Plant Physiol. (1997) 113: 1253–1263

RNase Activity Decreases following a Heat Shock in Wheat Leaves and Correlates with Its Posttranslational Modification[1]

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Heat shock results in a coordinate loss of translational efficiency and an increase in mRNA stability in plants. The thermally mediated increase in mRNA half-life could be a result of decreased expression and/or regulation of intracellular RNase enzyme activity. We have examined the fate of both acidic and neutral RNases in wheat seedlings that were subjected to a thermal stress. We observed that the activity of all detectable RNases decreased following a heat shock, which was a function of both the temperature and length of the heat shock. In contrast, no reduction in nuclease activity was observed following any heat-shock treatment. Antibodies raised against one of the major RNases was used in western analysis to demonstrate that the RNase protein level did not decrease following a heat shock, and the data suggest that the observed decrease in RNase activity in heat-shocked leaves may be due to modification of the protein. Two-dimensional gel/western analysis of this RNase revealed three isoforms. The most acidic isoform predominated in control leaves, whereas the most basic isoform predominated in leaves following a heat shock and correlated with the heat-shock-induced reduction in RNase activity and increase in mRNA half-life. These data suggest that RNase activity may be regulated posttranslationally following heat shock as a means to reduce RNA turnover until recovery ensues.

Following exposure to heat shock, gene expression undergoes substantial reprogramming, involving changes in transcription, translation, and RNA turnover (for review, see Lindquist, 1986). In addition to the well-studied alterations in transcriptional activity, there is a rapid disassembly of polyribosomes in many species that results in a reduction in the translation of nonheat-shock mRNAs (Storti et al., 1980; Lin et al., 1984; Gallie et al., 1995). Non-*hsp* mRNAs are not destroyed but are maintained in HSGs that include two major HSPs, HSP70 and HSP17 (Nover et al., 1983, 1989) and the mRNAs are subsequently recruited for translation upon recovery (Storti et al., 1980). The HSGs associate with the cytoskeleton, forming perinuclear complexes in plants (Nover et al., 1989; Apuya and Zimmerman, 1992), in invertebrates (Leicht et al., 1986; Arrigo, 1987), and in vertebrates (Collier and Schlesinger, 1986).

We have previously shown that heat shock causes a loss in the translational competence of nonheat-shock mRNAs, which is a consequence of the loss in the functional codependency between the 5′ cap structure and the 3′ poly(A) tail of these mRNAs (Gallie et al., 1995). We also observed that mRNA stability increases following a heat shock and that the increase is a function of the severity of the stress, resulting in an increase in the time over which a given mRNA is translationally active. Following a 15-min exposure to 37°C, the functional half-life of a reporter mRNA increased only 50%, but increased 5-fold following a 42°C heat shock, and nearly 9-fold following a 45°C heat shock. In contrast, no change in message stability was detected in heat-shocked mammalian cells (Gallie et al., 1995). The thermally induced increase in mRNA half-life in plants could be a result of two nonmutually exclusive possibilities: a reduction in the amount or activity of those RNases responsible for mRNA degradation, or a sequestration of mRNAs from RNase attack, perhaps through their incorporation into HSGs.

The regulation and cellular role of RNases in plants is still poorly understood. For instance, the RNase(s) involved in mRNA turnover have yet to be identified in plants or, indeed, in any higher eukaryote. However, RNase activities have been identified and, in some respects, characterized in several plant species (for review, see Green, 1994). Three RNase activities have been characterized in wheat leaves (Blank and McKeon, 1991a). An acidic RNase (approximately 20 kD) that is ubiquitous in plant species is often localized to the vacuole or ER (Baumgartner and Matile, 1976; Farkas, 1982; Abel and Glund, 1986, 1987; Loffler et al., 1993) or secreted from the cell (Brown and Ho, 1986; Jost et al., 1991). The other two are neutral RNases that differ both in size and in their catalytic requirements. One RNase is approximately 27 kD in size and is inhibited by salt or $MgCl_2$, whereas the other is composed of a group of RNases (the major species of which is 26 kD in size) in which the activity is stimulated by salt or $MgCl_2$ (Blank and McKeon, 1991a). All three RNase activities are induced as part of the senescence program (Blank and McKeon, 1991b), as has been shown for specific RNases in Arabidopsis and other species (Taylor et al., 1993; Green, 1994). The neutral RNases are present at a moderate level in normal wheat leaves but are induced to a high level during senescence (Blank and McKeon, 1991b). In addition to the regulation associated with senescence, the expression of other RNase genes has been shown to be regulated tissue-specifically (Yen and Green, 1991) and in

[1] This work was supported by a grant from the U.S. Department of Agriculture (NRICGP 95-37100-1618).

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Abbreviations: HSF, heat-shock factor; HSG, heat-shock granules; HSP, heat-shock protein; PVPP, polyvinylpolypyrrolidone.

response to phosphate limitation (Nurnberger et al., 1990; Bariola et al., 1993; Loffler et al., 1993). The S-RNases are a unique class of secreted RNases that are expressed specifically in the style tissue of species of the Solanaceae. They form the basis for gametophytic self-incompatibility, possibly by their uptake and subsequent destruction of cellular RNA within the pollen tube of incompatible pollen (for review, see Newbigin et al., 1993).

We have examined whether RNase expression or activity in wheat is subject to regulation following heat shock. We found that the steady-state level of RNase expression remains unchanged in wheat leaves following a heat shock but that RNase activity is reduced. RNase activity in extracts or following purification is heat-stable even following renaturation from boiling in the presence of SDS, demonstrating that the heat-mediated reduction in RNase activity in leaves is not a result of thermal lability of the enzyme itself. One of the neutral RNases was examined in greater detail using two-dimensional electrophoresis and was found to be present in multiple isoforms, the most acidic form of which predominated in nonstressed leaves. Following a heat shock, the distribution of isoforms was altered in such a way that the most basic isoform predominated. These data are consistent with a heat-induced dephosphorylation of RNase and correlate with the observed decrease in RNase activity and increase in mRNA stability that occurs as part of the heat-shock response.

MATERIALS AND METHODS

Heat Treatments

One gram of wheat leaves excised from 5-d-old seedlings was placed in a total volume of 3 mL of Gamborg's medium supplemented with 10 mM $CaCl_2$ (pH 5.6) in Petri dishes and subjected to heat shocks by immersing the dishes into either 37, 42, or 45°C water baths for the times indicated. The dishes were gently swirled in the water bath to achieve even heating. At specified time points, the tissue was removed, frozen in liquid nitrogen, and soluble protein extracts were obtained by grinding the tissue in grinding buffer (50 mM Hepes, pH 7.5, 5 mM magnesium acetate, 120 mM potassium acetate, and 1 mM PMSF). The extract was then centrifuged at 11,000*g* for 5 min at 4°C, the supernatant was collected, and the protein concentration was determined as described (Bradford, 1976).

