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AhSL28, a senescence- and phosphate starvation-induced S-like RNase gene in *Antirrhinum*

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Abstract

Several species of higher plants have been found to contain S-like ribonucleases (RNases), which are homologous to S-RNases controlling self-incompatibility. No S-like RNase genes have been isolated from self-incompatible *Antirrhinum*. To investigate the relationship between S- and S-like RNases, we cloned a gene named *AhSL28* encoding an S-like RNase in *Antirrhinum*. Amino acid sequence, genomic structure and phylogenetic analyses indicated that *AhSL28* is most similar to *RNS2*, an S-like RNase from *Arabidopsis thaliana* and formed a distinct subclass together with several other S-like RNases within the S-RNase superfamily. Unlike S-RNase genes in *Antirrhinum*, *AhSL28* is not only expressed in pistils but also in leaves, petals, sepals and anthers, in particular, showing a strong expression in vascular tissues and transmitting track. Moreover, its RNA transcripts were induced during leaf senescence and phosphate (Pi) starvation but not by wounding, indicating that *AhSL28* plays a role in remobilizing Pi and other nutrients, particularly when cells senesce and are under limited Pi conditions in *Antirrhinum*. Possible evolutionary relations of S- and S-like RNases as well as signal transduction pathways related to S-like RNase action are discussed.

Keywords: *AhSL28*; S-like RNase; Senescence; Phosphate starvation; *Antirrhinum*

1. Introduction

Recently, plant ribonucleases (RNases) have been extensively studied in several species and the best-characterized family is represented by enzymes typified by the fungal RNase T2 distributed throughout virus, bacteria, fungi, slime mold, *Drosophila*, oyster and plants [1]. Plant members of this family were first identified when sequences of proteins linked to gametophytic self-incompatibility (*S*) locus in the Solanaceae [2] were determined and found to be similar to fungal RNases [3]. The identification of these proteins, termed S-RNases, led to the discovery of related proteins in a variety of self-compatible or self-incompatible plant species. Based on their similarity to S-RNases, these enzymes were referred to as "S-like RNases" [4].

The S-like RNases have conserved sequences characteristic of the S-RNases. In general, their molecular masses are between 21 and 29 kDa, and most have been shown or predicted to be secreted proteins. Although S-like RNases are close molecular relatives to the S-RNases, they have important differences in structure, expression and function [4]. Most notably, they do not participate in the control of self-incompatibility. S-like RNase genes have been found in all plants that have been examined for their presence, indicating that they constitute an important family of RNA-degrading enzymes in plants. In contrast to S-RNase genes, whose expression is generally restricted to pistil, S-like RNase genes are often expressed in other organs under certain environmental conditions. For example, three tomato S-like RNase genes, *LV1*, *LV2* and *LV3*, are induced during phosphate (Pi)-starvation processes [5]. The mRNA levels of *Arabidopsis RNS1*, *RNS2* and *RNS3* increase dramatically during senescence [4]. Tomato RNase LE functions in plant wounding [6]. RNase X2 of *Petunia hybrida* [7], RNS2 of *Arabidopsis thaliana* [4] and RNase NE of *Nicotiana alata* [8] are expressed in pistil and likely function as pathogen defense proteins.

In gametophytic self-incompatible *Antirrhinum*, several S-RNases have been isolated, but no S-like RNase gene has been characterized. To investigate the evolutionary relationship between S- and S-like RNase genes in *Antirrhinum* in general and the latter's function in particular, we isolated an S-like RNase gene, named *AhSL28* from *Antirrhinum* in this

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paper. Phylogenetic and genomic structural analyses provided a close look at their relation. Furthermore, RNA expression analysis showed that it is specifically induced during senescence and Pi starvation with a higher expression in transmitting track and vascular tissue, suggesting that this gene plays a role in nutrition metabolism, especially in Pi scavenging.

2. Materials and methods

2.1. Plant material

An *Antirrhinum majus* line, M^{75} (self-compatible), and several self-incompatible lines derived by interspecific crosses between *A. majus* and *Antirrhinum hispanicum*, as well as an *S* alleles segregating population, were described previously [9].

2.2. Isolation and sequence of AhSL28 cDNA and genomic DNA

The initial *AhSL28* fragment was obtained by PCR amplification of the style sscDNA of *Antirrhinum* with the primers designed according to the most conserved region of S-RNase and then obtained its 3′ end using the method of 3′ RACE. A leaf cDNA library was constructed from an $S_2S_4(A^{16})$ line using a SMART cDNA Library Construction Kit (Clontech). The library was screened by plaque hybridization using PCR product containing 3′ part of *AhSL28* as a probe [10]. Positive plaques were purified, the positive cDNA clones were converted into a plasmid form according to the manufacturer's instruction and sequenced. *AhSL28* genomic DNA sequence was obtained by designing a series of primers (available upon request) for PCR of the genomic DNA of *Antirrhinum*. The sequences for *AhSL28* cDNA and its genomic sequences have been submitted to EMBL database under the accession numbers CAC50874 and AJ48 9249, respectively.

