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ORIGINAL ARTICLE

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Identification and evolutionary analysis of a relic S-RNase in *Antirrhinum*

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Abstract In several gametophytic self-incompatible species of the Solanaceae, a group of RNases named relic S-RNase has been identified that belong to the S-RNase lineage but are no longer involved in self-incompatibility. However, their function, evolution and presence in the Scrophulariaceae remained largely unknown. Here, we analyzed the expression of S-RNase and its related genes in *Antirrhinum*, a member of the Scrophulariacaeae, and identified a pistil-specific RNase gene; *AhRNase29* encodes a predicted polypeptide of 235 amino acids with an estimated molecular weight of 26 kDa. Sequence and phylogenetic analyses indicated that AhRNase29 forms a monophyletic clade with *Antirrhinum* S-RNases, similar to that observed for other relic S-RNases. Possible evolution and function of relic S-RNases are discussed.

Keywords Self-incompatibility · Relic S-RNases · S-RNases · Evolution · *Antirrhinum*

Introduction

Self-incompatibility (SI) is a genetic mechanism to prevent self-fertilization in many flowering species by allowing the pistil to reject pollen from genetically related individuals (de Nettancourt 2001). A common origin was once assumed for SI in flowering plants (de Nettancourt 2001). However, recent molecular evidence indicates that the molecular basis of SI is different in the three angiosperm families—Brassicaceae, Papaveracee and Solanaceae—demonstrating that SI had separate origins in evolution (for a recent review see McCubbin and Kao 2000). Nevertheless, SI in the Scrophulariaceae, Solanaceae and Rosaceae families has been shown to be controlled by a common protein: S-RNase (McCubbin and Kao 2000). Since their most recent common ancestor is the ancestor of ~75% of dicot plants, this indicates that S-RNases share a common origin in most dicot families (Igic and Kohn 2001; Steinbachs and Holsinger 2002). Additional evidence also supports a monophyletic origin of SI in the three families. First, all S-RNases have an intron of similar size in a conserved position, except in the genus *Prunus* of the Rosaceae, which has an additional unique intron located upstream of the first conserved region of S-RNases (Ishimizu et al. 1998), indicating that they all have a similar genomic structure. Second, it is considered unlikely that the same gene evolved a similar extra function by two or three independent convergent evolutionary events. Although it is still unclear how an ancestral RNase gene was recruited into SI, this must have occurred before the divergence of the three families (Xue et al. 1996; Igic and Kohn 2001).

An SI system requires at least three allelic genes to work (de Nettancourt 2001), but how an ancestral *S-RNase* gene evolved into three or more different allelic forms is unclear. It appears that point mutations and intragenic recombination contributed to allelic diversity of the *S-RNase* genes at the *S* locus (Wang et al. 2001). Recently, several *S-RNase*-related genes have been identified in the pistil of several self-incompatible and compatible species of the Solanaceae and Rosaceae (Ai et al. 1991; Lee et al. 1992; Kuroda et al. 1994; Golz et al. 1998; Katoh et al. 2002; Kondo et al. 2002) that belong to the *S-RNase* lineage but are no longer involved in SI and have been referred to as relic *S-RNases* (Golz et al. 1998). Golz et al. (1998) have also proposed that relic *S-RNases* may be associated with the partial duplication of an *S* allele in a self-incompatible species or may arise during the transition from SI to self-compatibility in a self-incompatible species.

So far, no relic *S-RNase* gene has been identified in *Antirrhinum*, a member of the Scrophulariaceae. In this study, we analyzed *S-RNase*-related genes expressed in the pistil of *Antirrhinum* and cloned a *RNase* gene: *AhRNase29*. Molecular and phylogenetic analyses

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showed that it represents a relic S-RNase found in the Scrophulariaceae.

Materials and methods

Plant material

Antirrhinum majus and self-incompatible lines derived from interspecific crosses between *A. majus* and *Antirrhinum hispanicum* have been described previously (Xue et al. 1996; Liang et al. 2002).

