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THE *RNS* FAMILY OF S-LIKE RIBONUCLEASES OF *ARABIDOPSIS THALIANA*: STRUCTURES, EXPRESSION AND FUNCTIONS

By

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ABSTRACT

THE *RNS* FAMILY OF S-LIKE RIBONUCLEASES OF *ARABIDOPSIS THALIANA*: STRUCTURES, EXPRESSION AND FUNCTIONS

By

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In the past decade, ribonucleases (RNases) have been found to be involved in many unexpected processes in different biological systems. One of these processes is self-incompatibility in some types of plants, in which the involvement of S-RNases has been shown to be crucial. This phenomenon prompted the identification of three genes homologous to the S-RNases in *Arabidopsis thaliana*, a self-compatible plant. The project described in this thesis involves the characterization of these genes and their expression, as well as experiments designed to provide insight into the roles of these proteins, termed S-like RNases, in self-compatible plants and plants in general. At the outset of the project, cDNAs for the three genes, *RNS1*, *RNS2* and *RNS3*, were isolated and sequenced. Extensive comparisons of the deduced amino acid sequences to those of other S-RNases and S-like RNases were made, revealing several regions where residues of either group were found to be unique in comparison to the other group. The *RNS* cDNAs were expressed in yeast and the resulting proteins assayed for RNase activity, which showed that the genes encode active RNases. Expression studies showed that although closely related at

the sequence level, these genes have quite different expression patterns. All are expressed in flowers, but among other organs expression varies. All three are induced to some extent during senescence, but only *RNS1* and *RNS2* are induced during starvation for phosphate (P_i). These observations led to the proposal that RNS1 and RNS2 have roles in P_i remobilization. Antibodies that recognize RNS2 specifically were made for use in the subcellular localization of RNS2. Immunogold electron microscopy of leaves and petals showed RNS2 present in the extracellular space, a location consistent with a role in P_i remobilization and possibly defense against pathogens. The final aspect of the project involved the generation of antisense and overexpression transgenic plants in which the ideas about the roles of these proteins could be tested. Attempts to generate antisense *RNS2* plants led to lines in which *RNS2* expression was only mildly inhibited. In contrast, antisense *RNS1* plants were obtained in which induction of *RNS1* in flowers and during P_i starvation is reduced substantially. These lines also exhibit high anthocyanin levels, which is a symptom of P_i starvation and stress. Further characterization will be required to determine the mechanism leading to induction of anthocyanins. However, these results demonstrate that decreasing the expression of one member of the *RNS* gene family is sufficient to induce an identifiable phenotype.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION: PLANT RIBONUCLEASES

Portions of this chapter are in press:

Bariola PA, Green PJ (1997) Plant Ribonucleases. In: D'Alessio G, Riordan JF (eds) Ribonucleases: Structure and Functions. Academic Press, Inc., Orlando, Florida. In press.

During the past few years, the study of plant ribonucleases (RNases) has advanced considerably due to renewed interest in the field and to technological advances. Prior to the early 1980's, the regulation and biochemistry of plant RNases were actively investigated as reviewed by Farkas (1982) and Wilson (1982), but subsequently interest in the field subsided because the methods available at the time were insufficient to elucidate the biological functions of individual enzymes. Much renewed interest was prompted by the discovery that genotype-specific ribonucleases, the S-RNases, are critical components of self-incompatibility (SI) mechanisms in some Solanaceous plants (McClure et al. 1989b; Lee et al. 1994). These findings, which exploited gene cloning and transgenic plant technologies, emphasized that plant RNases could participate in diverse and unexpected processes. Moreover, the discovery of the S-RNases led to the identification of a large number of related plant RNases that do not function in self-incompatibility, called the S-like RNases.

The goal of this chapter is to highlight current knowledge of the S-like RNases and other RNA-degrading enzymes in higher plants. Rather than attempt a comprehensive review, this chapter concentrates on findings of the last decade with emphasis on molecular analyses and work reported since the last review of this topic (Green 1994). The chapter is divided into sections dealing with the classes of plant RNases and with their regulation and functions.

CLASSES OF PLANT RNASES

Classifications Based on Early Biochemical Work

Before the widespread use of molecular biology and protein microsequencing, plant RNases were extensively characterized on the basis of their biochemical properties. An abundance of reports on RNases from a variety of plants facilitated the classification of plant RNases into four main groups: RNase I, RNase II, Nuclease I and Exonuclease I (Farkas 1982; Wilson 1982). RNase I proteins, or acid RNases, are RNA-specific, soluble endonucleases with molecular weights from 20 to 25 kDa and pH optima between 5.0 and 6.0. They are insensitive to EDTA and produce 3'-phospho (3'-P) nucleotides as end products. RNase II enzymes are also RNA-specific endonucleases, with molecular weights between 17 and 25 kDa. They too are EDTA-insensitive and produce 3'-P nucleotide end products, but unlike RNase I enzymes, they have pH optima of 6.0 to 7.0. They differ most from RNase I-type enzymes by their microsomal location. Both RNase I and RNase II enzymes preferentially cleave bonds adjacent to guanine. Nuclease I proteins degrade both RNA and ssDNA endonucleolytically, with a preference for bonds adjacent to adenine, and produce 5'-P nucleotide end products. Highly sensitive to EDTA, they have molecular weights of 31 to 39 kDa and pH optima of 5.0 to 6.5. Lastly, Exonuclease I enzymes are large exonucleases of more than 100 kDa, are capable of degrading both RNA and ssDNA, have pH optima of 7.0 to 9.0 and a high sensitivity to EDTA, and produce 5'-P end products.

The above designations are not comprehensive and did not include all known RNases even at the time of their publication (Farkas 1982; Wilson 1982). The classification of RNases was further complicated by common problems such as proteolytic degradation and aggregate formation during purification, as well as inconsistency in the assay procedures used in different laboratories. In addition, not every defining characteristic is investigated for each RNase reported. Despite these problems, many RNases reported more recently fit well into one of the four groups. Both *Arabidopsis thaliana* (Yen and Green 1991) and barley (Yen and Baenziger 1993) appear to have representatives of the RNase I, RNase II, and nuclease I classes, based on the properties of the RNA-degrading enzymes observed using activity gels. Additional reports from barley describe RNase I-type enzymes (Prentice and Heisel 1985; Kenefick and Blake 1986) and nucleases (Prentice and Heisel 1986; Brown and Ho 1987). Other RNase I-type enzymes have been identified in wheat (Blank and McKeon 1991a) and tomato (Abel and Glund 1987). A protein from wheat is a recent addition to the RNase II family (Yen and Baenziger 1993), and nucleases have been found recently in wheat (Kuligowska et al. 1988; Blank and McKeon 1989; Yen and Baenziger 1993), rye (Siwecka et al. 1989), zinnia (Thelen and Northcote 1989), and lentil (Kefalas and Yupsanis 1995).

Plant RNases in the T_2 Family

The family of plant RNases best characterized molecularly is a subset of the family of enzymes typified by the fungal RNase T_2 (Irie 1997). This family is the most

widespread RNase family known, with representatives in viruses (Schneider et al. 1993; Hime et al. 1995), bacteria (Meador, III and Kennell 1990; Favre et al. 1993), fungi (Irie 1996), slime mold (Inokuchi et al. 1993), *Drosophila* (Hime et al. 1995), oyster (Watanabe et al. 1993), cow (Irie 1993), and chicken (Irie 1993).

S-RNases: Plant members of the T_2 family were first identified when sequences of proteins genetically associated to gametophytic self-incompatibility in the Solanaceae family (Kao and Huang 1994) were determined and found to be similar to fungal ribonucleases (McClure et al. 1989b). Termed S-RNases, numerous examples of these proteins have been characterized in tobacco, tomato, potato, and petunia, each Solanaceous species (Newbigin et al. 1993). More recently, S-RNase families have been identified in snapdragon (the Scrophulariaceae family) (Xue et al. 1996), and in apple (Broothaerts et al. 1995) and pear (Norioka et al. 1995) of the Rosaceae family. However, other types of self-incompatibility are not controlled by S-RNases, such as that exhibited by the Brassicaceae family (Dodds et al. 1996b). The production of extracellular S-RNases in styles is both necessary and sufficient to confer plants with self-incompatibility, as was shown by transgenic plant studies in petunia (Lee et al. 1994) and tobacco (Murfett et al. 1994). It has been shown that the ribonuclease activity of the S-proteins is essential for the self-incompatibility phenotype (Huang et al. 1994). In vivo, rRNA is degraded in incompatible pollen tubes (McClure et al. 1990), leading to two popular models of ribonuclease action during self-incompatibility. In the first model, S-RNases are taken up into pollen tubes by a receptor whose gene is presumably closely

linked to the S-allele, degrading the RNA in the pollen tube, halting growth and thus preventing pollination. In the second model, S-RNases are allowed to enter pollen tubes non-specifically but inactivated once inside, unless the interaction is incompatible (Dodds et al. 1996b). Much work needs to be done to provide evidence for either model.

Other Plant Members of the T_2 family: The identification of the S-RNases led to the discovery of related proteins in a variety of self-compatible plant species. Based on their similarity to S-RNases, the latter group of enzymes was referred to as "S-like RNases" (Taylor and Green 1991; Taylor et al. 1993).

The S-like RNases have the two conserved histidine residues shown to be necessary for catalysis in RNase Rh, a related fungal RNase (Ohgi et al. 1992), and the five boxes of conserved sequence characteristic of the S-RNases (Ioerger et al. 1991), although these boxes in the S-like RNases tend to be less highly conserved. In general, the S-like RNases have molecular weights between 21 and 29 kDa, and most have been shown or predicted to be secretory proteins. Although the S-RNases and S-like RNases share many structural features (Green 1994), each group contains highly conserved residues not found in the other (Taylor et al. 1993). In fact, gene genealogies of these RNases indicate that most S-like RNases form a lineage distinct from that of the S-RNases (Bariola et al. 1994) (also see Chapter 2). Initially the term "S-like RNases" was used to describe RNases that are structurally similar to S-RNases but found in species not known to exhibit self-incompatibility. However, as more of these proteins were found in a wide variety of plant species, it became apparent that S-like RNases are

likely to play roles distinct from self-incompatibility, but fundamental to plants in general. Based on this hypothesis, self-incompatible plants would be expected to contain proteins of both the S-RNase and S-like RNase families. This is now known to be the case, as discussed below. In addition, some species that exhibit self-incompatibility contain proteins very closely related to S-RNases that are not involved in the self-incompatibility of the plant, as will be discussed. These enzymes lack the distinguishing structural features associated with S-like RNases and appear to have arisen as part of the S-RNase lineage after it diverged from the S-like RNase lineage. To avoid confusion, we propose that S-like RNases be defined as proteins from self-incompatible or self-compatible plants whose structures allow their placement into the evolutionary lineage shown in Figure 2-5 (see Chapter 2).

Comparison of the amino acid sequences of the proteins that form the S-like RNase lineage to those of the S-RNases reveals numerous residues that are conserved in S-like RNases but not in S-RNases (Green 1994). Conserved residues unique to the S-like RNases are mainly found between the conserved boxes that contain the two active-site histidine residues, as well as at the N-terminal end (see Green 1994, Figure 1).

The first S-like RNase genes to be identified were *RNS1*, *RNS2*, and *RNS3* of *Arabidopsis thaliana* (Taylor and Green 1991). These were cloned via the polymerase chain reaction (PCR) based on their containing the conserved active site regions of the T_2 family. The initial PCR products isolated in this reaction contained several residues conserved in the S-RNases. When the full sequences of their cDNAs were determined, however, several differences from the S-RNases were apparent (Taylor et al. 1993; Green

1994), laying the foundation for the designation of the S-like RNases as a separate group. One of the *RNS* genes, *RNS2*, is expressed in all organs examined with highest expression in flowers (Taylor et al. 1993). Immunocytological evidence indicates that RNS2 is extracellular (Chapter 4); RNS1 and RNS3 are also expected to be extracellular because they appear to contain typical N-terminal signal sequences for entry into the secretory pathway (Bariola et al. 1994).

RNS1 and RNS3 are quite closely related to RNases LE (Jost et al. 1991) and LX (Löffler et al. 1993) (see Figure 2-5) of the self-compatible tomato *Lycopersicon esculentum*, both of which were isolated from cell cultures due to their appearance upon starvation for phosphate (P_i) (Nürnberger et al. 1990; Löffler et al. 1992). Three other P_i-starvation induced RNases from tomato (Löffler et al. 1992) have also recently been sequenced at the protein level (Köck et al. 1995). These enzymes, designated LV1, LV2, and LV3, were isolated from tomato vacuoles. Interestingly, LV3 appears to be identical to RNase LE, an extracellular protein, and the regions of LV1 and LV2 that have been sequenced are identical to sequences of RNase LX. The latter enzyme has been shown to have an intracellular but extravacuolar location (Löffler et al. 1992). RNase LX contains a putative C-terminal endoplasmic reticulum retention signal (Löffler et al. 1993), which is missing in the LV2 peptide sequence (Köck et al. 1995). This observation may indicate that in the absence of this putative ER-retention signal, the protein is targeted to the vacuole. It has not been reported whether these proteins sharing long sequences are products of the same genes. Whether or not this is the case, regulating the location of

these RNases could be a novel mechanism for control of RNase activities and possibly RNase functions.

Another member of the Solanaceae, the self-compatible species *Nicotiana sylvestris*, was found to contain a stylar RNase with homology to the T_2 family (J. Golz, M. Anderson, E. Newbigin, manuscript in preparation). Tubers from the lotus species *Nelumbo nucifera* gave rise to another S-like RNase, identified as a storage protein (G. Day, Z. Chen, T. Chow, unpublished, Genbank accession number M83668). Two S-like RNases were identified in cultured zinnia leaf mesophyll cells undergoing xylogenesis (Ye and Droste 1996). These genes, *ZRNaseI* and *ZRNaseII*, are very closely related to the tomato and *Arabidopsis* RNases described above, but have different expression patterns; neither is induced in response to P_i starvation (Ye and Droste 1996).

Other studies have shown that the seeds of several cucurbit species contain S-like RNases. The first of these enzymes to be identified was RNase MC1 from the seeds of the bitter gourd *Momordica charantia* (Ide et al. 1991). Subsequently two RNases were isolated on the basis of their translational inhibitory properties in cell-free systems: cusativin, from cucumber seeds (Rojo et al. 1994a), and melonin, from seeds of the melon *Cucumis melo* (Rojo et al. 1994b). Both of these enzymes were found to have sequences characteristic of S-like RNases. Cusativin is known to accumulate only in the coat and cotyledons of dry seeds (Rojo et al. 1994a). Recently two related S-like RNases, LC1 and LC2, have been cloned from the seeds of *Luffa cylindrica*, the sponge gourd (T. Nakamura, K. Sasaki, G. Funatsu, submitted). As all of the above cucurbit species are

self-compatible it was suggested that the RNases play a role in protection of the seeds against pathogens ((Rojo et al. 1994a); T. Nakamura, K. Sasaki, G. Funatsu, submitted).

It is likely that S-like RNases will be found to be widespread among monocotyledonous plants as well. This is evidenced by the recent identification in rice, which is self compatible, of several cDNA sequences corresponding to S-like RNases by investigators participating in the Rice Genome Project (Genbank accession numbers D21885, D22272, D23641, and D24884). Based on these partial sequences it is not yet clear whether monocot S-like RNases will form their own sublineage within the S-like RNase lineage.

Interestingly, self-compatible species have been found to contain proteins structurally similar to the S-RNases. Two RNases, S_x and S_o, have been identified in a self-compatible cultivar of *Petunia hybrida* (Ai et al. 1992). Both their structural similarity to the S-RNases and breeding behavior suggest that these RNases are defunct S-RNases, possibly selected for in the breeding process that generated this self-compatible cultivar from its self-incompatible ancestors (Ai et al. 1992). A self-compatible variant of the potato *Lycopersicon peruvianum* contains a protein, S_c, whose sequence identifies it as a member of the S-RNase family, but which lacks RNase activity (Royo et al. 1994). A mutation of one of the highly conserved histidines in RNase S_c is thought to be responsible for the lack of activity and thus the self-compatibility of this normally self-incompatible plant. Like RNases S_x and S_o, the S_c protein is structurally more closely related to the S-RNases than the S-like RNases.

Several T_2-related enzymes other than the S-RNases have also been identified in self-incompatible plants. RNase NE, an S-like RNase with a sequence quite similar to those of RNase LE of tomato and RNS1 of *Arabidopsis*, was identified via PCR in anthers of *Nicotiana alata* (Dodds et al. 1996a). Similarly, styles of *Nicotiana alata* contain a member of the T_2 family, RNase MS1, thought to be unassociated with self-incompatibility (Kuroda et al. 1994), but the sequence of this enzyme has not been reported so it is unclear if this protein is an S-RNase or an S-like RNase. Other members of the T_2 family in self-incompatible plants bear stronger resemblance to the S-RNases, such as RNase X2 from *Petunia inflata* (Lee et al. 1992). Although abundant in pistils, the protein is not associated with self-incompatibility. RNase X2 may have diverged from the S-RNases, or alternately evolved from a common ancestor (Lee et al. 1992).

Few of the plant RNases described in this section have been categorized according to the traditional biochemical classifications referred to earlier. However, RNases LE, LX, LV1, LV2 and LV3 from tomato are all considered RNase I-type enzymes, based on their biochemical properties (Nürnberger et al. 1990; Jost et al. 1991; Löffler et al. 1992; Löffler et al. 1993). Most of the S-like RNases described in this section have molecular weights in the 20 to 25 kDa range specified for RNase I-type enzymes, but RNS2 from *Arabidopsis*, with a deduced molecular weight of 27.2 and two potential N-glycosylation sites (Taylor et al. 1993), is an exception. The tertiary structure of the S-like RNases has been investigated to a limited extent. Preliminary observations indicate that the tertiary structure of RNase LE (M. Irie, M. Köck, K. Glund, unpublished) exhibits several differences from that of RNase Rh from the fungus *Rhizopus niveus* (Kurihara et al. 1992;

Kurihara et al. 1996), the only other member of the T_2 family whose tertiary structure is currently available. In addition, RNase MC1 was recently crystallized (De and Funatsu 1992), so additional data on this enzyme may be forthcoming.

Pathogenesis-related protein group PR-10

Pathogenesis-related proteins (PR proteins) are enzymes induced in plants upon pathogen attack or in related situations (van Loon et al. 1994), and presumably have roles in the defense of plants against pathogens. The numerous families of PR proteins include such diverse members as chitinases (Legrand et al. 1987), glucanases (Kauffmann et al. 1987), and proteinase inhibitors (Geoffroy et al. 1990). PR proteins were recently linked to RNases with the report that a ginseng RNase with non-specific activity (Moiseyev et al. 1994) has protein sequence homology to two PR proteins from parsley (Somssich et al. 1988; van de Löcht et al. 1990). The parsley proteins in turn are known to be members of a large PR-protein family recently designated as PR-10 (van Loon et al. 1994; Walter et al. 1996 and references therein). The members of this family are present in a variety of plants, have molecular weights of 17 to 18 kDa, and are considered intracellular. In addition, proteins of a related group, the *Bet v* I family, the major pollen allergens in birch, are constitutively present at high levels in pollen (Swoboda et al. 1994). These pollen allergens constitute a large isoform family in birch and other plants (Swoboda et al. 1995).

Besides the ginseng RNase preparation (Moiseyev et al. 1994), which has recently been shown to be a mixture of two related proteins (G. Moiseyev, J. Beintema,

unpublished), the only other protein in the PR-10 family that has been reported to have RNase activity is *Bet v* I, reported in two separate papers (Swoboda et al. 1996; Bufe et al. 1996). Efforts to demonstrate activity for members of this family in potato (Constabel and Brisson 1995), parsley (I. Somssich, unpublished), and asparagus (S.A.J. Warner, J. Draper, unpublished) have been unsuccessful. Until more proteins of this type are shown to exhibit RNase activity, it may be premature to designate this family as another major family of plant RNases. However, if activity can be demonstrated in other PR-10 proteins, this family would be the first group of intracellular, non-specific RNases characterized molecularly in plants. In addition, it may then be possible to establish a direct link between specific RNase activities and plant defense against pathogens.

Group V Allergens

RNase activity has also been associated with a protein from timothy grass, *Phl p* Vb (Bufe et al. 1995), a member of a group of grass pollen allergens molecularly distinct from the *Bet v* I family described above. These proteins, the group V allergens, generally have molecular weights of 32 to 38 kDa, and include proteins targeted to the amyloplasts in rye grass (Singh et al. 1991; Knox 1993). However, the RNase activity of *Phl p* Vb should be interpreted with caution, as the activity is inhibited by human placental RNase inhibitor, which was previously found to inhibit only animal RNase A-type proteins among RNase groups tested (Lee and Vallee 1993). The sequences of group V allergens exhibit no readily apparent similarity to proteins in the RNase A superfamily. (Similar concerns about the use of human placental RNase inhibitor are also associated with the

cucumber S-like RNase cusativin (Rojo et al. 1994a), as well as with one of the *Bet v* I studies (Bufe et al. 1996), since the PR-10 proteins also have no obvious sequence similarities to RNase A). Whether the group V allergens comprise another major RNase family has yet to be determined.

Bifunctional Nucleases

Although many plant enzymes with the characteristics of nuclease I enzymes have been identified, little sequence information is available to confirm the relatedness of these proteins. However, there is a limited region of sequence identity between two nucleases from distantly related plants. One of these proteins is a 39 kDa nuclease I secreted from barley aleurone layers (Brown and Ho 1987). The secretion of this nuclease is induced by gibberelic acid (Brown and Ho 1986), a plant hormone which induces the secretion of a range of hydrolytic enzymes from aleurone layers to mobilize seed endosperm reserves for the germinating seedling (Jacobsen et al. 1995). Another nuclease, from zinnia, is induced during the differentiation of xylem elements (Thelen and Northcote 1989). Although the zinnia nuclease technically cannot be classified as a nuclease I (Wilson 1982) due to its 43 kDa molecular weight, its N-terminal sequence is similar to that of the barley nuclease, implying that these two enzymes are related. It has been noted (Fraser and Low 1993) that the partial barley nuclease sequence is similar to the amino-termini of S1 and P1 nuclease from the fungi *Aspergillus oryzae* (Iwamatsu et al. 1991) and *Penicillium citrum* (Maekawa et al. 1991), respectively. These fungal nucleases are part of a large family of single-strand-specific bifunctional (degrade both RNA and DNA)

nucleases (Gite and Shankar 1995) based on their biochemical properties. Mung bean nuclease (Laskowski 1980) is also considered to be a member of this family. The biochemical characteristics that define this group (Gite and Shankar 1995) are much broader than those that define the plant nuclease I enzymes (Wilson 1982), and nuclease I enzymes could easily be classified as part of the single-strand-specific nuclease family. (See Gite and Shankar, 1995, for an extensive discussion of the biochemical properties of the enzymes of this family.) Recently, a protein with limited homology to nuclease P1 has been purified from spinach chloroplasts (Yang et al. 1996); this protein will be discussed later.

In *Arabidopsis*, nucleases shown to have some of the properties of nuclease I enzymes have been identified using activity gels (Yen and Green 1991). A doublet of about 33 kDa that appears on both RNase and DNase activity gels led to the suggestion that the same 33 kDa enzymes can degrade both RNA and DNA. Recently this idea was confirmed through the analysis of altered RNase profile (*arp*) mutants of *Arabidopsis*. Six *arp* mutants that either lack or overproduce one or both of the 33 kDa doublet RNase activities were examined on DNase activity gels and shown to exhibit phenotypes identical to those on RNase activity gels (M.L. Abler, P.J. Green, manuscript in preparation). This result demonstrates genetically that the 33 kDa doublet represents a pair of bifunctional nuclease activities. The availability of these mutants may help elucidate the biological functions of these nucleases and reveal whether they are encoded by the same gene.

In addition to the nuclease I enzymes mentioned earlier (in the "Classifications based on Early Biochemical Work" section), a number of activities with some similarities to a tobacco pollen nuclease I (Matousek and Tupy 1984) have been identified in pollen from various species, with a nuclease from *Pinus nigra* best characterized (Matousek and Tupy 1985). Likewise, a nuclease with similar properties was identified in tobacco anthers (Matousek and Tupy 1987). Single-strand-specific nucleases have recently been found in spinach (Strickland et al. 1991), scallion (Uchida et al. 1993), wheat chloroplasts (Monko et al. 1994), and pea seeds (Naseem and Hadi 1987) and chloroplasts (Kumar et al. 1995). It will be interesting to determine if the nucleases described in this section, the plant enzymes classified as nuclease I mentioned earlier, and the single-strand-specific nucleases from fungi and plants are members of the same or multiple molecular families.

REGULATION AND FUNCTIONS OF PLANT RNASES

Phosphate Remobilization

Several S-like RNases have been shown to be upregulated in response to starvation for inorganic phosphate (P_i). The first of these reports showed that RNase LE from tomato is secreted in response to P_i limitation (Nürnberger et al. 1990). Subsequently, increased levels of the other tomato S-like RNases, LX, LV1, LV2, and LV3, all intracellular enzymes, were found during P_i starvation (Löffler et al. 1992). Most of this work was done at the protein level, but recently starvation for this nutrient has been shown to induce RNases LE and LX at the mRNA level (Köck et al. 1995). These results mirrored previous reports of two *Arabidopsis* RNase genes, *RNS1* (Bariola et al. 1994) and *RNS2* (Taylor et al. 1993), which were also found to be P_i-starvation inducible. In particular, the *RNS1* mRNA is dramatically upregulated from a low basal level (Bariola et al. 1994). This induction of *RNS1* also appears to occur at the protein level, because an RNase activity that comigrates with RNS1 produced in yeast increases in parallel with *RNS1* mRNA (Bariola et al. 1994; C.J. Howard and P.J. Green, manuscript in preparation). In addition, P_i starvation has recently been found to induce the gene for *Nicotiana alata* RNase NE (Dodds et al. 1996a). However, at least three S-like RNase genes, *RNS3* of *Arabidopsis* (Bariola et al. 1994) and *ZRNaseI* and *ZRNaseII* of *Zinnia* (Ye and Droste 1996), do not respond to P_i limitation, so P_i-starvation inducibility should not be considered characteristic of the S-like RNases. Limitation for P_i also leads to a large increase in the activity of a surface membrane-associated nuclease

in the trypanosome *Crithidia luciliae* (Gottlieb et al. 1988). Although the sequence of this protein is not available, the nuclease is considered similar to plant nuclease I enzymes due to its enzymatic properties (Neubert and Gottlieb 1990). It is conceivable that plant nuclease I genes could also be induced by P_i starvation. Indeed, extracts of plants grown on P_i-deficient medium exhibit increased RNase activity on activity gels in the area of the 33 kDa nuclease doublet (refer to Bariola et al. 1994, Figure 10).

It has been proposed that under P_i-limiting conditions, RNases could degrade RNA in conjunction with phosphatases and phosphodiesterases to release P_i, making it available for the plant to use in other processes (Glund and Goldstein 1993). This response is likely only a part of a broader effort by the plant to optimize P_i availability under conditions of scarcity (Goldstein et al. 1989). RNases could increase the efficiency of the plant to scavenge P_i in several ways. RNases secreted from roots into the soil could make P_i previously sequestered in RNA in organic matter in the soil available for uptake. The role of scavenging P_i from RNA in the growth environment is believed to be a main function of two fungal nucleases (Fraser and Low 1993). Extracellular RNases within the plant could rescue P_i from RNA that arises in the extracellular space from cells that have lysed due to senescence, damage or programmed cell death (discussed below). Finally, vacuolar RNases may participate in some aspect of intracellular RNA degradation, since short pieces of RNA exist in the vacuoles of plant cells (Abel et al. 1990). It is generally recognized that one main function of the plant vacuole is the turnover of cellular macromolecules, analogous to animal lysosomes (Boller and Wiemken 1986; Wink 1994). Since autophagy of small amounts of cytoplasm may occur

in plant cells (Boller and Wiemken 1986), this process may be a route for vacuolar uptake of cytoplasmic RNA. An increase in the RNase concentration in the vacuole could speed the process of the recycling of components of RNA.

Remobilization of P_i may take place during normal plant development. In barley seeds, a nuclease I is secreted from the aleurone layer upon treatment with gibberellin (Brown and Ho 1986), a hormone associated with seed germination (Jacobsen et al. 1995). The aleurone layer lays outside the endosperm, which stores macromolecules such as proteins and carbohydrates. Germination triggers the secretion of several hydrolytic enzymes (Jacobsen et al. 1995), which release nutrients in forms able to be used quickly by germinating seedlings. The nuclease I is proposed to degrade nucleic acids in endosperm, in conjunction with acid phosphatases, to release nucleosides and phosphate for use in new RNA synthesis in the seedling (Brown and Ho 1986). The widespread increase in RNase activities in germinating seeds (Farkas 1982) suggests that this may be a universal phenomenon in plants.

Senescence

The effect of senescence on plant RNase activity has been studied extensively. In general, RNase activities increase in plants during senescence, but in different systems the timing and extent of the increases vary (Farkas 1982). Many of these variations are likely due to differences in systems and experimental design (e.g. studies in attached leaves or excised leaves), but different patterns of induction may also contribute (Farkas 1982). It is now clear that senescence dramatically upregulates individual RNase

activities and genes. In wheat leaves, single-strand-specific nuclease activity increases during senescence (Blank and McKeon 1989), as does the activity of three RNases of 20 to 27 kDa (Blank and McKeon 1991b), monitored both biochemically and in activity gels. The three *Arabidopsis* S-like RNase genes, *RNS1*, *RNS2*, and *RNS3*, are each induced to different extents in leaves during senescence: *RNS1* mRNA levels increase only slightly (Bariola et al. 1994), whereas those of *RNS2* and *RNS3* increase more dramatically (Taylor et al. 1993; Bariola et al. 1994). *RNS2* is also known to be induced in senescing petals (Taylor et al. 1993).

