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ORIGINAL ARTICLE

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The rice tapetum-specific gene *RA39* encodes a type I ribosome-inactivating protein

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Abstract A tapetum-specific cDNA encoded by a rice gene, *RA39*, was isolated by cDNA subtractive hybridization, differential screening and rapid amplification of cDNA ends. *RA39* is a single-copy gene in the rice genome. mRNA in situ hybridization indicates that this gene is a tapetum-specific gene, and highly expressed in the tapetal cells at the meiosis and tetrad stages. The RA39 cDNA is 1,013 bp in length with an open reading frame encoding 298 amino acid residues. This cDNA sequence does not show significant homology to any known sequences in GenBank databases, but its deduced amino acid sequence (RA39) has between 19 and 34% sequence identity to ribosome-inactivating proteins (RIPs). Optimal alignment reveals that the five amino acid residues constituting the active site of the ricin A-chain (Tyr^{80}, Tyr^{123}, Glu^{177}, Arg^{180} and Trp^{211}), which are invariant among all RIPs published to date, are conserved in RA39. Recombinant RA39 protein expressed in *Escherichia coli* was purified to homogeneity. The purified protein exhibits the RNA *N*-glycosidase activity of RIPs. This demonstrates that RIPs occur in the reproductive organs of rice. The possible function of RA39 in anther development is discussed.

Keywords Rice · Tapetum-specific gene · Ribosome-inactivating proteins

Introduction

In flowering plants, the production of haploid male gametophytes occurs within the diploid sporophytic tissue of anthers. The formation and release of pollen requires the development of anthers. Male gametogenesis consists of three major developmental phases: (1) sporogenesis, which includes the differentiation of sporogenous cells and meiosis; (2) the development of the free uninucleate microspores; and (3) pollen maturation, which follows microspore mitosis and ends with the formation of mature pollen. Development of the anther, microsporogenesis and pollen maturation are dependent upon the coordinated expression of a complex array of genes (Goldberg et al. 1993). Both sporophytic tissues and male gametophytes of developing anthers have been known to express tissue-specific sets of genes in a highly regulated manner (Mascarenhas 1990). Identification of anther-specific genes is an essential step towards elucidation of the mechanisms underlying anther development and microsporogenesis.

In recent years, some anther- or pollen-specific genes at the stages of pollen development and maturation, such as *ZM13* of maize (Hamilton et al. 1998; Hanson et al. 1989), *TA29* of tobacco (Koltunow et al. 1990), *RA8*, *Osc4*, *Osc6*, *YY1* and *YY2* of rice (Tsuchiya et al. 1994; Hihara et al. 1996; Jeon et al. 1999), and *LAT52*, *LAT56* and *LAT59* of tomato (Ursin et al. 1989), have been identified in several laboratories. Two groups of genes have been recognized to be involved in pollen development based on the timing of their expression. The genes of the first group begin to express soon after meiosis, reaching maximum expression levels around microspore mitosis and decreasing thereafter. The genes of the second group become active after microspore mitosis, and their mRNAs increase in abundance up to maturity (Mascarenhas 1990). There is currently limited information about the function of these genes in anther and/or pollen development. Recently, several meiosis genes, such as *AtDMC1*, *SYN1* and *SPL* of *Arabidopsis* (Klimyuk and Jones 1997; Bai et al. 1999; Couteau et al. 1999; Yang et al. 1999) and *OsDMC1* of rice (Ding et al. 2001), have been identified.

Rice (*Oryza sativa*) is one of the most important crops in the world. Rice, wheat and maize together account for about half of the world's food production, and

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession number AB053261.

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rice itself is the principal food of half of the world's population. Grass genomes, including those of rice, wheat, maize, barley, rye and sorghum, share a large degree of synteny, making rice an excellent model cereal. Rice is also the easiest of the cereal plants to transform genetically (Gale and Devos 1998; Eckardt 2000). We are interested in genes that are preferentially or specifically expressed in anthers at the stage in which sporogenous cells differentiate and meiosis occurs in rice. These genes will be important not only for the understanding of gene regulation of cereal anther development, but also for their potential application in agriculture. For this purpose, a subtracted cDNA library was constructed in our laboratory by cDNA subtractive hybridization with spikelet cDNA (meiosis stage) as a tracer and young leaf cDNA as a driver. In this paper, we report the isolation and characterization of a tapetum-specific cDNA, RA39, identified from this subtracted cDNA library. *RA39* is a single-copy gene in the rice genome and is expressed specifically in the anther tapetal cells. The *RA39* gene product is identified as a novel type I ribosome-inactivating protein (RIP) by its sequence homology and enzyme activity.

