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ORIGINAL PAPER

Impact of transcriptional, ABA-dependent, and ABA-independent pathways on wounding regulation of *RNS1* expression

Melissa S. Hillwig · Nicole D. LeBrasseur · Pamela J. Green · Gustavo C. MacIntosh

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Abstract Injured plants induce a wide range of genes whose products are thought to help to repair the plant or to defend against opportunistic pathogens that might infect the wounded plant. In *Arabidopsis thaliana* L., oligogalacturonides (OGAs) and jasmonic acid (JA) are the main regulators of the signaling pathways that control the local and systemic wound response, respectively. RNS1, a secreted ribonuclease, is induced by wounding in *Arabidopsis* independent of these two signals, thus indicating that another wound-response signal exists. Here we show that abscisic acid (ABA), which induces wound-responsive genes in other systems, also induces RNS1. In the absence of ABA signaling, wounding induces only approximately 45% of the endogenous levels of *RNS1* mRNA. However, significant levels of RNS1 still accumulate in the absence of ABA signaling. Our results suggest that wound-responsive increases in ABA production may amplify induction of RNS1 by a novel ABA-independent pathway. To elucidate this novel pathway, we show here that the wound induction of *RNS1* is due in part to transcriptional regulation by wounding and ABA. We also show evidence of post-transcriptional regulation which may contribute to the high levels of *RNS1* transcript accumulation in response to wounding.

Keywords Abscisic acid · Post-transcriptional regulation · Promoter · Ribonuclease · Wounding

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M. S. Hillwig
Interdepartmental Genetics Program, Iowa State University,
Ames, IA 50011, USA

N. D. LeBrasseur
MSU-DOE Plant Research Laboratory,
Michigan State University,
East Lansing, MI 48824, USA

Present Address:
N. D. LeBrasseur
Weber Shandwick, 919 3rd Ave #1679,
New York, NY 10022, USA

P. J. Green
Delaware Biotechnology Institute and Department of Plant and Soil Sciences, University of Delaware,
Newark, DE 19716, USA

G. C. MacIntosh (✉)
Department of Biochemistry, Biophysics and Molecular Biology and Plant Sciences Institute, Iowa State University,
Ames, IA 50011, USA
e-mail: gustavo@iastate.edu

Abbreviations

ABA	Abscisic acid
ABRE	ABA-responsive element
DRE	Dehydration response element
JA	Jasmonic acid
OGA	Oligogalacturonides
RNase	Ribonuclease
SA	Salicylic acid

Introduction

Secreted ribonucleases (RNases) are enzymes located where RNA is not thought to be readily available, such as in the vacuole or outside the cell. The T_2 superfamily of secreted RNases, in particular, has been found in nearly every system examined for their presence, including fungi, viruses, bacteria, plants, and animals (Deshpande and

Shankar 2002). The ubiquitous distribution of T_2 RNases suggests that they have both an ancient origin and critical function(s) (Taylor and Green 1991).

Despite the apparent necessity for the activity of T_2 enzymes, very few has been demonstrated regarding their biological functions. The exception is S-RNases, a class of plant T_2 RNases whose activity is essential for the process of self-incompatibility in several plant families (reviewed in McCubbin and Kao 2000). Enzymes related to, but distinct from, S-RNases are also present in self-compatible plants and form a class known as S-like RNases (reviewed in Bariola and Green 1997). S-like enzymes are not involved in self-incompatibility, but seem to have important functions throughout the plant kingdom, as they are ubiquitous in plants. The *A. thaliana* genome contains five S-like genes, *RNS1* to *RNS5* (Taylor and Green 1991; G.C. MacIntosh, unpublished), and RNase activity has been demonstrated for the products of three of these (Taylor et al. 1993; Bariola et al. 1994).

Fluctuations in RNase activity levels or gene expression are useful for predicting RNase function. The discovery that growth on low concentrations of inorganic phosphate (P_i) induces expression of various RNases, including *Arabidopsis* RNS1 and RNS2 (Bariola et al. 1994, 1999) and tomato RNases LX and LE (Nürnberger et al. 1990; Bosse and Köck 1998), led to the hypothesis that S-like RNases are part of a rescue system that plants use to recycle P_i when environmental pools are limiting (Goldstein et al. 1989).

In addition to low inorganic phosphate concentration, RNases are also induced by wounding in several plant systems. For example, the transcript for RNase LE accumulates in wounded tomato leaves (Lers et al. 1998; Groß et al. 2004), and RNase NW is induced in wounded tobacco leaves (Kariu et al. 1998). We showed that RNS1 and several nuclease activities are coordinately regulated by wounding in *Arabidopsis* (LeBrasseur et al. 2002). The *RNS1* transcript was the most highly wounding-induced transcript 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, about half of which were hypothesized to be involved in RNA metabolism and turnover (Pérez-Amador et al. 2002). The strong *RNS1* transcript accumulation may indicate that RNS1 has a critical function during wounding. RNS1 transcript and activity are also increased in non-damaged tissue of wounded plants, where recycling of nutrients and degradation of bulk cellular nucleic acid, should not be necessary. We therefore proposed that RNS1 may also have a defensive function (LeBrasseur et al. 2002).

The induction of RNS1 and nuclease activities provides us with a unique perspective into *Arabidopsis* wound signaling mechanisms (LeBrasseur et al. 2002). 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 transcript accumulation and has been shown to be regulated by OGA elicitors probably released from injured plant cell walls (Rojo et al. 2003).

Although RNS1 and the three nuclease activities are strongly induced locally by wounding, they are not induced by treatments with OGA-rich fractions (LeBrasseur et al. 2002). The local response of RNS1 and the nucleases to wounding is also not controlled by the JA-dependent signaling pathway, as shown by the strong wound-induction of these activities in the JA-insensitive *coi1* mutant. It has been proposed that JA signaling controls the systemic wound response in *Arabidopsis* (Titarenko et al. 1997; León et al. 2001) but the systemic induction of *RNS1* did not depend on JA (LeBrasseur et al. 2002). To our knowledge, *RNS1* was the first gene in *Arabidopsis* shown to be induced systemically by wounding in a JA-independent manner and therefore indicates the existence of an alternative long-distance signaling pathway.

It is becoming clear that JA-independent pathways are important in the regulation of wounding response, however very little is known about the signal transduction pathways controlling these responses (Howe 2004). Several molecules have been proposed to act as signals in the wounding response in plants in addition to OGAs and JA (reviewed in de Bruxelles and Roberts 2001; León et al. 2001; Howe 2004), including abscisic acid (ABA, see review by Lorenzo and Solano 2005). ABA application induces the local and systemic expression of *PinII*, a wound-inducible gene, in potato, tomato and tobacco (Peña-Cortés et al. 1989). Analyses of ABA-deficient mutants of potato and tomato provided further evidence for a requirement for ABA in the wound-induction of *Pin* genes (Peña-Cortés et al. 1989, 1991), and ABA accumulates upon wounding (Peña-Cortés et al. 1991). However, the role of ABA in the wounding response is controversial. Birkenmeier and Ryan (1998) found that exogenous ABA induces *PinII* expression in tomato to a much lesser extent than either wounding or JA application, and that endogenous ABA levels only increase significantly at the wound site.