During senescence, cellular structures are disassembled and macromolecules in certain plant organs are broken down, freeing nutrients for relocation to other organs (Stoddart and Thomas 1982). This process is thought to occur to conserve minerals and nutrients for reuse. The breakdown and redistribution can occur both during vegetative growth, such as the rescue of minerals from senescing cotyledons, and also during reproductive growth, when in some plants all organs except the reproductive structures senesce and the vegetative organs serve as a source of nutrients for the reproductive structures (Noodén 1988a). RNases are likely some of the variety of hydrolytic enzymes induced during senescence (Borochov and Woodson 1989) that facilitate the breakdown of cellular components. The actions of RNases during senescence could lead to the recycling of P_i.

Cell Death Pathways

Programmed cell death is an essential part of developmental patterns and physiological processes in many organisms (Vaux 1993). In plants, cell death pathways are only beginning to be investigated (Greenberg 1996). Programmed cell death is associated with sex determination in maize (DeLong et al. 1993), the hypersensitive response (HR) against plant pathogens (Greenberg et al. 1994), and possibly pollination, senescence, and various developmental processes (Greenberg 1996). One of the best studied processes regarding cell death in plants is the differentiation of xylem, a major component of the plant vascular system. When isolated leaf mesophyll cells from zinnia are cultured in the presence of appropriate concentrations of the hormones auxin and cytokinin, they differentiate synchronously into elongated, lignified tracheary elements, the building blocks of the xylem (Fukuda 1996). The last part of the differentiation process involves strengthening of the cell wall and hydrolysis of the end walls between two differentiating cells to form a tube. Vacuoles lyse several hours after the secondary cell wall is formed, leading to degradation of cytoplasmic macromolecules. Finally, lysis of the protoplast occurs so that the cell wall can serve as a link in the channel that forms the xylem (Fukuda 1996).

A single-strand-specific nuclease of 43 kDa with homology to a nuclease I of barley (Brown and Ho 1987) appears during xylogenesis in the zinnia system (Thelen and Northcote 1989). In addition, several RNase activities of 17 to 25 kDa appear in zinnia cell extracts, and a 37 kDa nuclease accumulates in the culture medium. One of these RNases may correspond to ZRNase I, an S-like RNase with a predicted molecular weight

of 27 kDa, whose cDNA was isolated from zinnia mesophyll cells differentiating into xylem elements (Ye and Droste 1996). In zinnia cultured mesophyll cells, its mRNA first appears at high levels after about 48 hours of culture in differentiation-inducing medium, relatively late in the differentiation process (Ye and Droste 1996). This result was confirmed by tissue-print hybridization, in which *ZRNaseI* mRNA appears in differentiating xylem elements of stems (Ye and Droste 1996). A protease gene is also strongly induced at 48 hours of culture (Ye and Varner 1993). It is possible that these and other hydrolytic enzymes are involved either in killing the cell directly or in degrading cytoplasmic components during and after lysis in order to clear the xylem channel and facilitate nutrient reutilization. Nucleases could be involved in RNA degradation as well as the fragmentation of DNA that is one of the hallmarks of apoptotic cell death in animal systems (Zhivotovsky et al. 1994). Nuclear DNA fragmentation has been observed in pea root xylem cells undergoing cell death (Mittler and Lam 1995a). Less is known about the association of RNases and nucleases with other cell death pathways in plants. However, recent studies indicate that anther nucleases are highest during the first microspore division in tobacco (Matousek et al. 1994). This led to the suggestion that anther nucleases could participate in tapetal cell degeneration (Matousek et al. 1994).

Defense Against Pathogens

Increases in RNase activities in diseased plants are well-documented (Farkas 1982; Green 1994; Barna et al. 1989; Lusso and Kuc 1995). Several roles can be

proposed for RNases in plant disease and defense. First, if RNases are involved in cell death pathways in plants, as discussed in the previous section, they could play a role in the hypersensitive response of plants, which appears to involve programmed cell death (Greenberg et al. 1994). The HR involves death of plant cells shortly after pathogen infection in the immediate vicinity of the infection site, and the localized cell death is thought to contribute to the resistance of the plant to the disease (Keen 1992). Recently a DNase activity has been found to be induced in tobacco nuclei during cell death due to the HR (Mittler and Lam 1995b). This DNase, NUCIII, has been characterized as an endonuclease that cleaves both single-stranded and double-stranded DNA, but it has not been reported whether the protein has RNase activity (Mittler and Lam 1995b). It seems probable that DNA fragmentation occurs during the HR and the NUCIII could play a role in this process. In tobacco, an HR-like response that was for some reason induced by overexpression of a bacterial proton pump gene provided evidence for DNA fragmentation (Mittler et al. 1995).

A second role for plant RNases during pathogen attack could be to act as defense proteins in tissues potentially susceptible to infection. For example, the pistil, which is penetrated by the pollen tube during the fertilization process, is rich in extracellular nutrients that should make it susceptible to pathogen invasion. However, it is rarely infected. This resistance may be due to defense-related proteins that are present extracellularly in the pistil, such as proteinase inhibitors (Atkinson et al. 1993). Several T_2-type RNase genes have been shown to be expressed in pistils, so they are also candidate defense proteins. These include *X2* of *Petunia inflata* (Lee et al. 1992), *RNS2*

of *Arabidopsis* (Taylor et al. 1993), and *NE* of *Nicotiana alata* (Dodds et al. 1996a). It has been proposed that gametophytic self-incompatibility may have arisen via the recruitment of defense-related pistil RNases (Lee et al. 1992). Extracellular RNases may also play a part in the plant's defense against RNA viruses. Finally, RNases are known to accumulate in plant vacuoles (Farkas 1982; Wilson 1982). The defense-related proteins chitinase and β-1,3-glucanase, which can degrade fungal cell walls, are also known to be sequestered in vacuoles, increasing in abundance during pathogen attack (Mauch and Staehelin 1989). Mauch and Staehelin (1989) propose a model in which accumulation of large amounts of these proteins in vacuoles is an advantage: when fungi invade, cells lyse either due to the HR or direct pathogen invasion of the cell. Upon lysis, fungal hyphae would be flooded with defense-related proteins in high enough concentrations to lyse the hyphae. RNases may well be components of this onslaught of hydrolytic defense enzymes released from the vacuoles upon cell lysis.

Plants interpret mechanical wounding as a signal of attack, since pathogen infection and chewing by insects or other herbivores often result in wounding of tissues. Wounding induces defense-related genes in plants, such as those of proteinase inhibitors (Ryan 1990), peroxidases (Bowles 1990), and chitinases (Bowles 1990). Wounding is known to induce rapid increases in RNase activities (Farkas 1982). One zinnia S-like RNase gene, *ZRNaseII*, is rapidly induced upon mechanical wounding (Ye and Droste 1996). In *Arabidopsis*, wounding of stems resulted in induction of an RNase activity of about 34 kDa (M. Saitoh, M.L. Abler, P.J. Green, unpublished). Like the 33 kDa bifunctional nuclease activities discussed in the "Bifunctional Nucleases" section, the 34

kDa RNase comigrates with a DNase activity induced under the same conditions, so the protein is likely a bifunctional nuclease. Further examination of this enzyme and its wound-inducibility may provide insights into the roles of RNases in defense responses.

RNA Processing and Decay

Nuclear Activities: A number of events that take place in plant nuclei likely involve RNases, but very few such enzymatic activities have been identified. Presumably the major degradative process in the nucleus is the decay of introns and other sequences removed from precursors of mature mRNAs, rRNAs, and tRNAs. There are a few early reports of nuclear-associated RNase activities that might be involved in these processes in plants (reviewed in Farkas, 1982), but this area of research should be revisited because it is underdeveloped not only in plants but also in other eukaryotes (Stevens 1993; Ross 1995). The most convincing data for an RNase located in the nucleus comes from work on the 7-2/MRP RNA, which is known to be the RNA component of RNase MRP in mammalian cells. In plants and mammalian cells, 7-2/MRP RNA is found in nucleoli where it is likely to be involved in rRNA processing (Kiss and Filipowicz 1992; Kiss et al. 1992; Morrissey and Tollervey 1995; Lygerou et al. 1996).

Chloroplast and Mitochondrial Activities: Most of the organellar RNase activities that have been identified participate in the maturation of 5' and 3' ends of chloroplast and mitochondrial transcripts such as tRNAs. The ribonucleoprotein RNase P is responsible for the processing of the 5' end of pre-tRNAs (Altman et al. 1993); in plants, RNase P

enzymes have been identified in chloroplasts (Gegenheimer 1996) and mitochondria (Marchfelder and Brennicke 1994). Interestingly, the spinach chloroplast RNase P may not contain an RNA component (Wang et al. 1988; Gegenheimer 1996). Maturation of the 3' end of pre-tRNAs in eukaryotes involves an endonucleolytic cleavage in most cases, in contrast to the prokaryotic mechanism (Deutscher 1993b). 3' tRNA processing activities are detectable in plant nuclei, mitochondria, and chloroplasts (Oommen et al. 1992; Marchfelder and Brennicke 1994; Gegenheimer 1996). Preliminary characterization of RNase activities that affect 3' end maturation has also been achieved (Chen and Stern 1991). These include one or more 3' to 5' exoribonucleases and an endoribonuclease. The latter has been shown to cleave the spinach *petD* mRNA at the termination codon and at the mature RNA 3' end. Recently, an encoribonuclease has been purified from this system and its cDNA cloned (Yang et al. 1996). Interestingly, the protein has a region of similarity to nuclease P1 (Maekawa et al. 1991), placing it in the category of plant nucleases. In maize, a nuclear mutation, *crp1*, has been isolated that blocks the processing of the polycistronic precursor of *petD* mRNA, which appears to inhibit translation (Barkan et al. 1994). It is possible that the *CRP1* gene encodes a processing RNase or a protein that regulates such an activity.

Activities Implicated in Cytoplasmic mRNA Decay: Little is known about the RNase activities that facilitate the degradation of most plant mRNAs, since most degrade without generating easily identifiable intermediates. Two exceptions are the soybean *rbcS* (Tanzer and Meagher 1994; Tanzer and Meagher 1995), and the *PHYA* (Higgs and

Colbert 1994) mRNAs. Discrete fragments of the *rbcS* mRNA are observed *in vivo* and are produced in an *in vitro* decay system. The structures of these fragments suggest that they are produced by a stochastic endonuclease followed by exonuclease digestion in the 5' to 3' or 3' to 5' direction (Tanzer and Meagher 1994; Tanzer and Meagher 1995). In contrast, a continuous population of lower molecular weight fragments is observed for the *PHYA* mRNA, rather than discrete intermediates. Characterization of these fragments indicates that they most likely arise through the action of 5' to 3' and 3' to 5' exoribonucleases, although endoribonuclease models cannot be ruled out (Higgs and Colbert 1994). In yeast, many mRNAs are known to be degraded by a pathway involving deadenylation, possibly by a poly(A) nuclease, followed by decapping and digestion by XRN1, a 5' to 3' exoribonuclease. This type of pathway may explain the decay of about 25% of the *PHYA* mRNA, but the remainder (Higgs and Colbert 1994), as well as the *rbcS* mRNA (Tanzer and Meagher 1995), appears to degrade independent of deadenylation. Deadenylation-independent mRNA decay pathways, some of which involve cleavage by sequence-specific endoribonucleases (Brown and Harland 1990; Binder et al. 1994), also exist in yeast and animal systems (Beelman and Parker 1995).

Recent reports have identified sequences that can trigger rapid decay of reporter mRNAs in plants. These include the 3' untranslated region (UTR) of the *Arabidopsis SAUR-AC1* transcript (Gil and Green 1996), a dimer of the DST element (Newman et al. 1993), which is conserved among the 3' UTRs of unstable *SAUR* transcripts (McClure et al. 1989a), and AUUUA repeats (Ohme-Takagi et al. 1993), which also trigger mRNA decay in mammalian cells (Shaw and Kamen 1986; Vakalopoulou et al. 1991). However,

it is unknown whether these sequences serve as endonuclease-sensitive sites in plants or whether they trigger exonuclease digestion. By examining the effect of these instability elements in the *Arabidopsis* mutants that alter the RNase profile (*arp* mutants, described in the "Plant Nuclease" section) and other mutants that may become available, it may be possible to identify some of the RNases involved in general and specific mRNA decay pathways. In addition, *in vitro* systems (Byrne et al. 1993; Tanzer and Meagher 1994; Tanzer and Meagher 1995) may facilitate purification of RNases that act on specific mRNA transcripts, particularly in the case of the *PHYA* and *rbcS* transcripts.

Finally, it has been suggested that antisense RNA effects in plants may be mediated in part by the action of a double-stranded RNA degrading activity that is presumed to degrade the sense-antisense hybrid (Nellen and Lichtenstein 1993). This idea is based on the observation that in many cases accumulation of the sense RNA is diminished in plants engineered to produce a corresponding antisense RNA (Nellen and Lichtenstein 1993; Bourque 1995). In one case, this effect has been shown to be due to rapid decay of the sense RNA (Jiang et al. 1994). Although a considerable amount of dsRNase activity in plants may be extracellular (Matousek et al. 1994), perhaps the enzyme corresponding to an *Arabidopsis* expressed sequence tag (Genbank accession number Z18464) (Höfte et al. 1993), which has homology to RNase III, a bacterial intracellular dsRNase (Robertson 1982), is involved in this process.

DISSERTATION TOPIC AND THESIS OVERVIEW

The relationships among different RNases have become much clearer now that many have been cloned and sequenced. It seems apparent that the S-like RNases are among the major, if not the major, class of RNA-degrading enzymes in higher plants. Based on their gene expression and activity levels, the S-like RNases are likely to participate in fundamental physiological processes such as senescence, phosphate starvation responses and cell death pathways. My thesis project has involved characterizing three S-like RNases of *Arabidopsis thaliana* and using techniques designed to provide insight into their roles and purposes in plants. Chapters 2 and 3 detail my contributions to the initial analysis of S-like RNase structure and regulation mentioned in this introduction. My efforts to determine the subcellular location of RNS2 are described in Chapter 4, and the generation of transgenic plants with altered levels of RNS1 and RNS2 is described in Chapter 5.

CHAPTER 2

RNS2: A SENESCENCE-ASSOCIATED RNASE OF *ARABIDOPSIS* THAT DIVERGED FROM THE S-RNASES BEFORE SPECIATION

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Taylor CB, Bariola PA, del Cardayré SB, Raines RT, Green PJ (1993) RNS2: A senescence-associated RNase of *Arabidopsis* that diverged from the S-RNases before speciation. Proc. Natl. Acad. Sci USA 90:5118-5122

ABSTRACT

Several self-compatible species of higher plants, such as *Arabidopsis thaliana*, have been found to contain S-like RNases. These S-like RNases are homologous to the S-RNases that are involved in 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 yet understood. To address these questions, we have investigated the *RNS2* gene, which encodes an S-like RNase 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 subclasses formed by the S-RNases. RNS2 is quite similar to RNases LE (Jost et al. 1991) and LX (Löffler et al. 1993) of *Lycopersicon esculentum*, and to RNase NE of *Nicotiana alata* (Dodds et al. 1996a); both of these are Solanaceous species. The fact that RNases LE, LX and NE are 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*. Finally, *RNS2* is induced during infection by a bacterial pathogen, during both compatible and incompatible infection, although to a higher extent during compatible infection. 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.

INTRODUCTION

The identification of three T_2/S family RNase genes in a self-compatible plant, *Arabidopsis thaliana* (Taylor and Green 1991), was unprecedented. At the time, the only RNases of this type known were either fungal enzymes or enzymes associated with gametophytic self-incompatibility in certain Solanaceous plants. The logical first steps after the initial identification of the three *RNS* genes were to isolate the full-length cDNAs, examine their sequences, and determine their expression properties. These were intended to give clues as to the function of this type of RNase, which we termed "S-like RNases" due to their similarity to S-RNases, in self-compatible plants.

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 a class of fungal RNases (Taylor and Green 1991). This PCR product was used as a hybridization probe to isolate *RNS2* cDNA clones from the same library. 39 positive clones were initially identified in a screen of 250,000 plaques, and four were partially sequenced. Once the clone with the longest 3' untranslated region was identified, it was sequenced completely from both 5' to 3' and 3' to 5' directions (Figure 2-1). The nucleotide and deduced amino acid sequences of the longest clone containing an open reading frame have been deposited in the GenBank data base (accession number M98336) and are shown in Figure 2-2. Analysis of several independent cDNA clones showed that transcripts from the *RNS2* gene can be polyadenylated at multiple sites (Figure 2-2), a feature common to many plant genes (Dean et al. 1986). The 19 amino acids at the N terminus of the RNS2 protein are typical of a eukaryotic secretion signal sequence, as defined by a statistical analysis of known signal sequences (von Heijne 1986). This suggests that RNS2 is targeted to the secretory pathway in *Arabidopsis*, similar to the Solanaceous S-RNases, which are secreted enzymes.

To confirm that RNS2 is indeed an RNase, the coding sequence was expressed in *Saccharomyces cerevisiae* under the control of the *PHO5* promoter (Thill et al. 1983). 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

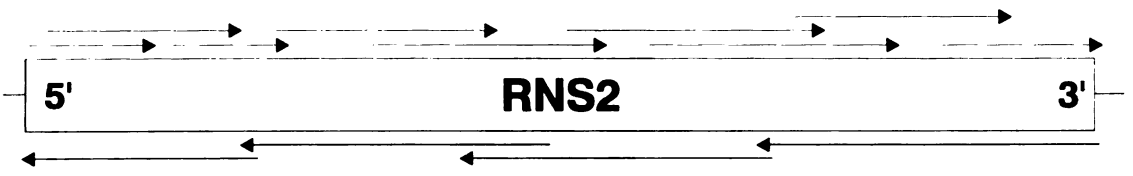


Figure 2-1 - Strategy for sequencing of *RNS2* cDNA. The rectangle represents the *RNS2* cDNA sequence, and arrows represent individual sequencing reactions read. Each base of the *RNS2* cDNA was read at least once from each direction.

```
  1 ATCGAATTAAAGTCAATGGCGTCACGTTTATGTCTTCTCCTTCTCGTTGCGTGTATCGCC
  1                M  A  S  R  L  C  L  L  L  L  V  A  C  I  A

                   ▼
 61 GGAGCATTTGCCGGAGACGTCATCGAACTCAATCGATCTCAGAGGGAGTTCGATTATTTC
 16 G  A  F  A  G  D  V  I  E  L  N  R  S  Q  R  E  F  D  Y  F

121 GCTCTATCTCTTCAATGGCCTGGAACCTATTGCCGTGGAACTCGCCATTGTTGCTCCAAA
 36 A  L  S  L  Q  W  P  G  T  Y  C  R  G  T  R  H  C  C  S  K

181 AACGCTTGCTGCAGAGGCTCCGATGCTCCAACTCAATTCACAATTCATGGGTTATGGCCT
 56 N  A  C  C  R  G  S  D  A  P  T  Q  F  T  I  H  G  L  W  P

241 GACTATAACGATGGTTCGTGGCCTTCATGTTGTTATCGATCTGACTTTAAAGAGAAGGAG
 76 D  Y  N  D  G  S  W  P  S  C  C  Y  R  S  D  F  K  E  K  E

301 ATTTCAACGTTGATGGATGGTCTTGAGAAGTACTGGCCTAGTCTCAGTTGTGGTTCTCCA
 96 I  S  T  L  M  D  G  L  E  K  Y  W  P  S  L  S  C  G  S  P

361 TCATCATGCAATGGTGGGAAAGGGTCATTTTGGGGCCACGAGTGGGAGAAACATGGGACT
116 S  S  C  N  G  G  K  G  S  F  W  G  H  E  W  E  K  H  G  T

421 TGTTCTTCTCCTGTTTTTCATGATGAGTATAATTACTTCCTTACCACACTTAATCTCTAC
136 C  S  S  P  V  F  H  D  E  Y  N  Y  F  L  T  T  L  N  L  Y

481 TTGAAGCATAATGTCACGGATGTCCTTTATCAAGCTGGCTATGTTGCTTCCAACAGTGAA
156 L  K  H  N  V  T  D  V  L  Y  Q  A  G  Y  V  A  S  N  S  E

541 AAGTATCCTCTAGGAGGTATCGTAACAGCCATTCAGAATGCATTTCATATCACCCCTGAA
176 K  Y  P  L  G  G  I  V  T  A  I  Q  N  A  F  H  I  T  P  E

601 GTGGTTTGCAAAAGAGATGCAATCGATGAAATACGTATATGCTTCTATAAAGATTTTAAG
196 V  V  C  K  R  D  A  I  D  E  I  R  I  C  F  Y  K  D  F  K

661 CCCAGGGACTGTGTTGGTTCACAAGATTTGACATCTAGAAAGTCATGCCCCAAGTACGTA
216 P  R  D  C  V  G  S  Q  D  L  T  S  R  K  S  C  P  K  Y  V

721 AGTTTGCCGGAATACACGCCATTAGATGGTGAAGCTATGGTTCTGAAGATGCCAACAGAA
236 S  L  P  E  Y  T  P  L  D  G  E  A  M  V  L  K  M  P  T  E

781 AGAGAAGCTCTTTGAATCGGAAAAGATGGGAGCTTTGTTATCTTCTGAGAGACAATACAT
256 R  E  A  L  *

841 ACATGTCTCTGATGTTGTAACTTTACTACCAAAACCTATAAAGATTGGCTTATTTCGTTC
                  ↘
901 TATTGGATATGTATCATCATTACTGGTAAATCAAGTTTCTTTCTAATAATGTAGAAGATC
                ↘                    ↘
961 AGAAAATCCATAAGAAGATATCAACATTTGAGTTCTATGGTAAAAAAAAAAA
```

Figure 2-2 - Primary structure and deduced amino acid sequence of *RNS2* cDNAs. Deduced amino acid residues are shown in one-letter notation below the nucleotide sequence. Triangle indicates the putative N-terminal end of the mature protein predicted by a statistical analysis (von Heijne 1986). Arrows indicate polyadenylation sites used in different cDNA isolates. The two putative N-glycosylation sites are underlined.

electrophoresis on RNase activity gels (Yen and Green 1991) (Figure 2-3). 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 cleavage of the N-terminal signal sequence). 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 (Ohgi et al. 1991) or differences in glycosylation (Tague and Chrispeels 1987) 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 (Yen and Green 1991), 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 deduced amino acid sequence of RNS2 with those of related plant RNases, the alignment shown in Figure 2-4 was generated as described in the Materials and Methods. The alignment demonstrates that the similarity of RNS2 to the S-RNases is dispersed throughout the coding region (Figure 2-4). Moreover, each of the five regions most conserved among the S-RNases [numbered C1-C5 by Kao and coworkers (Kheyr-Pour et al. 1990; Ioerger et al. 1991) and boxed in Figure 2-4] is also evident in RNS2. At the original time of publication of this sequence comparison (Taylor et al. 1993), RNS2 was compared to two other S-like RNases and 15 S-RNases, which comprised all of the plant T_2/S type RNases for which sequences were known. At present, the sequences of 12 S-like

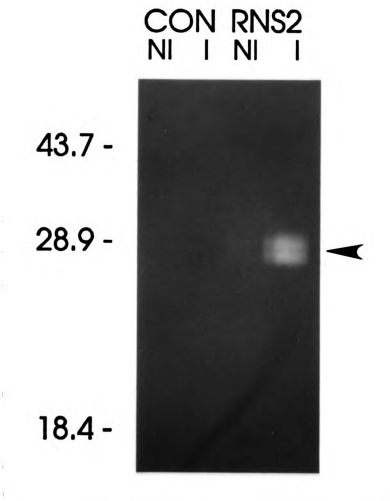


Figure 2-3 - Expression of RNS2 in yeast. Yeast cultures transformed with the control pWL (CON) or RNS2 (RNS2) constructs were grown under conditions that do not induce (NI) or do induce (I) transcription from the *PHO5* promoter. Supernatants from these cultures were run on RNase activity gels as described in the Materials and Methods. Positions of molecular mass markers (kDa) are shown to the left of the gel. The arrowhead indicates the RNS2 activity bands.

Legend to Figure 2-4 - Alignment of S- and S-like RNase amino acid sequences. S-like RNase sequences are LE (Jost et al. 1991) and LX (Löffler et al. 1993) of *Lycopersicon esculentum,* RNS1 and RNS3 of *Arabidopsis thaliana* (Bariola et al. 1994), RNS2 of *Arabidopsis thaliana* (Taylor et al. 1993), NE of *Nicotiana alata* (Dodds et al. 1996a), ZRN1 and ZRN2 of *Zinnia elegans* (Ye and Droste 1996), MC1 of *Momordica charantia* (Ide et al. 1991), LC1 and LC2 of *Luffa cylindrica* (T. Nakamura, K Sasaki, G Funatsu, submitted), and NNUC of *Nelumbo nucifera* (G Day, Z Chen, T Chow, unpublished, Genbank accession number M83668). S-RNase sequences are 1Stu, r1Stu, and 2Stu of *Solanum tuberosum* (Kaufmann et al. 1991), 1Pet, 2Pet and 3Pet of *Petunia inflata* (Ai et al. 1990), Ps2A, Ps3A and Ps1B of *Petunia hybrida* (Clark et al. 1990), 5Lyc of *Lycopersicon peruvianum* (Tsai et al. 1992), a, Z, F11 and 1Nic of *N. alata* (Kheyr-Pour et al. 1990), XPet and OPet of self-compatible *P. hybrida* (Ai et al. 1992), 2Nic, 3Nic and 6Nic of *N. alata* (Anderson et al. 1989), 2Sol and 3Sol of *Solanum chacoense* (Xu et al. 1990), 11Sc of *S. chacoense* (Saba-El-Leil et al. 1994), 5Lp of *L. peruvianum* (Rivers et al. 1993), 11Lp, 12Lp and 13Lp of *L. peruvianum* (Chung et al. 1994), fMdo of *Malus domestica* (Sassa et al. 1996), 2Mdo and 3Mdo of *M. domestica* (Broothaerts et al. 1995), 2Pp and 4Pp of *Pyrus pyrifolia* (Norioka et al. 1995), and 2Ant, 4Ant and 5Ant of *Antirrhinum* species (Xue et al. 1996). X2 of *P. inflata* (Lee et al. 1992) is not an S-RNase but was placed with this group because it is more closely related to the S-RNases than the S-like RNases (Lee et al. 1992). The sequences are aligned and numbered from predicted mature N-termini of the *N. alata* S-RNases (see Haring et al. 1990). Heavy-bordered boxes enclose conserved regions C1-C5 (Ioerger et al. 1991). Light shading indicates residues that are identical or functionally identical in at least 35 of the 47 sequences. Light-bordered boxes enclose residues that are identical or functionally identical in at least 9 of the 12 S-like RNase sequences but not highly conserved among the S-RNases. Dark shading indicates residues that are identical or functionally identical in at least 26 of the 35 S-RNase sequences but not highly conserved among the S-like RNases. Functionally identical residues are grouped as follows: A, S, T; I, L, M, V; H, K, R; F, W, Y; D, E; Q, N.

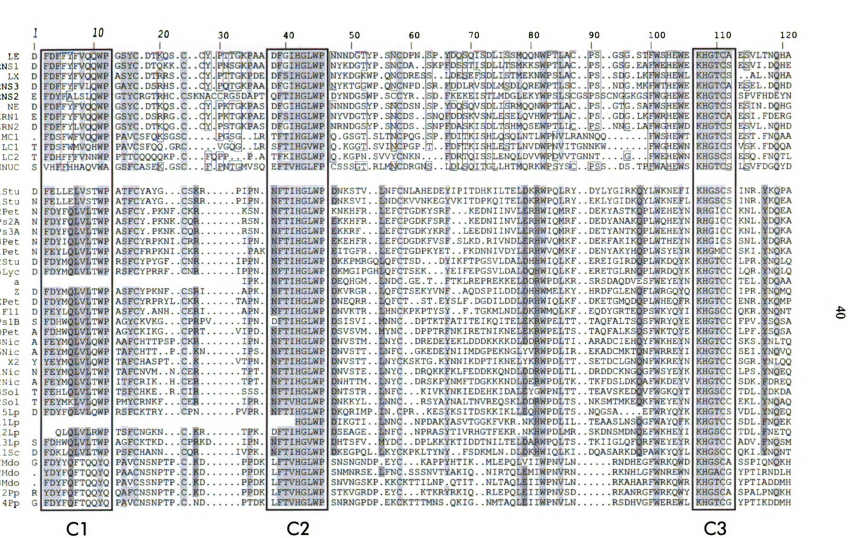


Figure 2-4 - Alignment of S- and S-like RNase amino acid sequences.

Legend to Figure 2-4 - Alignment of S- and S-like RNase amino acid sequences (second page). For the complete legend please see page 39.

Figure 2-4 - Alignment of S- and S-like RNase amino acid sequences (second page).

RNases have been reported, and the number of completely or partially sequenced S-RNases is approaching fifty. Figure 2-4 is an updated sequence comparison, including all of the known S-like RNase sequences and a representative majority of the S-RNase sequences. All of the features noted in the original published comparison (Taylor et al. 1993) are still valid with the newer sequences added.

