

Genome-wide survey of the RIP domain family in *Oryza sativa* and their expression profiles under various abiotic and biotic stresses

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Abstract Ribosome-inactivating proteins (RIPs) are *N*-glycosidases that inhibit protein synthesis by depurinating rRNA. Despite their identification more than 25 years ago, little is known about their biological functions. Here, we report a genome-wide identification of the RIP family in rice based on the complete genome sequence analysis. Our data show that rice genome encodes at least 31 members of this family and they all belong to type 1 RIP genes. This family might have evolved in parallel to species evolution and genome-wide duplications represent the major mechanism for this family expansion. Subsequently, we analyzed their expression under biotic (bacteria and fungus infection), abiotic (cold, drought and salinity) and the phytohormone ABA treatment. These data showed that some members of this family were expressed in various tissues with differentiated expression abundances whereas several members showed no expression under normal growth conditions or various environmental stresses. On the other hand, the expression of many RIP members was regulated by various abiotic and biotic stresses. All these data suggested that specific members of the RIP family in rice might play important roles in biotic and abiotic stress-related biological processes and function as a regulator of various environmental cues and hormone signaling. They may be potentially useful in improving plant tolerance to

various abiotic and biotic stresses by over-expressing or suppressing these genes.

Keywords Biotic stress · Abiotic stress · Ectopic expression · Ribosome-inactivating protein · Rice

Abbreviations

GUS β -glucuronidase
Mg *Megnaporthe grisea*
RIP Ribosome-inactivating proteins
Xoo *Xanthomonas oryzae* pv *oryzae*

Introduction

Ribosome-inactivating proteins (RIPs) were named due to their ability to inactivate ribosomes. These enzymes are *N*-glycosidases that can damage ribosomes in an irreversible manner by removing one or more adenine residues from rRNA and they may also depurinate other polynucleotides. It is now known that all RIPs are structurally related and were identified as a family. The early-identified RIPs were ricin, abrin and PAP (Pokeweed Antiviral Protein; Lin et al. 1971; Montanaro et al. 1973; Obrig et al. 1973). Subsequently, many other proteins with similar enzyme properties were identified from various plants, fungi, algae and bacteria (Nielsen and Boston 2001; Peumans et al. 2001; Girbes et al. 2004; Motto and Lupotto 2004; Park et al. 2004; Stirpe and Battelli 2006). Based on their structures, these family members can be grouped into two types: type 1 and type 2 (Girbes et al. 2004; Stirpe and Battelli 2006). The type 1 RIPs inhibit cell-free protein synthesis and contain only a single-chain with non-toxicity

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relatively to cells and animals. The type 2 RIP proteins consist of two chains with higher toxicity relatively. Chain A is similar to the chain in type 1 RIPs and chain B is a lectin chain. In a few reports (Nielsen and Boston 2001; Park et al. 2004), type 3 RIPs were proposed including maize protein b-32 (Walsh et al. 1991) and JIP60 (Reinbothe et al. 1994). However, the third type was considered as peculiar type 1 RIPs (Stirpe and Battelli 2006) therefore, we have used the old RIP nomenclature in this study.

The ability of *N*-glycosidase to depurinate the sarcin/ricin (S/R) loop of the large rRNA of prokaryotic and eukaryotic ribosomes is a common enzymatic activity for all RIPs. Besides this, RIPs have also been suggested to process nuclease (Peumans et al. 2001) and superoxide dismutase (SOD) activities (Li et al. 1996, 1997; Sharma et al. 2004; Barbieri et al. 2006). However, no conclusive results were reported about these activities.

RIP genes either exhibit tissue-specific expression or are expressed in various tissues of a plant. For example, a gene encoding ricin is expressed only in seeds of *Ricinus* plants, whereas another gene encoding saporin can be detected in all tissues of soapwort (Ferrerias et al. 1993). In rice, only one member of RIP family was identified and characterized (Ding et al. 2002). This gene was expressed only in tapetum layer of anther tissues. In some plants, RIP genes were up regulated in senescent tissues (Chaudhry et al. 1994; Stirpe et al. 1996; Rippmann et al. 1997). More interestingly, RIP genes were frequently reported to be regulated by various abiotic stresses including drought/polyethylene glycol (PEG) (Bass et al. 2004; Wei et al. 2005), salinity (Rippmann et al. 1997), H₂O₂ (Iglesias et al. 2005) and heat or osmotic stress (Stirpe et al. 1996). RIP genes were also regulated by various biotic stresses including wounding (Song et al. 2000), various viruses (Iglesias et al. 2005; Mendez and Girbes 2005; Girbes et al. 1996), Fungi (Vivanco et al. 1999; Wei et al. 2005; Xu et al. 2007), insects (Dowd et al. 1998; Gatehouse et al. 1990; Kumar et al. 1993; Zhou et al. 2000) and microorganisms (Wong et al. 1995). In addition, some hormones can be regarded as expression regulators of RIP genes including jasmonic acid (Reinbothe et al. 1994; Gorschen et al. 1997; Muller et al. 1997; Song et al. 2000; Vepachedu et al. 2003; Xu et al. 2007), abscisic acid (ABA) (Muller et al. 1997; Song et al. 2000; Xu et al. 2007) and gibberellic acid (Ishizaki et al. 2002).

