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RNS2: A senescence-associated RNase of *Arabidopsis* that diverged from the S-RNases before speciation

(RNase superfamily/senescence/phosphate starvation/S-like RNases)

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ABSTRACT **Several self-compatible species of higher plants, such as *Arabidopsis thaliana*, have recently been found to contain S-like RNases. These S-like RNases are homologous to the S-RNases that have been hypothesized to control self-incompatibility in Solanaceous species. However, the relationship of the S-like RNases to the S-RNases is unknown, and their roles in self-compatible plants are not understood. To address these questions, we have investigated the *RNS2* gene, which encodes an S-like RNase (RNS2) of *Arabidopsis*. Amino acid sequence comparisons indicate that RNS2 and other S-like RNases make up a subclass within an RNase superfamily, which is distinct from the subclass formed by the S-RNases. RNS2 is most similar to RNase LE [Jost, W., Bak, H., Glund, K., Terpstra, P., Beintema, J. J. (1991) *Eur. J. Biochem.* 198, 1–6.], an S-like RNase from *Lycopersicon esculentum*, a Solanaceous species. The fact that RNase LE is more similar to RNS2 than to the S-RNases from other Solanaceous plants indicates that the S-like RNases diverged from the S-RNases prior to speciation. Like the S-RNase genes, *RNS2* is most highly expressed in flowers, but unlike the S-RNase genes, *RNS2* is also expressed in roots, stems, and leaves of *Arabidopsis*. Moreover, the expression of *RNS2* is increased in both leaves and petals of *Arabidopsis* during senescence. Phosphate starvation can also induce the expression of *RNS2*. On the basis of these observations, we suggest that one role of RNS2 in *Arabidopsis* may be to remobilize phosphate, particularly when cells senesce or when phosphate becomes limiting.**

Fundamental insights into the relationship between protein structure and function and gene evolution have been gained from the study of members of the pancreatic RNase superfamily typified by RNase A (1). Another RNase family has recently been identified in the plant kingdom (2). RNases in this family are not homologous to the pancreatic RNase superfamily but rather share homology with a class of fungal RNases that includes RNase T_2 of *Aspergillus oryzae* (3). The plant members of this family include the S-RNases, proteins associated with a self-recognition response known as self-incompatibility (SI) in certain species of higher plants. In *Nicotiana alata* and other members of the Solanaceae, pollen carrying a particular allele at the *S* locus, which controls SI, are unable to fertilize plants carrying the same *S* allele. The mechanism by which S-RNases may participate in the rejection of incompatible pollen is unknown; however, their genes cosegregate with the *S* locus (4).

Initially it seemed plausible that the S-RNases were highly specialized polymorphic enzymes, without homologs in self-compatible species. However, several recent studies have shown that this is not the case. Among these are data obtained from PCR experiments performed with cDNA from the self-compatible crucifer *Arabidopsis thaliana*. By using primers directed against the regions most highly conserved between the fungal and S-RNases, amplification products corresponding to three *Arabidopsis* RNase genes, *RNS1*, *RNS2*, and *RNS3*, were identified (5). Further evidence for S-RNase homologs in self-compatible plants is indicated by the protein sequences of RNase LE, isolated from cultured cells of *Lycopersicon esculentum* (tomato) (6), and RNase MC, isolated from seeds of *Momordica charantia* (bitter gourd) (7); both share homology with fungal RNases and S-RNases. It has also been shown that two RNases isolated from tomato fruit, Tf1 and Tf2, have a number of biochemical properties in common with the S-RNases (8).

The finding of S-RNase homologs ("S-like" RNases) in self-compatible species indicates that enzymes related to the S-RNases play a fundamental role in RNA catabolism in higher plants. Plants contain a large number of RNase activities that are regulated in response to a variety of stimuli (9, 10). Several plant RNases have been purified and characterized biochemically, but nothing is known about the corresponding genes. It is therefore difficult to assess whether previously described RNase activities, such as those induced during senescence (11, 12), are encoded by S-like RNase genes. In an effort to gain a better understanding of the role of S-like RNases in plants and their relationships with the S-RNases, we have investigated *RNS2*, the most highly expressed *Arabidopsis RNS* gene (5).

MATERIALS AND METHODS

Isolation and Sequencing of *RNS2* cDNAs. The *Arabidopsis* cDNA library in λ Zap (13) that was initially used to detect the *RNS* genes by PCR amplification (5) was used to screen for *RNS2* cDNA clones by plaque hybridization (14) using the *RNS2* PCR product as a probe. Positive plaques were purified; the cDNA clones were converted into plasmid form (15) and sequenced (16).