For example, the histidine residues at positions 42 and 108 are absolutely conserved in all of these RNases. These residues have been shown to be important for catalysis in RNase T_2 of *Aspergillus oryzae* (Kawata et al. 1990). Other groups of notable residues are the conserved cysteines at positions 58 and 111 and positions 177 and 217 that have been shown to form disulfide bonds that are critical for maintaining the structure of RNase T_2 (Kawata et al. 1988). 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 (Figure 2-4). Some of the residues that are highly conserved among the S-RNases but not highly conserved in the S-like RNases are clustered between positions 134 and 167 (Figure 2-4). This region may constitute a domain required for the specialized functions of the S-RNases in self-incompatibility.

The most striking difference between the amino acid sequence of RNS2 and those of most of the other enzymes is the C-terminal extension of 20 amino acids. This sequence has some 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 (Chrispeels and Raikhel 1992; Neuhaus 1996). This idea will be discussed further in Chapter 4.

A broader illustration of the relationship of RNS2 to the other related RNases was obtained by constructing a gene genealogy based on the deduced amino acid sequences in Figure 2-4. Once again, the genealogy shown in the original publication of these results (Taylor et al. 1993) has been updated with new RNases (Figure 2-5), but the same trends are observed even with the many new sequences added. In the original figure, the S-RNases and S-like RNases were placed in separate lineages, which indicates that they form distinct categories of RNases in plants. This is consistent with the theory that the S-RNases and the S-like RNases derive from the same ancestral RNase, the S-RNases having acquired a specialized function in self-incompatibility (Taylor et al. 1993). In the updated figure, the S-like RNases still form a separate lineage. The S-RNases also form separate lineages; however, there are now three groups instead of one. These correspond to RNases from three plant families: the Solanaceae, the Rosaceae (apple and pear RNases), and the Scrophulariaceae (snapdragon RNases) (Figure 2-5). In the original figure, the lineage referred to as the S-RNase lineage was made up of only Solanaceous S-RNase sequences, thus forming only one lineage (Taylor et al. 1993). In contrast to this clear separation of proteins from different plant families, the S-like RNase lineage contains RNases from five

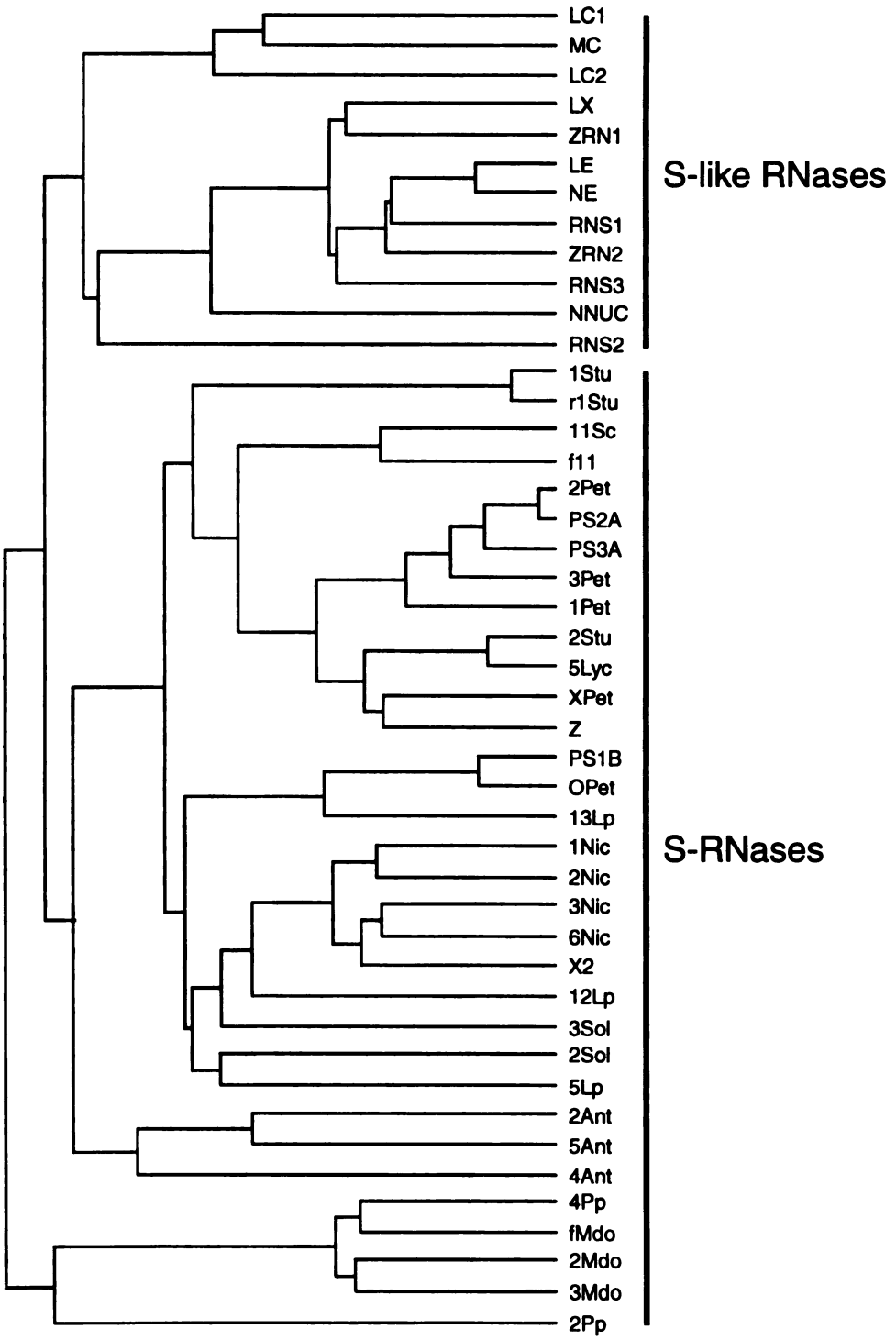


Figure 2-5 - Gene genealogy of S- and S-like RNases. The Genetics Computer Group program PILEUP (Devereux et al. 1984) was used to create the dendogram. Abbreviations of RNases are as described in the legend of Figure 2-4.

separate families, the Cucurbitaceae, Solanaceae, Asteraceae, Brassicaceae and Nelumbonaceae. This grouping is notable 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 the Solanaceous S-like RNases, LE (Jost et al. 1991) and LX (Löffler et al. 1993) of *Lycopersicon esculentum* and NE of *Nicotiana alata* (Dodds et al. 1996a). These RNases are placed on the same branch as S-like RNases from four other plant families and are therefore more closely related to these RNases than to any of the S-RNases isolated from Solanaceous species. The placing of the S-like RNases on a separate branch of the genealogy from those of the S-RNases and the close relationship of the Solanaceous S-like RNases to other non-Solanaceous S-like RNases strongly indicate that these two groups of RNases diverged prior to speciation. It should be noted that the plant S-like RNases form a lineage separate from not only that of the S-RNases, but also from those of other T_2-type proteins from viruses, bacteria, fungi, and animals, although only the plant lineage is shown in Figure 2-5.

As described in Chapter 1, we have since defined placement in this lineage indicated in Figure 2-5 as a requirement for categorization as an S-like RNase. This definition became necessary with the discoveries in self-incompatible plants of RNases very closely related to the S-like RNases, as well as enzymes more closely related structurally to the S-RNases but not playing roles in self-incompatibility.

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 (Figure 2-6). Similar to the S-RNases of the Solanaceae (Cornish et al. 1987), *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 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 results shown in Figure 2-6 demonstrate the *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 (Cornish et al. 1987). 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 (Matile and Winkenbach 1971). RNA was therefore isolated from *Arabidopsis* petals harvested at anthesis [stages 13 and 14 in Smyth et al. 1990] and during senescence [stage 16 in Smyth et al. 1990] and probed for the *RNS2* transcript. A clear increase in *RNS2* expression in senescing petals was observed in these experiments, as shown in Figure

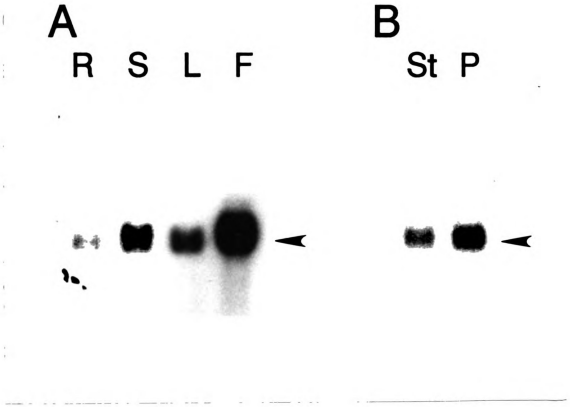


Figure 2-6 - Expression of *RNS2* in different organs of *Arabidopsis*. (A) Samples of 12 μg of total RNA isolated from roots (R), stems (S), leaves (L), and flowers (F) were hybridized to the *RNS2* probe following Northern blotting. (B) Samples of 5 μg of total RNA isolated from style and stigma (St) 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.

These experiments were performed by Crispin Taylor

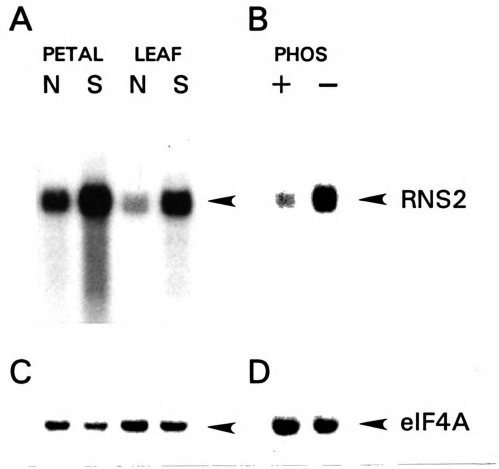


Figure 2-7 - Induction of *RNS2* during senescence and phosphate starvation. (A) Samples of 5 μg of total RNA isolated from nonsenescing (N) and senescing (S) petals and leaves, as indicated above the lanes, were hybridized to the *RNS2* probe following Northern blotting. The *RNS2* transcript is indicated by the arrowhead. (B) Etiolated *Arabidopsis* seedlings were incubated in the presence (+) or absence (-) of phosphate as described in the 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.

These experiments were performed by Crispin Taylor

2-7A. 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 (Figure 2-7A). As a control, the blot shown in Figure 2-7A 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 (Figure 2-7C). 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 (Woodson 1987). This includes the induction of a large number of hydrolytic enzymes (Borochov and Woodson 1989), which are thought to be involved in the recycling of nutrients from the vegetative to the reproductive organs (Kelly and Davies 1988).

A role for RNase LE in phosphate starvation rescue has been suggested because it is secreted from tomato cells following phosphate starvation (Nürnberger et al. 1990; Löffler et al. 1992). 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 hours. As shown in the Northern blot in Figure 2-7B, accumulation of the *RNS2* transcript increases markedly following phosphate limitation, while the level of the *eIF4A* transcript remains constant (Figure 2-7D). Examples of other enzymatic activities induced by phosphate starvation have been described in plants (Duff et al. 1991; Usuda and Shimogawara 1992), but in these cases it is unknown whether regulation is exerted at the mRNA or protein levels.

Ribonuclease activities are known to be higher in diseased plants than in healthy plants (Farkas 1982; Green 1994). To investigate whether an increase in RNS2 activity may contribute to this defense response, *Arabidopsis* plants were inoculated with one of two types of pathogens: avirulent pathogens, which induce the plant defense response termed the hypersensitivity response, or virulent pathogens, which can avoid activating the defense response and thus infect the plant. *Arabidopsis* leaves were infiltrated with *Pseudomonas syringae* pv. *syringae*, which is avirulent in this plant, *P. syringae* pv. *maculicola*, which is virulent in this plant, or buffer. After 48 hours, leaves infiltrated with *P. syringae* pv. *syringae* exhibited classic hypersensitivity response symptoms of necrotic dry lesions with the surrounding tissue appearing healthy. Leaves infected with *P. syringae* pv. *maculicola* had larger, water-soaked lesions and were chlorotic in the surrounding tissue. Control leaves appeared normal. Levels of *RNS2* mRNA appear to increase slightly over control leaves upon inoculation with the *P. syringae* strains (Figure 2-8), 1.7 times control with *P. syringae* pv. *syringae* and 2.7 times control with *P. syringae* pv. *maculicola*. In another experiment, levels of *RNS2* mRNA were examined in *Arabidopsis* leaves infiltrated with similar pathogens and harvested at different time points. *P. syringae* pv. *maculicola* was again used as the virulent strain, but the avirulent strain used was *P. syringae* pv. *tomato*. Leaves were infected with various titers of bacteria and harvested from 6 to 48 hours after infection. *RNS2* was once again induced slightly by attack by pathogens of both strains tested (Figure 2-9), with the maximum level of induction 2-2.4 times control in all cases. The avirulent *P. syringae* pv. *tomato* strain produced the highest levels of *RNS2* mRNA at 24 hours after infection. Maximal levels of *RNS2* mRNA in plants infected with *P.*

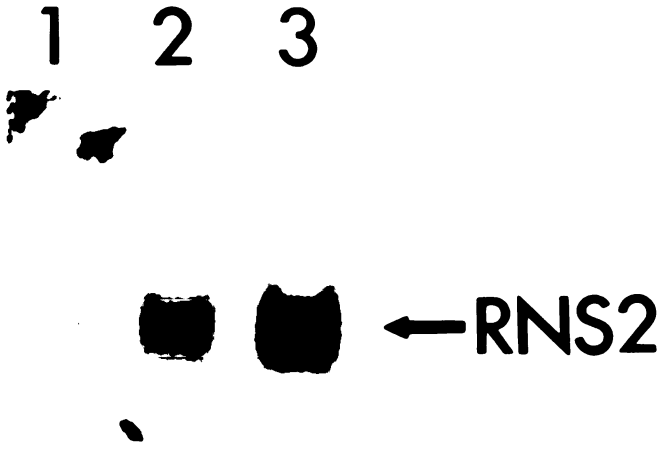


Figure 2-8 - Induction of *RNS2* by pathogens. Leaves of 4-week-old *Arabidopsis* plants were infiltrated with (1) buffer, (2) *Pseudomonas syringae* pv *syringae*, or (3) *P. syringae* pv *maculicola*. After 48 hours infiltrated leaves were harvested and their RNA was isolated. The RNA was subjected to Northern blot analysis and the resulting blot hybridized to the *RNS2* probe as described in Figure 2-6.

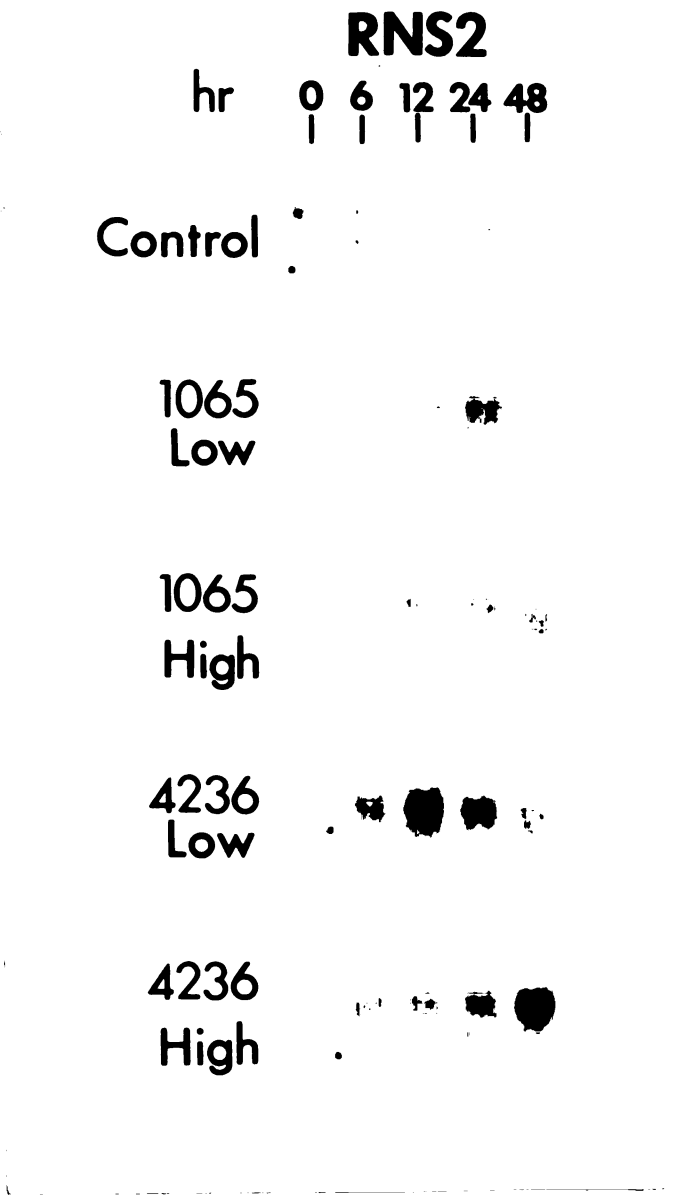


Figure 2-9 - Time course of *RNS2* induction by pathogens. Leaves of 4-week-old *Arabidopsis* plants were infiltrated with buffer (control) or low titer (OD_{600}=0.01) or high titer (OD_{600}=0.1) of *P. syringae* pv *tomato* strain 1065 or *P. syringae* pv *maculicola* strain 4236. Leaves were harvested at the times indicated and their RNA was isolated. The RNA was subjected to Northern blot analysis and the blot hybridized to the *RNS2* probe as described in Figure 2-6.

This experiment was performed by Xinnian Dong

syringae pv. *maculicola* depended on the titer of bacteria infiltrated, with a higher titer of infiltrated cells producing a later peak. Overall, there is a modest but reproducible induction of *RNS2* in *Arabidopsis* by all of the pathogens used in both experiments. The induction seems to appear only after 24 hours, suggesting that RNS2 is not directly induced by a specific bacterial component. It is possible that a peak in *RNS2* induction may have been missed because it occurs very soon after infection, as is true for phenylalanine-ammonia lyase (Dong et al. 1991). Another possibility, taking into account the induction of *RNS2* by senescence seen in Figure 2-7A, is that *RNS2* may be induced in infected leaves solely due to the senescence that accompanies bacterial infection. *RNS2* is induced to a similar level during these pathogen experiments (Figures 2-8 and 2-9) as during senescence (Figure 2-7A), and is higher during infection with the virulent pathogen, which produces more senescence in the affected plant.

Conclusions

RNS2 was the first senescence-associated RNase gene identified in higher plants. Senescence-associated RNase activites have been demonstrated in a number of plant species (Matile and Winkenbach 1971; Blank and McKeon 1991b), but it is not known whether they are encoded by S-like RNase genes. The idea that an RNase can be induced to rescue phosphate from RNA in the plant is supported by the induction of *RNS2* during senescence, phosphate starvation, and pathogen attack, during all of which the plant has a need to make the most efficient use of the resources it has available. Plants often grow under phosphate-

limiting conditions (Fried and Brosehart 1967), so the presence of such an induction pathway is not surprising. These ideas will be examined further in Chapter 3.

MATERIALS AND METHODS

Isolation and Sequencing of *RNS2* cDNAs

The *Arabidopsis* cDNA library in λZAP (Short et al. 1988) that was initially used to detect the *RNS* genes by PCR amplification (Taylor and Green 1991) was used to screen for *RNS2* cDNA clones by plaque hybridization (Sambrook et al. 1989) using the *RNS2* PCR product as a probe. Positive plaques were purified; the cDNA clones were converted into plasmid form (protocol provided by Stratagene) and sequenced (Sanger et al. 1977).

Expression of *RNS2* in *Saccharomyces cerevisiae*

An *RNS2* cDNA covering the entire coding region including the signal sequence was inserted between the yeast *PHO5* promoter and *GADPH* terminator (Rosenberg et al. 1984), and the resulting *PHO5-RNS2-GADPH* gene was then cloned into the yeast shuttle vector pWL (Del Cardayré et al. 1995). Transformants of *Saccharomyces cerevisiae* strain BJ2168 (*MAT***a** *prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52*) harboring these plasmids were grown in minimal dextrose liquid lacking tryptophan and containing 0.2 mM KH_2PO_4 (which induces expression from the *PHO5* promoter) (Thill et al. 1983) or 11 mM KH_2PO_4 (which does not induce the *PHO5* promoter) for two days at 30° C. Five μl of the culture supernatants were assayed by electrophoresis on RNase activity gels (Yen and Green 1991).

.Multiple Sequence Alignment and Gene Genealogy

The deduced amino acid sequences of RNS2, as well as those of RNS1 and RNS3 (see Chapter 3), were aligned with the amino acid sequences of all other S-like RNases known at present and sequences of the majority of S-RNases for which complete sequence data are available (see legend to Figure 2-4 for a list of the sequences aligned). The alignment was performed using the Genetics Computer Group program PILEUP (Devereux et al. 1984) with a gap weight of 3.0 and a gap length weight of 0.1, and adjustments to this alignment were made by eye. The RNase sequences were aligned and numbered from the positions corresponding to the presumptive mature N-termini of the *N. alata* S-RNases (Haring et al. 1990). 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

For organ analyses, *Arabidopsis thaliana* (L.) Heynh. ecotype RLD was grown in a 1:1:1 mixture of sphagnum moss, perlite, and fine vermiculite under conditions of 16 hr light / 8 hr dark with a relative humidity of 50% at 20° C. Roots, stems, leaves, and flowers of 4- to 5-week-old plants were collected. For the senescence experiments, flowers were staged on the basis of morphological characteristics as defined (Smyth et al. 1990). Senescing leaves were those showing visible signs of senescence, including chlorosis at the

leaf margins and wilting. To starve *Arabidopsis* for phosphate, one-week-old etiolated seedlings were removed from solid AGM medium [MS salts (Sigma) at 4.3 g/liter, sucrose at 30 g/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 medium with or without 1.25 mM KH_2PO_4 as described (Tewes et al. 1984) for 12 hr in the dark. For pathogen studies, *Arabidopsis thaliana* plants of ecotype RLD were grown in Metromix medium at 20° C under a 14 hour light / 10 hour dark photoperiod. *Pseudomonas syringae* pv *syringae* strain van Hall (Vincent and Fulbright 1983) and *Pseudomonas syringae* pv *maculicola* strain 4326 (Whalen et al. 1991) were grown overnight in King's B medium (King et al. 1954), washed twice with 10 mM potassium phosphate buffer at pH 6.9, and adjusted to OD_{640}=0.1 in the same buffer. Four-week-old *Arabidopsis* plants with well-expanded rosettes that had not yet bolted or that had bolts less than 2 cm tall were infiltrated with the above *Pseudomonas* strains as described (Tsuji et al. 1991). Infiltrated leaves were harvested after 48 hours. All of the above tissues were frozen in liquid nitrogen immediately upon collection and stored at -80° C before extraction.

Total RNA was isolated essentially as described (Puissant and Houdebine 1990). RNAs were denatured and separated on formaldehyde/agarose gels and transferred to Biotrace HP membrane (Gelman). The blots were hybridized 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. The probe was established to be specific to RNS2 by hybridization to DNA gel blots containing *Arabidopsis* genomic DNAs treated with various restriction

endonucleases, and subsequent comparison to patterns of blots probed with RNS1 and RNS3 fragments. Prehybridization was for five hours at 52° C in a buffer containing 5X SSC, 10X Denhardt's solution (Sambrook et al. 1989), 0.1% SDS, 0.1 M potassium phosphate (pH 6.8), and 100 μg/ml denatured salmon sperm DNA. Hybridization was for 16 hours at 52° C in the same buffer, except that 10 % (w/v) dextran sulfate and 30% (v/v) formamide were added, and the SDS was omitted. For use as an internal standard, an *Arabidopsis* probe for the ubiquitous, highly expressed translation initiation factor eIF4A (Owttrim et al. 1991) 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. Taylor, P. J. Green, unpublished). The probes were prepared using a random-primed labeling kit (Boehringer Mannheim) and hybridized to the membranes at final concentrations of 1 x 10^6 to 2 x 10^6 dpm / ml. The membranes were washed, with the final washes in 0.5X SSC, 0.1% SDS at 65° C, and then exposed to X-Omat AR film (Kodak) with intensifying screens. Radioactive bands were quantitated using a PhosphorImager model 400B.

CHAPTER 3

STUDIES ON *RNS1* and *RNS3*: *RNS1* IS TIGHTLY CONTROLLED IN RESPONSE TO PHOSPHATE STARVATION

Portions of this chapter were published in The Plant Journal:

Bariola PA, Howard CJ, Taylor CB, Verburg MT, Jaglan VD, Green PJ (1994) The *Arabidopsis* ribonuclease gene *RNS1* is tightly controlled in response to phosphate limitation. Plant J 6:673-685

ABSTRACT

Two stimuli that have been associated with nutrient remobilization in plants are phosphate (P_i) starvation and senescence. Little is known about how the nutrient remobilization machinery is induced at the molecular level, but in the case of P_i starvation, ribonucleases are considered to play important roles in the remobilization process. Here we investigate the control of two closely related ribonuclease genes of *Arabidopsis*, *RNS1* and *RNS3*. The *RNS1* gene is sharply induced during starvation for P_i, an effect specific among the major macronutrients, whereas *RNS3* transcript levels remain relatively constant. RNS1 and RNS3 produced in yeast comigrate with *Arabidopsis* ribonuclease activities that exhibit the same induction properties as the transcripts in both wild-type plants and the *pho1* mutant, which is defective in xylem loading of P_i. In contrast to what occurs during P_i starvation, both *RNS1* and *RNS3* are modestly induced during senescence, indicating that the two stimuli could trigger different signal transduction pathways. The characterization of *RNS1*, in particular, provides an important first step towards elucidating the mechanisms by which plants sense and respond to P_i limitation, a prominent condition in many soil types.

INTRODUCTION

Plants often grow under P_i-limiting conditions (Fried and Brosehart 1967). Phosphorus is commonly the most limiting nutrient in soil, after nitrogen (Salisbury and Ross 1992), and in some areas, P_i deficiency is a chronic situation (Sanchez and Uehara 1980). Despite the importance of this nutrient for plant growth, few details are known about the mechanisms by which plants sense and respond to the availability of P_i. It has been suggested that the extracellular rather than the intracellular P_i concentration elicits the induction of RNase activities in tomato (*Lycopersicon esculentum*). This proposal is based on the observation that following the transfer of cultured tomato cells to P_i-deficient medium, excretion of a P_i-starvation-inducible RNase (RNase LE) occurred well before intracellular P_i concentrations dropped (Glund and Goldstein 1993). The RNases induced under these conditions are thought to be part of a P_i starvation rescue system proposed to exist in higher plants (Goldstein et al. 1989). One function of this system would be to liberate P_i from RNA to facilitate its remobilization. In addition to RNase LE, P_i starvation has been reported to induce secretion of both phosphatases and phosphodiesterases in plant cells (Duff et al. 1991; Goldstein et al. 1988; Löffler et al. 1992; Ninomiya et al. 1977; Ueki and Sato 1971). The combined actions of these enzymes could contribute to the release of P_i (Löffler et al. 1992), in a form utilizable by plants.

A number of other plant enzymatic activities have also been reported to be affected by P_i limitation. In maize (*Zea mays*), P_i starvation results in altered activities of several enzymes involved in photosynthetic carbon metabolism (Usuda and Shimogawara 1992).

Studies of P_i starvation in cell cultures of black mustard (*Brassica nigra*) and periwinkle (*Catharanthus roseus*), as well as in tobacco plants, have demonstrated the enhancement or repression of the activities of numerous enzymes involved in respiratory metabolism (Nagano and Ashihara 1993; Paul and Stitt 1993; Theodorou and Plaxton 1993). Some of these findings conflict with each other, perhaps due to differences in culture methods or plant materials. In any case, very little information exists concerning whether regulation of activities affected by P_i starvation is exerted at the mRNA or protein levels in these studies as few of the corresponding genes have been isolated. Prior to this work only one cDNA had been found to be induced by P_i starvation, a tomato cDNA, but its protein product has yet to be identified (Glund and Goldstein 1993).

We have previously reported the identification of S-like RNase genes in *Arabidopsis thaliana*, a self-compatible plant (Taylor and Green 1991). One of these genes, *RNS2*, is induced during senescence and P_i starvation (Taylor et al. 1993) (also see Chapter 2). These observations suggest RNS2 may degrade RNA during senescence so that its P_i can be remobilized to non-senescing organs, as well as participate in P_i starvation rescue. However, it was unclear from this study whether or not induction was the result of a general pathway that could be triggered by starvation for other macronutrients. A fairly high level of *RNS2* expression was observed in the absence of induction, indicating that *RNS2* expression is not entirely dependent on either senescence or P_i starvation (Taylor et al. 1993). In this study we describe the cDNA cloning and expression of two related *RNS* genes: *RNS1*, which is highly inducible by P_i starvation, and *RNS3*, which is relatively insensitive to this stimulus. Among the major macronutrients, *RNS1* was preferentially

induced in response to starvation for P_i; other results indicate that the mechanisms leading to induction by P_i starvation and senescence may differ. The tight control of *RNS1* mRNA and associated RNase activity in response to P_i availability indicate this gene will provide an effective means for elucidating P_i starvation signal transduction pathways in plants.