Accumulated data imply that RIP genes may play important roles in response to various environmental stresses. In addition to these, data also showed that RIPs might act against viruses (Song et al. 2000; Iglesias et al. 2005; Huang et al. 2006). Thus, RIPs might be a potential antiviral agent and RIP genes might be utilized to improve plants in their resistances to various environmental stresses

by their ectopic/over-expressions. Expression of a Barley RIP gene leads to increased fungal protection in transgenic tobacco plants (Logemann et al. 1992). Transgenic tomato and potato plants carrying a pokeweed RIP gene *PAP* showed resistance to infection by various viruses (Lodge et al. 1993). The increased protection against fungal pathogen *Rhizoctonia solani* was reported by expressing a RIP gene in tobacco (Jach et al. 1995). Transgenic tobacco plants expressing the maize RIP gene *b-32* showed an increased tolerance against infection by a soil-borne fungal pathogen *Rhizoctonia solani* Kuhn (Maddaloni et al. 1997). Expression of *PAP* led to overproduction of pathogenesis-related (PR) proteins and conferred fungal resistance in transgenic tobacco (Zoubenko et al. 1997). Interestingly, the *PAP*-mediated resistance to pathogen infection might occur in the absence of ribosome binding, rRNA depurination and acidic PR protein production (Zoubenko et al. 2000). Ectopic expression of a RIP gene from iris bulbs (*IRIP*) in tobacco (*Nicotiana tabacum* cv. *Samsun*) also significantly lowered the number of lesions compared to control plants (Desmyter et al. 2003). The increased resistance by expressing a foreign RIP gene was also reported in transgenic rice carrying a RIP gene from *Trichosanthes kirilowii* (Yuan et al. 2002). However, Low or lack protection against infection by fungal pathogen *Erysiphe graminis* was observed in transgenic wheat plants expressing a barley seed RIP gene (Bieri et al. 2000). Similar result was reported in transgenic rice and tomato plants carrying maize or iris RIP gene, respectively (Kim et al. 1999; Schaefer et al. 2005).

Taken together, transgenic analyses were focusing on anti-viral resistance and no data showed the effect of RIP genes on other biotic and abiotic stresses. On the other hand, although more than 130 RIPs or their genes were purified or identified (Girbes et al. 2004), reported data were mainly focusing on the analyses of enzymatic activities, genome-wide identification and expression analysis under various biotic and abiotic stresses data were not reported so far. In this study, we reported the identification of the RIP family members in rice based on the complete genome sequence analysis. Subsequently, we analyzed their expression under biotic (bacteria and fungi) and abiotic (cold, drought and salinity) stresses as well as following the phytohormone ABA treatment. Our data showed that only type 1 RIP genes are present in rice genome and some of them might have evolved into pseudogenes since they show no expression under normal growth conditions or various environmental stresses. Expression analyses showed that some members of rice RIP family might play important roles in biotic stress-related biological processes and function as a regulator of various environmental cues and hormone signaling.

Materials and methods

Plant materials and growth conditions under normal and stress treatments

Rice (*Oryza sativa*) variety Nipponbare (*Japonica* rice) was used for all the experiments. Rice seeds were germinated in water and planted in greenhouse under natural growth conditions. For drought treatment, 2-week-old seedlings were treated with 30% PEG to simulate water stress and whole plants were collected at various time intervals (0, 0.5, 1, 2, and 3 h), and then frozen with liquid nitrogen prior to total RNA preparation. For salinity, cold and ABA treatments, the similar staged seedlings were subjected to 250 mM NaCl solution, 4°C condition, or 100 μM ABA-containing solution, respectively. Samples for RNA extractions were collected at 0, 2, 4, 8, and 16 h time intervals.

For biotic stress, similar staged seedlings were transferred to growth chamber under 14 h light/10 h dark and at 22–28°C conditions. After 2 days, plants were inoculated with *Megnaporthe grisea* (Mg) raceGUY11 (Kindly provided by Dr Naweed Isaak Naqvi) and samples were collected for RNA extractions at 0, 2, 4, 6, and 8-day intervals. About 4-week-old plants were inoculated with *Xanthomonas oryzae* pv *oryzae* (Xoo) strain PX099A (Kindly provided by Dr. Zhongchao Yin) and samples were collected in 0, 1, 2, 4, and 6-day intervals.

The *OSRIP18* promoter expression analysis

Around 2300 bp promoter region upstream of the *OSRIP18* gene was amplified from genomic DNA by PCR using primers anchoring the region upstream of the ATG start codon of corresponding genes (primer sequences were listed in Supplemental Table S1). After sequencing and verification, the amplified fragments were cloned upstream of GUS reporter gene, and then sub-cloned into pCAM-BIA1300 Ti-derived binary vector for transformation. This vector was transformed into scutellum derived rice calli via *Agrobacterium* mediated transformation. The plantlets regenerated were transferred into soil and T₀ plants were obtained. Both T₀ and T₁ plants were used for GUS histochemical analysis. Leaves, panicles and roots at different developmental stages were freshly collected from promoter-GUS transgenic plants and then incubated with histochemical staining solution (0.02 M 5-bromo-4-chloro-3-indolyl-bb-D-glucuronide, 0.1 M NaH₂PO₄, 0.25 M ethylenediaminetetraacetic acid (EDTA), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1.0% (v:v) Triton X-100, pH 7.0) for 24–36 h at 37°C as described by Jefferson (1987).

After GUS staining, transverse sections of anthers from WT and transgenic plants were prepared according to the manufacturer's instructions (Leica HistoResin Kit). Florets at different stages were dehydrated through an ethanol series and then embedded into Leica HistoResin and sectioned with Leica microtome. The 3–6 μm thick sections on slides were observed under Leica microscope, and photographed with a Nikon digital camera.

Real-time PCR and RT-PCR analysis

Total RNA preparation, cDNA synthesis, primer designing and cDNA real-time PCR analysis were carried out according to Jiang et al. (2007). All primer sequences were listed in the Supplemental Table S1. Two biological duplicates were carried out and three technical triplicates for quantitative assays for each of the duplicate were performed. For RT-PCR analysis, the first strand cDNA was synthesized using Invitrogen kit and was then used for templates. PCR amplifications were performed in 20 μl reaction mixtures with 20 ng of first strand cDNA, 200 μM of each dNTPs, 2.5 mM of MgCl₂, 0.5 μM each of primers, 1 unit of *Taq* DNA polymerase and 1 × PCR buffer provided by Qiagen. The temperature profile was 94°C for 2 min followed by 35 cycles at 94°C for 10 S, 59°C for 10 S and 72°C for 25 S using PTC-100 thermo-cyclers. The reaction was terminated with a 10 min extension step at 72°C. PCR products (10 μl) were visualized in 1.6% agarose gel and all pictures were taken in BIORAD UV-Gel documentation system using Quantity one 1D Analysis software.