Isolation and sequencing of AhRNase29 cDNA and genomic DNA

Several RNase sequences were obtained by PCR amplification of the single-strand cDNA from the style of *Antirrhinum* with primers designed according to the conserved C2 region of S-RNases (Xue et al. 1996). Among them, the 3'region of a cDNA named *AhRNase29* was obtained using rapid amplification of cDNA ends (RACE) as described previously (Liang et al. 2002). A leaf cDNA library was constructed from an S_2S_4 (A^{16}) line using a SMART cDNA Library Construction Kit (Clontech, Palo Alto, Calif.). The library was screened by plaque hybridization using a PCR product containing the 3' part of the *AhRNase29* cDNA as a probe. Positive plaques were purified and positive cDNA clones converted to plasmid form according to the manufacturer's instructions, and sequenced.

AhRNase29 genomic DNA sequence was obtained by designing two primers corresponding to the 5' and 3' ends of the cDNA for PCR using genomic DNA of *Antirrhinum*. *AhRNase29* cDNA and genomic sequences have been submitted to the EMBL database under the accession numbers of CAC50875 and AJ507660, respectively.

Molecular techniques

Nucleic acid isolation and hybridization, and in situ RNA hybridization were performed essentially as described previously (Liang et al. 2002). The phylogenetic tree was generated with Clustal W, using a neighbor-joining feature (http://www.ebi.ac.uk/).

Results

Identification of a pistil RNase gene from *Antirrhinum*

During cloning of *Antirrhinum S RNase* cDNA (Xue et al. 1996), one pistil cDNA fragment encoding a peptide with sequence similarity to *S RNases* was identified, and its full-length cDNA product was subsequently isolated. The cDNA encodes a predicted polypeptide of 235 amino acids with an estimated molecular weight of 26 kDa, and comparison of the cDNA sequence with its corresponding genomic sequence showed that the gene contains an intron of 141 bp in length (Fig. 1), similar to some of the *S RNases* found in *Antirrhinum* (Lai et al. 2002; Vieira and Charlesworth, 2002) and other plant species (Green 1994). Amino acid sequence comparison with three *Antirrhinum* S RNases (Xue et al. 1996) showed that it is more similar to S RNases than to an *Antirrhinum* S-like RNase called AhSL28 (Liang et al. 2002) (Fig. 2). Because this gene is not polymorphic between *S* alleles (see below), it is unlikely to play a direct role in the self-incompatible reaction and therefore was named *AhRNase29*. The five regions (C1–C5) that are highly conserved among S-RNases (Ioerger et al. 1991) are also present in AhRNase29 (Fig. 2). Apart from the histidine residues in the C2 and C3 regions – the active sites for ribonuclease activity – three cysteine residues in AhRNase29 at positions 46, 126 and 202 are also conserved (see Fig. 2), suggesting that it shares a common backbone fold with the S and S-like RNases (Kurihara et al. 1992). These results indicate that AhRNase29 is a typical T2-type RNase from *Antirrhinum* pistil.

Genomic organization of *AhRNase29*

To investigate genomic organization of *AhRNase29*, DNA blot analysis was carried out on genomic DNA from two self-incompatible lines (S_1S_5 and S_2S_4) using *AhRNase29* as a probe. Identical single fragments of 16 kb, 7.5 kb, and 5 kb in *Hin*dIII-, *Eco*RI- and *Bam*HI-digested DNA, respectively, were obtained with both *S* genotypes (Fig. 3), indicating that *AhRNase29* is a monomorphic and single copy gene. Linkage analysis using the *S* allele segregating populations developed previously (Xue et al. 1996) showed that *AhRNase29* detected similar hybridizing fragments as above, showing that an identical copy is present in plants with various *S* allele combinations (data not shown). Although we could not rule out the possibility that AhRNase29 is encoded in the *S* locus (it cannot be

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1     ATGTCAACTAATAAACAAACGCATATTTTCTTTCTGGTTGTGTGCCTTGTTCTCTTTCCT
       M  S  T  N  K  Q  T  H  I  F  F  L  V  V  C  L  V  L  F  P
61    GACTATGCTTTTACAGGCCGGCCTCCTGTTGGATTTGAGTACCTCAAGCTCTGGCTCCAA
       D  Y  A  F  T  G  R  P  P  V  G  F  E  Y  L  K  L  W  L  Q
121   TGGCCACCATCGTTCTGCAGCCTGTCTCGCGTAGCATGCGGGAGAGATCCTGTTCCTGCT
       W  P  P  S  F  C  S  L  S  R  V  A  C  G  R  D  P  V  P  A
181   GAATTTACGATACATGGACTTTGGCCTGATAATTACTCGCATGAACTGAATTATTGCAAA
       E  F  T  I  H  G  L  W  P  D  N  Y  S  H  E  L  N  Y  C  K
241   TCCAATAAACAACTCTCAGTTGTATAGACTTCAATCCCAACTTATTCCTGTCAATAATTT
       S  N  K  Q  L  S  V
301   CTCATTTTAAAGTTTTGTTTCTCAAGTATTAGATTACTTATATATTAGTTGTTATGTTGT