RESULTS

Features of *RNS1* and *RNS3* Sequences

To isolate *RNS1* and *RNS3* cDNA clones, a Lambda ZAP cDNA library of aerial tissue of *Arabidopsis* (ecotype Columbia) was screened with gene-specific PCR products corresponding to *RNS1* and *RNS3* as described in the Materials and Methods. The sequence strategies for the cDNAs are shown in Figure 3-1, and the deduced amino acid sequences of the longest clones identified are shown in Figure 3-2 (*RNS1*) and Figure 3-3 (*RNS3*). The *RNS1* and *RNS3* cDNAs contain open reading frames of 230 and 222 amino acids, respectively. Both contain putative signal sequences at their N-terminal ends, but no other known targeting sequences, and therefore are likely extracellular proteins. When the putative signal peptide sequences are removed at the sites predicted by statistical analysis (von Heijne 1986), the proteins have deduced molecular weights of 23.0 kDa for RNS1 and 23.3 kDa for RNS3. Neither protein contains potential N-glycosylation sites. The protein sequences of RNS1 and RNS3 are quite similar (Figure 3-4), with 61% identity and 75% similarity between them (Figure 3-5). In comparison, RNS2 (Taylor et al. 1993) is only 37% identical to RNS1 and 36% identical to RNS3. A notable feature of RNS1 is a putative P-loop sequence containing the sequence GINPDGKS between residues 133-141 (Figure 3-4). P-loops, which form ATP or GTP binding sites, have a consensus sequence of GXXXXGK[T/S] (where X refers to any amino acid), although the presence of this sequence is not necessarily confirmation of a nucleotide binding site (reviewed in Saraste et al. 1990).

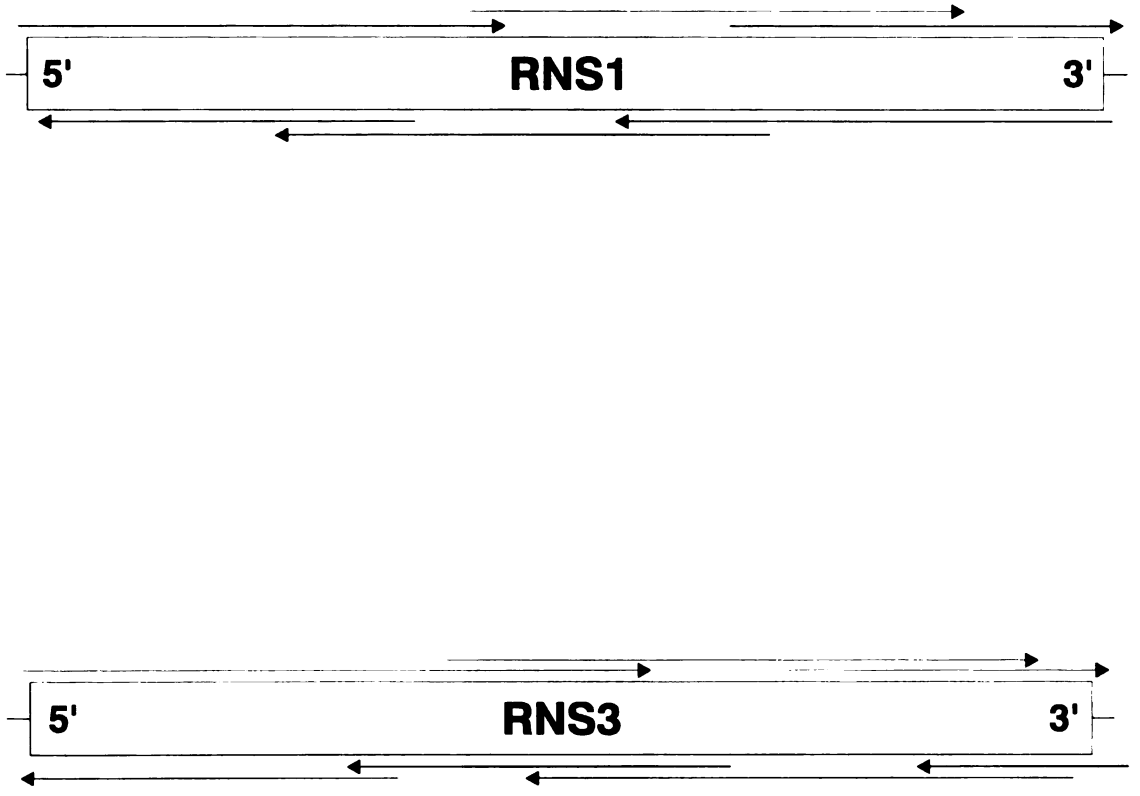


Figure 3-1 - Strategy for sequencing of *RNS1* and *RNS3* cDNAs. The large rectangles represent the *RNS1* and *RNS3* sequences, and arrows represent sequences read from individual primers. Each base of the *RNS1* and *RNS3* cDNAs were read at least once from each direction.

```
  1  ATTTCTCTTTATATATATTCACCCATTAACCATCTCAATCTTATAACCCTCAAAATCACA

 61  ATCTTCTCTTACAAAAAACTTTGAAAGATGAAGATTCTTCTAGCATCATTGTGTTTGATC
  1                              M  K  I  L  L  A  S  L  C  L  I

121  AGTCTTCTCGTAATCTTGCCTTCTGTCTTCTCTGCTTCTTCTTCCTCTGAAGATTTTGAT
 12  S  L  L  V  I  L  P  S  V  F  S  A  S  S  S  S  E  D  F  D

181  TTCTTCTACTTCGTCCAACAATGGCCAGGATCATACTGTGACACACAGAAGAAGTGTTGT
 32  F  F  Y  F  V  Q  Q  W  P  G  S  Y  C  D  T  Q  K  K  C  C

241  TATCCAAATTCAGGCAAACCAGCTGCTGATTTTGGCATTCATGGTCTTTGGCCTAACTAC
 52  Y  P  N  S  G  K  P  A  A  D  F  G  I  H  G  L  W  P  N  Y

301  AAAGATGGAACTTATCCATCTAACTGTGATGCCTCTAAACCATTCGATAGCTCAACGATA
 72  K  D  G  T  Y  P  S  N  C  D  A  S  K  P  F  D  S  S  T  I

361  TCAGATCTTCTCACTTCAATGAAGAAGAGCTGGCCAACACTGGCTTGCCCAAGCGGTTCA
 92  S  D  L  L  T  S  M  K  K  S  W  P  T  L  A  C  P  S  G  S

421  GGTGAAGCGTTTTGGGAGCACGAATGGGAGAAGCATGGTACTTGCTCTGAATCGGTTATC
112  G  E  A  F  W  E  H  E  W  E  K  H  G  T  C  S  E  S  V  I

481  GATCAACATGAATATTTCCAAACCGCTCTTAACCTTAAACAGAAAACCAATCTCCTTGGA
132  D  Q  H  E  Y  F  Q  T  A  L  N  L  K  Q  K  T  N  L  L  G

541  GCTCTAACCAAAGCCGGGATTAATCCGGATGGAAAATCTTACTCTTTGGAGAGCATAAGA
152  A  L  T  K  A  G  I  N  P  D  G  K  S  Y  S  L  E  S  I  R

601  GATTCGATAAAAGAGTCAATTGGTTTCACTCCTTGGGTTGAGTGTAACAGAGATGGTTCT
172  D  S  I  K  E  S  I  G  F  T  P  W  V  E  C  N  R  D  G  S

661  GGTAATAGCCAATTGTACCAAGTCTATCTTTGTGTTGACCGGTCTGGTTCCGGTTTAATC
192  G  N  S  Q  L  Y  Q  V  Y  L  C  V  D  R  S  G  S  G  L  I

721  GAATGTCCGGTTTTCCCACATGGGAAATGTGGAGCTGAGATCGAATTCCCTTCTTTTTAG
212  E  C  P  V  F  P  H  G  K  C  G  A  E  I  E  F  P  S  F  *

781  TTTCCAATTTCTAAATTCAGTCTCTTGAACCGGCATCGATCTTTTGTTCATGGAGATTGG

841  TTTGGTCAAAAAGAAGATTTAATGTAATAAAAATTTATGGGATATTGGATAATGATATAG

901  TTCAATTTCAATAAAAAAAAAAAAA
```

Figure 3-2 - Primary structure and deduced amino acid sequence of *RNS1* cDNA. Deduced amino acid residues are shown in one-letter notation below the nucleotide sequence. The triangle indicates the putative N-terminal end of the mature protein predicted by a statistical analysis (von Heijne 1986). The arrow indicates the polyadenylation site used.

```
   1 CGACAAATTTGATTCCCACAAATTATTTGATATCTTGAGGAAATGAAATTCTTCATTTTT
   1                                              M  K  F  F  I  F

  61 ATTCTAGCGTTACAACAACTCTACGTACAAAGTTTCGCCCAAGATTTCGATTTCTTCTAC
   7 I  L  A  L  Q  Q  L  Y  V  Q  S  F  A  Q  D  F  D  F  F  Y

 121 TTCGTTTTACAGTGGCCTGGAGCGTATTGTGATTCAAGACATAGTTGTTGCTATCCACAA
  27 F  V  L  Q  W  P  G  A  Y  C  D  S  R  H  S  C  C  Y  P  Q

 181 ACCGGTAAACCAGCTGCAGATTTTGGAATTCACGGTCTTTGGCCTAACTACAAAACCGGT
  47 T  G  K  P  A  A  D  F  G  I  H  G  L  W  P  N  Y  K  T  G

 241 GGATGGCCGCAAAATTGTAATCCTGACAGTCGATTTGATGATTTACGGGTTTCTGATCTG
  67 G  W  P  Q  N  C  N  P  D  S  R  F  D  D  L  R  V  S  D  L

 301 ATGAGCGATTTACAAAGAGAATGGCCAACGTTGTCGTGTCCGAGCAATGATGGTATGAAG
  87 M  S  D  L  Q  R  E  W  P  T  L  S  C  P  S  N  D  G  M  K

 361 TTTTGGACACATGAGTGGGAGAAACACGGTACGTGCGCTGAGTCCGAGCTTGACCAACAC
 107 F  W  T  H  E  W  E  K  H  G  T  C  A  E  S  E  L  D  Q  H

 421 GATTACTTCGAAGCTGGTCTCAAGCTCAAACAGAAAGCTAATCTCCTTCATGCCCTTACC
 127 D  Y  F  E  A  G  L  K  L  K  Q  K  A  N  L  L  H  A  L  T

 481 AATGCTGGGATCAAACCGGATGATAAATTTTATGAAATGAAAGATATTGAGAATACGATC
 147 N  A  G  I  K  P  D  D  K  F  Y  E  M  K  D  I  E  N  T  I

 541 AAACAAGTAGTTGGGTTTGCTCCGGGCATTGAATGTAACAAAGATTCGTCACATAATAGC
 167 K  Q  V  V  G  F  A  P  G  I  E  C  N  K  D  S  S  H  N  S

 601 CAACTCTATCAGATTTATTTATGTGTTGACACTTCGGCCTCCAAATTCATCAACTGTCCT
 187 Q  L  Y  Q  I  Y  L  C  V  D  T  S  A  S  K  F  I  N  C  P

 661 GTTATGCCGCATGGTCGATGCGACTCTCGAGTTCAATTTCCCAAGTTCTAAATTTTATGG
 207 V  M  P  H  G  R  C  D  S  R  V  Q  F  P  K  F  *

 721 CTACATTTTTTCTATGTATTTACATTCTACCTATTAATTTTTATTTGTGTTTCTTGCAAA

 781 ATTTAGTTGTAATAAACAGTGTATAATTGGACGATACCCGTGATTTGCATGGTAAAAAGA

 841 TGATAAAGGTTACTAAAAAAAAA
```

Figure 3-3 - Primary structure and deduced amino acid sequence of *RNS3* cDNA. Deduced amino acid residues are shown in one-letter notation below the nucleotide sequence. The triangle indicates the putative N-terminal end of the mature protein predicted by a statistical analysis (von Heijne 1986). Arrows indicate polyadenylation sites used in different cDNA isolates.

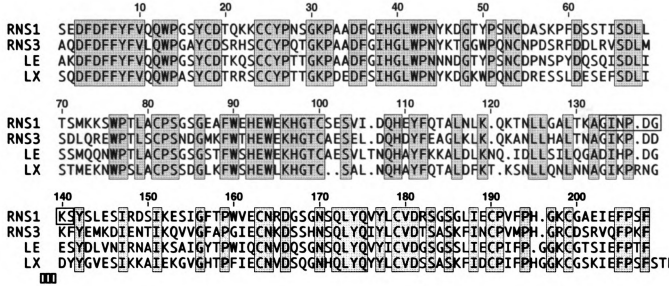


Figure 3-4 - Comparison of deduced amino acid sequences of RNS1 and RNS3 to sequences of RNases LE and LX of tomato. The alignment begins at the first residues of mature RNases LE (Jost et al. 1991) and LX (Löffler et al. 1993), omitting the putative signal sequences of RNS1 and RNS3. Shaded regions highlight residues identical in all four sequences. Gaps (represented by periods) were added where necessary to maximize alignment of similar residues. The putative P-loop sequence is boxed.

	RNS1	RNS2	RNS3	LE	LX
RNS1	-	62	75	79	77
RNS2	37	-	57	56	57
RNS3	61	36	-	75	78
LE	71	34	58	-	78
LX	64	35	62	65	-

Figure 3-5 - Amino acid similarities between the RNS proteins and RNases LE and LX. Sequences were compared using the Genetics Computer Group program GAP (Devereux et al. 1984). Shaded values indicate percent similarity; non-shaded values denote percent identity.

Comparison of RNS1 and RNS3 Protein Sequences With Those of Other S-Like RNases

Inspection of the RNS1 and RNS3 sequences reveals that both proteins are highly conserved members of the T_2/S family of plant ribonucleases. Many residues are conserved in the five regions of major homology (Ioerger et al. 1991), including histidines at positions 39, 92 and 97 and a carboxylic acid residue at position 93 (Figure 3-4), all of which were shown to be necessary for catalysis in RNase Rh of *Rhizopus niveus* (Ohgi et al. 1992; Ohgi et al. 1993). In addition, they contain several conserved cysteines, some of which are involved in disulfide bond formation in another homolog, RNase T_2 of *Aspergillus oryzae* (Kawata et al. 1988). (For comparison to other sequences see Figure 2-4). Also striking is the similarity of RNS1 and RNS3 to RNases LE and LX (Figures 3-4 and 3-5), for which protein and recently cDNA sequences have been determined. RNases LE and LX are induced in suspension-cultured cells of the self-compatible tomato *Lycopersicon esculentum* upon starvation for P_i (Löffler et al. 1992; Nürnberger et al. 1990). When compared on a percentage similarity basis, RNS1 and RNS3 are approximately as similar to RNases LE and LX as they are to each other (Figure 3-5).

The sequences of RNS1 and RNS3 have been added to the updated gene genealogy tree shown in Figure 2-5. As expected, when added to the dendrogram, these sequences fall into the S-like RNase lineage (Figure 2-5). Consistent with the data in Figure 3-5, this analysis demonstrates that RNS1 and RNS3 are more closely related to RNases LE and LX than to RNS2, suggesting that divergence within the S-like RNase lineage, as well as within the plant T2/S family, may have preceded speciation.

Ribonuclease Activity of RNS1 and RNS3

RNS1 and RNS3 have significant similarity to proteins known to have ribonuclease activity. To test whether they encode active RNases, their cDNAs were expressed in *Saccharomyces cerevisiae* using the yeast expression vector pWL (Del Cardayré et al. 1995; Taylor et al. 1993), as done for Figure 2-3. The proteins were targeted for secretion to avoid possible deleterious effects from expressing an RNase intracellularly. Because a heterologous signal sequence has been shown to function inefficiently in yeast (Ohgi et al. 1991), the sequences encoding the putative RNS1 and RNS3 signal peptides were replaced by that of the yeast α-factor protein (Brake et al. 1984). As described in the Materials and Methods, the promoter used in these constructs can be repressed or derepressed depending on the culture conditions. Following growth of yeast transformants under both conditions, the electrophoresis of media samples containing secreted proteins on RNase activity gels (Yen and Green 1991) revealed that RNS1 and RNS3 are RNases capable of degrading bulk RNA (Figure 3-6). As expected, expression levels are high under derepressing conditions and low under repressing conditions. No induction of any yeast RNase activity is visible. The bands of RNS1 and RNS3 activity, at 21.4 and 23.2 kDa, respectively, correspond well with the predicted values of 23.0 and 23.3 kDa for RNS1 and RNS3 taking into account removal of the putative signal peptides according to a statistical analysis (von Heijne 1986). The slight differences may be due to the non-reducing conditions of the RNase activity gels.

RNS1 and RNS3 comigrate with bands in the RNase profile of *Arabidopsis* aerial tissues (Figure 3-6). These bands appear to correspond to those referred to previously as the 22.6 and 23.7 kDa RNases (Yen and Green 1991). RNS2, which appears as a doublet when

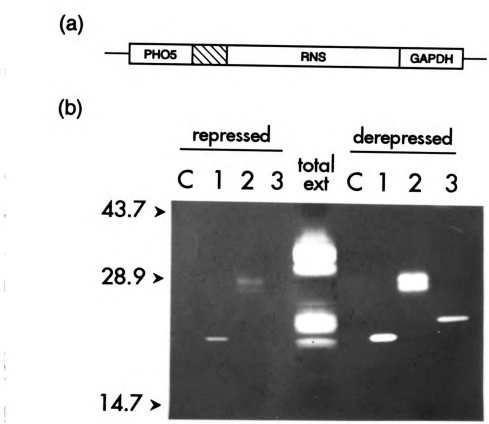


Figure 3-6 - Expression of RNS proteins in *Saccharomyces cerevisiae*.
(a) Schematic representation of yeast expression constructs. *RNS* cDNAs were fused between the yeast *PHO5* promoter and *GAPDH* terminator (Rosenberg et al. 1984) in the plasmid pWL (Del Cardayré et al. 1995) as described in the Materials and Methods. Hatch marks indicate the position of the signal peptide sequence included in each construct to facilitate secretion of the *RNS* gene products.
(b) RNase activity gel of the RNS proteins. Yeast cells containing the *RNS* expression constructs described in (a) were grown in liquid minimal dextrose medium (Thill et al. 1983) low in P_i to induce the *PHO5* promoter ("derepressed"), or medium high in P_i as controls ("repressed"). Samples of culture medium were electrophoresed on RNase activity gels. Lane C, vector control; lane 1, RNS1; lane 2, RNS2; lane 3, RNS3; total ext, protein extract of *Arabidopsis* above-ground tissues. Molecular weights of standards in kDa are shown to the left of the gel.

expressed in yeast (Figures 2-3 and 3-6), does not comigrate with any bands in the profile. This discrepancy may result from differential processing of the RNS2 protein in yeast, via glycosylation (possibly at its potential N-glycosylation sites), removal of the signal peptide, or other modifications that could lead to a molecular weight different from that of the endogenous plant protein. RNS1 and RNS3 have no putative N-glycosylation sites, and modifications other than glycosylation and signal peptide removal have not been reported for other members of the T_2/S RNase family. Therefore, it is likely that the *Arabidopsis* RNases that comigrate with the yeast-expressed proteins are RNS1 and RNS3.

RNS1 and *RNS3* Expression During Normal Development

To examine the expression patterns of *RNS1* and *RNS3* in different organs, blots of RNA isolated from roots, inflorescence stems, leaves, and flowers were hybridized to *RNS1* and *RNS3* probes. As shown in Figure 3-7, the *RNS1* transcript is present primarily in flowers and nearly undetectable in the other organs. In contrast, substantial levels of the *RNS3* transcript are observed in roots, inflorescence stems and flowers, but not in leaves. Both of these patterns differ from that of the *RNS2* transcript, which is present at a significant level in all four organs and most abundant in flowers (Figure 2-6). Although all three *RNS* genes are highly expressed in flowers, these results demonstrate that the genes are differentially controlled in other organs.

It was also of interest to test whether RNS1 and RNS3 might contribute to the well-documented increase in RNase activity reported to take place in senescing plant organs (reviewed in Farkas 1982 and Green 1994). Total RNA was isolated from healthy and

Figure 3-7 - *RNS* expression in organs of soil-grown *Arabidopsis*. Total RNA was isolated from roots (R), inflorescence stems (S), leaves (L), and flowers (F) of *Arabidopsis*. RNA gel blots containing 12 μg of these samples per lane were hybridized with *RNS1* (a) or *RNS3* (b) probes.

senescing *Arabidopsis* leaves at the stages described in Chapter 2. As shown in the gel blots in Figure 3-8, both *RNS1* and *RNS3* are induced during senescence. Although the levels of *RNS1* and *RNS3* transcripts in senescing leaves are modest compared to that of the *RNS2* transcript (Figure 2-7A), the induction of *RNS1* and *RNS3* during senescence is nevertheless reproducible. When the same blot was rehybridized with a probe for the eukaryotic translation factor *eIF4A* from *Arabidopsis* (CB Taylor, PJ Green, unpublished), no induction of the corresponding transcript was observed, indicating that equal amounts of RNA were loaded and that induction of the *RNS* transcripts during senescence cannot be accounted for by a general effect, such as an overall change in the ratio of mRNA to ribosomal RNA in senescing tissues.

RNS1 and *RNS3* Expression in Response to P_i Limitation

As discussed earlier, the S-like RNases induced during senescence may play a role in P_i remobilization. A similar argument can be made for those induced during P_i starvation. Previous reports of increases in RNase activities in tomato (Löffler et al. 1992; Nürnberger et al. 1990) and *RNS2* gene expression in *Arabidopsis* (Chapter 2) during P_i starvation could indicate that induction in response to this stimulus is common to all S-like RNases. To test this hypothesis with respect to *RNS1* and *RNS3*, total RNA was isolated from green seedlings grown on P_i-rich or P_i-deficient media after germination on P_i-rich medium. Gel blots of this RNA were hybridized with probes for *RNS1*, *RNS2*, *RNS3*, and the internal standard *eIF4A*. Figure 3-9 shows that *RNS1* transcript levels are extremely low in plants grown in P_i-rich medium, but are induced to a high level by P_i starvation. In

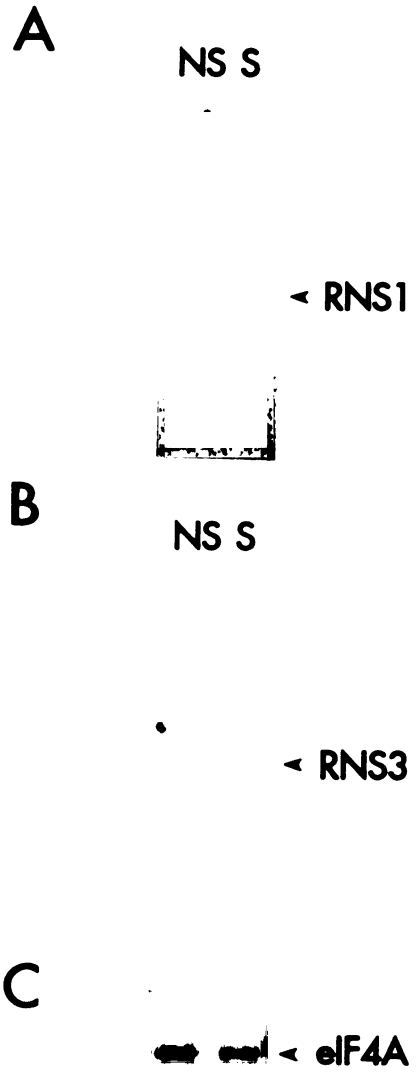


Figure 3-8 - *RNS* expression during senescence in leaves of *Arabidopsis*. Total RNA was isolated from non-senescing (NS) or senescing (S) leaves of *Arabidopsis*. An RNA gel blot with 5 μg of these samples per lane was hybridized sequentially to *RNS1* (a) and *RNS3* (b) probes, as well as to the translation initiation factor *eIF4A* (c) probe as an internal standard.

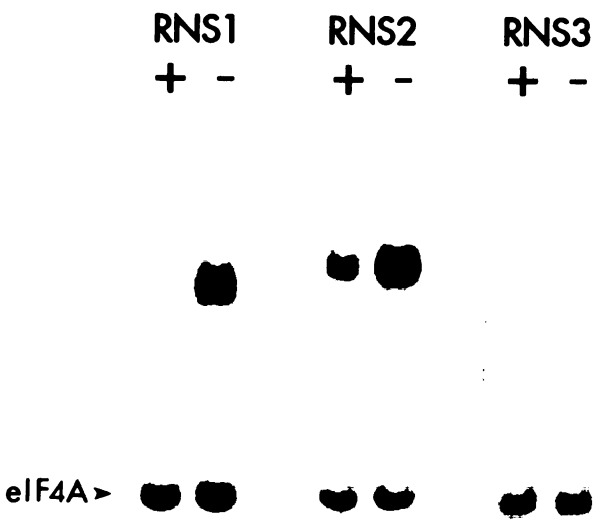


Figure 3-9 - *RNS* expression in plants grown under P_i-rich or P_i-deficient conditions. *Arabidopsis* plants were germinated on AGM medium, transferred three days after germination to media rich (+) or deficient (-) in P_i, and grown for an additional seven days. Total RNA was isolated from these plants, and RNA gel blots containing 10 μg of these samples per lane were hybridized to the *RNS1*, *RNS2*, or *RNS3* probes as indicated, and subsequently to the *eIF4A* probe.

Parts of this experiment were performed by Christie Howard and Crispin Taylor

contrast, *RNS3* transcript levels remain nearly unchanged during P_i starvation. *RNS2*, previously found to be induced in etiolated seedlings starved for P_i, is induced to essentially the same level in green seedlings starved for P_i (compare Figure 3-9 with Figure 2-7B).

Seedlings starved for P_i grow more slowly than those grown on P_i-rich medium, and appear somewhat stunted when compared to normal seedlings of the same age. Accordingly, *RNS1* induction during P_i starvation could be a general nutrient starvation response, not specific to P_i. This possibility was tested by subjecting seedlings to starvation for two other macronutrients, nitrogen and potassium, using methods analogous to the P_i starvation experiments. RNA gel blot analysis of RNA from these plants revealed that unlike P_i starvation, neither nitrogen nor potassium deficiencies lead to any large induction of *RNS1* expression (Figure 3-10). *RNS3* and *eIF4A* transcript accumulation is insensitive to starvation for all three macronutrients (Figure 3-10). The seedling samples used for Figures 3-9 and 3-10 were subjected to seven days of nutrient starvation. The *RNS1* transcript was also induced in seedlings starved for P_i for five and nine days, whereas seedlings starved for nitrogen or potassium for the same periods of time exhibited no change in *RNS1* transcript levels relative to unstarved plants (CJ Howard, PJ Green, unpublished).

Since the potassium starvation media described above contained a small amount of potassium, the effect of media containing no potassium were also tested on *Arabidopsis* seedlings. As shown in Figure 3-11, *RNS1* is induced to a small extent in seedlings grown on medium containing no potassium. The induction is 3.7-fold, as compared to the approximately 75-fold induction by starvation for P_i (Figure 3-11) (figures calculated after

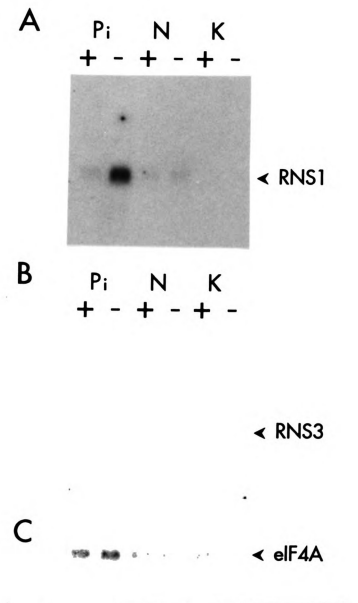


Figure 3-10 - *RNS1* and *RNS3* expression in plants starved for various nutrients. Plants were germinated on AGM medium, transferred three days after germination to media rich (+) or deficient (-) in phosphate (P_i), nitrogen (N), or potassium (K), and grown for seven more days. Total RNA was isolated from these seedlings, and an RNA gel blot containing 10 μg of these samples per lane was hybridized sequentially to the *RNS1* (a), *RNS3* (b), and *eIF4A* (c) probes.

Parts of this experiment were performed by Christie Howard and Crispin Taylor

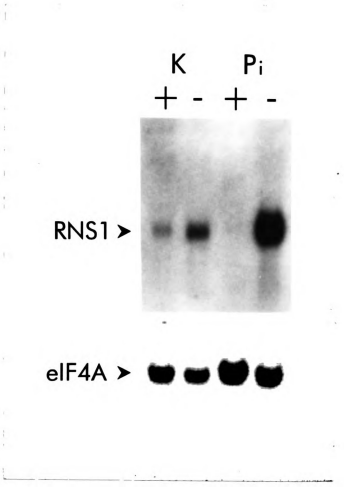


Figure 3-11 - *RNS1* expression in plants grown on media containing no potassium. Plants were germinated on AGM medium, transferred three days after germination to media rich (+) or deficient (-) in phosphate (P_i) or potassium (K), and grown for seven more days. Total RNA was isolated from these seedlings, and an RNA gel blot containing 10 μg of these samples per lane was hybridized sequentially to the *RNS1* and *eIF4A* probes.

Parts of this experiment were performed by Christie Howard

normalization to the signal of the *eIF4A* transcript in each lane). Potassium is a major cation involved in maintaining the cation-anion balance in plant cells (Poirier et al. 1991), therefore one possible explanation for the small induction of *RNS1* during potassium starvation is that lack of potassium allows less phosphate to be taken up into plants.

Poirier and co-workers (Poirier et al. 1991) have isolated an *Arabidopsis* mutant, *pho1*, impaired in delivery of P_i into the xylem after its uptake by root epidermal cells. Leaf P_i levels in this mutant are much lower than in wild type, so its leaves are constantly deprived of P_i. To examine the response of the *RNS* genes to the P_i limitation inherent in this mutant, total RNA was isolated from healthy, non-senescing leaves of the *pho1* mutant and of Columbia wild type. The expression patterns of *RNS1*, *RNS2*, and *RNS3* in wild type and the *pho1* mutant reflect their expression patterns in plants grown on P_i-rich and P_i-deficient media: *RNS1* is highly induced, *RNS2* is moderately induced, and *RNS3* transcript levels are nearly unchanged (Figure 3-12). These results show that exogenously applied and endogenous P_i deficiency result in similar *RNS* gene expression patterns.