Database searches

We employed several ways to search and predict genes encoding RIP domain-containing proteins. The representative amino acid sequences were also obtained from the Pfam database (<http://www.sanger.ac.uk/Software/Pfam/>) and their corresponding RIP domain sequences were used as query sequences. Both TBLASTN and BLASTP searches were carried out based on the following databases: RGP (Rice Genome research Program, <http://rgp.dna.affrc.go.jp/>), TIGR (<http://tigrblast.tigr.org/euk-blast/index.cgi?project=osa1/>), and NCBI (<http://www.ncbi.nlm.nih.gov>). TIGR and RiceGAAS (<http://ricegaas.dna.affrc.go.jp>) database were used for predicting coding regions and their proteins. In addition, the above databases were also searched using “RIP” as a keyword to retrieve more members.

Presence of RIP domain in the predicted members were confirmed by searching the Pfam program with E-value = 0.01 as the cutoff and the RIP domain

sequences were used for phylogenetic analysis. NCBI database was also employed to detect conserved domains. Proteins confirmed by the domain searches were regarded as putative RIP domain-containing proteins (referred to RIPs for convenience); otherwise, they were excluded from our data set. The full-length cDNA information of predicted members was retrieved from Knowledge-based *Oryza* Molecular biological Encyclopedia (KOME) (Kikuchi et al. 2003; <http://cdna01.dna.affrc.go.jp/cDNA/>).

Detection of duplicated genes

The chromosomal distributions of the predicted members were determined by searching the physical positions of their corresponding locus numbers in TIGR database. The data from previous publications (Goff et al. 2002; Yu et al. 2002, 2005) were used to determine whether any of the mapped genes were located in duplicated regions. Genes duplicated by segmental duplication were detected by searching the “Segmental genome duplication of rice” in TIGR database (http://www.tigr.org/tdb/e2k1/osa1/segmental_dup/index.shtml).

Sequence alignment and phylogenetic analysis

The DNASTAR program was used for the preliminary DNA and amino acid sequence manipulations. Only RIP domain sequences were used for further investigation. The sequence alignment was generated using ClustalX (Version 1.8) (Thompson et al. 1997) with manual adjustment. The aligned amino acid sequences formed the basis for the phylogenetic analysis using the program Mac PAUP 4.0b8 (ppc) (<http://www.paup.csit.fsu.edu>) as described by Jiang and Ramachandran (2006).

Results

Rice genome encodes 31 members of the RIP domain family

Following multiple cycles of searches and domain detections (see section Materials and methods), a total of 31 genes were detected from rice genome that encoded RIP domain-containing proteins (Supplemental Table S2). This is the first report by in silico genome-wide identifications in rice genome. These members were named as *Oryza sativa* *RIP1–31* (*OSRIP1–31*) for each of the predicted genes based on their orders and positions in corresponding chromosomes (See Supplemental Table S2). On the other hand, no type 2 RIP was detected in rice genome and type 3 is actually a peculiar type 1, suggesting that rice genome encodes only type 1 RIPs.

Large-scale duplications represent the major mechanism for the family expansion

Chromosomal localizations of all members of this family indicated a non-random distribution of many members (Supplemental Table S2). Chromosomes 5 and 6 were devoid of RIP genes whereas chromosome 3 encoded the highest density (12 of 31, 38.7%) of the RIP family members. Among these 12 members, 8 were clustered into 2 groups and each of them consisted of 4 tandemly duplicated members (*OSRIP8–11* and *OSRIP12–15*). No other tandem duplicates was detected for other members. This data suggested a low contribution of tandem duplications (8 of 31, 25.8%) to the family expansion. On the other hand, we have detected 64.5% of members that were located in large-scale duplicated regions, and none of members were located on segmentally duplicated regions. These data suggested that genome-wide duplications represent the major mechanism for this family expansion.

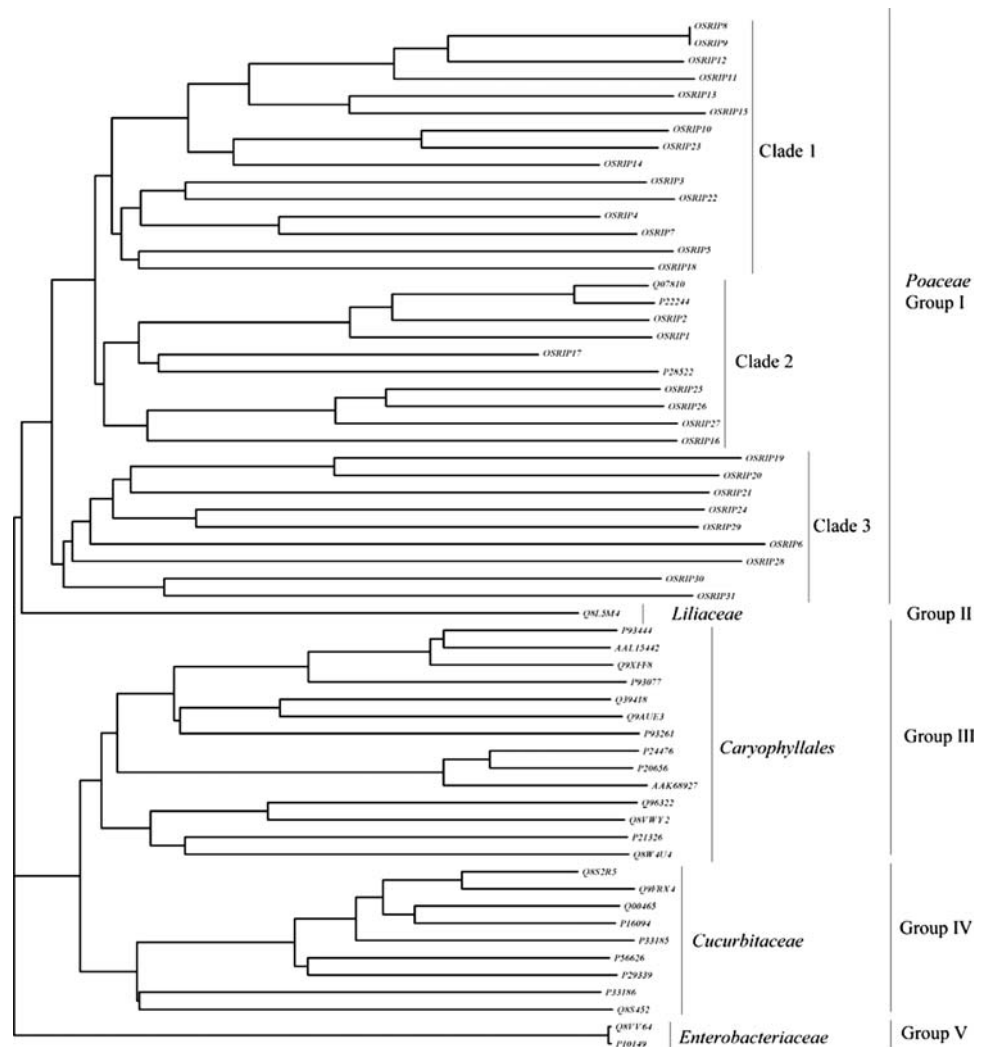
The RIP family might have evolved in parallel to species evolution

