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By

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ABSTRACT

REGULATION AND FUNCTION OF *ARABIDOPSIS THALIANA* SECRETED RIBONUCLEASES

By

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While secreted ribonucleases (RNases) have been well studied at the enzymatic and structural levels, little is known regarding their biological functions. One family of secreted RNases, the RNase T_2 family, is particularly widespread, with members throughout various kingdoms. In recent years, many plant members of this superfamily have been identified. Gametophytic self-incompatibility in several plant groups involves the activity of S-RNases, one subfamily of plant T_2 RNases. Another subfamily, the S-like RNases, are found in self-compatible as well as self-incompatible plant species, indicating that they have different functions than have S-RNases. Expression patterns of S-like RNases in many species led to the suggestion that these enzymes are involved in remobilization and recycling of phosphate from nucleic acid sources. The project described in this thesis involves the study of secreted RNases in the model plant *Arabidopsis thaliana*, which has five members of the S-like subfamily. I found that several Arabidopsis RNases are induced in wounded tissues through an as-yet unidentified signaling mechanism. One S-like member, RNS1, is induced both locally and systemically by wounding through a novel signal transduction pathway. The regulation of *RNS1* expression was studied using several reporter constructs. I found that

transcriptional regulation accounts for the majority of the wound response of this gene. Several other factors were shown to affect *RNS1* transcript levels, including abscisic acid, salt stress, and heat shock.

In addition to regulatory mechanisms controlling *RNS1* transcription, the function of RNS1 was also studied. Tools used for these studies include plants with constitutively high RNS1 activity and T-DNA mutant lines with insertions in the *RNS1* gene. One of these mutants, *rns1-2*, has little or no RNS1 activity. Conditions that affect *RNS1* transcript accumulation provided a starting point for the analysis of the mutant and overexpressor. The observation that the overexpressors grew better than wild type when supplied with RNA as the sole source of P_i supports the hypothesis that RNS1 is involved in P_i remobilization. Analysis of the mutant and overexpressor also revealed that RNS1 activity levels affect root length in Arabidopsis. This effect was not dependent on P_i concentration and indicated that secreted RNases are involved in unexpected processes. Certain phenotypes of the *rns1-2* plants resembled those of the *rny1*Δ mutant. Rny1, the only RNase T_2 enzyme found in *Saccharomyces cereviseae*, has been proposed to function in regulating aspects of membrane stability and permeability. Therefore, I propose that RNS1 may also be involved in similar processes.

Finally, the analysis of an Arabidopsis mutant with altered activity levels of several RNA-degrading enzymes is presented. Although one of the increased activities resembles RNS1, I demonstrate that this activity is not RNS1. The altered activities are specific to stems of the mutant, as are its morphological phenotypes. This mutant, *arp1*, provides an insight into the involvement of RNases in stem-associated processes and stem development.

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CHAPTER 1

INTRODUCTION: PLANT RNA-DEGRADING ACTIVITIES

The class of proteins known as ribonucleases (RNases) encompasses a diverse group of enzymes whose catalytic activity involves the degradation and catabolism of RNA. There are numerous ways in which one might categorize RNases, including primary structure homology, cellular localization, and enzymatic cleavage mechanism or other biochemical properties, to name a few. RNases are involved in many processes in cellular function. Perhaps their most obvious function is the controlled processing and degradation of mRNA as a mechanism of gene regulation, although to imagine that RNases are restricted to such processes is likely a major underestimation of their functions. In fact, in most systems, the genes encoding proteins involved in mRNA degradation are still unknown. In contrast, many RNA-degrading activities that are not likely to be involved in the processing or degradation of mRNA have been characterized and the genes encoding them cloned, as is the case in the field of plant RNases. Likely the most well-characterized of these activities are those that fall into the RNase T_2 superfamily.

More than one review has been published in recent years in the field of plant RNases and/or mRNA degradation (examples include Bariola and Green, 1997; Gutiérrez et al., 1999; and Johnson et al., 1998). Therefore, this chapter will not attempt to reiterate such reviews. Instead, it will focus on recent findings in regard to the expression patterns and putative functions of plant secreted RNases and the signaling mechanisms involved in their regulation, with particular attention paid to the S-like RNases, one class of plant T_2 RNases.

Secreted ribonucleases and the RNase T_2 family

Biochemists have long used secreted RNases for structural and enzymatic studies, due to the relative ease in their purification and the availability of large quantities of proteins.

RNase A, for example, is a low molecular weight, stable enzyme that has been purified in large quantities from many vertebrate species. Perusing any review on pancreatic RNases, which mostly focus on RNase A family members, will enlighten the reader on several aspects of protein structure and enzymatic mechanisms (see, for example, Cuchillo et al., 1997) and is therefore quite telling regarding the focus of past studies on these particular subjects. While much is known on base-specificity, protein folding structures, and role of specific amino acids in catalysis, little is known about the physiological functions of these enzymes. Further, even fortuitous findings of "special" biological actions of RNases (D'Alessio et al., 1991), including antitumor and angiogenic activities, have not been explained biologically.

The T_2 superfamily of RNases has also been well-studied at the enzymatic and structural levels (Irie, 1997). First identified in the fungus *Aspergillus oryzae* (Sato and Egami, 1957), RNase T_2 and its relatives are secreted acid endonucleases with no base-specificity, sharing two conserved stretches of amino acid residues important in the enzymes' catalytic sites (Irie, 1997). Unlike the RNase A superfamily, members of which have so far been identified only in vertebrates (Beintema et al., 1997), T_2 enzymes are incredibly widespread. To date, members of this family have been found in nearly every system examined for their presence, encompassing viruses, bacteria, plants, and animals (Irie et al., 1997). The ubiquitous distribution of T_2 RNases may be interpreted as the enzymes' having both an ancient origin and critical function(s) (Taylor and Green, 1991).

Function: the S-RNases

Despite the apparent necessity for the activity of these enzymes, very little has been demonstrated regarding their biological functions. The exception is the S-RNases, a class

of plant T_2 RNases, whose activity is required for the process of self-incompatibility in several plant families, including the Solanaceae (reviewed in McCubbin and Kao, 2000). Briefly, when the *S*-allele of a pollen grain growing through the style of the mother plant matches either of the two *S*-alleles in the pistil, growth of the pollen tube is inhibited. RNase activity has been shown to be essential for this incompatibility system (Lee et al., 1994). However, the mechanism through which the S-RNase imparts incompatibility is still unknown. Available evidence indicates that the S-RNases somehow act as cytotoxins in the incompatible tubes (McClure et al., 1989). The pollen component of the interaction has not been identified, nor is it known at what site the two components interact. The two leading models in the field predict that either 1) the S-RNases are specifically imported into incompatible pollen tubes or 2) nonspecific S-RNase uptake occurs, with subsequent specific inactivation of all compatible RNases or some other similar modification (Parry et al., 1997). Recent evidence supports the latter hypothesis, in that S-RNases appear to be taken up by pollen tubes in a genotype-independent manner (Luu et al., 2000).

In summary, the S-RNases are the sole members of the T_2 RNase superfamily who have been assigned a function. Yet, there is much to be understood regarding the mechanism of the incompatibility reaction.

The S-like RNase subfamily: general features

Soon after the identification of the S-RNases as members of the T_2 family, several groups reported similar proteins in self-compatible plant species, including Arabidopsis, tomato, and zinnia. Although these S-like RNases share certain structural features with the S-RNases, conserved regions specific to each group can also be found (for a comprehensive review of S-like RNases, see Bariola and Green, 1997). These enzymes

are obviously not involved in self-incompatibility, but appear to play important roles throughout the plant kingdom, as S-like members have now been identified in both self-compatible and self-incompatible species. The *Arabidopsis thaliana* genome contains five genes, *RNS1* to *RNS5*, with high similarity to the S-like RNase gene family (Taylor and Green, 1991; G.C. MacIntosh, N.D. LeBrasseur, and P.J. Green, unpublished), and RNase activity has been demonstrated for the products of three of the *RNS* genes (Taylor et al., 1993; Bariola et al., 1994).

Studies on S-like RNases have for the most part focused on gene expression or activity patterns, as will be discussed below. Limited biochemical and structural information is also available. As mentioned, the S-like RNases are secreted enzymes. While many are extracellular, including RNS1 (Bariola et al., 1999), some have been shown to be retained intracellularly. These include RNS2 (Bariola et al., 1999) and several activities in tomato that are localized to the vacuole or endoplasmic reticulum (Löffler et al., 1992; Lehmann et al., 2001). Alternate cellular localization indicates that multiple members of the S-like subfamily in the same plant may carry out separate functions or the same functions in different compartments.

The crystal structure of the tomato extracellular S-like RNase LE has been determined at 1.65 Å resolution (Tanaka et al., 2000). Other T_2 enzymes that have been crystallized include an S-like RNase from bitter gourd (Nakagawa et al., 1999) and the fungal enzyme RNase Rh (Kurihara et al., 1996). Comparison of these and other T_2 enzyme structures has revealed two structural groups: the animal/plant and the fungal subfamilies (Tanaka et al., 2000). However, the groups appear to share catalytic mechanisms despite these structural differences (Tanaka et al, 2000).

Plant nuclease I-type enzymes

Early classification of plant RNA-degrading activities based on biochemical properties of the enzymes identified several groups: RNase I, RNase II, nuclease I, and exonuclease I (Farkas, 1982; Wilson, 1982). While most T_2 RNases appear to fall within the RNase I class, many plant activities have been identified with characteristics placing them in the nuclease I family, including activities in tobacco, barley, mung bean, zinnia, and rye (reviewed in Bariola et al, 1997). Nuclease I enzymes are also extracellular heat-stable proteins, but unlike T_2 RNases, nucleases have the ability to degrade both RNA and single-stranded (ss) DNA. In general, they also have higher molecular weights, in the range of 31 to 42 kDa, are glycosylated, and have acidic pH optima. They are sensitive to EDTA and also require Zn^{2+} for activity and stability (Fraser and Low, 1993).

Until recently, sequence information was not available for many plant nucleases. Determination of the amino acid sequences of two fungal nucleases, P1 from *Penicillium citrum* (Maekawa et al, 1991) and S1 from *Aspergillus oryzae* (Iwamatsu et al, 1991), has allowed the cloning of genes encoding similar proteins in plants. Nuclease I cDNA sequences are now available for daylily (Panavas et al., 1999), celery (Yang et al., 2000), barley, zinnia, and Arabidopsis (Aoyagi et al., 1998; Pérez-Amador et al., 2000).

Regulation and putative functions of S-like and nuclease I enzymes in plants

To date, fluctuations in RNase activity levels or gene expression have provided the most useful data for predicting RNase function. Through elucidating the biotic and abiotic cues that correlate with increases in RNase activity, we have been able to postulate functions that secreted RNases play in the plant. The following sections will briefly highlight significant examples and recent findings concerning RNase and nuclease induction under

various environmental and developmental conditions and summarize conclusions drawn from these expression patterns.

RNases and phosphate starvation

Induction of S-like RNases and the genes encoding them is strongly associated with growth on low concentrations of inorganic phosphate (P_i). Pertinent examples include the Arabidopsis *RNS1* and *RNS2* genes and proteins (Bariola et al., 1994, 1999), and intra- and extracellular tomato activities RNases LX (Bosse and Köck, 1998) and LE (Nürnberger et al., 1990), respectively. In addition to the induction of enzymatic activity, it was shown that the *LE* and *LX* mRNAs also accumulate upon P_i starvation (Köck et al., 1995). The genes encoding the *Nicotiana alata* RNase NE (Dodds et al., 1996), and the *Prunus dulcis* RNase PD2 (Ma and Oliveira, 2000), both of which are highly similar to *RNS1*, are also responsive to low P_i.

In-depth analyses of signal transduction pathways controlling P_i-sensing and response to low P_i have only recently begun. Studies in the tomato cell culture system support the existence of both intracellular and extracellular mechanisms for sensing P_i levels and initiating RNase induction (Glund et al., 1990; Köck et al., 1998). Maintenance of P_i homeostasis in plants is probably controlled by at least two signaling mechanisms, one at the cellular level and another regulating multiple organs and likely originating from the shoots (Raghothama, 2000). There is little direct evidence that plant hormones are involved as primary signals in the P_i-response, but ethylene and auxin may be involved in altering root architecture and root hair growth (Lynch and Brown, 1997). However, cytokinins repress the expression of P_i-starvation inducible genes in Arabidopsis, but do not affect root hair alterations that are controlled by local P_i

concentrations, indicating that cytokinins are possibly involved in the repression of systemically controlled P_i-starvation responses (Martín et al., 2000). Cytosolic calcium levels and the activity of Ca^{2+}-ATPases are thought to be involved in regulating the response and adaptation of plants to low P_i (Lynch and Brown, 1997). Homeodomain leucine zipper proteins have been shown to bind to the phosphate response domain of the soybean *VspB* gene, placing these types of transcription factors as likely candidates for effectors of gene induction (Tang et al., 2001). Recent screens have identified mutants in the P_i-response pathway that should allow us to dissect the signal transduction pathways in detail in the future. At least one of these mutants is unable to induce RNS1 under low P_i conditions (Chen et al., 2000). Future screens for constitutive P_i-starvation response mutants, such as those proposed by Ticconi et al. (2001), will add to the tools available to aid us in the understanding of signal pathways involved in plant molecular and developmental responses to P_i limitation.

The ever-growing number of RNases demonstrated to be responsive to low P_i has led to the hypothesis that S-like RNases are part of a rescue system employed by plants to recycle P_i when environmental pools are limiting (Goldstein et al., 1989). In conjunction with acid phosphatases and phosphodiesterases, RNases could be involved in the generation of P_i through the hydrolysis of RNA (Glund and Goldstein, 1993). Extracellular secretion of a fungal phytase from Arabidopsis roots has been shown to increase phosphorous nutrition in the plant (Richardson et al., 2001). Similarly, RNases secreted from the root system to the rhizosphere could breakdown organic matter in the surrounding soil into P_i. A similar function has been attributed to two secreted fungal nucleases (Fraser and Low, 1993). Alternatively, RNases could be involved in recycling

cellular material from dead or dying cells, either internally (in the case of vacuolar and ER-localized RNases) or externally (in the case of secreted RNases).

Experiments done in Arabidopsis have supported this theory. RNS1 and RNS2 activities were diminished using antisense techniques, and each line was found to have increased anthocyanin contents. The phenotype was particularly dramatic when the seedlings were starved for P_i (Bariola et al., 1999). The authors suggested that this effect might be due to the plants' reduced abilities to acquire P_i, especially under low P_i-levels, although it could not be ruled out that the plants were somehow otherwise stressed, leading to high levels of anthocyanins.

Cell-death pathways

Programmed cell death (PCD) is an integral part of many plant developmental programs and environmental responses, including, but not limited to, senescence, the hypersensitive response (HR) during defense against pathogens, pollination, and germination (for recent reviews, see Beers and McDowell, 2001; Jones, 2001). There are numerous examples of S-like RNase and nuclease I-type activities induced during such processes.

The final stages of xylogenesis have been a paradigm for studying PCD mechanisms (for a review of xylogenesis, see Roberts and McCann, 2000). This process has been well-studied in the zinnia cell culture system, in which mesophyll cells can be induced to transdifferentiate into tracheary elements (TE; reviewed in Fukuda, 2000). This process is accompanied by induction of nuclease activities, including a 43-kDa protein that is apparently involved in autolysis, the final stage of vascular differentiation in plants (Thelen and Northcote, 1989). Two cDNA clones, ZRNaseI and ZRNaseII, have been isolated from differentiating zinnia cells and encode RNases with similarity to the S-like

RNase family. ZRNaseI is induced during late stages of TE differentiation (Ye and Droste, 1996). The in vivo function, as well as the substrates, of nuclease and RNase activities during TE formation is still a matter of speculation.

Nuclease and RNase activities are also associated with other cell death processes, such as senescence and the hypersensitive response. Pollination-induced petal senescence in petunia causes increases in RNase and ssDNase activities (Xu and Hanson, 2000). Activities of the nuclease I family are induced in senescing tissues in Arabidopsis and zinnia (Pérez-Amador et al., 2000). In Arabidopsis, *RNS2* and *RNS3* transcripts accumulate during leaf senescence (Taylor et al., 1993; Bariola et al., 1994). The gene encoding RNase LX is also induced in senescing tomato leaves (Lers et al., 1998), and the protein is expressed during xylem differentiation, germination, and senescence (Lehmann et al., 2001). In tobacco, the hypersensitive response resulting from both TMV and bacterial infection is associated with induction of extracellular nuclease activities (Mittler and Lam, 1997). Barley aleurone cells contain several nuclease activities and an S-like RNase that are induced by gibberellic acid and repressed by abscisic acid (Brown and Ho, 1987; Fath et al., 1999; Rogers and Rogers, 1999). The timing of induction of these activities correlates with hydrolysis of nuclear DNA and the progression of cell death. It has been hypothesized that the barley nucleases are involved in the degradation of the endosperm in response to seed germination (Fath et al., 1999).

It is possible that RNase and nuclease activities are critical components of PCD, required for the progression of the developmental pathway. It has been suggested that nucleases could be involved in the degradation of nuclear DNA during PCD (Fath et al., 1999; Mittler and Lam, 1997). However, most activities studied appear to be either

extracellular or associated with the vacuole or ER. Thus, they may be involved in late stages of the process, after the commitment step of vacuolar lysis, for example (Obara et al., 2001), or after lysis of the cell itself. In such a role, they could be components of recycling pathways engaged in order to mobilize useful molecules from dying cells to growing parts of the plant.

A more interesting hypothesis has been proposed by Lehmann et al. (2001). After demonstrating that RNase LX is retained in the ER, they suggested that hydrolytic enzymes, such as proteases and RNases, found in distinct ER domains or ER-derived structures such as ricinosomes play an important role in controlled degradation processes. Upon swelling and rupture of ER- or ER-derived membranes following disruption of the tonoplast (Fukuda, 1997), RNase LE and other hydrolytic enzymes would be brought in contact with its RNA substrate in the cytoplasm (Lehmann et al., 2001). Similar mechanisms may occur in other plant species during many degradative processes, such as senescence, endosperm mobilization, and xylem differentiation.

RNases and defense

Increases in RNase and nucleases activities during pathogen invasion have been found in several systems (for references, see Green, 1994). Two parsley pathogenesis-related (PR) proteins induced by a fungal pathogen show homology to a ginseng RNase (Moiseyev et al., 1994). These proteins are part of the large PR-10 family of intracellular plant PR proteins, and are not related to the S-like RNase or nuclease I family, but do support a role for RNases in defense responses. In *Nicotiana tabacum*, transcripts for the S-like RNase NE are induced by inoculation of leaves with the oomycete *Phytophthora* (Galiana et al., 1997). Interestingly, exogenous application of RNase A inhibited

development of this pathogen on the leaves by as much as 90%, without eliciting general defense responses such as HR. Additionally, RNase A inhibited the formation of lesions caused by tobacco mosaic virus (TMV; Galiana et al., 1997). A 30-kDa protein sharing similarity with a tomato RNase has been isolated from leaves of *Engelmannia pinnatifida* and shown to have broad-spectrum antifungal activity (Huynh et al., 1996). An extracellular RNase activity in rust-infected wheat leaves has also been reported (Barna et al., 1989), and as many as 20 isoforms of RNases are induced in pearl millet during SAR after challenge with downy mildew disease (Shivakumar et al., 2000). RNS1 antibody (Bariola et al., 1999) appears to cross-react with a protein induced by SAR in this system (P.D. Shivakumar and V. Smedegaard-Petersen, personal communication).

RNases are also induced by wounding in several systems. In tomato, for example, the transcript for RNase LE accumulates in wounded leaves (Lers et al., 1998). RNase NW, a tobacco protein with high sequence similarity to RNase NE, is also induced in wounded leaves (Kariu et al., 1998). Since both pathogen challenge and mechanical wounding have effects on RNase activity, it is possible that RNA-degrading enzymes have roles in defense responses. During wounding, RNases may function in a manner similar to their role in P_i-recycling, including recycling of nucleotides from damaged cells. They may also be involved in rebuilding of damaged vascular tissue, consistent with their induction during xylogenesis. RNases and nucleases may play a more specific role as defensive proteins to protect damaged or particularly susceptible tissues from pathogen invasion. This hypothesis is supported by the high expression levels of several RNase transcripts in pistils. Despite the fact that these structures are rich in nutrients, making them likely targets for pathogen invasion, they are rarely infected, possibly due to

the presence of various defense-related proteins (Atkinson et al., 1993). These defensive proteins may include S-like RNases, as S-like genes are expressed in pistils and flower structures of Arabidopsis (*RNS1*, *RNS2*, and *RNS3*; Taylor et al., 1993; Bariola et al., 1994), tobacco (*NE*; Dodds et al., 1996), and petunia (*X2*; Lee et al., 1992).

New ideas regarding secreted RNase functions

Recent results from our laboratory have prompted new ideas regarding functions for secreted RNases. These ideas have come from the study of the only member of the RNase T_2 family found in *Saccharomyces cerevisiae*. Inactivation of this RNase, Rny1, resulted in unexpected phenotypes: the mutant cells are larger than wild-type, grow as cell-aggregates, and are heat- and salt-sensitive. Complementation of the heat and salt-sensitivity phenotypes was accomplished using Rny1, three of the Arabidopsis RNSs, and the structurally unrelated enzyme RNase A. Mutation of RNase A that caused a loss of RNase activity but retained the protein's stability and structure resulted in the inability of the protein to complement the growth phenotype (MacIntosh et al., 2001). These phenotypes, in combination with recent experiments that demonstrated that specific RNAs can bind to membranes and thereby alter membrane permeability (Khvorova et al., 1999), led to the hypothesis that secreted RNases can control membrane stability and permeability by regulating the amount of RNA in the membrane (MacIntosh et al., 2001). More recent experiments have also shown that exogenous application of RNase A to the culture medium complements the flocculation phenotype of the *rny1*Δ cells (G.C. MacIntosh and P.J. Green, unpublished). Thus, it appears that an extracellular RNase activity can affect membrane characteristics in yeast.

Dissertation topic and thesis overview

In summary, T_2 RNases and nucleases I enzymes are widely distributed in nature and tightly regulated in response to a variety of signals. Yet in most cases, their biological roles and substrates remain elusive. The main objective of my researach was to determine the biological significance of extracellular RNases in plants. To address this question, several methods have been employed: studying the mode of regulation of the Arabidopsis S-like RNase, RNS1; identifying signals that direct the expression of *RNS1*; analyzing the effects of mutation and overexpression of *RNS1*; and examining a mutant with altered RNase activities. Identifying signals in addition to P_i starvation that affect *RNS1* expression should help us deduce the processes in which RNS1 activity is important. Analysis of mutant and overexpressing plants during these processes may then reveal functions of RNS1. Chapter 2 will detail my findings that RNS1 and other RNA-degrading activities are regulated by wounding in Arabidopsis through a novel signaling pathway. The regulation of RNS1 will be further analyzed in Chapter 3, including other signals that control *RNS1* expression and their effects on the *RNS1* promoter. Functional questions will be addressed in Chapter 4, in which I will summarize recent data derived from the analysis of plants lacking RNS1 and of plants with constitutively high RNS1 activity. Finally, in Chapter 5, I will present my contribution to the analysis of an Arabidopsis mutant that has high levels of several RNase activities.

CHAPTER 2

LOCAL AND SYSTEMIC WOUND INDUCTION OF RNASE AND NUCLEASE ACTIVITIES IN ARABIDOPSIS: RNS1 AS A MARKER FOR A JASMONIC ACID-INDEPENDENT SYSTEMIC SIGNALING PATHWAY

A version of this chapter is in press:

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ABSTRACT

Induction of defense-related genes is one way plants respond to mechanical injury. We investigated whether RNases are involved in the wound response in *Arabidopsis thaliana*. As in other plant systems, several activities are induced with various timings in damaged leaves, stems, and seedlings in Arabidopsis, including at least three bifunctional nucleases capable of degrading both RNA and DNA, as well as RNS1, a member of the ubiquitous RNase T_2 family of RNases. The strong induction of *RNS1* expression is particularly interesting because it occurs both locally and systemically following wounding. The systemic induction of this RNase indicates that members of this family may be involved in defense mechanisms in addition to their previously hypothesized functions in nutrient recycling and remobilization. Additionally, the systemic induction appears to be controlled independently of jasmonic acid, and the local induction of RNS1 and the nuclease activities is independent of both JA and oligosaccharide elicitors. Consequently, a novel systemic pathway, likely involving a third signal, appears to exist in Arabidopsis.