Materials and methods

Plant materials

Rice plants (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) were grown under standard greenhouse conditions. Spikelets containing anthers in meiosis and young leaves were used as sources of RNA for cDNA subtractive hybridization to screen genes preferentially or specifically expressed in spikelets. Young leaves were collected from 2-week-old plants. Roots and buds were harvested from seeds germinated on sterile-water soaked papers. Carpels at anthesis stage and anthers at tetrad and uninucleate microspore stages were collected.

Extraction of nucleic acids

Total RNA was isolated from spikelets, leaves, roots, buds, carpels and anthers with a Trizol kit (Gibco-BRL) according to the manufacturer's protocol, and were then treated with RNase-free DNaseI (TaKaRa) to remove residual genomic DNA. Genomic DNA was extracted from young leaves by cetyltrimethylammonium bromide (CTAB) according to the method of Murray and Thompson (1980).

Isolation of RA39 cDNA clone by PCR-mediated cDNA subtractive hybridization

PCR-mediated cDNA subtractive hybridization was performed according to the method of Foote et al. (1997) with several modifications. Leaf and spikelet RNA (0.5 μg) were reverse-transcribed to first-strand cDNA with SuperScriptII RNase H^- reverse transcriptase (Gibco-BRL). A TdT-tailing step was carried out to attach a poly(A) sequence to the 5′ end of the first-strand cDNAs. cDNA of spikelets was used as a "tracer". Biotinylated cDNA of leaves was used as a "driver".

The subtractive hybridization was conducted as follows. Tracer (0.2 μg) was mixed with 4 μg of the biotinylated driver and the mixture was co-precipitated with ethanol and resuspended in 4 μl 1.25× EE buffer (Straus and Ausubel 1990; Lisitsyn et al. 1993). The solutions were overlaid with mineral oil (Sigma), heated in a boiling bath for 10 min to ensure complete denaturation, and hybridized for 48 h at 68°C. After hybridization, 100 μl EE buffer was added to the sample. Streptavidin (Promega, 5 μl at 4 μg/μl in EE buffer) was mixed with the hybridized cDNA solution and incubated for 10 min at room temperature to form streptavidin-biotinylated cDNA complexes. The complexes were removed by extraction with an equal volume of phenol/chloroform. The phenol/chloroform extraction step was repeated until there were no visible protein-DNA complexes at the interface between the organic and aqueous phases. After the resultant aqueous phase was extracted with diethyl ether, 5 μl of this aqueous phase was used as template for PCR reamplification (Foote et al. 1997) and, after purification, 0.2 μg of this PCR product was subjected to a second round of subtraction, repeating subtraction steps three times. The subtracted cDNA was amplified by PCR (Foote et al. 1997), and 390 inserts were successfully cloned into the pGEM-T vector (Promega) and identified by a PCR check with a T7/SP6 primer pair.

The inserts of these clones were separately fractionated on 0.8% agarose gels, and blotted onto Hybond N^+ nylon membrane (Amersham Pharmacia). Hybridization screening was performed at 65°C for 12 h with leaf and spikelet cDNA probe pools labeled with digoxigenin (DIG)-dUTP by following the manufacturer's protocols (DIG high primer DNA labeling and detection starter kit, Roche). Of the 390 clones, 120 were found to be spikelet-specific; S039 was designed as RA39.

Identification of cDNA ends

The 5′ region of the *RA39* transcript was obtained by rapid amplification of cDNA ends (RACE) (Frohman et al. 1988) using a Gibco-BRL 5′ RACE kit and two gene-specific primers P1: 5′-AAGAACCTGAGAGCTTCGCAG-3′ and P2: 5′-ATCGGTTGAGGTAATGTAG-3′. The 5′ cDNA fragment was gel-purified and ligated into the pGEM-T vector (Promega) and confirmed by sequencing. The full-length RA39 cDNA was generated by PCR using a high-fidelity PCR system (Roche), cloned into the pGEM-T vector and designated as pRA39. The sense and antisense orientations of pRA39 were determined by sequencing.

Southern blot hybridization

Genomic DNA (30 μg) was separately digested with *Hin*dIII, *Xba*I or *Pst*I. After the digested DNA was subjected to electrophoresis on a 0.8% agarose gel, the DNA fragments were blotted onto a Hybond N^+ nylon membrane (Amersham Pharmacia). Hybridization was carried out overnight at 65°C with a DIG-labeled RA39 cDNA probe according to the manufacturer's protocol (Roche).

