsPAB1* through *OsPAB7*) of the *PAB* gene family in rice. These can be divided into two groups based on sequence identity and expression pattern. The four genes in the first clade—*OsPAB2*, *OsPAB4*, *OsPAB6*, and *OsPAB7*—share strong homology with *AtPAB2*, and are expressed in all the organs that were examined here. The remaining three, *OsPAB1*, *OsPAB3*, and *OsPAB5*, show the specific expression pattern for flowering. *OsPAB1* is expressed throughout floral development, although its level decreases as the flowers mature. In contrast, expression of *OsPAB3* is evident in older flowers and developing seeds, while *OsPAB5* transcript is present only in mature flowers. All the genes in the second clade are expressed in carpels and stamens, but not in the paleae and lemmae of spikelets. In addition, we have identified a T-DNA insertional line, in which the *gus* reporter gene is fused to *OsPAB1* to produce the PAB1-GUS fusion protein. In those experiments, GUS staining was strong in the ovaries and anthers, demonstrating that *OsPAB1* is preferentially expressed in the reproductive organs.

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1. Introduction

Eukaryotic mRNAs are polyadenylated at their 3' ends in the nuclei; most are then exported to the cytoplasm. They function in association with poly(A) binding (PAB) proteins that attach to the poly(A) tail. These PAB proteins are involved in regulating mRNA stability [1–3]. For example, in yeast, a PAB protein apparently plays an essential role in degrading mRNA by shortening the 3' poly(A) tail [4]. This is followed by mRNA decapping and degradation [5–7]. In contrast, in a mammalian cell-free system, a decrease in mRNA stability results from PABP depletion [8]. Likewise, short-lived transcripts are degraded after losing their poly(A) tail [9], which indicates that this protein protects polyadenylated mRNAs from nucleolytic degradation.

PABP may also be involved in translation initiation. Positive cooperation between the cap and the poly(A) tail suggests an interaction between the cap-binding protein complex and the PAB protein [10,11]. In yeast, PAB1 appears to be related to poly(A) tail formation [12], poly(A) tail length maturation [13], mRNA stabilization [7,14], and translation initiation [2,15]. The PAB protein integrates with the translation initiation factor eIF4G [16], creating a functional link between the 5' cap and the 3' poly(A) tail by interacting with eIF4G (in yeast and plants) and with eIF4B (in plants) [4,17,18]. In the yeast system, The PAB protein–poly(A) tail complex assists eIF4E in bringing eIF4G to the mRNA and the 40 S ribosomal subunits in order to form the 48 S initiation complex [19,20]. These results suggest that the PAB protein may interact, directly or indirectly, with initiation factors that are located in the 5' cap, thereby prompting the complex to activate translation [15,18,21,22].

PAB proteins have been identified in diverse eukaryotic organisms. Their N-terminals contain four well-conserved RNA-recognition motifs (RRMs), each of

Abbreviations: GUS, β -glucuronidase; IF, initiation factor; PABP, poly(A) binding protein.

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which includes two RNA-binding motifs (RNPs) that play a role in contacting single-stranded RNA and unpaired ribonucleotides [23]. These PAB-specific sequences distinguish them from other RNA-binding proteins. The C-terminal regions are diverged in PAB proteins [24].

Approximately 20 different *PAB* genes are present in *Arabidopsis* [25–27], with the expression patterns of four of them having been studied so far. *AtPAB1*, *AtPAB3*, and *AtPAB5* genes are expressed organ-preferentially. *AtPAB3* and *AtPAB5* transcripts are present in floral organs, especially immature flowers, while the *AtPAB1* gene is expressed in roots and, weakly, in immature flowers [25,26]. In contrast, *AtPAB2* is expressed ubiquitously, including the roots, stems, leaves, flowers, pollens, and siliques [27,28]. Researchers have also used yeast *PAB* mutants to conduct functional studies of *PAB* genes in plants. For example, the loss of viability due to the disrupted molecular functions of the yeast *pab1* gene was complemented by the wheat *PAB1* gene as well as the *Arabidopsis PAB2* and *PAB5* genes [17,26,29]. The objective of the study presented here was to identify *PAB* genes in rice (*Oryza sativa*), based on their sequence homology with *AtPAB* genes. We also wished to elucidate the expression patterns of those rice genes in order to deduce their putative functional roles during plant development.

2. Materials and methods

2.1. Protein sequence analysis

The tblastn program (<http://tigrblast.tigr.org/eukblast>) was used to identify the *OsPAB* family genes in rice. The TIGR rice database offered seven clones that were highly homologous to *AtPAB5*. Using the genome automated annotation system (<http://ricegaas.dna.affrc.go.jp>), seven sequences were annotated. The amino acid sequences were aligned with the BCM search launcher program (<http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html>), and alignments were prepared with BOXSHADE (<http://www.ch.EMBLnet.org>). The phylogenetic tree was generated with Clustal X version 1.81 (<http://www-igbmc.u-strasbg.fr/BioInfo>) and MEGA version 2.1 (<http://www.megasoftware.net>).