2.3. Southern and northern analyses

Genomic DNA was isolated from leaves of *Antirrhinum* using a cetyltrimethylammonium bromide (CTAB) extraction method [11]. The DNA (10 μg) was digested, separated on 0.8% agarose gel and transferred onto Hybond N^+ (Amersham) membrane. Prehybridization, hybridization,

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                                                                                                C1
AhSL28      MAVLTARPLNPAAIQCACFVILWIGLLCVNVGINGSGDLGEKLGANQRDFDYFHLALQWPGTFCRRTRHCCP
RNS2        MA----SRL--------CLLLL---VACIAGAF--AGDVIE-LNRSQREFDYFALSLQWPGTYCRGTRHCCS
RNase LC1   MAMAKREIVL---------VFV---LTILFPMVK-----SQT-------FDSFWMVQHWPPAVCSFQQGRCV
RNase LC2   M--ATKVLLLAT------IVLL---LTILFLGAE-----SQT-------FDHFFFVQQWPPTTCQQQQKPCF
S2 RNase    MATVQKSQHSH-------FFLL---VGCIVHLSNFCSTTTAQ-------FDYFKLVLQWPNSYCSLKTTHCP
S4 RNase    MAMIKKNRKVNP------LSLL---VVCVVPL-NCCSTIIAK-------CDYLKLVLQWPKSFCLINSRKCQ

                                *C2
AhSL28      TNGCCRGSNAPAEFTIHGLWPDYNDGSWPSCCTGKKFEEKEIST--LLGDLNKYWPSLSCGSPSNCHGGKGL
RNS2        KNACCRGSDAPTQFTIHGLWPDYNDGSWPSCCYRSDFKEKEIST--LMDGLEKYWPSLSCGSPSSCNGGKGS
RNase LC1   GQG------LRS-FTIHGVWPQKGGTSVIN-CPGPTFDFTKISH--LESTLNVDWPNV-------ITGNNKW
RNase LC2   QPP------PAT-FKIHGLWPQKGPNSVVY-C-NKNFDRTQISS--LENQLDVVWPDV-------VTGNNTG
S2 RNase    RTR------LPSQFTIHGLWPDNKSWPLSN-CRDTSADVLKITDKGLIQDLAVHWPDLT---RRQRKVPGQK
S4 RNase    RNP------LPSNFTIHGLWPDNYTRQAPQSC--TTNNFQRFTDTDIEQRMEESWPDLK---QQSIAGLSYN

                C3    *                          C4
AhSL28      FWEHEWEKHGTCSSSVTGAEYNYFVTALKVYFKYNVTEVLREAGYVASNSEKYPLGGIVTAIQNAFHATPEL
RNS2        FWGHEWEKHGTCSSPVFHDEYNYFLTTLNLYLKHNVTDVLYQAGYVASNSEKYPLGGIVTAIQNAFHITPEV
RNase LC1   FWGHEWNKHGICSVSKFDQQ-AYFQMAINMRNSIDLLSALRVGGVVPNGRSKAR-QRVQSAIRAQLGKEPVL
RNase LC2   FWEHEWNKHGSCSESQFNQT-LYFQTAINMMNKVNLLKALGKGGITSDERTKSS-QTMQKVLLAQFGNQPFL
S2 RNase    FWVTQWKKHGACALPMYSFN-DYFVKALELKKRNNVLDMLSRKSLTPGD-QRVDVSDVNGAITKVTGGIAIL
S4 RNase    FWQDQWRKHGSCCFPPHESE-IYFLKALELKDRLDVLTILENNNFNPGTPQPFSVLRVFNTISRAIGKTPIL

                        C5
AhSL28      KCSGDAVE----ELYLCFYKN-FEPRDCATK---SNKKSCPRYVSLPEYSSLKMANDGNEVSESALDSDI
RNS2        VCKRDAID----EIRICFYKD-FKPRDCVGSQDLTSRKSCPKYVSLPEYTPL--DGEAMVLKMPTEREAL
RNase LC1   RCRGTGRQSRLLEIVMCFDDDGVTLINCN-----PANSNCPN--S----------------------FI-F
RNase LC2   RCKKVGQQFWLLEIVMCFKDDGVTMINCQ-----PSKVSCPP--N----------------------FI-F
S2 RNase    KC----PEGYLTEVIICFDPSGFPVIDC------PGPFPCKD--DPLEFQVLS----------RRKFQDL
S4 RNase    KC----AQSYLKEVVICVDNNGASVVHC------PRSRPRPR--PRRDPCPFS----------DVKFP--
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Fig. 1. Alignment of predicted polypeptide sequences of AhSL28 with two *Antirrhinum* S-RNases (S_2- and S_4-RNase) and three S-like RNases of *Arabidopsis* (RNS2) and *L. cylindrica* (RNase LC1 and LC2). The conserved (C1–C5) domains as well as the activity sites in C2 and C3 are indicated by an asterisk.

and washing of the blot were performed as recommended by the manufacturer. Total RNA was extracted from different tissues with the protocol according to RNeasy Plant Mini Kit (Qiagen). RNA samples were separated on 1% agarose/formaldehyde gels and transferred to Hybond N^+ (Amersham), and prehybridization, hybridization and washing of the blot were performed as recommended by the manufacturer. Probes were labeled with ^{32}P by random priming using Prime-α-Gene Labeling system (Promega). The blots were exposed to X-ray films (Kodak).