To classify the family members and explore their evolutionary relationships, the amino acid sequences from RIP domains of all rice members and some members from other species were used for sequence alignment and a phylogenetic tree was constructed based on these aligned sequences (Fig. 1). The phylogenetic tree showed that RIP genes from the same family usually clustered together. All RIP genes can be classified into 4 groups. The group I consisted of members from the family *Poaceae*, which contained all rice RIP genes and those from wheat (Q07810), and barley (P22244) and maize (P28522). This group can be subgrouped into 3 clades. All three non-rice RIP genes from *Poaceae* were grouped into the clade 2. This fact suggests that the biological functions among these 3 clades may be different and both clade 1 and clade 3 may be specific for rice. The group II contained only one member, which was from *Liliaceae* since only one gene was selected from this family for phylogenetic analysis. The group III consisted of members from *Caryophyllales*, which contained 4 sub-families. The members of group IV were all from the family *Cucurbitaceae*. The group V belonged to bacterial members suggesting that RIP genes might have evolved in parallel to the evolution of their corresponding species.

Members of rice RIP gene family were differentially expressed in various tissues

To explore expression patterns of this family members, real-time PCR was carried out using cDNA templates from

Fig. 1 Phylogenetic analysis and classification of genes encoding RIP domain proteins in rice and other plants. The phylogenetic tree was generated using the bootstrap method with a heuristic search of the PAUP 4.0b8 program (Mac). Groups in the phylogenetic tree are marked by the roman numbers I–V. In rice, three subgroups were clustered, designated as clade 1–3. Besides amino acid sequences from rice RIP domains, some members from other species were also used for the tree construction and were listed using their protein accession numbers



6 different rice tissues including young and mature leaves, young and mature panicles, young and mature roots. Among the 31 RIP members, 20 (64.5%) showed expressions in 1–6 tissues under normal growth conditions. No significant expression signal was detected for the remaining 11 members under these conditions. However, 3 of the 11 members including *OSRIP7*, *OSRIP17* and *OSRIP21* showed expression under various stressed conditions (see below). Thus, only 8 members (25.8%) showed no expression under normal or stressed conditions in this study.

Under normal growth conditions, 7 genes were mainly expressed in young leaf with at least 2-fold expression levels compared to other tissues. These genes were *OSRIP1*, *OSRIP24–27*, and *OSRIP29–30* as shown in Fig. 2a–g. Another set of 7 genes showed panicle-specific expression. Among them, 4 genes were mainly expressed in young panicle with at least 40-fold increases in their relative mRNA amount compared to other tissues (Fig. 2h–k). These genes included *OSRIP10*, 18, 22 and 31. The

remaining 3 genes were mainly expressed in mature panicles including *OSRIP13*, *OSRIP14* and *OSRIP16* (Fig. 2l–n). Besides the young leaf or panicle-specific genes, another set of genes displayed mature root-specific expression as shown in Fig. 2o. Thus, among 20 expressed genes, 15 genes (75%) showed the highest expression in single tissue specific expression pattern. The remaining 5 genes showed expression in all 6 tissues with relatively higher levels in two or more tissues (Fig. 2p–t).

The expression of many RIP members was regulated by various stress conditions

Since RIP genes were frequently reported to respond to various stresses, we investigated expression patterns of these family members under various biotic and biotic stresses as well as ABA treatment. Based on statistic analysis (*t*-test), more than one third of the family members (11 of 31, 35.5%) were up/down-regulated by various stresses (Table 1). Most of the regulated genes were