RT-PCR analysis of RA39 mRNA accumulation

The sense and antisense primers specific to RA39 cDNA were P3: 5′-TTAATTGCACGAGCTCAGCCG-3′ and P4: 5′-TGCGTCTAGATTAGCTAGGAGGG-3′. The rice tubulin gene *tubA* was used as a control (Ding et al. 2001).

First-strand cDNA was synthesized by following the conditions described by Ding et al. (2001). PCR was performed in a 50 μl mixture containing 1 μl of first-strand cDNA, 10 pmol each of the gene-specific primers, 200 μM dNTPs, 1× PCR buffer and 2.5 U DNA polymerase (5 U/μl, Sangon) for 25 cycles. The PCR products were separated on 1% agarose gels.

In situ hybridization

Anthers at different developmental stages were fixed at room temperature for 16 h in a solution of 50% ethanol, 5% acetic acid and 3.7% formaldehyde, and dehydrated in a graded ethanol series to

100% ethanol prior to infiltration with xylene and embedding in paraffin (Sigma). The paraffin blocks were sliced into 10 µm sections that were mounted onto poly-L-lysine-treated glass slides (Sigma). The paraffin-treated and rehydrated sections were processed according to the in situ hybridization procedure described by Meyerowitz (1987). DIG-labeled sense and antisense RNA probes were synthesized using T7 or SP6 RNA polymerase (Roche) from linearized pRA39. Hybridization was performed for 20 h at 42°C in 50% formamide, 5% dextran sulfate, 1% blocking reagent (Roche), 150 mg/ml tRNA (Sigma), 500 mg/ml poly(A) RNA (Sigma), 300 mM NaCl, 1 mM EDTA and 10 mM Tris pH 7.5. The hybridization signal was detected with a DIG nucleic acid detection kit (Roche) in accordance with the manufacturer's instructions.

Purification of recombinant RA39 protein

A set of primers, 5′-GATGAATTCCACCAGCTTAGCCGTC-3′ (P5) and 5′-GATCGTCGACACACCTTGCATTAATACAACCA-3′ (P6), were used to amplify the fragment corresponding to the ORF of the putative RA39 protein. After the sequence was confirmed by DNA sequencing, the amplified cDNA fragment was digested with *Eco*RI and *Sal*I, and ligated into the glutathione-*S*-transferase (GST) gene fusion vector pGEX-4T-3 (Amersham Pharmacia). The resultant plasmid was designed as pGEA39.

Escherichia coli strain BL21, transformed with pGEA39, was cultured in 5 ml LB medium at 37°C. After reaching exponential phase, the culture was induced immediately with 1 mM IPTG and then incubated for 2 h at 37°C with vigorous shaking. Cells were collected by centrifugation and resuspended in 400 µl lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM NaCl, 1 mM PMSF). Enzymatic lysis of cells was performed according to the method of Worrall (1996). Inclusion bodies were washed twice with the lysis buffer supplemented with 4 M urea and 0.5% Triton X-100. The washed inclusions were solubilized in the lysis buffer supplemented with 8 M urea and 1 mM DTT. Protein refolding was performed according to Kohno et al. (1990). The refolded RA39 protein was purified by affinity chromatography using glutathione sepharose 4B (GST Purification Modules; Amersham Pharmacia) and then used to assay for RNA *N*-glycosidase activity.

SDS-PAGE was performed with 4.5% stacking and 12% separating acrylamide gels (Laemmli 1970). A low-molecular-mass protein marker kit (14–97 kDa, Sangon) was used to determine approximate protein sizes. Proteins were visualized with 0.1% Coomassie Brilliant Blue G-250 in 50% ethanol and 10% acetic acid.

Assay for RNA *N*-glycosidase activity

Rabbit reticulocyte ribosomes (Promega) were used as the substrate for RA39 protein. The RNA *N*-glycosidase activity of the protein was determined by the method of Van Damme et al. (2000). RA39 protein was added to the rabbit reticulocyte lysate in 25 mM Tris-HCl pH 7.4 containing 25 mM KCl and 5 mM $MgCl_2$, and incubated for 30 min at 37°C. Afterwards, total ribosome RNA was extracted from the reaction mixture using 0.5% SDS and phenol, and precipitated with ethanol. After redissolving in diethylpyrocarbonate (DEPC)-treated H_2O, the recovered RNA was divided into two equal aliquots. One aliquot was used as a control, the other was incubated with an equal volume of 1 M acidic aniline (pH 4.5) for 15 min at room temperature in the dark. RNA was fractionated in a 1.2% agarose gel and stained with ethidium bromide.