2.4. In situ RNA hybridization

Dig-labeled sense and anti-sense RNA probes were prepared following the manufacturer's recommendation (Boehringer Mannheim). Tissue fixation and embedding, in situ hybridization and signal detection were essentially performed as described [12].

2.5. Leaf senescence, Pi starvation and wounding experiments

For leaf senescence experiments, leaves were staged on the basis of morphological characteristics as defined in Smyth et al. [13]. Senescing leaves were those showing visible signs of chlorosis at leaf margins and wilting. To starve *Antirrhinum* plants for Pi, 1-month-old seedlings were removed from soil and cultured in liquid media with or without 1.00 mM NaH_2PO_4 for 12 h in the dark [4]. As for plant wounding experiment, detached leaves were cut into stripes and left at room temperature for 5, 15, 30 and 60 min, respectively.

3. Results

3.1. Identification of an S-like RNase from Antirrhinum

During the isolation of stylar cDNA encoding S-RNases in *A. hispanicum* × *majus* [11], one cDNA fragment homologous to S-RNases was identified and its full-length product was cloned and referred to as *AhSL28*. It encodes a predicted polypeptide of 276 aa with an estimated molecular weight of 30 kDa, slightly bigger than S- and S-like RNases from several plant species [1]. Amino acid sequence comparison with two S-RNases from *Antirrhinum* and several S-like RNases including those from *Arabidopsis* and *Luffa cylindrica* separately showed that its similarity to them is dispersed throughout (Fig. 1). The five regions highly conserved among the S-RNases (C1–C5) [14] are also evident in the AhSL28 including the histidine residues in C2 and C3 regions, important for RNase activity (Fig. 1). Two pairs of cysteine residues at positions 71 and 154 and positions 216 and 246, respectively, known to form disulfide bonds, are also present [15]. The results indicated that AhSL28 is highly homologous to S-RNases. Linkage analysis to the *S* locus in *Antirrhinum* showed that *AhSL28* is a monomorphic gene (data not shown but see below) and therefore encodes an S-like RNase. We also found that AhSL28 have longer N and C ends than other RNases (Fig. 1). It is unclear why it has the longer terminal ends.

3.2. Genomic organization of AhSL28

To investigate the genomic organization of *AhSL28*, DNA blot analysis was carried out on genomic DNA from two self-incompatible lines (S_1S_5 and S_2S_4). It detected three identical *Hin*dIII (0.9, 3.9 and 5.5 kb) and three *Eco*RI (2.9, 7.2 and 9.5 kb) fragments between the two *S* genotypes (Fig. 2). The three respective hybridizing fragments were predicted from the *AhSL28* genomic DNA sequence because two internal *Eco*RI and *Hin*dIII restriction sites are found respectively, showing that *AhSL28* is a unique copy and monomorphic gene in *Antirrhinum*. Similar results were obtained using genomic DNA from *A. majus* (data not shown), suggesting that *AhSL28* is highly conserved in *Antirrhinum*.

3.3. AhSL28 is closely related to Arabidopsis RNS2

To identify which group of S-like RNases AhSL28 belongs to, a phylogenetic analysis was performed based on deduced amino acid sequences from available S- and

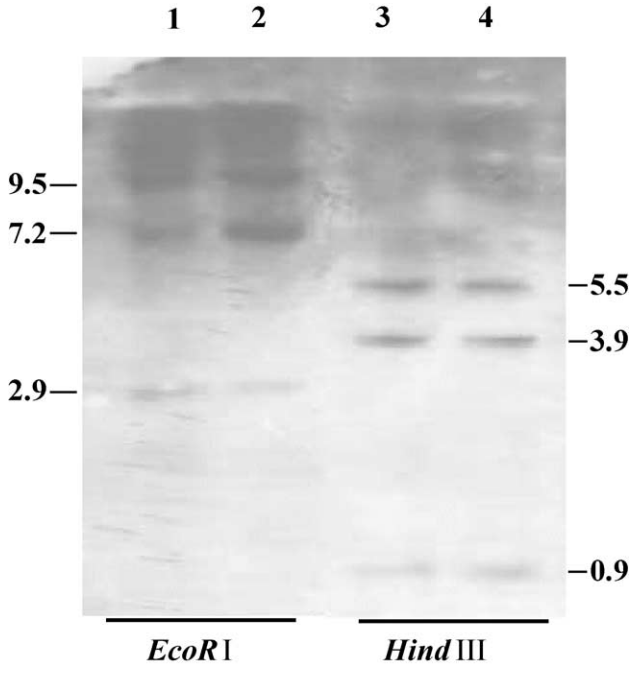

Fig. 2. DNA blot analysis of *AhSL28*. Ten micrograms of genomic DNA from various *S* allele-containing lines was restricted by *Hin*dIII and *Eco*RI, respectively. After separation by agarose gel electrophoresis and blotting, the DNA was hybridized with *AhSL28* cDNA. Lanes 1 and 3 represent plants with S_2S_4 and lanes 2 and 4 plants with S_1S_5, respectively. The numbers indicate the sizes of hybridizing fragments in kilobases.