361   TCTTAACCTAGCTAATTACAAATAATTAACTTGTATACGCAGCAAATTGAAGACATTGGA
                                                 Q  I  E  D  I  G
421   GAGTGGCTGGATAAAGATTGGCCAGATCTAATGAAACAAGCCACAGTGAACCCGGATAAG
       E  W  L  D  K  D  W  P  D  L  M  K  Q  A  T  V  N  P  D  K
481   GGGTTTTATGAAGAACAATGGAGAAAACATCGAATATGCTCTTCAAATATCTTCACTCCC
       G  F  Y  E  E  Q  W  R  K  H  R  I  C  S  S  N  I  F  T  P
541   AAAGAATACTTCACCCTCGGAATGAAGTTAAAGAAAGCACGTAATCTACTGCAAGTATTT
       K  E  Y  F  T  L  G  M  K  L  K  K  A  R  N  L  L  Q  V  F
601   CACCAGAATGAAATATATGAGAGTCAATTCTCCTCTATCTCACGTATAAATAAAGCTATA
       H  Q  N  E  I  Y  E  S  Q  F  S  S  I  S  R  I  N  K  A  I
661   AAAATCATTACAGGGAGACAATCCCCAATAGTGAAATGCTCCCGCCACCCCCAAAAAGGG
       K  I  I  T  G  R  Q  S  P  I  V  K  C  S  R  H  P  Q  K  G
721   TCTTTATTGACAGAAGTCATCCTATGCTTTGACCTAAAGGGAGACTACTTCAAAAATTGT
       S  L  L  T  E  V  I  L  C  F  D  L  K  G  D  Y  F  K  N  C
781   ACAGACCCGTTTGGCCGTGCATGCCCGAAAAGTACCAATGTTTTTTTTCCTAAGAAGGTC
       T  D  P  F  G  R  A  C  P  K  S  T  N  V  F  F  P  K  K  V
841   ATTGTTTAACTGCTAGTAGTGTTAGGAAGAATATTGTTACATCATATGTGTTGCCCATAC
       I  V  *
901   TAAAGATGTACAATATTAATATCTAAATTATTACAGCTCCAGTTGACAAAAAAAAAA
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Fig. 1 The DNA and amino acid sequences of *AhRNase29*. The intron is *bold* and *underlined*. The nucleotide sequence is numbered from the *A* of the initiation codon