2.2. Isolation of cDNA clones

Rice (*O. sativa* var. japonica cv. Hwayoung) plants were used for RNA isolation. A tri-reagent (MRC Inc. Cincinnati, OH, USA) was used for isolating total RNA from anthers before heading. Total RNAs were treated with RNase-free DNaseI at 37 °C for 20 min, followed by heating at 65 °C for 10 min. Afterward, 3 µg of

DNA-free RNAs were reverse-transcribed in a total volume of 25 µl reaction buffer (Promega Co., Madison, WI, USA) that contained 10 ng of oligo (dT)_{12–18} primer, 2.5 mM dNTPs, and 200 U of Moloney murine leukemia virus RT (New England Biolabs, Beverly, MA, USA). The samples were incubated at 37 °C for 1 h, then heated at 100 °C for 5 min. Sets of primers were designed to isolate the *OsPAB1*, *OsPAB3*, and *OsPAB5* clones. For *OsPAB1*, the sense primers were (5'-GCAGCCTTCAAAGGAACTG-3'), (5'-CAGTTG-CAGGCACAATTACG-3'), and (5'-CAAAGCTGTC-CAACACATGG-3'); and the antisense primers were (5'-ATGTTCGATTTGTCCCTGCTC-3'), (5'-CCATG-TGTTGGACAGCTTTG-3'), and (5'-GCGTACAGC-TGGA-ACAACAA-3'). For *OsPAB3*, primers included (5'-TGTACGTGGGAGACCTGGAA-3') and (5'-GC-AGAAGCCAAGGAAGTCTG-3'); for *OsPAB5*, the primers were (5'-GCAACTCCCTCAGATATGG-3') and (5'-TTGGCGTTTACACCAGCAGA-3'). The reaction was performed at 94 °C for 5 min, followed by 33 cycles of PCR (94 °C, 45 s; 57 °C, 45 s; and 72 °C, 90 s), and a final 10 min at 72 °C.

2.3. RT-PCR analysis

Total RNAs were isolated from vegetative and reproductive organs of wild-type rice plants [30], and cDNA templates were synthesized using total RNA and reverse transcriptase. Reverse transcriptase-mediated PCR (RT-PCR) was performed in a 50-µl solution containing a 1-µl aliquot of the cDNA reaction, 0.2 µM of gene-specific primers, 10 mM dNTPs, and 1 U of rTaq DNA polymerase (TakaRa Shuzo, Shiga, Japan). The reaction included an initial 5-min denaturation at 94 °C, followed by 25 cycles of PCR (94 °C, 30 s; 56 °C, 30 s; and 72 °C, 1 min 30 s), and a final 10 min at 72 °C. Ten microliters of the reaction mixture was separated on a 1.2% agarose gel, then transferred to a Hybond N+ nylon membrane (Amersham, Buckinghamshire, UK). The blot was hybridized at 60 °C for 12 h in Church buffer [31], using a P³²-labeled probe. Membranes were washed once for 10 min with 0.2 × SSC and 0.1% SDS at 25 °C, and twice, for 10 min each, with 0.2 × SSC and 0.1% SDS at 58 °C. These washed membranes were then exposed at RT for 30 min on X-ray film. The following oligonucleotide primer sets were designed based on the genome sequence, and were used for gene-specific amplification of the transcripts: *OsPAB1*, (5'-GCAG-CCTTCAAAGGAACTG-3') and (5'-ATGTTCGATT-GTCCCTGCTC-3'); *OsPAB2*, (5'-GTTGAGGGAG-CTTTTGTCTG-3') and (5'-CGTCATTCAGCGACA-GAGAA-3'); *OsPAB3*, (5'-CTTTTGGCACAATCCT-CTCC-3') and (5'-TTCTCTCCTCCTTCCGTTGA-3'); *OsPAB4*, (5'-TGTACTCAAACCGTGACCCA-3') and (5'-ATTGGAGCAGGTGACAAACC-3'); *OsPAB5*, (5'-AAGTGGGGTTGGAAATGTG-3') and (5'-

GAATTCTTTAGCTGGCTGCG-3'); *OsPAB6*, (5'-TGTACTCAAACCGTGACCCA-3') and (5'-ATTG-GAGCAGGTG-ACAAACC-3'); *OsPAB7*, (5'-CGCC-TATGTCAACTTCAGCA-3') and (5'-ACCATA-GG-ACGCATTTGTG-3'); and actin, (5'-TCCATCTTGG-CATCTCTCAG-3') and (5'-GTACCCGCATCAGG-CATCTG-3').