S-like RNases from several plant species (Fig. 3A). In the plant lineage, S- and S-like RNases form two distinctive ones as found previously [12] consistent with a specialized function for S-RNases in self-incompatibility. The single S-like RNase lineage implies that their sequences, and thus presumably their function, are evolutionarily conserved across the plant species. AhSL28 is most related to RNS2 from *Arabidopsis* with an overall similarity of 63.4%, suggesting that AhSL28 plays a similar role to RNS2 [4].

To investigate its genomic structure, we obtained a genomic DNA sequence of *AhSL28* of 4915 bp corresponding to its cDNA. Seven introns with the lengths of 1053, 207, 722, 719, 902, 339 and 151 bp and eight exons with the lengths of 236, 30, 74, 104, 94, 163, 78, and 238 bp are

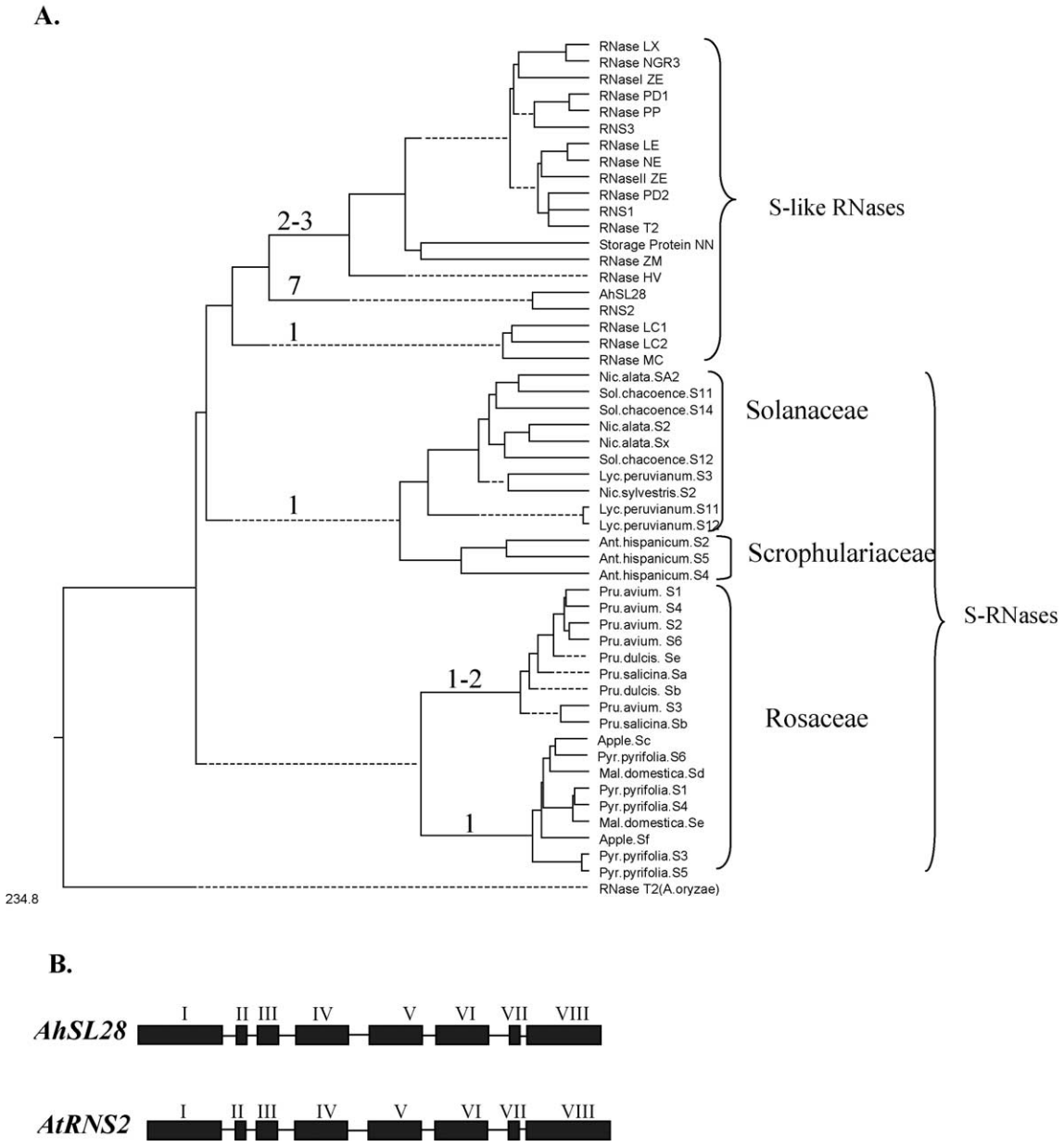

Fig. 3. (A) A phylogenetic tree of plant RNases derived from the alignment of 21 S-like RNases and 31 S-RNases using Clustal W. The plant S-like RNase sequences included: PD1 and PD2 of *Prunus dulcis* [26]; RNS1, RNS2, and RNS3 of *A. thaliana*; T2 of *Cicer arietinum*; LE and LX of *Lycopersicon esculentum*; NE of *N. alata*; ZE of *Zinnia elegans*; HV of *Horrdeum vulgare*; PP of *Pyrus pyrifolia*; NN and NGR3 of *Nicotiana glutinosa*; ZM of *Zea mays* (accession number M83668); LC1 and LC2 of *L. cylindrica* (accession numbers D64011 and D64012); MC of *Momordica charantia* and AhSL28 of *Antirrhinum*; RNase T2 of *Aspergillus oryzae* (accession number S01668). The plant S-RNase sequences are derived from the Solanaceae, Scrophulariaceae and Roasaceae, respectively. Lyc: *Lycopersicon*; Mal: *Malus*; Nic: *Nicotiana*; Pis: *Pisum*; Pru: *Prunus*; Pyr: *Pyrus*; Sol: *Solanum*; Lineages with the numbers of the introns are also indicated. (B) The genomic DNA sequence structure of *AhSL28* from *Antirrhinum* and *RNS2* from *Arabidopsis*. Boxes indicate exons and lines represent introns (intron not drawn to scale).