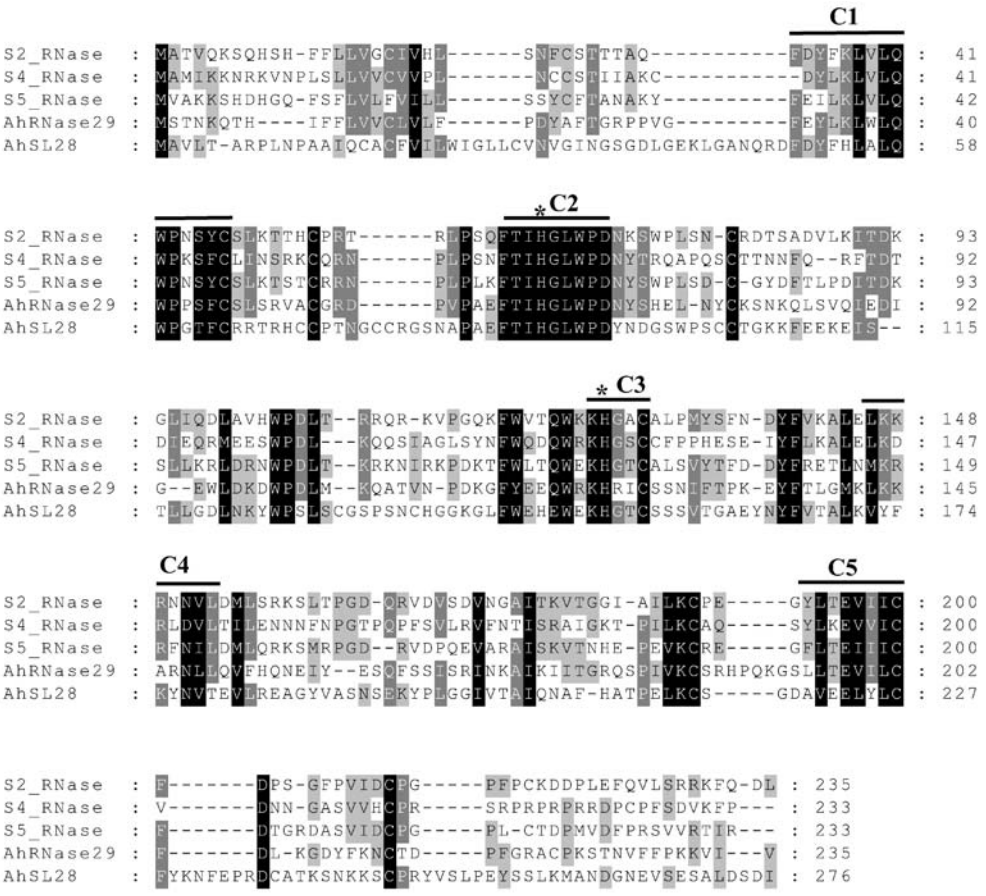

Fig. 2 Alignment of predicted polypeptide sequences of S, S-like and relic S-RNases in *Antirrhinum*. The S RNase sequences (S2-, S4- and S5-RNase) are from Xue et al. (1996) and AhSL28 is from Liang et al. (2002). Conserved domains *(C1–C5)* are indicated. *Asterisks* Activity sites in C2 and C3

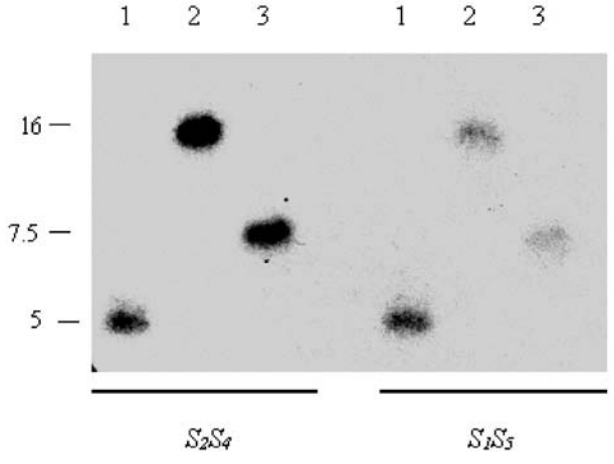

Fig. 3 DNA blot analysis of *AhRNase29*. Genomic DNA (10 μg) from S_2S_4- and S_1S_5-containing lines was digested with *Bam*HI, *Eco*RI, or *Hin*dIII (lanes *1*, *2* and *3*, respectively). After separation by agarose gel electrophoresis and blotting, the DNA was hybridized with *AhRNase29* cDNA. The sizes of hybridizing fragments (kb) are indicated

mapped due to lack of restriction fragment length polymorphism between *S* alleles), its identical allelic presence shows that it plays no direct role in SI, indicating that *AhRNase29* likely represents a relic *S-RNase* (Golz et al. 1998).

AhRNase 29 is specifically expressed in pistil

The possibility that *AhRNase29* belongs to the relic *S-RNases* suggested that it might be specifically expressed in the pistil. To investigate this possibility, *AhRNase29* expression was examined by northern blot experiments. A transcript of 1.1 kb was detected in the pistil, but not in leaf, petal, sepal, or anther (Fig. 4), showing that *AhRNase29* is a pistil-specific gene. To confirm this, in situ RNA hybridization was performed. An antisense *AhRNase29* probe detected transcripts in the transmitting tract of the pistil (Fig. 5), which is also the site of expression of *S-RNases* in *Antirrhinum* (Xue et al. 1996). The expression pattern appeared to support the hypothesis that *AhRNase29* is a relic *S-RNase* gene.