2.4. Isolation of the T-DNA tagging line and flanking sequences

T-DNA tagged rice (*O. sativa* var. japonica cv. Hwayoung) plants were generated by the *Agrobacterium*-mediated co-cultivation method with the pGA2707 vector [32,33]. GUS positive lines in floral organs were selected. Genomic DNA was extracted from immature leaves of the GUS positive lines [34] using the MM300 Mixer Mill homogenizer (Retsch, Haan, Germany). The genomic sequence flanking T-DNA was isolated by inverse PCR [35]. Briefly, 1 µg of the genomic DNA was digested with *Pst*I. After overnight digestion, the sample was ethanol-precipitated and dissolved in water. Self-ligation of the cut DNA was performed in a 50 µl volume containing 1 U of ligase (Roche) and 5 µl of 10 × buffer at 8 °C for 12 h. About 20 ng of the ligated DNA was used for template. PCR was performed with an initial 5-min denaturation at 94 °C, followed by 35 cycles (each cycle: 94 °C, 1 min; 62 °C, 1 min; and 72 °C, 3 min); then a final 10 min at 72 °C. The PCR primer sequences were 5'-TTGGGGTTTCTACAGGACG-TAAC-3' and 5'-GAACCCGCTCGTCTGGCTAA-GATC-3'.

2.5. Genotyping

A T-DNA border-specific primer, GUS1 (Primer *c*, 5'-TTGGGGTTTCTACAGGACGTAAC-3'), and gene-specific primers [the sense, (Primer *a*, 5'-TTGAT-GCCATAACCAGCGTA-3') and the antisense, (Primer *b*, 5'-ATCACAGGGATCCCCACATA-3')] were used for genotyping. The GUS1 primer and the sense primer amplified the 1.2-kb genomic DNA, while the sense and antisense primers amplified the 1.6-kb band. PCR parameters were as follows: 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 2.5 min; followed by 72 °C for 10 min.

2.6. Assay for GUS activity

Histochemical GUS assay was performed as published previously [36]. Flowers and leaves were stained using β-glucuronidase (GUS) with 2.4 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc) [37] in the buffer [100 mM sodium phosphate (pH 7.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.5% Triton-X 100, 10 mM EDTA (pH 8.0), 0.1% X-

gluc, 20% MeOH, and 2% DMSO]. Each sample was incubated in the GUS assay solution at 37 °C for 12 h. Chlorophyll was removed in 70% ethanol, and the samples were examined under a dissecting microscope.

2.7. Microscopic techniques

The GUS-stained samples were dehydrated in series with 50, 75, 85, 95, and 100% EtOH, then infiltrated serially with 25% xylene/75% EtOH, 50% xylene/50% EtOH, 75% xylene/25% EtOH, and 100% xylene. Following the infiltration with xylene alone, the samples were embedded in paraffin (Paraplast X-tra, OXFORD Labware) [38,39]. Ten-micrometer sections were attached to gelatin-coated glass slides and de-paraffinized in xylene. After mounting the samples with permount solution, light microscopy was performed with a Nikon COOLPIX 5000 (Nikon Co., Tokyo, Japan).

3. Results and discussion

3.1. Isolation of PAB genes

Rice genome sequences at the TIGR rice database were searched for putative *PAB* genes, using *AtPAB5* as quarry. From this analysis, seven candidates were selected and their putative products were deduced (Fig. 1). These genes were designated as *OsPAB1*–*OsPAB7* (Table 1). The cDNA clones for *OsPAB2*, *OsPAB4*, *OsPAB6*, and *OsPAB7* are present in the rice EST database (<http://www.ncbi.nlm.nih.gov/blast>) (Table 1). Because EST data are not available for the other three, their cDNAs were obtained from anthers, via PCR with gene-specific primers located at the 5' and 3' ends of each ORF (Fig. 1). The amino acid sequences of the cDNAs were consistent with the predicted products.

Aligning the amino acid sequences of the seven PAB proteins revealed that *OsPAB1* is distinctive from, and longer than, the other six. Whereas *OsPAB2* through *OsPAB7* comprise 456–672 residues, *OsPAB1* is 956 residues long. N-terminal regions are conserved (Fig. 1) and contain four RRMs, in which the RNP2 and RNP1 domains are conserved [40,41]. However, the RNP2 motif (LYVGD L) in the first RRM is not visible in *OsPAB1*, *OsPAB3*, or *OsPAB6*. Therefore, it is evident that the RNP2 motif in the first RRM was altered during evolution. Because the residue appears to be invariant within RRM, this alteration may be associated with functional diversification of the proteins [41].

3.2. Distance analysis of rice PAB genes

To determine the evolutionary relationships among rice PAB proteins and compare them with *Arabidopsis* PAB proteins, phylogenetic analysis was performed