RNase Profiles of P_i-starved and *pho1* Plants

RNS1 and RNS3 proteins produced in yeast comigrate with major activities in the *Arabidopsis* RNase profile (Figure 3-6). If the comigrating *Arabidopsis* RNases are indeed RNS1 and RNS3, then they might be expected to exhibit the same responses to P_i starvation observed for the *RNS1* and *RNS3* genes. To investigate this possibility, protein extracts from seedlings grown on P_i-rich and P_i-deficient media were resolved on RNase activity gels. Samples from the P_i-starved plants exhibit a strong band of activity comigrating with

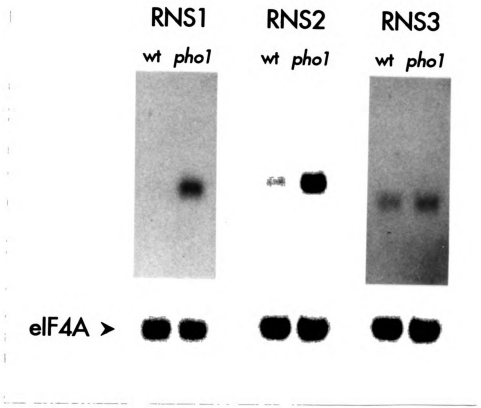


Figure 3-12 - *RNS* expression in the *pho1* mutant of *Arabidopsis*. Columbia wild-type (wt) and *pho1* plants were germinated on AGM medium and transferred to soil ten to twelve days after germination. Total RNA was isolated from leaves of these plants nine to ten days after transfer to soil. RNA gel blots containing 10 μg of these samples per lane were hybridized to the indicated probes as in Figure 3-9. The same blot was hybridized with both the *RNS1* and *RNS3* probes.

Parts of this experiment were performed by Christie Howard and Crispin Taylor

yeast-produced RNS1 (Figure 3-13). Since the band is not visible in samples from plants grown on P_i-rich medium, it likely corresponds to an increase in the amount of RNS1 protein present in P_i-starved plants, coinciding with the increase in *RNS1* mRNA. A similar result was obtained when protein samples from *pho1* mutant plants were examined. These plants, which have increased levels of *RNS1* mRNA (Figure 3-12), exhibit increased activity of the band comigrating with yeast-produced RNS1, as compared to wild type. Changes are less obvious in the area of the RNS3 band in both P_i-starved and *pho1* samples, which was expected because little change in *RNS3* transcript levels is observed in these plants (Figures 3-9 and 3-12). However, it should be noted that there are multiple activities in this region. The apparent increases in activity of the band comigrating with yeast-produced RNS1 in P_i-starved and *pho1* mutant plants support the contention that this RNase is the *RNS1* gene product. These experiments also suggest that induction of *RNS1* could be part of the plant P_i starvation rescue response.

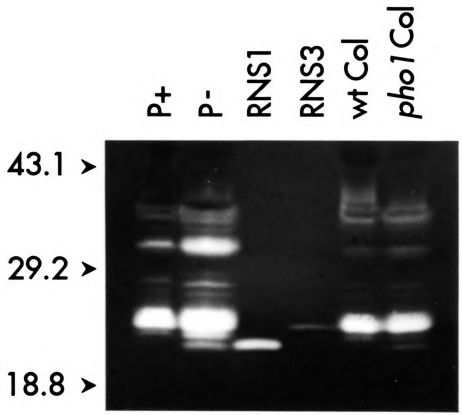


Figure 3-13 - RNase activity profiles of P_i-starved plants and *pho1* mutant plants. Protein extracts were prepared from the same plant materials used for RNA analysis (see the Materials and Methods) and resolved on RNase activity gels. Samples were: protein extracts from seedlings grown on media rich (P+) or deficient (P-) in P_i; RNS1 and RNS3 expressed in yeast; and protein extracts of leaves from Columbia wild-type (wt Col) or *pho1* (*pho1* Col) plants. Molecular weights of standards in kDa are shown to the left of the gel.

Parts of this experiment were performed by Vanita Jaglan and Christie Howard

DISCUSSION

In this study we examined two *Arabidopsis* genes, *RNS1* and *RNS3*, which encode RNases in the T_2/S superfamily, a major family of RNA-degrading enzymes in higher plants. Our results demonstrate that control of *RNS1* differs from that of *RNS3* in non-floral organs and in response to P_i starvation, but is similar during senescence. The marked induction of *RNS1* under P_i-limiting conditions was of particular interest because of its potential for providing insight into P_i starvation-inducible signal transduction mechanisms.

Structural Features of RNS1 and RNS3

Comparison of the amino acid sequences of RNS1 and RNS3 revealed many similarities with other members of the T_2/S RNase family. Most significant was the finding that RNS1 and RNS3 are more closely related to S-like RNases from tomato, zinnia, and tobacco than to any other known RNases, including RNS2 of *Arabidopsis*. This result indicates that the lineages giving rise to the former enzymes and RNS2 (Figure 2-5) diverged before speciation. RNS1 and RNase LE are 71% identical at the amino acid level and both are induced by P_i starvation. Because tomato and *Arabidopsis* are in different families, the high degree of similarity between RNases LE and RNS1 suggests that homologs of these RNases may be fundamental components involved in RNA degradation in diverse plant species.

RNS1 contains a putative P-loop sequence, which constitutes an interesting distinction between RNS1 and all other T_2/S RNases with published sequences. P-loops are

present in several families of proteins that specifically associate with nucleoside triphosphates, such as some ATP synthases, kinases, elongation factors, and myosins (Saraste et al. 1990). In addition, an unrelated RNase, 2-5A-dependent RNase, utilizes P-loops to bind an oligoadenylate activator (Zhou et al. 1993). It is not known how a P-loop could function in RNS1. One possibility is that RNS1 activity is controlled by a nucleotide effector; alternatively, a P-loop could conceivably contribute to an RNA binding domain on RNS1.

Induction of the *RNS* Genes During Senescence and P_i Starvation is Differential and not a General Nutrient Starvation Response

Our analyses have shown that all three *Arabidopsis RNS* genes are induced by senescence, albeit to different extents. The effect of senescence is most apparent in the case of *RNS2*, which is strongly induced in aging flower petals and leaves (Figure 2-7A). Induction of *RNS1* and *RNS3* in senescing leaves of *Arabidopsis* is modest in comparison to *RNS2*, but nevertheless significant.

A second stimulus shown to induce *RNS* genes in *Arabidopsis* is P_i starvation. It was important to examine whether this effect was related to the control of *RNS* genes during senescence, because mineral deficiency often induces senescence-like responses in plants (Noodén 1988). If both P_i starvation and senescence trigger the same signal transduction pathway, then one might expect the hierarchy of control of the three genes to be similar in response to both stimuli. However, this does not appear to be the case. The effect of P_i starvation is large for *RNS1*, which is highly induced from a low basal level, and minimal

for *RNS3*, which is expressed at similar levels with or without the stimulus of P_i starvation. *RNS2* exhibits an intermediate effect; it is clearly induced during P_i starvation, but also displays a fairly high basal level. In contrast, during senescence *RNS1* and *RNS3* are both modestly induced from a low basal level. *RNS2* is highly expressed during senescence, but the induction ratio is modest due to a relatively high basal level. The simplest way to explain the differential effects of senescence and P_i starvation on *RNS* gene expression is that the two stimuli operate via different signal transduction mechanisms, perhaps with some common components. However, alternative models involving multiple pathways for one or both stimuli or gene-specific repressors of a common pathway cannot be excluded.

It was also of interest to investigate whether the effects of P_i starvation on *RNS1* expression were mediated through a general pathway that responds to multiple forms of nutrient deficiency in *Arabidopsis*. To this end, we examined transcript levels following nitrogen and potassium starvation. As shown in Figures 3-10 and 3-11, neither treatment resulted in large induction of the *RNS1* or the *RNS3* transcripts. Induction due to nitrogen deficiency might have been expected because RNA could be viewed as a source of nitrogen as well as P_i. Nevertheless, *RNS1* induction was preferentially induced under P_i starvation conditions relative to the other nutrients tested. This argues that the strong induction of *RNS1* during P_i starvation is not a general nutrient starvation response.

In spite of the importance of P_i as a nutrient for plant growth, very little is known about the molecular components that mediate the response of plants to P_i-limiting conditions. The regulatory properties of *RNS1* indicate that this gene will provide an excellent entry point into elucidation of P_i starvation-inducible signal transduction

pathways. For example, if regulation is controlled by the 5' flanking sequences of *RNS1*, then the *RNS1* promoter may be an effective tool to identify the transcription factors that mediate responses to P_i starvation. Other signal transduction components could be identified genetically through the isolation of mutants that alter *RNS1* expression under P_i-rich or P_i-deficient conditions. Another important advantage of using *RNS1* in these studies is that regulation can be examined at both the mRNA and protein levels. Figure 3-13 indicates that the *RNS1* gene product is likely responsible for the band of RNase activity induced in *pho1* and P_i-starved wild-type plants that comigrates with RNS1 produced in yeast. Although we cannot rule out the possibility that the RNase activity of RNS1 does not change in P_i-starved plants and another RNase the same size as RNS1 is induced under identical conditions, this seems unlikely.

Roles of the *RNS* Gene Products in Higher Plants

The expression properties of the *RNS* genes strongly suggest that the corresponding RNases participate in P_i remobilization in *Arabidopsis*. Induction of all three *RNS* genes during senescence is consistent with previous observations that senescence induces a number of hydrolytic enzymes (Borochov and Woodson 1989). These enzymes presumably degrade macromolecules of dying cells, freeing them for remobilization to reproductive structures (Kelly and Davies 1988). Senescence has been correlated with the induction of RNase and nuclease activities in other plant systems (Farkas 1982), but the genes for these RNases have not been isolated so it is unclear whether regulation occurs at the RNA or protein levels. Another situation in which P_i remobilization could play a key

role is under conditions of P_i limitation, when *RNS1* and *RNS2* are induced. As discussed earlier, plants commonly grow under P_i-limiting conditions, so understanding their responses to this situation is of fundamental importance. It has been proposed, on the basis of the experiments performed primarily with cultured tomato cells, that plants have a P_i starvation rescue system involving not only RNases, but also phosphodiesterases and phosphatases (Goldstein et al. 1989). Presumably, this system would allow plants to establish different priorities for P_i use during conditions of nutrient limitation and abundance.

Beyond their role in P_i remobilization, the *RNS* gene products may also participate in other processes in plants, including cell death and defense against pathogens. Programmed cell death in animal systems is often associated with the induction of nucleolytic activities (Collins and Rivas 1993), and this has also been reported to occur in plants during xylogenesis. When mesophyll cells of *Zinnia elegans* are induced to differentiate into xylem cells, a nuclease and several RNases accumulate (Thelen and Northcote 1989). A recent screen for xylogenesis-associated cDNAs (Ye and Varner 1993) led to isolation of zinnia homologs of RNS1 and RNS3 (Ye and Droste 1996); this may suggest that RNS1 and/or RNS3 participate in xylem maturation in *Arabidopsis*. With respect to defense against pathogens, RNase X2 of *Petunia inflata* has been suggested to participate in plant defense because it is specifically localized to the pistil (Lee et al. 1992). The pistil is a floral structure that is potentially vulnerable to infection but is rarely invaded, perhaps due to the presence of RNases, protease inhibitors, and other proteins that could be deleterious to pathogens (Lee et al. 1992; Atkinson et al. 1993 and references therein).

RNS1, *RNS2*, and *RNS3* could contribute to this effect because all three genes are expressed in flowers, and *RNS2* expression has been further localized to *Arabidopsis* pistils (Chapter 2).

MATERIALS AND METHODS

cDNA Isolation

RNS1 and *RNS3* were first identified via the polymerase chain reaction (PCR) from rescued plasmid DNA of a Lambda ZAP library as previously described (Taylor and Green 1991). The PCR products were then used as hybridization probes to screen the same Lambda ZAP library for full-length cDNAs as described in Chapter 2. cDNAs were sequenced by the dideoxy chain termination method (Sanger et al. 1977). Multiple clones of *RNS1* and *RNS3* were isolated; DNA sequences of the longest *RNS1* and *RNS3* cDNAs identified were deposited into the EMBL, Genbank, and DDBJ databases with accession numbers of U05206 (*RNS1*) and U05207 (*RNS3*). Nucleotide position numbers referred to in this chapter correspond to these sequences.

Expression Analyses

Plant Material: A. thaliana (L.) Heynh. ecotype RLD was grown in soil as described in Chapter 2. For organ analysis, samples of roots, inflorescence stems, leaves, and flowers of four- to five-week-old plants were collected. Senescing leaves were harvested as described in Chapter 2. For nutrient starvation analyses, seeds were sterilized and plated on solid AGM medium (see Chapter 2 for formulation) on a layer of Nitex 300 μm nylon mesh (Tetko Inc., Briarcliff Manor, New York). Plating density was 100-200 seeds per 100 x 25 mm plate. Plants were germinated under conditions of 16 hour light/8 hour dark, at 20^0C. Three days after plating, when radicles had appeared but no shoot growth was yet apparent,

the seedlings were transferred on the nylon mesh to growth medium rich or deficient in phosphate, nitrogen or potassium (see formulations below), allowed to grow for seven days, and then harvested. Seeds of the P_i-uptake mutant *pho1*, mutant line PL9 (Poirier et al. 1991), a derivative of the Columbia ecotype, were kindly provided by Yves Poirier. For examination of *RNS* expression in the *pho1* mutant, seeds of both this line and Columbia wild-type were surface-sterilized and sown on solid AGM medium. After ten to twelve days, seedlings were transferred to soil and grown under conditions for soil-grown plants described in Chapter 2. Nine to ten days after transfer, healthy leaves of each line were harvested. All plant tissue was frozen immediately in liquid nitrogen and stored at -80^{0}C until use.

Media Formulations for Nutrient Starvation Experiments: The composition of media for the nutrient starvation experiments was as described for AGM medium (Chapter 2) with several changes. The minimal organics salts of Linsmaier and Skoog (Linsmaier and Skoog 1965) were substituted for the MS salts, with the following modifications: In all cases, $CuSO_4$ and $CoCl_2$ were omitted and the $FeSO_4 \times 7H_2O$ concentration was increased to 0.05 g l^{-1}, as recommended by Tewes et al. (1984). In P_i-deficient medium, the KH_2PO_4 was omitted. In both N-rich and N-deficient media, 1.4 g l^{-1} KCl was substituted for the KNO_3. N-rich medium also contained 1.7 g l^{-1} $(NH_4)NO_3$. In both K-rich and K-deficient media, 1.6 g l^{-1} $NaNO_3$ was substituted for the KNO_3. K-rich medium also contained 1.4 g l^{-1} KCl. In K-deficient medium, 0.17 g l^{-1} NaH_2PO_4 was substituted for the KH_2PO_4. Medium with no potassium was the same as K-deficient medium except MES was omitted and the molar

equivalent amount of NaI was substituted for the KI. The pH of all media was adjusted to 5.7, using KOH for media rich and deficient in P_i and N, and NaOH for K-rich and K-deficient media.

RNA Manipulation: Total RNA was isolated and Northern blots were prepared and probed as described in Chapter 2. The *RNS1* probe used was a 0.76 kb EcoRI fragment from the 5' end of the cDNA, including 87 bp of the 5' untranslated region and 673 bp of the coding region. *RNS1* and *RNS3* probes were prepared using ^{32}P-labeled dCTP and a random-primed labeling kit (Boehringer Mannheim). For the *RNS3* probe, a 0.66 kb EcoRV-XhoI fragment from the 5' end of the cDNA, consisting of 14 bp of the 5' untranslated region and 643 bp of the coding region, was used. On genomic DNA blots, both probes showed the same hybridization patterns as the gene-specific *RNS1* and *RNS3* PCR probes (Taylor and Green 1991, and unpublished results). As an internal standard, the blots were hybridized with a probe for the *Arabidopsis* translation initiation factor *eIF4A* (CB Taylor, PJ Green, unpublished). For sequential hybridizations, blots were stripped between hybridizations in a solution of 0.1X SSC, 0.1% SDS that was brought to greater than 90^{0}C and shaken for 20 min at room temperature. Blots were checked for residual activity using Phosphorimager analysis before rehybridization.

Expression of the *RNS* cDNAs in Yeast

RNS1 and *RNS3* cDNAs were inserted into the yeast expression vector pWL (Del Cardayré et al. 1995) under the control of the inducible *PHO5* promoter (Arima et al. 1983;

Vogel and Hinnen 1990) and the *GAPDH* terminator (Rosenberg et al. 1984). Sequences encoding the putative signal peptides of RNS1 and RNS3 upstream of nucleotide positions 135 and 84, respectively, were replaced by the signal peptide of the yeast α-factor signal sequence (Brake et al. 1984). *Saccharomyces cerevisiae* cells transformed with these constructs via the lithium acetate method (Ito et al. 1983) were grown in liquid minimal dextrose high-P_i (repressing conditions) and low-P_i (derepressing conditions) media (Thill et al. 1983) as described in Chapter 2. RNase activities secreted into the culture medium were then assayed as described below.

RNase activity gels

RNase activity gels were electrophoresed and developed as described (Yen and Green 1991). Plant material was grown, harvested and stored in the same manner as for RNA analyses, and protein extracts were prepared essentially as described (Yen and Green 1991), except that the concentrated extraction buffer consisted of 250 mM $NaPO_4$ pH 7.4, 5 mM EDTA, 4 mM PMSF, 25 μg ml^{-1} leupeptin, and 25 μg ml^{-1} antipain. *Arabidopsis* above-ground tissues consisted of all tissues growing above the soil. Each lane contained 100 μg of *Arabidopsis* protein samples. For yeast expression studies, cultures of cells expressing a given *RNS* gene or control were centrifuged for two minutes at 14,000 *g*, and the supernatants were harvested. Supernatant samples of 5 μl for RNS1 and 10 μl for RNS2, RNS3, and control were then resolved on RNase activity gels.

CHAPTER 4

IMMUNOLOCALIZATION OF RNS2

ABSTRACT

As discussed in Chapter 2, RNS2 is a highly abundant RNase whose gene is induced by both senescence and P_i starvation. The deduced amino acid sequence of RNS2 contains a C-terminal extension that has some features in common with plant C-terminal vacuolar targeting signals. To examine whether or not RNS2 is vacuolar, antibodies that recognize RNS2 were produced for use in determining its subcellular location. Generated against a peptide corresponding to a region unique to RNS2, the antibodies recognize the protein specifically. Use of the antibodies in immunoblots showed that RNS2 is present in all major organs of the plant, and increases in abundance during P_i starvation, as predicted by RNA gel blot analysis (Chapter 2). Immunogold electron microscopy of leaf and petal sections using these antibodies revealed that RNS2 is present in the cell wall. This extracellular location is consistent with a role for RNS2 in remobilization of P_i from RNA released from lysed cells or RNA stored as reserves in seeds, as well as in defense against pathogens.

INTRODUCTION

As described in Chapter 2, the deduced amino acid sequence of RNS2 contains, in addition to an N-terminal secretory signal sequence, a C-terminal extension of about 20 amino acids as compared to the C-terminal ends of other S- and S-like RNases (Figure 2-4). When coupled with an N-terminal secretory signal sequences, some C-terminal extensions target plant proteins to the vacuole (Bednarek and Raikhel 1992). At the onset of the experiments described in this chapter, no consensus sequence had been deduced for plant C-terminal vacuolar targeting sequences, but a common feature among those known was short stretches of hydrophobic amino acids, and it was therefore thought that this feature could form the core of the signal recognized by the vacuolar sorting system in plants (Bednarek and Raikhel 1992). The RNS2 C-terminal extension has such stretches of hydrophobic amino acids (Figure 2-4), and it was thus proposed that RNS2 could be a vacuolar protein. This idea seemed plausible, since plant vacuoles contain large amounts of RNase activity (Boller and Kende 1979; Abel and Glund 1987). More recently, two C-terminal vacuolar targeting sequences from plant proteins were analyzed by mutagenesis, and the hydrophobic stretches present in both were found not to be required for proper targeting (Dombrowski et al. 1993; Neuhaus et al. 1994). Since these reports, progress has been slow in determining whether any consensus sequence or structure is necessary for vacuolar targeting by C-terminal extensions (Neuhaus 1996).

One of the major goals of this thesis project was to elucidate roles of the RNS proteins in plants. Since determining the subcellular location of a protein can aid greatly in

deducing its biological role, it was decided that determining the subcellular location of RNS2 was essential. To do this, antibodies recognizing RNS2 were needed, so the initial efforts for this project were to obtain antisera specific for RNS2.

RESULTS

Production of Antibodies that Specifically Recognize RNS2

In order to determine the subcellular location of RNS2, antibodies that specifically recognized this protein were necessary. One method to obtain antibodies specific to one protein out of a family of closely related proteins is to use as antigens synthetic peptides with regions unique to one protein. In the initial attempt to obtain such antibodies for RNS2, three peptide regions were chosen from RNS2 based on their uniqueness in comparison to the protein sequences of RNS1 and RNS3. Peptides SP616 and SP618 correspond to internal sequences of RNS2, whereas JK374 encompasses its C-terminal extension (Figure 4-1). These peptides (Figure 4-1) were synthesized with a lysine residue added at their N-terminal ends to facilitate coupling to carrier proteins. After coupling to keyhole limpet hemocyanin, the peptides were injected into rabbits for antibody production. Sera were tested for reactivity against the antigens using dot blots containing samples of the peptides.

Injection of peptides SP616, SP618 and JK374 led to the production of antibodies that reacted specifically with each peptide. Unfortunately, the antibodies that recognized peptides SP616 and SP618 on dot blots did not recognize either RNS2 produced heterologously in yeast (see Chapter 2) or RNS2 in *Arabidopsis* extracts (data not shown). Antibodies generated against peptide JK374 recognized yeast-produced RNS2, but reacted with no proteins in *Arabidopsis* extracts (data not shown). The latter observation raises the possibility that the C-terminal extension of RNS2 is removed *in vivo*, but this idea was not

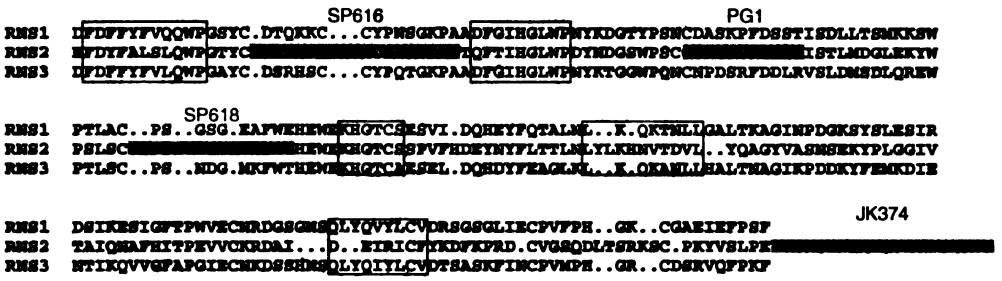


Figure 4-1 - Synthetic peptides used for producing anti-RNS2 antibodies. Deduced amino acid sequences of RNS1, RNS2 and RNS3 are aligned, beginning with the first residues shown in Figure 2-2. Peptides are shaded and their designations indicated above the sequences. Boxes highlight the conserved regions described in Figure 2-2.

explored further. The failure of antibodies generated against synthetic peptides to recognize the whole protein is not uncommon, as the conformation of the free peptide or peptide-carrier protein conjugate may be different than that of the corresponding region in the protein (Harlow and Lane 1988).

After the above efforts, an attempt was made to generate antibodies against heterologously-produced RNS2, in the hopes that the structure of the RNS2 protein is different enough from RNS1 and RNS3 so that specific antibodies would be possible. The system chosen to produce RNS2 in this case was *E. coli*, utilizing pET-based vectors that added a 6X-histidine tail to RNS2 (see Materials and Methods), for ease in the purification of large amounts of protein. In this system, RNS2 formed preferentially an insoluble, inactive form; however, the protein could be solubilized in 6 M urea and purified on a nickel column. After elution from the column, RNS2 was further purified by SDS-PAGE and electroelution. Injection of this RNS2 preparation into rabbits did not lead to the production of anti-RNS2 antibodies. This failure was probably due to the presence in the RNS2 preparation of some component that inhibited strong emulsion formation with the adjuvant. A poorly-formed emulsion fails to protect the antigen from rapid catabolism, leading to a shortened time of exposure of the antigen to the immune system and thus possibly a poor immune response (Harlow and Lane 1988). The emulsion-inhibiting component could not be removed from the preparation by passing it through a desalting column.

Finally, another attempt was made to produce specific anti-RNS2 antibodies using a peptide antigen. The sequence corresponding to peptide PG1 was chosen using the criteria

of high hydrophilicity, increasing the chances that the sequence appears on the surface of the protein (Harlow and Lane 1988), and uniqueness in relation to RNS1 and RNS3. In addition, the peptide was chosen such that it began with a cysteine, which had two advantages: it can be used in coupling to carrier proteins, and this cysteine in particular is part of the true sequence of the peptide, potentially increasing accuracy in the antibody formation. At the time of synthesis this peptide sequence was unique in the Genbank database. Injection of PG1 coupled to keyhole limpet hemocyanin into rabbits led to the production of sera rich in antibodies that strongly recognize RNS2, both yeast-produced and in *Arabidopsis* extracts (Figure 4-2). These antibodies do not recognize RNS1 or RNS3 (Figure 4-2), and pre-immune serum from the same rabbit exhibits very low reactivity with proteins in *Arabidopsis* extracts (data not shown). The anti-RNS2 antibodies detect a single band of approximately 32 kDa in *Arabidopsis* protein extracts (Figure 4-2). This size corresponds well with the predicted molecular weight of RNS2 of 27.2 kDa, given removal of the putative secretion signal sequence and glycosylation at one or both of the potential N-glycosylation sites (see Chapter 2). Since extensive efforts were made using PCR to identify any additional RNases of the RNS family in *Arabidopsis* (data not shown), and, as of November 1996, no other RNS-like sequences have appeared in the Genbank database as a result of the large-scale *Arabidopsis* cDNA sequencing projects, it is reasonably certain that RNS1, RNS2 and RNS3 comprise the entire gene family of this type of RNase in *Arabidopsis*. Therefore, it is likely that these antibodies recognize only RNS2.

Figure 4-2 - Immunoblot characterization of anti-RNS2 antibodies. Proteins were resolved by SDS-PAGE, transferred to PVDF membrane, and immunodetected with anti-RNS2 serum. RNS1, RNS2, RNS3: approximately 300 ng each of the indicated RNase, in supernatant from RNase-expressing yeast cells (see Chapter 3). Arab: 50 μg of proteins extracted from above-ground tissues of five-week old *Arabidopsis* Columbia wild-type plants.

Patterns of RNS2 Expression

Protein extracts were made from several organs of *Arabidopsis* to determine where in the plant RNS2 is present. Immunoblot analysis showed that RNS2 is present in all major organs of the plant (Figure 4-3A). RNS2 is abundant in roots, stems, leaves and flowers, as predicted by analysis of *RNS2* mRNA levels in Chapter 2 (Figure 2-6). Among these organs, it appears that RNS2 is most abundant in roots as a percentage of total protein in each extract (Figure 4-3A), which conflicts with the relative *RNS2* abundance seen at the mRNA level (Figure 2-6). However, it is known that a large percentage of the proteins in green tissues is RuBisCo protein, for example up to 50% of the soluble protein in leaves (Kung 1976) (note the large bands of about 55 and 15 kDa in leaf, stem and flower extracts in Figure 4-3B). Roots contain no chloroplasts and therefore no RuBisCo protein. When comparisons are made using equal protein amounts, in extracts of green tissues such a large percentage of the proteins are RuBisCo that a smaller proportion of all other proteins can be loaded in relation to the proportion in root extracts. Thus it may appear that RNS2 is more abundant in roots, whereas the relative amounts of RNS2 per cell may be very different from the relative amounts in total soluble protein of different organs. In addition to the above organs, RNS2 is also present in significant amounts in extracts of siliques and seeds. This observation indicates that RNS2 may have a role in the reproductive process.

RNS2 abundance was examined in protein extracts from seedlings grown on media rich or deficient in P_i. As shown in Figure 4-4A, RNS2 is more abundant in P_i-deprived seedlings. This confirms the assumption made in Chapter 2, that induction of the *RNS2* gene during P_i starvation leads to increased accumulation of RNS2 protein in *Arabidopsis*.

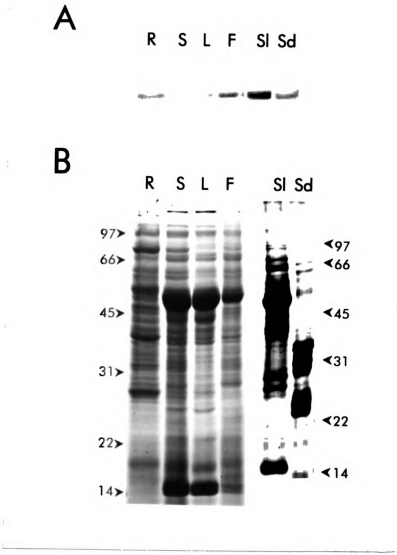


Figure 4-3 - Distribution of RNS2 among various organs of *Arabidopsis*. Protein extracts (30 μg per lane) were made from organs of four- to five-week-old Columbia wild-type plants and resolved by SDS-PAGE. (A) Protein extracts transferred to PVDF membrane and immunodetected with anti-RNS2 serum. R, roots; S, stems; L, leaves; F, flowers; Sl, siliques; Sd, seeds. (B) Protein extracts visualized with Coomassie staining. Molecular weights of standards in kDa are shown to the sides of the gels.