Recent evidence suggests that *RNS1* may be controlled by ABA signaling. A mutant screen identified an mRNA cap-binding protein, ABH1, as a negative modulator of ABA signaling in stomata (Hugouvieux et al. 2001). DNA chip analyses comparing gene expression in WT and *abh1* plants identified *RNS1* as one of a few transcripts that are down-regulated in *abh1*. These genes might function in early ABA signaling, as their

transcripts represent putative targets for ABH1-dependent mRNA processing (Hugouvieux et al. 2001). As ABA is a proposed regulator of the wounding response in other plants, it could also control the OGA- and JA-independent pathway defined by *RNS1* expression in *Arabidopsis*. In addition, the *abh1* results indicate that ABA might post-transcriptionally stabilize *RNS1* mRNA after wounding. Here, we show that ABA induces *RNS1* expression with a timing that is similar to that of wounding. We also show that ABA is necessary to produce the full wounding response. However, ABA is only part of the signaling pathways controlling RNS1 induction in wounded *Arabidopsis* plants. Our results indicate that an as-yet uncharacterized ABA-independent pathway, independent of JA and OGA as well, also contributes to *RNS1* induction during the wounding response. We found evidences that this novel pathway acts synergistically with ABA to regulate *RNS1* induction at the transcriptional level. The possibility of post-transcriptional regulation is also discussed.

Materials and methods

Plant materials and treatments

Unless otherwise stated, the Columbia-0 ecotype of *Arabidopsis thaliana* L. was used throughout this study. Soil-grown plants were grown in chambers under 16 h of light in 60% relative humidity at 21°C. For seedling experiments, seeds were surface-sterilized and germinated on *Arabidopsis* growth medium as described (Taylor et al. 1993). The *aba1-1*, *abi1* and *abi2* seeds were kindly provided by Dr. Michael Thomashow (Michigan State University), *abi4* and *abi5* seeds were obtained from the Arabidopsis Biological Resource Center (ABRC). For wounding treatments, leaves of 4- to 6-week-old plants or leaves of 14-day-old seedlings were wounded using ridged flat-tipped tweezers, harvested at subsequent timepoints, and treated as previously described (LeBrasseur et al. 2002). ABA treatments were conducted on 14-day-old seedlings grown on MS-agar plates covered with plastic mesh. Seedlings were transferred to 0.5× MS medium (Sigma, Saint Louis, MO, USA) with or without 100 μM ABA (Sigma, Saint Louis, MO, USA) and harvested at subsequent timepoints. WT controls for the ABA mutant experiments were performed with the ecotype Landsberg erecta (Ler), Columbia-0 (Col) or Wassilewskija (Ws) as indicated. Experiments were performed a minimum of three times. Representative blots or gels are shown.

Plants were transformed by vacuum infiltration as previously described (Bariola et al. 1999). For each experiment, at least three independently transformed lines were used. Representative results are shown.

Cloning and sequence analysis

Standard cloning techniques were used throughout. The *RNS1* promoter region was isolated previously (Howard 1996) and contains 2.6 kb of DNA upstream of the *RNS1* initiation codon. This includes DNA from chromosome 2 coordinates 870957 (5′) to 873663 (3′), based on the current AGI annotation as shown at TAIR (http://www.arabidopsis.org), which corresponds to the TAIR 7 version of the *Arabidopsis* genome, released in April 2007. The promoter region was cloned into a Bluescript II vector (Stratagene, La Jolla, CA, USA) containing the *β*-glucuronidase (*GUS*) protein sequence (Jefferson et al. 1987) with a *rbcS-E9* polyadenylation sequence (Fang et al. 1989). We removed the short stretch of 5′ UTR sequence that was present in this construct to create a 3′ end on the *RNS1* promoter that corresponds to coordinate 873563. This construct was then cloned into an *A. tumefaciens* shuttle vector containing a kanamycin resistance gene as described before (Gil and Green 1996) and designated p2081. In plasmid p2082 the *GUS* coding region was replaced by *luciferase* (Millar et al. 1992). Construct p848 (35S-GUS-E9), containing the cauliflower mosaic virus 35S promoter in place of the *RNS1* promoter, was constructed in a similar manner (Howard 1996).

The *nos* (nopaline synthase) promoter was amplified by PCR from pBI-121 using the primers PG-454 (5′-gatcatctgcagagaattaagg) and PG-453 (5′-gttcaaccatgggaaacgatcc). The nos-globin-E9 construct was made by replacing the 2× 35S promoter of p1185 (Diehn et al. 1998) with *nos* to make p2031. The *RNS1* cDNA (Bariola et al. 1994) was then inserted in place of *globin* to make p1966. The entire nos-RNS1-E9 cassette was then cloned into the plant transformation vector pCambia 1301 (GenBank accession number AF234297), which has the hygromycin resistance plant selection marker. This clone was named p1975. The nos-globin-E9 cassette was cloned into pCambia 2301 (GenBank accession number AF234316), which confers kanamycin resistance to transformed plants, and was named p1995. The entire *RNS1* transcribed region, including the full 5′ UTR and introns, was PCR-amplified. PCR products were sequenced to assure no errors were introduced and then inserted in place of *globin* in p2031. The orientation of the insert was confirmed and then the nos-preRNS1-E9 cassette was ligated into pCambia 2301.

Computational analysis of the proximal 1,000 nt of the promoter sequence was performed using two internet-accessible databases, PlantCARE (Lescot et al. 2002) and PLACE (Higo et al. 1999). Only elements in which the core is absolutely conserved are reported here.

RNA and protein extraction and analysis

Total RNA from *Arabidopsis* samples was extracted and analyzed as previously described (LeBrasseur et al. 2002). RNA blots were hybridized using a ^{32}P-labeled *RNS1* probe. To control for loading, the same RNA blots were stripped 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). The *COR6.6* gene, kindly provided by Dr. Michael Thomashow (Michigan State University), was used as a positive control for ABA treatments (Hajela et al. 1990). *GUS* and *globin* probes were prepared by PCR. The *nos* probe was prepared by polynucleotide kinase end-labeling of an antisense oligonucleotide using ^{32}P-ATP (sequence: GATCCAGATCCGGTGCAGATTA TTTGGATTGAGAGTGAATAT). All blots were exposed for 16 h, except RNS1p-GUS constructs that were exposed for 3–5 days. Blots were quantified using PhosphorImager. *RNS1*, *GUS* and *nos* expression data were normalized using *EF-1α* values (as *RNS1/EF-1α*; *GUS/EF-1α; nos/EF-1α*). The ratios from at least three independent experiments were used for the expression data shown in Figs. 2b, 5b and 6c, d. For the nos-RNS1 constructs, both the individual bands and the doublet as a whole were quantified. Results for the doublet are reported, but individual bands gave similar results.