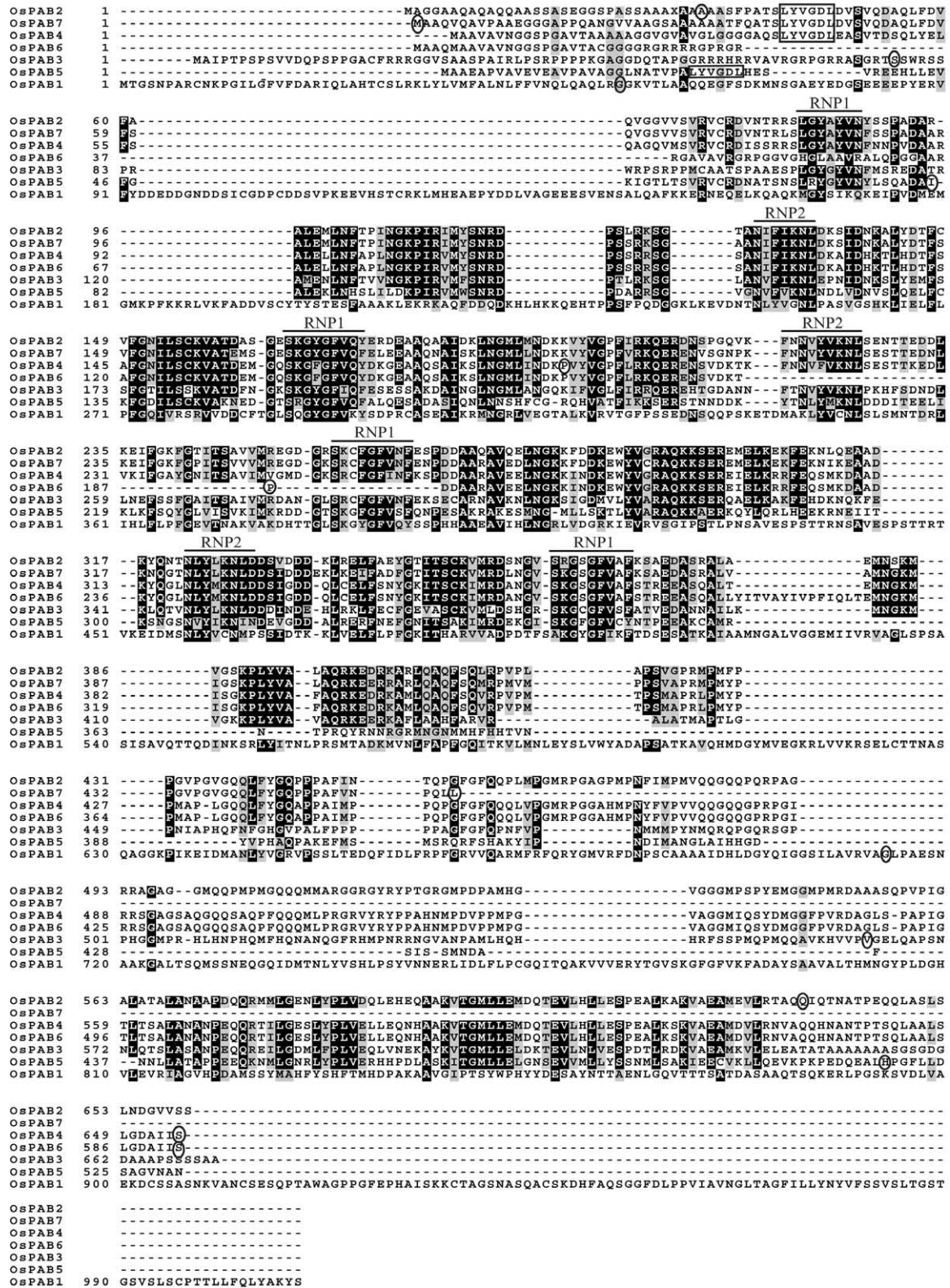


Fig. 1. Alignment of seven putative poly(A) binding proteins in rice. Conserved residues are black-shaded; functionally conserved residues, gray-shaded. The lines above conserved sequences indicate the RNP2 and RNP1 motifs, respectively. Rectangular boxes (LYVGDL) show the RNP2 motif of the first RRM domain in OsPAB2, OsPAB4, OsPAB5, and OsPAB7. Ovals encircling an amino acid sequence indicate the start and end points of the EST clones or the amplified transcripts.

Table 1
Seven rice PAB genes, their accession numbers and EST names present in rice databases

Gene name	Accession number	EST name
<i>OsPAB1</i>	AL732352	NF
<i>OsPAB2</i>	AP005569	AU032638, OD101C01
<i>OsPAB3</i>	AP003622	NF
<i>OsPAB4</i>	AL606604	AU057898, AU076143
<i>OsPAB5</i>	AC083942	NF
<i>OsPAB6</i>	AL731638	AU057899
<i>OsPAB7</i>	AP004228	AU075435

NF: Not found.

(Fig. 2). *Arabidopsis* PAB proteins had previously been divided into two clades: (1) AtPAB2, and (2) AtPAB3 and AtPAB5 [29,41]. Between these two clades, approximately 50% similarity is present. In this study, we found that four of our rice proteins—*OsPAB2*, *OsPAB4*, *OsPAB6*, and *OsPAB7*—also could be grouped with AtPAB2, which suggests that these rice proteins may play roles similar to that of AtPAB2. Although their divergence was substantial, *OsPAB1*, *OsPAB3*, and *OsPAB5* ultimately were grouped with *Arabidopsis* second-clade AtPAB3 and AtPAB5. Again, as noted above, the *OsPAB1* protein showed extreme divergence in sequence compared with the other PAB proteins and there was no obvious counter part of *OsPAB1* in *Arabidopsis*.

