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Cloning and Characterization of an RNase-Related Protein Gene Preferentially Expressed in Rice Stems

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RNase-related proteins (RRPs) are S- and S-like RNase homologs lacking the active site required for RNase activity. Here we describe the cloning and characterization of the rice (*Oryza sativa*) RRP gene (*OsRRP*). A single copy of *OsRRP* occurs in the rice genome. *OsRRP* contains three introns and an open reading frame encoding 252 amino acids, with the replacement of two histidines involved in the active site of RNase by lysine and tyrosine respectively. *OsRRP* is preferentially expressed in stems of wild-type rice and is significantly down-regulated in an increased tillering dwarf mutant *ext37*.

Key words: *Oryza sativa*; RNase-related protein; S-like RNase; S-ribonuclease; stem

Plant members of the RNase T2 family are classified into two subfamilies, S-RNases and S-like RNases. S-RNases are encoded by the S-gene and function as self-incompatibility factors with RNase activity, whereas S-like RNases are homologous to S-RNases but are not involved in self-incompatibility.[1,2] A group of enzymatically inactive S- and S-like RNase homologs, known as RNase-related proteins (RRP),[3] has been recently found in barley,[4] *Calystegia sepium*,[3] rice,[5] and wheat.[6] RRPs share high homology in amino-acid sequence with S- and S-like RNases, but lack one[3] or two[4–6] histidine residues located in the conserved regions that are required for RNase activity.[7–10] While it is well established that S-RNases are involved in the control of self-incompatibility,[11–15] there is growing evidence that S-like RNase genes are associated with processes in which RNA turnover might be involved, *e.g.*, phosphate starvation,[16–19] senescence,[19–21] wounding,[22–25] and defense against pathogens.[24–27] But the function of RRPs in plants remains largely unknown.

In this report we present the cloning and characterization of the *OsRRP* gene encoding an RNase-related protein of rice (*Oryza sativa* L.). *OsRRP* contains three introns and occurs as a single gene in the rice genome. In wild-type rice plants, *OsRRP* is preferentially expressed in stems. In an increased tillering dwarf mutant, *ext37*,[28] the expression of *OsRRP* is significantly down-regulated in plants at the three-leaf seedling stage and the booting stage.

The increased tillering dwarf mutant rice *ext37* was obtained from EMS-mutagenized JX89 rice seeds.[28] The average tiller number per plant of mutant *ext37* is 21, while wild-type JX89 plants have 7 tillers. The mutant also shows lower height (average 33 cm) as compared with the wild-type (average 89 cm). To identify genes that are differentially expressed between the mutant *ext37* and its wild-type JX89, suppression subtractive hybridization (SSH)[29] was performed. With *ext37* mRNA as tester and JX89 mRNA as driver, 16 clones were isolated and sequenced from a forward-subtractive cDNA library prepared from stems of rice plants at the eight-leaf stage. Among these clones, five cDNAs (E1, E13, E56, E92, and E131) showed high homology with three rice EST clones (AU163522, AU163523, and BE230627) in the GenBank database. Their deduced peptides all share homology to RNase-related protein (RRP). Assembling sequences of these five cDNAs and the three EST clones resulted in a putative full-length cDNA with an open reading frame encoding a polypeptide highly homologous to the RRP in the GenBank database (Fig. 1a). Based on this assembled sequence, PCR primers (forward: 5′ GCC-TAGAGAGTACT ATGGAGCAG 3′; reverse: 5′ CT-TAGCTAGCAAATTAAAGGC 3′) were designed to amplify the putative rice RNase-related protein (*OsRRP*) cDNA from rice JX89. A 999-bp *OsRRP* cDNA was obtained and sequenced (GenBank accession no. AY056038).

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Abbreviations: EMS, ethyl methane sulfonate; *OsRRP*, *Oryza sativa* RNase-related protein; RRP, RNase-related proteins; RT-PCR, reverse transcription-polymerase chain reaction