RNase and Nuclease Activity Gels

RNase activity gels were performed using 12% SDS-PAGE gels containing 100 $\mu g\ ml^{-1}$ wheat rRNA as described (Blank et al., 1982) with minor modifications. After electrophoresis the SDS was removed by incubating the gels in 25% isopropanol containing either 10 mM imidazole, pH 7.5 (for the neutral RNase activities), or 10 mM sodium acetate, pH 5.5 (for the acidic RNase activity). The gels were then washed for 10 min in either 10 mM imidazole, pH 7.5, or 10 mM sodium acetate, pH 5.5, depending on the assay, to remove the isopropanol. The gels were incubated at 50°C for 20 to 30 min either in 100 mM imidazole, pH 7.5 (for the salt-inhibited neutral p27 RNase activity), 100 mM imidazole, pH 7.5, 200 mM KCl, and 10 mM $MgCl_2$ (for the salt-stimulated neutral p26 RNase activity), or 100 mM sodium acetate, pH 5.5 (for the acidic p20 RNase activity), to permit in situ degradation of the RNA. The gels were then washed for 10 min in either the 10 mM imidazole buffer (for the neutral RNase activities) or the 10 mM sodium acetate buffer (for the acidic RNase activity) to allow any residual digested RNA fragments to diffuse from the gel. The gel was stained for 10 min in toluidine blue, followed by a wash in the appropriate buffer to remove excess dye, and the bands representing RNases were observed as cleared regions in the gel.

Nuclease activity analyses were performed using 12% SDS-PAGE gels containing 10 $\mu g\ ml^{-1}$ single-stranded herring sperm DNA as described (Compton and Cidlowski, 1987) with minor modifications. After electrophoresis, the SDS was removed by incubating the gels in 25% isopropanol containing 10 mM Tris, pH 7.5, for 10 min, which was repeated once. The gels were then washed twice for 10 min each in 10 mM Tris, pH 7.5, 2 mM $MgCl_2$ to remove the isopropanol. The gels were incubated at 37°C for 60 min in 40 mM Tris, pH 7.5, 2 mM $MgCl_2$ to permit in situ degradation of the DNA. The gel was then washed for 30 min in the same buffer to allow any residual digested DNA fragments to diffuse from the gel. The gel was stained for 10 min in ethidium bromide, followed by a wash in water to remove excess dye, and bands representing nucleases were visualized by UV illumination. The gels were then incubated overnight in 0.1% SDS at room temperature and stained once again with ethidium bromide to verify that the bands had resulted from in situ digestion of the DNA and not from masking by an abundant protein band.

Purification of RNases

Both the acidic and neutral RNases were purified from wheat leaves. Ten-day-old wheat seedling leaves were excised and immediately frozen in liquid nitrogen. The frozen tissue was ground in liquid nitrogen supplemented with 10% (w/w) PVPP. For every 100 g of ground tissue, 300 mL of buffer A (10 mM Tris, pH 7.5, 5 mM EDTA, and 1 mM PMSF) was added and homogenized for an additional 20 min. Following centrifugation at 6500*g* for 30 min at 4°C and filtration through eight layers of cheesecloth, protein fractions were precipitated from the crude extract using ammonium sulfate. The 40 to 60% ammonium sulfate precipitate containing the acidic RNase activity was resuspended in buffer A and dialyzed against 10 mM sodium acetate, pH 6.0 (buffer B). The dialysate was clarified by centrifugation and applied on an Affi-gel Blue column that had been previously equilibrated with buffer B. Protein was eluted using NaCl stepwise elution, the fractions containing the acidic RNase were identified using RNase activity gels, and the protein was precipitated using acetone. The 60 to 80% ammonium sulfate precipitate containing the neutral RNase activities was dialyzed in buffer A, and following clarification by centrifugation, it was loaded on a DEAE-Sephacel column that had been previously equilibrated with buffer A. Protein was eluted using a KCl gradient buffer A from 0.12 M to 0.3 M. Fractions containing

neutral RNase activity were identified using RNase activity gels. Fractions containing RNase activity were pooled and precipitated using acetone. For both the acidic and neutral RNases, the pelleted protein was resuspended in SDS loading buffer without reducing agents and fractionated by preparative SDS-PAGE using a Prep Cell (Bio-Rad) electrophoresis apparatus. Fractions with acidic or neutral RNase activity were collected for the immunization of rabbits.

Antibody Preparation, Two-Dimensional Gel Electrophoresis, and Western Analysis

The antisera used for western analysis were generated in rabbits (Robert Sargeant, Ramona, CA). For two-dimensional gel electrophoresis, the protein samples were precipitated with acetone and resuspended in 9.5 M urea with 2% ampholites (a 3:1 mixture of pH 5.0–8.0:pH 3.0–10.0 ampholites). Five to fifty micrograms of protein (depending on the analysis being performed) was loaded onto an IEF tube gel and electrophoresed at 500 V for 4 h. Following electrophoresis, the tube gels were equilibrated in SDS buffer and the proteins were resolved in the second dimension using SDS-PAGE. For RNase activity assays, RNA was cast into the SDS-PAGE gel to a final concentration of 100 $\mu g\ ml^{-1}$ and the gel was processed for RNase activity as described above following the resolution of the protein in the second dimension. For western analysis, the protein was transferred to nitrocellulose membrane using semi-dry transblotting. The nitrocellulose membrane was blocked for 30 min in TPBS (Tween 20/PBS) with 5% reconstituted dry milk and incubated with anti-RNase antibodies diluted in TPBS with 1% milk for 1.5 h. The blots were then washed with TPBS, incubated with goat anti-rabbit antibody conjugated to horseradish peroxidase (Southern Biotechnology, Birmingham, AL) for 1 h, and the RNase signal was detected using chemiluminescence (Amersham).

RESULTS

The Expression of Wheat RNases Is Regulated Tissue-Specifically

Three RNase activities in wheat leaves, previously designated as WL_A, WL_B, and WL_C, have been characterized (Blank and McKeon, 1991a). The acidic RNase, WL_A, is 20 kD in size and exhibits a pH optimum of approximately 5.7. A group of neutral RNases (referred to as WL_B in Blank and McKeon, 1991a) is composed of a major RNase activity of 26 kD, a minor activity of 28 kD, and a pair of minor activities 24 kD in size. This group of RNases requires high ionic conditions (200 mM KCl and 10 mM $MgCl_2$) for activity and has a broad pH optimum encompassing the neutral range. A second type of neutral RNase, WL_C, is 27 kD in size and also has a pH optimum of approximately 7.0 to 7.6, but is inhibited by high salt (Blank and McKeon, 1991a).