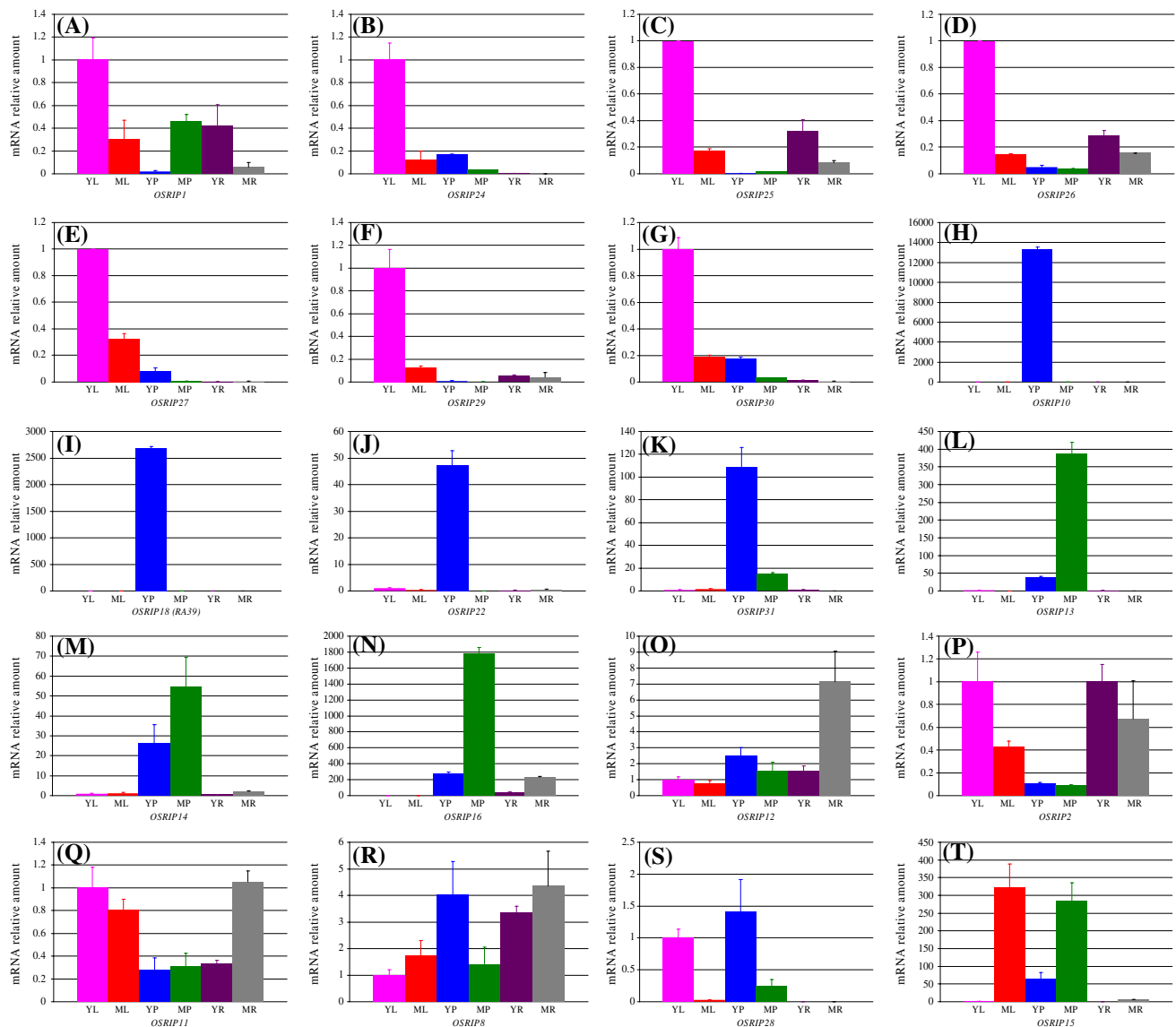


Fig. 2 Expression patterns of rice RIP genes in various tissues shown by real-time PCR analysis. The mRNA relative amount was calculated as described in section “Materials and methods”. Only 20 of 31 RIP members were presented since very low or no signal can be detected for the remaining genes in tested tissues under normal growth conditions. (a)–(g) Genes mainly expressed in young leaves. (h)–(k) Genes mainly detected in young panicles. (l)–(n) Genes

which showed expression mainly in mature panicles. (o) The gene *OSRIP12* with the highest expression levels in mature leaves. (p)–(t) Genes which showed expression in 6 different tissues with higher abundance in two or more tissues. YL, young leaf; ML, mature leaf; YP, young panicle; MP, mature panicle; YR, young root; MR, mature root

responsive to one or two stresses (9 of 11, 81.8%) and the remaining 2 genes including *OSRIP1* and *OSRIP2* were regulated by 3 or more stresses. For example, the *OSRIP1* gene was down regulated by cold stress but was up regulated by all other 5 stresses (Table 1). On the other hand, the highest percentage of genes (19.4%) were responsive to bacterium *Xanthomonas oryzae* pv *oryzae* (Xoo) infection whereas only 6.5–12.9% (2–4 genes of 31) of genes were regulated by other stresses.

Among 6 genes regulated by Xoo infection, all of them showed the highest expression level after 1 day of

infection and the expression levels were dramatically reduced after 2 days of infection although the level was still higher than that of the control (Fig. 3a–f). In the meantime, these members showed differences in relative mRNA amount induced by this stress. More than 12,000-folds expression was induced 1 day after infection for the gene *OSRIP10* (Fig. 3c). However, *OSRIP14* was induced only around 6 times higher than that of the control (Fig. 3f). In contrast, only two genes were observed to be significantly regulated under Mg infection including both *OSRIP1* and *OSRIP17* (Fig. 3g, h). The

Table 1 A summary of expression patterns of the RIP family members under various abiotic and biotic stresses as well as ABA treatment shown by real-time PCR analysis

Gene	Cold	PEG	Salinity	ABA	Xoo ^a	Mg ^a
<i>OSRIP1</i>	–	+	+	+	+	+
<i>OSRIP2</i>	NS	+	+	+	NS	NS
<i>OSRIP3</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP4</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP5</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP6</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP7</i>	NS	NS	+	NS	+	NS
<i>OSRIP8</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP9</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP10</i>	NS	NS	NS	NS	+	NS
<i>OSRIP11</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP12</i>	NS	NS	NS	NS	+	NS
<i>OSRIP13</i>	NS	NS	NS	NS	+	NS
<i>OSRIP14</i>	NS	NS	NS	NS	+/-	NS
<i>OSRIP15</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP16</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP17</i>	–	NS	NS	NS	NS	+/-
<i>OSRIP18</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP19</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP20</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP21</i>	NS	NS	+	NS	NS	NS
<i>OSRIP22</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP23</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP24</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP25</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP26</i>	NS	+	NS	NS	NS	NS
<i>OSRIP27</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP28</i>	NS	NS	NS	+	NS	NS
<i>OSRIP29</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP30</i>	NS	NS	NS	NS	NS	NS
<i>OSRIP31</i>	NS	NS	NS	NS	NS	NS
Total (%)	2 (6.5%)	3 (9.7%)	4 (12.9%)	3 (9.7%)	6 (19.4%)	2 (6.5%)

^a Xoo, treated with *Xanthomonas oryzae* pv *oryzae*; Mg, treated with *Megnaporthe grisea*. “NS” indicates no significant difference in their expression upon stresses shown by *t*-test; “+” indicates significant up regulation in their expression; “–” indicates significant down regulated expression. “+/-” indicates up and then down regulated expression

former was up regulated in various samples and the latter was up regulated after 4-day infection followed by down regulation of the expression.