AhRNase29 belongs to the S-RNase lineage in the Scrophulariaceae

To identify the relationship of AhRNase29 with other S, S-like and relic S-RNases, a phylogenetic analysis was performed based on the deduced amino acid sequences

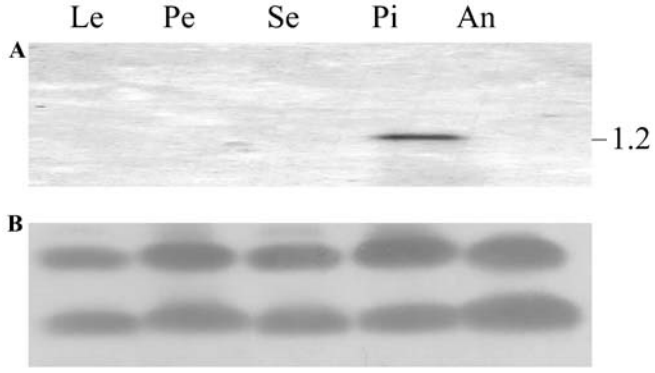

Fig. 4A, B RNA blot analysis of *AhRNase29*. **A** RNA from leaf (*Le*), petal (*Pe*), sepal (*Se*), pistil (*Pi*), and anther (*An*) was hybridized with the full-length *AhRNase29* cDNA. **B** The same blot was hybridized with rDNA. The transcript size (kb) is indicated

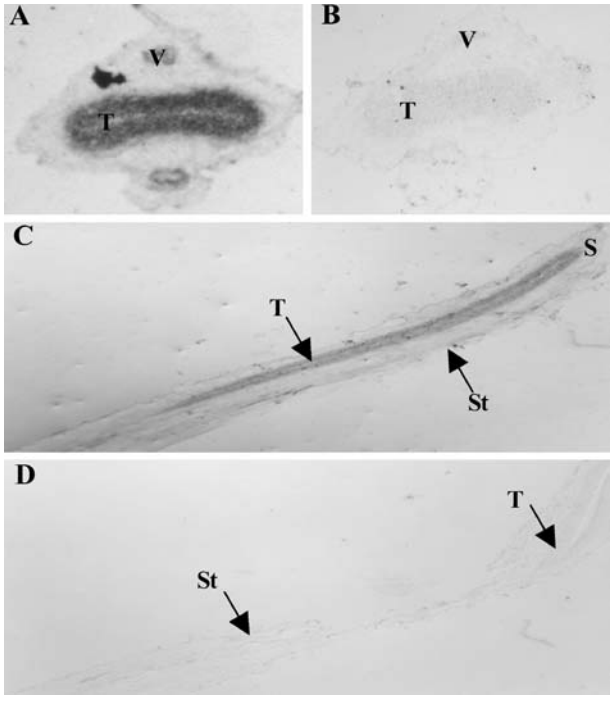

Fig. 5A–D In situ RNA hybridization analysis of *AhRNase29*. Expression of *AhRNase29* in pistil was detected by RNA in situ hybridization with sense or antisense probes specific for *AhRNase29*. **A**, **B** Transverse sections of pistil hybridized with antisense (**A**) or sense (**B**) probes. **C**, **D** Longitudinal sections of the style hybridized with antisense (**C**) or sense (**D**) probes. *Red color* Hybridization signal, *V* vascular tissue, *T* transmitting track, *S* stigma, *St* style

from a number of S, S-like RNases and relic S-RNases of several plant species using fungal RNase as an outgroup (Fig. 6). The result showed that S-RNases and relic S-RNases form a monophyletic lineage distinctive from the S-like RNase lineage. Like other relic S-RNases from the Solanaceae (Golz et al. 1998), AhRNase29 is clustered together with the three *Antirrhinum* S-RNases, indicating that AhRNase29 represents a new relic S-RNase present in the Scrophulariaceae.

Discussion

In this study, we have identified a pistil-specific *RNase* gene, *AhRNase29*, in *Antirrhinum* and shown that it is a relic *S-RNase*. *AhRNase29* represents the first relic *S-RNase* gene found in the Scophulariaceae.