We first examined whether these RNase activities were developmentally regulated by comparing those RNases active in mature dry wheat seed with those expressed in germinating seed and seedling tissues. Wheat seeds were germinated for 10, 24, 48, and 72 h. The seeds that were germinated for 0, 10, and 24 h were analyzed as whole seed, whereas the 48- and 72-h-old seedlings were dissected into root, shoot, and endosperm tissues. The RNase activities present in each sample were determined from soluble protein extracts resolved on RNA-containing SDS-PAGE gels processed at either an acidic or neutral pH (with or without salt) to reveal the three types of RNase activities. The presence of the SDS inhibits RNase activity, which is necessary to prevent the digestion of the RNA, because the RNases migrate through the gel during electrophoresis. Only upon removal of the SDS from the gel by isopropanol, followed by the removal of the isopropanol, does the RNase regain activity.

Although RNase activity gels are only semi-quantitative, they are useful in detecting large differences in RNase activities, and their main advantage is in their separation of the individual RNase activities. Equivalent amounts of protein extracted from each tissue were loaded onto an RNase activity gel (Fig. 1). In mature dry seed, only one acidic RNase (p20) predominated (Fig. 1C). The higher molecular weight RNases in Figure 1C are the neutral RNases (i.e. p26 and p27) also seen in Figure 1, A and B. Because of their broad pH optimum, the neutral RNases are usually seen on the acidic RNase activity gels as well as on the neutral activity gels. An acidic p20 RNase activity was present in all tissues tested at all stages of germination and varied little between the tissues (Fig. 1C). An RNase considerably smaller than the acidic p20 RNase was observed, particularly in the endosperm tissue of the 48- and 72-h-old seedlings (Fig. 1, lanes 5 and 8). This may represent a degradation product of the acidic RNase, which is no longer detectable in the endosperm of 72-h-old seedlings. In contrast to the acidic p20 RNase, the salt-sensitive p27 RNase (Fig. 1B) was not detected and the salt-stimulated p26 RNases (Fig. 1A) were present only at a low level in mature dry seed or up to 24 h of germination (Fig. 1, A and B, respectively). Note that the acidic p20 RNase activity can also be seen in the neutral activity gel of Figure 1, A and B, although its activity is lower because of the neutral pH of the assay for these panels. Both types of neutral RNase activities were present in root tissue in 48- and 72-h-old seedlings (Fig. 1, A and B, lanes 4 and 7). Note that by loading sufficient amounts of protein in each lane so that the RNase activities in the seed, coleoptile, and leaves could be revealed, the level of the salt-stimulated and salt-sensitive neutral RNases in the root tissue (Fig. 1, A and B, lanes 4 and 7) grossly exceeded the linear range of the activity gel, suggesting that this tissue contains high levels of these RNases.

In contrast, the level of the acidic p20 RNase in root tissue (Fig. 1, lanes 4 and 7) was not substantially different than in the other tissues, suggesting that the neutral RNases are differentially expressed at high levels in root tissue. All three groups of RNases also appeared in coleoptile tissue of 48- and 72-h-old seedlings (Fig. 1, lanes 6 and 9). The salt-stimulated neutral p26 RNase, which was detected at a low level in seed extract up to 24 h post-imbibition, was observed at higher levels in the endosperm (a dead tissue at this stage) of 48- and 72-h-old seedlings.

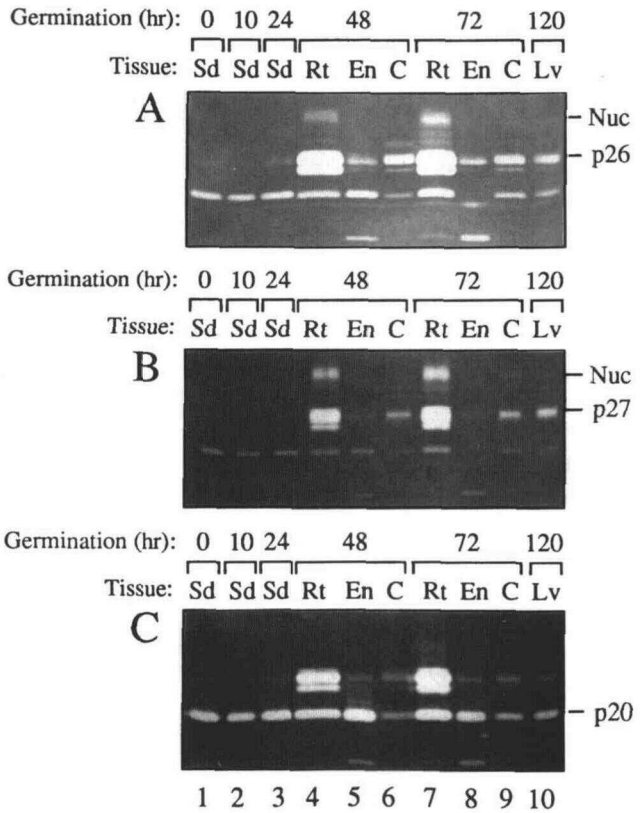

Figure 1. Tissue-specific expression of RNases in wheat. Wheat seeds were germinated for 10, 24, 48, and 72 h (indicated at the top of each panel). The RNase activities present in the soluble protein fraction of each sample were observed following their resolution on an RNase activity gel (a 12% SDS-PAGE gel containing 100 μg mL^{-1} wheat rRNA). Once the gels were stained with toluidine blue, which stains RNA a dark blue, bands representing RNase activities appeared white because of the absence of intact RNA. A, The neutral, salt-stimulated RNases were revealed by processing the gel (see "Materials and Methods") at pH 7.5 in the presence of 200 mM KCl and 10 mM $MgCl_2$. p26 is the predominant neutral, salt-stimulated RNase. B, The neutral, salt-inhibited RNases were revealed by processing the gel at pH 7.5 in the absence of salt. p27 is the predominant neutral, salt-inhibited RNase. C, The acidic RNases were revealed by processing the gel at pH 5.5 in the absence of salt. p20 is the predominant acidic RNase. Ten micrograms (A), 2 μg (B), and 5 μg (C) of protein extract were loaded in each lane. Sd, Seed; Rt, root; En, endosperm; C, coleoptile; Lv, leaf.

RNases that are secreted from the aleurone into the endosperm as part of the mobilization of the storage reserves during germination have been characterized in several cereal grains (Wilson, 1982; Brown and Ho, 1986). This salt-stimulated neutral RNase activity that appears in the endosperm following 48 h of germination may represent such a secretory RNase. A low level of a salt-sensitive neutral RNase was also observed in endosperm of 48- and 72-h-old seedlings. These data suggest that the acidic RNase activity is present in both seed and vegetative tissue, whereas the expression of the salt-stimulated or salt-sensitive neutral RNases is regulated in a tissue-specific manner following germination.