INTRODUCTION

One of the mechanisms by which plants respond to wounding, either mechanical or feeding-related, is the activation of transcription of a variety of genes. The products of these genes include defensive proteins such as proteinase inhibitors (Boulter, 1993; Ryan, 1990), components of signal transduction pathways, for example, systemin in tomato and potato (Ryan and Pearce, 1998), proteins that may be involved in wound healing (Bowles, 1990), and other proteins whose functions in the wound response are as yet unknown.

While the expression of some of these genes is proximal to the injury site, others are expressed at greater distances and constitute the systemic response (Green and Ryan, 1972). In tomato, multiple signals appear to be involved in activating gene expression. Local responses are thought to be controlled by carbohydrate signals released from injured plant cell walls (Farmer and Ryan, 1992). Long-distance effects are initiated by the small peptide systemin (Ryan and Pearce, 1998). Both oligosaccharides and systemin initiate the de novo production of abscisic acid (ABA) and jasmonic acid (JA), which leads to accumulation of the transcripts of wound-responsive (WR) genes (León et al., 2001). Ethylene also appears to be involved in the amplification of systemin-activated signaling (O'Donnell et al., 1996). In addition, electrical signals have been implicated in the systemic response (Wildon et al., 1992). In general, wound signals in tomato are thought to constitute a unified reaction that mounts both local and systemic defense and repair responses (León et al., 2001), although a JA-independent WR gene has been identified (O'Donnell et al., 1998).

In Arabidopsis, the emerging picture has striking differences in comparison to the

tomato system. In Arabidopsis, two distinct signaling pathways can be identified. Some genes whose transcripts accumulate upon wounding are independent of JA, while the expression of others requires jasmonate synthesis and perception (Titarenko et al., 1997). Recently, the JA-independent response was shown to be activated by oligosaccharides (Rojo et al., 1999). It was suggested that the two pathways work in an antagonistic manner, so that the inhibition of the JA-dependent pathway occurs in local wounded leaves through the induction of ethylene by the oligosaccharide elicitors. Conversely, only JA-dependent genes would be activated systemically, due to the limited diffusion range of the oligosaccharides (Rojo et al., 1999). However, a JA-dependent WR gene has been found that is strongly expressed both locally and systemically in Arabidopsis (Kubigsteltig et al., 1999).

In other systems, RNases have been shown to accumulate in response to mechanical wounding or pathogen attack, as discussed in Chapter 1. Although several wound-responsive RNases have been identified, the signaling pathways responsible for RNase induction are rarely addressed. In order to investigate the effects of wounding on the expression of the S-like family of Arabidopsis RNases, we examined the RNase profile of wounded plants. We found not only T_2 enzymes, but also multiple activities increase in damaged tissues in response to mechanical wounding, including several with both RNase and DNase activity. Within the RNase T_2 family, RNS1 activity increases, and the *RNS1* transcript accumulates in both wounded and systemic unwounded leaves of wounded plants. Unexpectedly, we found that the regulation of these activities by wounding is independent of both jasmonic acid and oligosaccharides, indicating that a novel, unidentified wound signaling pathway may be operating in Arabidopsis. Additionally, the

systemic induction of *RNS1* suggests that the enzyme may have a defensive function.

RESULTS

Wounding induced several RNase activities in Arabidopsis

To examine the effect of wounding on RNase expression in Arabidopsis, 2-week-old seedlings, or stems or leaves of 4-week-old plants were wounded. Samples were harvested at subsequent time points, and protein extracts were analyzed on RNase activity gels (Figure 2-1). Significant alterations in RNase activities were seen in the activity gels, including the activity of one well-characterized member of the RNase T_2 family, RNS1, and several other activities not likely to be related to this family. In Figure 2-1A, equivalent timepoints were taken from leaves of wounded and unwounded plants to check for any possible light regulation of RNase activities. Several activities can be seen even in unwounded leaves, the most prominent of which is a strong band of activity at 25 kDa; no RNases appear to be regulated in by light (Figure 2-1A, control lanes). Similar controls were taken for stems and seedlings, and there, too, no light/dark regulation is evident (data not shown). In contrast, in leaves, stems, and seedlings (Figures 2-1A, 2-1B, and 2-1C, respectively), multiple activities were induced by wounding, including an activity that migrated at approximately 35 kDa (Figures 2-1A – 2-1C, top arrow), two at 33 kDa (middle arrow; separation of the two 33-kDa bands can be seen in Figures 2-2A and 2-2B), and a fourth of approximately 23 kDa (bottom arrow, labeled RNS1).

Past studies have indicated that the activity of a well-characterized RNase T_2 enzyme, RNS1, is induced by phosphate starvation in seedlings and migrates at 23 kDa on RNase activity gels (Bariola et al., 1994, 1999). In the wounded samples, a 23-kDa

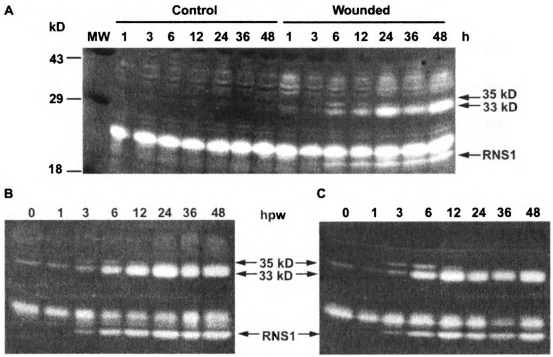


Figure 2-1. Multiple RNase activities are induced by wounding
Activity gels showing the increase in RNase activity in Arabidopsis at various time points after wounding. Bands seen on the gel are areas in which RNA cast in the gel has been degraded. Each lane contains 100 μg of protein from wounded leaves **(A)**, stems **(B)**, and seedlings **(C)**. Time, in hours from wounding to harvesting, is indicated above each lane of the gels. Arrows to the right of the gels indicate increased activities. Control lanes contain proteins from unwounded leaves. MW, molecular weight marker (Gibco-BRL). Sizes of the MW markers in kDa are shown to the left in **(A)**. hpw, hours post wounding.

activity was also induced (Figures 2-1A – 2-1C; bottom arrow, labeled RNS1). This activity was seen within 3 h and remained elevated for at least 48 h after wounding. RNA gel blot analysis indicated that the *RNS1* transcript was induced in wounded leaves (see below). Additionally, in an insertional knock out mutant of *RNS1*, the 23-kDa activity was not induced after wounding (see Chapter 4). Taken together, RNA gel blot analysis and mutant phenotypes confirmed that the 23-kDa activity induced by wounding was indeed RNS1. Thus, as in other plant systems, RNase T_2 enzymes are also induced by wounding in Arabidopsis. However, our results also demonstrate that RNase activities not related to T_2 enzymes respond to wounding in Arabidopsis.

A pair of 33-kDa activities induced in all three wounding experiments has a time course of induction similar to that of RNS1. In leaves, the doublet of activity at 33 kDa is induced within 6 h (Figure 2-1A, middle arrow). In stem tissue and in seedlings, the upper band of the doublet is evident even in unwounded samples. Still, both bands are enhanced after wounding, reaching an apparent maximum by 24 h (Figures 2-1B and 2-1C), and high levels of these activities can be found as late as 48 h post wounding (hpw).

Interestingly, unlike the sustained induction of the 23- and 33-kDa RNases, a fourth activity, at 35 kDa, is induced transiently. In leaves, this activity appears within 6 h of wounding (Figure 2-1A). In stems and in seedlings, the 35-kDa activity appears more rapidly, with induction evident within 3 h (Figures 2-1B and 2-1C). In all three cases, the activity is no longer visible by 24 hpw. Unlike RNS1, these wound-inducible activities exhibit characteristics that distinguish them from the RNase T_2 family, as discussed below.

Multiple bifunctional nuclease activities are increased by wounding

Previous studies using the gel assay system shown in Figure 2-1 demonstrated that a doublet of bifunctional nucleases capable of degrading both RNA and DNA migrate at 33 kDa; these nucleases are normally expressed at low levels but are upregulated in a recently isolated mutant of Arabidopsis (M.L. Abler, N.D. LeBrasseur, L.R. Danhof, D.M. Thompson and P.J. Green, manuscript in preparation; see Chapter 5). To investigate whether the 33-kDa RNase activities increased by wounding are bifunctional nucleases, protein extracts of wounded stem tissue were run on 9% acrylamide RNase and DNase gels. While other activity gels in this study contained 11% acrylamide, the lower acrylamide percentage used in these gels results in better separation of the two activities that migrate at approximately 33 kDa, as can be seen by comparing Figures 2-1B and 2-2A. Additionally, the increase in the intensity of the bands seen in the RNase activity gel at 33 kDa is mirrored in the DNase gel (Figure 2-2A), confirming that this doublet has bifunctional nuclease activity.

In addition to the 33-kDa doublet, another activity that increases upon wounding appears to have bifunctional activity. The transient 35-kDa RNase activity seen in Figure 2-1 also degrades DNA (Figure 2-2A). Therefore, wounding causes an induction not only of T_2 RNases in Arabidopsis, but also of several nuclease activities, including a previously unidentified nuclease at 35 kDa.

Past studies of RNase expression profiles in Arabidopsis have shown that cultivar variation is present. Specifically, the lower band of the 33-kDa doublet can normally be seen at low levels in the cultivar RLD but is absent in Columbia (M.L. Abler, L.R. Danhof, and P.J. Green, unpublished). However, the lower band can be induced in

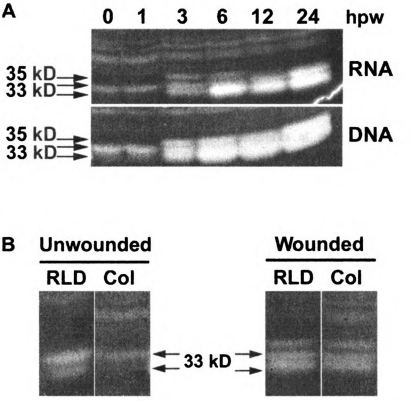


Figure 2-2. Bifunctional nuclease activities are induced by wounding.
(A) Protein samples (100 μg for RNase gel; 75 μg for DNase gel) from wounded stems were run on activity gels containing either RNA (upper panel) or DNA (lower panel) as a substrate. Both gels contain 9% acrylamide in order to separate the two activities at 33 kDa. Arrows to the left of the gels indicate wound-regulated bifunctional activities. hpw, hours post wounding.
(B) Stem protein samples (100 μg) from both Columbia (Col) and RLD (RLD) ecotypes were run on RNase activity gels. Unwounded samples are shown on the left, and stem samples taken 3 h after wounding are on the right. The lower band (lower arrow) is not found in unwounded Columbia stems but is present after wounding. Arrows indicate the upper and lower bands of the 33-kDa nuclease doublet.

Columbia upon wounding (Figure 2b). Thus, wounding indicates that although this activity is differentially regulated under nonwounded conditions in Columbia and RLD, it is not altogether absent from Columbia.

RNS1 activity is induced by wounding locally and systemically

As seen in Figure 2-1, an RNase activity that migrates at approximately 23 kDa is induced during wounding. During phosphate starvation in Arabidopsis seedlings, a 23-kDa activity is also induced (Bariola et al., 1994). The increase in this activity is due to an increase in the protein levels of RNS1 (Bariola et al., 1999) and corresponds to an increase in transcript accumulation (Bariola et al., 1994). Additionally, as mentioned, wounded leaves of an *RNS1* T-DNA insertion mutant lack the 23-kDa activity, (see Chapter 4). Therefore, we were interested in examining whether the *RNS1* transcript also accumulates in response to wounding. Rosette leaves of adult plants were wounded and then harvested at subsequent timepoints. RNA gel blot analysis demonstrated that the *RNS1* transcript accumulated within 3 h and was still elevated as late as 48 hpw (Figure 2-3A). Immuno-blot analysis confirmed that RNS1 protein accumulated with a time course similar to that of the activity increase (data not shown).

We also investigated whether the Arabidopsis response to wounding includes systemic induction of RNase or nuclease activities in non-wounded tissue. Unwounded rosette leaves adjacent to wounded leaves were harvested, and RNA was examined by RNA gel blot. The *RNS1* transcript was induced in unwounded leaves. The increase was transient and rapidly disappeared between 6 and 8 hpw (Figure 2-3B), unlike the more sustained increase of *RNS1* in wounded leaves (Figure 2-3A). Wounded seedlings (9 hpw; Figure 2-3B, lane W) were included in the blot for a comparison of transcript levels

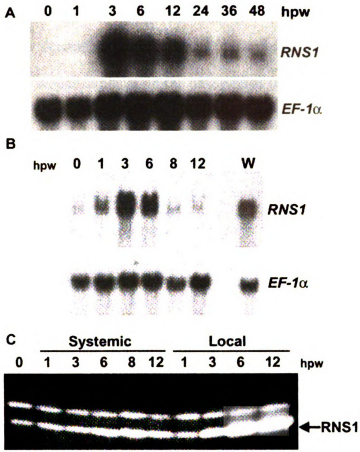


Figure 2-3. RNS1 activity and transcript increase transiently in wounded and in systemic, non-wounded leaves.
(A) Northern blot of wounded leaves. Arabidopsis leaves were wounded and harvested at subsequent timepoints. RNA was extracted, and 10 μg was separated by electrophoresis, blotted, and probed with *RNS1*. Blots were then stripped and reprobed with *EF-1α* to control for loading. hpw, hours post wounding.
(B) Systemic induction of the *RNS1* transcript shown by RNA gel blot analysis. Unwounded rosette leaves adjacent to the wounded leaves were harvested at the timepoints indicated above the lanes. The gel contained 10 μg total RNA per lane. RNA from seedlings harvested 9 h after wounding was included for comparison (W). The blot was stripped and reprobed with *EF-1α* as a loading control. hpw, hours post wounding.
(C) RNase activity gel showing systemic induction of RNS1 activity. Each lane contains 100 μg protein. The arrow indicates the RNS1 band. No other induced activities were detected in systemic leaves. Protein samples from wounded leaves were included for comparison of RNS1 activity levels.

induced locally and systemically. Additionally, protein extracts were examined by RNase activity gel assay to determine whether RNS1 or nuclease activities are induced in unwounded leaves of wounded plants. In the unwounded leaves, the 23-kDa RNS1 band was induced (Figure 2-3C), indicating that RNases may play a role in the systemic wounding response in Arabidopsis. As in wounded leaves, the systemic RNS1 activity increase could be seen slightly later than the transcript accumulation: in multiple experiments, we observed a slight increase in this activity between approximately 6 and 12 hpw. Wounded samples were included in the gel for comparison (W, Figure 2-3C). No other bands aside from RNS1 were induced systemically (not shown). Note that the activity gel in Figure 2-3C was incubated at pH 6.0, instead of pH 7.0, to enhance RNS1 activity, since no other activities were seen to be induced systemically at pH 7.0 (see Materials and Methods). As a result, the relative activity of RNS1 is higher compared to the constitutive 25-kDa band. It is interesting that the systemic activity increase does not appear to reflect the amount of transcript induced (compare 3 and 6 h in Figure 2-3B with 6 and 12 h in Figure 2-3C). The discrepancy may be due to a possible posttranscriptional regulation of RNS1.

The induction of *RNS1* and the nucleases is independent of jasmonic acid

Wounding responses in Arabidopsis are controlled by jasmonic acid (JA)-dependent and -independent signaling pathways (Titarenko et al., 1997). The presence of distinct wounding pathways in Arabidopsis has led to the proposal that the function of multiple signals is to control local and systemic gene expression differentially. Specifically, it has been suggested that the JA-dependent pathway controls induction of systemic responses, while an oligosaccharide-dependent pathway directs gene activation locally at the

Figure 2-4. RNS1 and nuclease induction by wounding is independent of jasmonic acid.
(A) Top panel; activity gel showing induction of all four wound-responsive activities in both the wild type (WT) and the JA-insensitive *coi1* mutant (*coi1*). Arrows to the right indicate the induced activities, including the nuclease at 35 kDa (top arrow), the doublet at 33 kDa (middle arrow), and RNS1 (bottom arrow). Bottom panel; systemic induction of RNS1 in the *coi1* mutant. hpw, hours post wounding.
(B) Northern blot analysis of wounded wild type (WT) and *coi1* (*coi1*) plants. The blot contains RNA (10 μg) from unwounded leaves (U), local 6-h wounded leaves (L), and unwounded leaves of wounded plants 3 h after wounding (S). The blot was probed with *RNS1*, stripped, and reprobed with *EF-1α* as a loading control.
(C) Activity gels containing 100 μg of protein from leaves of JA-treated (JA) or untreated (control) plants. Plants were treated with methyl jasmonate or buffer alone, and leaves were harvested at 0, 3, 6, and 24-h timepoints. W, protein from leaves 24 h after wounding. Arrows to the right of the gel indicate the running positions of wound-inducible activities.
(D) Northern analysis of RNA (10 μg) from leaves of JA-treated (JA) or untreated (control) plants. Plants were treated as in **(c)**. 2- and 24-h timepoints are shown. Similar results were seen at 3 and 6 h (data not shown). W, RNA from leaves 6 h after wounding. Blots were probed with *RNS1*, then sequentially stripped and reprobed with *AOS* to control for JA-treatment and with *EF-1α* as a loading control.

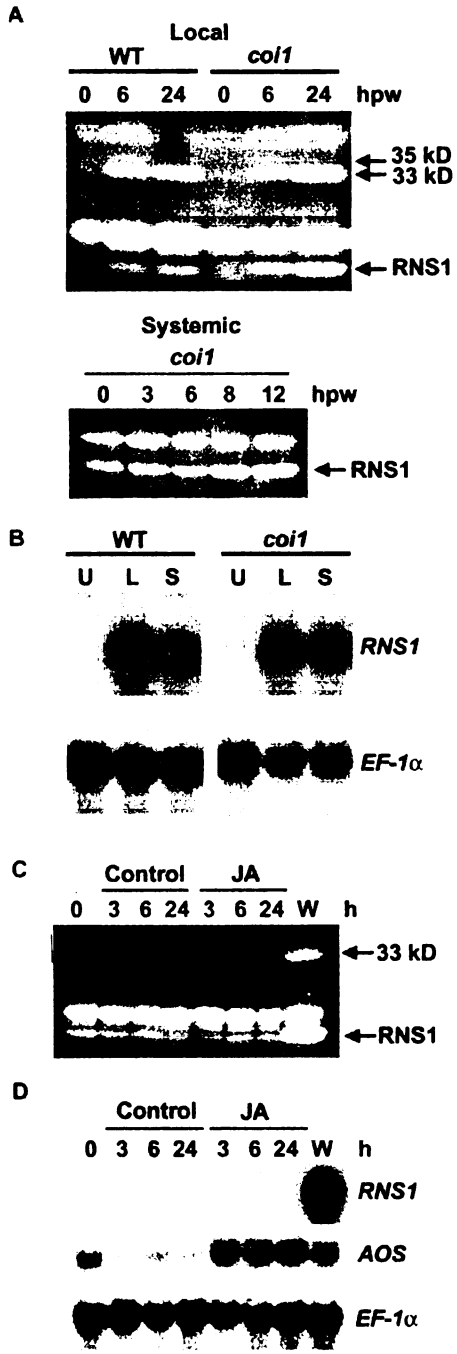


Figure 2-4. RNS1 and nuclease induction by wounding is independent of jasmonic acid.

site of damage (Rojo et al., 1999). Under this assumption, *RNS1* would be expected to be regulated by the JA-dependent pathway, as its transcript is induced systemically. In contrast, the bifunctional nuclease activities, which are not systemically wound-responsive, would be regulated independently of JA. The JA-insensitive mutant *coi1* (Feys et al., 1994) has been widely used to demonstrate dependence of the wound-induction of specific transcripts on JA (*e.g.*, Reymond et al., 2000; Titarenko et al., 1997). We investigated whether the wound induction of RNS1 or the nuclease activities are regulated by the JA-dependent pathway by wounding leaves of *coi1* plants. As expected, like the wild type, wounded *coi1* plants displayed increased levels of the 35- and 33-kDa activities (Figure 2-4A). The transient 35-kDa nuclease was seen in both wild-type and mutant plants at 6 hpw, and the nuclease bands at 33 kDa were induced at 6 and 24 hpw in both wild-type and *coi1* plants. However, wounded *coi1* leaves unexpectedly also had increased RNS1 activity. In fact, in contrast to systemic responses previously characterized in Arabidopsis, both local and systemic wound induction of *RNS1* is independent of JA. RNS1 activity was induced in *coi1* local wounded tissue (Figure 2-4A, upper panel) and in unwounded leaves of the same plants (Figure 2-4A, lower panel). RNA gel blot analysis demonstrated that the *RNS1* transcript also accumulated in the *coi1* mutant upon wounding (Figure 2-4B). Local 3-h and systemic 6-h timepoints are shown (Figure 2-4B, L and S, respectively). Systemic *RNS1* levels at 6- and 24-h timepoints were also similar in *coi1* and wild-type plants (data not shown).

To confirm that JA does not affect the induction of RNS1 or the nucleases, young plants were treated with methyl jasmonate (MeJA) and examined for RNase activity (Figure 2-4C). Efficacy of the MeJA treatment was shown by the accumulation of the

allene oxide synthase transcript (*AOS*), a component of the jasmonate pathway known to be induced by JA (Laudert and Weiler, 1998; Figure 2-4D). However, MeJA treatment did not cause *RNS1* accumulation after 3, 6, or 24 h of treatment (Figure 2-4D). Likewise, the 35- and 33-kDa nucleases, as well as RNS1, were not induced by MeJA at the activity level (Figure 2-4C). Thus, we have demonstrated that the wound-inducible RNase and nuclease activities are not controlled by the JA-dependent signaling pathway. Recent results from an independent microarray experiment also demonstrated that the local expression of *RNS1* is induced in a JA-independent manner (Reymond et al., 2000), supporting our observations.

***RNS1* is not induced by the oligosaccharide pathway**

It has been suggested that the JA-independent wound response in Arabidopsis is controlled by oligosaccharide elicitors. Transcripts whose accumulation after wounding is not dependent on JA can be induced by oligogalacturonic acids (OGAs), proteinase-inhibitor inducing factor (PIIF) fractions from tomato leaves, and chitosan (Rojo et al., 1999), all of which are rich in oligosaccharides. To test if one of these known inducers of certain JA-independent wound-inducible genes would also induce *RNS1* transcript accumulation, an OGA-rich fraction known as 'TFA-PIIF' (generously provided by Dr. E.E. Farmer; purified according to Bishop et al., 1984) was used to treat detached rosette leaves. Leaves were floated in an MS medium either with or without the addition of TFA-PIIF and harvested at subsequent timepoints. *RNS1* transcript levels were analyzed by RNA gel blot analysis (Figure 2-5A).

As seen in Figure 2-5A, the slight wounding involved in detaching leaves caused a

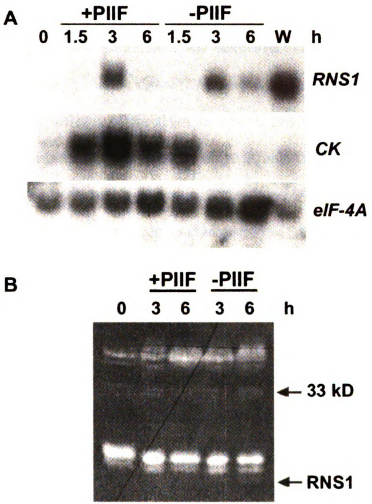


Figure 2-5. OGA-rich fractions do not induce RNase activities.
Leaves were detached from 4-week-old wild type plants and floated in buffer with (+PIIF) or without (−PIIF) the addition of TFA-PIIF. Samples were harvested at timepoints indicated above the lanes (in hours).
(A) Northern blot containing 10 μg RNA per lane. W, RNA from leaves 6 h after wounding. The blot was probed with *RNS1*, then sequentially stripped and reprobed with *Choline kinase* (*CK*) to control for PIIF-treatment and with *eIF-4A* as a loading control.
(B) Activity gel containing 100 μg protein per lane. Arrows to the right of the gel indicate the normal running positions of the wound-inducible activities.

higher than normal basal level of transcript in the samples after 3 h. However, in contrast to most known Arabidopsis JA-independent wound-induced transcripts, the presence of TFA-PIIF in the medium did not induce *RNS1* compared to the control (Figure 2-5A). Blots were also probed with choline kinase (*CK*), an OGA-induced transcript (Rojo et al., 1999), to demonstrate efficacy of the OGA treatment. The wounding involved in the detachment technique induced *CK* expression at the 90 min timepoint, regardless of the presence or absence of OGAs and consistent with the timing of *CK* wound-induction seen previously (Rojo et al., 1999). However, as expected, the TFA-PIIF-treated samples had increased levels of *CK* compared to the control samples at both 3- and 6-h timepoints (Figure 2-5A). Thus, while *CK* levels increased due to the treatment, *RNS1* levels were not affected by the PIIF fraction. Interestingly, the wound-inducible nuclease activities, as well as RNS1 activity, were also not induced by the treatment, as seen by RNase activity gel assay (Figure 2-5B). Throughout the time course, the only activity detected was a major band at 25 kDa, which was not regulated by wounding. This activity was also not affected by the TFA-PIIFs, and no other activities were induced, including the wound-responsive 35- and 33-kDa nucleases and RNS1.