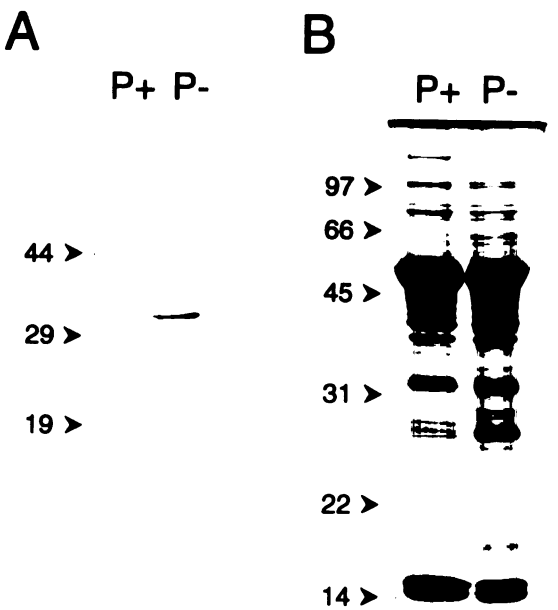


Figure 4-4 - Increase in RNS2 abundance during phosphate starvation. Protein extracts (25 µg per lane) were made from seedlings grown on media rich (P+) or deficient (P-) in P_i and resolved on SDS-PAGE. (A) Protein extracts transferred to PVDF membrane and immunodetected with anti-RNS2 serum. (B) Protein extracts visualized with Coomassie staining. For both A and B, molecular weights of standards in kDa are shown to the left of the gels.

Extensive efforts were made to determine the relative abundance of RNS2 in senescing and non-senescing leaves, analogous to the experiments done at the mRNA level in Figure 2-7A. No conclusions could be made since, during senescence, proteins are being rapidy degraded (Noodén 1988b), which made accurate protein quantitation and thus equal loading of proteins very difficult (data not shown).

The anti-RNS2 antibodies generated using peptide PG1 as an antigen recognize a protein of approximately the same size as the predicted RNS2 protein, which increases in abundance during P_i starvation, as does *RNS2* mRNA. These antibodies do not recognize RNS1 or RNS3, and were produced in high titer. For these reasons it was decided that they were of sufficient quality to use in immunogold electron microscopy to determine the subcellular location of RNS2.

Immunolocalization of RNS2

The anti-RNS2 antibodies described earlier were used in immunogold labeling at the ultrastructural level to determine the subcellular location of RNS2. In the initial attempts, *Arabidopsis* tissues were fixed with a typical fixation mixture of glutaraldehyde and formaldehyde (see Materials and Methods). Treatment of these sections with anti-RNS2 antibodies produced very little labeling. This problem was traced to the use of glutaraldehyde as a fixative. Glutaraldehyde reacts with free amino groups (Harlow and Lane 1988). Since the PG1 antigen, against which the anti-RNS2 antibodies were generated, contains two lysine residues, treatment of the *Arabidopsis* tissues with glutaraldehyde likely resulted in the modification of the PG1 epitope(s) on RNS2 such that

the antibodies were unable to bind to the protein. Glutaraldehyde also effectively masked RNS2 detection on immunoblots (data not shown).

To overcome this problem, *Arabidopsis* tissues were treated with a fixation mixture containing formaldehyde. Omission of the glutaraldehyde at this step allowed labeling of RNS2 as well as good preservation of cell structures. Extensive labeling of RNS2 was observed in leaf tissues (Figures 5A and 5B). Unexpectedly, RNS2 labeling appeared in cell walls. Treatment of leaf tissues with pre-immune serum resulted in a low background level of labeling; however, a small amount of labeling, much less extensive than that seen with anti-RNS2 serum, was seen in cell walls (Figures 5C and 5D). RNS2 labeling was also observed in petal tissue (Figure 6A and 6B), although not as strongly as in leaf tissue. Again, the labeling is specific, as treatment with pre-immune serum results in very little labeling (Figures 6C and 6D).

The appearance of RNS2 in cell walls in electron micrographs of leaf and petal tissues does not imply that RNS2 is a cell-wall intrinsic protein, since small extracellular proteins are able to move freely within the porous structure of the plant cell wall. Since RNS2 is found in substantial quantities in the soluble fraction of crude plant extracts, it is likely that this protein is a free-floating extracellular protein. Experiments are underway to confirm by cell fractionation studies the extracellular localization of RNS2 by electron microscopy.

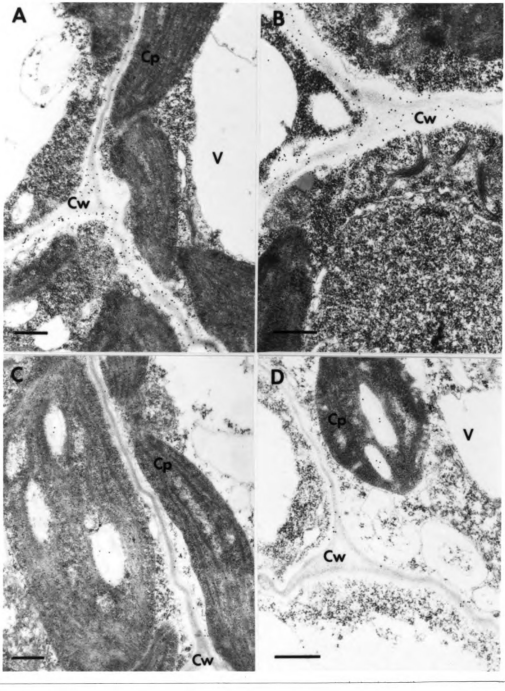


Figure 4-5 - Immunocytochemical localization of RNS2 in leaves of *Arabidopsis* using immunogold labeling. Sections of leaf cells were treated with (A & B) anti-RNS2 antibodies or (C & D) pre-immune serum. Cw, cell wall; V, vacuole; Cp, chloroplast. Bars = 0.5 μm.

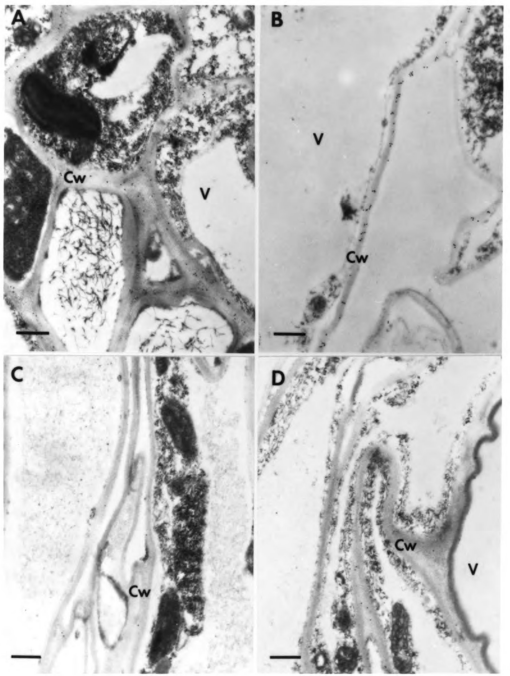


Figure 4-6 - Immunocytochemical localization of RNS2 in petals of *Arabidopsis* flowers using immunogold labeling. Sections of petal cells were treated with (A & B) anti-RNS2 antibodies or (C & D) pre-immune serum. Cw, cell wall; V, vacuole. Bars = 0.5 μm.

DISCUSSION

A surprising finding in determining the subcellular location of RNS2 was that RNS2 appears to be an extracellular protein. Although unexpected, this result is not inconsistent with data from other T_2/S type proteins in plants. For example, the S-RNase S_2 of *Nicotiana alata* has been localized to the intercellular matrix and cell wall in pistils using methods like those described in this chapter (Anderson et al. 1989), and the S-like RNase LE of tomato was purified from culture medium after secretion from suspension-cultured cells (Nürnberger et al. 1990).

The plant cell wall contains numerous species of hydrolytic enzymes under normal conditions, among them proteases, glycosidases, and phosphatases (Cassab and Varner 1988), as well as some types of ribosome-inactivating proteins (Barbieri et al. 1993). Roles in defense against pathogens have been proposed for many of the above enzymes (Cassab and Varner 1988), and proven in some cases (Alexander et al. 1994). Since the cell wall is one of the first barriers pathogens encounter upon infection of a plant, a cell wall location for defense-related enzymes seems appropriate. Bolstering this idea is the observation that several types of proteins that are specifically upregulated during defense responses are extracellular proteins (Alexander et al. 1994).

Following this logic, since RNS2 is a cell-wall localized hydrolytic enzyme, it is possible that it participates in the defense response of plants against pathogens. A direct role in attack on pathogens seems difficult to reconcile, as the protein would be separated from substrate RNAs by a physical barrier. However, RNS2 could act in concert with other

defense-related enzymes. For example, β-1,3-glucanases, which attack fungal cell walls, may initiate fungal cell lysis, after which RNS2 may degrade fungal RNA. Indeed, the cytotoxicities of a barley ribosome-inactivating protein, a chitinase and a β-1,3-glucanase against fungal hyphae are synergistically enhanced when their actions are combined (Leah et al. 1991). Alternately, RNS2 could aid in the protection of plants against infection by RNA-based viruses, although a mechanism by which RNS2 could gain access to the viral RNA is obscure. It should be noted that the S-RNase S_2 of *Nicotiana alata*, which is known to be a secreted protein (Anderson et al. 1989), is taken up into the cytoplasm of pollen tubes when applied *in vitro* (Gray et al. 1991). The exact mechanism by which S-RNases contribute to self-incompatibility is unknown, but one current model incorporates the idea of allele-specific uptake of S-RNases into the pollen tube cytoplasm (Kao and McCubbin 1996). Although the idea that RNS2 could be taken up into cells of plant pathogens seems unlikely, it cannot be ruled out, due to the lack of knowledge about the target(s) of action of most T_2/S type RNases.

As described above, it is possible that RNS2 may be a defense-related enzyme, but the observation that the *RNS2* gene is not induced strongly by attack with some pathogens (Figures 2-8 and 2-9) suggest that RNS2 may have alternate or additional roles. The RNS2 protein is relatively abundant in all organs examined (Figure 4-3), and increases in abundance during P_i starvation (Figure 4-4). In addition, the *RNS2* gene is induced during senescence (Figure 2-7). As discussed in Chapter 2, these results are consistent with a role for RNS2 in the recovery of phosphate from RNA. However, since RNA is not normally present extracellularly, this idea seems unusual. Interestingly, acid phosphatase is the most

abundant cell wall hydrolase in some tissues (Lamport and Catt 1981), and, like RNA, the substrates for acid phosphatases are not usually found outside of cells (Cassab and Varner 1988). As plants often grow under nutrient-limiting conditions, they may maintain a hydrolytic extracellular environment to maximize recycling of cellular components released during such processes as programmed cell death (see Chapter 1) and wounding. Another role is suggested by the presence of RNS2 in dry seeds (Figure 4-3). The RNase may participate in the mobilization of nucleotides and phosphate upon germination from RNA stored in the endosperm.

In conclusion, RNS2 is an abundant extracellular protein that may have multiple roles in plants. To attempt to confirm some of these ideas, it will be necessary to examine transgenic plants with decreased RNS2 levels. Progress toward these experiments is described in Chapter 5.

MATERIALS AND METHODS

Anti-RNS2 Antibody Production

Antigen Preparation: Synthetic peptides SP616, SP618 and JK374 were prepared at the W. M. Keck Facility at Yale University. Synthetic peptide PG1 was prepared at the Michigan State University Department of Biochemistry Macromolecular Structure Facility. The purity of peptides was monitored by analytical reversed-phase HPLC and mass spectrometry. Peptide sequences were as follows: SP616, KRGTRHCCSKNACCRGSDAP; SP618, KGSPSSCNGGKGSFWG; JK374, KYTPLDGEAMVLKMPTEREAL; PG1, CYRSDFKEKE. Peptides SP616, SP618 and JK374 were coupled to keyhole limpet hemocyanin (KLH) (Pierce) using the crosslinkers disuccinimidyl suberate (DSS) and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Pierce), in separate reactions. Peptide PG1 was coupled to KLH using maleimide-activated KLH (Pierce).

Heterologously-produced RNS2 was made using the *E. coli* pET system. The *RNS2* cDNA was altered via site-directed mutagenesis (Kunkel et al. 1987) to create an Nco I site just after the putative signal sequence (see Chapter 2) and an Xho I site at the 3' end of the coding sequence. The mutagenized cDNA was inserted into the pET-22b+ vector (Novagen) between the corresponding sites. The resulting gene encodes a fusion protein with a pelB leader and a 6X-histidine tail. *E. coli* strain BL21 (DE3) (Novagen) containing the above plasmid was grown in Luria broth containing 100 mg/l carbenicillin until mid-log phase, at which point IPTG was added to a final concentration of 1 mM. After two hours,

cells were collected by centrifugation. Growth at either 37° C or 28° C resulted in RNS2 forming in insoluble inclusion bodies (data not shown). To purify RNS2, the cells were lysed in a solution of 10 mg/ml lysozyme, 1% Triton X-100, 50 mM Tris pH 7.5, 2 mM EDTA at 30° C for 30 minutes, followed by passing the lysate repeatedly through a 22-gauge needle and centrifuging at 20,000 g. The resulting pellet was solubilized in 6 M urea, 5 mM imidazole, 0.5 M NaCl, 20 mM Tris pH 7.9 and purified on a nickel column (His-Bind Resin; Novagen) according to manufacturer's instructions. Eluted RNS2 was further purified by separation on SDS-PAGE, visualization with $CuCl_2$ (Lee et al. 1987), isolation of the band corresponding to RNS2, and electroelution. The purity of the protein was monitored by SDS-PAGE and silver staining (Bio-Rad Silver Stain kit). The protein was desalted on a PD-10 column (Pharmacia), concentrated, and the buffer replaced with phosphate-buffered saline (PBS) (Sambrook et al. 1989) before injection.

Anti-peptide antibodies were isolated from whole sera via affinity purification on Affi-Gel 10 or Affi-Gel 15 columns (Bio-Rad), depending on the pI of the peptide. Peptides were coupled to the support in DMSO as per the manufacturer's suggestions, with 7.5-20 μmol peptide per ml of resin. Anti-peptide antibodies were bound to and eluted from the affinity column as described (Harlow and Lane 1988). After elution, antibodies were concentrated in Centricon-10 units (Amicon) to the same volume as the serum originally applied to the column.

Injection of Antigens and Antibody Isolation: Female New Zealand white rabbits were used for all antibody production, and pre-immune serum was collected from each rabbit. All

antigens were emulsified in Titer Max adjuvant (Vaxcel, Inc.) for subcutaneous injection. Approximately three weeks after the initial injections, rabbits were re-injected with the same antigens as boosts, followed ten days later by collection of blood. Rabbits were bled at two-week intervals thereafter. Peptides SP616, SP618 and JK374 coupled to KLH via DSS or MBS were injected in amounts of 40-160 μg total protein, in both initial and boost injections. These peptides were also injected uncoupled, 5 mg per initial injection or boost. Approximately 100 μg of RNS2 purified via the pET system was injected initially, followed by 140 μg boosts. Peptide PG1 coupled to KLH was administered at 267 μg in the first injection and 400 μg in boosts. Sera were screened by testing various dilutions on immunoblots containing yeast-produced RNS2 (Chapter 2).

Protein Gels and Immunoblot Analysis

All *Arabidopsis* tissues described in this chapter are from the Columbia ecotype. Roots, stems, leaves and flowers from soil-grown plants, above-ground tissues from soil-grown plants, and plants grown on P_i-rich and P_i-deficient media were grown and harvested as described in Chapter 3. Green siliques from 5 to 15 mm in length were collected from four-week-old plants. Seeds used for protein extraction were viable, dry seeds that had been stored at room temperature for approximately one year. Proteins were extracted from harvested tissues as described in Chapter 3. Glycerol was added to 10% v/v in all protein extracts and samples were frozen at -80° C in small aliquots.

Protein extracts were mixed with sample buffer and boiled for five minutes before being separated on 11% SDS-PAGE gels (Laemmli 1970) cast with an

acrylamide:bisacrylamide ratio of 30:0.8. Proteins were blotted onto PVDF membrane (Immobilon-P; Millipore) using semi-dry electrophoretic transfer as described (Harlow and Lane 1988). After transfer but before drying, membranes were autoclaved in transfer buffer for 20 minutes at 120° C as described (Swerdlow et al. 1986), since autoclaving increases the signal strength of RNS2 detection by the antibodies described above (data not shown). Blots were processed for RNS2 signal detection using one of two protocols: one with a TBS-Tween buffer (Birkett et al. 1985) and another with a Blotto/Tween buffer (Harlow and Lane 1988) as blocking agents. Anti-PG1 (anti-RNS2) serum (described above) from rabbit 11192 was diluted 1:2000 for detection, and goat-anti-rabbit IgG:alkaline phosphatase conjugate was used as the secondary antibody. Signals were developed using nitroblue tetrazolium and 5-chloro-4-bromo-3-indolyl phosphate (Harlow and Lane 1988), incubating in development buffer for 10 minutes. Dot blots with peptides were prepared by spotting 1 μl of 5 mg/ml solutions of peptides onto strips of nitrocellulose membrane. For signal detection, the dot blots were incubated with various dilutions of sera and processed as described above.

Immunocytochemistry

In initial experiments, *Arabidopsis* tissues (ecotype Columbia) from healthy, soil-grown plants were fixed in 2% formaldehyde/1% glutaraldehyde in 50 mM sodium phosphate buffer with 0.1 M sucrose (pH 7.2) and vacuum infiltrated for two hours at room temperature. In subsequent experiments, *Arabidopsis* leaves and petals were fixed in 4% formaldehyde in the same buffer and vacuum infiltrated as described above. After fixation,

all tissues were washed three times in 10 mM sodium phosphate buffer with 0.5 M sucrose (pH 7.2) for 10 minutes each. The tissue was postfixed in 1% OsO_4 + 10 mM sodium phosphate + 0.05 M sucrose (pH 7.2) for one hour and then rinsed in distilled water three times for 10 minutes each. Following dehydration in an ethanol series, the tissue was infiltrated with London Resin White acrylic resin (Polysciences, Warrington, PA) and polymerized at 58° C under vacuum overnight. Thin sections were prepared on an Ultracut E microtome (Reichert-Jung, Vienna, Austria) and mounted on formvar-coated nickel grids (Polysciences, Warrington, PA). Immunocytochemistry was performed as described (Schroeder et al. 1993). Affinity purified anti-PG1 (anti-RNS2) antibodies or pre-immune serum were diluted 1 to 4. Goat anti-rabbit IgG (1:1 dilution) was used as an amplifying bridge. Protein A conjugated to colloidal gold of 15 nm diameter (EY Lab Inc., San Mateo, CA) was diluted 1 to 50. Thin sections were examined on a JEOL 100CXII transmission microscope (Tokyo, Japan).

CHAPTER 5

GENERATION OF TRANSGENIC PLANTS WITH DECREASED AND INCREASED AMOUNTS OF *RNS1* AND *RNS2*

ABSTRACT

To gain insight into the *in vivo* roles of the RNS1 and RNS2 proteins, antisense constructs for the *RNS1* and *RNS2* genes were transformed into plants and the transformants screened for decreased protein or RNA of the corresponding gene. Several constructions were used in the attempt to produce antisense *RNS2* lines. The most affected antisense lines had leaf *RNS2* mRNA levels of 39-60% that of control plants and exhibit no obvious altered phenotype. In contrast, antisense *RNS1* lines were obtained that had *RNS1* mRNA levels as low as 10% of that of control plants under P_i-deficient conditions. These lines have high anthocyanin contents, which is a response seen during starvation for P_i. A project was also begun to overexpress *RNS1* in roots such that RNS1 would be secreted into the soil, degrading rhizospheric RNA in combination with phosphatases and thus increasing the P_i available to the plant. Transgenic plants with increased root *RNS1* expression were obtained, but the expression level is comparable to that of the endogenous *RNS1* gene under P_i-deficient conditions.

INTRODUCTION

To determine the function of a protein in any biological system, some of the best approaches to use are gene inactivation techniques. The most definitive methods of this type, targeted gene replacement or deletion, have not been extensively developed for use in plants. Instead, the method most widely used in plants involves the techniques of antisense RNA (Bourque 1995; Dougherty and Parks 1995). This method has the advantage that expression can be downregulated over a wide range, from completely abolished to only slightly affected, making it possible to not only see the effects of varying amounts of the protein but also to obtain plants affected in proteins whose complete suppression would be lethal. In addition, expression of a family of closely related genes can sometimes be suppressed *en masse* with antisense.

In Chapters 3 and 4, it was proposed that RNS1 and RNS2 had roles in the mobilization and recycling of P_i during such phenomena as senescence and P_i starvation. Obtaining antisense plants with decreased expression of *RNS1* and *RNS2* seemed an attractive first step to test these theories. Antisense techniques have recently been used to show that S-RNases are essential in the rejection of self pollen during the self-incompatibility response (Lee et al. 1994), demonstrating that this method can succeed with genes of the T_2/S family of RNases in plants. Since no clear guidelines exist regarding the best way to construct antisense gene fusions, as a starting point the full-length *RNS1* and *RNS2* cDNAs were fused in reverse orientation to a strong promoter and transformed into *Arabidopsis*.

Another project using similar techniques but with a different objective was begun in collaboration with Dr. Marcel Bucher to overexpress *RNS1* in roots of *Arabidopsis*. Some soils contain abundant organic matter and therefore contain RNA, a rich source of phosphate. Plants are known to secrete organic acids into the rhizosphere, increasing the solubility of inorganic phosphates and thus their availability to the plant (Johnson et al. 1996a and references therein). In addition, secretion of acid phosphatases into the rhizosphere by plant roots is thought to aid in the mobilization of P_i from organic phosphates (Duff et al. 1994). Some fungi are known to secrete nucleases, thought to facilitate absorption of P_i from their environment (Fraser and Low 1993). However, it is not known whether plants secrete RNases or nucleases from their roots. The secretion of RNases from roots should give plants a competitive advantage in extracting P_i from soils that are rich in organic matter but otherwise poor in P_i. To release P_i from RNA, RNase action needs to be combined with phosphatase action, and there is evidence that phosphatases are indeed secreted from *Arabidopsis* roots (M. L. Abler, unpublished). An observation was made that *RNS1*, dramatically upregulated in seedlings during P_i starvation, is induced to a lesser extent in roots of P_i-starved plants (C. J. Howard, P. J. Green, unpublished). Increasing RNS1 secretion from roots may give plants the competitive advantage described above. To test this theory, the *RNS1* cDNA was fused to a root-specific promoter and *Arabidopsis* plants transformed with this construction, in the hopes that transformed plants would secrete from their roots abundant RNS1 protein. This project was designed to explore whether such engineering would be useful for crop plants, so that less chemical fertilizer use would be needed.

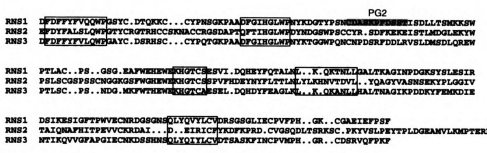
RESULTS AND DISCUSSION

Antisense Inhibition of the *RNS1* Gene

Production of anti-RNS1 antibodies: To confirm that putative antisense *RNS1* plants contained low levels of RNS1 protein, anti-RNS1 antibodies were essential. Because the use of a unique synthetic peptide as an antigen worked well with RNS2 (Chapter 4), this approach was also tried for RNS1. The same region of the protein that was used to design the synthetic peptide for anti-RNS2 antibodies was used to make a peptide specific for RNS1. The peptide, PG2, shown in Figure 5-1A, was unique in the Genbank database at the time of synthesis, with its closest relative a ribonuclease from zinnia, ZRNaseI (Ye and Droste 1996). Peptide PG2 was prepared and injected just as done for the anti-RNS2 peptide PG1, but the antibodies generated against it produced unexpected results. These antibodies recognize a protein of about 17 kDa, which is significantly smaller than the predicted molecular weight of 23.0 kDa for RNS1 (Figure 5-1B). In addition, the abundance of this protein actually decreases during P_i starvation, in stark contrast to the strong induction of the *RNS1* gene during this stimulus (Figure 3-9). Finally, the protein appears abundant in stems and leaves, unlike the *RNS1* transcript (Figure 3-7). Since it appears that the anti-PG2 antibodies recognize a protein that is not RNS1, these antibodies were not used in further studies. This type of problem is common when using synthetic peptides as antigens (Harlow and Lane 1988).

The next approach used to make anti-RNS1 antibodies was to use as an antigen RNS1 protein produced in a heterologous system. The yeast overexpression system used to

A



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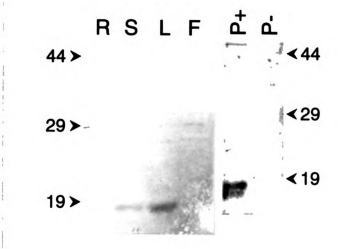


Figure 5-1 - Attempts to prepare anti-RNS1 antibodies using a peptide antigen. (A) Location of peptide PG2 in the amino acid sequence of RNS1. Deduced amino acid sequences of RNS1, RNS2 and RNS3 are aligned, beginning with the first residues shown in Figure 2-2. Boxes highlight the conserved regions described in Figure 2-2. (B) Protein samples were resolved by SDS-PAGE, transferred to PVDF membrane, and immunodetected with anti-PG2 antiserum. Roots (R), stems (S), leaves (L), and flowers (F) were prepared as for Figure 4-3, and seedlings grown on P_i-rich (P+) or P_i-deficient (P-) media were prepared as for Figure 4-4.

produce RNS1 (described in Chapter 3) was chosen because RNS1 can be generated in quantities up to 9 mg/liter of supernatant, and RNS1 is the dominant protein secreted so it can be easily purified. Concentrated supernatant in which yeast cells secreting the RNS1 protein were grown was passed over an anion exchange column and bound proteins eluted with an increasing salt gradient. At pH 6.0, RNS1 bound to the anion exhange column and eluted at a fairly narrow range, such that it was possible to separate the protein from all major contaminant proteins. The purity of the eluted RNS1 preparation was estimated at >95%, as monitored by one-dimensional SDS-PAGE and silver staining (Figure 5-2A) as well as isoelectric focusing (data not shown). Injection of RNS1 protein into rabbits led to the production of antisera of high titer that recognize a protein of about 25 kDa, close to the predicted size of 23.0, in extracts of P_i-starved *Arabidopsis* seedlings (Figure 5-2B). These antibodies are not entirely specific for RNS1; RNS3 is also detected at approximately 10-fold lower efficiency (data not shown; the RNS3 band is faintly visible in Figure 5-2B). However, RNS1 and RNS3 have slightly different electrophoretic mobilities, so these antibodies are adequate for use in detection of RNS1 on immunoblots.

Generation of antisense RNS1 *plants:* To obtain antisense *RNS1* plants, a T-DNA transformation vector was constructed containing the entire *RNS1* cDNA fused in reverse orientation between the strong cauliflower mosaic virus 35S promoter and the *nos* terminator (Figure 5-3). Transgenic plants were made using *Agrobacterium tumefaciens*-mediated transformation with the method of vacuum infiltration. With this method, large numbers of independent transformants can be obtained easily, which was an advantage for

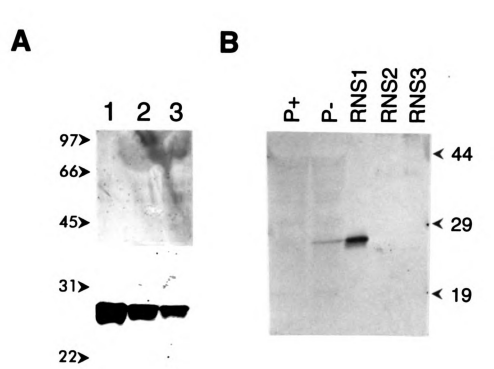


Figure 5-2 - Anti-RNS1 antibody preparation using heterologously-produced RNS1 as an antigen. (A) Various amounts of a preparation of yeast-produced RNS1 that was purified by anion exchange chromatography, resolved by SDS-PAGE and silver stained. 1, 200 μg ; 2, 600 ng; 3, 200 ng. (B) Protein samples were resolved by SDS-PAGE, transferred to PVDF membrane, and immunodetected with anti-RNS1 antiserum. RNS1, RNS2, RNS3: approximately 70 ng each of the indicated RNase, in supernatant from RNase-expressing yeast cells (see Chapter 3). P+, P-: 30 μg of total proteins from Columbia wild-type seedlings grown on media rich (P+) or deficient (P-) in P_i as described for Figure 5-2. For both (A) and (B), molecular weights of standards in kDa are shown to the sides of the gels.

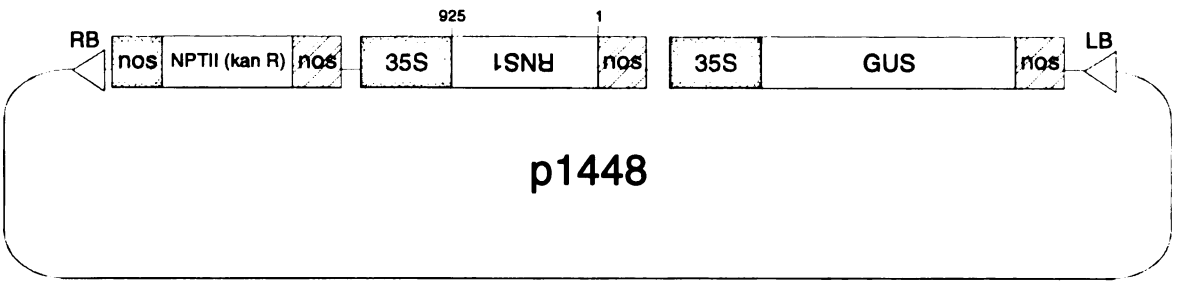


Figure 5-3 - Structure of antisense *RNS1* plant transformation vector. The entire *RNS1* cDNA is fused to the cauliflower mosaic virus 35S promoter and a *nos* terminator. nos, nopaline synthase; NPTII, neomycin phosphotransferase; GUS, β-glucuronidase; RB and LB, right border and left border, respectively, of the T-DNA. All elements except for *RNS1* are as described (Jefferson 1987). Shaded elements indicate promoters, hatched elements indicate terminators, and empty boxes indicate coding regions.

this project since it was expected that many transformants would need to be screened to obtain plants in which the antisense effect was very strong. Transgenic control lines were made by transforming plants with the pBI121 vector.