Expression of RNS2 in Yeast. An *RNS2* cDNA covering the entire coding region including the signal sequence was inserted between the yeast *PHO5* promoter and *GADPH* terminator (17), and the resulting *PHO5–RNS2–GADPH* gene was then cloned into the yeast shuttle vector pWL. The detailed structure of pWL, originally designed to facilitate the expression of RNase A in yeast, will be published elsewhere (S.B.d.C., D. J. Quirk, W. J. Rutter, and, R.T.R.). Transformants of *Saccharomyces cerevisiae* strain BJ2168 (*MATa prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52*) harboring these plasmids were grown in minimal dextrose liquid lacking

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Abbreviation: SI, self-incompatibility.

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§The nucleotide sequence of the *RNS2* cDNA has been deposited in the GenBank data base (accession number M98336).

tryptophan and containing 0.2 mM KH_2PO_4 (which induces expression from the *PHO5* promoter) (18) or 11 mM KH_2PO_4 (which does not induce the *PHO5* promoter) for 2 days at 30°C. Five microliters of the culture supernatants was assayed by electrophoresis on RNase activity gels (19).

Multiple Sequence Alignment. The amino acid sequence of RNS2 deduced from the cDNA clones was aligned with the amino acid sequences of 15 Solanaceous S-RNases (20–25), 4 fungal RNases (3, 26–28), and 2 S-like RNases for which protein sequence data are available (6, 7), using the Genetics Computer Group program PILEUP with a gap weight of 3.0 and a gap length weight of 0.1 (29). The RNase sequences were aligned from the positions corresponding to the presumptive mature N termini of the *N. alata* S-RNases (4). The gene genealogy was also generated using PILEUP, which produces a similarity score for each possible pair of sequences. The genealogy is a representation of these similarity scores, which are used to order the alignment. The horizontal branch distances in the genealogy are proportional to the similarities between the sequences.

Expression Analyses. *A. thaliana* (L.) Heynh. ecotype RLD was grown under conditions of 12 hr light/12 hr dark with a relative humidity of 50% at 20°C. Total RNA was isolated from roots, stems, leaves, and flowers of 4- to 5-week-old *Arabidopsis* plants as described (5). For the senescence experiments, flowers were staged on the basis of morphological characteristics as defined in Smyth *et al.* (30). Senescing leaves were those showing visible signs of senescence, including chlorosis at the leaf margins and wilting. To starve *Arabidopsis* for phosphate, 1-week-old etiolated seedlings were removed from solid AGM medium [MS salts (Sigma) at 4.3 g/liter, sucrose at 30 g/liter, glycine at 2 mg/liter, *myo*-inositol at 100 mg/liter, pyridoxine at 0.5 mg/liter, nicotinic acid at 0.5 mg/liter, thiamine hydrochloride at 0.1 mg/liter, buffered with 2.5 mM Mes at pH 5.7] and shaken in liquid media with or without 1.25 mM KH_2PO_4 as described in (31), for 12 hr in the dark. Northern blots were prepared and hybridized as described (5) to a 0.7-kb gene-specific probe for *RNS2*, corresponding to the *Eco*RI–*Xba* I fragment of the longest *RNS2* cDNA clone. This probe includes 15 bp of the 5′ untranslated region and the coding region up to nucleotide position 696. For use as an internal standard, an *Arabidopsis* probe for the ubiquitous, highly expressed (32) translation initiation factor eIF4A was generated by using PCR. This probe corresponds to amino acids 197–323 of *Nicotiana tabacum* eIF4A2, and its deduced amino acid sequence is 96.5% identical to the latter (C.B.T., and P.J.G., unpublished observations).

RESULTS AND DISCUSSION

RNS2 was initially identified as a PCR product amplified from an *Arabidopsis* cDNA library using primers corresponding to the regions most conserved between the S-RNases and