DNA sequence analysis and computer programs

DNA was sequenced by the dideoxy chain termination method with an automated sequencer (Applied Biosystems model 377). The degree of DNA and amino acid sequence homology was determined with the BLAST program (Altschul et al. 1997). Multiple sequence alignment was performed with the DNAMAN version 4.0 (Lynnon Biosoft) using PAM series matrix, an open gap penalty of 10, an extend gap penalty of 0.05 and a divergent delay of 40%. Protein sorting signals and localization sites were analyzed with the PSORT program (http://psort.nibb.ac.jp) Protein motifs were sought using the PPSEARCH program (http://www.ebi.ac.uk).

Results

Molecular cloning and characterization of RA39 cDNA

To identify mRNAs that preferentially or specifically accumulate in the reproductive organs of rice, PCR-mediated cDNA subtractive hybridization was performed with cDNA of meiotic pollen-producing spikelets as a tracer and biotinylated cDNA of leaves as a driver, according to the method of Foote et al. (1997). After subtractive hybridization, the subtracted cDNA was cloned into the pGEM-T vector. By differential hybridization to cDNA probe pools from spikelets and leaves, 120 clones were identified that had a hybridization signal with the spikelet cDNA probe, but no signal with the leaf cDNA probe. Of these clones, S039 was chosen for further characterization because of its high organ specificity. The insert of S039 is 435 bp long including a poly(A) tail of 23 bp and an ORF encoding 119 amino acid residues, suggesting that it is a 3′ cDNA fragment representing a mRNA; S039 was renamed RA39. The 5′ end of the RA39 transcript was obtained by 5′ RACE. Assembling the two cDNA fragments formed the full-length RA39 cDNA of 1,013 bp in length with a 5′ UTR of 48 bp, a 3′ UTR of 68 bp, and an ORF of 897 bp. A putative poly(A) signal, AATATA, was localized at nucleotide positions 972–977. The poly(A) tail started at position 990. BLAST analyses revealed that RA39 did not show significant homology to any known sequences in GenBank databases.

In order to determine the copy number of *RA39* in the rice genome, genomic DNA was digested to completion with either *Hin*dIII, *Xba*I or *Pst*I, which do not digest the RA39 cDNA probe, and subjected to Southern blot analysis. Hybridization with the cDNA probe showed one band in all cases (Fig. 1), indicating that *RA39* exists as a single copy gene in the rice genome.

RA39 mRNA accumulates specifically in anthers

We analyzed RA39 mRNA accumulation with RT-PCR conducted with equal amounts of first-strand cDNA prepared from equal amounts of total RNA individually isolated from spikelets, leaves, roots, buds, carpels and anthers. Figure 2 shows that RA39 transcripts were detected in both spikelets and anthers, but not in leaves, roots, buds or carpels.

RA39 mRNA accumulation was also analyzed using global RT-PCR. The global RT-PCR [also termed poly(A) PCR] procedure was designed to enable amplification of the entire poly(A) mRNA population from a

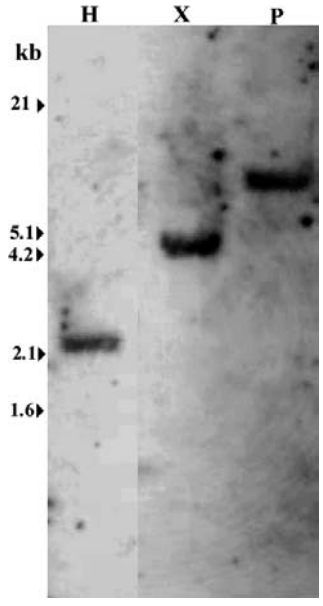

Fig. 1 Southern blot analysis of the *RA39* gene. Genomic DNA (30 µg) was digested separately with *Hin*dIII (*H*), *Xba*I (*X*) or *Pst*I (*P*), and separated on a 0.8% agarose gel. Hybridization was performed with a digoxigenin (DIG)-labeled RA39 cDNA probe. The position of DNA size standards (kb) are shown on the left