Thus, although the treatment was effective, induction of RNase activity by wounding is not mediated by oligosaccharides. These results were unexpected, considering that all the activities were also independent of JA. Therefore, we hypothesize that in addition to oligosaccharides and JA, an additional signal exists that directs expression of nuclease and RNase activities in wounded tissue.

DISCUSSION

In this chapter, we have demonstrated that several Arabidopsis RNase and nuclease

activities are coordinately regulated by wounding. Unlike other wound-inducible proteins previously studied in Arabidopsis, these activities are not controlled by jasmonic acid signaling or by oligosaccharide elicitors. The systemic induction of RNS1 makes this ribonuclease especially intriguing. The systemic induction of RNS1 not only supports the existence of a novel pathway for the regulation of systemic wound responses, but is also suggestive of a defensive role for this RNase.

The increases in all the activities occur in a coordinated manner and provide us with a unique perspective into Arabidopsis wound signaling mechanisms. Our understanding of the wound response in Arabidopsis is currently highlighted by the presence of two distinct, antagonistic pathways: JA-dependent and -independent. The JA-independent pathway controls local induction of transcript accumulation and has been shown to be regulated by OGA elicitors probably released from injured plant cell walls (Rojo et al., 1999). The three nuclease activities and RNS1 are strongly induced locally by wounding (Figures 2-1 and 2-2). However, they are not induced by treatments with OGA-rich fractions (Figure 2-5). In fact, in several repetitions of the OGA treatments, the slight wound-induction of *RNS1* caused by detaching the leaves appeared to be inhibited by the presence of the OGAs (for example, compare +PIIF and −PIIF at 6 h in Figure 2-5A). Given that the RNase and nuclease activities are not induced by the PIIF treatments, we conclude that another signal besides OGAs is likely operating to direct the local induction of these activities upon wounding.

The local response of RNS1 and the nucleases to wounding was also not controlled by the JA-dependent signaling pathway, as shown by the strong wound-induction of these activities in the *coi1* mutant (Figure 2-4A). Further, *RNS1* transcript accumulated to high

levels in unwounded leaves of wounded plants. JA is an important systemic signaling molecule during wound-induced gene induction in Arabidopsis (Titarenko et al., 1997). However, the systemic induction of *RNS1* did not depend on JA (Figure 2-4B). To our knowledge, *RNS1* is the first gene in Arabidopsis shown to be induced systemically by wounding in a JA-independent manner and therefore indicates the existence of an alternate long-distance signaling pathway.

It has been proposed that dehydration itself might be the causal induction factor of some JA-independent wound-inducible genes, including *RNS1*, which was shown by microarray analysis to be induced by dehydration (Reymond et al., 2000). However, the dehydration experiments performed involved detaching the rosette leaves from the roots, which itself induces *RNS1* (see Figure 2-5). My results indicate that dehydration itself does not induce *RNS1* expression but may potentiate the wound induction (see Chapter 3). The induction of certain wound-inducible genes in potato and tomato are affected by ABA levels (Peña-Cortés et al., 1989). Therefore, ABA could be considered a possible signal for the wound induction of *RNS1*. The involvement of ABA in the wound response will be discussed further in Chapter 3.

Given its involvement in the wound response in solanaceous plant species and in the regulation of various programmed cell death processes (Jones, 2001), ethylene is another candidate for the signal involved in the induction of *RNS1* both locally and systemically. However, the wound induction of *RNS1* also occurs in the *ein2* mutant, suggesting that ethylene perception is not required (Reymond et al., 2000). Salicylic acid (SA) is required for many plant-pathogen defense responses (reviewed in Alvarez, 2000), and interaction between SA, JA, and ethylene pathways is emerging as an important regulatory method

for activating multiple resistance mechanisms (reviewed in Pieterse and Van Loon, 1999). However, SA does not appear to be involved in the regulation of *RNS1* by wounding, as the transcript is still induced in SA-deficient, *NahG*-expressing plants (E.E. Farmer, personal communication). Additionally, analysis of AFGC microarray data in the Stanford Microarray Database available on the web (http://genome-www4.stanford.edu/ MicroArray/SMD/; Wisman and Ohlrogge, 2000) indicates that *RNS1* is not induced by treatment with the SA-analog BTH or by various pathogens that induce systemic acquired resistance, which is known to cause the accumulation of SA within the plant (Alvarez, 2000).

Other possible signals for the induction of *RNS1* include reactive oxygen species (ROS). ROSs are commonly produced in plants in response to both pathogen and herbivore attacks (Grant and Loake, 2000; Orozco-Cárdenas and Ryan, 1999). In tomato, H_2O_2 acts as a second messenger for the induction of defense–related genes induced systemically at later timepoints than the earlier, signaling-related genes (Orozco-Cárdenas et al., 2001). The timing of RNase and nuclease responses coincides with these later responses that are dependent on H_2O_2 signaling. The involvement of ROSs, as well as other possibilities, for example, electrical signals, in RNase induction during the Arabidopsis wound response will prove an important avenue of study for defining alternate pathways regulating defense responses.

The systemic increase in RNS1 appears to be regulated at multiple levels. Studies with reporter constructs controlled by the *RNS1* promoter region indicate that the local increase in *RNS1* transcript caused by wounding is likely due to transcriptional regulation (see Chapter 3). However, while the transcript increases to significant levels systemically,

the activity increase is not as great (compare Figure 2-3B, 3 and 6 h, with Figure 2-3C, 6 and 8 h). It is possible that a post-transcriptional mechanism may also exist that regulates the amount of RNS1 protein translated or the activity of the translated protein in the unwounded tissue.

In addition to its interesting regulatory properties, the systemic induction of RNS1 implies that the RNase may have an important function during the wound response. It is striking that the *RNS1* transcript was the most highly induced in local wounded tissue of all transcripts examined in two independent microarray experiments: one examined 150 genes enriched for those implicated in defense responses (Reymond et al., 2000), and the second examined 600 genes, approximately half of which were hypothesized to be involved in RNA metabolism and RNA turnover (M.A. Pérez-Amador and P.J. Green, unpublished). The high level of transcript accumulation of *RNS1* may be indicative of an important role for the protein product of this gene. Additionally, the transcript and the activity are induced in non-damaged tissue, where recycling of nutrients and degradation of bulk cellular nucleic acid, two of the proposed functions of secreted RNases, should not be necessary. Instead, it is possible that RNS1 has a defensive function. Normally, *RNS1* is expressed solely in flowers (Bariola et al., 1994). The presence of RNases in the pistil may contribute to protection of the structure from pathogens (Bariola et al., 1994). In fact, it has been demonstrated that application of an extracellular RNase to tobacco leaves inhibits growth of both TMV and a fungal pathogen (Galiana et al., 1997). Likewise, local and systemic induction of RNS1 could contribute to protection of the plant against invasion of pathogens following wounding. Although it is not known how RNases could achieve such defensive roles, wound signal molecules are known to

produce membrane-associated changes within the plant cells, including membrane depolarization (Thain et al., 1995). Recently, it has been proposed that T_2 RNases can affect membrane stability or permeability (MacIntosh et al., 2001), suggesting a possible link. The role of RNS1 in defense mechanisms will be readily testable in a recently-isolated *RNS1* T-DNA insertional mutant and in plants that overexpress RNS1 activity (see Chapter 4).

In contrast to the systemically-induced RNS1, the bifunctional activities were induced only in local, damaged tissue. These activities may also have defensive roles in the local tissue, or they may be functioning in other aspects necessary during the wound response, such as recycling of nucleotides from damaged cells or rebuilding of damaged vascular tissue. Cells damaged by wounding would contain phosphate and other molecules that could be recycled for use in active, growing parts of the plant. Similar functions have been hypothesized for T_2 RNases that are induced by phosphate-starvation and senescence in several species, including tomato and tobacco (reviewed in Howard et al., 1998). Activities at 33 kDa, as well as RNS1, are induced in P_i-starved seedlings (see Figure 10 in Bariola et al., 1994), and bifunctional nucleases are induced by senescence in Arabidopsis (Pérez-Amador et al., 2000). Possibly, RNS1 and the nucleases are induced during wounding as part of this wide-spread recycling mechanism.

It is possible that the systemic induction of *RNS1* could be explained as a sort of "priming" of the plant for nucleotide recycling in as-of-yet undamaged tissue. However, the nucleases, as well as *RNS2* and *RNS3*, which encode two additional RNases shown to be induced during conditions requiring nutrient recycling, such as P_i starvation and senescence (Bariola et al., 1994; Taylor et al., 1993), are not induced locally or

systemically by wounding, at least indicating that other hydrolytic enzymes thought to be involved in P_i recycling and remobilization are not generally activated by wounding. In fact, *RNS1* is the only one of the five Arabidopsis genes related to the RNase T_2 family that is induced in either local or systemic tissue by wounding (not shown). Such specificity indicates that the enzyme may have an important role in the wound response.

Although local induction of hydrolytic activities by wounding has been found in several other plant species (e.g., Galiana et al., 1997; Lers et al., 1998), little is known regarding the signal pathways involved in their regulation. We have now identified a wide array of activities that are regulated by wounding in Arabidopsis, including RNase and nuclease activities that show both sustained and transient patterns of induction. Given its unique responses to wounding, independent of all known wound regulators, *RNS1* will be a valuable marker for identifying novel signals that operate in the Arabidopsis wound response. More importantly, to our knowledge, RNS1 is the first RNase shown to be induced systemically by wounding. This result points us toward new ideas regarding the function of the widespread T_2 family of RNases. Specifically, in addition to having a role in P_i recycling as has been previously hypothesized, RNS1 may have a more direct defense-related function, possibly involved in protection of the plant from further attack after a wound-stimulus has been detected.

MATERIALS AND METHODS

Plant materials and treatments

Unless otherwise stated, the Columbia ecotype of *Arabidopsis thaliana* was used throughout this study. Soil-grown plants were grown in chambers under 16 h of light in 50% relative humidity at 20°C. For seedling experiments, seeds were surface-sterilized

and germinated on Arabidopsis growth medium as described (Taylor et al., 1993). The *coi1* seeds were kindly provided by Dr. J.G. Turner (University of East Anglia, Norwich, UK). Mutant *coi1* plants were selected by germinating on MS medium supplemented with 50 μM methyl jasmonate as described (Feys et al., 1994). The plants were then transferred to soil and grown for an additional 4 weeks before wounding treatments were performed.

Stems or leaves of 4- to 6-week-old plants or leaves of 14-day-old seedlings were wounded using ridged flat-tipped tweezers and harvested at subsequent timepoints. For non-wounded material, leaves on either side of the wounded leaf were harvested. All samples were frozen in liquid N_2 immediately after harvesting and stored at −80°C until used for RNA or protein extractions. Wounding experiments were performed a minimum of three times. Representative blots or gels are shown.

Jasmonic acid treatments were conducted on 4-week-old plants that were placed in enclosed boxes. Methyl Jasmonate (MeJA; Bedoukian Research, Inc., Danbury, CT) was diluted 1:25 in ethanol to a final concentration of 190 mM. A cotton-tipped applicator was soaked with 50 μL of the MeJA solution and placed in the container with the plants. An applicator soaked with ethanol alone was placed in a separate container with control plants. Four boxes were used in each of two experiments, for 2- and 24-h timepoints of both MeJA-treated and control plants. Two additional experiments were conducted for 3-, 6-, and 24-h timepoints, for a total of four replications. Representative blots and gels are shown in Figures 1-4c and 1-4d.

For OGA-treatments, rosette leaves of 4-week-old plants were removed by slicing the petiole with a razor blade. Approximately 20 leaves were floated on 40 mL of MS

medium (Life Technologies, Rockville, MD) supplemented with 0.5% (w/v) sucrose in each of two 250-mL flasks. One flask also contained 250 μg/mL of the OGA-rich TFA-PIIF fraction (provided by Dr. E. Farmer, Université de Lausanne, Switzerland). Flasks were shaken on a rotary platform at ~50 R.P.M. Six or seven leaves were removed from the flasks at 1.5-, 3-, and 6-h timepoints and immediately frozen in liquid nitrogen. OGA treatments were performed twice using this method, and the northern blot and activity gel in Figure 2-5 are representative of this method. Additionally, two replicates of treatments of seedlings grown in liquid culture, according to Rojo et al. (1999), were performed using a PIIF fraction kindly provided by Dr. G. Howe (Michigan State University). Similar results were obtained in all four repetitions; however, the greatest induction of the positive control, choline kinase, was seen using the TFA-PIIF treatments of detached leaves.

RNA extraction and RNA gel blot hybridization

Total RNA from Arabidopsis samples was extracted as previously described (Newman et al., 1993). RNA (10 μg per lane) was separated by electrophoresis in 3% (w/v) formaldehyde/1.2% (w/v) agarose gels and blotted to Nytran Plus nylon membrane (Schleicher and Schuell, Keene, NH). The RNA blots were hybridized as described in Taylor and Green (1991) using a ^{32}P-labeled *RNS1* probe. To control for loading, the same RNA blots were stripped in distilled water at 90-95°C for at least 20 minutes and then hybridized with a ^{32}P-labeled probe for the Arabidopsis translation elongation factor *EF-1α* (EST accession number R29806) or translation initiation factor *eIF-4A* (Taylor et al., 1993). A choline kinase probe, kindly provided by Dr. J. Sánchez-Seranno (Universidad Autónoma de Madrid, Spain), was used as a positive control for OGA

treatments (Rojo et al., 1999). A probe for allene oxide synthase (Laudert et al., 1996) was used as a positive control for MeJA treatments. Quantitation of hybridization was performed using Phosphorimager (Molecular Dynamics, Sunnyvale, CA) analysis to confirm increased *CK* and *AOS* levels in response to PIIF- and JA-treatments, respectively, as well as increased *RNS1* levels in local wounded and systemic leaves (data not shown).

Protein extraction and detection of RNase and DNase activities

Total protein was extracted as described (MacIntosh et al., 1996). Homogenates were clarified by centrifugation, and soluble protein was quantified by the method of Bradford (1977). 100 μg total protein was loaded in each lane.

RNase and DNase activities were assayed using activity gels as described (Yen and Green, 1991), with minor modifications. After the isopropanol wash, before incubation, gels were washed in 100 mM Tris-HCl containing 2 μM $ZnCl_2$ for 20 min in order to restore Zn^{2+} required for certain RNase and DNase activities. Gels were washed and incubated at pH 7.0, except for the RNase gels of systemic activity in Figures 2-3C and 2-4A (lower panel), which were incubated at pH 6.0 to enhance resolution of RNS1 activity, which is most active between pH 5.0 and 6.0 (data not shown). Similar results were seen at pH 7.0 (data not shown). Separating gels contained 11.3% (w/v) acrylamide, except the gels in Figure 1-2, which contained 9% acrylamide for increased separation of the doublet at 33 kDa.

CHAPTER 3

REGULATION OF *RNS1* EXPRESSION

ABSTRACT

RNS1 activity and transcript have been shown to accumulate in response to wounding (Chapter 2) and P_i starvation (Bariola et al., 1994, 1999). I examined whether the induction was a transcriptional or posttranscriptional process using nuclear run-on analysis (in the case of P_i starvation) and transgenic plants expressing promoter fusion constructs (wounding). Whereas the run-on analyses were inconclusive, I demonstrated that the *RNS1* promoter was responsive to wound stimuli, as shown by accumulation of the reporter transcript. I then used the promoter constructs to examine other signals that might direct transcription of *RNS1*. Through analyses of these transgenic plants and RNA blot analyses of endogenous *RNS1* transcript, I found that both ABA and NaCl induced *RNS1* expression. Interestingly, however, ABA was not required for the wound induction of RNS1 activity. Additionally, heat shock appeared to have a negative effect on *RNS1* induction by wounding. In contrast to previous reports, I determined that dehydration did not cause increases in *RNS1* expression, indicating that another signal directs the induction of *RNS1* by wounding.

INTRODUCTION

As seen by results presented in Chapter 2 of this thesis, the levels of *RNS1* transcript are responsive to external stimuli. In addition to wounding, *RNS1* also responds to the P_i status of its environment as well as to internal cues, as shown by the increase in *RNS1* transcript in senescing tissues (Bariola et al., 1994). In addition to *RNS1*, genes encoding RNases in many plant species are regulated in response to P_i starvation, senescence, and wounding.

Although numerous conditions that affect the levels of various RNase activities have been identified in many plant species, little is known regarding the regulatory pathways that control these responses. Previous studies addressed the regulation of *RNS1* in response to P_i starvation; in particular, run-on analyses and transcriptional fusions were used to determine whether *RNS1* transcript accumulates as a result of transcriptional or posttranscriptional regulatory mechanisms (Howard, 1996). No difference could be detected by nuclear run-ons between seedlings grown on full and P_i-deficient media. However, these results were inconclusive, because *RNS1* transcript levels were not above background. Transcriptional increases were detected by promoter fusion constructs, but the increases were not sufficient to explain the large increase in *RNS1* levels. Therefore, I repeated nuclear run-on analyses of P_i-starved and P_i-supplied seedlings to examine the level of regulation of *RNS1* under these conditions.

Additionally, since *RNS1* transcript is so abundant after wounding, I was interested in how these levels are regulated during the wound response. I was also particularly interested in other signals involved in regulating *RNS1* gene expression, given that known Arabidopsis wound pathways are not involved in the response of this gene during

wounding (see Chapter 2).

RESULTS

Run-on analysis of *RNS1* expression

Run-on analyses were attempted to determine whether P_i starvation leads to transcriptional induction of *RNS1*. Comparison of run-on transcription assays and *RNS1* transcript accumulation in seedlings grown in the presence or absence of P_i should indicate the contribution of transcriptional regulation to the increase in *RNS1* resulting from P_i starvation. Seedlings were plated on Arabidopsis germination medium (AGM) on mesh circle inserts. On the day of germination (approximately 2.5 days after plating), the mesh inserts were used to transfer seedlings to new plates that either contained (P+) or lacked (P-) P_i. After 7 d of growth, seedlings were harvested and used to isolate active nuclei and total RNA. RNA was used for RNA gel blot analysis to confirm that *RNS1* transcript levels were increased (Figure 3-1B).

Nuclei prepared from the two seedling samples were used for nuclear run-on analyses. Labeled nascent transcripts were purified and used to probe slot blots containing ssDNA of plasmids of *RNS1*, *eIF4A*, and empty Bluescript (*BSK*). The *BSK* plasmid was used as a control for background hybridization, and *eIF4A* was used as a loading control, as transcription of this gene is unaffected by the P_i status of the seedlings. As seen in Figure 3-1A, hybridization of *RNS1*, whose expression is regulated in response to P_i starvation, is below detection in samples from P_i-starved and unstarved seedlings. In multiple repetitions, the hybridization signals obtained were not above background levels detected by the *BSK* control, although *eIF4A* signals were significantly above background. The results indicate that although the nuclei were active, *RNS1*

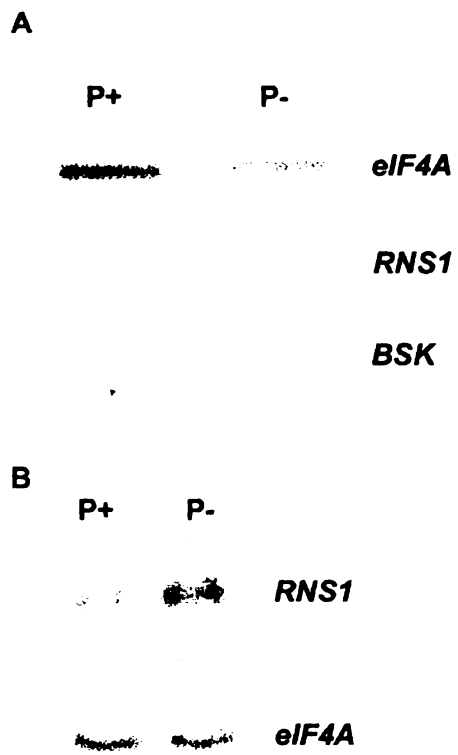


Figure 3-1. Run-on analysis of *RNS1* in response to P_i starvation.
(A) Nuclear run-on analysis was performed to compare transcriptional activity of the *RNS1* gene in Arabidopsis seedlings grown on medium containing (P^+) or lacking (P^-) P_i.
(B) Accumulation of *RNS1* transcript is shown by Northern blot analysis of RNA from the same samples used to isolate nuclei in **(A)**.

transcription was not at sufficient levels to measure by run-on analysis. As a result, I could not conclude anything concerning the transcriptional regulation of *RNS1* in response to P_i starvation.

Regulation of *RNS1* expression in response to wounding

Since *RNS1* is so strongly induced by wounding (see Chapter 2), I were interested in whether this regulation is the result of transcriptional control of the *RNS1* promoter or of a posttranscriptional mechanism. Several constructs were used to examine the regulation of *RNS1* (Figure 3-2). The first, p1975 (referred to as nos-RNS1cDNA), included the *RNS1* cDNA fused between the constitutive, low expressing *nos* promoter and the pea *E9* terminator. This cDNA contains the open reading frame (ORF) plus approximately 60 bases of the 5' UTR (Bariola et al., 1994). The full 5' UTR, as determined by primer extension analysis, contains an additional 40 bases (Howard, 1996). Therefore, I made a second construct, p2019 (referred to as nos-preRNS1), in which intron sequences and the missing bases of the 5' UTR not found in the cDNA were included. Transgenic plants were made using *Agrobacterium tumefaciens*-mediated transformation via the method of vacuum infiltration (Bechtold et al., 1993). Additionally, transgenic control plants were made by transformation with a control plasmid, p1995, which consists of the *nos* promoter driving the *globin* reporter coding region (nos-Globin). To examine the regulation of the transgenes by wounding, leaves of the transformed plants were wounded and harvested 3 h later. Unwounded leaves were also harvested to provide control samples. RNA was prepared from the samples and used for RNA gel blot analysis. Blots were probed with an oligo complementary to the transcribed *nos* sequences to distinguish between *RNS1* transcribed from the transgene and the endogenous copy. The RNA gel

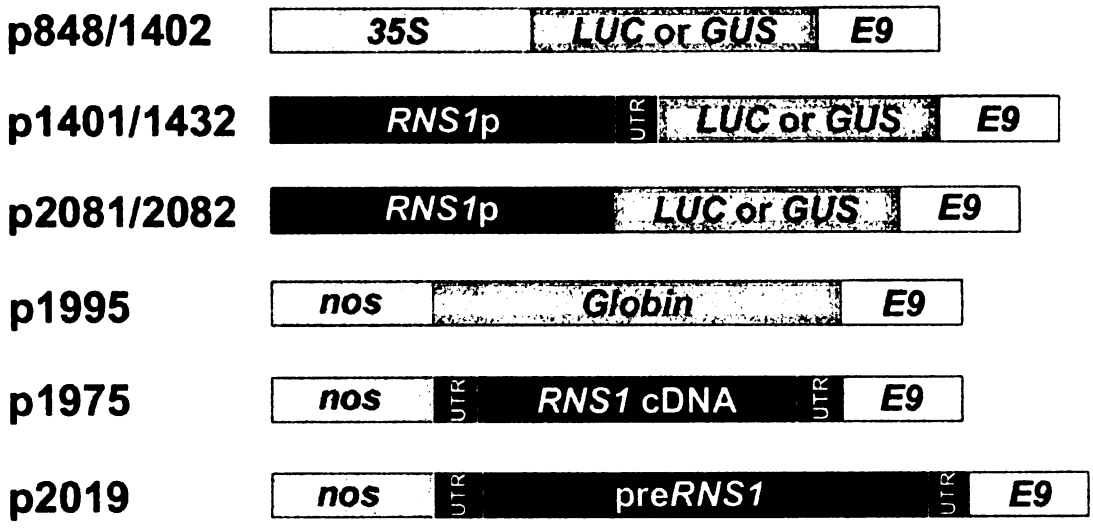


Figure 3-2. Constructs used to examine the regulation of *RNS1*.
Several constructs were used to transform wild-type Arabidopsis plants. Transgenic lines were then used to analyze the expression of the reporters under various conditions. Clone identification numbers are shown to the left. *LUC*, *Luciferase* coding region; *GUS*, *β-glucuronidase* coding region; *35S*, CaMV *35S* promoter; *nos*, nopaline synthase promoter; *RNS1*p, *RNS1* promoter; *E9*, 3' end of the pea *E9* gene; pre*RNS1*, transcribed region of *RNS1*; The *RNS1* cDNA was isolated by Bariola et al. (1994).

blots indicated no difference between *nos* signal in wounded and unwounded leaves of plants expressing the nos-RNS1cDNA construct, although endogenous levels of *RNS1* were clearly elevated by wounding, as shown by hybridization with the *RNS1* cDNA (Figure 3-3; center panels). However, in the nos-preRNS1 plants, a slight but reproducible increase in *nos* signal was seen (Figure 3-3; left panels). It is therefore possible that a certain amount of posttranscriptional regulation of *RNS1* mRNA exists, which requires either the entire 5' UTR region or intron sequences, or both, whereas the cDNA sequence alone is not sufficient. However, this slight increase of approximately 2.5-fold does not account for the high levels of *RNS1* transcript found in wounded tissues.