Under normal growth conditions, the flower is the only major organ of the plant in which *RNS1* mRNA is abundant (see Figure 3-7). For this reason, flowers were selected as the organ to assay for decreased RNS1 activity. (Screening for lowered *RNS1* mRNA levels was ruled out due to the difficulty of gathering enough flowers from individual plants to obtain sufficient total flower RNA.) The initial generation of transgenic plants was grown in soil and proteins extracted from flowers gathered from each of 120 independently transformed plants were resolved on RNase activity gels. Lowered RNS1 activity was judged by comparing the intensity of the RNS1 activity band in plants containing the antisense *RNS1* construction with that of control plants. As discussed in Chapter 3, this band is very likely to be that resulting from the RNS1 protein. Extensive efforts to prove this were done by attempting to transfer proteins from stained RNase activity gels to blots and detect RNS1 with anti-RNS1 antibodies (described above), but these did not succeed due to technical problems. However, to date all fluctuations in RNS1 activity observed due to various growth conditions are mirrored at the mRNA level (C. J. Howard, P. J. Green, manuscript in preparation); therefore the use of this band to assay RNS1 activity in putative antisense plants seemed justified. Of the 120 individual transformants screened in this way, 13 putative *RNS1* antisense plants were identified.

To confirm lowered RNS1 activity in the 13 putative antisense *RNS1* lines, one antibiotic-resistant progeny plant (T2 generation) from each line was grown in soil and its

flowers assayed as done for the T1 generation. Of these lines, five displayed lowered RNS1 activity in the T2 generation. T3 plants of several of these lines were assayed in the same way, which eliminated two of the lines due to normal levels of RNS1 activity. The final test done to confirm lowered RNS1 levels lines was to grow T3 and T4 seedlings of these lines on media rich or deficient in P_i, as described for wild-type seeds in Chapter 3. Seedlings were harvested after seven days of growth on these media, taking care to remove antibiotic selection-sensitive seedlings, and these plants were analyzed for RNS1 activity on RNase activity gels.

The RNase activity profiles of the lines most affected in *RNS1* are shown in Figure 5-4. The antisense effect is most easily seen in extracts of seedlings grown under P_i-deficient conditions, due to the low level of RNS1 activity in seedlings (antisense and control) grown with abundant P_i. Lines 8d.5.2, 8d.5.3 and 23g.4 all have significantly lower RNS1 activity than controls when grown on P_i-deficient medium. 8d.5.2 and 8d.5.3 have a similar amount of RNS1 activity under these conditions, probably due to their being sibling lines. 23g.4 exhibits even lower RNS1 activity under P_i-deficient conditions, and in addition appears to have less activity in high-P_i conditions than all the other lines in Figure 5-4. The profiles of these lines are shown in comparison to several controls, including the wild-type plants and two transgenic lines. Because all the antisense lines were grown in the presence of kanamycin, the wild-type is not the best control line for this situation, but it is shown for comparison. Line 34d.3, a transgenic line containing only the pBI121 vector, exhibits the expected large amount of RNS1 activity during P_i starvation. Line 36h.3.2 contains the antisense *RNS1* construction but has near-normal levels of RNS1 activity in

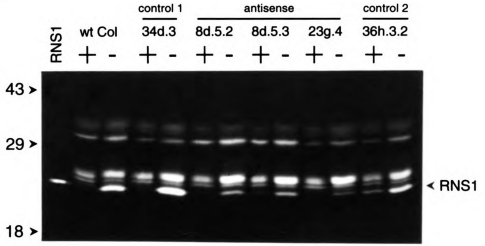


Figure 5-4 - Decreased RNS1 activity in *RNS1* antisense lines. T3 or T4 seedlings of antisense *RNS1* lines were germinated on AGM medium, transferred two days after germination to media rich (+) or deficient (-) in P_i, and grown for an additional seven days. Protein extracts were prepared from all kanamycin-resistant seedlings and 50 μg of each sample was resolved on RNase activity gels. Control 1, transgenic line containing pBI121 vector; control 2, transgenic line containing construction p1448 but which has a near-normal amount of RNS1 activity; RNS1, RNS1 protein produced in yeast. The RNS1 activity band is indicated. Molecular weights of standards in kDa are shown to the left of the gel.

low-P_i conditions, like the majority of the lines containing this construction that were screened.

RNS1 gene expression in the above lines was examined by hybridizing total RNA isolated from the same batches of tissue harvested for Figure 5-4 with an *RNS1* RNA probe that hybridizes only with the sense *RNS1* mRNA (Figure 5-5A). Once again, the antisense effect is most clearly seen in P_i-deprived seedlings. As *RNS1* levels seem to be similar under high-P_i conditions in all lines shown, antisense suppression of *RNS1* was quantitated by calculating the fold induction of *RNS1* by P_i starvation (after normalization with *eIF4A*). As shown in Figure 5-5B, the 34d.3 control line is induced 11.3 fold by this stimulus, whereas the 8d.5.2, 8d.5.3 and 23g.4 lines are only induced 3.1, 2.4 and 1.1 fold, respectively. Although *RNS1* expression is not completely abolished in 23g.4, its *RNS1* mRNA level during P_i starvation is only 10% that of the 34d.3 control, making this line an excellent choice for future studies.

The reduction in RNS1 protein levels expected in the antisense *RNS1* lines was confirmed using the anti-RNS1 antibodies described earlier with immunoblots containing the same protein extracts used in Figure 5-4. RNS1 protein is clearly detected in P_i-starved extracts of the wild-type, 34d.3 and 36h.3.2 lines (Figure 5-6), all of which have more RNS1 activity (Figure 5-4) and *RNS1* mRNA (Figure 5-5) than the antisense lines. However, little, if any, labeling of RNS1 is observed in extracts of the three antisense lines (Figure 5-6). This lack of labeling, which is in contrast to the reduced but visible levels of RNS1 activity seen in Figure 5-4, is probably due to the differences in sensitivity between

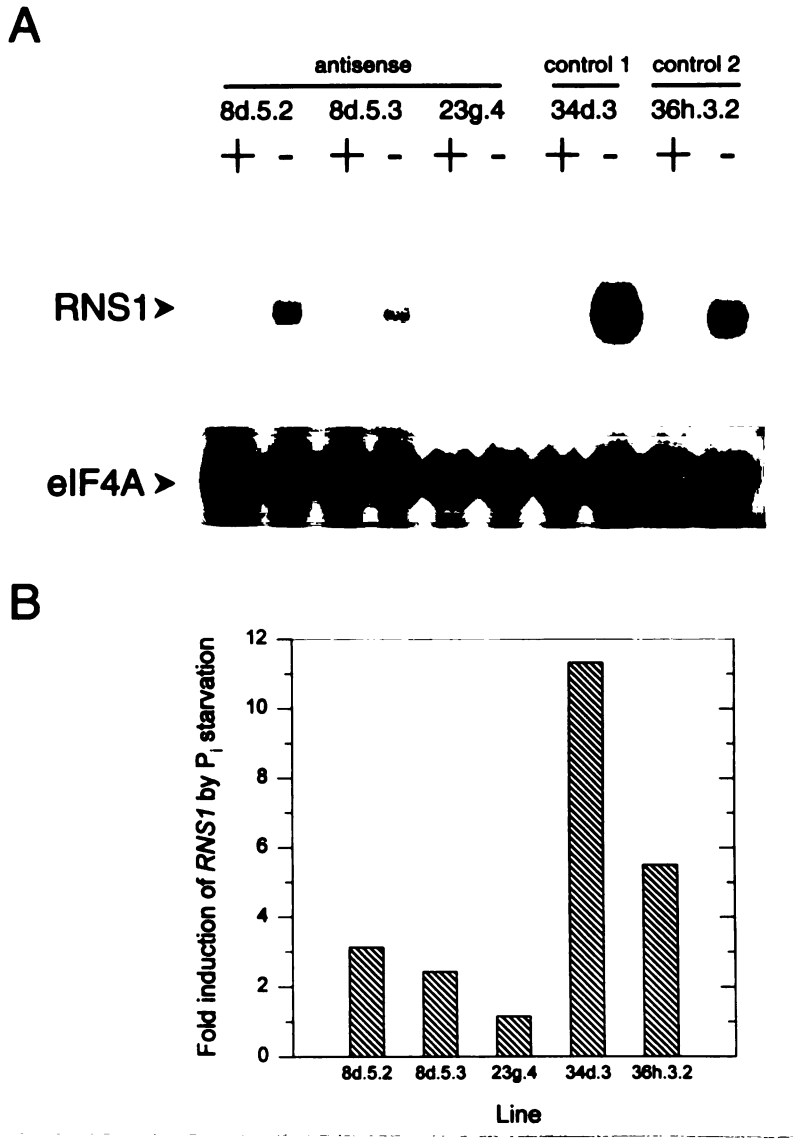


Figure 5-5 - *RNS1* mRNA levels in *RNS1* antisense lines. Seedlings of antisense *RNS1* lines were grown as described in Figure 5-4, and total RNA was isolated from all kanamycin-resistant seedlings. (A) RNA gel blots containing 10 μg of these samples per lane were hybridized to an *eIF4A* probe and subsequently to the antisense *RNS1* probe. +, seedlings grown on P_i-rich medium; -, seedlings grown on P_i-deficient medium; antisense, antisense lines; control 1, empty vector control line; control 2, line containing construction p1448 but with a near-normal level of RNS1 activity. (B) *RNS1* and *eIF4A* counts were quantitated by Phosphorimager analysis, and *RNS1* counts were divided by *eIF4A* counts for each lane. The normalized counts for the - lanes were divided by those of the + lanes, and the result plotted to show relative differences in *RNS1* mRNA levels in these lines.

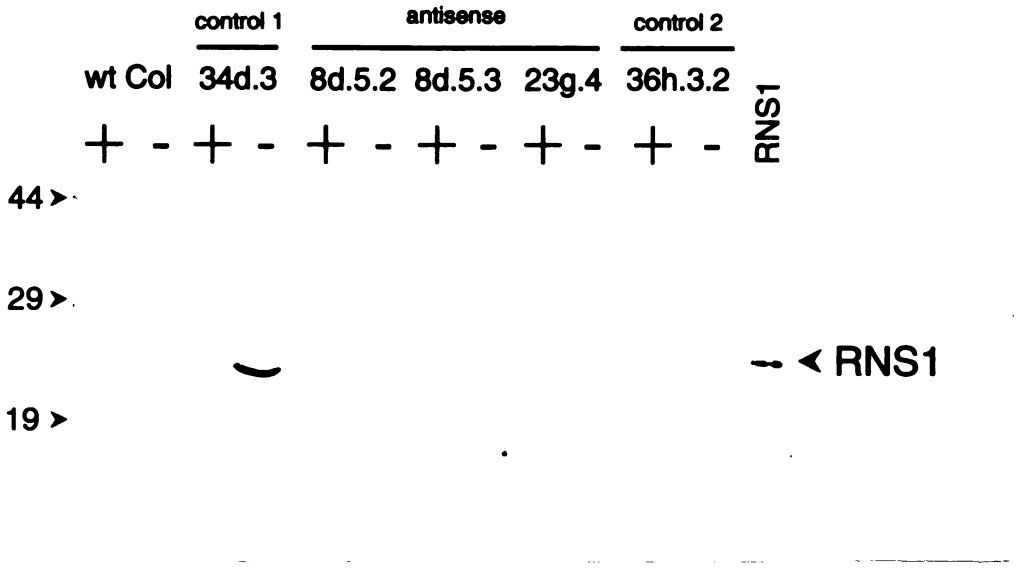


Figure 5-6 - RNS1 protein levels in *RNS1* antisense lines. Seedlings of antisense *RNS1* lines were grown as described in Figure 5-4, and proteins were isolated from all kanamycin-resistant seedlings. 100 μg of each plant protein extract were separated by SDS-PAGE, transferred to PVDF membrane, and immunodetected with anti-RNS1 antiserum. +, grown on P_i-rich medium; -, grown on P_i-deficient medium; RNS1, yeast-produced RNS1 control (see Chapter 3). The RNS1 band is indicated. Molecular weights of standards in kDa are shown to the left of the gel.

RNase activity gels and immunoblots. In any case, it is clear that the antisense lines have drastically reduced amounts of RNS1 protein.

An interesting phenotype displayed by the three antisense *RNS1* lines is increased accumulation of anthocyanins. When grown for seven days on P_i-deficient medium, the antisense lines contain from 2.6 to 5.1 times the amount of anthocyanins present in the wild-type and 34d.3 control plants (Figure 5-7). In addition, lines 8d.5.2 and 8d.5.3, when grown on P_i-rich medium, contain amounts of anthocyanins comparable to those seen in P_i-deprived control plants. Interestingly, line 36h.3.2, which has *RNS1* mRNA levels between those of the antisense lines and the control lines (see Figure 5-5), also has anthocyanin levels between those of the two other groups of plants. The chalcone synthase gene, which encodes an enzyme involved in anthocyanin biosynthesis, is induced by many stress-related stimuli, such as high light intensity, pathogen attack, and wounding (reviewed in Mol et al. 1996). However, anthocyanin accumulation is also a symptom of P_i starvation (Dedaldechamp et al. 1995; Marschner 1986). Although the elevated anthocyanin levels in the antisense *RNS1* lines is not proof that their tissues are more starved for P_i than control plants are under the same conditions, the possibility is intriguing.

Line 23g.4 has a phenotype in addition to the increased anthocyanin accumulation described above. In the original T1 plant, as well as in some, but not all, of the descendants of this plant, the following traits were observed in comparison to wild-type: smaller leaves, shorter and thinner stems, reduced seed set, and a preference for multiple stems to appear during the initial phase of bolting instead of the one dominant bolt that usually appears. Interestingly, the *pho1* mutant, examined in Chapter 3, has a similar phenotype, including

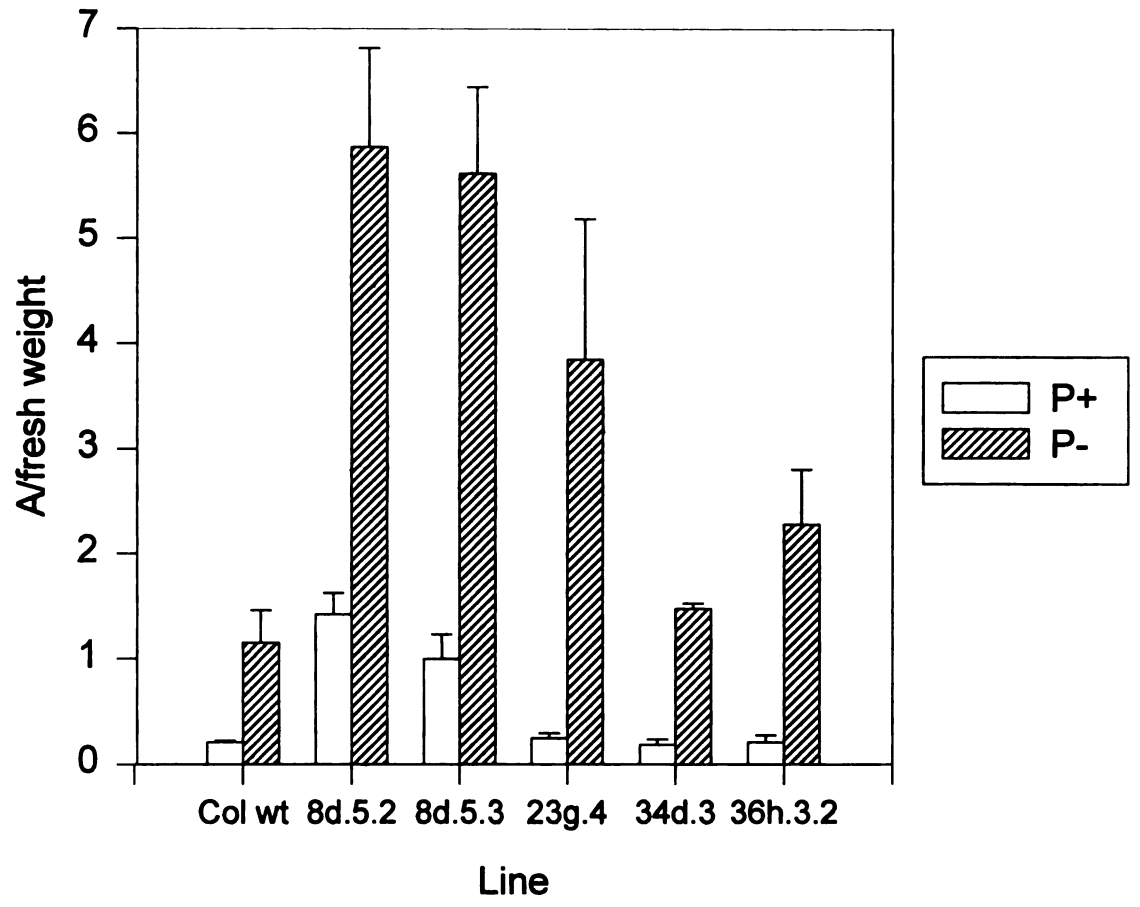


Figure 5-7 - Quantitation of anthocyanin levels in seedlings of *RNS1* antisense lines. Seedlings of antisense *RNS1* lines were grown as described in Figure 5-4, and anthocyanins were extracted as described in the Materials and Methods. Each bar encompasses four independent readings. Absorbance at 530 nm minus absorbance at 657 nm was taken as a measure of anthocyanin content, and was normalized to the fresh weight of each sample. Empty bars, seedlings grown on P_i-rich medium; hatched bars, seedlings grown on P_i-deficient medium.

elevated anthocyanin levels, smaller leaves, thinner stalks, reduced seed set, less secondary inflorescences and delayed flowering (Poirier et al. 1991). The similarities in these phenotypes are an intriguing indication that the tissues of plants in the 23g.4 line may be P_i starved.

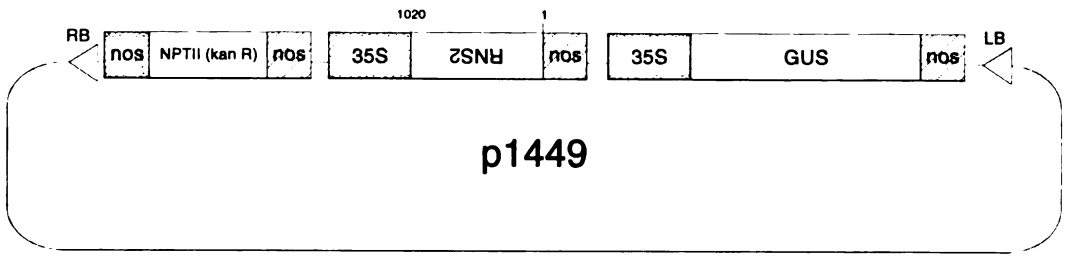
As mentioned above, this phenotype has incomplete penetrance in the 23g line. It is not known how many copies of the transgene were present in the intial 23g T1 plant, but segregation data suggest that it contained more than one transgene locus. One of three progeny plants (T2 generation) grown in soil, a sibling plant to the 23g.4 line, displayed the altered phenotype, although the 23g.4 plant itself did not. Finally, of three descendants (T3 generation) of the 23g.4 plant grown in soil, one displayed the altered phenotype. These observations are consistent with the effects of varying transgene dosage. Since the molecular mechanisms of transformation by vacuum infiltration have not been elucidated, it is not known whether this method gives rise to plants that are hemizygous or homozygous for the transgene(s). A plant hemizygous for multiple transgenes may give rise to progeny that contain more or less transgenes than it had itself, and if antisense effects are proportional to the amount of antisense transcript, as some studies suggest (Dougherty and Parks 1995), progeny with a greater number of transgenes should display a more affected phenotype. This idea is a simple explanation for the transient nature of the 23g phenotype, but more complicated models cannot be ruled out. The above model would also explain another phenomenon observed with both the 8d and 23g lines: only some of the progeny of these lines exhibit reduced amounts of RNS1 activity. It may be possible to test this model using Southern blot analysis.

The isolation of the antisense *RNS1* lines described in this section is an important step in determining the role of RNS1 during P_i starvation in plants. The visible phenotypes of increased anthocyanin accumulation and, for the 23g line, a *pho1*-like phenotype, are further clues that RNS1 could be a major part of the phosphate rescue and recycling system that has been proposed to exist in plants (Goldstein et al. 1989). These phenotypes also suggest that the three RNS proteins do not have redundant functions, since reducing the expression of only *RNS1* has a marked effect. One priority for the near future is to select a line homozygous for the transgene(s), in which the antisense *RNS1* effect is strong and stably inherited. Such a line could then be crossed to other pertinent lines in the future, for example an antisense *RNS2* line. Another interesting experiment would be the quantitation of free P_i in the antisense *RNS1* lines. If the tissues of these lines are indeed starved for P_i, there may be an effect on the amount of free P_i in the plant as compared to the amount of P_i sequestered in nucleic acids.

Antisense Inhibition of the *RNS2* Gene

The same approach used to produce antisense *RNS1* plants was used in the initial attempt to obtain antisense *RNS2* plants. As shown in Figure 5-8A, the entire *RNS2* cDNA was fused in reverse orientation into a T-DNA construction analagous to that made for the antisense *RNS1* construction. 119 independent transformants of this plasmid were screened by assaying leaf proteins of soil-grown plants for lowered RNS2 levels with immunoblots and anti-RNS2 antibodies (see Chapter 4). In this population of transgenic plants, RNS2

A



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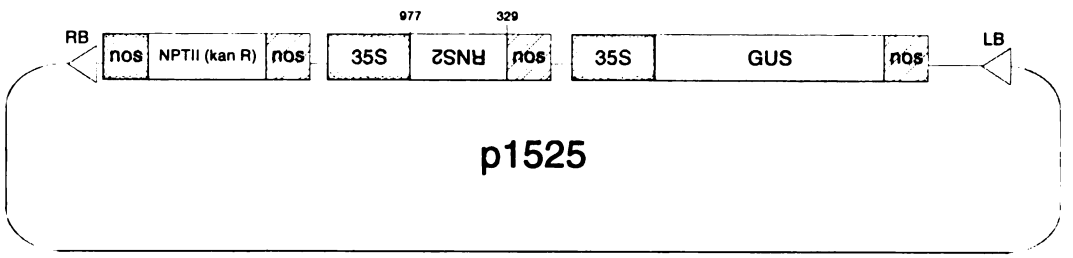

C

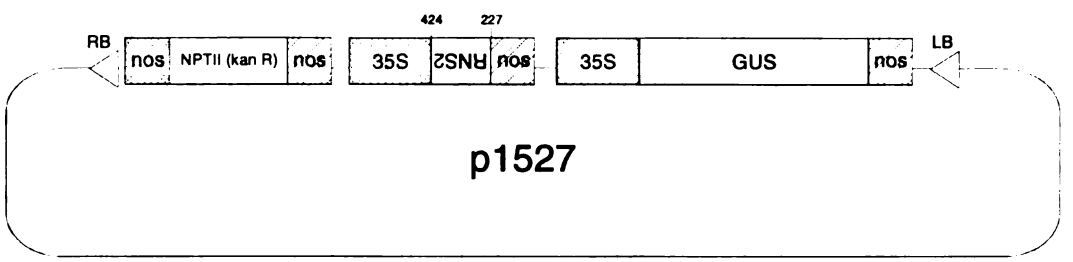

Figure 5-8 - Structure of antisense *RNS2* plant transformation vectors. Various portions of the *RNS2* cDNA are fused to the cauliflower mosaic virus 35S promoter and a *nos* terminator, in three separate constructions. All other elements are as described in Figure 5-1. Shaded elements indicate promoters, hatched elements indicate terminators, and empty boxes indicate coding regions. A) Construction p1449. B) Construction p1525. C) Construction p1527.

levels varied to a small extent, but no plants with dramatically lowered amounts of RNS2 were observed.

There is no consensus in the literature as to which portions of genes to use to obtain the best results with antisense techniques in plants (Bourque 1995). The size and portion of an open reading frame which results in the greatest antisense effects appears to be gene-dependent. For this reason, the above results were not interpreted to mean that no antisense *RNS2* plants could be obtained, and so two additional portions of the *RNS2* cDNA were fused into antisense constructions for plant transformation in the hopes that these portions would be more successful in suppressing RNS2 production. The first construction, p1525 (Figure 5-8B) included the final two-thirds of the *RNS2* cDNA, a fragment of about 650 base pairs, and the second, p1527 (Figure 5-8C), incorporated a fragment of approximately 200 base pairs between and including the conserved active site regions (see Chapter 2).

Independent transformants, 74 of p1525 and 63 of p1527, were screened by analyzing leaf RNAs of soil-grown plants for lowered amounts of *RNS2* mRNA. This method replaced the immunoblotting method used earlier because of the greater relative ease of quantitation and normalization of the *RNS2* signal. *RNS2* signal strength was divided by that of the *eIF4A* signal in the same sample, and this number was compared to the corresponding number from a control sample on the same blot. Of the 137 plants screened, only 19 had leaf *RNS2* mRNA levels less than or equal to 70% that of wild-type plants, and of the 19, only four had less than 35% that of wild-type. Several antibiotic-resistant progeny plants (T2 generation) from each of these 19 plants were grown in soil and leaf mRNAs were analyzed for *RNS2* as done for the T1 generation. Progeny of only four

of the 19 T1 plants showed lowered leaf *RNS2* mRNA levels; RNA gel blot analysis of several of these progeny plants is shown in Figure 5-9. In these plants, levels of *RNS2* mRNA range from 39% to 68% of that in wild-type. Three of these four lines, resulted from transformation with plasmid p1527.

The lines shown in Figure 5-9 display no unusual phenotype when grown under normal conditions in soil. Progeny from two of the four lines were observed to have leaf anthocyanin levels slightly higher than normal, but this effect was not examined quantitatively. Due to a lack of time, it was not possible to test the reactions of these plants to phenomena known to affect *RNS2* expression, such as P_i starvation and inoculation with pathogens. The lack of obvious phenotypes in these plants may be due to the incomplete suppression of *RNS2* expression: the inhibition of *RNS2* to only 39 to 68% that of wild-type may not be sufficient to affect the plant because an adequate amount of RNS2 protein is still being produced. Alternately, the reduction of RNS2 levels may be compensated by the actions of other RNases in plants. In *E. coli*, for example, it is known that molecularly distinct RNases can participate in similar processes, and that the knockout of all the functionally overlapping RNases is required to produce a measurable effect (Deutscher 1993a).

It is possible that the lines shown in Figure 5-9 could be manipulated to contain lower levels of RNS2 than they do at present. It is unknown whether these T2 lines are hemizygous or homozygous for the transgene, although all are progeny of hemizygous T1 plants. Selection of homozygous progeny of these lines could lead to plants in which antisense effects are stronger due to a higher transgene dosage. Along similar lines, it has

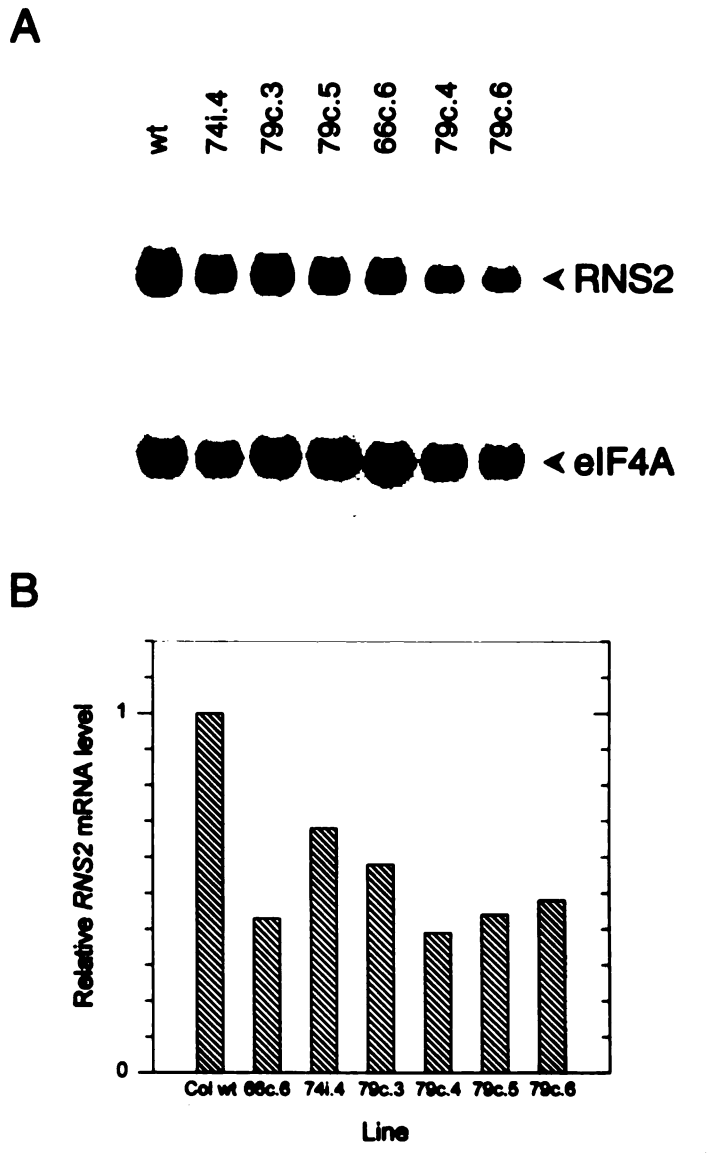

Figure 5-9 - *RNS2* mRNA levels in *RNS2* antisense lines. (A) Total RNA was extracted from leaves of four-week-old, kanamycin resistant, soil-grown antisense *RNS2* lines. RNA gel blots containing 6 μg of these samples per lane were hybridized to an antisense *RNS2* probe and subsequently to the *eIF4A* probe. (B) *RNS2* and *eIF4A* counts for the bands shown in (A) were quantitated by Phosphorimager analysis. *RNS2* counts were divided by *eIF4A* counts for each lane, and these results were divided by the *RNS2*/*eIF4A* ratio for the wild-type control lane to show relative differences in *RNS2* mRNA levels. wt, Col wt: Columbia wild-type samples.

been shown that crossing of two independent antisense plants and thus increasing transgene dosage can result in a greater antisense effect (Pennisi 1996). It remains to be seen whether these techniques will lower RNS2 levels further.