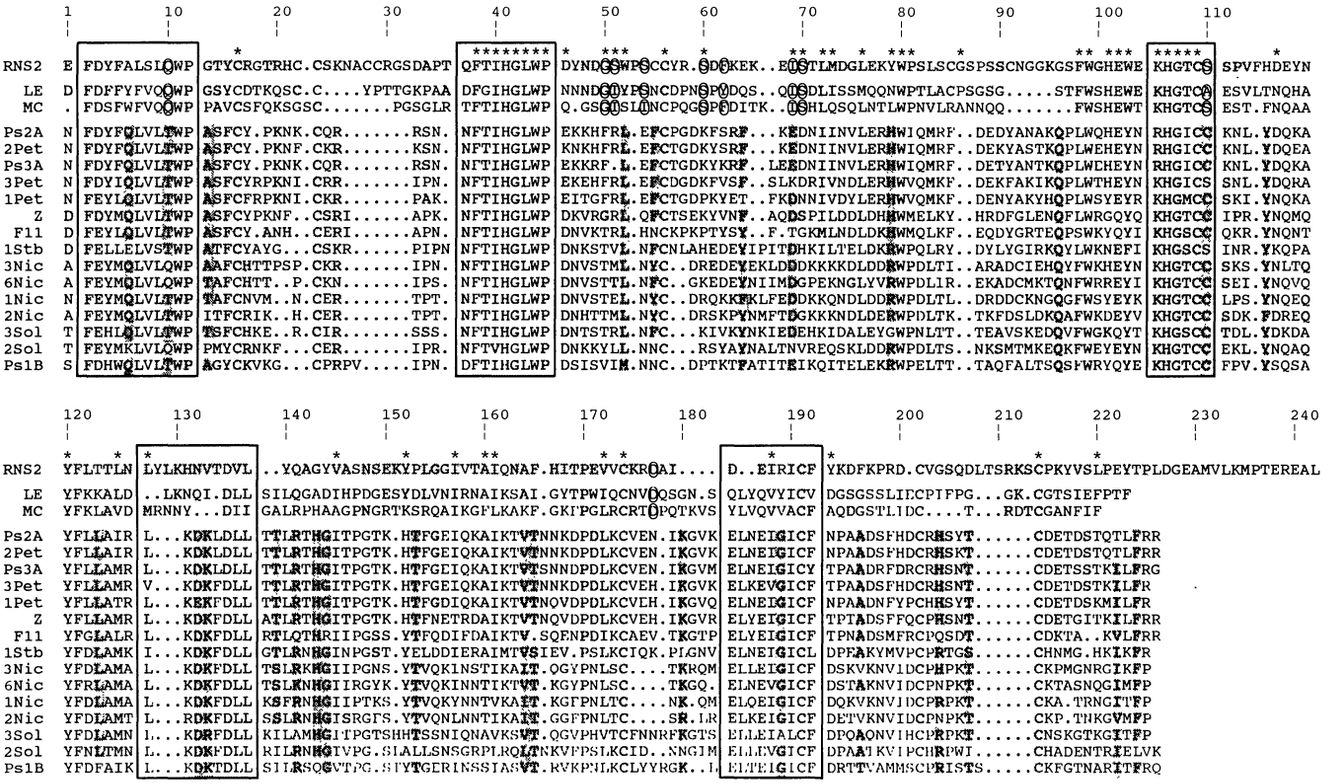

FIG. 1. Alignment of S- and S-like RNase amino acid sequences. The alignment was performed as described in *Materials and Methods* using the Genetics Computer Group package (29). Sequences are of RNS2 of *Arabidopsis*, LE from *L. esculentum* (6), MC from *M. charantia* (7), *N. alata S* alleles 2, 3, and 6 [2Nic, 3Nic, and 6Nic (20)] and 1, F11, and Z [1Nic, F11, and Z (21)], *Petunia inflata S* alleles 1, 2, and 3 [1Pet, 2Pet, and 3Pet (22)], *Solanum chacoense S* alleles 2 and 3 [2Sol and 3Sol (23)], *Petunia hybrida S* alleles 1, 2, and 3 [Ps1B, Ps2A, and Ps3A (24)], and *Solanum tuberosum S* allele 1 [1Stb (25)]. The RNases are aligned from the presumptive mature N termini of the *N. alata S* alleles, as in ref. 4. Light shading indicates residues in any sequence that are identical or functionally identical to a residue at the same position in the RNS2 sequence, ellipses denote residues that are identical or functionally identical in the three S-like RNases (RNS2, LE, and MC), and heavy shading denotes residues that are identical or functionally identical in at least 12 of the 15 S-RNases. Conserved regions C1–C5 of Ioerger *et al.* (34) are boxed. Asterisks above the RNS2 sequence denote residues in the fungal RNases T_2 (3), Rh (26), M (27), and Trv (28) that are identical or functionally identical to the RNS2 residues. Functionally identical residues are grouped as follows: A,S,T; I,L,M,V; H,K,R; F,W,Y; D,E; Q,N. The deduced amino acid sequence of RNS2 includes the following residues that precede the sequence shown in the alignment: MASRLCLLLLVACIAGAFA ↓ GDVIELNRSQR. The arrow indicates the most likely site for cleavage of the signal sequence, based on a statistical comparison of amino acid sequences in the vicinity of known cleavage sites (35).

a class of fungal RNases (5). This PCR product was used as a hybridization probe to isolate *RNS2* cDNA clones from the same library. The nucleotide and deduced amino acid sequences of the longest clone containing a full-length coding region have been deposited in the GenBank data base (accession number M98336). Analysis of several independent cDNA clones showed that transcripts from the *RNS2* gene can be polyadenylylated at multiple sites, a feature common to many plant genes (33). The 19 amino acids at the N terminus of the RNS2 protein (see legend to Fig. 1) are typical of a eukaryotic signal sequence. This indicates that RNS2 is targeted to the secretory pathway in *Arabidopsis*, similar to the S-RNases of the Solanaceae which are secreted enzymes.