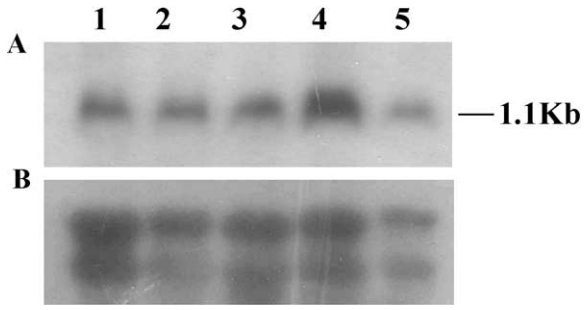

Fig. 4. RNA blot analysis of *AhSL28*. (A) RNA from petal (1), sepal (2), leaf (3), anther (4), and pistil (5) was hybridized with the full-length cDNA of *AhSL28*. (B) The same blot was hybridized by rDNA.

identified respectively, similar to *RNS2* which also contains seven introns with the lengths of 218, 118, 120, 377, 263, 268, 117 bp and eight exons with the lengths of 202, 30, 74, 104, 94, 163, 87 and 258 bp, respectively (Fig. 3B) [4]. Plant T2-type RNases have been grouped into three major classes according to their intron structures. RNS2 from *Arabidopsis* and many apparently orthologous genes from other angiosperms belong to Class II, which have unique sequence motif near the 5′ end with two pairs of double cystein residues as well as many introns [16]. In comparison, AhSL28 possesses these characteristics and is a member of Class II T2-type RNases from *Antirrhinum*.

3.4. AhSL28 is induced during leaf senescence and Pi starvation

The close similarity of *AhSL28* to *RNS2* suggested that *AhSL28* is involved in leaf senescence and Pi scavenge [4]. To investigate these, *AhSL28* expression was detected by northern blot analysis. A similarly sized transcript of 1.1 kb was detected in leaf, petal, sepal, pistil and anther with an ubiquitous expression pattern (Fig. 4). To confirm this, in situ RNA hybridization was performed. An anti-sense probe

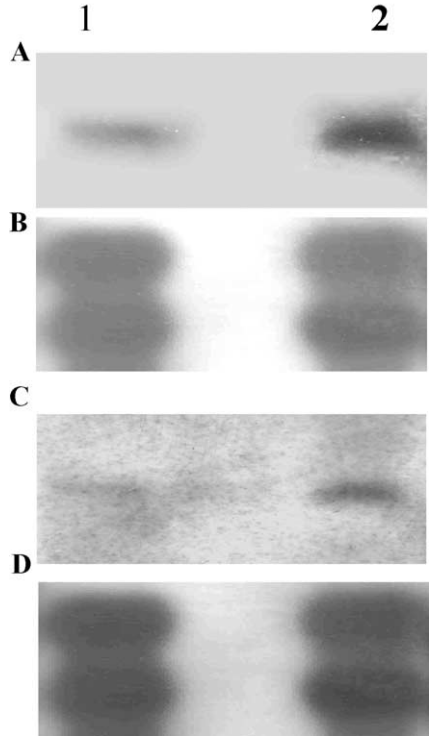

Fig. 6. *AhSL28* is induced by senescence and Pi starvation. (A) RNA from young (1) and senescent (2) leaves was hybridized with the full-length cDNA of *AhSL28*. (B) The same blot was hybridized by rDNA. (C) Leaf RNA from plants grown on normal medium (1) or medium without Pi (2) was hybridized with the full-length cDNA of *AhSL28*. (D) The same blot was hybridized by rDNA.

of *AhSL28* detected its transcripts in the petal, sepal, pistil and anther from an S_2S_5 line with a higher expression in vascular tissues and transmitting track (Fig. 5).