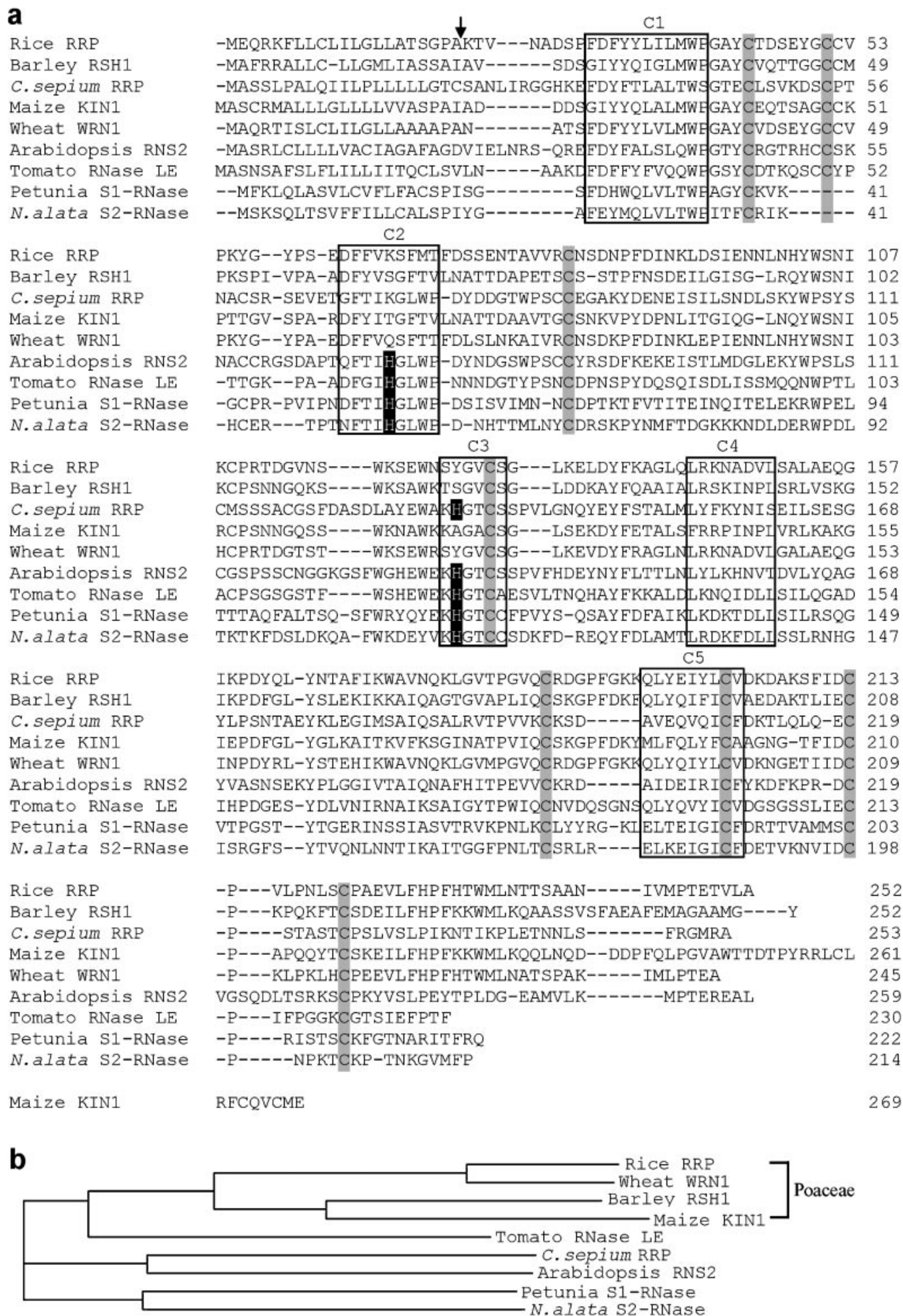

Fig. 1. Comparison of the Amino Acid Sequences of RNase-Related Proteins, S-Like RNases, and S-RNases.

a, Multiple sequence alignment using the ClustalW program.[31] The GenBank accession nos. of aligned sequences are as follows: rice RRP, RNase related protein from *Oryza sativa* (AY056038); barley RSH1, RNase S-like homolog from *Hordeum vulgare* (AF182197); *C. sepium* RRP, RNase related protein from *Calystegia sepium* (AF139660); maize KIN1, S-like RNase from *Zea mays* (U66241); wheat WRN1, S-like RNase from *Triticum aestivum* (AF495872); Arabidopsis RNS2, S-like RNase from *Arabidopsis thaliana* (M98336); tomato RNase LE, RNase from *Lycopersicon esculentum* (X79337); petunia S1-RNase, S1 self-incompatibility RNase from *Petunia hybrida* (U07362), and *N. alata* S2-RNase, S-RNase from *Nicotiana alata* (U08860), Dashes indicate gaps that were introduced to maximize alignment. The predicted signal peptide cleavage site is marked with a vertical arrow. Five conserved domains for plants, C1 to C5, are boxed. Black boxes indicate two histidine residues important for RNase activity.[10] Shaded boxes indicate conserved cysteine residues. b, Phylogram constructed with the ClustalW program,[31] based on the amino acid sequence alignment shown in a.