3.3. PABP expression patterns

Expression patterns of the rice *PAB* genes were studied with RT-PCR analysis, using gene-specific primers and various cDNA templates. Actin primers were used as controls to show that a similar amount of cDNA was used. The experiment demonstrated that the first-clade genes, i.e. *OsPAB2*, *OsPAB4*, *OsPAB6*, and *OsPAB7*, were expressed in all the organs examined (Fig. 3). This result is consistent with a previous report [28] that the first-clade *Arabidopsis* PAB gene, *AtPAB2*, also is expressed in both vegetative and reproductive tissues. The similarity in protein sequences and expression patterns suggests that first-clade rice PAB genes either are functionally redundant or may assemble in the same cell type for different molecular functions.

Second-clade rice *PAB* genes were expressed preferentially in reproductive tissues. *OsPAB1* transcript was present throughout panicle and seed development, with levels being highest when the panicles were young, but decreasing with their further development. *OsPAB3* transcript was not visible when panicles were < 8 cm, but began to accumulate when those tissues were 8–13 cm long. Its level then remained relatively at a more or less similar level until the stages of flower maturation and seed development. In contrast, the expression pattern of *OsPAB5* was distinct, in that it was specifically expressed in mature panicles. In the spikelets, all three rice PAB genes were expressed in both stamens and carpels, but not in the paleae and lemmiae. Similar

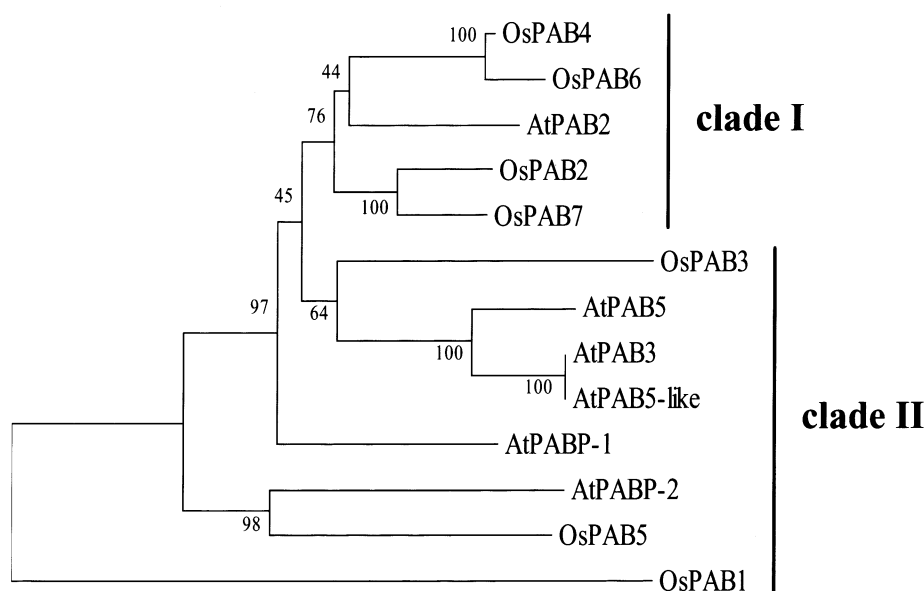


Fig. 2. Phylogenetic analysis of PAB proteins from rice and *Arabidopsis*. Rice and *Arabidopsis* PABPs are divided into clade I and II according to the previous report [41]. Clade I proteins are related to AtPAB2, whereas Clade II proteins are related to AtPAB3 and AtPAB5.