Since posttranscriptional regulation could not account for the increase of *RNS1* by wounding, I also examined the ability of the *RNS1* promoter to confer wound-inducibility to a reporter gene. Constructs were available (Figure 3-1) that contained 2.7 kb of *RNS1* promoter plus ~60 bases of *RNS1* 5' UTR sequence fused to either *β-glucuronidase* (*GUS*; p1401) or *luciferase* (*LUC*; p1432) reporter genes, as well as control constructs with the same reporters driven by the constitutive CaMV *35S* promoter (p848 and p1402, respectively) (Howard, 1996). These constructs were used to transform Arabidopsis to analyze their regulation by wounding.

Regulation of the *GUS* constructs was analyzed by RNA gel blot. In unwounded leaves of plants transformed with the RNS1p-GUS construct, little or no *GUS* transcript could be detected. In contrast, 3 h after wounding, wounded leaves contained high levels of *GUS* (Figure 3-4A). *LUC* lines revealed that luciferase activity also increased following wounding. Transformed RNS1p-LUC lines were wounded and examined for LUC activity 5 h after wounding. Unwounded RNS1p-LUC and wounded and

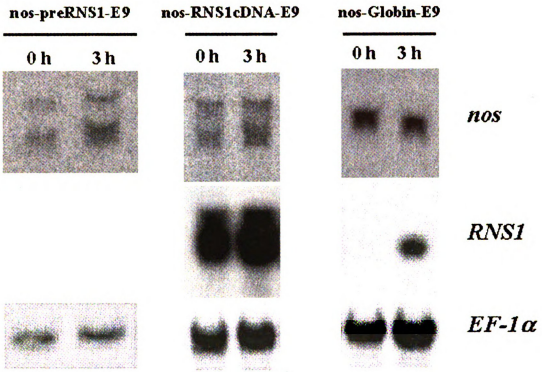


Figure 3-3. Differential response of *RNS1* transcribed sequences to wounding. Pools of T_2 Arabidopsis seedlings expressing either the entire *RNS1* transcribed region (left panels), the *RNS1* cDNA (center), or the globin transcript (right) under the constitutive *nos* promoter were wounded and harvested 3 h later. An oligonucleotide corresponding to a transcribed portion of *nos* was used as a probe in order to distinguish the transgene from endogenous *RNS1*. Blots were then stripped and reprobed with *RNS1* (to control for wounding) and *EF-1α* (to control for loading).

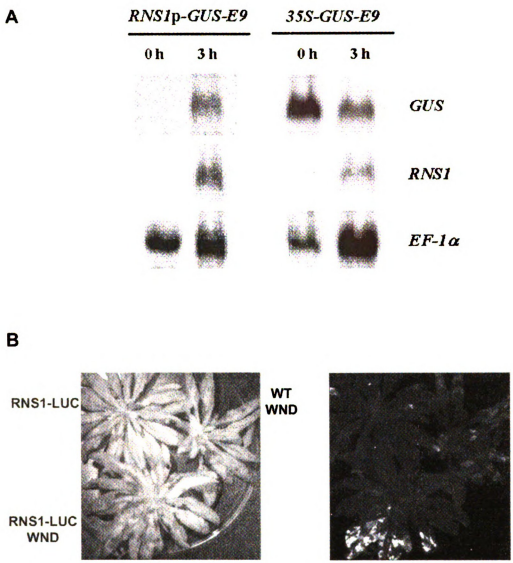


Figure 3-4. The *RNS1* promoter confers wound inducibility to reporter transcripts.
(A) Leaves of transgenic Arabidopsis plants expressing the *GUS* reporter under the control of either 2.7 kb of genomic sequence upstream of the *RNS1* transcription start site plus 60 bases of 5' UTR sequence or the constitutive *35S* promoter were harvested before (0 h) or 3 h after wounding. Blots were probed with *GUS*, then stripped and reprobed with *RNS1* (to control for wounding) and *EF-1α* (to control for loading). 35S-GUS plants were used as controls to demonstrate that *GUS* is not stabilized by wounding.
(B) Images of wild type (WT) plants and plants expressing *LUC* under control of the same upstream *RNS1* region as in **(A)** (RNS1-LUC). WND, wounded plants 5 h after wounding. The lower left leaves of the rosette were wounded. Left panel, plants in visible light; right panel, luciferase activity.

unwounded WT Col plants were used as controls, since no 35S-LUC lines were ever found to have LUC activity, either with or without wounding (data not shown). As seen in Figure 3-4B, only wounded RNS1p-LUC plants have LUC activity. These results indicate that *RNS1* is transcriptionally induced by wounding. However, since the promoter constructs used contained approximately 60 bases of 5' UTR sequence, I removed this sequence to confirm that the promoter sequence alone confers wound-inducibility to the *GUS* and *LUC* reporters. These constructs, p2081 and p2082, are shown in Figure 3-2. As expected, plants transformed with these constructs also displayed wound-inducibility of the transgenes. RNA gel blot analysis revealed increased *GUS* levels 3 h following wounding of leaves of transformed plants (Figure 3-5A), while LUC activity was again seen only following wounding of p2082 lines (Figure 3-5B). My results confirm that wounding causes a transcriptional induction of *RNS1*.

Use of promoter constructs to analyze signals mediating *RNS1* induction

One unique aspect of the wound-induced expression of *RNS1* is the fact that JA and OGAs are not required (Chapter 2 and LeBrasseur et al., in press). The *LUC* reporters offered us the opportunity to screen for various signals that might induce *RNS1* expression. I used the lines to examine several aspects of *RNS1* expression, including response to hormone treatments, interaction of the wound-induction with these hormones, and effects of other external stimuli.

Since dehydration has been proposed to be the signal for the wound induction of JA-independent genes (Reymond et al., 2000), I first assessed whether dehydration caused induction of *RNS1*. Figure 3-6 shows that dehydration did not cause induction of LUC activity in adult plants left unwatered for over two weeks, until leaves of the plants

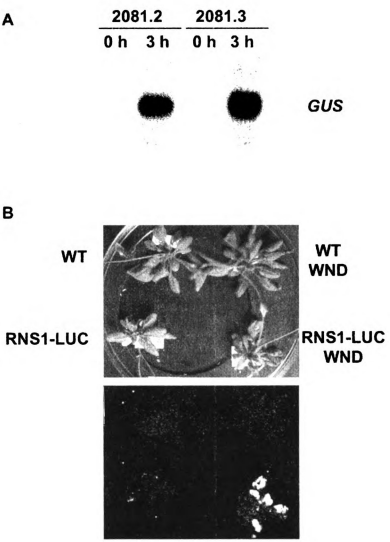


Figure 3-5. 5' UTR sequences are not required for the wound responsiveness of the *RNS1* promoter.
(A) Northern blot analysis of RNA from unwounded (0 h) and wounded (3 h) leaves of T_2 plants (2081.2 and 2081.3) expressing the *GUS* reporter controlled by 2.7 kb of the *RNS1* promoter with no 5' UTR sequence.
(B) Luciferase activity (bottom panel) in control and wounded (WND) wild-type (WT) and transgenic plants expressing the *LUC* reporter under control of the *RNS1* promoter (RNS1-LUC) as in **(A)**.

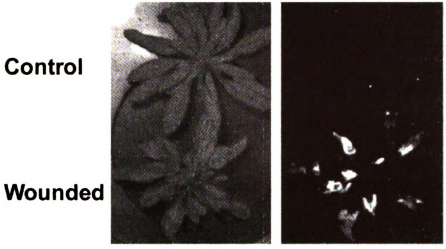


Figure 3-6. Dehydrated plants do not induce the *RNS1* promoter.
Plants were dehydrated as described in the text. Luciferase activity (right panel) is seen only after wounding the dehydrated plants. No *RNS1* transcript was detected by Northern blot analysis of dehydrated WT seedlings (not shown).

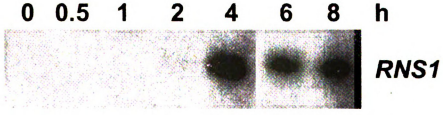


Figure 3-7. *RNS1* transcript accumulates in response to salt stress.
Ten-day-old wild-type Arabidopsis seedlings were transferred to plates containing 250 mM NaCl and subsequently harvested at the time points indicated. RNA (15 μg) was analyzed by Northern blot using an *RNS1* probe.

This blot was kindly provided by Daniel Cook.

appeared purple from high levels of anthocyanins (not shown). Wounded dehydrated plants were used as a control and had high LUC activity. The activity in the wounded dehydrated plants was the strongest seen in any of the LUC experiments performed in this work. Therefore, I speculate that although dehydration does not cause *RNS1* induction, it may potentiate the wound-induction. To confirm that *RNS1* does not respond to dehydration, I probed a blot containing RNA from dehydrated seedlings. No *RNS1* signal was detected on the blot (not shown), which contained RNA from time points 0-8 h of dehydration, although typical dehydration-responsive transcripts were induced (D. Cook, personal communication). Since both the *LUC* reporter and endogenous *RNS1* transcript did not respond to the dehydration treatment, I conclude that dehydration is not the signal that causes induction of *RNS1* by wounding.

As discussed briefly in Chapter 1, *S. cereviseae* has only one member of the RNase T_2 family, Rny1. Since the *RNY1* transcript accumulates in response to salt stress (MacIntosh et al., 2001), I were interested in examining the response of *RNS1* to a similar treatment. RNA blots containing RNA samples from Arabidopsis seedlings transferred to plates containing 250 mM NaCl were probed with *RNS1*. As seen in Figure 3-7, like *RNY1*, *RNS1* also accumulated in response to high NaCl concentrations. Transcript was detected by 2 h of treatment and remained high at 8 h.

Recently, a mutant deficient in ABA signaling has been isolated that affects an mRNA cap-binding protein and is suggested to affect *RNS1* levels through a posttranscriptional mechanism (Hugouvieux et al., 2001). In an attempt to identify any effects of ABA on *RNS1* expression, I treated the *LUC* lines with ABA. LUC activity was seen 5 h after addition of ABA to seedlings growing in liquid medium (Figure 3-8A). I

also probed a blot containing RNA from ABA-treated seedlings and found that endogenous *RNS1* transcript accumulated beginning 2 h after transfer of seedlings to plates containing 100 μM ABA (Figure 3-8B). *RNS1* levels remained high after 8 h of treatment. Thus, ABA caused induction of *RNS1*. Most likely, this induction is transcriptional, since I detected LUC activity upon ABA-treatment. These results point toward a possible signal for the wound induction of this gene.

Since ABA induced *RNS1*, I examined whether ABA signaling is required for the wound induction of this gene. Several different mutants in the ABA pathway were wounded and analyzed for RNase activity. Two of the mutants, *abi1* and *abi2*, are blocked in ABA signal transduction, and a third, *aba1-1*, is deficient in ABA biosynthesis, (for a review on ABA signal transduction, see Leung and Giraudat, 1998). Twenty-four hours following wounding, all three mutants had elevated levels of RNS1 activity (Figure 3-9). Thus, although ABA is able to induce *RNS1* transcription, it is not required for the wound induction. The 33-kDa activities were also induced in the mutants, indicating that the bifunctional nucleases are also upregulated independently of ABA.

Previous studies in this laboratory indicated that wounding and heat shock may interact to regulate *RNS1* expression. I used the *LUC* and *GUS* reporters to examine the effects of these two stimuli on *RNS1* transcription. In multiple repetitions, LUC activity was not seen after wounding when combined with treatment at high temperatures (Figure 3-10A). High temperature alone had no effect on LUC activity. Since it has been reported that high temperature can affect RNase activity posttranslationally (Chang and Gallie, 1997), I tested whether the *RNS1* transcript was affected by heat shock.

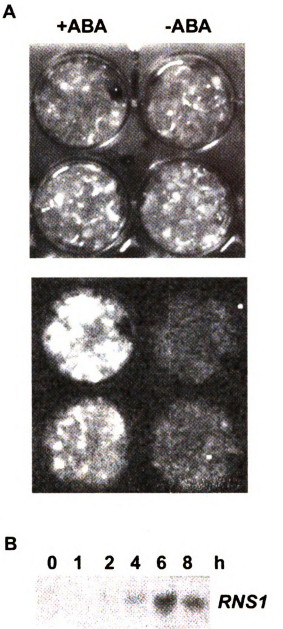


Figure 3-8. ABA induces the *RNS1* promoter.
(A) Luciferase activity (bottom panel) in seedlings grown in liquid culture 5 h after addition of 5 μM ABA (+ABA) or buffer alone (−ABA).
(B) Northern analysis of RNA isolated from seedlings treated with ABA for the indicated times.

*RNA gel blot in **(B)** provided by Daniel Cook.*

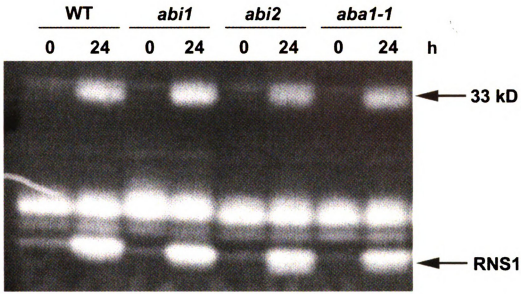


Figure 3-9. Mutants in ABA signaling and biosynthesis induce RNS1 by wounding. Wild type (WT) and mutants *abi1*, *abi2*, and *aba1-1* were examined for RNase activities in unwounded (0) and wounded (24) 2-week-old seedlings. Induction of RNS1 and the 33-kDa activities is seen in all four lines.

For this experiment, stems of wild-type plants were wounded, then cut and floated in a buffer at either 22 or 42°C. The wound induction of *RNS1* is diminished by the heat treatment (Figure 3-10B). These results indicate that heat shock inhibits the wound response of *RNS1*.

DISCUSSION

The findings in Chapter 2 of this thesis revealed that *RNS1* responds to wounding through a pathway not previously identified in Arabidopsis. This has led to the obvious question as to what signal transduces the wound stimulus to cause increased levels of *RNS1*? I addressed this question by examining the level of regulation of this gene and found that *RNS1* is transcriptionally activated by wounding. I next examined several other factors that might affect *RNS1* transcription and found that, although ABA induces *RNS1* expression, it is not required for the wound induction.

Sequence requirements for the wound induction of *RNS1*

The accumulation of *RNS1* following wounding occurs within 2 h in Arabidopsis leaves (refer to Figure 3-10B). In fact, previous microarray analyses of wounded leaves found that the fold-induction of *RNS1* at 2 h was higher than any other of the 600 spots examined (M.A. Pérez-Amador and P.J. Green, unpublished). In the unwounded state, this transcript normally can not be detected or is at very low levels in leaves of adult Arabidopsis plants (Bariola et al., 1994). Thus, wounding apparently causes a strong response of *RNS1*. The accumulation of *RNS1* could be due to either a transcriptional induction or a posttranscriptional mechanism, such as an increase in stability of the transcript. Posttranscriptional regulation of this transcript has been suggested by previous experiments that showed that although P_i starvation caused increased transcription of

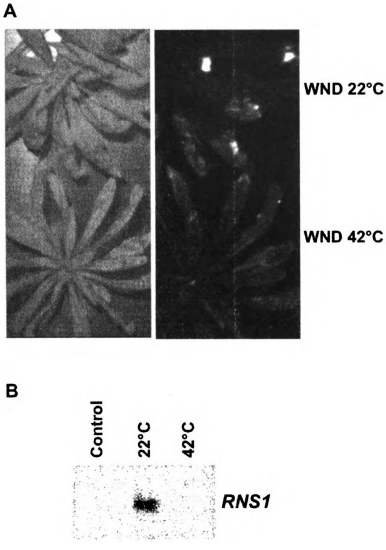


Figure 3-10. Heat shock inhibits the wound-induction of luciferase activity.
(A) Transgenic plants expressing the RNS1p-LUC construct were wounded and placed at either 22 or 42°C for 5 h. Luciferase activity (right panel) was seen only in the wounded plants left at 22°C. Similar results were seen in seedlings (not shown).
(B) *RNS1* accumulation in unwounded (control) and wounded stems incubated at 22 or 42°C for 2 h after wounding.

*Experiment in **(B)** was performed by Gustavo MacIntosh*

RNS1 promoter fusion reporters, transcriptional regulation could not account fully for the levels of *RNS1* under these conditions (Howard, 1996).

In contrast to these results, I found that increased transcription of *RNS1*, as directed by 2.7 kb of promoter sequence, causes the majority of the increased levels of this transcript by wounding. However, I did detect small effects of the transcript alone upon wounding. This effect was only seen when the introns and full 5' UTR sequence were included in the reporter; the cDNA sequence alone did not confer wound-inducibility. On the other hand, the full 3' UTR is not required for the wound induction (see Chapter 4). In conclusion, the *RNS1* promoter contains the sequences most critical for the regulation of the induction of the RNS1 enzyme by wounding.

Response of *RNS1* to various biotic and abiotic stimuli

Previous work had shown that *RNS1* transcript accumulates in P_i-starved seedlings and in senescent leaves (Bariola et al., 1994). Work in this thesis has shown that multiple other signals direct expression of this gene. Wounding, as seen here and in Chapter 2, strongly influences *RNS1* transcription. This effect is not directed by the known wound signals jasmonic acid and OGA elicitors. Thus, I was interested in what other internal or external cues alter *RNS1* levels. To examine this question, I used the *LUC* and *GUS* lines to screen many different stimuli.

Using this screen, I found that ABA causes increased transcription of *RNS1* and the *LUC* reporter. ABA has been suggested to affect *RNS1* posttranscriptionally (Hugouvieux et al., 2001). A posttranscriptional mode of regulation was suggested because the authors isolated a mutant in a gene that encodes a cap-binding protein. The mutant shows ABA-hypersensitivity and down-regulates several transcripts implicated in ABA signaling.

RNS1 is one of the transcripts with lower basal levels in this mutant. These results may be slightly misleading, since *RNS1* transcript is usually just at or below the level of detection, suggesting that the wild-type plants used for Northern and microarray analyses in their study may have been stressed. Nonetheless, if *RNS1* is affected in this mutant, it is logical to infer that this transcript is normally posttranscriptionally regulated by ABA. I found that ABA causes transcriptional induction of a reporter controlled by the *RNS1* promoter (Figure 3-8A); however, I cannot rule out the possibility that certain ABA effects are carried out through a posttranscriptional mechanism. Treatment of the nos-RNS1cDNA or nos-preRNS1 lines with ABA should address this possibility.

Although ABA does affect transcription of *RNS1*, it is not required for the wound induction of RNS1 activity. Similarly, the wound-induction of the 33-kDa activities does not require ABA signaling or biosynthesis (Figure 3-9). It is possible that local RNS1 induction is independent of ABA, while systemic induction requires ABA. The systemic regulation should be addressed in future experiments.

One interesting result derived from the dehydration experiments was a particularly strong induction of the LUC activity in wounded dehydrated plants. Dehydration is known to induce ABA production in plants (Thomashow, 1999). Since ABA caused increased LUC activity in the transgenic lines, it is possible that the combined effect of wounding and increased ABA levels may have an amplification effect on the induction of the reporter. This effect may explain the increased *RNS1* transcript levels identified in the dehydration experiment by Reymond et al. (2000), since dehydration was achieved by detaching rosette leaves from the roots, possibly causing a weak wound response that was amplified by the dehydration. It appears, however, that the dehydration-elicited ABA

increase alone is not sufficient for *RNS1* induction, since both luciferase activity in the transgenic lines (Figure 3-6) and *RNS1* transcript in WT plants (not shown) did not accumulate in response to dehydration.

The differential control of *RNS1* by ABA and dehydration is particularly interesting since overexpression of *CBF1* or *CBF2* results in accumulation of *RNS1* (M.F. Thomashow, personal communication). CBF1 and CBF2 are transcription factors rapidly induced by cold temperature in Arabidopsis (Gilmour et al., 1998), and overexpression of *CBF1* results in increased cold tolerance (Jaglo-Ottosen et al., 1998). Interestingly, *RNS1* is not induced by cold treatment (not shown), nor is it induced by overexpression of *CBF3* (M.F. Thomashow, personal communication), another in the cold-induced *CBF* family (Gilmour et al., 1998). Therefore, cross-talk between pathways that control dehydration, cold, and ABA responses appears to regulate *RNS1* accumulation in a highly specific manner. Alternatively, overexpression of the transcription factors may cause secondary effects that result in increased RNS1 accumulation.

While dehydration may potentiate the wound-induction of *RNS1*, we found that heat shock inhibited this response. Posttranslational effects on RNase activity by heat shock have been suggested to be one mechanism by which plants reduce mRNA turnover until recovery has occurred (Chang and Gallie, 1997). RNS1 is an extracellular enzyme and therefore is not expected to be involved in mRNA decay. Additionally, this effect is apparently not posttranslational, according to the reduced *RNS1* transcript levels by heat shock (Figure 3-10B). The wound-induced 35-kDa nuclease activity was also inhibited by the heat treatment (G.C. MacIntosh and P.J. Green, unpublished). I am currently using the *GUS* reporter lines to determine whether *RNS1* is transcriptionally affected by heat

shock. Further, effects on the 33-kDa nuclease activities will be examined.

During the screen, I also examined several other factors that did not cause increased LUC activity (not shown). These include auxin and cytokinin. Additionally, phosphatase and kinase inhibitors did not affect the induction of the *LUC* reporter by wounding. It is possible that the treatments were not effective and that further studies will uncover regulation by these factors; however, it currently appears that these two hormones and inhibitors of other Arabidopsis wounding signal transduction pathways (Rojo et al., 1998) are not involved in the wound induction of *RNS1*.

In conclusion, transcription of the *RNS1* gene is regulated by several factors. Wounding, senescence, and P_i-starvation were already known to control *RNS1* levels (LeBrasseur et al., in press; Bariola et al., 1994). I have now shown by promoter fusion analysis that the wound induction is largely a transcriptional response. ABA and NaCl are other factors that regulate *RNS1* transcription, while stresses such as cold and dehydration do not. Although dehydration does not cause *RNS1* induction, it may amplify the induction by wounding. In contrast, heat shock has an inhibitory effect on *RNS1* wound-induction. There appear to be multiple networks controlling the expression of *RNS1*. These may be important for tightly regulating RNS1 activity levels under various stress conditions. Understanding of the conditions regulating RNS1 activity is important in order to elucidate the function of this and related enzymes in plants. This question will be addressed in the following chapter.

MATERIALS AND METHODS

Nuclei preparations and run-on analyses

Seedlings for nuclei purification were grown on P^+ and P^- media as described (Bariola et

al., 1994). Nuclei were purified as follows. Frozen 7-day-after-transfer seedlings were ground in Nuclear Isolation Buffer (NIB; 1.0 M sucrose, 5 mM $MgCl_2$, 10 mM Tris pH 7.2) to a powder. The ground tissue was filtered through two layers of miracloth, Triton X-100 was added to 0.5% (v/v) to lyse the chloroplasts, and the filtrate was spun at 1,000 × g at 4°C for 10 min. The pellets were resuspended in 0.5 ml NIB plus 0.5% Triton and spun as above, for 15 min. Pellets were then resuspended in Nuclei Storage Buffer (50% glycerol, 5 mM $MgCl_2$, 2 mM DTT, 20 mM HEPES pH 7.2) and frozen at −80°C until used for run-on analysis. An aliquot of nuclei from each preparation was DAPI-stained, and nuclei were counted to estimate the number of nuclei purified.