To confirm that RNS2 is indeed an RNase, the coding region of the *RNS2* cDNA including the putative signal sequence was expressed in *S. cerevisiae* under the control of the *PHO5* promoter (18). RNase activity secreted into the culture medium by yeast transformed with the vector control (CON) or the RNS2 expression construct (RNS2) was then detected following electrophoresis on RNase activity gels (19) (Fig. 2). Under inducing conditions, two bands of RNS2 activity were observed that have apparent molecular masses of 28–33 kDa, which is slightly higher than the predicted molecular mass of 27 kDa (assuming the RNS2 signal sequence is cleaved as indicated in the legend to Fig. 1). This slight difference in molecular mass and the presence of two RNS2 bands may result from processing of the RNS2 signal sequence at multiple sites (36) or differences in glycosylation (37) that are known to affect the mobility of heterologous proteins produced in yeast. It should also be noted that the RNase activity gels are run under nonreducing conditions (19), which may contribute to the differences. However, both RNase bands are specific to the RNS2 clone and correlate with the induction of the *PHO5* promoter as expected. This demonstrates that the *RNS2* gene encodes an active RNase.

Comparison of RNS2 to Related RNases. To compare the similarity of the deduced amino acid sequence of RNS2 with those of related plant RNases, the alignment shown in Fig. 1 was generated as described in *Materials and Methods*. The alignment demonstrates that the similarity of RNS2 to the S-RNases is dispersed throughout the coding region (Fig. 1). Moreover, each of the five regions most conserved among the S-RNases [numbered C1–C5 by Kao and coworkers (21, 34) and boxed in Fig. 1] is also evident in RNS2. In contrast to the extensive similarity of RNS2 with the S-RNases, its similarity to the related fungal enzymes is highest in the central region of the protein (positions 37–110) but is markedly less pronounced towards the N and C termini (see asterisks above sequence in Fig. 1). Twenty-five residues are

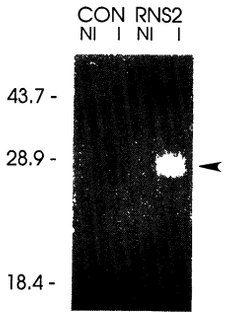

FIG. 2. Expression of *RNS2* in yeast. Yeast cultures transformed with the control pWL (CON) or RNS2 (RNS2) constructs were grown under conditions that do not induce (NI) or do induce (I) transcription from the *PHO5* promoter. Supernatants from these cultures were run on RNase activity gels as described in *Materials and Methods*. Positions of molecular mass markers (kDa) are shown to the left of the gel. The arrowhead indicates the RNS2 activity bands.

absolutely conserved among all the RNases of this superfamily. The most prominent of these are the histidine residues at positions 41, 101, and 106 in Fig. 1, which have been shown to be important for catalysis in RNase Rh of *Rhizopus niveus* (38). Others include the pairs of cysteine residues at positions 56 and 109 and positions 174 and 214 that have been shown to form disulfide bonds that are critical for maintaining the structure of RNase M of *Aspergillus saitoi* (28).

Residues at a number of positions are conserved in mutually exclusive sets, either only among the S-RNases or only among the S-like RNases. Many of the residues that distinguish these subclasses are clustered and therefore correspond to regions within the S-RNases and the S-like RNases that are potentially related to their disparate functions. The majority of the residues that are conserved among the S-like RNases but absent from the S-RNases fall between the histidine residues of the putative active site and most are also conserved in all of the fungal enzymes (Fig. 1). Some of the residues that are highly conserved among the S-RNases but absent from the S-like RNases are clustered between positions 132 and 165 (Fig. 1). This region may constitute a domain required for the specialized functions of the S-RNases in SI. At present it is not possible to test this hypothesis directly, as a functional test for S-RNase action in SI has not yet been developed.

The most striking difference between the amino acid sequence of RNS2 and those of all the other enzymes is the C-terminal extension of 20 amino acids. This sequence has features in common with C-terminal vacuolar-targeting signals from other plant proteins, such as lectins and seed storage proteins—namely, a preponderance of hydrophobic amino acids, especially in stretches of three to four (39). If RNS2 is targeted to the vacuole, as these sequences imply, this would constitute a significant distinction between RNS2 and the 18 related RNases in Fig. 1, all of which are thought to be extracellular (4, 6, 7).