How RNase-based SI evolved is still unknown. In this respect, it is interesting to define the evolutionary relationship between the relic *S-RNases* and *S-RNases* because of their close similarities. Two scenarios are possible: either an ancestral *S-RNase* was a direct evolutionary product of a relic *S-RNase*, or vice versa. It appears to be more likely that an ancestral *S-RNase* gave rise to an ancestral relic *S-RNase*. First, relic *S-RNases* have a similar genomic structure to that of *S-RNases*, with an intron of similar size at a conserved position as well as a similar pistil-specific expression pattern, indicating that they share a common origin (Golz et al. 1998; Igic and Kohn 2001). Second, based on the constructed phylogenetic tree, S-RNases and relic S-RNases form a monophyletic lineage distinct from that of S-like RNases from the Solanaceae and Scrophulariceae (Fig. 6). If relic *S-RNases* were the ancestor of *S-RNases*, they would be more closely related to *S-RNase* from the Rosaceae because the ancestor of the latter predates the divergence between the Solanaceae and Scrophulariaceae (Igic and Kohn 2001; Steinbachs and Holsinger 2002). The fact that relic *S-RNases* are always more closely related to the *S-RNases* from the same family (see Fig. 6) is consistent with the suggestion that *S-RNase* was the ancestor of relic *S-RNase* (Golz et al. 1998; Kondo et al. 2002). Nevertheless, their divergence occurred within separate families (Fig. 6). Interestingly, we have identified a cDNA sequence identical to *AhRNase29* in *Antirrhinum mollissimum* (data not shown, but see Vieira and Charlesworth 2002), indicating that such a transition from an *S-RNase* and to a relic *S-RNase* occurred before speciation within the Scrophulariaceae.

Because of their similar structure and expression, Golz et al. (1998) have proposed that a duplication and translocation event of the *S* locus was involved in the evolution of relic *S-RNases*. However, the presence of *Antirrhinum* relic *S-RNase* genes is not associated with SI. It is possible that only part of the *S*-locus, including the *S-RNase* but without the pollen *S* component, was duplicated and evolved into relic *S-RNases* (Golz et al. 2001). Therefore, detailed comparative analysis of the genomic structure of relic *S-RNase* and *S-RNase* may provide some clue to their evolutionary pathway(s).

The function of relic S-RNases remains unclear. Their presence in the three gametophytic self-incompatible families suggests that they may play a conserved role. The extensive similarities to S-RNases also suggest that relic S-RNases might play a role in SI, but a direct role can be ruled out because of their monomorphic nature. Apart