To verify the identity of the two bands obtained with the nos-RNS1 reporter constructs, 3′ rapid amplification of cDNA ends (RACE) was performed using the 3′ RACE System (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocols. Gene specific primer for initial PCR was the *nos* probe describe above. Primers for nested PCR were GTGTTTGATCAGTCTTCTCGTAATCTTGC (RNS1) and CTGATGCATTGAACTTGACGAACGTTGTCG (E9).

Total protein was extracted and RNase activities were assayed as described previously (LeBrasseur et al. 2002). Equal loading of protein gels was confirmed by Coomassie Blue staining of standard SDS-PAGE loaded with the same volume of protein extracts used for activity assays. All blots and gels are representative of at least three independent experiments.

Histochemical GUS staining and luciferase imaging

Histochemical localization of GUS activity was determined using a *β*-Glucuronidase Reporter Gene Staining Kit (Sigma) according to manufacturer's recommendations. Luciferase activity was analyzed using a CCD camera (ChemiPro System, Roper Scientific, Trenton, NJ, USA) as described by Chinnusamy et al. (2002); exposure time was 20 min.

Results

RNS1 expression is induced by ABA

It has been suggested that ABA may regulate *RNS1* transcript accumulation, as loss of an mRNA-binding protein, ABH1, that downregulates ABA responses leads to reduced *RNS1* transcript levels (Hugouvieux et al. 2001). Transcriptional regulation of gene expression by ABA has been characterized to a large degree. Thus, we analyzed the *RNS1* promoter sequence to identify putative regulatory elements (Fig. 1a, Electronic supplementary Fig. S1). A search for regions with homology to known regulatory elements identified three putative ABA-responsive elements (ABREs; Yamaguchi-Shinozaki and Shinozaki 1993, 1994), and one MYB- and three MYC-binding regions. Some members of the MYC and MYB transcription factor families are induced by drought and ABA (Abe et al. 1997). A dehydration response element (DRE) was also found in the *RNS1* promoter. This element has been shown to be sufficient for a rapid response to dehydration without the involvement of ABA (Yamaguchi-Shinozaki and Shinozaki 1993, 1994). In addition, several wounding-responsive elements were found in the *RNS1* promoter: a W-box (Eulgem et al. 1999) and two WUN elements (Pastuglia et al. 1997).

To test whether ABA could in fact induce *RNS1* expression, we treated *Arabidopsis* seedlings with ABA and

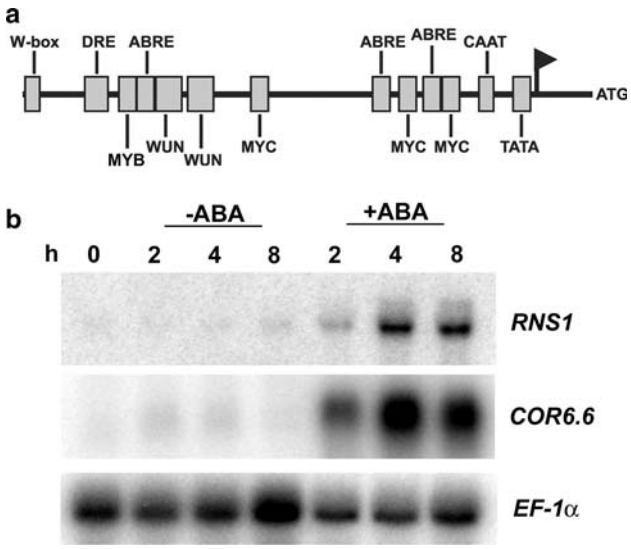

Fig. 1 ABA induces *RNS1* expression. **a** Structure of the *RNS1* promoter. Motifs with significant similarity to previously identified *cis*-acting elements are shown (*grey boxes*). These include CAAT and TATA boxes, wound-responsive elements (W-box, *WUN*), a dehydration-responsive element (*DRE*), ABA-responsive elements (*ABRE*), and MYB and MYC binding sites. **b** Northern analysis of RNA isolated from seedlings treated with 100 μM ABA for the indicated times (h). The *COR6.6* probe was used as a control for the ABA treatment, and *EF-1α* as a control for loading

samples were harvested at different time points. Mock-treated plants were harvested as control. Figure 1b shows that *RNS1* is induced in seedlings treated with ABA with kinetics similar to that of *COR 6.6*, a known ABA regulated gene (Gilmour et al. 1992). Using the ABA-insensitive mutant *abi2* (see below) we also showed that the induction of *RNS1* by ABA is regulated by the ABI2 pathway (Supplementary Fig. S2a). In the *abi2* mutants ABA is unable to induce *RNS1* accumulation.

We also analyzed the induction of RNS1 activity by ABA using an *in gel* activity assay. In this assay extracts are resolved by semi-denaturing SDS-PAGE using gels containing RNA, later incubated in activity buffer, and finally stained to detect RNA. Clear bands represent ribonuclease activities (Supplementary Fig. S2b). Twelve hours after ABA treatment an increase in RNS1 activity is clearly observed, and it maintains similar levels after 24 h. Thus, ABA is able to induce *RNS1* mRNA accumulation followed by an increase in RNS1 activity.

Both ABA-dependent and -independent pathways control *RNS1* induction by wounding

Because *RNS1* expression is induced by ABA, we tested whether ABA is the signal that controls the wounding pathway resulting in the accumulation of *RNS1* transcript and protein. To address this question, we took advantage of a series of mutants deficient in ABA production and signaling. *ABI1* and *ABI2* encode protein phosphatases that participate in the transmission of the ABA signal (Leung et al. 1994, 1997). Mutant plants carrying the *abi1* and *abi2* alleles are insensitive to ABA. In addition, *aba1-1* mutant plants possess a non-functional zeaxanthin epoxidase and cannot produce ABA (Rock and Zeevaart 1991); consequently, ABA-dependent processes are inhibited in these plants.

RNA blot analyses revealed that the wounding induction of *RNS1* transcript accumulation in *abi1*, *abi2*, and *aba1-1* mutants is only 32–48% that of the WT plants (Fig. 2a, b). Our results indicate that an ABA-dependent pathway is required for the full induction of *RNS1* after wounding. However, wounding still induces *RNS1* expression in these mutants; thus, an as-yet uncharacterized ABA-independent pathway is responsible for the induction of *RNS1* in the absence of ABA signaling. As described previously (LeBrasseur et al. 2002), this pathway is also independent of JA and OGA, the two signals commonly associated with wounding responses in *Arabidopsis*.