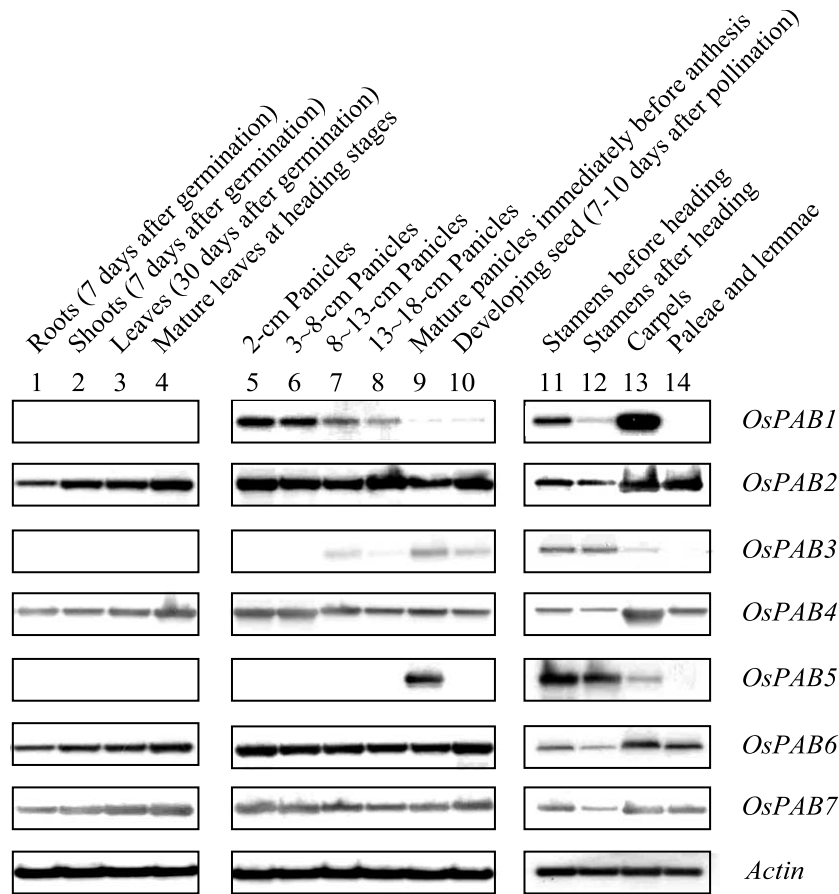


Fig. 3. RT-PCR analyses of the transcript levels of seven rice PAB genes. PCR was performed with cDNA prepared from various vegetative and reproductive organs as indicated in the figure.

floral organ-specific expression patterns have been observed from the second-clade *Arabidopsis* genes, *AtPAB3* and *AtPAB5* [25,26].

3.4. Analysis of transgenic plants carrying the *OsPAB1*–*gus* gene fusion

We selected the *OsPAB1* gene for further analysis because it is expressed preferentially in floral organs, and its protein structure is distinctive from the other PAB proteins. Among the characterized *Arabidopsis* PAB genes, *OsPAB1* is most closely related to *AtPAB5*. It has been speculated that *AtPAB5* is associated with reproductive development, based on observations of oocyte or sperm differentiation in animal species [25,42,43]. To study the detailed expression pattern of *OsPAB1*, we screened T-DNA tagged rice lines that were generated by the binary T-DNA vector pGA2707 [33]. The vector contains three splicing donor/acceptor sites and the promoter-less *gus* gene immediately next to the right border, so that insertion of T-DNA within an exon or intron could generate a fusion transcript that is capable of producing a protein containing GUS activity [33,36,44]. After GUS-screening 11 182 insertional lines,

we identified *gus*-positive lines that showed flower-preferential expression pattern. Sequencing T-DNA

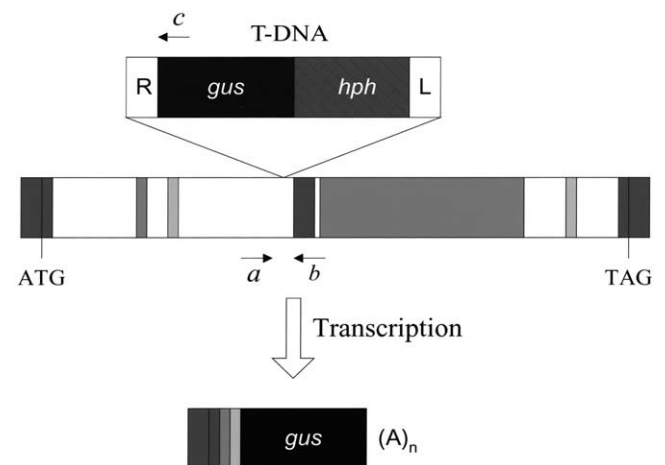


Fig. 4. Schematic diagram of *OsPAB1* and the position of T-DNA insertion. The seven exons are shaded, and six introns are indicated with open boxes. T-DNA integrated into the 3rd intron is indicated above the *OsPAB1* gene. The T-DNA contained *gus* and hygromycin resistance (*hph*) genes between the right (R) and left (L) borders. The arrows indicate three primers (*a*, *b*, and *c*) used for genotyping of the progeny.