Wheat RNases Are Thermally Stable in Vitro but Exhibit Reduced Activity in Vivo following a Short Heat Shock

The response to heat shock is complex and varies with the temperature of the stress, its duration, and the rate of heating. In wheat, induction of the heat-shock response occurs between 28 and 31°C as measured by the onset of HSP synthesis (Hendershot et al., 1992). Induction of HSP expression was detected following a heat treatment at 37°C for as little as 7 to 10 min (Krishnan et al., 1989; Weng and Nguyen, 1992; Weng et al., 1993) and it reaches a maximum level following a 30-min treatment (Vierling and Nguyen, 1992). Treatment up to 37°C constitutes a mild heat shock, 40 to 43°C is moderate, and 45°C and above is considered severe. A 2-h treatment at 51°C reduced the viability of germinating wheat seed approximately 50% (Abernethy et al., 1989).

To test whether RNase activity is altered following a heat shock and whether changes in its activity correlate with the heat-induced increase in mRNA stability, wheat leaves were subjected to a range of heat treatments ranging from mild to severe, and the effect on RNase activity was measured by RNase activity gels. The length of each heat treatment was short, ranging from 10 to 90 min, and varied from 37 to 45°C. Although these treatments are not lethal, they are sufficient to cause a reduction in the translational efficiency of non-HSP mRNAs and an increase in their stability (Gallie et al., 1995). Following the heat treatments, the leaves were frozen and equal amounts of the soluble protein fraction were resolved on SDS-PAGE gels containing wheat RNA.

The activities of all detectable RNases decreased in leaves following a heat shock and occurred as a function of the length and severity of the heat treatment (Fig. 2). The decrease in activity can be most clearly seen in Figure 2, lanes 16 through 19, which represent 90-min treatments at 24, 37, 41, and 45°C, respectively. The activities of both the major (26 kD) and the minor RNases (24 and 28 kD) that comprise the salt-stimulated p26 class of neutral RNases were coordinately reduced following the heat stress (Fig. 2A). The activity of the minor RNases decreased with increasing exposure to the 37°C treatment and were undetectable at any of the treatments at 41 or 45°C. Note that a low level of the acidic p20 RNase activity can be seen in Figure 2, A and B, because of residual activity of this RNase at the neutral pH. The p27 class of neutral RNases (Fig. 2B) and the acidic p20 RNase (Fig. 2C) also decreased following a heat shock. The decrease in activity for each type of RNase was small following the 37°C heat treatment (compare lane 17 with lane 16 in Fig. 2, A–C , for the 90-min exposure to the 37°C heat shock) but became more apparent as the temperature of the heat shock increased (compare lanes 18 and 19 with lane 16 in each panel). These changes in RNase activity following a heat shock correlate well with the increase in message stability observed in vivo, where the functional mRNA half-life increased by only 50% following a 37°C heat shock, but increased 5-fold following a 42°C heat shock, and nearly 9-fold following a 45°C heat shock (Gallie et al., 1995).

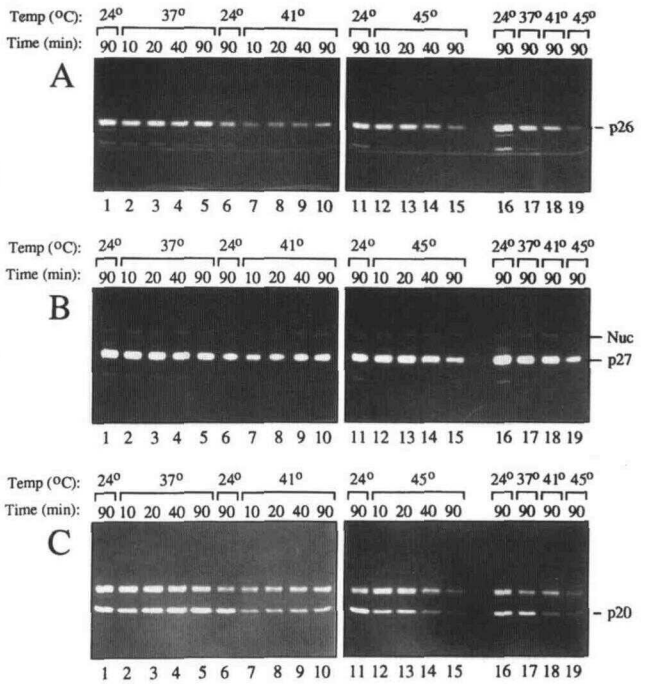

Figure 2. The effect of heat shock on RNase activity in wheat leaves. Wheat leaves were subjected to heat treatments at 37, 41, or 45°C (indicated at the top of each panel) for 10 to 90 min (indicated above each lane) and the RNase activities present in equal protein amounts were compared with that in control leaves using 12% SDS-PAGE RNase activity gels. In lanes 16–19, the RNase activities from leaves subjected to 90 min heat treatments at 24, 37, 41, or 45°C are compared together. Once the gels are stained with toluidine blue, which stains RNA a dark blue, bands representing RNase activities appeared white because of the absence of intact RNA. A, The neutral, salt-stimulated p26 RNases were revealed by processing the gels at pH 7.5 in the presence of 200 mM KCl and 10 mM $MgCl_2$. The faint band that runs across the gel below the p26 RNase is a minor RNase contaminant present in the purified RNA used in the making of the RNase activity gel and is sometimes observed under neutral conditions. B, The neutral, salt-inhibited p27 RNases were revealed by processing the gel at pH 7.5 in the absence of salt. C, The acidic p20 RNase was revealed by processing the gel at pH 5.5 in the absence of salt. Only the RNase that is assayed under its optimal enzyme condition is indicated in each panel. However, the acidic p20 RNase can be observed as a faint band below the neutral RNases in A and B. The neutral RNase activities can also be observed running above the acidic p20 RNase in C. In B, the nucleases (Nuc) are also indicated.