We also analyzed the role of ABA on the wound-dependent increase in RNS1 activity by *in gel* RNase activity assay. Figure 2c shows that the increase in RNS1 activity observed after ABA treatment is absent in the *abi2* mutant. In addition, a modest decrease in activity (although this

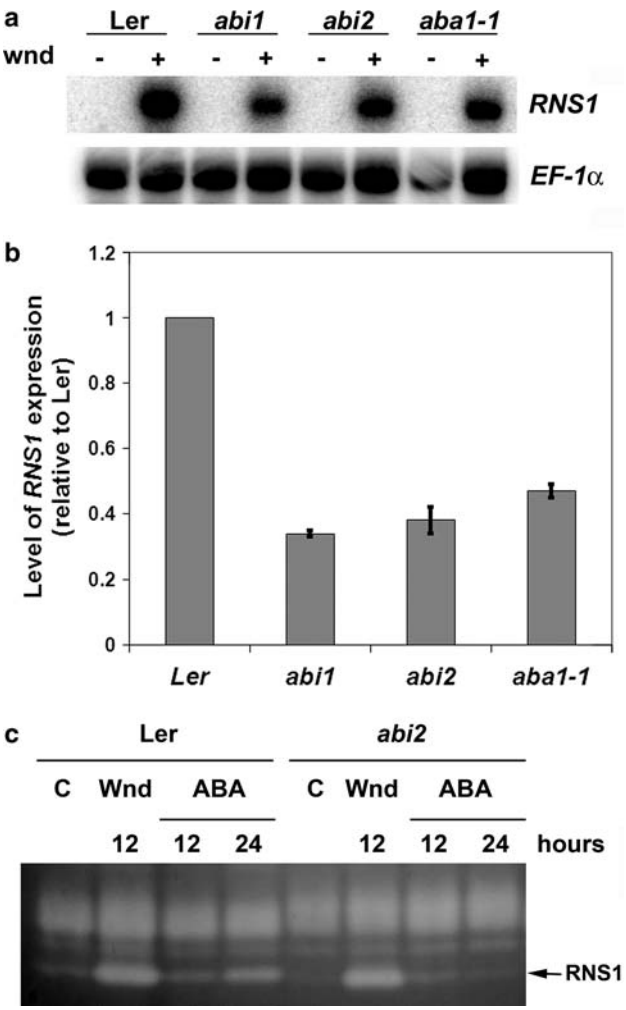

Fig. 2 Participation of ABA in the wound signaling pathway that controls *RNS1* expression. Wild type (*Ler*) and mutants in ABA signaling (*abi1*, *abi2*) and biosynthesis (*aba1-1*) were examined for induction of *RNS1* after wounding. **a** Northern blot analysis of RNA extracted from seedlings 4 h after wounding. **b** Average values of the quantification of the results obtained in three experiments as the ones described in **a**. Three independent experiments were performed; for each experiment individual bands were quantified and normalized using EF-1α as loading control, these values from the three experiments were then averaged and standard error was calculated. **c** Increase in RNS1 activity in response to ABA and wounding is compromised in the *abi2* mutant. Wild type (*Ler*) and *abi2* 2-week-old seedlings were examined for RNase activities. Plants were wounded for 12 h, treated for 12 and 24 h with ABA or left untreated as control. Twenty micrograms of proteins were loaded in each lane

assay is not truly quantitative we estimated a reduction of ~25%) can be observed in wounded *abi2* plants with respect to wounded WT plants. These results are similar to those obtained by northern blots, and confirm the existence of two pathways that control the expression of *RNS1* and the increase in RNS1 activity in response to wounding. RNS1 induction by wounding is paralleled by an increase in several nuclease activities that degrade both RNA and

DNA (LeBrasseur et al. 2002). Both the sustained induction of the 33-kD activities and the transient increase in the 35-kD activity still occur in all the tested ABA mutants (data not shown), indicating that the uncharacterized ABA-independent pathway is also at least partially responsible for the induction of other nuclease activities after wounding.

In an initial attempt to dissect the ABA-dependent pathway controlling *RNS1* expression we analyzed whether any of the most common transcription factors involved in regulation of ABA-dependent transcription was necessary for wound induction of *RNS1*. Three different classes of transcription factors have been identified through genetic screenings of plants with reduced sensitivity to ABA (reviewed by Finkelstein et al. 2002). The *abi3* mutation corresponds to a B3-domain transcription factor (Giraudat et al. 1992), while *abi4* and *abi5* correspond to APETALA2 domain (Finkelstein et al. 1998) and bZIP domain (Finkelstein and Lynch 2000) transcription factors, respectively. Microarray analyses indicate that ABI3 does not control *RNS1* expression (Suzuki et al. 2003). Thus, we tested whether ABI4 or ABI5 were responsible for ABA-dependent induction of *RNS1*. WT and mutant *abi4* and *abi5* plants were wounded and RNA was extracted 4 h later. We found that neither ABI4 nor ABI5 are necessary for full induction of *RNS1* by wounding (Supplementary Fig. S3). These results suggest that another transcription factor is responsible for ABA-dependent induction of *RNS1* by wounding. Alternatively, post-transcriptional processes could be invoked to explain this regulation.

Evidence for transcriptional and post-transcriptional control of *RNS1* by wounding and ABA

As a first step toward dissecting the regulatory mechanisms that control *RNS1* gene expression, we investigated whether *RNS1* transcript accumulation is controlled at the transcriptional and/or the post-transcriptional level. To this end we made transgenic *Arabidopsis* lines carrying the constructs depicted in Fig. 3. Transcriptional regulation was analyzed using the construct RNS1p-GUS (Fig. 3b), in which a 2.6-kb fragment corresponding to the *RNS1* promoter region controlled the expression of the *β-glucuronidase* (*GUS*) reporter gene. The same reporter driven by the CaMV *35S* promoter was used as control (35S-GUS; Fig. 3a). Transformed plants were analyzed by RNA gel blots.

As shown in Fig. 4, *RNS1* is regulated at the transcriptional level. In untreated leaves of plants transformed with the RNS1p-GUS construct, the *GUS* transcript is not detected. But 4 h after wounding, the *GUS* transcript is clearly expressed in wounded leaves. The control 35S-*GUS* lines showed no response to wounding. Similarly, *GUS* accumulation is also observed in plants treated with ABA. These results show that the *RNS1* promoter is sufficient to provide a transcriptional response to wounding and ABA.