A broader illustration of the relationship of RNS2 to the other related RNases was obtained by constructing a gene genealogy (see Fig. 3) based on the deduced amino acid sequences in Fig. 1. This genealogy initially divides the RNase superfamily into two lineages, the fungal RNases and the plant RNases. The most significant feature, however, is the placing of the S-like RNases and the S-RNases into two discrete lineages, an observation that indicates that they form distinct categories of RNases in plants. This arrangement is consistent with current models proposing a specialized function for the S-RNases in SI (4). Had the S-RNases and S-like RNases been intermingled in the genealogy, a specialized role for the S-RNases would have been more difficult to reconcile. The grouping of the three S-like RNases is also of note because it implies that their sequences, and thus presumably their function, may be evolutionarily conserved across a broad range of plant species. This is most clearly illustrated by RNase LE from *L. esculentum*, a Solanaceous species. RNase LE is placed on the same branch as RNases RNS2 and MC and is therefore more closely related to these RNases than it is to any of the S-RNases (Fig. 3) that were isolated from other Solanaceous species. Moreover, preliminary sequence data (P.A.B., C.B.T., and P.J.G., unpublished) indicate that RNS1, one of the other RNases of *Arabidopsis*, is more similar to RNase LE than it is to any of the other RNases, including RNS2. The placing of the S-like RNases on a separate branch of the genealogy from the S-RNases and the close relationship of LE to other S-like RNases from non-Solanaceous species strongly indicate that these two groups of RNases diverged prior to speciation. These conclusions greatly extend the information gleaned from the gene genealogy reported previously by Ioerger *et al.* (40) for the S-RNases. This earlier genealogy did not include any S-like RNases and thus did not address the relationships between S-

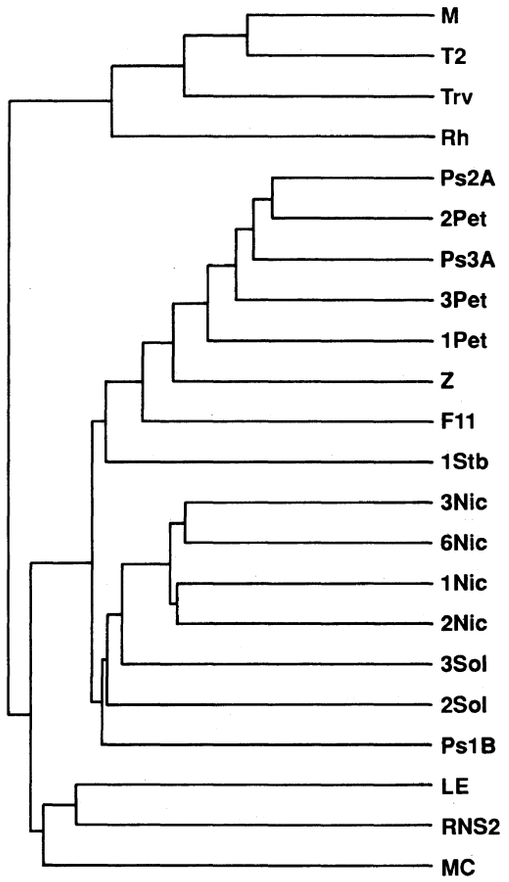

FIG. 3. Gene genealogy of the RNase superfamily. The genealogy was generated from the alignment depicted in Fig. 1 as described in *Materials and Methods*. Horizontal branch lengths represent overall similarity between the RNases. Designations for each RNase are as in Fig. 1. T2, Rh, M, and Trv are the fungal RNases (refs. 3, 26, 27, and 28, respectively).

and S-like RNases. However, the genealogy of Ioerger *et al.* (40) included most of the S-RNases that were analyzed in the present study, and the genealogy in Fig. 3 is consistent with the evolutionary relationships proposed (40) for those enzymes.