Besides biotic treatments, abiotic stresses including cold, PEG and high salinity were also observed to regulate expression of some RIP members. Upon cold stress, both *OSRIP1* and *OSRIP17* genes were down regulated (Fig. 3i, j). Three genes were induced by PEG treatment including *OSRIP1*, *OSRIP2* and *OSRIP26* (Fig. 3k–m). Two of them (*OSRIP1* and *OSRIP2*) were also up regulated under 250 mM NaCl (Fig. 3n, o). Apart from these two genes, both *OSRIP7* and *OSRIP21* were also observed to be

induced by salinity stress (Fig. 3p, q). Since biotic and abiotic stresses were regulated by ABA-dependent and -independent signal pathways (Shinozaki et al. 2003), we also analyzed the expression of RIP members following ABA treatment. The result showed that only 3 genes *OSRIP1*, *OSRIP2* and *OSRIP28* were up regulated by the treatment (Fig. 3r–t).

OSRIP18 showed tapetum-specific expression

BLAST searches showed that *OSRIP18* shared 100% homology at the nucleotide level with the *RA39* gene

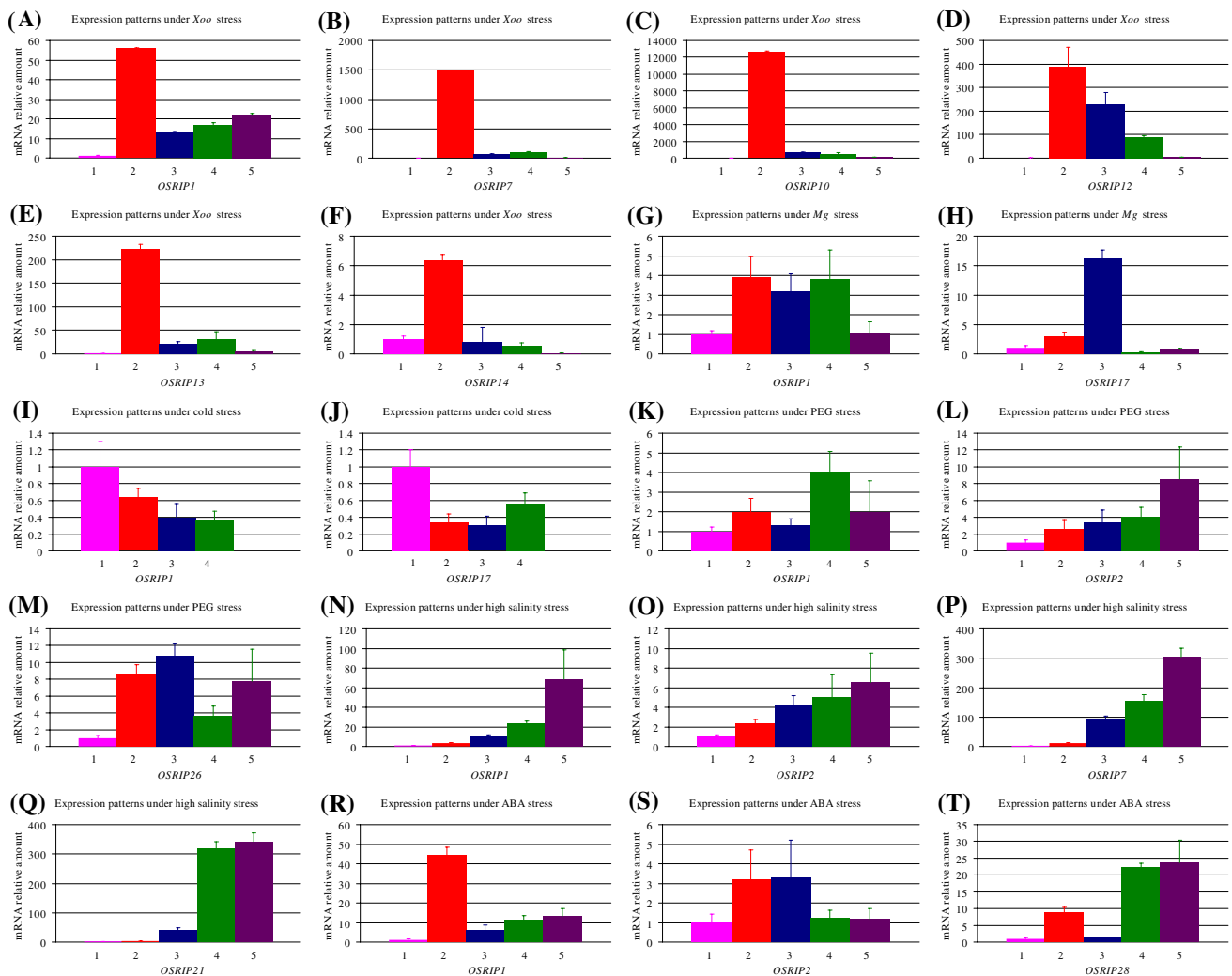


Fig. 3 Expression patterns of rice RIP genes under various abiotic and biotic stresses as well as ABA treatment shown by real-time PCR analysis. The mRNA relative amount (Y axis) was calculated as described in section “Materials and methods”. (a)–(f) Expression patterns and mRNA levels of genes following *Xoo* infection. (g) and (h) Expression analysis of both *OSRIP1* and *OSRIP17* upon Mg

treatment. (i) and (j) Expression of both *OSRIP1* and *OSRIP17* under cold stress. (k)–(m) Expression of *OSRIP1*, *OSRIP2* and *OSRIP26* under PEG treatment. (n)–(q) Expression patterns of 4 salinity-responsive RIP genes under high salinity stress. (r)–(t) Expression patterns of 3 ABA-dependant genes

previously reported by Ding et al. (2002). This report showed that *RA39* was expressed in tapetum specific manner by in-situ hybridization. Our data showed that *OSRIP18* was mainly expressed in young panicle by real-time PCR analysis (Fig. 2i), we then analyzed its expression in detail. Firstly, 9 different stages of panicles were freshly collected for RNA preparation and were then subjected to Northern blot analysis using the *OSRIP18*-specific probe. These results showed that the gene was expressed only in uni-nucleate stage with the highest expression at the early uni-nucleate stage of panicles (Fig. 4a). To investigate the expression patterns at cellular level, the transgenic plants harboring the *OSRIP18* promoter-GUS construct was then analyzed by

staining the panicles at different stages of development. The analysis confirmed that the gene was expressed in anthers only at the uni-nucleate stage (Fig. 4b). The GUS-stained florets were then used for plastic section for further observation under microscope. The result showed that the activity of *OSRIP18* promoter was restricted to the tapetum layer (Fig. 4c). Thus, the gene was regarded as a tapetum-specific member.