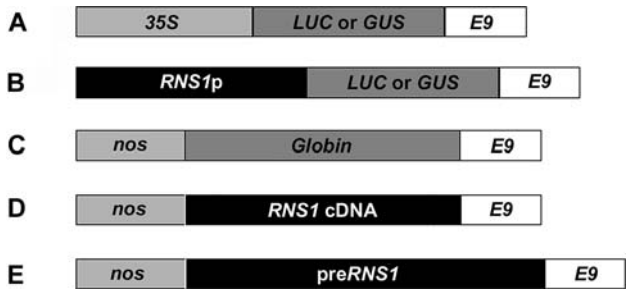

Fig. 3 Constructs used to examine the regulation of *RNS1*. Several constructs were used to transform wild-type *Arabidopsis* plants. Transgenic lines were then used to analyze the expression of the reporters under various conditions. *LUC Luciferase* coding region, *GUS β-glucuronidase* coding region, *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* including UTRs and introns

Although endogenous levels of *RNS1* transcript are induced both by wounding and ABA, after 4 h endogenous *RNS1* expression is fivefold higher in wounded plants than in ABA-treated plants (Fig. 5a, b). Side-by-side comparison of the levels of *GUS* reporter transcript showed that *GUS* expression is similar or higher in RNS1p-GUS plants treated with ABA compared to those that were wounded (Fig. 5a, b). Thus, although the *RNS1* promoter used in these studies is sufficient to provide transcriptional control in response to both stimuli, other regulatory mechanisms seem to contribute to the different levels of induction of the *RNS1* transcript from the native gene.

To examine the possibility of post-transcriptional regulation by wounding and ABA, we designed constructs containing either the *RNS1* cDNA or genomic DNA under the control of the nopaline synthase (nos) promoter (Fig. 3d, e). Specifically, we fused the mature *RNS1* transcribed region (RNS1cDNA) or a genomic clone corresponding to the coding region plus 5′ and 3′ UTR and intron sequences of *RNS1* (pre-RNS1) to the nos promoter (designated nos-RNS1cDNA and nos-preRNS1, respectively; Fig. 3d and e). As a control for this set of constructs, we used the human *β-globin* gene under the control of the *nos* promoter (nos-globin, Fig. 3c). These constructs contain a ‘tag’ of 42 nucleotides transcribed from the *nos* promoter. Thus, blots were probed with an oligonucleotide complementary to the *nos* tag to distinguish between *RNS1* transcribed from the transgene and the endogenous *RNS1* copy.

Analysis of the nos-RNS1cDNA lines revealed no difference between *nos* signal in wounded and unwounded leaves (Fig. 6a, c), although endogenous *RNS1* was induced by wounding (not shown). In the nos-preRNS1 plants, however, a reproducible increase in *nos* signal was seen (Fig. 6a, c). The two bands detected in the preRNS1 and

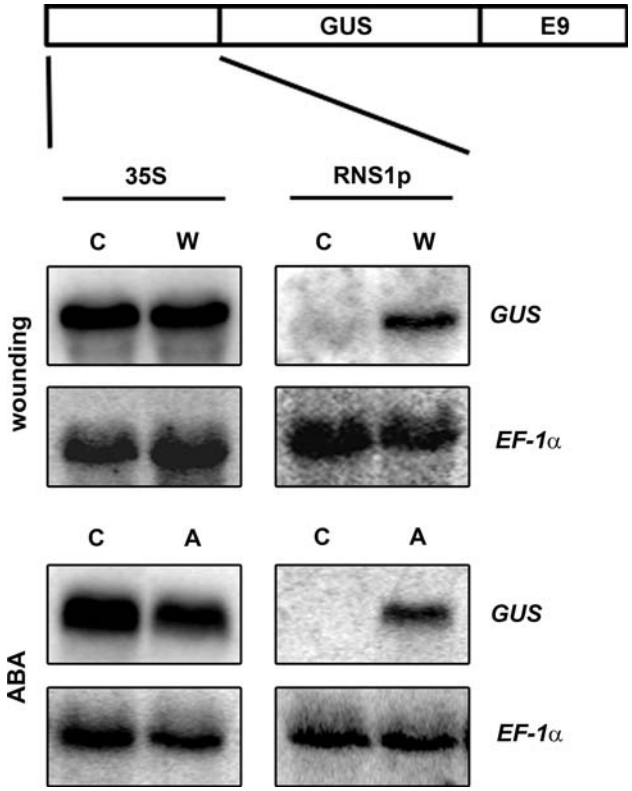

Fig. 4 The *RNS1* promoter confers wound- and ABA-inducibility to reporter transcripts. Leaves of transgenic *Arabidopsis* plants expressing the *GUS* reporter under the control of either 2.6 kb of genomic sequence upstream of the *RNS1* transcription start site or the constitutive 35S promoter were harvested 4 h after wounding or ABA treatment (W and A, respectively). Untreated plants were used a control (*C*) for wounding and buffer treated plants (*C*) were used as control for ABA treatments. Blots were probed with *GUS*, then stripped and probed with *EF-1α* (to control for loading). 35S-GUS plants were used as controls to demonstrate that *GUS* is not stabilized by wounding. For each experiment, at least three independently transformed lines were used. Representative results are shown