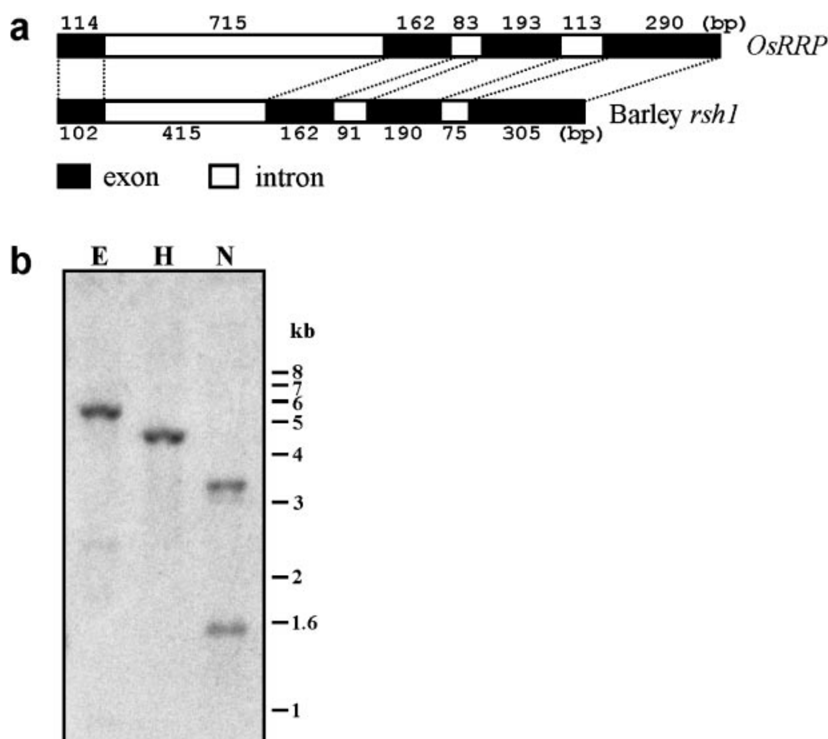

Fig. 2. Genomic Organization of *OsRRP*.

a, Comparison of the lengths of exons and introns as between *OsRRP* and barley *rsh1*.[4] The numbers indicate the sizes of exons and introns in base pair (bp). b, Southern blot analysis of *OsRRP*. Twenty µg of rice genomic DNA from JX89 digested with *Eco*RI (E), *Hin*dIII (H), or *Nco*I (N) was separated by agarose gel electrophoresis, blotted onto a Hybond-N membrane, and hybridized to a ^{32}P-labeled *OsRRP* genomic fragment (nt 324-1358 of AF439449), as described previously.[32]

The predicted OsRRP had 252 amino acids, a calculated molecular mass of 28.4 kDa, and a pI value of 5.25, with a putative signal peptide of 21 residues at the N-terminus (Fig. 1a). Sequence comparison of OsRRP with homologues from other plant species revealed that the five highly conserved regions (Fig. 1a, C1 to C5) and the eight conserved cysteins among S-RNases, S-like RNases, and RNase-related proteins[1,3,30] are also present in OsRRP. But the two histidines located in regions C2 and C3, which are involved in the active site of RNase,[7,8,10] were replaced respectively by lysine and tyrosine residue in OsRRP. Using purified protein, Van Damme *et al.*[3] demonstrated that RNase-related protein from *Calystegia sepium* (CalsepRRP), which lacks one active-site histidine residue in region C2, has no RNase activity. OsRRP lacks the two active-site histidines, and therefore it should be considered an enzymatically inactive homolog of S-like RNase. The other three RNase-related proteins or S-like RNase homologs from Poaceae species (wheat, barley, and maize) also lack the two active-site histidines (Fig. 1a). These four S-like RNase inactive homologs form a Poaceae-specific group in a phylogenetic tree (Fig. 1b).

To obtain the genomic sequence of *OsRRP*, a 1.9-kb DNA fragment was PCR-amplified from rice genomic DNA with the same primers used for amplification of *OsRRP* cDNA. Sequence analysis revealed that the *OsRRP* gene (GenBank accession no. AF439449), like its barley homolog *rsh1*,[4] contains three introns in the protein-coding region (Fig. 2a). Although the sizes of exons are highly conserved between rice and barley, the first intron of *OsRRP* is much larger in rice (715 bp) than in barley (415 bp) (Fig. 2a). Unlike these two genes of Poaceae species, *CalsepRRP* from *Calystegia sepium* has seven introns.[3]