In addition to RNase activities, there are also several single-stranded-preferring nucleases (32 to 38 kD in size) present in wheat leaves that have expression regulated by senescence and light (Blank and McKeon, 1989). These nucleases have not been well characterized but are known to degrade both RNA and DNA. They exhibit low sequence specificity because they are detected following the degradation of herring sperm DNA in a nuclease activity gel (an SDS-PAGE gel containing single-stranded DNA, see "Materials and Methods"). To determine whether the activity of these single-stranded-preferring nucleases are repressed by heat shock, as observed for the RNases, nuclease activities present before and after the heat treatments were followed using nuclease activity gels. Because the wheat leaf nucleases exhibit similar pH and ion requirements (Blank and McKeon, 1989), the activity of all nucleases could be followed using one set of conditions. Two prominent nucleases were observed in extracts from wheat leaves (Fig. 3) and several minor activities could be detected upon longer incubation of the gel (data not shown), confirming the earlier identification of the nuclease activities in wheat leaves (Blank and McKeon, 1989). Nuclease activity was not significantly affected by the heat treatments (Fig. 3). This is most easily seen in lanes 16–19, which represent the 90-min treatments at each heat-shock temperature, although some reduction in activity was noted following the most severe treatment. As with RNase activity gels, nuclease gels are only semi-quantitative, so small alterations in nuclease activity might not be detected. Nevertheless, the nuclease activities can serve as useful internal controls against which the changes in RNase activities can be compared. Because these nucleases digest RNA as well, their activity can also be seen in the neutral activity gel of Figure 2B, where their levels of activity also were not affected following the heat treatments.

These data suggest that either the activity of the RNases or the level of RNase protein is reduced in heat-shocked wheat leaves. Because the reduction in RNase activity was observed soon after the onset of the heat shock, it may occur too rapidly for a heat-mediated decrease in RNase gene expression. However, the loss in RNase activity observed following a heat shock might be explained by the thermal instability of the RNases. To determine whether this was true, wheat leaf extracts enriched for the neutral RNase and nuclease activities were treated at temperatures ranging from 4 to 100°C in SDS loading buffer before assaying on an RNase activity gel. When assayed at a neutral pH, no difference in the neutral p26 or p27 RNase

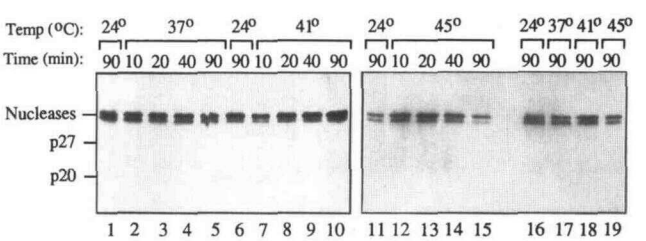

Figure 3. The effect of heat shock on nuclease activity in wheat leaves. Wheat leaves were subjected to heat treatments at 37, 41, or 45°C (indicated at the top of each panel) for 10 to 90 min (indicated above each lane) and the nuclease activities present in equal protein amounts were compared with that in control leaves using 12% SDS-PAGE DNase activity gels. In lanes 16–19, the nuclease activities from leaves subjected to 90-min heat treatments at 24, 37, 41, or 45°C are compared together. Bands representing nucleases, in this case defined as nonspecific DNases, were revealed by processing the gel at pH 7.5 in the absence of salt (see "Materials and Methods"). The sizes of the p20 and p27 RNases are indicated for reference, although these RNases are not seen in the DNase activity gel because they are specific for RNA. Once the gel is stained with ethidium bromide, which causes DNA to fluoresce under UV light, bands representing DNase activity appeared dark (nonfluorescing) because of the absence of intact DNA.

activities was observed (Fig. 4, A and B), suggesting that these RNases are thermally stable enzymes that can easily renature following denaturation by the heat-SDS treatment. A minor RNase activity not previously detected that migrates slightly faster than the acidic p20 RNase (which can be seen in Fig. 4A because of the residual activity of this RNase at the neutral pH) was observed. Whether this represents a unique RNase that appears only upon enrichment for neutral RNase activities, or whether it is a proteolytic product of one of the known RNase activities that occurs during the enrichment process, is unknown. Like the other neutral RNases, however, its activity remains unaffected by the heat treatments. In contrast, the nuclease activities (Fig. 4, A and B) were increasingly inhibited as the temperature of the in vitro heat treatment increased. Of the three DNA-specific nuclease activities seen in Figure 4, A and B, the lowest molecular weight nuclease was more sensitive to the heat treatments than the other nucleases. The activity of the acidic RNase was also unaffected by the in vitro heat treatments (Fig. 4C). The neutral p26 and p27 RNase activities can also be seen on the acidic RNase gel because of their enrichment in the fraction used for this study. The observation that the wheat leaf RNases retain full activity following heat treatments as high as 100°C suggests that their in vivo loss of activity following heat treatments ranging from only 37 to 45°C must result from a reduction in expression or activity of the RNases and not from thermal lability.

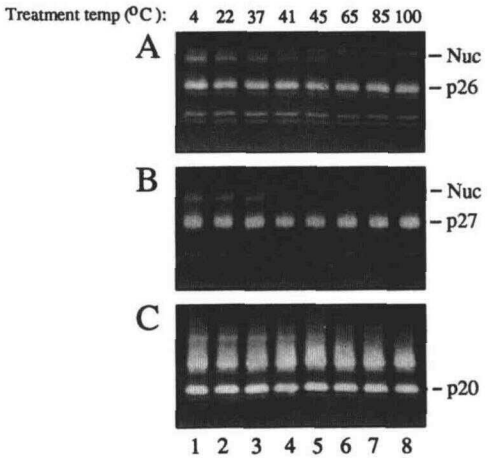

Figure 4. Thermal stability of wheat leaf RNases in vitro. Aliquots from a soluble protein fraction from wheat leaves that was enriched for the neutral RNases (i.e. p26 and p27) were added to an equal volume of SDS-loading buffer and subjected to a 3-min heat treatment in vitro at the temperature indicated above each lane. The protein was then resolved on a 12% RNase activity gel. A, The neutral, salt-stimulated p26 RNases were revealed by processing the gels at pH 7.5 in the presence of 200 mM KCl and 10 mM $MgCl_2$. B, The neutral, salt-inhibited p27 RNases were revealed by processing the gel at pH 7.5 in the absence of salt. C, The acidic p20 RNase was revealed by processing the gel at pH 5.5 in the absence of salt. Only that RNase that is assayed under its optimal enzyme condition is indicated in each panel. However, the acidic p20 RNase can be observed as a faint band below the neutral RNases in A and B. Because the extract used was enriched for the neutral RNases, they appear as an abundant RNase band above the acidic p20 RNase in C. In A and B, the nuclease activities (Nuc) are also indicated.