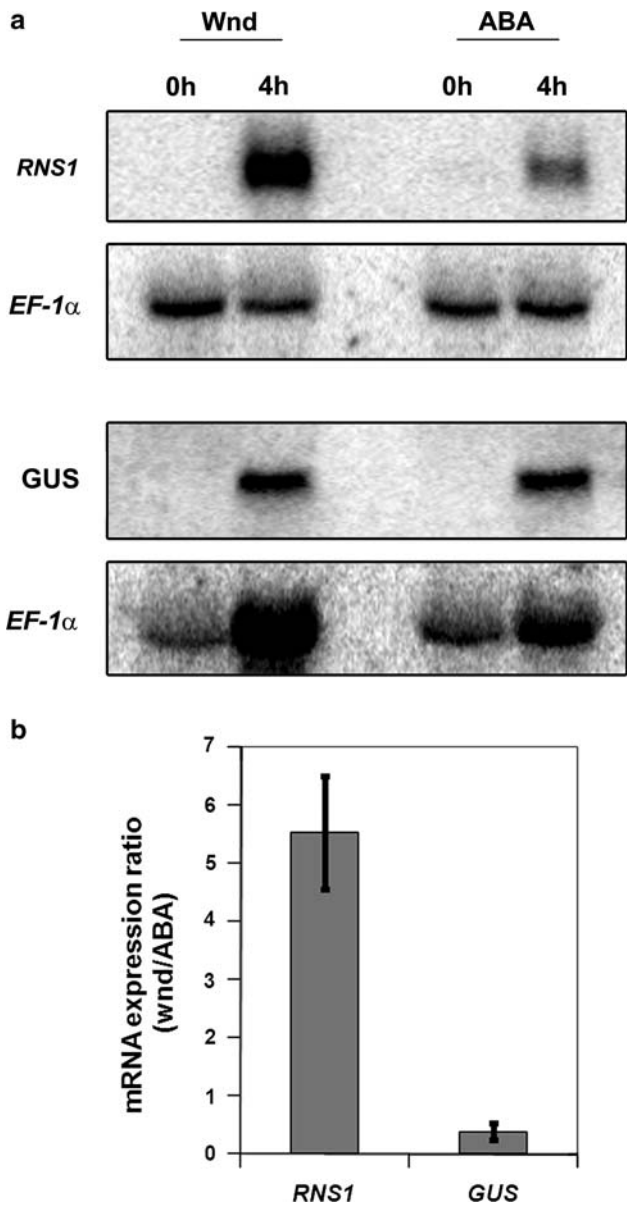

Fig. 5 Differential accumulation in response to wounding and ABA of endogenous or reporter genes under the control of the *RNS1* promoter. **a** Northern blot analysis of transcript accumulation corresponding to the endogenous *RNS1* (*upper panels*) or the *GUS* reporter under the control of the *RNS1* promoter (*lower panels*). Blots were treated as in Fig. 4. **b** Quantification of data shown in **a**. Data represent the average normalized ratio from at least four independent experiments involving eight independent transgenic plant lines. For each blot, the normalized values were calculated by dividing the *GUS* (or the *RNS1*) transcript level in response to wounding and to ABA to that of the *EF-1α* transcript. Only the +wounding or +ABA transcript level (each divided by that of *EF-1α*) was used to calculate the normalized ratio for a given experiment

RNS1cDNA lanes could be the result of alternative polyadenylation sites, as these constructs contain both the endogenous *RNS1* and *E9* 3′ end polyadenylation signals. This was confirmed by 3′RACE analysis, which identified two transcript ends corresponding to the two alternative polyadenylation sites (data not shown). Control nos-globin lines indicate that wounding does not induce the *nos* promoter (in fact, a slight but reproducible repressive effect was seen). It is therefore possible that some level of post-transcriptional regulation of *RNS1* mRNA exists that requires either the entire UTR regions or intron sequences or both, whereas the cDNA sequence alone is not sufficient. This increase of approximately 2.5-fold (Fig. 6c) might provide a second layer of induction in addition to the transcriptional effect described above.

Post-transcriptional regulation was not observed after treatment with ABA (Fig. 6b, d). Transcript levels in ABA-treated nos-preRNS1 and nos-RNS1cDNA plants resemble those in untreated plants. This disparity in post-transcriptional regulation might explain why endogenous *RNS1* is induced to higher levels by wounding than by ABA.

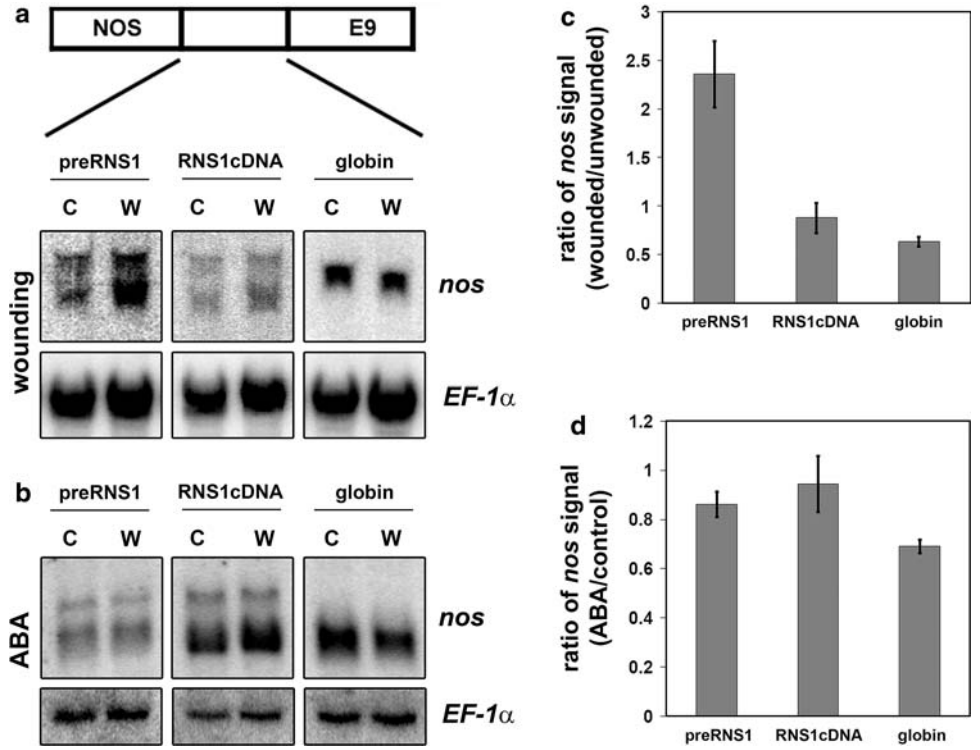

Fig. 6 Differential response of *RNS1* transcribed sequences to wounding and ABA. **a** Pools of T_2 *Arabidopsis* seedlings expressing either the entire *RNS1* transcribed region (*left panels*), the *RNS1* cDNA (*center*), or the *globin* transcript (*right*) under the control of the constitutive *nos* 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 probed with *RNS1* and *EF-1α* (to control for loading). **b** Same as (**a**) except that the plants were treated with 100 μM ABA and harvested 4 h later. **c** Average values of the quantification (see Fig. 2) of the results obtained in three experiments as the ones described in **a**. *Nos* signal was corrected for loading differences (NOS/EF-1α); and it is shown as ratio of wounded versus unwounded expression [(NOS/EF-1α)wounded/(NOS/EF-1α)unwounded]. **d** Average values of the quantification of the results obtained in three experiments as the ones described in **b**. *Nos* signal is shown as ratio of ABA-treated versus buffer-treated expression. For each experiment, at least three independently transformed lines were used. Representative results are shown

Tissue specific, developmental and stress regulated activity of the *RNS1* promoter

Analysis of specific patterns of expression can also provide clues to dissect the mechanisms that control *RNS1* expression. Analysis of *RNS1* promoter activity could also be used to identify transcription factors with similar expression patterns that may participate in this control. To analyze promoter activity, we used plants expressing the RNS1p-GUS construct described in Fig. 3. Plants expressing a similar construct in which the GUS reporter was replaced by luciferase (RNS1p-LUC) were also made.

Plants at different stages, from germination to maturity, were subjected to GUS staining (Fig. 7a–f). In the absence of stress the *RNS1* promoter is active early during germination (Fig. 7a–c). GUS staining was detected in cotyledons as early as one day after germination (Fig. 7a), and almost disappeared 3–4 days after germination. Seven-day-old seedlings showed expression in root tips (Fig. 7c) and hydathodes (Fig. 7b), and some expression could be observed in vascular tissue (Fig. 7b). In adult leaves, *RNS1* expression was limited to hydathodes (Fig. 7d). In flowers, *RNS1* expression was only observed in anthers (Fig. 7e–f).