OSRIP18 was up regulated by both PEG and salinity stresses in panicles

The expression of *OSRIP18* was panicle specific in WT plants and no mRNA could be significantly detected in

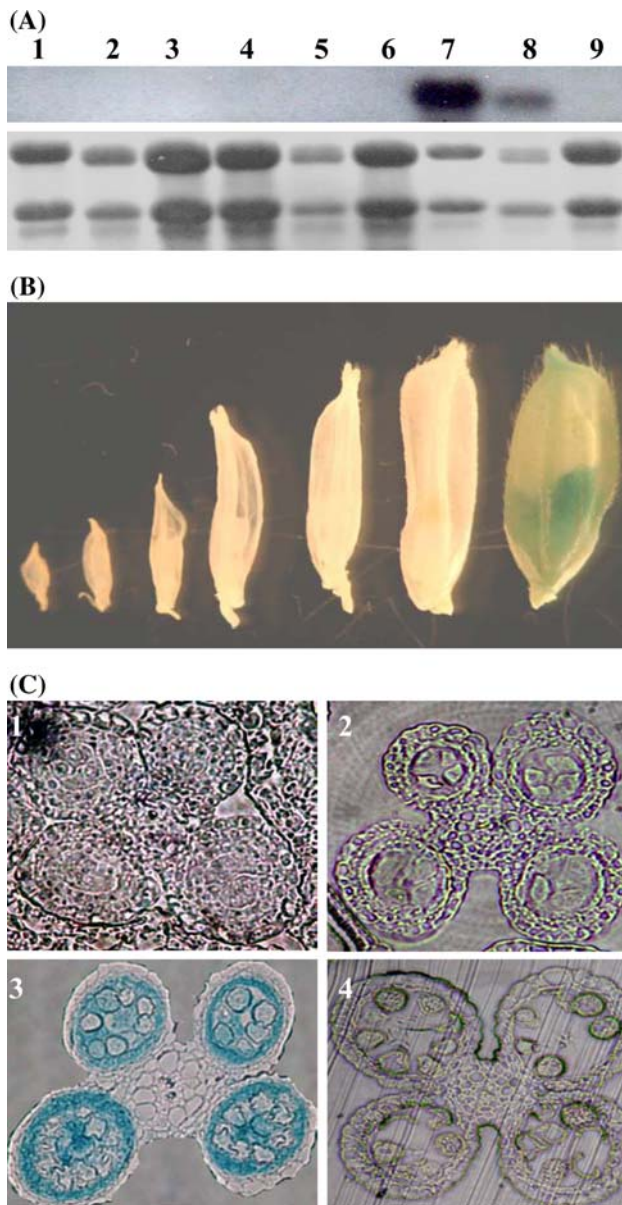


Fig. 4 Tapetum-specific expression of *OSRIP18* shown by Northern blot and the promoter-GUS analysis. (a) Northern blot analysis using coding region of *OSRIP18* as a probe. 1, young leaf; 2, mature leaf; 3, young root; 4, mature root; 5, young panicle with 1–3 cm in length; 6, young panicle with 3–6 cm in length; 7, young panicle of 6–10 cm in length (early uni-nucleate stage); 8, young panicle of 8–13 cm in length (late uni-nucleate stage); 9, flowering panicle of more than 13 cm in length. Bottom panel shows total RNA staining image after being transferred in nylon membranes for loading control. (b) GUS activity at the uni-nucleate stage of florets. (c) GUS stained images of WT and transgenic plants harboring *OSRIP18* promoter-GUS construct. The 1, 2, 3 and 4 represent sections of transgenic anther at pollen mother cell stage, tetra stage; uni-nucleate stage and WT anther at uni-nucleate stage as a negative control, respectively

other tissues tested including leaves and roots (Fig. 2i). On the other hand, the expression of *OSRIP18* was not regulated by PEG and high salinity stresses during seedling

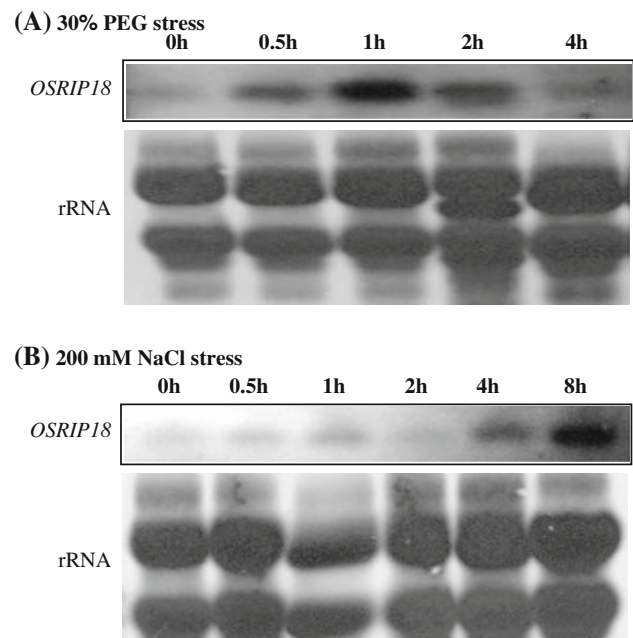


Fig. 5 Northern blot analysis of *OSRIP18* in WT plants at uni-nucleate stage of pollen development under drought and salt stresses. WT plants at the stage of uni-nucleate pollen development were subjected to 30% PEG solution (a) and 200 mM NaCl solution (b). The total RNA samples were prepared from different time-points and then transferred into nylon membrane for hybridization using *OSRIP18* as a probe. Bottom panels in (a) and (b) show total RNA staining image after being transferred in nylon membranes for loading control

stage, as shown by real-time PCR (Table 1), suggesting that this gene may not play a role during this stage of plant development. In order to test if this gene is regulated by PEG and high salinity stresses in panicle tissues we performed Northern blots on total RNA samples isolated from panicle tissues from plants subjected to drought or salinity stresses. Northern blot analysis showed that this gene was induced by 30% PEG treatment with the highest expression level after 1 h of treatment (Fig. 5a). Similarly, the gene was also up regulated by 200 mM NaCl stress with highest expression levels after 8 h of salt treatment (Fig. 5b). These results suggested the role of this gene in response to drought and high salinity during panicle development.