Control of *RNS2* Expression. As a first step toward elucidating the function of RNS2, the expression of *RNS2* in roots, leaves, stems, and flowers of *Arabidopsis* was investigated by Northern blotting (Fig. 4*A*). Similar to the S-RNases of the Solanaceae (41), *RNS2* is most highly expressed in flowers of *Arabidopsis*. However, *RNS2* is also expressed in other organs, notably leaf and stem, albeit at a much lower level

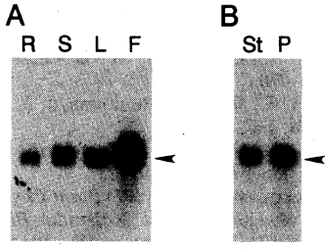

FIG. 4. Expression of RNS2 in different organs of *Arabidopsis*. (*A*) Samples of 12 μg of total RNA isolated from roots (lane R), stems (lane S), leaves (lane L), and flowers (lane F) were hybridized to the RNS2 probe following Northern blotting. (*B*) Samples of 5 μg of total RNA isolated from style and stigma (St) and petals (P) dissected from *Arabidopsis* flowers were subjected to Northern blotting and hybridization to the *RNS2* probe. The *RNS2* transcript is indicated by the arrowhead.

than in flowers. The expression of *RNS2* in all organs that were examined implies that RNS2 is a fundamental component of the RNA degradation machinery in *Arabidopsis*. To localize further *RNS2* expression in flowers of *Arabidopsis*, RNA was isolated from *Arabidopsis* pistils (stigma and style) and petals harvested at anthesis. The data shown in Fig. 4*B* demonstrate that *RNS2* is expressed in both of these flower organs, with slightly higher expression apparent in petals. This is in contrast to the expression of the S-RNases, which is restricted to the gynoecium and is most prominent in the transmitting tissue of the style (41). Thus, in analogy with the sequence data described above, there are some distinct similarities between the expression properties of *RNS2* and the S-RNases but also some significant differences.

The presence of *RNS2* transcripts in petals indicates that RNS2 may contribute in part to the increase in RNase activity that is known to occur in petals during senescence in plants (11). RNA was therefore isolated from *Arabidopsis* petals harvested at anthesis (stages 13 and 14 in ref. 30) and during senescence (stage 16 in ref. 30) and probed for the *RNS2* transcript. A clear increase in *RNS2* expression in senescing petals was observed in these experiments, as shown in Fig. 5*A*. The increase in *RNS2* expression during senescence is even more evident in leaves, where the basal level of the *RNS2* transcript is lower than in petals (Fig. 5*A*). As a control, the blot shown in Fig. 5*A* was stripped of the *RNS2* probe and hybridized with a probe for the translation factor eIF4A from *Arabidopsis* (see *Materials and Methods*). The levels of the *EIF4A* transcript are approximately equal in each pair of samples (Fig. 5*C*). Moreover, the level of the *Arabidopsis CAB-1* transcript, which encodes the chlorophyll a/b binding protein (42), decreased in leaves during senescence (C.B.T. and P.J.G., results not shown). These results confirm that the senescence-induced accumulation of *RNS2* mRNA is a specific effect. RNS2 is therefore likely to be a component of the major change in gene expression that is associated with the onset of senescence (43). This includes the induction of a large number of hydrolytic enzymes (44), which are thought to be involved in the recycling of nutrients from the vegetative to the reproductive organs (45).

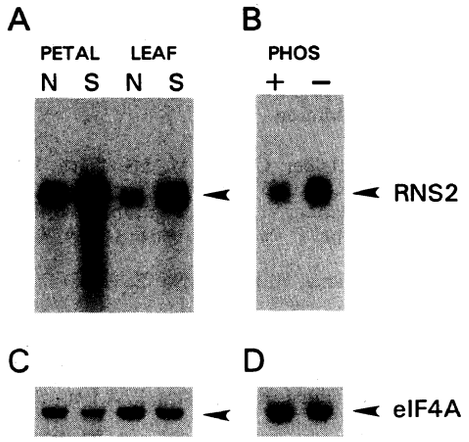

FIG. 5. Induction of the *RNS2* transcript during senescence and phosphate starvation in *Arabidopsis*. (*A*) Samples of 5 μg of total RNA isolated from nonsenescing (N) and senescing (S) petals and leaves, as indicated above the lanes, were hybridized to the *RNS2* probe following Northern blotting. The *RNS2* transcript is indicated by the arrowhead. (*B*) Etiolated *Arabidopsis* seedlings were incubated in the presence (+) or absence (−) of phosphate as described in *Materials and Methods*. Samples of 10 μg of total RNA isolated from these seedlings were hybridized to the *RNS2* probe following Northern blotting. (*C* and *D*) The Northern blots shown in *A* and *B* were stripped of the *RNS2* probe and hybridized with an *EIF4A* probe (see *Materials and Methods*) to generate *C* and *D*, respectively. The arrowhead shows the *EIF4A* transcript.

A role for RNase LE in phosphate rescue has been suggested because it is secreted from tomato cells following phosphate starvation (46, 47). To test whether phosphate limitation could induce the expression of the *RNS2* gene, RNA was isolated from *Arabidopsis* seedlings that had been placed in phosphate-free medium or in medium containing 1.25 mM phosphate for 12 hr. As shown in the Northern blot in Fig. 5*B*, accumulation of the *RNS2* transcript increases markedly following phosphate limitation, while the level of the *EIF4A* transcript remains constant (Fig. 5*D*). Examples of other enzymatic activities induced by phosphate starvation have been described in plants (48, 49), but in these cases it is unknown whether regulation is exerted at the mRNA or protein levels.