Overexpression of *RNS1* in Roots

To overexpress *RNS1* in roots, the full-length *RNS1* cDNA was fused to a root-specific promoter from a soybean proline-rich protein (Suzuki et al. 1993). Specific expression in the root was desired to avoid any potential deleterious effects of overexpression of RNS1 in other organs of the plant. It was expected that the endogenous RNS1 N-terminal sequence would be sufficient to target the protein for secretion. Although it has not been proven that RNS1 is an extracellular protein, its N-terminal sequence has a high score as a secretory signal sequence according to a statistical analysis (von Heijne 1986). In addition, RNS1 has been purified from culture medium of *Arabidopsis* seedlings grown under P_i-deficient conditions (J. Paz-Ares, personal communication).

The vector (shown in Figure 5-10) was transformed into *Arabidopsis* as described above for the antisense constructions. Primary transformants were grown in soil, their seeds collected, and this generation (T2 generation) screened for increased *RNS1* transcript levels in roots. Pooled T2 seedlings from each T1 line were grown on standard growth medium containing antibiotic selection, with the plates placed vertically to maximize root growth such that more tissue was available for analysis. After about four weeks of growth, roots were collected and total RNA extracted for RNA gel blot analysis. All of the lines screened contained measurably higher *RNS1* mRNA levels than control plants. A selection of lines

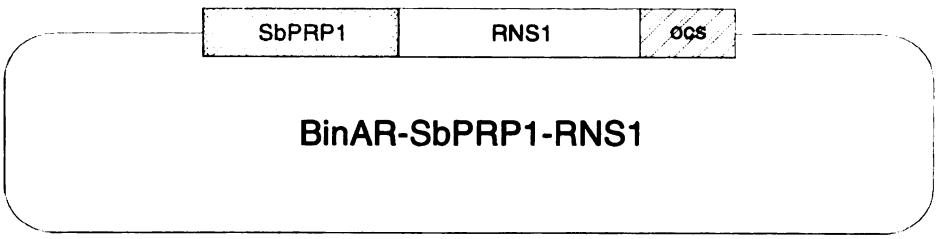

Figure 5-10 - Structure of *RNS1* root overexpression transformation vector. The entire *RNS1* cDNA is fused to the root-specific SbPRP1 promoter and an *ocs* terminator. SbPRP1, soybean proline-rich protein promoter (Suzuki et al. 1993); ocs, octopine synthase. Shaded box indicates a promoter, hatched box indicates a terminator, empty box indicates a coding region.

This plasmid was constructed by Dr. Marcel Bucher

with a representative range of the *RNS1* expression levels seen is shown in Figure 5-11: these lines express *RNS1* between a range of 2.3 to 6.6 times that of the control line. None of the lines showed an unusual phenotype when grown in soil, although lines 58B and 59C, the lines with the highest root *RNS1* expression, did have slightly smaller rosettes than other lines.

To determine whether lines with these amounts of *RNS1* expression would be more competitive in P_i-poor soils, it was first necessary to compare their expression to the endogenous expression of *RNS1* in roots of P_i-starved plants. *Arabidopsis* wild-type seeds were grown on media rich or deficient in P_i (media formations given in Chapter 3) on plates placed vertically as done for the *RNS1* root overexpression lines. Seedlings grown on P_i-rich medium appeared to grow normally, much like control plants growing on AGM medium, but seedlings grown on P_i-deficient medium had both stunted green tissue and stunted roots. P_i-starved roots were approximately 20% the length of roots grown on P_i-rich medium, and often grew partially off the medium into the air, an unusual response. It is known that P_i starvation can have large effects on root morphology in other plants (Johnson et al. 1996b). After about four weeks of growth, roots were harvested and *RNS1* expression was measured using RNA gel blots as described above, and shown in Figure 5-12A. Under P_i-deficient conditions, *RNS1* is induced about 6.6-fold, much less than the induction of approximately 75-fold that is seen in total seedling RNA (compare with Figure 3-11). This level of induction is very similar to that of the most efficient *RNS1* root overexpression lines shown in Figure 5-10. Proteins from root tissues of the same batch were resolved on RNase

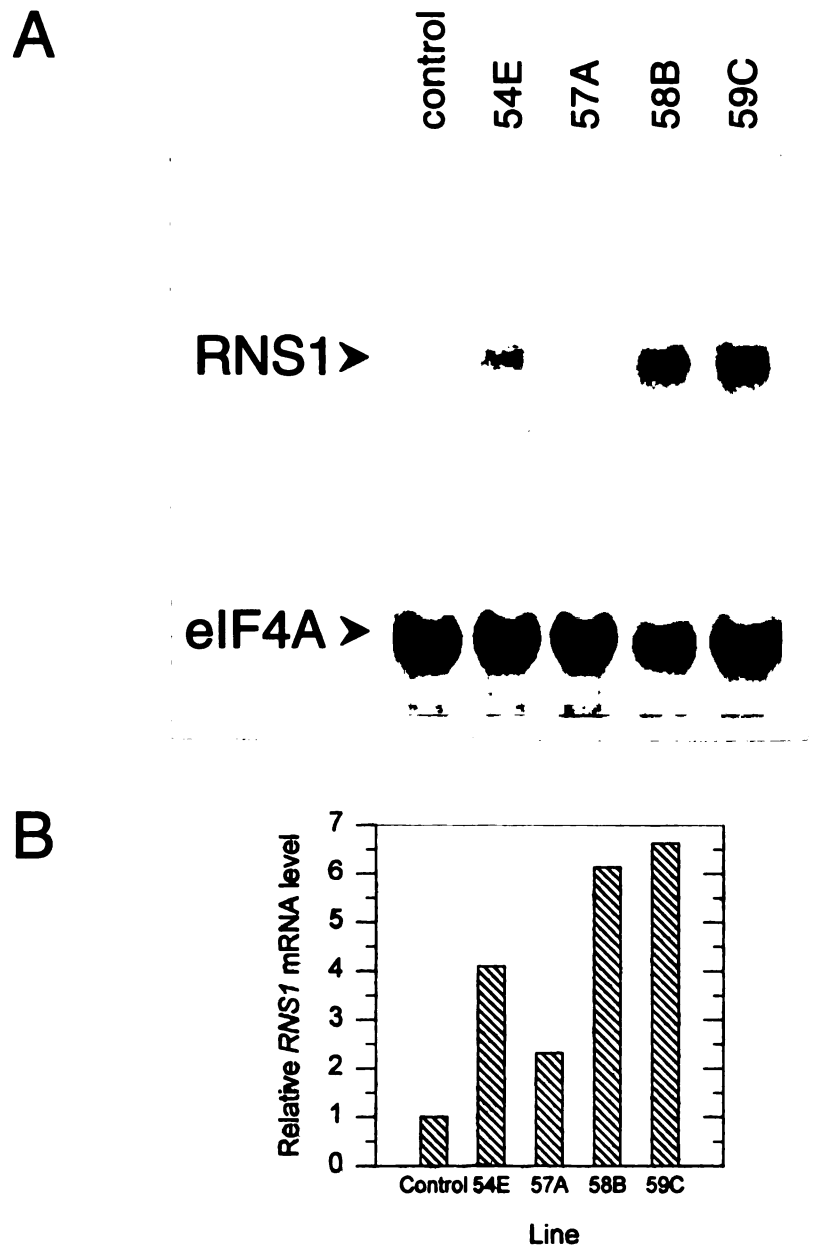

Figure 5-11 - *RNS1* mRNA levels in *RNS1* root overexpression lines. (A) Total RNA was extracted from roots of four- to five-week-old transgenic plants containing the *RNS1* root overexpression construction that were grown on kanamycin-containing medium. RNA gel blots containing 9 μg of these samples per lane were hybridized to the *RNS1* probe and subsequently to the *eIF4A* probe. (B) *RNS1* and *eIF4A* counts for the bands shown in (A) were quantitated by Phosphorimager analysis. *RNS1* counts were divided by *eIF4A* counts for each lane, and these results were divided by the *RNS1/eIF4A* ratio for the wild-type control lane to show relative differences in *RNS1* mRNA levels. Control, transgenic line 34d.3, which contains the pBI121 vector.

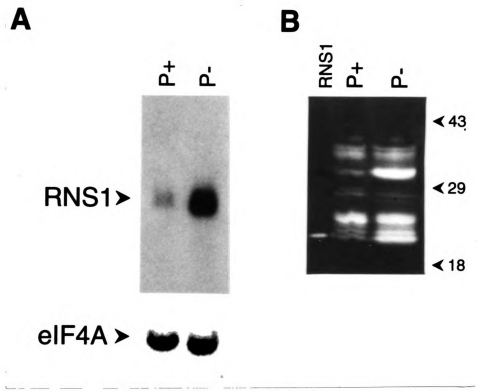

Figure 5-12 - Effect of P_i starvation on *RNS1* mRNA levels and RNS1 activity in roots. (A) Total RNA was extracted from roots of wild-type plants grown on P_i-rich or P_i-deficient media for four to five weeks. RNA gel blots containing 10 μg of these samples per lane were hybridized to the *RNS1* probe and subsequently to the *eIF4A* probe. (B) Proteins were extracted from the same batches of roots and 50 μg of each sample were resolved on an RNase activity gel. RNS1, RNS1 protein expressed from yeast. Molecular weights of standards in kDa are shown to the right of the gel.

activity gels, as shown in Figure 5-12B, and the extent of the induction of RNS1 activity is comparable to that observed at the RNA level.

Since the attempt to increase *RNS1* expression in *Arabidopsis* roots resulted in plants that express *RNS1* at about the same level as wild-type plants under P_i-deficient conditions, this strategy may not be useful in engineering crop plants able to tolerate low-P_i conditions in organic soils. However, *RNS1* root expression in these lines may be different under more natural conditions, such as in soil, or in other plants. At present, the effect of the *RNS1* overexpression construction is being tested in tobacco plants by Dr. Marcel Bucher. In addition, this idea may be more feasible if one were to use a stronger root-specific promoter, should one become available.

An interesting observation made during these studies can be seen in Figure 5-12. An RNase activity of about 33 kDa is induced strongly, and to a much greater extent than RNS1, in P_i-starved roots. This activity is in the range of a pair of bifunctional nuclease activities, believed to be secreted, that are observed in *Arabidopsis* stem extracts. The strong upregulation of this activity during P_i starvation may constitute part of the endogenous plant system to scavenge P_i from the rhizosphere. This possibility merits further study.

MATERIALS AND METHODS

Plasmid Constructions

p1448: The entire *RNS1* cDNA was excised with Bam HI and Sal I from plasmid p1184 and inserted into pT7T3α19 (BRL) between the Bam HI and Sal I sites to make p1392. A Pst I fragment of p1392, containing the *RNS1* cDNA, was fused between the Pst I sites of p1079 (provided by Wan-Ling Chiu), and a plasmid with the cDNA in the antisense orientation relative to the 35S promoter was designated p1398. p1398 was partially digested with Hind III and the ends of linearized forms of the plasmid converted to blunt ends with T4 DNA polymerase (Sambrook et al. 1989), after which the DNA was recircularized. A plasmid was selected such that the Hind III site flanking the cDNA was destroyed; this plasmid was designated p1444. The Hind III cassette of p1444, containing the *RNS1* cDNA fused between the 35S promoter and *nos* terminator, was inserted into the Hind III site of pBI121 (Jefferson 1987). A plasmid in which the *RNS1* cassette was oriented in the same direction relative to the *GUS* cassette of pBI121 was designated p1448.

p1449: The entire *RNS2* cDNA was excised with Bam HI and Kpn I from plasmid p1127 and inserted into pUC118 (Sambrook et al. 1989) between the Bam HI and Kpn I sites to make p1393. A Sal I fragment of p1393, containing the *RNS2* cDNA, was fused between the Sal I sites of p1079, and a plasmid with the cDNA in the antisense orientation relative to the 35S promoter was designated p1399. p1399 was partially digested with Hind III and the ends of linearized forms of the plasmid converted to blunt ends with T4 DNA polymerase

(Sambrook et al. 1989), after which the DNA was recircularized. A plasmid was selected such that the Hind III site flanking the cDNA was destroyed; this plasmid was designated p1445. The Hind III cassette of p1445, containing the *RNS2* cDNA fused between the 35S promoter and *nos* terminator, was inserted into the Hind III site of pBI121 (Jefferson 1987). A plasmid in which the *RNS2* cassette was oriented in the same direction relative to the *GUS* cassette of pBI121 was designated p1449.

p1525: A 648 bp Sca I-Eco RV fragment of the *RNS2* cDNA, comprising approximately the last two-thirds of the cDNA and including sequences encoding the conserved regions C3-C5 (see Figure 2-4), was excised from plasmid p1127. This fragment was inserted into the p1079 backbone, which had been digested with Pst I and whose ends had been converted to blunt ends using T4 DNA polymerase (Sambrook et al. 1989). A plasmid containing the fragment inserted in the antisense orientation relative to the 35S promoter was designated p1524. The Hind III cassette of p1524, containing the above fragment fused between the 35S promoter and *nos* terminator, was inserted into the Hind III site of pBI121 (Jefferson 1987). A plasmid in which the cassette was oriented in the same direction relative to the *GUS* cassette of pBI121 was designated p1525.

p1527: A 208 bp Eco RI-Xho I fragment of the *RNS2* cDNA, comprising sequences encoding the conserved regions C2 and C3 (see Figure 2-4) and the sequences between them, was excised from plasmid p1010-8. This ends of this fragment were converted to blunt ends using T4 DNA polymerase (Sambrook et al. 1989), and it was inserted into the

p1079 backbone between blunted Pst I sites. A plasmid containing the fragment inserted in the antisense orientation relative to the 35S promoter was designated p1526. The Hind III cassette of p1526, containing the above fragment fused between the 35S promoter and *nos* terminator, was inserted into the Hind III site of pBI121 (Jefferson 1987). A plasmid in which the cassette was oriented in the same direction relative to the *GUS* cassette of pBI121 was designated p1527.

BinAR-SbPRP1-RNS1: Plasmid provided by Marcel Bucher. See Figure 5-10.

Generation of Transgenic Plants

Plasmids p1448, p1449, p1525, p1527 and BinAR-SbPRP1-RNS1 were transformed into *Agrobacterium tumefaciens* strain GV3101 C58C1 Rif^r (pMP90) (Koncz and Schell 1986) via electroporation using a Gene-Pulser apparatus (Bio-Rad) as per manufacturer's recommendations. The plasmids were inserted into *Arabidopsis thaliana* ecotype Columbia with a vacuum infiltration method of *Agrobacterium*-mediated transformation. Protocols for this method by N. Bechtold (Bechtold et al. 1993), A. Bent (Bent et al. 1994), and T. Araki were modified or combined. Briefly, rosettes of four-week old plants with bolts of 5-15 cm were submerged in a solution of *A. tumefaciens* containing the plasmid of interest and subjected to a vacuum of 400 mm Hg for five minutes. The vacuum was quickly broken and the plants were allowed to recover and set seed under normal growth conditions. Details of this protocol can be viewed on the World Wide Web (http://www.bch.msu.edu/pamgreen/green.htm#prot). Seeds from these plants were plated

on kanamycin-containing medium, and one antibiotic-resistant seedling from each plant that was originally infiltrated was transferred to soil, to ensure that all plants analyzed were the results of unique integration events. To ensure unbiased selection of transformants to be transferred to soil, the antibiotic-resistant plant closest to the edge of the plate was selected in each case.

Anti-RNS1 Antibody Preparation

Synthetic peptide PG2 was prepared at the Michigan State University Department of Biochemistry Macromolecular Structure Facility, and its purity was monitored by analytical reversed-phase HPLC and mass spectrometry. Peptide PG2 was coupled to maleimide-activated KLH (Pierce) before injection. RNS1 protein used as an antigen was heterologously produced in yeast as described in Chapter 3. 250 ml of liquid minimal dextrose low-P_i medium (Thill et al. 1983) were inoculated with *S. cerevisiae* strain BJ2168 containing plasmid p1270 and grown until saturation (2.5 days). Cells were removed by centrifugation, the supernatant was concentrated to 0.5% of its original volume in Centriprep-10 units (Amicon), and the buffer replaced with 20 mM MES pH 6.0. The predicted amino acid sequence of RNS1 is predicted to bind to an anion exchange column at pH 6.0 (analysis done using the Prosis program). The proteins in the supernatant were bound to a Mono Q FPLC column (Pharmacia), eluted with a gradient of 0 to 0.25 M NaCl, and fractions analyzed by SDS-PAGE and silver staining. RNS1 eluted between 0.15-0.18 M NaCl. Pooling of the RNS1-containing fractions resulted in a preparation >95% pure in

RNS1, as monitored by SDS-PAGE and silver staining. Purity was also checked by isoelectric focusing and silver staining.

Proteins were injected subcutaneously into female New Zealand white rabbits and blood collected as described in Chapter 4. Two rabbits were used for each antigen. The first injections of PG2 coupled to KLH were of 280 μg, and after three weeks, boosts of 350 μg were administered. The first injections of heterologously-produced RNS1 protein were of 80 μg, followed by 300 μg boosts. Sera were screened for anti-RNS1 binding ability by testing various dilutions on immunoblots containing yeast-produced RNS1 (Chapter 3).

Expression Analyses

Antisense RNS1 *plants:* 120 kanamycin-resistant independent transformants of p1448 were analyzed for reduced RNS1 activity. Original transformants (T1 generation) as well as wild-type plants were grown in soil under conditions described in Chapter 3. Flowers were collected from four- to five-week-old plants on one day only per plant, collecting all flowers on the plant that were in the range of development from buds with petals showing to fully-open flowers that do not yet have developing siliques protruding. Flowers were frozen on dry ice and stored at -80° C. Flower proteins were extracted as described for other tissues in Chapter 3. RNS1 activity was analyzed by electrophoresing 20 μg of flower proteins from each transformed line on RNase activity gels (Yen and Green 1991); the intensity of the RNase activity band likely to be RNS1 was compared in transformed lines and wild-type. For those lines with lower flower RNS1 activity, seeds were collected, one kanamycin-resistant progeny plant (T2 generation) was grown in soil and their flowers analyzed for

RNS1 activity just as done for the T1 generation. Seeds from those lines that still appeared to have lowered flower RNS1 activity were once again selected for kanamycin resistance, and several resistant plants (T3 generation) were moved to soil and flowers screened for lowered RNS1 activity. In some cases, the T4 generation was screened in the same way.

Seeds of promising T3 or T4 lines were plated on AGM containing kanamycin on mesh circles, moved after two days to kanamycin-containing P_i-rich or P_i-deficient media, and harvested seven days later, as described for wild-type seeds in Chapter 3. Control lines included in these experiments were Columbia wild-type (grown on media without kanamycin) and transgenic lines containing just the pBI121 vector. Any kanamycin-sensitive seedlings were removed prior to harvesting. Harvested seedlings were frozen in liquid nitrogen for RNA extraction as described in Chapter 2, or on dry ice for protein extraction, as described in Chapter 3. RNA gels were prepared and blotted to membrane as in Chapter 2, and then RNA gel blots were probed first with the *eIF4A* probe as described in Chapter 2. The blots were then stripped and re-probed with an *RNS1* RNA probe such that only sense RNA strands would be detected. The RNA probe was made using a Riboprobe kit (Promega), with plasmid p1184 linearized with Bam HI, and T7 RNA polymerase. Quantitation of signal in *RNS1* and *eIF4A* bands was done using Phosphorimager analysis. Proteins were extracted from seedlings and electrophoresed on RNase activity gels as described in Chapter 3, and were electrophoresed and blotted to membrane for immunoblots as described in Chapter 4. For immunoblots, a 1:1000 dilution of serum from rabbit 55315 was used.

Antisense RNS2 *plants:* For the first strategy, 119 kanamycin-resistant independent transformants of p1449 were analyzed for lowered amounts of RNS2 by immunoblotting. Original transformants (T1 generation) as well as wild-type plants were grown in soil under conditions described in Chapter 3. Several healthy, non-senescing leaves were collected from four- to five-week-old plants on one day only per plant, frozen on dry ice and stored at -80° C. Proteins were extracted from the leaves as described in Chapter 3. 50 μg of leaf proteins from each transformed line, as well as wild-type, were electrophoresed on SDS-PAGE gels, blotted to PVDF membrane and immunodetected with anti-RNS2 antibodies as described in Chapter 4. Judgements were made by eye as to whether RNS2 bands for each lane were less intense than the wild-type RNS2 band on the same blot.

In the next screen for antisense *RNS2* plants, 74 kanamycin-resistant independent transformants of p1525 and 63 of p1527 were screened for lowered *RNS2* mRNA levels by RNA gel analysis. Original transformants (T1 generation) and wild-type plants were grown in soil and leaves harvested as for the p1449 transformants, except that the harvested leaves were frozen in liquid nitrogen. Total RNA was extracted from leaves and RNA gel blots prepared as described in Chapter 2. RNA gel blots were probed first with the *eIF4A* probe as described in Chapter 2, and were then stripped and re-probed with an *RNS2* RNA probe such that only sense RNA strands would be detected. This probe was made using a Riboprobe kit (Promega), with plasmid p1127 linearized with Eco RI, and T3 RNA polymerase. Levels of *RNS2* and *eIF4A* mRNA were measured by Phosphorimager analysis, the *RNS2*/*eIF4A* ratio was calculated for each line, and these numbers were divided by the *RNS2*/*eIF4A* ratio of the wild-type sample on the same blot to calculate the

relative level of *RNS2* mRNA in putative antisense *RNS2* lines. Lines in which the level of *RNS2* mRNA was less than or equal to 70% of that in wild-type were selected for re-screening. Several kanamycin-resistant progeny (T2 generation) were grown in soil, leaves harvested, and relative *RNS2* mRNA levels calculated as done for the T1 generation.

RNS1 *root overexpression plants:* Independent initial transformed lines (T1 generation) containing the BinAR-SbPRP1-RNS1 construction were grown in soil. Seeds (T2 generation) from ten of these lines were screened for overexpression of *RNS1* in roots, as compared to a transgenic line containing only the pBI121 vector. Approximately 200 seedlings from each line were grown on AGM medium containing kanamycin; the plates were placed vertically such that roots grew vertically down the surface of the medium. After four to five weeks of growth at 22° C and a daylength of 16 hours light/8 hours dark, roots were excised, frozen in liquid nitrogen and stored at -80° C. RNA extraction was done and RNA gel blots were prepared as described in Chapter 2. RNA gel blots were probed first with an *RNS1* random-primed probe, and then stripped and re-probed with the *eIF4A* probe, as described in Chapter 3. Levels of *RNS1* and *eIF4A* mRNA were measured by Phosphorimager analysis, the *RNS1/eIF4A* ratio calculated for each line, and these numbers divided by the *RNS1/eIF4A* ratio of the control line on the same blot to calculate the relative level of *RNS1* mRNA in putative *RNS1* root overexpression lines.

To compare endogenous *RNS1* expression levels in roots with the transgenic lines described above, Columbia wild-type seeds were grown on P_i-rich and P_i-deficient media (Chapter 3) on plates placed vertically under the same conditions as above. Roots were

collected from four- to five-week-old plants. From one portion of each set, RNA was extracted and RNA gel blots were prepared and probed as described above. Proteins were extracted from another portion of each set and resolved on RNase activity gels as described in Chapter 3.

Anthocyanin Assays

For assay of anthocyanin content in antisense *RNS1* lines, seeds of T3 or T4 lines were grown on P_i-rich or P_i-deficient media and harvested seven days after transfer as described in the previous section. Fresh weight was recorded for each sample. Seedlings were frozen in liquid nitrogen, lyophilized in 13 ml plastic test tubes (Sarstedt) and pulverized with 3 mm diameter glass beads (Fisher). Anthocyanin content from each line was measured using a protocol based on the methods of Rabino and Mancinelli (1986), Feinbaum and Ausubel (1988), and Kubasek et al (1992). Ground tissue was shaken in 2.5 ml of 1% HCl/methanol for two hours at room temperature. 2 ml of chloroform were added and the mixture vortexed, after which 5 ml of H_2O were added and the vortex repeated. After separating the phases by centrifugation, 1 ml of the aqueous/methanol phase was assayed using a Beckman DU Series 600 spectrophotometer. Absorbance at 530 nm minus absorbance at 657 nm was used as a measure of anthocyanin content; values were normalized to the fresh weight of each sample. Two separate experiments were done, each including four plates for each line (two on P_i-rich and two on P_i-deficient media) such that four readings were incorporated in each instance.

CHAPTER 6

CONCLUSIONS AND FUTURE PROSPECTS

The discovery of genes for S-like RNases in plants (Taylor and Green 1991) was the first indication that T_2/S type RNases are present in self-compatible plants, and opened up many questions about the roles of these types of RNases in plants. An initial goal of this thesis project was to compare the protein structures and gene expression of the RNS family in *Arabidopsis* with those of each other and other plant T_2/S RNases. These studies revealed important structural differences between the S-RNases and the S-like RNases (Chapter 2). Moreover, the *RNS* genes are induced during senescence, and *RNS1* and *RNS2* are induced during P_i starvation (Chapters 2 and 3). The induction of *RNS2* by starvation for P_i was one of the first reports of a P_i-starvation inducible transcript, and the first for which a physiological role could be proposed (in remobilization of P_i). The *RNS1* and *RNS2* cDNAs have since been used in several other laboratories as molecular markers for P_i starvation and senescence (e. g. Callard et al. 1996).

These observations led to the next phase of this thesis project, in which experiments designed to further elucidate potential roles of the RNS proteins were begun. The localization of RNS2 to the cell wall (Chapter 4) is an important first step in pinpointing the cellular role(s) of RNS2. If antisense plants with lower amounts of *RNS2* than those described in Chapter 5 can be obtained, possibly by crossing separate antisense lines or selection of homozygous lines, the proposals that RNS2 is involved in phosphate and/or nucleoside remobilization, and possibly plant defense, can be more easily tested. A selection of bacterial and fungal plant pathogens should be tested, since defense reactions to different pathogens can vary. Should it not be possible to obtain antisense plants with significantly decreased amounts of RNS2, it may be feasible to obtain a transgenic line

whose *RNS2* gene has been disrupted by T-DNA insertional mutagenesis, using a recently developed method (Krysan et al. 1996).

The *RNS1* gene is much more strongly induced during P_i starvation than the *RNS2* gene, and so may have a more crucial role in the response of the plant to this stress condition. Antisense plants with low amounts of RNS1 have been obtained and preliminary characterization on the lines performed (Chapter 5). Some characteristics of these lines, namely increased anthocyanin deposition and a sometimes-seen *pho1*-like phenotype, indicate that these plants may be impaired in the P_i starvation response, but more characterization of the lines is needed. Possible future experiments would be the determination of free P_i levels in antisense and control plants grown in media containing varying concentrations of P_i, or perhaps media in which RNA is included as the sole P_i source. Similarly, a dose-response experiment in which plants would be grown on media containing decreasing amounts of P_i would be useful for monitoring with other molecular markers that indicate P_i starvation, such as the *PAP1* acid phosphatase gene of *Arabidopsis* (T. McKnight, personal communication). In this way, it may be possible to determine if antisense *RNS1* plants begin to show symptoms of starvation for P_i at higher P_i levels than control plants.

With the completion earlier this year of the sequencing of the yeast genome, a yeast member of the T_2/S RNase family has recently been identified. Targeted inactivation (Rothstein 1991) of this gene is in progress. Characterization of the effects of this inactivation, if any, and subsequent complementation with the plant *RNS* genes, may provide insight into the cellular roles of the RNS group of RNases.

The study of plant RNases has the potential to provide much information on plant RNA metabolism and nutrient remobilization. In addition to these contributions to basic sciences, RNases could have applied significance. For example, male sterile maize (Mariani et al. 1990) and corresponding restorer lines (Mariani et al. 1992) have been created by expressing heterologous RNase and RNase inhibitor genes in appropriate cell types. Heterologous expression of a double-stranded RNA activated RNase system in plants has shown promise in protecting plants against RNA viruses (Ogawa et al. 1996). RNases is also being explored as anti-tumor (Wu et al. 1993) and anti-AIDS virus (Saxena et al. 1996) agents. As most of the families of plant RNases have yet to be characterized molecularly, further research in this field should contribute to basic and possibly applied science.

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LIST OF REFERENCES

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