Activity of the *RNS1* promoter in response to wounding and ABA was analyzed using RNS1p-LUC plants. As described previously, the *RNS1* promoter responds to wounding and ABA stimuli. Luciferase expression was observed throughout ABA-treated plants (Supplementary Fig. S4c), and in local and systemic tissues of wounded plants (Supplementary Fig. S2d). Note that *RNS1* promoter activity is higher next to the wound (the wounds can be observed in the visible light picture, Supplementary Fig. S2b). We were unable to detect significant luciferase activity upon dehydration of plants expressing RNS1p-LUC. However, wounding combined with dehydration produced a stronger luciferase signal than did wounding alone (data not shown).

Discussion

The regulation of *RNS1* transcript accumulation defines a novel pathway for the wounding response in *Arabidopsis*.

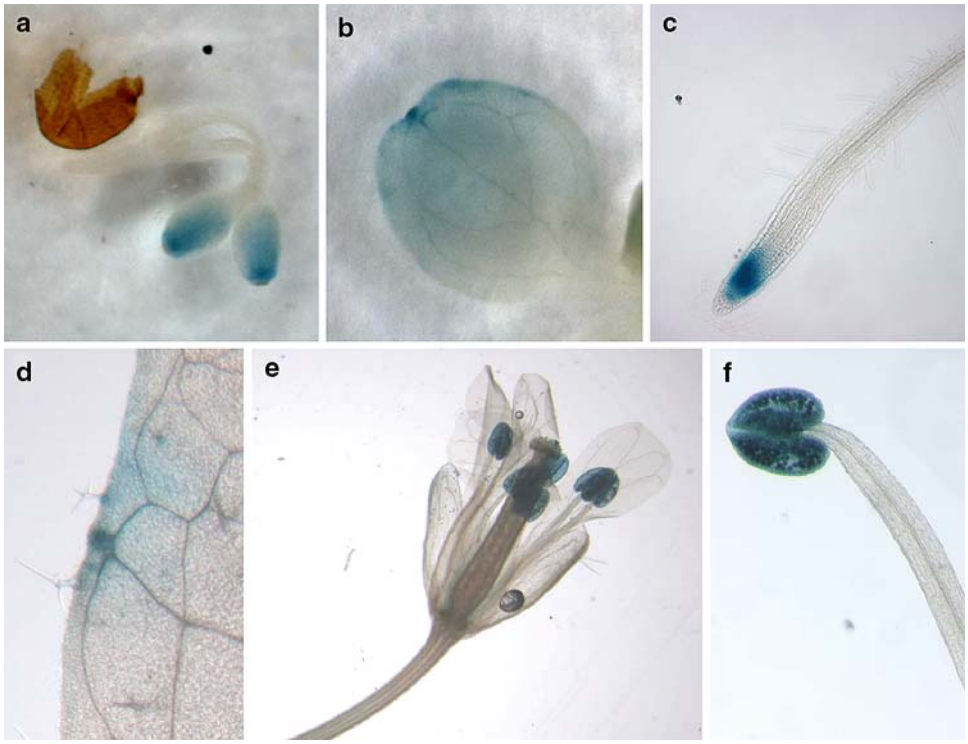

Fig. 7 Tissue specific, developmental and stress regulated activity of the *RNS1* promoter. Patterns of *RNS1* promoter-driven GUS expression in seedlings at different ages or in different tissues: (**a**) 1-day-old seedling, (**b**–**c**) cotyledon and root of 7-day-old seedling, respectively, (**d**) 4-week-old leaf, (**e**–**f**) mature flower

The induction of RNS1 activity is independent of the two signals that have been proposed to control wounding responses in this plant—JA and OGAs. This pathway is also independent of other defense response regulators like ethylene (Reymond et al. 2000) and salicylic acid (SA; LeBrasseur et al. 2002). In this report we examined whether ABA controls this novel pathway. We showed that treatment of plants with ABA or wounding induces the expression of *RNS1* within the same timeframe. Accumulating evidence, points to ABA as a component of the wounding response in plants. Although the exact nature of its contribution has not been defined, it is known that ABA accumulates upon wounding (Peña-Cortés et al. 1991). Our results with ABA mutants indicate that ABA is necessary for full induction of *RNS1* during the wounding response. Thus ABA role during wounding seems to be to regulate the amplitude of the wounding response for *RNS1* and likely other genes that could be co-regulated by the same pathway.

ABA is also likely to mediate the induction of dehydration-responsive genes that occurs locally following wounding. The cDNA microarray analysis carried out by Reymond et al. (2000) suggests that dehydration may also directly control wound gene induction, at least in *Arabidopsis*. Many of the wound-induced genes identified in that study were also induced by dehydration. It is likely that the extent of tissue damage incurred by the plant will determine the extent to which dehydration and ABA influence gene expression during a wound response (de Bruxelles and Roberts 2001). An alternative view was presented by Cheong et al. (2002), who suggested that drought and cold response pathways are activated in response to wounding. Their hypothesis is based on microarray experiments showing that the transcription factor DREB1B/CBF and several of its downstream targets are induced after wounding.

The use of mutants that either do not produce or cannot respond to ABA allowed us to show that ABA is one part of a signaling cascade that mounts a comprehensive wound response. ABA mutants consistently showed a weaker induction of *RNS1* expression, indicating that ABA is necessary for full *RNS1* induction. However, this hormone is not absolutely necessary for *RNS1* wound induction, as 40–50% of the increase still occurs in the absence of ABA signaling (Fig. 2b). These results indicate the existence of an ABA-independent pathway that is responsible for a substantial portion of the induction of RNS1 and nuclease activities after wounding. Based on our and others' previous results (Reymond et al. 2000; LeBrasseur et al. 2002), this pathway is also non-responsive to JA, OGAs, SA or ethylene. The existence of this pathway led to the previous assertion that wounding control of *RNS1* was independent of ABA (LeBrasseur et al. 2002). However, it is now clear that intact ABA production and ABA signaling pathways are necessary for full induction of *RNS1*, as indicated by the results obtained with *aba1-1*, and *abi1* and *abi2*, respectively.

Our experiments indicate that there is a synergy between different signals contributing to the induction of *RNS1* expression. Although *RNS1* is not significantly induced by

dehydration, there seems to be a stronger induction by wounding when plants are dehydrated. It is possible that the putative DRE is not functional, or alternatively, this element present in the *RNS1* promoter may function only in a cooperative manner with other elements in the promoter. Synergistic interactions have been described before. For example, the stress-responsive gene *RD29A* is rapidly induced after dehydration by an ABA-independent pathway, which is followed by a strong ABA-dependent induction. This regulation was explained by the existence of separate *cis*-acting elements in the *RD29A* promoter, including DRE and ABRE elements (Yamaguchi-Shinozaki and Shinozaki 1993, 1994).