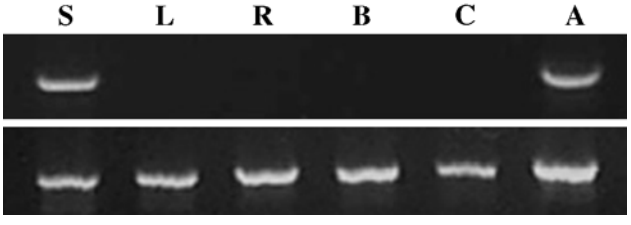

Fig. 2 RT-PCR analysis of RA39 mRNA accumulation. First-strand cDNA was synthesized with total RNA from spikelets at the meiosis stage of pollen mother cells (*S*), leaves (*L*), roots (*R*), buds (*B*), carpels at anthesis stage (*C*), and anthers at tetrad and uninucleate microspore stage (*A*), and used as templates for PCR with a gene-specific primer set (see Materials and methods). PCR products were separated on 1% agarose gels. *Top* Organ specificity of RA39 mRNA accumulation, *Bottom* control with rice tubulin gene *tubA*

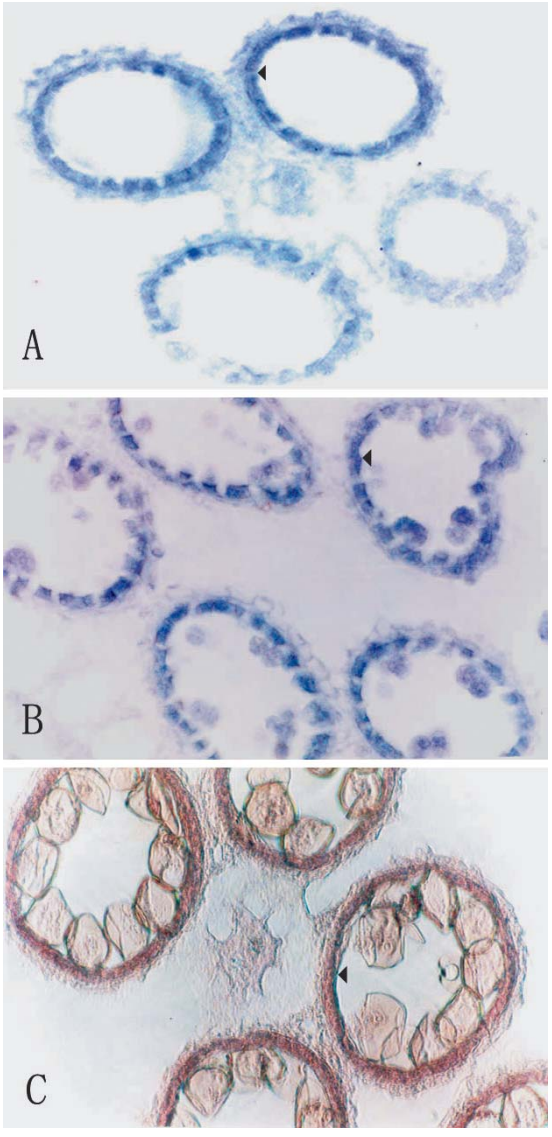

Fig. 3A–C In situ localization of RA39 mRNA in developing anthers. **A** Meiosis stage, **B** tetrad stage, **C** uninucleate pollen stage. Tapetal cells and hybridization signals are indicated by *arrowheads*

few cells while preserving the relative representation of cDNAs in the population (Varmuza and Tate 1992; Brady and Iscove 1993; Chwetzoff and d'Andrea 1997). cDNA population was synthesized by global RT-PCR with RNA from the organs listed above, separated by electrophoresis, transferred to nylon membrane, and hybridized with DIG-labeled RA39 cDNA. The hybridization results were identical to those of RT-PCR above (data not shown). These results indicate that RA39 is an anther-specific mRNA.

In situ hybridization localizes RA39 mRNA in anther tapetal cells

To determine in which anther tissue RA39 mRNA accumulates, in situ hybridization was performed on cross-sections of rice anthers. When hybridized to the RA39 antisense probe, intense signals were detected in the tapetal cells of the anther of meiosis and tetrad stages, whereas no signal was observed in other parts of the anther. No hybridization signal was detected in meiotic cells, tetrads or uninucleate pollen (Fig. 3). Weak signals were observed in several dyads (Fig. 3B). These results indicated that RA39 is a tapetum-specific mRNA. The tapetum-specific expression of the gene is developmentally regulated. RA39 mRNA was present at high levels at the meiosis stage, low levels at the tetrad stage, with still lower levels at the uninucleate pollen stage (Fig. 3). The sense control probe failed to produce any hybridization signal (data not shown).