To determine the gene copy number of *OsRRP*, Southern blot analysis of rice genomic DNA digested with restriction enzymes *Eco*RI, *Hin*dIII, and *Nco*I was carried out with the *OsRRP* genomic fragment (nt 324-1358 of AF439449) as a probe. Only a single hybridizing band was detected in samples digested with either *Eco*RI or *Hin*dIII, which do not cut within the probe region (Fig. 2b, lanes E and H). When the genomic DNA was digested with *Nco*I that cut once within the probe region, two hybridizing bands were observed (Fig. 2b, lane N). These results indicate that the rice genome contains only a single copy of the *OsRRP* gene. This is consistent with the BLAST search result of *OsRRP* genomic sequence against the Rice Genome Project Database (http://btn.genomics.org.cn:8080/rice/). Only contig2959 contains a single copy of *OsRRP*, which is on scaffold005125 of indica rice genomic DNA (GenBank accession no. AAAA01005125). In the japonica rice genome, *OsRRP* is located on chromosome 9 (GenBank accession nos. AP006067 and AP006174).

Northern blot analysis was performed to determine

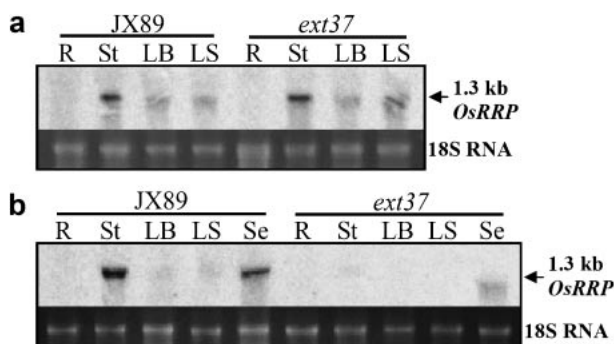

Fig. 3. Northern Blot Analysis of *OsRRP* mRNA in Wild-Type and Mutant Rice Plants.

Each lane contains 20 µg of total RNA extracted from different tissues of wild-type (JX89) and mutant (*ext37*) rice plants at the eight-leaf stage (a), and the three-leaf seedling stage and the booting stage (b). The blots were probed with random-primed ^{32}P-labelled *OsRRP* cDNA, as described previously.[32] 18S ribosomal RNA (18S RNA) bands stained with ethidium bromide are shown (bottom panel) to indicate the amount of total RNA loaded per lane. LB, leaf blade; LS, leaf sheath; R, root; Se, seedling (whole plant at the three-leaf stage); St, stem.

the expression of *OsRRP* in different tissues and at different developmental stages of wild-type (JX89) and mutant (*ext37*) rice plants (Fig. 3). *OsRRP*, originally identified as up-regulated in stems of *ext37* plants at the eight-leaf stage, could not be confirmed as an overexpressed gene by northern blot analysis (Fig. 3a). *OsRRP* mRNA was more highly expressed in stems than in leaves, and was undetectable in the roots of plants at the eight-leaf stage (Fig. 3a). A similar level and pattern of *OsRRP* expression was observed in both JX89 and *ext37* plants at the eight-leaf stage. In contrast, *OsRRP* expression was significantly reduced in *ext37* plants at the three-leaf seedling stage and at the booting stage (Fig. 3b) as compared to JX89 plants. As in wild-type plants at the eight-leaf stage, *OsRRP* was also preferentially expressed in the stems of wild-type plants at the booting stage (Fig. 3b).

The barley RRP gene (*rsh1*) was found specifically expressed in leaves and was induced by light, but its role has not yet been defined.[4] Catalytically inactive *C. sepium* RRP (*CalsepRRP*) accumulates exclusively in the rhizomes and has been considered to be a vegetative storage protein, with no other function than a storage role.[3] Expression of the wheat RRP gene (*WRN1*) has been detected in leaves, roots, and dry seeds, and was down-regulated by natural- and dark-induced senescence.[6]

Since our first submission of the *OsRRP* sequences to GenBank, 11 more cDNA sequences of rice S-like RNase, which are essentially the same as that of *OsRRP* described in this report, have been deposited in the GenBank database under UniGene Cluster Os.9417. Only one of them, drought-induced S-like RNase (RNase DIS, GenBank accession no. AY061961), was characterized, and it found to be the most strongly up-regulated protein in rice leaves during drought stress.[5] We found, however, that *OsRRP* transcription was not induced by drought stress, as determined by northern blot analysis (data not shown). Given that *OsRRP* is preferentially expressed in stems and down-regulated in increased tillering dwarf mutant plants at the three-leaf seedling stage and the booting stage, it will be interesting to investigate further whether OsRRP plays a role in rice shoot development. To investigate the function of OsRRP, further studies are required to localize OsRRP precisely in stems and to correlate the expression levels of *OsRRP* in transgenic rice plants overexpressing or silencing *OsRRP* with the corresponding phenotypic changes.

Acknowledgments

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