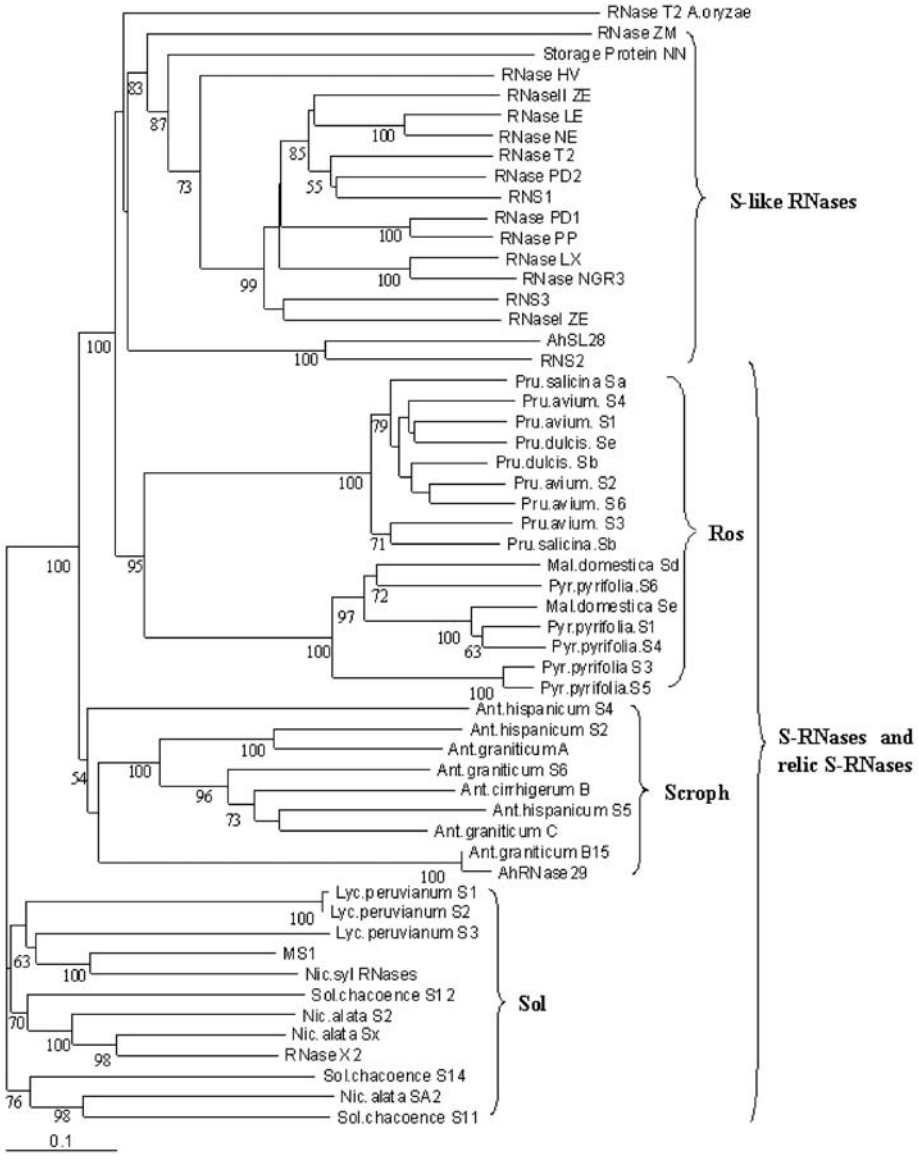

Fig. 6 A phylogenetic tree derived from the alignment of multiple S, S-like RNases and relic S-RNases using Clustal W plus a neighbor-joining feature. RNase T2 of *Aspergillus oryzae* (accession number S01668) was used as an outgroup. The plant S-like RNase sequences included: *PD1*, *PD2* (*Prunus dulcis*; Ma and Oliveira 2000); *RNS1*, *RNS2*, *RNS3* (*Arabidopsis thaliana*; Taylor et al. 1993); *T2* (*Cicer arietinum*; AJ012689); *LE*, *LX* (*Lycopersicon esculentum*; Löffler et al. 1993); *NE* (*Nicotiana alata*; accession no. U13256); *ZE* (*Zinnia elegans*; U19923); *HV* (*Hordeum vulgare*; AY120886); *PP* (*Pyrus pyrifolia*; D49529); *NGR3* (*Nicotiana glutinosa*; AB032256); *ZM* (*Zea mays*; M83668); *AhSL28* (*Antirrhinum*; Liang et al. 2002). The plant S-RNase sequences are derived from the Solanaceae (*Sol*) (Anderson et al. 1989; Ai et al. 1991; Saba-el-Leil et al. 1994; Ishimizu et al. 1998), Scrophulariaceae (*Scroph*) (Xue et al. 1996; Vieira and Charlesworth 2002) and Rosaceae (*Ros*) (Sassa et al. 1996; Ushijima et al. 1998; Wiersma et al. 2001). *Lyc* Lycopersicon *Mal* Malus, *Nic* Nicotiana, *Pru* Prunus, *Pyr* Pyrus, *Sol* Solanum, *Ant* Antirrhinum. The plant relic S-RNases included *MS1* (*Nicotiana alata*; Kuroda et al. 1994), *Nic.syl RNase* (*Nicotiana sylvestris*; Golz et al. 1998), and *RNase X2* (*Petunia*; Lee et al. 1992)

from possible roles in defense against pathogen attack and in nutrition metabolism, like S-like RNases (Green 1994; Liang et al. 2002), it will be interesting to investigate the role, if any, of relic S-RNases in the self-incompatible reaction through transgenic and biochemical approaches.

AhRNase29 is the first relic *S-RNase* gene isolated from the Scrophulariaceae, suggesting that relic *S-RNases* likely have a wider distribution in many RNase-based SI species. Several phylogenetic analyses, including this study, have revealed that S-like RNases form a monophyletic clade independent of the S-RNase and relic S-RNase clade (Golz et al. 1998; Igic and Kohn 2001; Liang et al. 2002; Steinbachs and Holsinger 2002), indicating that *S-RNases* were the ancestor of S-like RNases and their separation occurred during an early period of angiosperm radiation. The available evidence also indicates that an ancestral RNase was recruited into SI at an early stage of angiosperm evolution. Subsequent to this,

some ancestral *S-RNases* lost their newly acquired function and became an ancestral *S-like RNase* prior to family diversification, and some evolved into an ancestral relic *S-RNase* after the appearance of the family. Further studies are required to piece together some details of these processes.

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