RA39 mRNA encodes an RIP

RA39 encodes a putative 298 amino acid polypeptide (RA39) of 33.8 kDa with a calculated pI of 7.91. The

translated RA39 protein is a relatively hydrophilic protein with a hydrophobic N-terminal region and an internal major hydrophobic region (Fig. 4A). Analyses with the PSORT program revealed that RA39 protein contains a putative signal peptide of 17 amino acids (Met[1]–Ala[17]) and a putative transmembrane region of 17 amino acids (Val[194]–Ile[210]), corresponding to each of the two hydrophobic regions identified in Fig. 4A. In addition, RA39 protein contains a cytoplasmic tail of 88 amino acids (Arg[211]–Tyr[298]), suggesting that RA39 might be a transmembrane protein. The predicted localization of RA39 using the PSORT program was in endoplasmic reticulum. The deduced polypeptide contained proposed protein kinase phosphorylation sites: (1) a cAMP-/cGMP-dependent protein kinase phosphorylation site (R-R-Q-T) at amino acid position 167, (2) three PKC phosphorylation sites (S/T-X-R/K) at positions 128, 138 and 189, and (3) seven CK2 phosphorylation sites (S/T-X-X-D/E) at positions 42, 66, 128, 178, 200, 225, 227. Database searches by the BLASTP program revealed that RA39 has 19% to 34% sequence identity to RIP. The optimal alignment of the amino acid sequence of RA39 with those of a type I RIP barley endosperm RIP1 (HvRIP1), a type III RIP maize endosperm b-32 (Zmb-32), and an endosperm type II RIP ricin A-chain (ricin) of castor bean is shown in Fig. 4B. RA39 had 21.7% overall sequence similarity to HvRIP1, 21.2% to Zmb-32 and 19.8% to ricin, the structure of which has been resolved by X-ray diffraction analysis (Katzin et al. 1991). The active site of ricin comprises the residues Tyr[80], Tyr[123], Glu[177], Arg[180] and Trp[211] (Katzin et al. 1991), invariant among all RIPs published to date (Bass et al. 1992; Van Damme et al. 2000), all of which were conserved in RA39 (Tyr[115], Tyr[150], Glu[203] Arg[206] and Trp[235] in RA39). Other residues located in the vicinity of the active site, which should be involved in maintenance of the catalytic conformation of the active site (Katzin et al. 1991), were also conserved in RA39 (Fig. 4B).

To examine whether the RA39 protein has RIP activity, GST-tagged recombinant RA39 was expressed in *E. coli*. SDS-PAGE analyses revealed that the recombinant protein was not present in the soluble fraction, and accumulated in inclusion bodies within the cells under standard conditions (with 1 mM IPTG at 37°C). Lowering of the growth temperature (to 15°C or 20°C) and the IPTG concentration (0.1 mM) did not result in production of soluble RA39. We purified active recombinant RA39 by solubili-

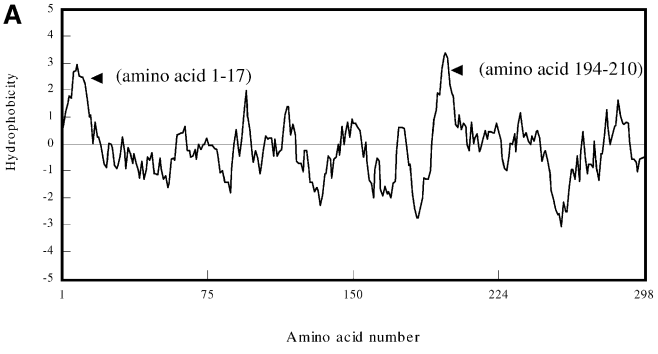

Fig. 4 Hydrophobicity profile of RA39 (**A**) and multiple sequence alignment of RA39 and three other ribosome-inactivating proteins (RIPs) (**B**). **A** The hydrophobicity profile of RA39 was determined by the Kyte-Doolittle (1982) method. The putative signal peptide and transmembrane domain are indicated. **B** Multiple sequence alignment of RA39, HvPRP1, Zmb-32 and ricin. Gaps introduced to maximize the alignment are marked with *dots*. Numbers on right margin indicate the positions of the amino acid residues of each polypeptide. Identical amino acid residues are highlighted with *black blocks*, otherwise homologous amino acid residues are indicated with *open blocks*. The five amino acid residues (Tyr[80], Tyr[123], Glu[177], Arg[180] and Trp[211]) that form active site of ricin, invariant among RIPs published to date, are indicated by *solid arrowheads*. The sources and accession numbers of the amino acid sequences used in this comparison are: RA39, *Oryza sativa* (this study), AB053261; HvRIP1, *Hordeum vulgare* (Leah et al. 1991), P22244; Zmb-32, *Zea mays* (Bass et al. 1992; Krawetz and Boston 2000), P25891; ricin, ricin A-chain of *Ricinus communis* (Katzin et al. 1991), 494726