The Steady-State Level of a Neutral RNase Remains Unaffected by Heat Shock

To determine whether the steady-state level of RNase was altered following thermal stress, we measured the amount of protein for one of the major neutral RNases in wheat leaves before and after a heat shock. The acidic and the salt-inhibited neutral RNases were purified from wheat leaves. The acidic p20 RNase was preferentially precipitated in a 40 to 60% saturated solution of ammonium sulfate (Fig. 5, left, lane 2), whereas the neutral p27 RNase was preferentially precipitated in a 60 to 80% saturated solution of ammonium sulfate (Fig. 5, middle, lane 3). The acidic and neutral RNases were further purified following fractionation using Affi-gel Blue and DEAE Sephacel columns, respectively (Fig. 5). Following their subsequent fractionation on a preparative SDS-PAGE apparatus, the purified RNases were injected into rabbits. The acidic p20 RNase proved to be poorly antigenic. However, highly specific antibodies were obtained to the neutral p27 RNase. No cross-reactivity was detected between the anti-p27 neutral RNase antibodies and the acidic p20 RNase (Fig. 5, right), suggesting that these two types of RNases are quite distinct immunologically. Moreover, no cross-reactivity was detected between the anti-p27 neutral RNase antibodies and the single-stranded nucleases (Fig. 5, right).

The same extracts used in Figure 2 were used for western analysis with the anti-p27 neutral RNase antibodies to determine whether the levels of this RNase decrease following a heat shock. A single 27-kD protein was detected that corresponds to the major neutral p27 RNase used for the antibody production (Fig. 6). However, no consistent decrease in the level of this RNase was detected in extracts made from heat-shocked wheat leaves compared with the control, regardless of the length or severity of the stress. In fact, a slight increase in p27 RNase protein level following a heat shock can be seen in Figure 6. These data suggest that the decrease in p27 RNase activity that follows a heat shock is not due to a decrease in the steady-state level of the RNase.

Heat Shock Alters the Distribution of the RNase Isoforms

The observation that the steady-state level of p27 RNase remains unchanged following a heat shock suggests that its reduced activity following a thermal stress may be due to modification of the p27 RNase itself. Phosphorylation is one type of protein modification that is often observed as the means by which the activity or specificity of the gene expression machinery is regulated following a heat shock. An example of this is the activation through phosphorylation of the heat shock factor, the transcriptional activator of the *HSP* genes (for review, see Sorger, 1991). In addition, changes in the phosphorylation of several translational initiation factors result in their inhibition and are responsible for the global repression of translation of nonheat-shock mRNAs that ensues following a heat shock (for

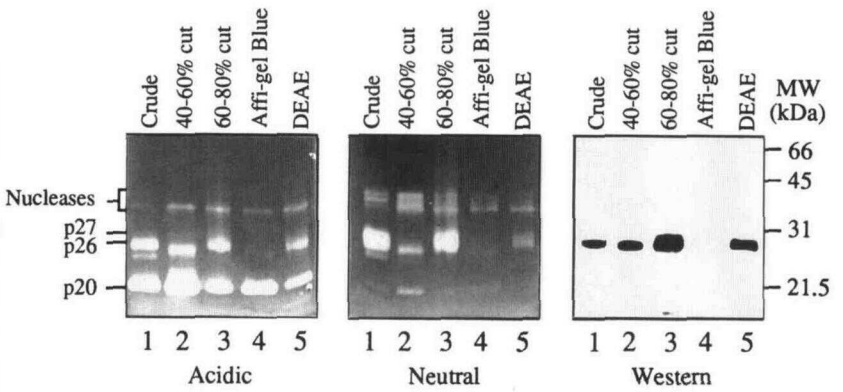

Figure 5. Partial purification of RNases from wheat leaves and immunological analysis. The neutral and acidic RNases were enriched using the procedures indicated above each lane. The RNases present in each enriched fraction were assayed with RNase activity gels at pH 5.5 (left), which is optimal for acidic RNase activity, or pH 7.5 (middle), which is optimal for neutral RNase activity. The acidic p20 RNase was preferentially precipitated in 40 to 60% ammonium sulfate (lanes 2) and was further fractionated on an Affi-gel Blue column (lanes 4). The salt-inhibited neutral p27 RNase was preferentially precipitated in 60 to 80% ammonium sulfate (lanes 3) and was further fractionated on a DEAE-Sephacel column, where some acidic p20 RNase activity copurified (lanes 5). The neutral p27 RNase was further purified using preparative SDS-PAGE and antibodies against it were raised. Western analysis of this p27 RNase in each fraction is shown in the right panel. The western signal was detected using chemiluminescence. MW, Molecular weight.

review, see Duncan, 1995). To determine whether the acidic and neutral RNases in wheat leaves are present in multiple isoforms, protein extract from control wheat leaves was resolved on two-dimensional RNase activity gels where IEF was used for the first dimension and an RNA-containing SDS-PAGE gel was used for the second dimension. The two-dimensional gel was then processed to reveal the RNase activities. Two isoforms were observed for the acidic p20 RNase (Fig. 7A) even when a high level of RNase activity was resolved (Fig. 7B). Up to six isoforms of the neutral p27 RNase were observed that spanned a pI range of 5.1 to 6.2 (Fig. 8A). The most acidic isoform exhibited the greatest activity, resulting in some degree of digestion of the RNA during the migration of this isoform in the second dimension and causing vertical streaking above the final position of the RNase. The activity of each p27 isoform decreases as the isoforms become progressively more basic. The three most basic isoforms (group B) exhibited substantially less activity than did the three most acidic isoforms (group A), which can be seen better in Figure 8B, where less extract was loaded. The activity of the most acidic isoform still exceeded the linear range of the RNase activity gel and caused some digestion of RNA above the final position of the RNase spot during its migration in the second dimension (Fig. 8B). However, the activity of each isoform decreases as it becomes more basic and the activities of the three most basic isoforms (group B) are barely detectable in Figure 8B.

Although the two-dimensional RNase activity gels clearly show multiple RNase isoforms, it is difficult to use two-dimensional activity gels for quantitative purposes. This is in part due to possible changes in both the abundance of each isoform as well as any possible impact that the modification of each isoform may have on its activity. We therefore used the anti-p27 neutral RNase antibodies to first determine which of these isoforms were recognized. Extract from control leaves was resolved using IEF/SDS-PAGE two-dimensional electrophoresis, and the proteins were then transferred to nitrocellulose membrane and probed using the anti-p27 neutral RNase antibodies. The three most acidic of the six RNase isoforms were recognized by the antibodies, whereas the three most basic isoforms were not (Fig. 8C), even following a long exposure of the membrane (data not shown), indicating that these two groups may be immunologically distinct.