To detect its expression during leaf senescence, RNA was isolated from young and senescing leaves and probed with *AhSL28* cDNA (Fig. 6). The result showed about 10-fold

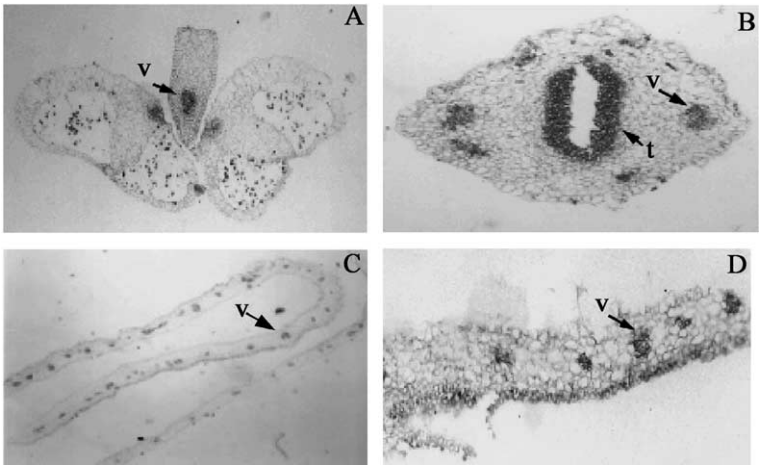

Fig. 5. In situ RNA hybridization analysis of *AhSL28*. The expression of *AhSL28* in various organs was detected by RNA in situ hybridization with an anti-sense probe specific for *AhSL28*. (A) Anther; (B) pistil; (C) petal; (D) sepal. The blue color shows the hybridization signal. v: vascular tissue; t: transmitting track.

increase in *AhSL28* expression in senescing leaves, suggesting that *AhSL28* plays a role in leaf senescence. To investigate the role of *AhSL28* in Pi rescue, RNA was isolated from *Antirrhinum* leaves cultured on a medium containing 1 mM Pi or Pi-free for 12 h and analyzed by northern blot (Fig. 6). The *AhSL28* transcript increased about 10 times on the Pi-free medium. Taken together, these results clearly showed that both leaf senescing and Pi starvation induce the mRNA expression of *AhSL28*.

The induction of *AhSL28* expression in the senescing leaves suggested that its expression is also likely to be wound inducible. To test this, detached leaves were cut into stripes and left at room temperature for 5, 15, 30 and 60 min, respectively, and RNA extracted for northern blot analysis. The result showed that the *AhSL28* transcripts were not altered during these time intervals (data not shown), showing that *AhSL28* is not induced by wounding. However, it is unknown whether the expression of *RNS2* of *Arabidopsis* was affected by wounding.

4. Discussion

In this study, we cloned an S-like RNase gene—*AhSL28*, the first cDNA encoding an S-like RNase to be identified in *Antirrhinum*. So far, both several self-compatible plant species, such as *A. thaliana*, and self-incompatible ones, such as *N. alata* [8], have been found to contain S-like RNases. However, their evolutionary relationships to S-RNase as well as among themselves are not clear.

Plants have evolved separate mechanisms controlling self-incompatibility in different families, but in three distantly related families—the Solanaceae, Scrophulariaceae, and Rosaceae, S-RNases have been found to control gametophytic self-incompatibility [16]. Although previous phylogenetic analyses of S-RNases have indicated that they share a common ancestry in the Solanaceae and Scrophulariaceae, it is not clear whether the S-RNase of the Rosaceae results from convergence or they are truly a homologous system. First, although almost all the S-RNases have five conserved domains(C1–C5) and two hypervariable regions (HVa and HVb), the Rosaceae S-RNases lack HVb [17]. Second, in the genus of *Prunus* of the Rosaceae, their S-RNases have an additional unique intron when compared with all other S-RNases. Third, the Rosaceae is distantly related to the Solanaceae and Scrophulariaceae because it belongs to the subclass Rosaceae and the other two belong to the subclass Asteridae [16]. A recent phylogenetic analysis of 67 T2-type RNases by Igic and Kohn [16] has shown that the S-RNase-based self-incompatibility was an ancestral state, suggesting that the three families' GSI were derived from a homologous system rather than due to convergence and S-RNase divergence predates speciation in the Rosaceae, Scrophulariaceae and Solanaceae. Phylogenetic analyses have shown that the Rosaceae acquired SI before the Scrophulariaceae and Solanaceae. A single or two separate events have been proposed to explain the recruitment of RNases into SI during angiosperm evolution. Based on the available evidence [16], it appears that a single event was more likely to have occurred.