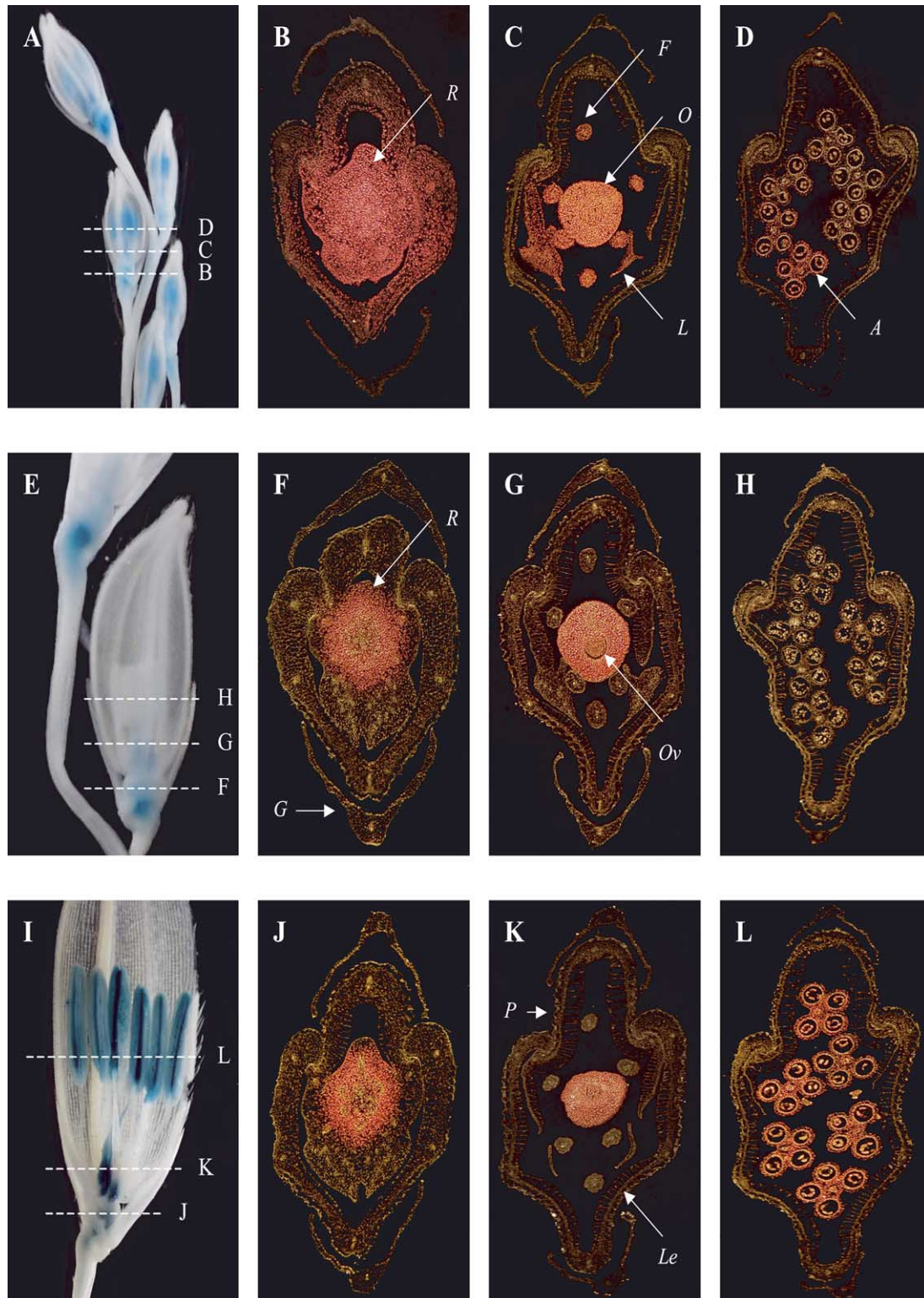


Fig. 5. Analysis of GUS activity in the spikelets of *OsPab1-gus* transgenic plants. Spikelets were harvested from 3 to 8-cm panicles (A–D), 8 to 13-cm panicles (E–H), and 13 to 18-cm panicles (I–L), and were GUS-stained and examined under a dissecting microscope (A, E, and I). Spikelets were sectioned transversally at the bottom (B, F, and J), middle (C, G, and K) and upper (D, H, and L) portions of the spikelets as indicated in Figures A, E, and I. *A*, anther; *F*, filament; *G*, glume; *L*, lodicule; *O*, ovary; *Ov*, ovule; *P*, palea; *Le*, lemma; *R*, rachilla. GUS activity under the dark field appeared as pink or red (B–D, F–H, J–L).

junction regions of the flower-preferential lines resulted in identification of one line containing a T-DNA insertion into the *OsPABI* gene. The insertion occurred in the 3rd intron of *OsPABI* and the direction of the *gus* reporter gene was the same as that of *OsPABI* (Fig. 4).

Because our RT-PCR analysis had also revealed that *OsPABI* expression was confined to floral tissue, we analyzed transgenic flowers that carried a tag in *OsPABI* to examine whether the *OsPABI*–*gus* gene fusion could produce a functional GUS enzyme. Histochemical GUS analysis showed that GUS activity was observed in the inner floral organs throughout spikelet development (Fig. 5A, E, and I). However, the reporter gene was not expressed in vegetative organs, indicating that the *OsPABI* gene is not active in non-reproductive organs (data not shown).

To study the detailed expression pattern of the gene, the stained spikelets were sectioned transversally. In young spikelets (panicle size of 3–8 cm), GUS staining was detected in the rachilla, ovaries, lodicules, filaments, and anthers (Fig. 5B–D), and persisted in the rachilla and ovaries from spikelet development until the mature stage. In contrast, expression was diminished in lodicules, filaments, and anthers in the spikelets of 8–13-cm panicles (Fig. 5G, H). Interestingly, reporter gene expression was again detected in anthers within the spikelets of 13–18-cm panicles (Fig. 5L). These staining patterns indicated that GUS activity was present in active cells during flower development, an observation

that agrees with previous reports of PAB genes being preferentially expressed in active cells. In *Arabidopsis*, the *AtPAB2* gene also is strongly expressed in actively dividing cells [28]. Other studies have shown that PAB mRNA is up-regulated in a rapidly dividing pancreatic cancer cell line [45]. Based on our results, we believe that the *OsPABI* gene may play significant roles in the highly active cells of spikelets, i.e. sites where post-transcriptional regulation, including poly(A) tail processing and translation initiation, vigorously occurs [26,29,43].