We next examined whether the distribution of the acidic isoforms of this neutral p27 RNase is altered following heat shock. Extract from wheat leaves that were heat-shocked for 90 min at 41°C was resolved and probed using two-dimensional gel/western analysis. Whereas the most acidic

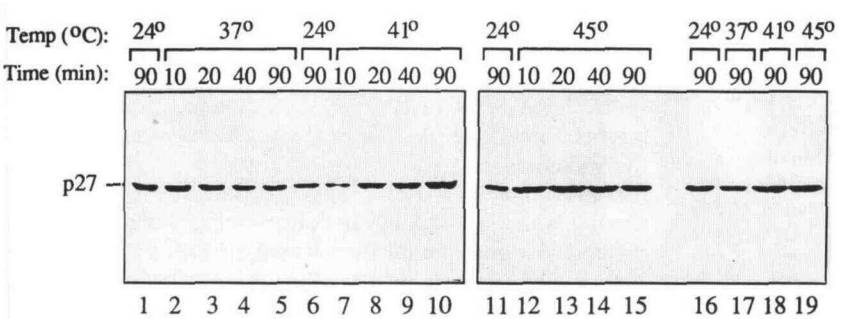

Figure 6. The effect of heat shock on the steady-state level of the neutral p27 RNase in wheat leaves. The leaves were subjected to heat treatments at 37, 41, or 45°C (indicated at the top of each panel) for 10 to 90 min (indicated above each lane) and crude protein extracts made from leaves were resolved on a 12% SDS-PAGE gel. In lanes 16–19, the p27 RNase activities from leaves subjected to 90-min heat treatments at 24, 37, 41, or 45°C are compared together. Following transfer to nitrocellulose, the membrane was probed with the anti-p27 RNase antibodies and the signal was detected using chemiluminescence.

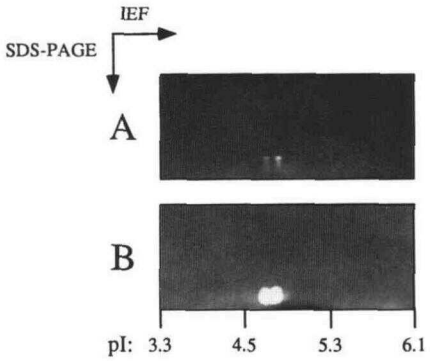

Figure 7. Analysis of the acidic p20 RNase isoforms present in wheat. A fraction enriched for the acidic p20 RNase was resolved in the first dimension by IEF and in the second dimension by 12% SDS-PAGE. A low (A) and a high (B) level of acidic p20 RNase activity was resolved. Following resolution of the proteins in both dimensions, the gels were processed to reveal the acidic RNase activities. Once the gels are stained with toluidine blue, which stains RNA a dark blue, the spots representing p20 RNase activities appear white because of the absence of intact RNA. The pI along the first dimension is indicated below the panels.

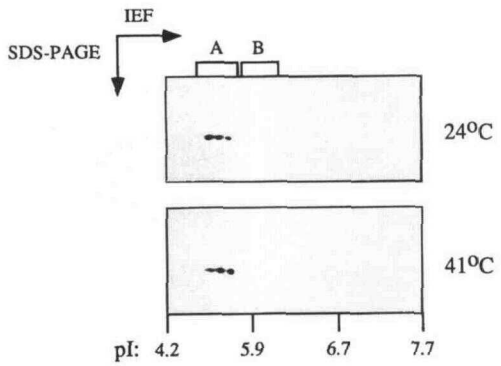

Figure 9. Redistribution of the neutral p27 RNase isoforms following a heat shock. Crude protein extract from control (top) and heat-shocked (bottom) wheat leaves was resolved in the first dimension by IEF and in the second dimension by 12% SDS-PAGE. The protein was then transferred to nitrocellulose membrane and probed with the anti-p27 RNase antibodies, and the signal was detected using chemiluminescence. The pI along the first dimension is indicated below the panels. Proteins that are more acidic (toward the left) are more negatively charged, which can be a consequence of phosphorylation.

isoform had predominated in control leaves (Fig. 9, top), in heat-shocked leaves the most basic isoform of this group predominated and the most acidic isoform was now the least abundant form (Fig. 9, bottom). The observation that none of the isoforms in the B group were detectable following the shift within the A group to more basic isoforms also suggests that they are immunologically distinct. These data suggest that a heat shock causes a conversion of the most acidic isoform of this p27 RNase into its more basic isoform in a manner that is consistent with a heat-mediated dephosphorylation event and that correlates with the reduction in activity for this RNase following a heat shock.

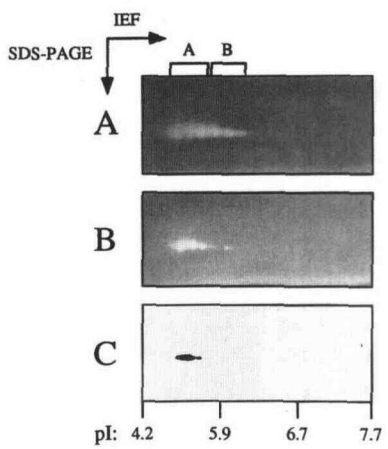

Figure 8. Analysis of the isoforms present in wheat leaves for the neutral p27 RNase. A fraction enriched for the p27 RNases was resolved in the first dimension by IEF and in the second dimension by 12% SDS-PAGE. For the top and middle panels, the gel contained RNA and was subsequently processed to reveal the neutral p27 RNase activities. For the bottom panel, the protein was transferred to nitrocellulose membrane following resolution in the second dimension, probed with the anti-p27 RNase antibodies, and the signal was detected using chemiluminescence. The pI along the first dimension is indicated below the panels.

The Recovery of RNase Activity following a Heat Shock

Exposure to heat shock results in the repression of translation that occurs coordinately with the increase in message stability (Gallie et al., 1995). We have observed that translational repression persists during recovery from a heat shock and the length of time needed to recover normal translational activity is a function of the severity of the stress (D. Gallie, unpublished observations). To determine the length of time required before RNase activity recovers to its pre-heat-shock level, wheat leaves were subjected to a heat shock and allowed to recover at 24°C. Leaves were taken at time points during recovery and their RNase activities were analyzed on RNase activity gels. As seen in Figure 2, a reduction in the activity of the RNases was observed following a heat shock at 41°C. Recovery began between 20 and 35 h after the cessation of the heat shock and was completed by approximately 35 h (Fig. 10). Recovery following a more severe heat shock (45°C) was only detectable by 35 h post-heat-shock (data not shown), suggesting that the length of the time required for recovery of RNase activity increases with the severity of the thermal stress. The time required for the recovery of the pre-heat-shock level of RNase activities following a heat shock might be needed for either renewed RNase synthesis or for the re-activation of the pre-existing RNase activities that had been inactivated by the heat shock. Regardless of the mechanism involved in the recovery of RNase activity, these data demonstrate that a heat shock can have long-term consequences on RNase activity in wheat leaves. These observations also correlate well with the previous finding that the increase in mRNA stability following a

heat shock is also a function of the severity of the thermal stress (Gallie et al., 1995).