Conclusions and Future Prospects. To our knowledge, *RNS2* is the first senescence-associated RNase gene identified in higher plants. Senescence-associated RNase activities have been demonstrated in a number of plant species (11, 12, 50), but it is not known whether they are encoded by S-like RNase genes. Since plants often grow under phosphate-limiting conditions (51), it is not surprising that a RNase gene might be induced to facilitate the recovery of phosphate from dying cells. A vacuolar localization for RNS2 would not be inconsistent with this hypothesis, as small fragments of RNA have been found in this organelle (52). It is also possible that RNS2 may participate in the remobilization of phosphate in nonsenescing cells (for example, during phosphate starvation). At present it is unknown whether RNS2 corresponds to any of the major RNase activities identified previously from *Arabidopsis* (19). Our initial PCR experiments indicate that *RNS2* is one of at least three closely related RNase genes in *Arabidopsis* (5). In the future, it may be possible to compare the RNS2 sequence to all the related *Arabidopsis* RNases in order to identify regions unique to each enzyme. This will facilitate the production of monospecific antisera that could be used to differentiate among the *RNS* gene products.

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1. Beintema, J. J., Schüller, C., Irie, M. & Carsana, A. (1988) *Prog. Biophys. Mol. Biol.* **51,** 165–192.
2. McClure, B. A., Haring, V., Ebert, P. R., Anderson, M. A., Simpson, R. J., Sakiyama, F. & Clarke, A. E. (1989) *Nature (London)* **342,** 955–957.
3. Kawata, Y., Sakiyama, F. & Tamaoki, H. (1988) *Eur. J. Biochem.* **176,** 683–697.
4. Haring, V., Gray, J. E., McClure, B. A., Anderson, M. A. & Clarke, A. E. (1990) *Science* **250,** 937–941.
5. Taylor, C. B. & Green, P. J. (1991) *Plant Physiol.* **96,** 980–984.
6. Jost, W., Bak, H., Glund, K., Terpstra, P. & Beintema, J. J. (1991) *Eur. J. Biochem.* **198,** 1–6.
7. Ide, H., Kimura, M., Arai, M. & Funatsu, G. (1991) *FEBS Lett.* **284,** 161–164.
8. McKeon, T. A., Lyman, M. L. & Prestamo, G. (1991) *Arch. Biochem. Biophys.* **290,** 303–311.
9. Farkas, G. L. (1982) in *Encyclopedia of Plant Physiology N.S.*, eds. Boulter, D. & Parthier, B. (Springer, Berlin), Vol. 16, pp. 224–262.
10. Wilson, C. M. (1982) in *Isozymes: Current Topics in Biological and Molecular Research*, eds. Ratizzi, M. C., Scandalios, J. G. & Whitt, G. S. (Liss, New York), Vol. 6, pp. 33–54.
11. Matile, P. & Winkenbach, F. (1971) *J. Exp. Bot.* **22,** 759–771.
12. Blank, A. & McKeon, T. A. (1991) *Plant Physiol.* **97,** 1409–1413.
13. Short, J. M., Fernandez, J. M., Sorge, J. A. & Huse, W. D. (1988) *Nucleic Acids Res.* **16,** 7583–7600.
14. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Plainview, NY), 2nd Ed.
15. Delauny, A. J. & Verma, D. P. S. (1990) in *Plant Molecular Biology Manual*, eds. Gelvin, S. B., Schilperoort, R. A. & Verma, D. P. S. (Kluwer, Dordrecht, The Netherlands), pp. A14/1-A14/23.
16. Sanger, F., Nicklen, S. & Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
17. Rosenberg, S., Barr, P. J., Najarian, R. C. & Hallewell, R. A. (1984) *Nature (London)* **312,** 77–80.
18. Thill, G. P., Kramer, R. A., Turner, K. J. & Bostian, K. A. (1983) *Mol. Cell. Biol.* **3,** 570–579.
19. Yen, Y. & Green, P. J. (1991) *Plant Physiol.* **97,** 1487–1493.