However, it is not clear what evolutionary relationships S-RNase and S-like RNase had during SI evolution. Though they share conserved domains, the most conspicuous differences between S-RNase and S-like RNase are their expression site and function. S-RNases are all pistil-specific expression genes, whereas most of S-like RNases express ubiquitously. Furthermore, all the pistil-specific RNases (S-RNase and relic S-RNase) have a single intron at a conserved position except those in the genus *Prunus* of the Rosaceae with an unique additional intron located upstream of the C1 region. In contrast, most S-like RNases genes have more introns except for *RNase NE* having a single intron at a position similar to that of S-RNase, for example, *RNase LE* from *L. esculentum* has two introns and *AhSL28* from *Antirrhinum* and *RNS2* from *Arabidopsis* have seven introns. Two possibilities exist with respect to the evolutionary relationship between S-RNase and S-like RNase: one is that an S-RNase was a direct evolutionary product of an S-like RNase or vice versa. It appears to be more likely that an ancestral S-RNase gave rise to an ancestral S-like RNase. First, according to the phylogenetic tree (Fig. 3A), S-RNases from the Rosaceae, Solanaceae and Scrophulariceae are more closely related to the RNase from fungi, indicating that the emergence of an S-RNase was before that of an S-like RNase. Second, most S-like RNase genes have more introns than that of S-RNases, which, in most cases, possess only a single intron with its position and length highly conserved. If an S-like RNase was the ancestor of an S-RNase, they would have to keep losing their introns during its evolution into an S-RNase. If this was the case, an S-like RNase or S-RNase missing the conserved intron found in the S-RNases would have been formed. However, till now, all of the S-like RNase genes possess an intron similar to that in the S-RNase genes, indicating that the simple S-RNase genomic structure is an ancestral one. Third, as for the intron evolution in eukaryotes, a major dispute concerns an intron early (IE) vs. intron late (IL) debate [18], but available evidence indicate that most introns are gained during a long evolution period and the intron insertion is an ongoing process [18]. The genomic structure analysis of more S-RNase and S-like RNase genes will help to provide a better picture about their evolution relation.

Previous experiments on these genes indicate that S-like RNases function in several physiological processes including Pi starvation, senescence, wounding, cell death pathway, defense against pathogens and light signal pathway [19]. Senescence is an important and complex phase in the plant life cycle and is thought to contribute to fitness through the recycling of nutrients to actively growing cells. It is also a highly regulated developmental process during which many hydrolytic enzymes are activated to remobilize cell compo-

nents. Among the hydrolytic enzymes, those with RNase activity are important for the degradation of ribonucleic acid [20]. In addition, under Pi-limiting conditions, RNases could degrade RNA together with phosphatases and phosphodiesterases to release Pi [21], making it available for the plant to use in other processes. These two responses seem to be coordinated in plants because some plant S-like RNase gene expressions are induced during both senescence and Pi starvation. Consistent with this, *AhSL28* is likely involved in these two processes. These S-like RNases are likely to be a component of a Pi remobilization system that recycles Pis during senescence or Pi starvation.

But, there are RNases that can only be induced by either of the two stimuli. The expression of RNase NE of *N. alata* is not detected during leaf senescence and its response to Pi limitation varies depending on tissue types [8]. RNS3 of *A. thaliana* is induced during senescence but not during Pi starvation [22]. These data suggest that senescence and Pi starvation induce separate signal transduction pathways for such a RNase gene expression. Tomato RNase LE is induced by senescence and wounding [6], indicating that senescence overlaps with the wounding signaling pathway. However, *AhSL28* is induced by senescence but not wounding, suggesting that the two stimuli also work through separate signal transduction pathways.

How these signal transduction pathways are integrated is not clear. Efforts have been made to dissect the senescing and Pi-starvation signaling pathways. Several Pi-starvation-response mutants in *Arabidopsis* were isolated and characterized. The experimental results indicated that the Pi signal is transduced from an as yet unidentified Pi sensor to transcriptional activators of phosphatase5 (PHO5) via a cyclin/cyclin-dependent kinase complex [23]. Other experiments suggested that senescence likely acts through plant hormones including ethylene and abscisic acid. The ethylene accelerates plant senescing, and several genes have been found to be involved in the ethylene response pathway through a protein kinase cascade [24]. It is likely that many S-like RNases are target genes of these signaling pathways. When plant senesces or grows in a Pi-starved environment, the sensor(s) activates transcription activators of many response genes like PHO5 or many S-like RNases via the protein kinases and induces the expression of them. Many plant hormones such as ethylene and abscisic acid also show to function in plant wounding process [25]; they also likely induce the expression of S-like RNases during both senescence and wounding.

The in situ RNA hybridization analysis showed that *AhSL28* is highly expressed in transmitting track and vascular tissues which are sites rich in nutrients, consistent with the role played by *AhSL28* to degrade nucleic acid to supply Pi or other nutrients.

AhSL28 is the first senescence- and Pi starvation-induced RNase found in *Antirrhinum*. Its further functional characterization including detailed regulatory mechanisms of its expression will help dissect the signaling pathways of senescence and Pi starvation and their integration with other signaling networks.