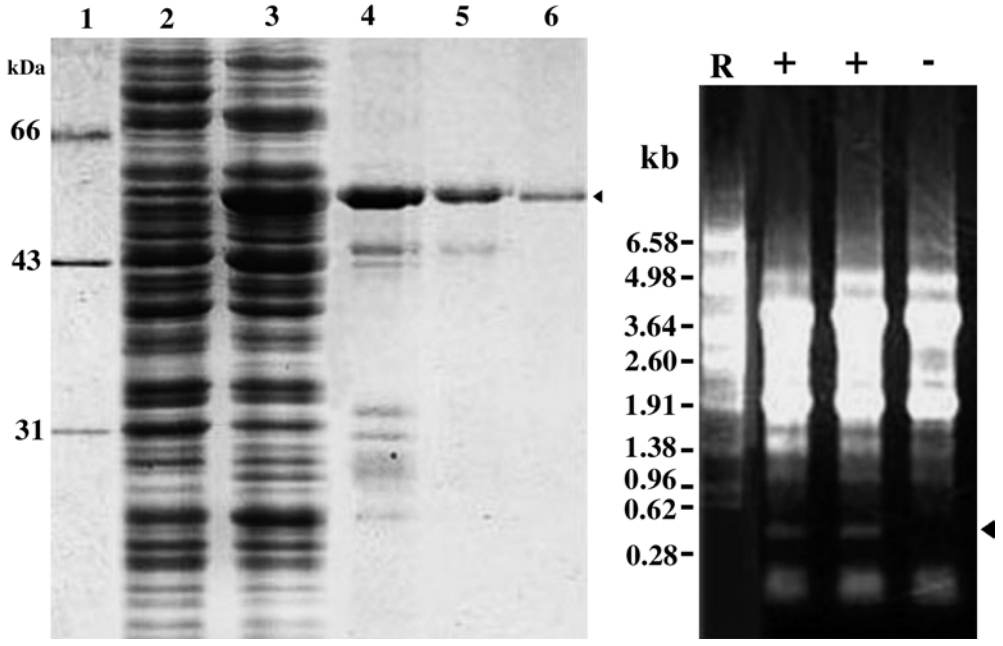

Fig. 5 Purification (**A**) and RNA *N*-glycosidase activity assay (**B**) of RA39 protein. **A** Purification and SDS-PAGE of recombinant RA39. GST-tagged RA39 was expressed in *Escherichia coli*. After removal of contaminating proteins by washing with the lysis buffer supplemented with 4 M urea and 0.1% Triton-X-100, the inclusion proteins were solubilized in lysis buffer supplemented with 8 M urea and 1 mM DTT and then refolded (see Materials and methods). The refolded proteins were loaded onto a glutathione sepharose 4B column for further purification. Lanes: *1* Molecular mass standards, *2* total proteins from cells not induced with IPTG, *3* total proteins from cells induced with IPTG, *4* inclusion proteins before washing, *5* inclusion proteins after washing, *6* purified RA39. *Arrowhead* GST-tagged recombinant RA39 protein. **B** RNA *N*-glycosidase activity of RA39 toward rabbit reticulocyte ribosomes. Ribosome were incubated with RA39. After incubation, rRNA was extracted, treated with acidic aniline, and separated on an agarose gel. RNA bands were visualized by ethidium bromide staining. + Aniline treatment, – no aniline treatment, *arrowhead* position of the 400 bp cleavage product released from ribosomes by aniline treatment of modified rRNA, *R* RNA markers (Promega) in kb

zing the inclusion bodies, refolding the protein and conducting affinity chromatography (Fig. 5A). The possible RNA *N*-glycosidase activity of the recombinant active RA39 on eukaryotic ribosomes was determined using rabbit reticulocyte ribosomes as a substrate. RIP-mediated depurination of the large rRNA renders the RNA sugar-phosphate backbone susceptible to hydrolysis at the depurination site (Endo and Tsurugi 1987). When depurinated rRNA is treated with aniline, cleavage occurs at the depurinated site and a ~400 bp fragment is released (Stirpe and Barbieri 1986; Endo and Tsurugi 1987). RA39 was incubated with the rabbit ribosomes, rRNA was extracted, treated with aniline, and analyzed by gel electrophoresis. RA39 apparently exhibited RNA *N*-glycosidase activity because a ~400-bp RNA fragment was observed in rRNA electrophoretograms upon treatment with aniline (Fig. 5B). These results strongly suggest that RA39 is a RIP.