Discussion

The RIP family members were ancient but not ubiquitous

BALST searches against rice and other genomes showed that this family was not only present in rice but also in at least 58 other plant species, 11 bacteria and 39 viruses (data not shown). These results indicated that RIP family was ancient.

Interestingly, we could not detect any RIP member in *Arabidopsis* genome although the genome sequencing was complete when BLAST searches were carried out using TIGR, TAIR (<http://www.arabidopsis.org/>), and MATDB (<http://mips.gsf.de/proj/thal/>) databases. On the other hand, although a RIP-like protein named TRIP was purified from tobacco (*Nicotiana tabacum*) leaves and showed strong N-glycosidase activity, TRIP showed no sequence similarity compared with known RIPs (Sharma et al. 2004). Until now, no RIP domain-containing protein sequence from tobacco was deposited into public databases based on our BLAST searches. Furthermore, a RIP-type activity has been detected in mammalian tissues (Barbieri et al. 2001). However, no other animal RIP was either reported or available in public databases. These facts suggest that RIP family is not ubiquitously present in all organisms and this family has evolved with species-specificity suggesting specific functions of this family.

Evolution of the RIP family

Our data showed that rice genome encodes only type 1 and no type 2 RIP genes were detected. It seems that type 1 RIPs could generally be more abundant than type 2 RIPs (Girbes et al. 2004). Only a few reports described coexist of type 1 with type 2 RIPs in a same organism (Girbes et al. 2004). However, data showing the evolution of RIP family with special distributions among organisms is not yet available. In this study, the RIP domain sequences from only type 1 RIP members were retrieved for evolutionary analysis since no type 2 RIPs were detected in many species. Our data showed that the RIP family has evolved together with the differentiation of a species and large-scale genome duplications represent the major mechanism for the family expansion. Although a large data set has been reported on identifications and characterizations of RIPs, no data showed the evolution of this family. To our knowledge, this is the first report on the evolutionary mechanism for this family. However, our data still cannot explain why *Arabidopsis* contain no RIP member. Toxicity of RIPs may not explain the lack of the family members in plants since many of the type 1 RIPs are non- or low-toxic and *Arabidopsis* still can survive after carrying a rice RIP gene (data not shown). Since type 1 RIPs were present in both dicot and monocot plants, it is reasonable to extrapolate that there should be at least one RIP gene in the common ancestor of monocot and dicot plants. On the other hand, expression analysis showed that some of the family members in rice may have evolved into pseudogenes, suggesting the possibility of the loss of RIP genes during long evolutionary history. Taken together, loss of genes might contribute to the lack of the family

members in some plant species even no direct evidence was reported.

Tissue-specific and stress-induced expression patterns coincide with the developmental stages sensitive to various environmental factors

To our knowledge, this is the first report on genome-wide expression analysis of these family members in plants. Our analysis indicated that most of rice RIP genes were mainly expressed in one tissue either in young leaf or young/mature panicle, suggesting a tissue-specific function of these family genes. On the other hand, rice plants generally show more sensitivity to various environmental factors including drought and salt stresses at the seedling and the reproductive stage when compared to the other developmental stages (Lutts et al. 1995; Lafitte et al. 2004; <http://www.knowledgebank.irri.org/>). Thus, the sensitive stages of rice plants to various stresses are parallel to the highest mRNA relative amount detected in these stages. Furthermore, some of rice RIP genes also exhibit varying responses to various stresses. Taken together, the data suggest that the family members might play important roles in response to various stresses.

OSRIP18 may play a role in pollen development by sensing different environmental cues

Environmental stresses including drought and high salinity were frequently reported to affect reproductive development of plants (Reddy and Goss 1971; Saini 1997; Lauchli and Grattan 2007; Barnabas et al. 2008). These reports include the identification of a few stress-related genes or quantitative trait loci (QTL). For example, a total of 77 QTL were identified to be related to reproductive development under drought stress (Lanceras et al. 2004). Additionally, Over-expression of stress responsive gene *STRESS-RESPONSIVE NAC 1* (SNAC1) significantly enhances drought resistance in transgenic rice in the field under severe drought stress conditions during the reproductive stage (Hu et al. 2006). The *OSRIP18* gene exhibited panicle-specific expression by real-time PCR analysis and tapetum-specific expression by in-situ hybridization (Ding et al. 2002) and by analyzing the promoter-GUS transgenic plants (Fig. 4c). These results suggested roles of this gene in pollen development. The purified RA39 protein also exhibited the RNA N-glycosidase activity, confirming this protein as a RIP (Ding et al. 2002). Furthermore, this gene was regulated by PEG and salinity treatment during panicle development (Fig. 5a, b). On the other hand, our data also showed that this gene was expressed at the stage with more sensitive stage to environmental stresses as shown in Fig. 4. Thus, all these data suggest that the gene may play important roles as a member of natural

defense system against varying environmental conditions during pollen development.

OSRIP18 and other rice RIP genes may be potentially useful for developing new plant varieties with higher tolerance to various stresses

Although several reports showed expression patterns of RIP genes under various abiotic stresses, all efforts were focusing on improving plants against virus infection by transgenic techniques and no data was reported whether these RIP genes can be used for improving plants with higher tolerance to drought and salt stresses. Our expression data suggest that some of rice RIP genes may be regarded as potential candidates for improving and developing rice as well as other plant varieties with increased tolerance to various abiotic and biotic stresses by over-expressing or suppressing these genes.

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