Nuclear run-on reactions were begun by adding each of the following to a 50-μl aliquot of nuclei for a final volume of 100 μl: 1 × transcription salts (10 mM $MgCl_2$, 100 mM ammonium sulfate, 20 mM HEPES pH 7.9), 10 μl glycerol, 1 μl 0.1 M DTT, 0.5 mM ATP, GTP, and CTP, 70 units of RNase inhibitor (Promega), and 100 μCi ^{32}P-UTP (3000 Ci/mmol). After 20 min, 5 μl DNase RQ1 (Promega) was added to each tube, and reactions were placed at 30°C for 5 min. Reactions were stopped with 300 μl phenol/chloroform (50:50 v/v) and 200 μl stop solution (1% SDS, 20 mM EDTA, 100 mM LiCl, 10 mM aurintricarboxylic acid). The tubes were spun in a microcentrifuge for 10 min at 4°C. The supernatant was transferred, and 75 μl of 10 M ammonium acetate and 940 μl EtOH was added. After precipitation, the transcripts were resuspended and used to probe slot blots containing 2.5 μg ssDNA of the appropriate plasmids.

ssDNA was purified using a procedure from Stratagene. Briefly, a single colony of bacteria (*E. coli* strain DH5α F') carrying the appropriate clone was inoculated into 5 ml of 2 × YT medium containing 50 μg/ml ampicillin and 1×10^8pfu/ml VCM13 helper

phage and grown overnight at 37°C. Cells were pelleted, and 1 ml of supernatant plus 150 μg 20% PEG and 2.5 M NaCl was precipitated on ice for 15 min. After centrifugation, the pellet was resuspended in 400 μl 0.3 M sodium acetate (pH 6.0) and 1 mM EDTA. The ssDNA was extracted with phenol/chloroform and precipitated.

Cloning

The *RNS1* cDNA was digested with *Sma*I and blunt-end ligated into a nos-*globin* gene construct, p2031, with the *globin* gene insert removed. This construct, p1966, was then digested with *Pst*I and ligated into the *Pst*I site of the plant transformation vector pCambia 1301, which has the hygromycin resistance plant selection marker. This clone was named p1975. The nos-globin control in the plant vector pCambia 2301 was named p1995 and confers kanamycin resistance to transformed plants. To clone the entire *RNS1* transcribed region, including the full 5' UTR and introns, primers (PG749 and PG750) were designed for PCR amplification using wild-type genomic DNA. Each primer contained a *Bam*HI recognition sequence. The PCR product was isolated from an agarose gel (Qiagen) and ligated into pGEM-T Easy (Promega). The insert was sequenced to confirm the sequence of the PCR product and ligated with *Bam*HI/*Bgl*II-digested p2031. The orientation of the insert was confirmed, the *nos-RNS1* insert was retrieved by digestion with *Pst*I and ligated into the *Pst*I site of pCambia 2301.

Constructs p848, p1401, p1402, and p1432 were made previously (Howard, 1996). To remove 5' UTR sequences from these constructs, primers corresponding to the transcription start site (3' primer PG866) and to a site in the promoter approximately 420 bp upstream of the transcription start site (5' primer PG853) were synthesized. The 3' primer included a *Bgl*II restriction site. The PCR product was isolated and ligated into

pGEM-T Easy (p2028), sequenced to confirm the PCR product sequence, and digested with *Bgl*II and *Spe*I to isolate the correct 3' end of the promoter fragment. This 383-bp fragment, which lacks the 5' UTR sequences present in constructs p1401 and p1432, was used to replace the 475-bp *Bgl*II/*Spe*I fragment in the original vectors. These pMON-derivative plasmids carry a kanamycin resistance gene.

Plant transformation

All plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101 C58C1 Rif^r (pMP90) (Koncz and Schell, 1986) by electroporation, using a Gene-Pulser apparatus (Bio-Rad) as per the manufacturer's instructions. *Arabidopsis thaliana* ecotype Columbia plants were transformed by vacuum infiltration according to the method of Bechtold et al. (1993). Seeds from transformed plants were plated on selection medium containing either kanamycin at 50 μg/ml or hygromycin at 30 μg/ml. Resistant seedlings were transferred to soil after 2 weeks. Expression of the transgene was tested in adult plants by Northern analysis (not shown). Lines expressing the transgenes were used for further studies.

Plant treatments

Plants used for various treatments were selected on medium with appropriate drugs and transplanted to soil. Seedlings grown in liquid culture were sterilized for 7 min. in 50% bleach/0.02% Triton X-100, washed three times in ddH_2O, and resuspended in 0.1% agarose. Approximately 25 – 30 seeds were added to each well in 12-well ELISA plates. Each well contained 4 mL of MS medium (Life Technologies, Rockville, MD) supplemented with 0.5% (w/v) sucrose. Plates were shaken on a rotary platform at ~50 R.P.M. in a growth chamber at 16 h light and 22°C. Treatments were performed on 10- or

14-day-old seedlings.

ABA was added to the liquid medium from a 5-mM stock solution dissolved in 1N NaOH. Final ABA concentration was 5 μM. Controls were treated with equal amounts of 1N NaOH. Wounding was done using ridged flat-tipped tweezers. Approximately 30% of the leaf surface was wounded.

RNA and protein analyses

RNA and protein extraction, RNase activity assays, and Northern hybridization were performed as described in Chapter 2.

Luciferase analysis

After each set of treatments, *LUC*-expressing and control plants were sprayed with the substrate luciferin (Promega; 5 μg/ml in 0.011% triton). In some cases, luciferin was added directly to the liquid medium instead of being sprayed onto the plants. Luciferase activity was examined using a photonic camera 40 min after addition of the luciferin substrate.

CHAPTER 4

RNS1 FUNCTION: ANALYSIS OF AN RNS1 MUTANT AND OVEREXPRESSOR

ABSTRACT

I used a T-DNA insertional mutant, *rns1-2*, and plants overexpressing the *RNS1* gene to analyze the function of the RNS1 ribonuclease. The *rns1-2* mutant lacked detectable RNS1 activity, even in wounded leaves. Plant morphology was examined. Unlike antisense *RNS1* lines, *rns1-2* plants did not have elevated anthocyanins. However, in support of a previous hypothesis regarding the function of S-like RNases in P_i-remobilization (Goldstein et al., 1989), the overexpressors were better able to utilize RNA as a source of P_i. I also found an unexpected effect of RNS1 activity on root length in the plants. While the mutant had longer roots, the overexpressing plants had shorter roots than the wild type. These results indicate that RNS1 is involved in processes other than recycling P_i, since the phenotypes were seen independently of P_i concentrations in the medium.

INTRODUCTION

For decades, RNases have been used as models for structural and enzymatic studies. RNase A in particular has been extensively analyzed, and crystallized structures of wild type as well as enzymatic mutants have been determined (Gilliland, 1997). Despite the knowledge gained on enzymatic activity, little is known concerning physiological functions of secreted ribonucleases. In the T_2 family, the exception is the S-RNases involved in self-incompatibility in the Solanaceae (Parry et al., 1997). Previously, antisense methods were used to reduce Arabidopsis S-like RNase activity to address the function of members of this family. These experiments revealed that seedlings with low RNS1 or RNS2 activity had high anthocyanin content, an effect that was amplified by P_i starvation (Bariola et al., 1999). These results are consistent with the hypothesis that S-like RNases are involved in the mobilization and recycling of P_i during P_i starvation and senescence. However, in the antisense experiments, *RNS1* transcript levels were never reduced below 10% of that of the wild type (Bariola et al., 1999). Also, the segregation of kanamycin resistance in the antisense lines indicated that they may have had silencing or T-DNA rearrangement complications (P.A. Bariola, G.C. MacIntosh, and P.J. Green, unpublished). Recent results, including the systemic induction of RNS1 during wounding (LeBrasseur et al., in press) and phenotypes of the insertional mutant of the yeast T_2 RNase (MacIntosh et al., 2001), have indicated new directions regarding the functions of secreted RNases. This chapter will focus on the use of insertional mutagenesis (Krysan et al., 1999) to examine the function of the RNS1 enzyme. Along with this project, I also overexpressed *RNS1* in plants to examine the effect of increased RNS1 activity. Previous attempts to overexpress *RNS1* in roots did not lead to increased levels of the transcript

beyond that induced by P_i starvation (Bariola, 1996). In this study, I expressed the *RNS1* cDNA throughout the plant under the control of the strong cauliflower mosaic virus (CaMV) *35S* promoter.

RESULTS

Isolation of *rns1* mutants and *RNS1* overexpressing plants

To get a more accurate assessment of the function of RNS1, I screened the University of Wisconsin T-DNA-tagged lines for insertions within the *RNS1* gene. PCR primers complementary to sequences in the promoter region of *RNS1* (650 bp upstream of the transcription start site) and 450 bp 3' of the end of the cDNA were synthesized Two putative insertion lines identified in the primary screen were pursued further. The locations of the insertions are shown in Figure 4-1. The alleles were named *rns1-1* and *rns1-2*. The *rns1-1* allele lies within the 3' UTR, while the *rns1-2* allele is 85 bp 5' of the translation start site in the 5' UTR. PCR analysis revealed that the left border (LB) of the T-DNA flanked both ends of the insertion in both alleles (not shown). Plants homozygous for each allele were identified by PCR analysis. To analyze RNS1 function further, I transformed plants with the *RNS1* gene under the control of the strong constitutive CaMV *35S* promoter (Figure 4-1).

Once plants homozygous for the T-DNA insertions were identified, I examined RNS1 activity in the two mutants. Protein extracts from unwounded and 6-h wounded leaves were run on RNase activity gels. The *rns1-1* plants still had significant levels of wound-induced RNS1 activity (Figure 4-2A), as might be expected since the T-DNA insertion lies in the 3' UTR. Not only was there still activity, but also regulation of the gene appeared to be unaffected by the insertion: there was no activity in the absence of

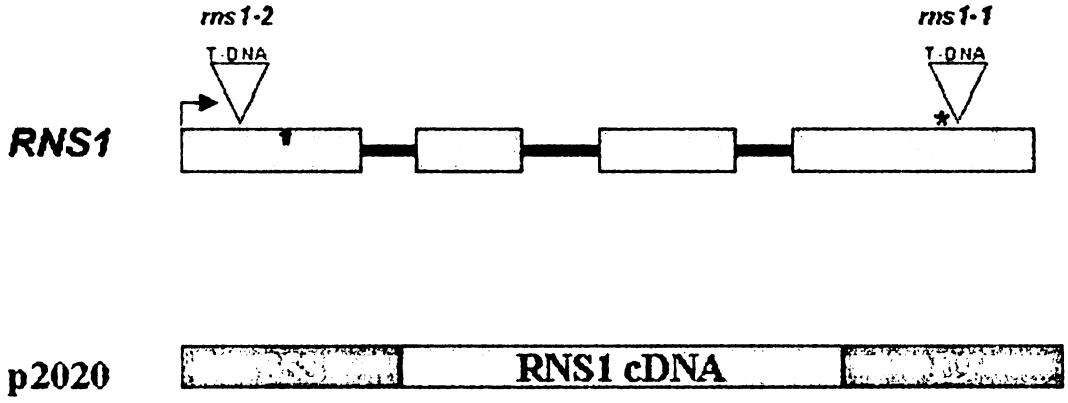


Figure 4-1. *rns1* mutants and overexpressor constructs.
Two *RNS1* T-DNA insertion alleles were identified. The first insertion, *rns1-1*, is downstream of the RNS1 polyadenylation site (asterisk). The second, *rns1-2*, lies in the 5' UTR, 85 bases 3' of the translation start site (arrow). The bent arrow indicates the transcription start site. Boxes represent exons, and lines represent introns. To overexpress *RNS1*, the cDNA was cloned between the CaMV *35S* promoter and the pea *E9* 3' end. This construct was named p2020.

wounding, and strong activity after wounding. The regulation in the mutant is consistent with sequences required for the wound-induction, as demonstrated in Chapter 3.

In contrast, *rns1-2* mutants lacked RNS1 activity, even after wounding (Figure 4-2B). I therefore studied this allele further. To confirm that the *rns1-2* line was homozygous for the T-DNA insertion, DNA gel blot analysis was performed. Genomic DNA digested with *Eco*RI was subjected to DNA gel blot analysis using an *RNS1* probe. While the probe hybridized to a 5-kb band in wild-type DNA, only a 2-kb band was seen in the *rns1-2* lane (Figure 4-3B). The absence of the 5-kb band in this lane indicates that the *rns1-2* allele is in the homozygous state in this line. The blot was also hybridized with a T-DNA probe to determine the number of T-DNA insertions in the genome. Sizes of the expected bands, based on restriction maps of the T-DNA vector and genomic region surrounding *RNS1*, are 1.0, 1.7, 4.6, and 5.1 kb. Bands of these sizes were observed (Figure 4-3A), and no other bands were found on the blot, indicating that the T-DNA had inserted at only one site in the *rns1-2* line. However, at least two copies of the T-DNA were present at this allele, since PCR fragments were obtained using primers corresponding to the LB of the T-DNA and to either the 5' or 3' ends of the *RNS1* genomic region (not shown). The exact number of T-DNA copies inserted was not determined.

Since this insertion lies in the 5' UTR of the gene and the gene might therefore produce transcripts including the full open reading frame, I performed RNA gel blot analysis of wounded and unwounded leaves from the mutant. Several aberrantly sized bands could be detected using an *RNS1* probe (Figure 4-3C). These were found only upon wounding, confirming previous results that the 5' UTR is not required for wound

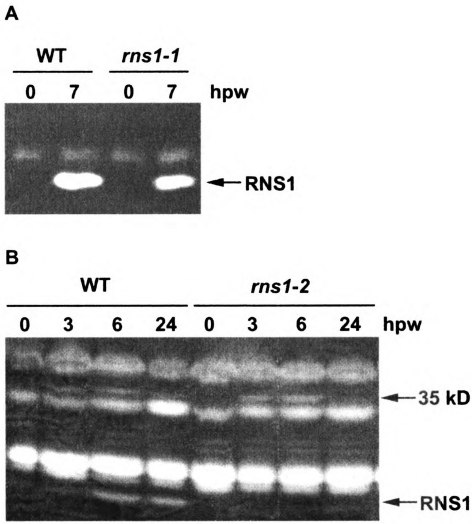


Figure 4-2. RNS1 activity in the *rns1-1* and *rns1-2* mutants.
(A) Wounded (7 hours post wounding, hpw) and unwounded (0) leaves of 4-week-old *rns1-1* and wild-type (WT) Ws Arabidopsis plants were analyzed by RNase activity gel assay.
(B) Leaves of WT Ws and *rns1-2* mutants were wounded, harvested at timepoints indicated, and analyzed by RNase activity gel assay. RNS1 activity (bottom arrow) can be found only in WT wounded samples. Induction of the 35-kDa RNase activity (top arrow) provides a positive control for the wound response of the *rns1-2* leaves. hpw, hours post wounding.

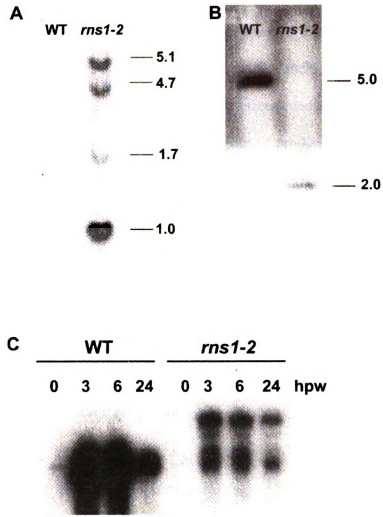


Figure 4-3. DNA and RNA gel blot analyses of *rns1-2* mutant plants.
Genomic Southern analysis of wild-type (WT) and *rns1-2* plants using the T-DNA **(A)** or *RNS1* **(B)** probe. Estimated sizes of hybridizing bands are shown to the right.
(C) Northern analysis of wounded WT and *rns1-2* leaves harvested at the indicated timepoints using an *RNS1* probe. hpw, hours post wounding.

induction of *RNS1* (see Chapter 3). The various sizes could be due to irregular splicing within the T-DNA and *RNS1*. One of the smaller bands was approximately the same size as the wild-type transcript; however, RT-PCR analysis of RNA from wounded *rns1-2* leaves did not detect any polyadenylated transcript that included the start of the open reading frame (not shown). Additionally, *RNS1* transcript induced in the wild type was significantly more abundant than the aberrant bands produced. Thus, although it is possible that a small amount of wild-type transcript is made and properly translated in this mutant, I predict the amount to be minimal.

Plants with high RNS1 activity in the absence of wounding were identified from a T_1 population of the 35S-RNS1 lines. Leaves were harvested and analyzed by activity gel assay. Multiple T_1 plants had high RNS1 activity; two examples are shown in Figure 4-4. Seeds from a homozygous T_2 progeny plant of line 2020.2 were used in further studies.

The *rns1-2* mutant does not have increased levels of anthocyanins

Since *RNS1* antisense lines have high levels of anthocyanins, particularly when grown on P_i^- medium, I looked for a similar effect in the *rns1-2* mutant, which had lower RNS1 activity induced by wounding than found in antisense plants subjected to P_i-starvation (compare Figure 6 in Bariola et al., 1999 and Figure 4-2b of this thesis). Thus, I expected a similar or more severe anthocyanin phenotype to be associated with the *rns1-2* mutant.

The anthocyanin content of *rns1-2* seedlings was measured after 7 d of growth on medium either containing (P^+) or lacking (P^-) P_i. Antisense *RNS2* lines, which have high anthocyanin levels similar to antisense *RNS1* plants (Bariola et al., 1999), and wild-type Ws seedlings were used as positive and negative controls, respectively. As expected, the

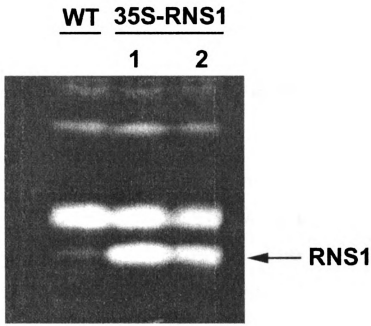


Figure 4-4. RNS1 activity in overexpressing plants.
Protein extracts of unwounded leaves of wild-type (WT) and two T_1 35S-RNS1 plants were analyzed by RNase activity gel assay. High RNS1 activity (arrow) is seen only in the overexpressing plants.

antisense line had a modest increase in anthocyanins when grown on P^+ medium and a stronger increase on P^- medium. However, the *rns1-2* mutant had anthocyanin contents similar to wild-type seedlings (Figure 4-5). This unexpected result was seen regardless of whether the *rns1-2* seedlings were grown on kanamycin-containing selection medium (kanamycin selection was used for the antisense lines in Bariola et al., 1999). Thus, it appears that the antisense lines have high anthocyanins for a reason other than the absence of RNS1 or RNS2 activity.

RNS1 activity appears to affect plant growth on RNA

Expression patterns of *RNS1* during P_i-starvation indicated that the RNS1 enzyme may be involved in nutrient recycling during P_i stress (Bariola et al, 1999). Therefore, I examined the effect of low P_i concentrations in the growth medium on *rns1-2* and 35S-RNS1 seedlings. No obvious morphological difference between wild-type and transgenic populations grown on concentrations of P_i varying from 0 to 1250 μM was found (not shown).

Further studies were conducted to examine the ability of the mutant lines to grow when provided with RNA as the sole source of phosphate. In the initial experiment, seeds were plated densely on AGM medium with either 1250 μM P_i (P^+) or 0.6 mg/ml RNA. On RNA-containing medium, the 35S-RNS1 seedlings were larger and bolted and flowered sooner than the WT Col seedlings, while the *rns1-2* mutants were smaller and lagged developmentally compared to WT Ws (Figure 4-6A). I then repeated the analysis of the 35S-RNS1 and WT Col seedlings with only 12 seedlings per plate to increase spacing between the plants. Once again, the overexpressing lines grew larger on the RNA plates (Figure 4-6B). While 35S-RNS1 seedlings were larger on RNA medium, on P^+

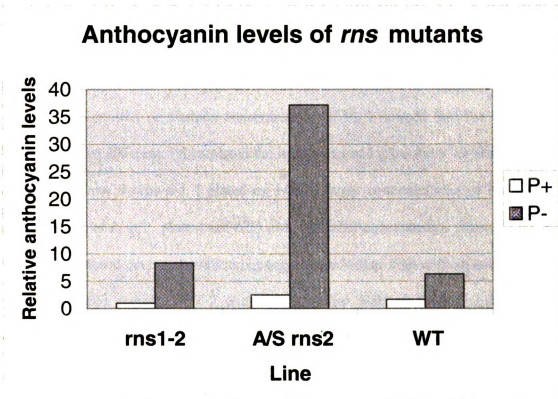


Figure 4-5. Anthocyanin content in mutant *rns* lines.
Anthocyanin contents of *rns1-2*, wild-type Ws (WT), and antisense RNS2 (A/S rns2) lines grown in the presence (white bars) or absence (gray bars) of P_i. Like antisense RNS1 seedlings, the antisense RNS2 line had high anthocyanin levels, particularly in the absence of P_i. The *rns1-2* line had anthocyanin levels similar to that of the wild type.

medium, as determined by fresh-weight analysis, the 35S-RNS1 seedlings were smaller than WT Col (Table 4-1; see below for more on this effect), indicating that high levels of RNS1 activity aid the overexpressor line in growing on RNA.

RNS1 activity affects plant size

When I first examined the *rns1-2* mutant, I expected the mutant to be less able to grow on medium containing low concentrations of P_i. I did not observe such effect, at least based on morphological examination (not shown). However, during a test in which I plated the wild type and mutant on various concentrations of P_i, I noticed that the roots of the mutant appeared different. I then plated the lines again and grew them vertically, to allow the roots to grow downward. I plated on two different concentrations of P_i: 1250 μM (concentration of P_i in P^+ plates) and 62.5 μM. The latter concentration allowed the plants to grow but caused severe starvation phenotypes, including high anthocyanin levels and severely reduced growth. *rns1-2* mutants grown on both of these P_i concentrations appeared to have longer roots than the wild type Ws seedlings (Figure 4-7A). To quantitate these differences, roots were measured and analyzed by *t*-test (Table 4-2). In each of five repetitions, the differences in root length were statistically significant ($P<0.001$). They were also different independent of the P_i concentration in the medium, indicating that this phenotype is not related to the putative function of RNS1 in P_i remobilization.

Since RNS1 activity affected root length in the mutant, I also examined the 35S-RNS1 roots. In two repetitions, the 35S-RNS1 seedlings had significantly shorter roots than the Columbia wild type (Figure 4-7B; $P<0.001$). Therefore, it appears that levels of RNS1 activity negatively correlate with root length in Arabidopsis.

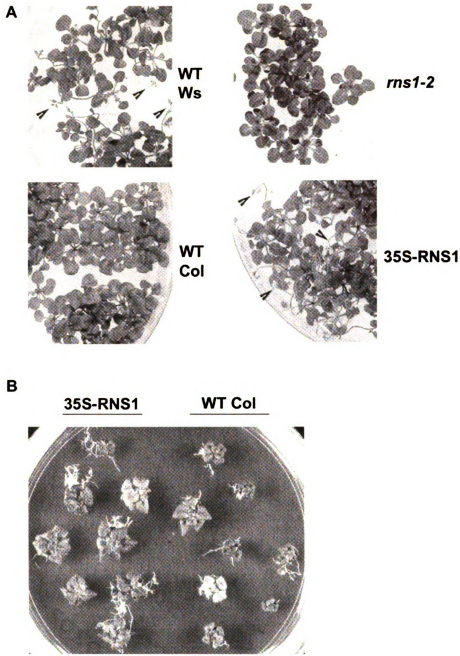


Figure 4-6. Growth of *rns1-2* and 35S-RNS1 seedlings on RNA-containing medium.
(A) 35S-RNS1, wild-type Columbia (WT Col), *rns1-2*, and wild-type Ws (WT Ws) seeds were grown on P_i^- plates containing 0.6 mg/ml RNA. After 34 days, the transgenic lines exhibited differences from their wild-type counterparts. While the WT Ws plants had bolted, *rns1-2* plants had not. Similarly, the WT Col seedlings, which normally bolt later than Ws, had still not yet bolted, while many of the 35S-RNS1 plants had bolted and flowered. Arrowheads point to flowers in the Ws and 35S-RNS1 plants.
(B) 35S-RNS1 and WT Col seedlings grown on P_i^- plates containing 0.6 mg/ml RNA.