We found that *RNS1* induction by wounding and ABA is controlled, at least in part, at the transcriptional level. The best characterized transcription factors that participate in ABA regulation are ABI3, ABI4 and ABI5. Although their activity has been mostly studied during seed development, it is clear that these transcription factors can also act in vegetative tissues (Arenas-Huertero et al. 2000; Rohde et al. 2000; Brocard et al. 2002). However, our results and those of Suzuki et al. (2003) indicate that ABI3, ABI4 and ABI5 are not responsible for transcriptional control during wounding induction of *RNS1*. Recently, other transcription factors with the ability to bind ABREs have been described (see review by Yamaguchi-Shinozaki and Shinozaki 2005). Analysis of *RNS1* expression patterns can provide clues to identify transcription factors that could regulate its expression. Comparison of *RNS1* promoter activity with known expression patterns of other ABRE binding proteins shows a striking similarity between *RNS1* and *AREB1* expression (Fujita et al. 2005). AREB1 is a basic domain/leucine zipper factor that binds ABREs and functions as a *trans*-activator to regulate ABRE-dependent ABA signaling that enhances drought tolerance in vegetative tissues (Fujita et al. 2005). As *RNS1*, *AREB1* is expressed in roots, hydathodes and anthers (Fujita et al. 2005); thus, it is possible that AREB1 also participates in the control of *RNS1* expression. In addition, the MYB transcription factor PHR1 has been shown to regulate *RNS1* expression in response to phosphate-starvation conditions (Rubio et al. 2001).

The *RNS1* promoter alone is able to provide some wound and ABA responsiveness to reporter genes. The *RNS1* promoter has a modular structure similar to that of other ABA-responsive genes, suggesting that similar synergistic interactions control *RNS1* expression. The promoter contains ABA-responsive elements, such as ABRE, MYB and MYC regions, and an ABA-independent, dehydration-responsive DRE element, which might mediate a quick response to dehydration even before the peak of ABA production is reached (Yamaguchi-Shinozaki and Shinozaki 1993, 1994). The binding of DREB1B to this element after its wound induction (Cheong et al. 2002) might provide a direct link between wounding and dehydration responses. These promoter elements could also cooperate with several wounding-responsive elements in the *RNS1* promoter to produce the full induction of *RNS1* after mechanical damage, as shown for other genes (Narusaka et al. 2004). In addition to this synergy, ABREs might be promiscuous; signals other than ABA might activate ABRE-mediated transcription (reviewed by Nambara and Marion-Poll 2003). It is thus possible that ABREs act as nodes in signaling crosstalk. This possibility is supported by recent work showing that ABREs are over-represented in the promoter regions of genes corresponding to several different stress cDNA collections (Mahalingam et al. 2003). However, the presence of multiple ABRE elements in a promoter is not a random event, since only 137 genes out of 26,207 genes in the *Arabidopsis* genome have multiple ABREs (Huang and Wu 2006). Interactions between ABA and JA signaling during the wound response might be mediated by AtMYC2 (review by Lorenzo and Solano 2005). This hypothesis would explain microarray results that show overlaps between wounding responses and those observed after pathogen attacks, abiotic stress, and hormonal treatments (Reymond et al. 2000; Cheong et al. 2002).

The transcriptional responsiveness of the reporter constructs shown in Figs. 3 and 7 supports the functionality of the ABA- and wounding-responsive elements in the *RNS1* promoter. However, transcriptional activity of the promoter is insufficient to explain the differences in transcript accumulation of the endogenous *RNS1* after wounding and ABA treatment (Fig. 5). Analysis of the expression of the *RNS1* transcript, including UTRs and introns and driven by a constitutive promoter, showed that untranslated sequences also respond to wounding (Fig. 6a, b). The simplest explanation of this result is that sequences in the *RNS1* mRNA stabilize the transcript in response to wounding. In plants, several stress and hormonal responses affect mRNA stability (reviewed by Gutiérrez et al. 1999). Our results might be the first evidence of post-transcriptional control during the wounding response since, to our knowledge, changes in mRNA stability in response to this stress have not been described before.

A transcription regulatory element in the transcribed region, however, cannot be ruled out. For example, a transcriptional enhancer could be located in the *RNS1* transcribed region. Most studies on transcriptional regulation and promoter elements that control ABA and wounding responses have focused on the promoter region upstream of the transcription start site (see, for example, reviews by Farmer et al. 2003; Yamaguchi-Shinozaki and Shinozaki 2005).

Several recently identified ABA-hypersensitive mutants, such as *abh1*, have mutations in RNA-binding proteins.

A double-stranded RNA-binding protein, HYL1 (Lu and Fedoroff 2000), and an Sm-like snRNP protein, SAD1 (Xiong et al. 2001), control ABA regulation of seed germination. Plants carrying homozygous mutations in either of these genes are hypersensitive to ABA, suggesting that both proteins are negative regulators of ABA signaling. Another RNA-binding protein, AKIP1, was identified as a specific target of the ABA-activated protein kinase AAPK (Li et al. 2002). These results prompted the idea that ABA signaling is linked to RNA metabolism (reviewed by Fedoroff 2002).

The induction of the RNS1 RNase by ABA is further support for ABA-regulation of RNA metabolism. Interestingly, although *RNS1* is induced by ABA, its expression is downregulated in the *abh1* mutant. Based on this finding, Hugouvieux et al. (2001) proposed that *RNS1* and other downregulated transcripts could be negative regulators of ABA signaling. Following this hypothesis, RNS1 would be induced by ABA early during the wounding response, and work in a negative feedback loop to regulate such response. In addition, in view of the reduced level of *RNS1* in the *abh1* mutant, it was suggested that *RNS1* itself is a target for post-transcriptional regulation by ABA (Hugouvieux et al. 2001). We were unable to detect regulation of the *RNS1* cDNA or pre-RNA by ABA. However, our experimental set-up could have interfered with this regulation. ABH1 is a cap-binding protein; therefore interactions with the 5′ UTR of target transcripts could be expected. Our transgene transcripts carry a 5′ nos tag to differentiate them from endogenous *RNS1*. This tag could disrupt interaction between ABH1 or an associated factor with the 5′ UTR of *RNS1*. Thus, more experiments will be necessary before we can discard a role of post-transcriptional regulation of *RNS1* by ABA.

Our initial results and the tools developed in this work open a new avenue to the study of post-transcriptional regulation during wounding, an area mostly overlooked so far. It also provides a means to test directly the commonly accepted idea that ABA regulation has a large post-transcriptional component. In addition to helping us to dissect the complex signaling pathways leading to RNS1 induction, our work has begun to address the role of ABA in the regulation of wounding response and the function of RNS1 as part of the signal or response to wounding and ABA.

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