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References

[1] P.J. Green, The ribonucleases of higher plants, Annu. Rev. Plant Physiol. Plant Mol. Biol. 45 (1994) 421–445.
[2] M.A. Anderson, E.C. Cornish, S.-L. Mau, E.G. Williams, R. Hoggart, A. Atkinson, I. Bonig, B. Grego, R. Simpson, P.J. Roche, J.D. Haley, J.D. Penschow, H.D. Niall, G.W. Tregear, J.P. Coghlan, R.J. Crawford, A.E. Clarke, Cloning of cDNA for a stylar glycoprotein associated with expression of self-incompatibility in *Nicotiana alata*, Nature 321 (1986) 38–45.
[3] B.A. McClure, V. Haring, P.R. Ebert, M.A. Anderson, R.J. Simpson, F. Sakiyama, A.E. Clarke, Style self-incompatibility gene products of *Nicotiana alata* are ribonucleases, Nature (Lond.) 342 (1989) 955–958.
[4] C.B. Taylor, P.A. Bariola, S.B. DelCardayré, R.T. Raines, P.J. Green, RNS2: a senescence-associated RNase of *Arabidopsis* that diverged from the S-RNases before speciation, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 5118–5122.
[5] A. Löffler, K. Glund, M. Irie, Amino acid sequence of an intracellular, phosphate-starvation-induced ribonuclease from cultured tomato (*Lycopersicon esculentum*) cells, Eur. J. Biochem. 214 (1993) 627–633.
[6] A. Lers, A. Khalchitski, E. Lomaniec, S. Burd, P.J. Green, Senescence-induced RNases in tomato, Plant Mol. Biol. 36 (1998) 439–449.
[7] H.-S. Lee, A. Singh, T. Kao, RNase X2, a pistil-specific ribonuclease from *Petunia inflata*, shares sequence similarity with solanaceous S proteins, Plant Mol. Biol. 20 (1992) 1131–1141.
[8] P.N. Dodds, A.E. Clarke, E. Newbigin, Molecular characterization of an S-like RNase of *Nicotiana alata* that is induced by phosphate starvation, Plant Mol. Biol. 31 (1996) 227–238.
[9] Y. Xue, R. Carpenter, H.G. Dickinson, E.S. Coen, Origin of allelic diversity in *Antirrhinum S* locus RNases, Plant Cell 8 (1996) 805–814.
[10] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York, 1989.
[11] S.O. Rogers, A.J. Bendich, Extraction of DNA from plant tissues, in: S.B. Gelvin, R.A. Schilperoort, D.P.S. Verma (Eds.), Plant Molecular Biology Manual, Kluwer Academic Publishers, Dordrecht, 1990, pp. A6/1–A6/10.
[12] Z. Lai, W.S. Ma, B. Han, L.Z. Liang, Y.S. Zhang, G.F. Hong, Y.B. Xue, An F-box gene linked to the self-incompatibility (*S*) locus of *Antirrhinum* is expressed specifically in pollen and tapetum, Plant Mol. Biol. 50 (2002) 29–41.
[13] D.R. Smyth, J.L. Bowman, E.M. Meyerowitz, Early flower development in *Arabidopsis*, Plant Cell 2 (1990) 755–767.
[14] T. Kao, S. Huang, Gametophytic self-incompatibility: a mechanism for self/nonself discrimination during sexual reproduction, Plant Physiol. 105 (1994) 461–466.
[15] M. Irie, K. Ohgi, M. Yoshinaga, T. Yanagida, Y. Okada, N. Teno, Roles of lysine1 and lysine7 residues of bovine pancreatic ribonuclease in the enzymatic activity, J. Biochem. (Tokyo) 100 (1986) 1057–1063.

[16] B. Igic, J.R. Kohn, Evolutionary relationships among self-incompatibility RNases, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 13167–13171.
[17] K. Ushijima, H. Sassa, R. Tao, H. Yamane, A.M. Dandekar, T.M. Gradziel, H. Hirano, Cloning and characterization of cDNAs encoding S-RNases from almond (*Prunus dulcis*): primary structural features and sequence diversity of the S-RNases in Rosaceae, Mol. Gen. Genet. 260 (1998) 261–268.
[18] L. Duret, Why do genes have introns? Recombination might add a new piece to the puzzle, Trends Genet. 17 (2001) 172–175.
[19] P.A. Bariola, P.J. Green, Plant ribonucleases, in: G. D'Alessio, J.F. Riordan (Eds.), Ribonucleases: Structures and Functions, Academic Press, New York, NY, 1997, pp. 163–190.
[20] G.L. Farkas, Ribonucleases and ribonucleic acid breakdown, Encycl. Plant Physiol. 14B (1982) 224–262.
[21] K. Glund, A.H. Goldstein, Regulation, synthesis, and excretion of a phosphate starvation inducible RNase by plant cells, in: D.P.S. Verma (Ed.), Control of Plant Gene Expression, CRC Press, Boca Raton, FL, 1993, pp. 311–323.
[22] T. Shimizu, T. Inoue, H. Shiraishi, A senescence-associated S-like RNase in the multicellular green alga *Volvox carteri*, Gene. 274 (2001) 227–235.
[23] D.L. Chen, C.A. Delatorre, A. Bakker, S. Abel, Conditional identification of phosphate-starvation-response mutants in *Arabidopsis thaliana*, Planta. 211 (2001) 13–22.
[24] J.J. Kieber, The ethylene response pathway in *Arabidopsis*, Annu. Rev. Plant Physiol. Plant Mol. Biol. 48 (1997) 277–296.
[25] J. Leon, E. Rojo, J.J. Sanchez-Serrano, Wound signaling in plants, J. Exp. Bot. 52 (354) (2001) 1–9.
[26] R.-C. Ma, M.M. Oliveira, The RNases PD2 gene of almond (*Prunus dulcis*) represents an evolutionarily distinct class of S-like RNase genes, Mol. Gen. Genet. 263 (2000) 925–933.