Figure 4-7. Root phenotype of *RNS1* mutant and overexpressor.
(A) Root length of 12-day-old *rns1-2* and wild-type Ws (WT Ws) seedlings grown on vertical plates containing either 1250 or 62.5 μM P_i.
(B) Root length of 12-day-old 35S-RNS1 and wild-type Columbia (WT Col) seedlings grown on 1250 μM P_i.

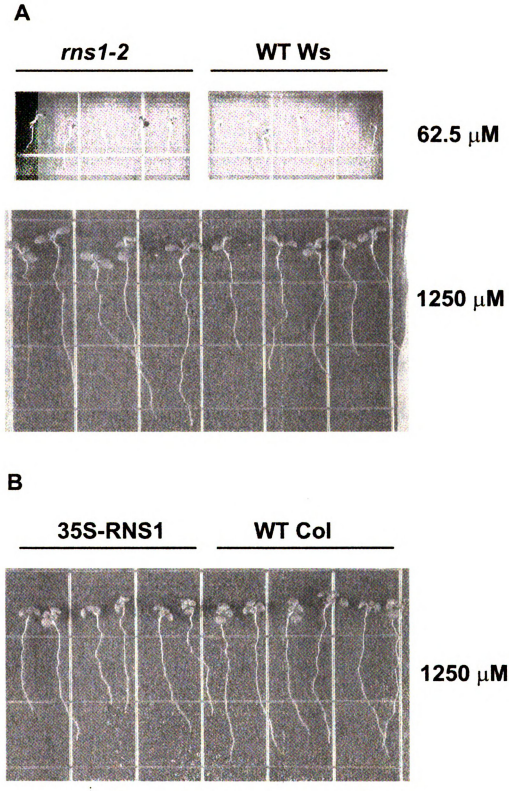


Figure 4-7. Root phenotype of *RNS1* mutant and overexpressor.

Table 4-1. Statistical analysis of fresh weight of WT, *rns1-2*, and 35S-RNS1 seedlings[1]

Line	Ave. weight per seedling (mg)	*t*	P
WT Col P^+	12.9	2.3	<0.05
35S-RNS1 P^+	8.8		
WT Ws P^+	9.6	4.3	<0.01
rns1-2 P^+	12.9		
WT Col RNA	10.8	4.8	<0.01
35S-RNS1 RNA	15.7		

[1]The *t*-test was used to determine statistical significance of the weight differences between 9-day-old WT Col and 35S-RNS1 and WT Ws and *rns1-2* seedlings grown on P^+ medium and between 37-day-old WT Col and 35S-RNS1 seedlings grown on P^- medium containing 0.6 mg/ml RNA. Results shown are from one experiment. A P-value of 0.05 or less is considered statistically significant.

Table 4-2. Statistical analysis of root length in *rns1-2*, WT Ws, 35S-RNS1 and WT Col seedlings[1]

Test	Line	P_i (μM)	Age (days)	Ave Root length (mm)	*t*	P
1	*rns1-2*	62.5	13	10.0	12.5	<0.001
	WT Ws			6.6		
	rns1-2	1250		48.3	12.6	<0.001
	WT Ws			31.0		
2	*rns1-2*	62.5	12	7.1	8.1	<0.001
	WT Ws			3.1		
	rns1-2	1250		37.4	8.0	<0.001
	WT Ws			29.5		
3	*rns1-2*	62.5	7	4.5	6.0	<0.001
	WT Ws			3.6		
	rns1-2	1250		16.9	5.3	<0.001
	WT Ws			13.5		
4	*rns1-2*	1250	8	36.8	5.3	<0.001
	WT Ws			29.1		
5	35S-RNS1	1250	12	41.2	4.4	<0.001
	WT Col			50.3		
6	*rns1-2*	1250	9	24.6	5.2	<0.001
	WT Ws			18.3		
	35S-RNS1	1250		19.5	5.2	<0.001
	WT Col			24.3		

[1]The *t*-test was used to determine statistical significance of the differences in root lengths in seedlings grown on two different P_i concentrations. A P-value of 0.05 or less is considered statistically significant.

In addition to the roots, the size of the green tissue of the *rns1-2* and overexpressor seedlings appeared to differ from the size of the wild types. I quantitated this difference by growing the four lines on P^+ medium and weighing the seedlings. I removed the roots to eliminate any effect of the differences I observed in root tissue. *T*-test analysis of the weights indicated that *rns1-2* seedlings were significantly larger than WT Ws seedlings ($P<0.01$), and 35S-RNS1 seedlings were smaller than WT Columbia ($P<0.05$) (Table 4-1). These results are preliminary but are consistent with the phenotype seen in root length.

The difference in root length could be due to cell size, cell number, or a combination of the two. To address this issue, I examined the roots by laser confocal microscopy. Roots of the four lines were fixed and stained using two dyes, DAPI to stain nucleic acid and calcofluor to stain the cell wall. I did not observe a large increase in size of the *rns1-2* cells or smaller 35S-RNS1 cells (not shown), but a small difference may be difficult to visualize using this technique. A more involved experiment, including measurements of hundreds of cells, will be a more thorough method to assess any differences.

During the analysis of these roots, I noticed a difference in the rate at which the *rns1-2* and wild-type roots absorbed the dyes. Five minutes after adding dyes to the WT roots, the intensity of the stain was low (Figure 4-8). After 45 min, the staining was stronger, and a larger portion of the root could be viewed. However, even at this point, the intensity of the WT root was not as high as the *rns1-2* root only 5 min after the dyes were added. These results are preliminary and require repetitions to ensure that the effect was not an artifact of the staining preparation procedure. However, as will be discussed

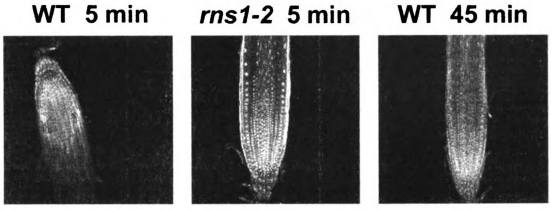


Figure 4-8. Absorption of dyes in wild-type Columbia (WT) and *rns1-2* mutant root cells.
The dyes calcofluor and DAPI were added to fixed intact roots and photographed at the indicated times. Staining of the mutant root cells was greater in 5 min than that of the WT cells even after 45 min.

These photos were taken by Gustavo MacIntosh and Kirk Czymmek.

below, the differential penetration of the dyes was consistent with the phenotype seen in a yeast mutant in the *RNY1* gene, the only homolog of *RNS1* in *S. cereviseae*, and supports the hypothesis that RNase T_2 enzymes are involved in regulation of membrane permeability (MacIntosh et al., 2001).

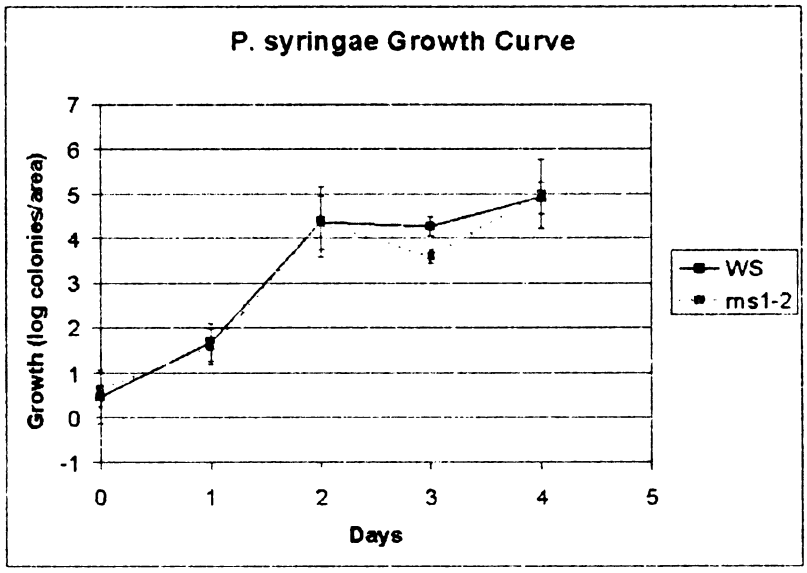
Absence of RNS1 does not increase susceptibility of the plant to a bacterial pathogen

As discussed in Chapter 2, the induction of RNS1 activity by wounding in unwounded leaves is not easily explained as part of a nucleotide-recycling mechanism, since this part of the plant has not been injured and should not require such a system. I hypothesized that RNS1 might also play a role in defense of the plant from opportunistic pathogens that invade after wounding. Thus, as a first examination of this putative function, I tested the ability of a bacterial pathogen, *Pseudomonas syringae* pv tomato (*Pst*) DC3000 to infect the *rns1-2* and 35S-RNS1 plants in comparison to their respective WT.

Leaves of the *rns1-2*, WT Ws, WT Col, and 35S-RNS1 plants were inoculated with *Pst*, and growth of the pathogen was monitored for three days. In two repetitions, growth of the pathogen on the *rns1-2* mutant was similar to that on WT Ws (Figure 4-9A). Unlike *rns1-2*, the overexpressor appeared to have an effect on the growth of the bacteria; in four independent experiments, I consistently detected a lower *Pst* growth rate in 35S-RNS1 compared to that in WT Col (Figure 4-9B). However, error bars indicated that variation between growth on the two lines was too great to make a statistically valid conclusion. Additionally, analysis of variance indicated no significant difference between growth of the bacteria on the 2nd day after infection. Further repetitions of this experiment, particularly focusing on day two, may indicate valid differences. However, I currently cannot conclude that RNS1 activity affected growth of this pathogen.

Figure 4-9. Growth of *Pst* DC3000 on leaves of 35S-RNS1 and *rns1-2* plants.
(A) Leaves of 4-week-old wild-type Ws (Ws) and *rns1-2* mutants were infiltrated with *P. syringae*. Starting on the day of infiltration (day 0), bacterial growth was measured until day 3. Representative results of one of two experiments are shown.
(B) Growth of *P. syringae* on wild-type Columbia (Col) and 35S-RNS1 plants was monitored as in (A). Averaged results from four independent experiments are shown.

A



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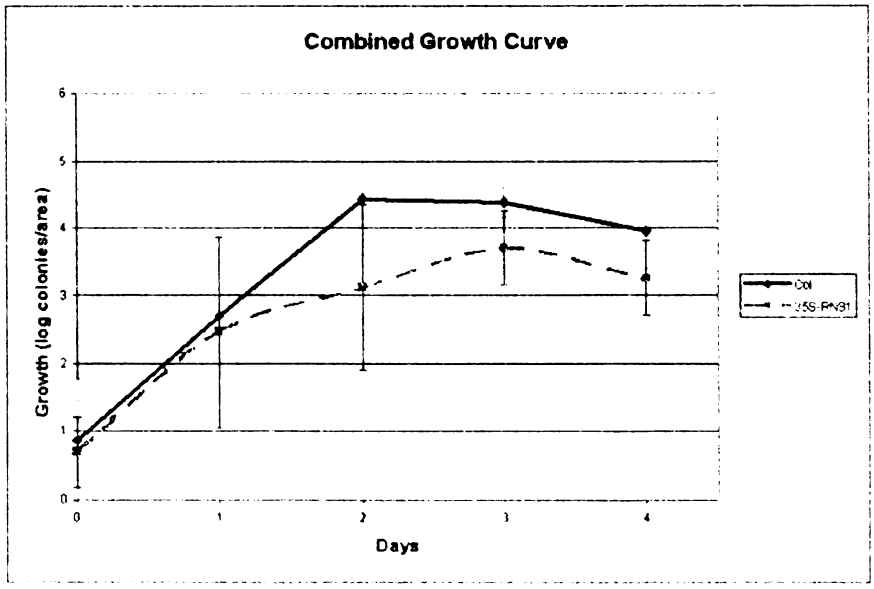


Figure 4-9. Growth of *Pst* DC3000 in leaves of 35S-RNS1 and *rns1-2* plants.

DISCUSSION

The vast majority of studies done on extracellular RNases, including the RNase T_2 family, have focused on structural or enzymatic studies, or have addressed regulatory questions. Gene expression or protein induction patterns have been particularly useful in providing insights into the function of S-like RNases, but many questions still remain. I have attempted to gain further information on the function of plant secreted RNases through basic plant biology techniques, namely, overexpression and gene knock out of *RNS1*. The phenotypes of these plants have not only reinforced previous ideas concerning the role of this family of enzymes, but also indicate that they may perform unexpected functions in the plant.

Involvement of RNS1 in phosphate remobilization

Previous data, including gene expression patterns and the phenotype of antisense *RNS1* plants, have been consistent with the idea that RNS1 is involved in the remobilization of P_i from nucleic acid sources, either in the extracellular space or possibly from an intracellular source (Bariola et al., 1999). I therefore expected similar insights to be gained by studying the *rns1-2* null mutant and *RNS1* overexpressor. One phenotype of the plants studied here is consistent with this previously proposed function: the overexpressing plants appear better able to grow when provided with RNA as the only source of phosphate. This phenotype is interesting because it is the first evidence that RNS1 may be secreted from the roots in order to degrade organic material in the surrounding rhizosphere to provide the roots with P_i for uptake. Introduction and secretion of an *Aspergillus* phytase activity into Arabidopsis has been shown to improve the plants' ability to utilize phosphorus from phytate, a major component of soil organic

phosphorus (Richardson et al., 2001). By extension, induction of secreted RNases may be an endogenous method plants use to acquire P_i from other organic sources. It will be important to study this effect further, to quantitate the differences in plant weight and to ensure that this effect is specific to growth on RNA by using DNA-containing medium as a control. Although I did not have the opportunity to quantitate differences, the observed growth effects on RNA are in contrast to the sizes of the plants grown on high P_i, in which case the 35S-RNS1 plants appear to be smaller than the WT, indicating that the effect of the RNA overcomes the differences in growth on medium containing high P_i.

I did not detect any differences in the sizes of the plants grown on low concentrations of P_i that are consistent with a role for RNS1 in P_i-recycling. One possible interpretation of this result is that the presence of RNS2, which is also induced by P_i starvation, provides the *rns1-2* seedlings with sufficient RNA-degrading activity. However, the *rns1-2* mutant was less able to grow on RNA plates than the wild type. The different localizations of the two enzymes may cause these effects; RNS1 is extracellular, and RNS2 appears to be intracellular, most likely either vacuolar or ER-associated (Bariola et al., 1999). Therefore, RNS2 may be unable to degrade RNA in the medium because the substrate is not accessible.

I was also surprised that the *rns1-2* mutant did not have high levels of anthocyanins, either in the presence or absence of P_i. The antisense lines had significantly higher anthocyanin contents, and this was shown to be specific for reduced RNS1 or RNS2 activity; the vector controls did not have high anthocyanin content (Bariola et al., 1999). Therefore, there appears to be another reason for the accumulation of anthocyanins in the antisense lines. For example, as mentioned in the introduction to this chapter, kanamycin

segregation indicates that there may be an unusual silencing of the transgenes in the antisense lines. The reduced kanamycin resistance may somehow result in increased anthocyanins, even in the kanamycin resistant seedlings. Alternatively, the sequences used to silence *RNS1* or *RNS2* may have an effect on the accumulation of *RNS4* and/or *RNS5*, which may then cause increased anthocyanins. Expression of *RNS2* was not affected in the antisense *RNS1* lines, and vice versa (Bariola et al., 1999), but *RNS4* and *RNS5* had not been identified at the time of the analysis of the antisense lines. The expression of these genes in the antisense lines was therefore not explored.

Is RNS1 involved in defense mechanisms?

One interesting hypothesis borne from the observation that RNS1 is induced systemically upon wounding is the idea that this enzyme may be involved in defense of the plant. My initial results do not support this hypothesis. The *rns1-2* mutant did not have increased susceptibility to *Pst*. However, *RNS1* is not normally induced by infection with this pathogen (not shown). Therefore, the mutant would not be expected to have increased sensitivity. Additionally, *Pst* is virulent in Ws, and therefore, I may not be able to observe further susceptibility. Interestingly, though, the *RNS1* overexpressing plants appeared to have an increased resistance to *Pst*. This effect is only preliminary; further replications must be done for this to be considered a reliable result.

Other pathogens may be more likely to reveal that the presence or absence of RNS1 can have an impact on defense mechanisms. For instance, it was shown that application of RNase A in the extracellular space of tobacco leaves prevented the growth of the oomycete *Phytophthora parasitica* (Galiana et al., 1997). Inoculation of tobacco leaves with this pathogen also induced expression of the gene encoding RNase NE, another S-

like RNase. In the future, a pathogen more closely related to *P. parasitica*, such as *Peronospora parasitica*, may be a better tool to analyze the role of RNS1 in defense.

RNS1 activity negatively correlates with root length

Possibly the most surprising phenotype that resulted from altering the expression levels of *RNS1* was the difference in root length. This effect may extend to the green tissue of the plant as well, as preliminary evidence suggests that the weight of this tissue is also increased in the mutant and reduced in the 35S-RNS1 plants (not shown). These effects are particularly unexpected because *RNS1* transcript was not detected in roots or leaves by Northern blot (Bariola et al., 1994). Under non-inducing conditions, *RNS1* was detected only in flowers. Most likely, RNS1 is expressed in pistils, not petals, based on microarray analysis of the *apetala2* and *agamous* mutants (SMD experiment ID 12302; http://genome-www5.stanford.edu/MicroArray/SMD/). However, *RNS1* can be detected in leaves by RT-PCR (not shown), indicating that some activity may also be present in tissues other than flowers. Perturbation of this small amount of activity in roots and possibly throughout the plant is evidently sufficient for the phenotypes I observed. Similarly, although the yeast enzyme Rny1 cannot be detected by activity gel assay in wild-type cells, the knock-out still shows the enlarged cell-size phenotype (MacIntosh et al., 2001).

Examination of the cells in the roots did not reveal gross changes in the cell size. It is possible that there is a small difference, e.g., 10%, in the cell size between the different lines. Differences on this order would be difficult to detect by eye. As such, more in depth analysis of the size of the cells is required.

I hypothesized that cells in *rns1-2* would be larger than WT cells based on results

seen in a mutant in the *RNY1* gene of *S. cereviseae*. In this case, the mutant cells were approximately 10-fold larger in diameter than the WT cells and had enlarged vacuoles (MacIntosh et al., 2001). Thus, I postulated that a similar effect may be causing the *rns1-2* roots to be larger. However, the presence of the cell wall or factors regulating cell-cycle may limit the expansion of the *rns1-2* cells. As such, the cells may not be enlarged, but may instead divide more rapidly. We plan to compare cell division rates in the mutant and overexpressor lines by microscopic video analysis of the root cells during growth.

During the analysis of the root cells, we also noticed that *rns1-2* cells seemed to absorb the dyes more rapidly than the WT cells (Figure 4-8B). This effect has also been seen in the *rny1*Δ mutant using syro13, a dye that does not normally enter cells, but which is taken up by the *rny1*Δ cells (G.C. MacIntosh and P.J. Green, unpublished observations). These results are also preliminary; however, two of the phenotypes in the yeast mutant are consistent with my observations of the plant knock out: the yeast *rny1*Δ cells and the *rns1-2* mutant are bigger under normal growth conditions, and both appear to be more permeable to stains. MacIntosh et al. (2001) hypothesized that natural RNAs may exist in the membrane, possibly as an ancient mechanism to control membrane stability and permeability in the "prebiotic world". They further postulated that secreted ribonucleases may be used to control the amount of RNA in the membrane. In the *rny1*Δ mutant, this control is lacking, resulting in excess RNA in the membrane and a corresponding increase in membrane permeability. The cells may compensate for this change by increasing in size and enlarging the vacuole. Similarly, the *rns1-2* mutant is lacking an extracellular RNase activity in the same family as Rny1. The cells may be more permeable, and consequently, the plant is larger.

My results indicate that several phenotypes result from altering the expression of *RNS1*. It will be important in the future to confirm these results by complementing the phenotypes using a genomic fragment of *RNS1*. The *rns1-2* mutant has already been transformed with a construct that consists of the *RNS1* coding region plus 1.4 kb of upstream promoter sequence and 1.8 kb of sequence downstream of the stop codon, and primary transformants have been selected. In the near future, seeds from these plants and from plants transformed with the vector control should be plated for analysis of root length. If lack of RNS1 causes longer roots, the complemented plants should have roots of WT length. This result is expected, since the 35S-RNS1 roots are shorter than roots of Columbia wild type.

Since the *rns1-2* line has a minor *ap2* phenotype as a result of the T-DNA used in the mutagenesis, we are in the process of isolating additional alleles. The T-DNA insertions in these *rns1-3* and *rns1-4* alleles lie in the *RNS1* coding region (+602) and promoter (−80), respectively. Since the T-DNA lies between the two conserved histidine residues highly conserved in S- and S-like RNases, the *rns1-3* allele may prove to be a valuable null mutant. Any phenotypes seen in *rns1-2* should also be present in new alleles identified.

MATERIALS AND METHODS

Plant materials

Throughout this chapter, *Arabidopsis thaliana* ecotypes Columbia or Ws were used. The Ws ecotype was used as a WT control for the *rns1* mutants, since Ws was used to generate the Wisconsin T-DNA collection. The 35S-RNS1 construct was transformed into the Columbia ecotype; therefore, Columbia was used as a control for experiments

involving these lines.

For plating, seeds were sterilized as described (Pérez-Amador et al., 2000). Selection medium contained 50 μg/ml kanamycin. RNA-containing medium was made according to Chen et al. (2001).

Root length was determined by growing seedlings vertically in a chamber (Percival) at 22°C. Lengths were measured at different ages, including 9, 12, and 14 days. Four repetitions of *rns1-2* versus WT Ws and two repetitions of 35S-RNS1 versus WT Columbia were performed. The first three sets of *rns1-2*/Ws seedlings were grown on 1250 and 62.5 μM P_i. In total, more than 320 seedlings each of *rns1-2* and WT Ws and more than 40 seedlings each of 35S-RNS1 and WT Columbia lines were measured and analyzed for a statistically significant variation by *t*-test. Values of *t* for each of the nine sets exceeded the cut-off value for a probability of 0.1% ($P<0.001$).

Fresh weight of green tissue was determined by excising this tissue from the root base. Seedlings were measured in sets based on the plate they were grown on. The total weight per line per plate was measured and a per seedling weight was calculated for each plate. A *t*-test was used to determine whether the differences between the weights of the four lines were significant. Using at least 37 seedlings per line, $P<0.05$ for the 35S-RNS1 vs. WT Columbia set and $P<0.01$ for the *rns1-2* vs. WT Ws set.

Plasmid construction

A clone containing the *RNS1* cDNA fused between a doubly enhanced copy of the cauliflower mosaic virus *35S* promoter and the *nos* terminator was constructed previously (Bariola, 1996). This plasmid was digested with *Xba*I to remove the entire promoter/cDNA/terminator cassette, and the fragment was ligated into the *Xba*I site of

the plant transformation vector pCambia 2301. WT *Arabidopsis thaliana* ecotype Columbia plants were transformed with this construct, p2020, by vacuum infiltration (Bechtold et al., 1993).

Identification of *rns1* T-DNA insertional knock-out mutants

The University of Wisconsin T-DNA insertion line collection was screened using primers PG904 (positioned at −572 from the *RNS1* transcription start site) and PG905 (+1645). Primers were tested on WT Ws genomic DNA and resulted in the amplification of a 2.2-kb fragment. These primers were sent to the University of Wisconsin for PCR-screening of their T-DNA insertion line collection using each of the two primers and a primer corresponding to the left border (LB) of the T-DNA. An aliquot of each reaction was electrophoresed on 0.8% (w/v) agarose gels and transferred to Nytran Plus nylon membrane (Schleicher and Schuell, Keene, NH). The RNA blots were hybridized as described in Taylor and Green (1991) using a ^{32}P-labeled *RNS1* probe. The *RNS1* probe hybridized strongly with the PCR products from two reactions: LB/PG904 reaction 15 and LB/PG905 reaction 19. Bands corresponding to these signals were gel-isolated and sequenced to confirm the position of the insertion. The LB/PG904 insertion (*rns1-1*) lay 20 bases 3' of the stop codon, and the LB/PG905 insertion (*rns1-2*) caused a deletion of 21 bases in the 5' UTR, 65 to 86 bases upstream of the *RNS1* translation start site.

Seeds were obtained from Wisconsin for the appropriate 25 pools of 9 insertion lines each. Seeds were grown and harvested as pools for PCR analysis to identify the pools that contained the desired insertions. Seedlings from those two pools were then transferred to soil, and individual plants were screened by PCR analysis to identify single plants harboring at least one copy of the *rns1* alleles. At least one heterozygous plant was

identified for both alleles. These plants were allowed to self-fertilize, and their progeny were analyzed similarly to identify a homozygous line. Seeds from the homozygous lines were used for further analyses.