Because T-DNA insertion into a gene may destroy its function, we genotyped T2 progeny of the line carrying that T-DNA insertion in the *OsPABI* gene (Fig. 6A). DNA prepared from seedlings was PCR-amplified using Primers *a* and *b*, which are located in the 3rd intron of *OsPABI*, and Primer *c*, located in T-DNA near the right border (Fig. 4). The first two were able to amplify the 1.6-kb fragment if no insertion was made between them (Fig. 6A; Plants 3, 5, 7, 8, 10, and 11). However, if the plant carried the T-DNA insertion in the *OsPABI* gene, the primers could not amplify a band because the distance between the two was approximately 7.9 kb. In that case, Primers *a* and *c* amplified the 1.2-kb fragment instead (Fig. 6B; Plants 1–6, 9, and 11). Moreover, when both the 1.2- and the 1.6-kb bands were amplified, the plants were heterozygous for the T-DNA insert (Plants 3, 5, and 11), but, if only the 1.6-kb band was amplified, the resultant plants were wild-type segregants (Plants 7,

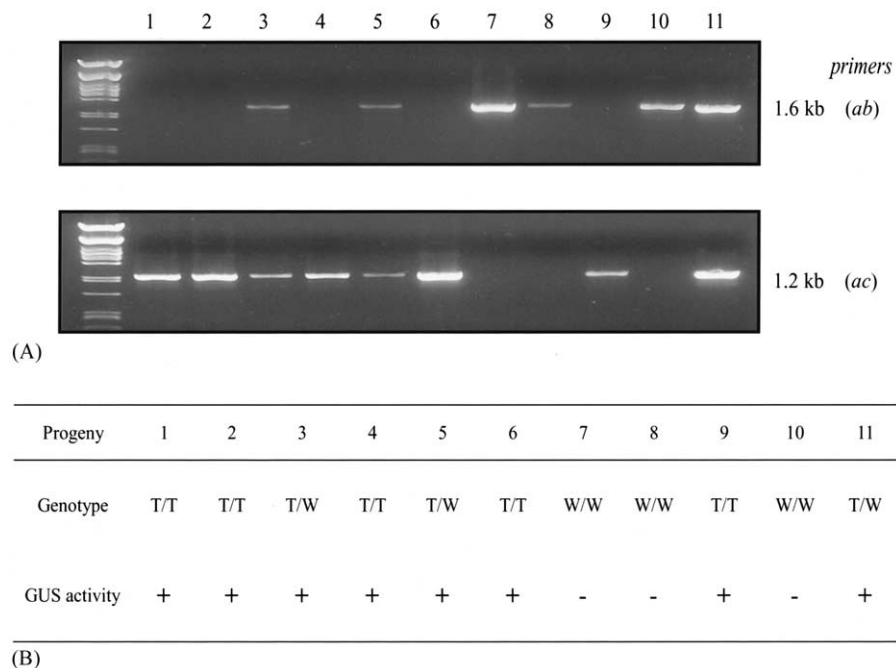


Fig. 6. Genotyping and GUS activity in progeny of the *OsPABI*–*gus* tagged line. (A) Eleven plants from the *OsPABI*–*gus* tagged line were genotyped with Primers *a* and *b* (upper) or Primers *a* and *c* (lower). The 1.6-kb bands are PCR products using Primers *a* and *b*, and the 1.2-kb bands are PCR products using Primers *a* and *c*. Lanes 1, 2, 4, 6, and 9 are homozygous; Lanes 3, 5, and 11, heterozygous; Lanes 7, 8, and 10, wild-type plants. (B) Comparison of genotypes and GUS activity. T/T, T/W, and W/W indicate homozygote, heterozygote, and wild-type plants, respectively. GUS-positive, (+); GUS-negative, (–).

8, and 10). Those with the 1.2-kb band were homozygous for T-DNA (Plants 1, 2, 4, 6, and 9).

Our GUS analysis of developing spikelets also showed that the homozygous and heterozygous plants were GUS-positive whereas the wild types were GUS-negative, thereby demonstrating that GUS expression cosegregates with T-DNA (Fig. 6B). Because of the malfunction of the OsPAB1 protein, we had expected a phenotypic alteration in the T-DNA-inserted progenies. However, we could detect no drastic changes in appearance among either the homozygous or the heterozygous plants growing under normal conditions. Between the mutants and the corresponding wild type segregants, there were no differences in seed fertility and morphology of reproductive organs (Fig. 5). It is possible that the T-DNA insertion within the third intron of *OsPAB1* did not completely inhibit expression of the gene by correctly splicing out the intron carrying the T-DNA. Alternatively, the other PAB genes complement the defects because the *OsPAB2*, *OsPAB5*, *OsPAB6*, and *OsPAB7* genes all were ubiquitously expressed in the flowers throughout spikelet development. It is also possible that the mutant phenotype may become visible when the knockout plants are grown in a certain stress environment, though we did not examine the phenotypic changes under various stress conditions.

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