Discussion

In this paper, we describe the isolation and characterization of an anther-specific cDNA, which we designated RA39. This cDNA encodes a 298 amino acid polypeptide (RA39), and had no hits in databases by BLAST search. RT-PCR analysis indicates that the RA39 mRNA is restricted to anthers. In situ hybridization reveals RA39 to be a tapetum-specific mRNA, active transiently during pollen meiosis. RA39 is an early tapetum mRNA, as compared with Osc4, Osc6, YY1 and YY2, which are late tapetum mRNAs (Tsuchiya et al. 1992, 1994; Hihara et al. 1996).

BLASTP analysis reveals that the deduced amino-acid sequence of RA39 has only 19–34% identity to RIPs, but the active site and the vicinity of the active site of RIPs, invariant among all RIPs published to date (Katzin et al. 1991; Bass et al. 1992; Van Damme et al. 2000), are highly conserved in the RA39 protein (Fig. 4B). Recombinant RA39 expressed in *E. coli* has the RNA *N*-glycosidase activity of RIPs. The protein shares some features common to type I RIPs, i.e., it is a monomeric enzyme, each subunit having an approximate molecular mass of 30 kDa and a basic isoelectric point. Based on homology analysis of amino acid sequences among RA39 and other RIPs, and enzyme activity assay, we conclude that RA39 is a novel type I RIP. RIPs are a widely distributed group of plant proteins and have been identified in seeds, roots, leaves and bulbs of many monocots (except rice, until now) and dicots (Bass et al. 1992; Vivanco et al. 1999; Hao et al. 2000; Krawetz and Boston 2000; Kwon et al. 2000; Song et al. 2000; Van Damme et al. 2000). The discovery of RA39 demonstrates that RIPs also occur in the anthers of higher plants.

RIPs are toxic *N*-glycosidases that depurinate the universally conserved α-sarcin loop of large rRNAs. This

depurination inactivates the ribosome, thereby blocking its further participation in protein synthesis. RIPs have been linked to defense, with antiviral, antifungal, and insecticidal properties demonstrated in vitro and in transgenic plants (Nielsen and Boston 2001). Because the inner space of the flower is thought to be converted from sterile to non-sterile conditions at the time of flowering (Takakura et al. 2000), it is possible that the RA39 expressed in tapetal cells could be secreted into the outer wall layers of anthers and/or the wall of developing pollen, and constitute a natural defense system against pathogens and viruses during flowering and fertilization.

RA39 protein may also play an important role in the regulation of tapetal development. During microspore development, the tapetum is considered to have a nutritive function for the maturing microspores and thus plays a major role in microsporogenesis by providing enzymes, hormones and other food materials to the developing pollen mother cells and microspores (Kaul 1988; Mascarenhas 1990; Goldberg et al. 1993). In normal pollen development, shortly after microspore release from the tetrad, and before mitosis, tapetal cells begin to degenerate (Wu and Cheung 2000). The cell components from the degenerate tapetum are also important nutrients for the growth and maturation of the spores (Kaul 1988; Wu and Cheung 2000). Tapetum degradation appears to be a type of programmed-cell-death (PCD) (Piffanelli et al. 1998; Papini et al. 1999; Wu and Cheung 2000). The initial features of PCD have been observed in the tapetal cells of the meiosis stage of angiosperms *Lobivia rauschii* Zecher and *Tillandsia albida* Mez et Purpus (Papini et al. 1999). The temporal and spatial regulation of *RA39* expression in the tapetum coincides with the temporal regulation of breakdown of the tapetal cells (Fig. 3). The PSORT program predicts that RA39 is localized in endoplasmic reticulum, which is the site of protein synthesis. In animal cells, RIPs have been linked to PCD, or apoptosis (Nielsen and Boston 2001). Based on these results, it could be speculated that RA39 protein might be related to the temporal regulation of the degeneration of tapetal cells.

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