20. Anderson, M. A., McFadden, G. I., Bernatzky, R., Atkinson, A., Orpin, T., Dedman, H., Tregear, G., Fernley, R. & Clarke, A. E. (1989) *Plant Cell* **1,** 483–491.
21. Kheyr-Pour, A., Bintrin, S. B., Ioerger, T. R., Remy, R., Hammond, S. A. & Kao, T.-h. (1990) *Sex. Plant Reprod.* **3,** 88–97.
22. Ai, Y., Singh, A., Coleman, C. E., Ioerger, T. R., Kheyr-Pour, A. & Kao, T.-h. (1990) *Sex. Plant Reprod.* **3,** 130–138.
23. Xu, B., Mu, J., Nevins, D., Grun, P. & Kao, T.-h. (1990) *Mol. Gen. Genet.* **224,** 341–346.
24. Clark, K. R., Okuley, J. J., Collins, P. D. & Sims, T. L. (1990) *Plant Cell* **2,** 815–826.
25. Kaufmann, H., Salamini, F. & Thompson, R. D. (1991) *Mol. Gen. Genet.* **236,** 457–466.
26. Horiuchi, H., Yanai, K., Takagi, M., Yano, K., Wakabayashi, E., Sanda, A., Mine, S., Ohgi, K. & Irie, M. (1988) *J. Biochem. (Tokyo)* **103,** 408–418.
27. Inada, Y., Watanabe, H., Ohgi, K. & Irie, M. (1991) *J. Biochem. (Tokyo)* **110,** 896–904.
28. Irie, M., Watanabe, H., Ohgi, K. & Harada, M. (1986) *J. Biochem. (Tokyo)* **99,** 627–633.
29. Devereux, J., Haeberli, P. & Smithies, O. (1984) *Nucleic Acids Res.* **12,** 387–395.
30. Smyth, D. R., Bowman, J. L. & Meyerowitz, E. M. (1990) *Plant Cell* **2,** 755–767.
31. Tewes, A., Glund, K., Walther, R. & Reinbothe, H. (1984) *Z. Pflanzenphysiol.* **113,** 141–150.
32. Owttrim, G. W., Hofmann, S. & Kuhlemeier, C. (1991) *Nucleic Acids Res.* **19,** 5491–5496.
33. Dean, C., Tamaki, S., Dunsmuir, P., Favreau, M., Katayama, C., Dooner, H. & Bedbrook, J. (1986) *Nucleic Acids Res.* **14,** 2229–2240.
34. Ioerger, T. R., Gohlke, J. R., Xu, B. & Kao, T.-h. (1991) *Sex. Plant Reprod.* **4,** 81–87.
35. von Heijne, G. (1986) *Nucleic Acids Res.* **14,** 4683–4690.
36. Ohgi, K., Horiuchi, H., Watanabe, H., Takagi, M., Yano, K. & Irie, M. (1991) *J. Biochem. (Tokyo)* **109,** 776–785.
37. Tague, B. W. & Chrispeels, M. J. (1987) *J. Cell Biol.* **105,** 1971–1979.
38. Ohgi, K., Horiuchi, H., Watanabe, H., Iwama, M., Takagi, M. & Irie, M. (1992) *J. Biochem. (Tokyo)* **112,** 132–138.
39. Chrispeels, M. J. & Raikhel, N. V. (1992) *Cell* **68,** 613–616.
40. Ioerger, T. R., Clark, A. G. & Kao, T.-h. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9732–9735.
41. Cornish, E. C., Pettitt, J. M., Bonig, I. & Clarke, A. E. (1987) *Nature (London)* **326,** 99–102.
42. Millar, A. J. & Kay, S. A. (1991) *Plant Cell* **3,** 541–550.
43. Woodson, W. R. (1987) *Physiol. Plant.* **71,** 495–502.
44. Borochov, A. & Woodson, W. R. (1989) *Hortic. Rev.* **11,** 15–43.
45. Kelly, M. O. & Davies, P. J. (1988) *CRC Crit. Rev. Plant Sci.* **7,** 139–173.
46. Nürnberger, T., Abel, S., Jost, W. & Glund, K. (1990) *Plant Physiol.* **92,** 970–976.
47. Löffler, A., Abel, S., Jost, W., Beintema, J. J. & Glund, K. (1992) *Plant Physiol.* **98,** 1472–1478.
48. Duff, S. M. G., Plaxton, W. C. & Lefebvre, D. D. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 9538–9542.
49. Usada, H. & Shimogawara, K. (1992) *Plant Physiol.* **99,** 1680–1685.
50. Woolhouse, H. W. (1982) in *The Molecular Biology of Plant Development*, eds. Smith, H. & Grierson, D. (Blackwell, Oxford), pp. 256–281.
51. Fried, M. & Brosehart, H. (1967) *The Soil–Plant System in Relation to Organic Mineral Nutrition* (Academic, New York).
52. Abel, S., Blume, B. & Glund, K. (1990) *Plant Physiol.* **94,** 1163–1171.