Protein and RNA analyses

Protein and RNA purification and analyses were performed as described in Chapter 2.

DNA extraction and DNA gel blot hybridization

For genomic Southern analysis, DNA was extracted from rosette leaves of mature plants using Plant DNAzol (GibcoBRL) according to the manufacturer's instructions. DNA was quantified by ethidium staining and comparison against a known amount of Lambda DNA. After digestion with *Eco*RI, 3 μg DNA was separated by electrophoresis in a 1.0% (w/v) agarose gel and blotted and hybridized as above using a ^{32}P-labeled *RNS1* or T-DNA probe. The T-DNA probe was a PCR product of the entire transferred region of the T-DNA in the *rns1-2* allele.

For genomic DNA extraction for PCR, a shorter extraction protocol was used to isolate DNA from one to two leaves of young plants. Tissue was pulverized and incubated for 30 min at 65°C in 500 μl CTAB buffer [2% (w/v) CTAB (Sigma); 1.4 M NaCl; 0.2% (v/v) β-mercaptoethanol; 20 mM EDTA; 100 mM Tris, pH 8.0]. The solution was extracted twice with chloroform:IAA [24:1 (v/v)], precipitated, and resuspended in 100 μl ddH_2O containing RNase A (1 μg/μl). PCR was performed according to the University of Wisconsin web site (www.biotech.wisc.edu/Arabidopsis/Guidelines.html) using 1 μl of DNA for each reaction.

Anthocyanin assays

Anthocyanin contents of WT Ws, *rns1-2*, and *RNS2* antisense lines were measured according to Bariola et al. (1999). Levels of *rns1-2* and WT anthocyanins were determined twice, by growing the lines once on medium lacking selection and once on kanamycin selection medium (except WT). The *RNS2* antisense line was included in the latter as a positive control for high anthocyanin content. Representative anthocyanin content of one experiment is shown; results from the other were very similar. Error bars are not included because the *RNS2* antisense line was examined only once.

Bacterial growth curves

Inoculations with *Pseudomonas syringae* pv. tomato DC3000 were performed as described by Li et al. (2000). Samples of inoculated tissue from four different leaves of each line were taken daily by excision with a cork borer (area/leaf = 0.250 cm^2). Bacteria inside the leaf discs were released by grinding the tissue in a microfuge tube in sterile water and plated on LB medium. The bacterial population was determined based on the numbers of colonies formed on LB plates, as described by Bertoni and Mills (1987). Mean results from four repetitions of the 35S-RNS1 and WT Col lines are shown. Representative results of two repetitions of the *rns1-2* and WT Ws lines are shown.

CHAPTER 5

ANALYSIS OF AN *ARABIDOPSIS THALIANA* MUTANT WITH AN ALTERED RNASE PROFILE

ABSTRACT

Plants produce a complex series of ribonucleases (RNases), but little is known about their biological function and regulation at the molecular level. In Arabidopsis, stem tissues are particularly rich in RNases, suggesting that RNase regulation and function may be linked at the molecular level to stem growth and development. To address this possibility and to generate tools to study RNase function, we screened stem extracts of Arabidopsis to identify mutants with altered RNase profiles (*arp* mutants). Several mutants affecting different size classes of RNA-degrading enzymes were isolated. Of particular interest was *arp1*, which overproduces a doublet of 33-kDa RNases. Based on our analysis, we were able to demonstrate genetically that these activities are bifunctional nucleases that degrade both RNA and DNA. Additionally, *arp1* overproduces a previously unidentified small RNase of approximately 23 kDa that comigrates with the well-characterized RNase, RNS1. The elevated RNase activity caused by the *arp1* mutation is observed only in stem tissue and is not found in leaves or in seedlings. Other phenotypes of *arp1* are also stem-associated, including overproduction of the 33-kDa nucleases, short stature, and increased branching. These data indicate that the *ARP1* locus constitutes a novel regulator of the production of several RNase and nucleases in stems and that ARP1 may be an important factor in stem growth and development.

INTRODUCTION

Although RNases have been used for decades as models for protein structure studies, the biological roles of these enzymes and the mechanisms by which they are regulated in plants and in other higher eukaryotes are largely unknown. In particular, functional analyses of secreted RNases that do not appear to be involved in mRNA or rRNA processing are limited. To date, fluctuations in RNase activity levels or gene expression have provided the most useful data for predicting RNase function, although the machinery involved in these regulatory pathways has not been elucidated. Additionally, organ-specific expression patterns of RNases can be found that may lend clues to their functions *in vivo*. For instance, the most complex pattern in mature Arabidopsis plants is found in stem extracts (Yen and Green, 1991). Our knowledge of the molecular pathways controlling stem-specific processes in plants, e.g., branching, or other processes prominent in stem tissue, such as tracheary element formation (for reviews, see Schmitz and Theres, 1999, and Roberts and McCann, 2000, respectively), is limited. However, the high level of activity found in stems suggests that RNases could play important roles in such processes. The induction of multiple nuclease activities that occurs in zinnia cells undergoing differentiation into tracheary elements (Fukuda, 2000) is consistent with this argument.

In contrast to the many studies on the expression patterns of RNases, very little is known concerning the factors that regulate these pathways at the molecular level. For example, while P_i starvation in Arabidopsis induces *RNS1* and *RNS2*, but not *RNS3* (Bariola et al., 1994) or *BFN1* (Pérez-Amador et al., 2000) expression, senescence induces all four of these activities (Taylor et al., 1993; Bariola et al., 1994; Pérez-Amador

et al., 2000). Evidently, there is a high degree of specificity of RNase regulation by different stimuli, but the molecular components controlling these patterns are not known, neither in plants, nor in most higher eukaryotic systems. The identification of regulators is the first step in the elucidation of the mechanisms plants use to mobilize certain sets of enzymes in response to developmental or environmental cues.

To further our understanding of the regulation of RNases and thereby gain insight into their potential functions, we designed a screen to isolate mutants of Arabidopsis with altered RNase profiles. Through a gel-based screen of EMS-mutagenized M_2 plants, we identified several mutants that show altered expression of at least one RNase activity. In particular, one mutant with altered levels of at least three activities provides a useful tool for the elucidation of regulatory pathways involved in RNase expression in Arabidopsis. This alteration of RNase patterns appears to be specifically associated with the stem, and physiological phenotypes of the mutant are also related to modifications in stem morphology, possibly offering new insights into processes involved in stem development and differentiation.

RESULTS

An RNase-activity gel screen for mutants affecting the Arabidopsis RNase profile

To screen for mutants with altered RNase profiles, a gel-based assay for RNase activity (Yen and Green, 1991) was used to analyze protein extracts from the stems of EMS-mutagenized M_2 Arabidopsis plants. Stem extracts were assayed because the stem RNase profile is more complex, containing more bands of RNase activity, than the leaf RNase profile (Yen and Green, 1991). It was previously reported that up to 13 bands of RNase activity could be distinguished on a gel containing Arabidopsis stem extracts (Yen and

Green, 1991). Eight bands of RNase activity were consistent and intense enough to screen for alterations in their activities. Protein extracts from 2,500 M_2 plants were scored on the RNase activity gels to identify plants whose RNase profiles differed from those of wild-type stems. Figure 5-1 depicts an example of the identification of a this type of mutant (compare the intensity of bands at approximately 33 kDa in lane 2657 with their intensities in other lanes). In the primary screen, 112 putative mutants with alterations in the Arabidopsis RNase profile were identified. The phenotypes of 60 of the 112 putative mutants could be reproduced on subsequent gels. To determine whether the phenotypes observed in such potential mutants were heritable, several progenies (M_3) of each were grown, stem extracts were prepared, and the extracts were examined using the activity gel assay. Those mutants with heritable phenotypes were studied further.

In total, nine altered RNase profile (*arp*) mutants that showed the original phenotypes in all M_3 progeny have been isolated. This chapter focuses on those mutants that affect the activity of a doublet of 33-kDa RNase activities for several reasons. Six of the nine mutants isolated display alterations in the levels of these activities. Previously, 33-kDa activities were seen on both RNase and DNase activity gels (Yen and Green, 1991). The doublet is also interesting because the RNase activities are induced in seedlings starved for inorganic phosphate (P_i) (see Figure 10 in Bariola et al., 1994) and by wounding (Chapter 2). One of the six mutants affecting the 33-kDa activities was plant 2657, first identified in Figure 5-1 as showing increased RNase activity of the doublet. The phenotype of 2657 was seen more clearly in a lower percentage acrylamide gel that resolved the two bands of the doublet (Figure 5-2A). In contrast to the increased activity of the 33-kDa RNases in line 2657, relative to the wild-type activity (first and last lanes),

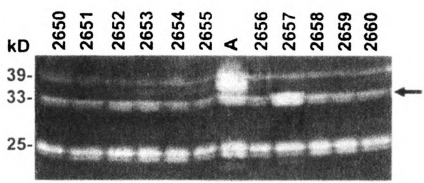


Figure 5-1. *arp* mutants are altered in RNase activity profiles
Primary identification of an M_2 RNase activity mutant. Stems of plant 2657 showed increased levels of two bands of RNase activity at 33 kDa. Plant numbers are shown above the gels. Approximate molecular weights (kDa) are indicated to the left. Arrows on the right point to the RNase activities affected in the mutant extract. 100 µg of protein was loaded in each lane. An extract from aerial portions of wild-type plants (leaves, stems, flowers), indicated by "A", was included as a standard.

This experiment was performed by Michael Abler and Linda Danhof.

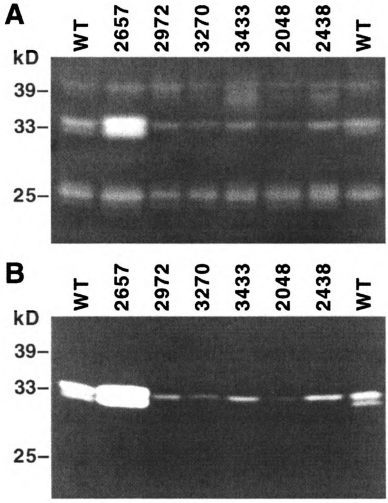


Figure 5-2. The 33-kDa RNase activities also degrade DNA.
(**A**) RNase activity gel of stem samples from the six lines in which the 33-kDa RNase activities are affected. Plant numbers are shown above the gels and approximate molecular weights (kDa) are indicated to the left. WT, wild-type RLD stem extracts.
(**B**) DNase activity gel of the six mutants in which the 33-kDa RNase activities are affected. Denatured single-stranded DNA was substituted for RNA as the substrate in the gel.

This experiment was performed by Michael Abler.

plants 2972, 3270, 3433, 2048, and 2438 all greatly diminish or abolish the activity of the lower band of the doublet (Figure 5-2A).

The 33-kDa RNase activities are bifunctional nucleases

Previous data from our laboratory indicated that there is also a doublet of DNase activity at 33 kDa, but it was not known whether both the RNase and DNase activities were derived from the same enzyme(s) (Yen and Green, 1991). The availability of several plants affecting the 33-kDa RNase activities allowed us to test this hypothesis by analyzing the DNase activities in these mutants.

Figure 5-2A shows the RNase gel phenotypes for the six plants affected in the activity of the 33-kDa doublet. Shown in Figure 5-2B, protein extracts from the same plants were run in a gel containing denatured single-stranded DNA as the substrate for the enzyme activity. The profile of DNase activities in Arabidopsis stem extracts is far simpler than the RNase profile, and the 33-kDa doublet is the most abundant of the DNase activities in wild-type stems. In each of the plants, the alteration in the DNase gel phenotype corresponds with that in the RNase gel for the 33-kDa activities. Because all of the mutations affect the DNase and RNase activities in the same manner, these data demonstrate genetically that the 33-kDa doublet corresponds to a pair of bifunctional nuclease activities, capable of degrading either RNA or DNA.

Genetic characterization of the *arp* mutants

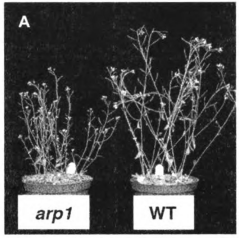
Genetic analyses of the plants affected in the 33-kDa activities were performed. Each line was backcrossed to WT RLD to determine the inheritance patterns of the mutations. The RNase profiles of the F_1 plants from each of the crosses were indistinguishable from that of the wild type, thereby indicating that all nine mutations were recessive (Abler et al.,

manuscript in preparation). Segregation of the mutant phenotype in the F_2 generation showed that the mutations were inherited in a Mendelian fashion, fitting a 3:1 ratio of wild type to mutant (Abler et al., manuscript in preparation).

Complementation tests of the six mutants affecting the 33-kDa nuclease activities indicated that the mutations correspond to two loci and that the 2657 mutation affects a locus distinct from that affected in the other four mutants (Abler et al., manuscript in preparation). Accordingly, the mutant locus generating the gel phenotype exhibited by plant 2657 (increased 33-kDa activities) was designated *arp1*. During subsequent mapping experiments, it was determined that the gel phenotype of mutant lines 2048, 2438, 2972, 3270, and 3433 resembled the pattern of stem RNase activities in the Columbia ecotype of Arabidopsis (not shown and LeBrasseur et al., in press). From this observation and subsequent RFLP analysis, we concluded that these lines were in fact Columbia contaminants in the screen, which had been performed using the ecotype RLD. Nevertheless, our results demonstrate that a single locus confers ecotype-specific control of the lower band of the 33-kDa doublet in wild-type stems.

Morphology of the *arp1* mutant

Examinations of growth and development indicated that a visible phenotype is associated with altered expression of the 33-kDa nuclease activities in *arp1*. As shown in the example in Figure 5-3A, *arp1* plants appear shorter and more branched than wild type. To quantify these visible phenotypic differences, two blind studies were performed in which approximately 40 plants each (wild type and *arp1*) were scored. Seeds from the *arp1* line and from wild-type plants were planted in pots, and the order of the pots was



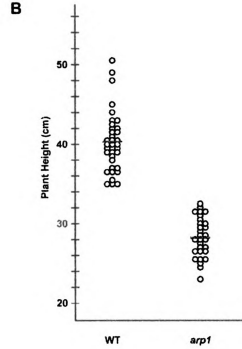


Figure 5-3. Morphological differences in the phenotypes of wild-type and *arp1* mutant plants.
(A) Six-week-old *arp1* and wild-type (WT) plants are shown. The *arp1* plants appear shorter and more branched than wild type plants.
(B) Scatter plot of final plant height of wild-type and *arp1* plants. Individuals in the respective populations are represented by circles. The average plant heights for the populations are represented as horizontal bars.

This experiment was performed by Michael Abler.

randomized. The time for each plant to reach certain developmental stages (*e.g.*, germination, flowering) was measured, as were physical (*e.g.*, plant height) characteristics of the plants. Differences between populations were analyzed by the Mann-Whitney rank-sum test.

Significant growth and developmental differences between wild-type Arabidopsis and *arp1* plants were confirmed in the blind study. Figure 5-3B depicts the differences in height between *arp1* and wild type. The data in Table 5-1 demonstrate statistically that *arp1* plants were more heavily branched and had shorter siliques than did wild type. The differences between the wild-type and *arp1* plants were evident in plant height, number of branches, and silique length. Developmental processes (days to bolting, appearance of first flower and branch flower) did not differ between the populations. The altered morphological phenotypes of the *arp1* mutants are likely due to the *arp1* mutation itself, since these phenotypes have always cosegregated with the gel phenotypes through four generations of backcrosses (not shown).

The *arp1* mutation induces an RNase activity in addition to the 33-kDa nuclease activities

Another difference observed in the RNase profiles of *arp1* and wild-type inflorescence stems was the appearance of an approximately 23-kDa band of RNase activity in *arp1* plants. Like the morphological phenotypes, the 23-kDa band of RNase activity consistently cosegregated in plants showing the increased 33-kDa nuclease *arp1* phenotype through four generations of backcrosses. Initially, this 23-kDa band was not scored because it was not seen consistently in wild-type Arabidopsis plants. However, as shown in Figure 5-4, RNS1 produced in yeast comigrates with the 23-kDa RNase activity

Table 5-1. Statistical analysis of morphological differences between WT RLD and *arp1* plants[a]

	arp1 vs. WT	
Parameter[b]	T	P
Plant height (final)	6.89	<0.000001
	6.93	<0.000001
Number of branches	3.56	0.00037
	4.26	0.000020
Silique length	9.03	<0.000001
	11.51	<0.000001

[a]The Mann-Whitney rank-sum test was used to determine the statistical significance of the morphological differences between populations. Parameters with reproducibly significant differences are listed in the first column.

[b]Other parameters (measured in days from planting) that were not significantly different include germination, 2, 4, and 6 rosette leaves, bolting, first flower, 10-cm bolt, 15-cm bolt, branch flower, and axillary stem flower.

This analysis was performed by Michael Abler

that is elevated along with the 33-kDa doublet in *arp1* plants. Both the original mutant (*arp1* M6) and a mutant recovered after four generations of backcrosses (*arp1* BC4⊗) have RNase activities that comigrate with yeast-produced RNS1. Neither the WT plants (WT), nor plants from the BC4⊗ generation with a wild-type *ARP1* allele (*ARP1* BC4⊗), have activities that comigrate with RNS1 (Figure 5-4).

Since the 23-kDa band comigrates with RNS1, RNA gel blot and immunoblot analyses were performed to determine whether *RNS1* mRNA or protein levels were affected in *arp1*. For RNA blot analysis, RNA was isolated from stems of 6-week-old *arp1* and WT RLD plants. Poly(A)+ RNA was purified from total RNA preparations so that small effects might be detected. *RNS1* transcript was not detected in either total or poly(A)+ RNA in WT RLD or *arp1* stems by RNA gel blot analysis using an *RNS1* probe (Figure 5-5A). RNA from wounded seedlings was included as a positive control for *RNS1* hybridization [Wnd and Wnd Poly(A)+].

Since we did not detect increased *RNS1* mRNA levels in *arp1* stems, we were interested in whether the RNS1 protein was elevated because of a possible posttranscriptional effect on RNS1. To examine this possibility, proteins from *arp1* and WT RLD stems and seedlings were separated by SDS-PAGE and subjected to immunoblot analysis (Figure 5-5B). RNS1 purified from yeast cells and protein from P_i-starved and P_i-supplied seedlings were included as controls (RNS1, P-, and P+, respectively). Although the RNS1 antibody (Bariola et al., 1999) detects the yeast-produced RNS1 and RNS1 in P_i-starved seedlings, no RNS1 was detected in *arp1* stems. Therefore, significant increases in *RNS1* transcript as well as protein could not be found in *arp1* stems compared to WT.

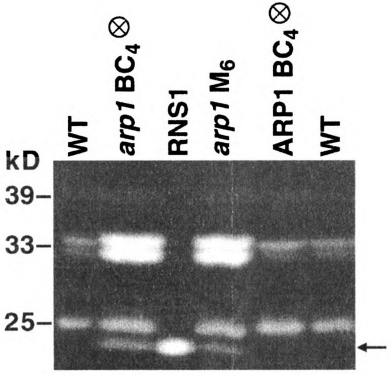


Figure 5-4. The *arp1* mutant has increased levels of an activity that comigrates with RNS1.
Approximate molecular weights are shown to the left of the gel. 100 µg of protein was loaded in the *arp1* and wild-type RLD (WT) lanes. Lane RNS1 contains an aliquot of culture medium from yeast cells expressing the Arabidopsis *RNS1* gene from a yeast secretion vector as described (Bariola et al., 1994). ARP1 BC4⊗, F1 progeny plant of the fourth backcross of *arp1* with wild-type RLD displaying wild-type levels of the 33-kDa nucleases; *arp1* BC4⊗, F_1 progeny plant of the fourth backcross of *arp1* with wild-type RLD displaying increased levels of the 33-kDa nucleases; *arp1* M_6, sixth generation of mutant line 2657.

This experiment was performed by Michael Abler.

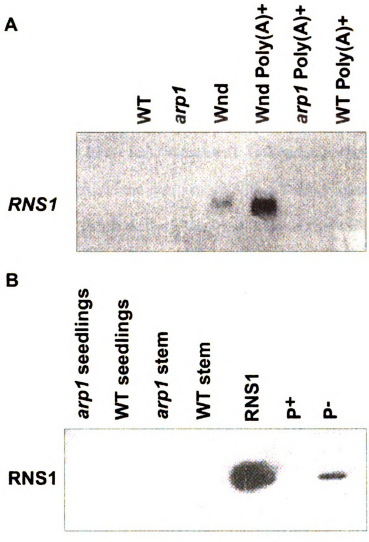


Figure 5-5. *RNS1* transcript and protein is not detected in *arp1* stems.
(A) RNA gel blot analysis of RNA isolated from wild-type (WT) and mutant (*arp1*) stems. Total and poly(A)$^+$ RNA samples from wild-type wounded (9 h) seedlings (Wnd) are included as positive controls. Lanes contain 20 μg total RNA or 1 μg poly(A)$^+$ RNA.
(B) Immunoblot analysis of 100 μg of protein from stems and seedlings of wild-type and mutant lines. An aliquot of RNS1 purified from yeast (RNS1) and 30 μg protein from P_i-supplied (P^+) and P_i-starved (P^-) seedlings were used as controls.

These results raised the possibilities that the increased activity at 23-kDa was due to either a posttranslational effect on RNS1 or an increase in another, unknown RNase activity that comigrates with RNS1. Biochemical characteristics of the 23-kDa band, such as pH optimum, EDTA-sensitivity, Zn-requirements, and the ability to degrade poly(A)$^+$ RNA, resembled those of RNS1 (data not shown). To determine whether the small RNase was indeed RNS1, we crossed the *arp1* mutant with a T-DNA insertional mutant of the *RNS1* gene. The T-DNA in this mutant allele, *rns1-2*, lies in the 5' UTR of the *RNS1* gene (see Figure 4-1 of this thesis). Plants homozygous for this insertion do not produce RNS1, as shown by activity gel assay of wounded leaves, a condition known to induce RNS1 transcript and activity (Figure 4-2B of this thesis and LeBrasseur et al., in press).

The *arp1* mutant, in the RLD background, was crossed with *rns1-2*, which is in ecotype Ws, and stem samples of segregating F_3 progeny were analyzed by activity gels. Three F_3 progeny lines in particular, lines 8, 13, and 24, were analyzed further to determine *RNS1* zygosity. DNA gel blot analysis was used to confirm that these three progenies were homozygous for the *rns1-2* mutation. Genomic DNA was extracted from wild type, *rns1-2*, and progeny plants 8, 13, and 24, digested with *Eco*RI, and analyzed by DNA gel blot analysis. A 5-kb band is seen in wild-type DNA, while only the expected 2-kb band can be seen in the *rns1-2* mutant as well as in progeny plants 8, 13, and 24 (Figure 5-6A). Since the 5-kb band is not seen in these plants, we conclude that the progeny plants are homozygous for the *rns1-2* mutant locus. Next, we analyzed the gel phenotype of these lines (Figure 5-6B). The position of RNS1 (arrow) is shown in wounded wild-type Ws stems (Ws wnd). This band is absent in unwounded Ws and RLD stem samples (Ws and RLD). Progeny plant 8 has at least one copy of the wild-type

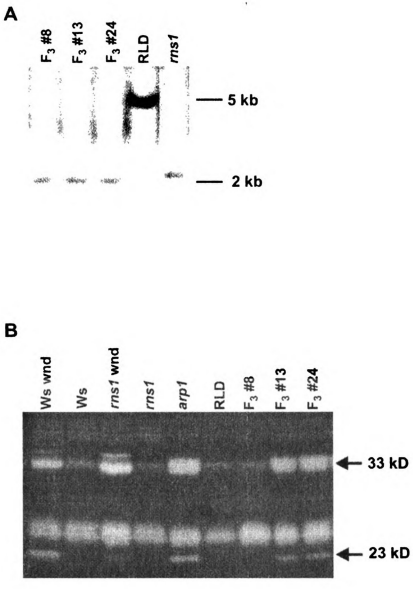


Figure 5-6. A previously unidentified 23-kDa band is increased in *arp1*.
(A) Southern analysis demonstrating that the F_3 progeny examined have no wild-type allele of *RNS1*. EcoRI-digested DNA was probed with an *RNS1* fragment. Only WT DNA hybridizes to the expected 5-kb fragment.
(B) RNase activity gel of stem protein extracts from wild type (Ws and RLD), *rns1-2* (*rns1*), *arp1*, and F_3 progeny of a cross between *arp1* and *rns1-2* (F_3 #8, F_3 #13, and F_3 #24). Wounded stem samples were harvested 7 h after wounding (Ws wnd and *rns1* wnd) and are shown as controls for RNS1 levels in the *rns1-2* mutant. F_3 progeny 13 and 24 are homozygous for the *arp1* mutation, as shown by the increase in the 33-kDa nucleases (upper arrow). Although 13 and 24 are homozygous for the *rns1-2* allele, they still have high levels of the 23-kDa band (lower arrow).

ARP1 allele, as seen by the normal levels of the 33-kDa nuclease doublet (F_3 #8). In contrast, progeny plants 13 and 24 are homozygous for the *arp1* mutation, resulting in an increase in the 33-kDa doublet (F_3 #13 and F_3 #24). Like the *arp1* mutant (*arp1*), stem samples of plants 13 and 24 still display increased levels of a 23-kDa activity, although they lack a normal copy of the *RNS1* gene. Therefore, we conclude that the 23-kDa band that is increased in *arp1* stems is not RNS1. Wounded stems of the *rns1-2* mutant have slight levels of an activity at 23 kDa (Figure 5-6B, compare *rns1* and *rns1* wnd). Two explanations could account for this result. It is possible that the 23-kDa band in *arp1* stems is also wound-inducible, but is not normally seen due to the high levels of RNS1, which runs at the same position. Alternatively, slight amounts of wild-type *RNS1* transcripts may be made and translated in wounded *rns1-2* stems. Small amounts of aberrantly sized wound-induced transcripts are detected by Northern blot in the mutant; however, we were unable to detect full-length polyadenylated *RNS1* transcript in *rns1-2* plants by RT-PCR (data not shown). Thus, although we cannot exclude the latter possibility, we believe the former is more likely. Nevertheless, the levels of the 23-kDa *arp1* band are significantly higher than the wound-induced 23-kDa band in *rns1-2*, indicating that the two bands are different RNase activities.

The *arp1* RNase phenotype is stem associated

Because the visible phenotypes of the *arp1* plants are affected predominantly in aspects of stem morphology, such as plant height and branching, the RNase profiles of several tissues in the mutant were examined to determine whether this phenotype was stem-specific. The 33-kDa doublet and 23-kDa RNase activities, which are increased in the stems of *arp1*, are at wild-type levels both in leaves and in 10-day-old seedlings of the

mutant (Figure 5-7). Phosphate-starved seedlings, in which RNS1 and the 33-kDa doublet activities are induced, have been included for comparison of the running positions of these activities. Thus, it appears that the *arp1* RNase phenotype, along with the morphological *arp1* phenotypes, are associated with the stem.

DISCUSSION

Similar to other eukaryotes, plants produce a number of major RNase activities that have been implicated in various processes on the basis of their regulation. However, the genes involved in the regulation of eukaryotic RNases are largely unknown. In this study, we used a novel brute-force genetic screen to identify several Arabidopsis mutants with alterations in the RNase profile (*arp* mutants). Some of these mutants affect multiple RNase activities, indicating that they have defects in RNase regulatory components rather than RNase structural genes. The mutant characterized in the most detail, *arp1*, has unusually high levels of two 33-kDa activities, which degrade both RNA and DNA, and a smaller RNase. These data together with the recessive character of *arp1* prompt us to suggest that the mutation may inactivate a regulatory component common to the control of these three RNA-degrading activities. An interesting and unique feature of *arp1* is that the elevated RNase levels and other morphological phenotypes of this mutant are prominent in the stem. Therefore, the *arp1* mutation may alter a repressor of these RNase activities and other functions required for normal stem growth and development.

Mutants affecting single and multiple RNase activities

The screen of 2,500 M_2 plants ultimately yielded four plants with different heritable alterations in the stem RNase profile and identified an ecotype polymorphism in

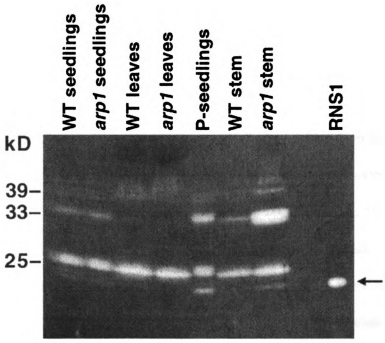


Figure 5-7. The *arp1* RNase phenotype is stem specific.
The *arp1* increase in RNS1 and the 33-kDa activities is seen in stems but not in seedlings or leaves. P_i-starved seedlings are shown as a control for the running position of RNS1 (arrow). 100 µg protein was loaded in all lanes except P^-, in which 30 µg was loaded. RNS1 purified from *RNS1*-expressing yeast (Bariola et al., 1994) is shown to the far right. Approximate molecular weights are indicated on the left. WT, wild-type RLD samples.

Columbia compared to Ws. RNase activities of different molecular weights were affected in the mutants. In particular, plant 2657 exhibited an increase in both activities of the 33-kDa doublet, whereas in the Columbia polymorphism represented by plants 2972, 3270, 3433, 2048, and 2438, the lower 33-kDa activity was decreased or eliminated. The varieties of RNase phenotypes identified in this study differ markedly from previous plant mutants shown to affect RNases. Both the variegated *immutans* mutant of Arabidopsis and the *Opaque2* mutant of maize, which were isolated on the basis of other phenotypes (Redei, 1967; Wilson and Alexander, 1967; Dalby and Davies, 1967) appear to elevate all major RNase activities in the gel profile (Wetzel et al., 1994; Wilson and Alexander, 1967; Dalby and Davies, 1967). Because all of the mutants isolated in this work affect only a subset of major Arabidopsis RNases, they have the potential to provide insight about specific regulatory networks and functional associations.

The 33-kDa activities are bifunctional nucleases

Previous evidence indicated that a doublet of RNase activity was the same activity as seen in DNase activity gels. In addition to the fact that the doublets run at approximately 33 kDa on both gels, both degradative activities had similarities in EDTA-sensitivity, pH optima, and cation requirements (Yen and Green, 1991). Still, it was possible that the activities were in fact products of separate genes. The isolation of *arp1* and the identification of the Columbia polymorphism in this study provide firm evidence that the 33-kDa activities are bifunctional nucleases that can degrade both RNA and ssDNA. Each plant examined affected in one or both of the bands of RNase activity also shows the same alteration in the DNase activity at the same position. It is theoretically possible that *arp1* and the Columbia polymorphic allele both affect separate DNase and RNase

activities, which coincidentally are similarly sized, in the same manner. However, this seems extremely unlikely, and we therefore conclude that the RNase and DNase activities are derived from the same protein products. The potential relationship between the upper and lower bands of the 33-kDa doublet is unknown. Other experiments have determined that both bands are wound-inducible and that the lower band normally missing in Columbia but found in RLD can be induced by wounding in Columbia (LeBrasseur et al., in press).

Possible functions for the 33-kDa nucleases

Very little is known about the biological functions of RNases in plants. To investigate the possibility that the 33-kDa activities were involved in mRNA degradation, mutant plants were transformed with constructs containing *globin* reporter genes. The mutant plants showed little or no effect on the accumulation of *globin* compared to wild type, whether or not *globin* was destabilized (Abler et al., manuscript in preparation). Thus, it appears the mutations do not affect global mRNA turnover or the turnover of specific unstable or stable reporter transcripts. These results are not unexpected, as most of the RNase activity in plants is localized to the vacuole (Boller and Kende, 1979; Abel and Glund, 1986), or secreted from the cell, and therefore is not likely to function in the cytoplasmic degradation of mRNA (Bariola and Green, 1997).

Certain characteristics of the activities in the 33-kDa doublet can aide in their classification. For instance, their size, acidic pH optima, sensitivity to EDTA, and requirement for divalent cations (Yen and Green, 1991), as well as ability to degrade both RNA and DNA, place them within the nuclease I-type class of enzymes (Wilson, 1982). In general, nuclease I enzymes are secreted glycoproteins (Fraser and Low, 1993).

Although it is not known whether the 33-kDa activities are secreted, extracellular washes of stem and leaf extracts show an increase of activity in this range (M.L. Abler and P.J. Green, unpublished). Additionally, while we do not have evidence that the 33-kDa activities are glycosylated, one explanation of the doublet of activity is differential glycosylation of the same protein. In this case, the ecotype-specific polymorphism and the *arp1* effect on the 33-kDa activities may be explained by altered regulation of the glycosylation of these proteins. Analyses of other plant nuclease I enzymes provide insight into possible functions for the 33-kDa activities. Nuclease I enzymes have been characterized biochemically in many species, and cDNAs encoding these enzymes have been isolated recently from several species (see Chapter 1).

Putative functions for nuclease I enzymes can be hypothesized based on their expression patterns. Plant nuclcase I enzymes and the genes encoding them are induced during various plant growth and developmental conditions, including germination, tracheary element formation, senescence, and various stress responses (for a review, see Bariola and Green, 1997). The 33-kDa activities altered in the *arp1* mutant are strongly induced by wounding (LeBrasseur et al., in press) and phosphate starvation (see Figure 10 in Bariola et al., 1994). These activities appear to have many characteristics of nuclease I-type enzymes and may be functioning similarly to other members of this family, which have been proposed to be involved in remobilizing the constituents of nucleic acids from dying tissue for use in other parts of the plant (Bariola and Green, 1997). Additionally, they may play a role in vascular formation, either in killing the cell directly, in degrading components left after cell lysis to clear the xylem channel, or in nuclear degradation during PCD of tracheary elements (Bariola et al., 1997; Fukuda,

2000). The fact that altering these activities in *arp1* correlates with stem-associated phenotypes is consistent with the induction of these enzymes during tracheary element formation.

arp1 is a regulatory link between the 33-kDa nucleases and an RNase activity

The *arp1* mutation is particularly interesting because several activities are affected. In addition to the 33-kDa nucleases, a previously unidentified 23-kDa RNase activity is also increased. Since multiple activities are upregulated, *ARP1* is not likely to encode a structural RNase, but rather a regulatory protein. It is interesting to consider in what pathways *ARP1* might regulate RNases. An obvious possibility is the P_i-starvation pathway, as both 33-kDa and smaller RNase activities are increased in seedlings starved for P_i (Bariola et al., 1994). However, in the case of P_i-starvation, RNS1 activity and transcript are induced to high levels, a situation not seen in *arp1*. Additionally, the visible phenotypes of *arp1* plants do not resemble those associated with P_i-deficiency and the P_i-loading defective *pho1* mutant, which include increased anthocyanin levels and delayed flowering (Marschner, 1996; Poirier et al., 1991). The *arp1* plants and seedlings do not have high levels of anthocyanins (not shown), nor is flowering time affected (refer to Table 5-1). Finally, the P_i-starvation response is clearly visible in seedlings, whereas the *arp1* RNase phenotype is not seen in seedlings (Figure 5-7). Thus, it is more likely that the *ARP1* locus is involved in the regulation of RNases in other pathways, as will be discussed further below.

The _arp1_ phenotypes are associated with stems

Insight into the function of ARP1 is provided by the phenotypes seen in the mutant. The mutation obviously causes pleiotropic effects. Interestingly, many phenotypes seem to be

associated with the stem; the increased activities can be seen in the stem tissue, while leaves and seedlings do not exhibit this increase, stems of *arp1* are shorter than wild-type, and branching is increased in *arp1* stems.

Considering the obvious stem-related mutant phenotypes seen in *arp1*, an intriguing possibility is that ARP1 is involved in the control of developmental pathways in stems. RNase and nuclease activities are abundant in stems and may constitute an important part of stem growth and development. The induction of nucleases during zinnia tracheary development (Thelen and Northcote, 1989) implies that nucleases are involved in stem-specific growth and maturation processes. It appears that normal function of ARP1 results in low activity of the three enzymes in stems under standard growth conditions. The product of the *ARP1* gene may be involved in upregulating these enzymes only under specific conditions, for instance, during stem growth and tracheary element formation. The increase in levels of the 23- and 33-kDa activities may be indicative of deviations from the normal developmental patterns in the stem. Cross sections of *arp1* and wild-type stems did not reveal any gross alterations in cell structure or organization of the tracheary elements (M.L. Abler and P.J. Green, unpublished), but further analysis may reveal abnormalities. The *ARP1* locus has been mapped to the top of chromosome 5 (L.R. Danhof, M.A. Johnson, and P.J. Green, unpublished), and the cloning of this gene should provide us with an interesting tool with which to study stem-related developmental processes, an important area of study that remains largely unexplored. Additionally, this mutant will be a useful tool for isolating activities to provide us with more plant RNase sequences. Progeny of the *arp1* and *rns1-2* cross will be particularly useful, since RNS1 will not interfere with purification of the new 23-kDa activity in these lines.

MATERIALS AND METHODS

Plant materials

Arabidopsis thaliana ecotype RLD was used in this study. Ethyl methanesulfonate-mutagenized M_2 seed was provided by Lehle Seeds (Round Rock, Texas). Plants were grown under conditions of 16 h light/8 h dark and 50% RH at 20°C and 100 μE light intensity. Seedlings were grown on AGM plates as described (Taylor et al., 1991). P_i-starved seedlings were germinated on complete medium and transferred to P^- medium as described (Bariola et al., 1994). A lateral branch was harvested from each M_2 plant to provide tissue samples and still allow the plant to grow and set seed.

RNase and DNase activity gels

Protein samples were prepared essentially as described (Yen and Green, 1991) with the exception that frozen plant tissues were homogenized on ice. Soluble protein concentrations were measured by the method of Bradford (1976) and an equal amount (100 μg) of total protein was loaded into each lane of the polyacrylamide gels, except for P_i-starved seedlings, for which 30 μg of protein was loaded. Polyacrylamide gels containing RNA or DNA as substrate were prepared as described (Yen and Green, 1991), except the concentration of acrylamide in the separating gels of Figures 5-2 and 5-4 was reduced from 11% to 9% in order to better separate the 33-kDa bands of activity. The lower acrylamide concentration necessitated a higher initial RNA concentration in the gel, so 5.0 mg/ml of RNA was used in the 9% gels instead of 2.4 mg/ml RNA.

Mutant growth and development

Pots containing *arp1* or wild-type seeds were randomly assigned to three flats. Individual

pots were identified only by number, in order to perform a blind study. Parameters measured (in days from planting) for each plant included germination, 2, 4, and 6 rosette leaves, bolting, first flower, 10-cm bolt, 15-cm bolt, branch flowering, and axillary stem flowering. Physical characteristics measured for each plant included final plant height, number of branches, and silique length. Silique length was determined by measuring length of the fifth, sixth, and seventh siliques on the primary inflorescence stem for each plant. The Mann-Whitney rank sum test was used to determine significant morphological differences between the wild type and mutant populations.

DNA and RNA extraction and gel blot analyses

RNA methods were performed as described in Chapter 2. Genomic DNA procedures were performed as described in Chapter 4, except DNA was extracted from 2-week-old seedlings.

Immunoblot analysis

Protein for Western analysis was extracted as described in MacIntosh et al. (1996) and quantified using the Bradford assay (Bio-Rad). Protein extracts were mixed with loading buffer and boiled for 5 min before separation on 11% SDS-PAGE gels (Laemmli, 1970). Lanes contained 100 μg of stem or seedling extracts from *arp1* or wild-type plants or 75 μg of protein extracts from seedlings grown on P^+ or P^- medium. An aliquot of RNS1 produced in yeast (Bariola et al., 1994) was included as a positive control. For RNS1 signal detection, proteins were transferred to PVDF membrane (Immobilon-P, Millipore), and membranes were processed as described (Bariola et al., 1999), except detection was performed using an ECL+Plus Western blotting detection system (Amersham) according to the manufacturer's instructions.

CHAPTER 6

CONCLUSIONS AND FUTURE PROSPECTS

When I began this thesis project, the general consensus in the field was that S-like RNases were involved in degrading nucleic acids in order to supply the plant with P_i. The nucleic acid, it was thought, might be from within the plant itself, or perhaps from external sources. This more hypothetical idea that RNases could be secreted from roots into the surrounding soil to degrade organic compounds for uptake by the plant was dubbed by our lab as the "dead bird hypothesis." While many, including even members of my lab, are skeptical of this idea, it seems that results in this thesis are consistent with this concept. The fact that plants overexpressing *RNS1* grow larger on medium containing RNA as the only phosphate source (while on P_i-supplied medium, these plants are smaller) seems most readily explained by secretion from the roots of the RNS1 enzyme, which is then able to degrade the RNA and thereby provide the seedlings with P_i. While I cannot rule out alternative theories, I can only conceive of those that are less intuitive than the "dead bird." For instance, the increase in RNS1 might cause membrane permeability issues in the plant that somehow affect the ability of the roots to utilize complex forms of phosphate.

Given the wide range of processes with which induction of S-like RNases correlates, it certainly seems likely that they are involved in recycling and remobilization processes. In fact, findings in this thesis that nucleases and RNS1 are induced during wounding might at first seem mundane, another example of RNases used as maid-service for the plant. However, this family of enzymes may be involved in more than that. The similarities between the *rns1* and *rny1*Δ mutants indicate that the lost activities must have a common function in both systems, which does not appear to include recycling phosphate.

Although the model proposed by MacIntosh et al. (2001) has been discussed in Chapters 1 and 4, I would like to extend the discussion here. Their proposal is integral to the extension of hypothesized functions for RNS1. Figure 6-1 summarizes their model for the function of secreted RNases in controlling membrane permeability. In this model, the amount of RNA in the membrane is one factor that determines the amount of water and ions that enter the cells. This idea is based on the finding that specific, synthetic RNAs can bind both artificial and natural membranes and in the process, alter the permeability of those membranes (Khvorova et al., 1999). The use of RNA to alter membrane characteristics may have been a function of this nucleic acid in the hypothetical RNA world. If RNA was once used in this capacity, one could envision that during evolution, an extracellular RNase might have been recruited to control the amount of RNA in the membrane; the widespread existence of T_2 RNases supports an early evolutionary origin and basic requirement for these enzymes. During stress conditions, including heat shock and osmotic stress, the RNase activity is induced by the cell in order to degrade some of the RNA in the membrane. As a result, the cell is less permeable, more "closed" to the environment. In an RNase mutant, such as *rny1*Δ, this control is missing. Therefore, RNA accumulates in the membrane, causing it to be more permeable. To compensate, the cell expands, and the vacuole is enlarged, two phenotypes seen in the yeast mutant.

Similar phenotypes are seen in the plant mutant, as discussed in Chapter 4. Namely, the roots, and possibly the above-ground tissue, of *rns1-2* seedlings are larger than the WT. Interestingly, the overexpressors have the opposite phenotype: smaller roots. The other phenotype the yeast and plant mutants may have in common is an increase in

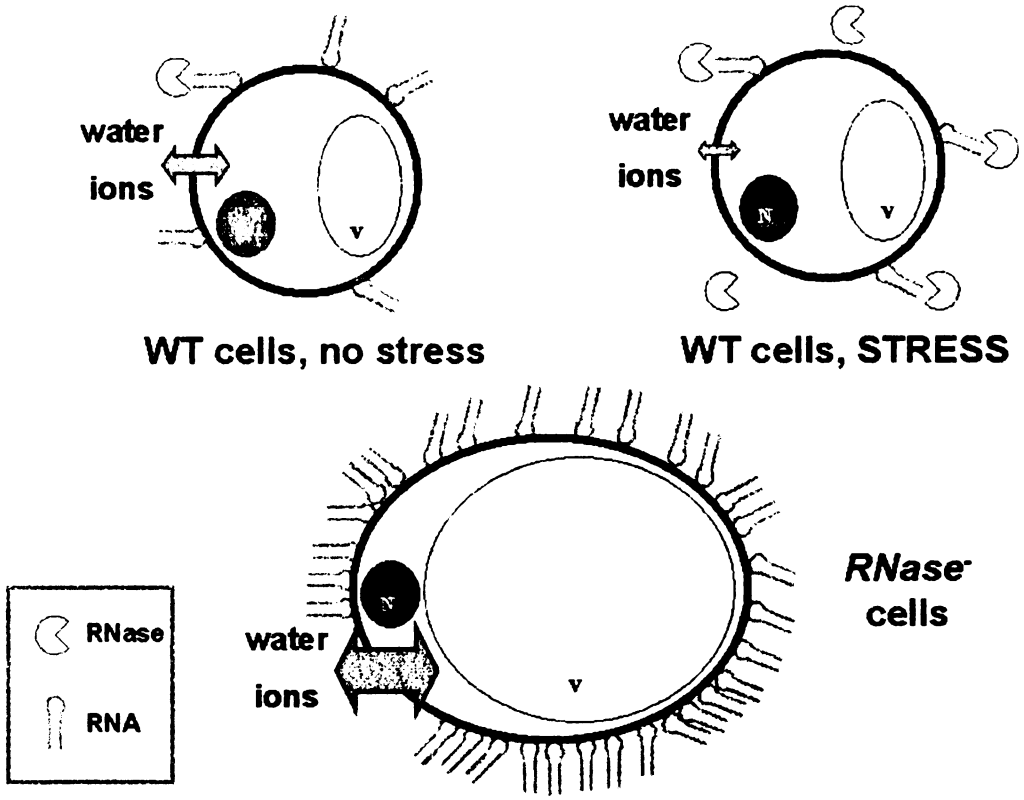


Figure 6-1. Model for the control of membrane permeability by RNases.
In this model, modified from one provided by G.C. MacIntosh and P.J. Green, RNA can normally be found in the membrane in a given amount. Under conditions of stress, extracellular RNase activity increases, degrading some of the RNA in the membrane and reducing cell permeability. In the mutant cells, such as *rny1*Δ and *rns1-2*, the secreted RNase is missing, leading to high amounts of RNA in the membrane. In the case of the yeast mutant, the cell compensates by increasing the size of the vacuole and overall cell-size. n, nucleus; v, vacuole.

permeability. Dyes used to stain either cell type seem to be taken up by the mutant cells more readily than in their WT counterparts. Additionally, the regulation patterns of the two enzymes share some similarities; for instance, both are induced by salt stress. As yet, the *rns1-2* mutant does not appear sensitive to high salt, as is the yeast mutant (MacIntosh et al., 2001). [The effect of osmotic stress on the *rns1-2* mutant should be tested in the future by transferring the mutant to medium containing various concentrations of sorbitol, for example, and monitoring the response of the root cells to the changes. If the mutant cells are more permeable, they should also be more sensitive to external osmotic changes.] Additionally, RNS1 is not induced by heat stress, another variation from the yeast enzyme. Thus, differences exist. Obviously, we do not imagine that RNA is the only, or even the most important, control of membrane permeability; protein ion transport channels play the critical role in this process. Even so, remnants of an ancient use of RNA may still exist. The yeast mutant phenotypes identified were specific to one strain of *S. cereviseae*. It is possible that this strain is somehow compromised in another aspect of membrane regulation, making it particularly susceptible to alterations in levels of RNA in the plasma membrane (MacIntosh et al., 2001). Similarly, Arabidopsis has various mechanisms to control membrane traits, and the loss of RNS1 may not have a strong effect in many circumstances. However, the loss of whatever small amount of activity is normally present in roots is sufficient to cause a significant increase in the length of the roots.

Interestingly, we can also reconcile this membrane-permeability hypothesis with the systemic induction of RNS1 upon wounding. We have not yet identified the signal involved in regulating *RNS1* by wounding. Electrical signals have been proposed to cause

systemic gene induction in tomato (Stankovic and Davies, 1996) and could affect *RNS1* as well. Not only might the induction of *RNS1* be mediated through electrical stimuli, it is conceivable that by altering the permeability of cell membranes, RNS1 could be involved in either potentiating or shutting down the plants' response to the signal.

One key question to be addressed in order to support this theory will be the identification of natural RNAs that are present in membranes. The yeast mutant will provide a useful tool for these searches, which are currently being conducted by G.C. MacIntosh and P.J. Green. If they exist, these RNAs are most likely highly specific species produced by the cell and targeted to the membrane. Current searches in the lab to identify Arabidopsis RNAs that do not encode proteins as their final product, but instead function as RNA, may provide a fortuitous overlap with the function of secreted RNases, in that some of the so-called non-coding RNAs identified may turn out to be membrane-associated RNAs.

As mentioned briefly in Chapter 1, secreted RNases have several "special" actions that have not been explained at a biological level. For example, bovine seminal RNase exhibits a wide range of cytotoxic activities, including immunosuppressive, antiviral, embryotoxic, and antitumor effects (reviewed in D'Alessio et al., 1997). Angiogenin, a relative of RNase A, has the unique ability to induce blood vessel formation (reviewed in Riordan, 1997). How extracellular RNases cause such responses has not been elucidated, but the field might one day benefit from a reevaluation of special biological actions of RNases with these new ideas in mind.

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