DISCUSSION

We have previously observed an inverse correlation between the effect of a heat shock on translational efficiency and its effect on mRNA stability. Heat shock caused a significant reduction in the rate of translation at the same time that message stability increased (Gallie et al., 1995). In contrast, no increase in mRNA stability was observed in animal cells, suggesting that plants differ significantly from animals in this aspect of their response to heat shock (Gallie et al., 1995). The changes in protein yield from a given mRNA in plants following a heat shock are a result of the combined impact of stress on translation and mRNA stability. For instance, a moderate heat shock results in a severalfold increase in mRNA stability but only a moderate loss in translational efficiency such that the final protein yield from a given species of mRNA can be greater than that same pool of mRNA in nonstressed cells. In contrast, even with nearly an order of magnitude increase in the stability of an mRNA following a more severe heat shock, the reduction in translational efficiency is so great that the final yield of protein from a given species of mRNA is far less than that from an identical pool of mRNA in nonstressed cells (Gallie et al., 1995). This increase in mRNA stability may result from either an increase in protection from the mRNA degradatory apparatus and/or a reduction in the expression or activity of those RNases responsible for mRNA turnover. Although these observations concerning the effects of heat shock on mRNA stability may apply to many mRNAs, there are important exceptions. mRNAs encoding for secreted proteins such as α-amylase in barley aleurone cells, for instance, are selectively destabilized following a heat shock (Brodl and Ho, 1991; Lanciloti et al., 1996), perhaps as a consequence of the heat-mediated disruption of the ER, which is normally required for the translation of these mRNAs (Belanger et al., 1986). For such mRNAs specific degradatory mechanisms may have evolved as a means by which some tissues can effectively respond to a heat shock.

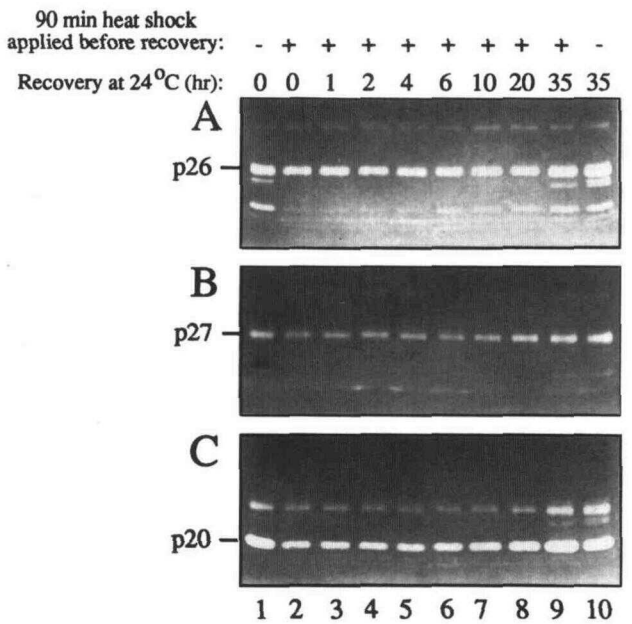

Figure 10. Recovery of RNase activity following a heat shock. Leaves were subjected to a 90-min heat treatment at 41°C and allowed to recover at room temperature up to 35 h. Leaf samples were collected at time intervals and frozen. Protein extracts made from leaves were assayed on 12% RNase activity gels and the RNase activities present in the heat-shocked leaves were compared with those present in control leaves. A, The neutral, salt-stimulated p26 RNases were revealed by processing the gel (see "Materials and Methods") at pH 7.5 in the presence of 200 mM KCl and 10 mM $MgCl_2$. B, The neutral, salt-inhibited p27 RNases were revealed by processing the gel at pH 7.5 in the absence of salt. C, The acidic p20 RNase was revealed by processing the gel at pH 5.5 in the absence of salt. The neutral RNase activities (e.g. p26 and p27) can be observed running above the acidic p20 RNase in C. The acidic p20 RNase can also be observed running below the neutral RNases in A and faintly in B. +, Heat-treated leaves; −, control leaves.

Although the RNase(s) responsible for the turnover of mRNAs in higher eukaryotes have not been identified, we observed that the activity of all RNases detected in wheat leaves was coordinately reduced following a heat shock and that the extent of the decrease was a function of the severity of the stress. The changes in RNase activity contrasted with the lack of change in the observed nuclease activities, suggesting that the leaf RNases were, as a group, specifically regulated. We had observed previously that the increase in mRNA stability following a heat shock is rapid in that a heat treatment as short as 5 to 10 min was sufficient to result in an increase in mRNA half-life (Gallie et al., 1995). Temporally, the reduction in RNase activity paralleled the increase in mRNA stability. Such a rapid loss in RNase activity suggests that changes in the activity of the RNA degradatory machinery may occur following a heat shock rather than an alteration in RNase gene expression. The RNases themselves did not exhibit thermolability in vitro nor does there appear to be any heat-induced protease specific for RNases, because the RNase activities in extracts from heat-shocked leaves were stable if incubated in vitro (data not shown). This prediction was supported by the observation that the steady-state level of protein of the neutral p27 RNase remained unaffected by heat shock as determined by western analysis. Given that the RNases themselves are thermally stable in vitro, even following boiling in the presence of SDS, the reduction in RNase activity in cells treated from only 37 to 45°C may be due to modification of the RNase protein itself. This conclusion was supported by the several isoforms that were observed for the neutral p27 RNase on two-dimensional activity gels and following two-dimensional/western analysis. The shift in the relative abundance of the isoforms from a predominance of the acidic isoform in control leaves to a predominance of the basic isoform in heat-shocked leaves correlated with the changes in RNase activity that were observed following a heat shock, data suggesting that the reduction in activity of this RNase following thermal stress may be a consequence of dephosphorylation. Al-

though the data presented in this study suggest that a decrease in RNase activity following a heat shock might be responsible for the increase in mRNA stability, a direct cause-and-effect relationship remains to be demonstrated. Moreover, our observations concerning the heat-mediated regulation of RNase activity do not exclude the possibility that mRNAs are also sequestered into HSGs following a heat shock as an additional means by which non-heat-shock mRNAs might be stored (Nover et al., 1983, 1989) until their subsequent recruitment for translation during recovery.

How might dephosphorylation of an RNase affect its function? It is possible that a change in the modification of an RNase might determine its subcellular compartmentalization or its association with a regulatory subunit. However, in the present study, soluble protein extracts were obtained by grinding the tissue and would consequently disrupt compartmentalization. Moreover, the resolution of the extracts on SDS-PAGE gels should also disrupt any association that an RNase might have with any putative regulatory subunit. Therefore, neither of these possibilities can explain the decrease in RNase activity in heat-shocked wheat leaves detected with RNase activity gels. It should be noted, however, that these observations do not rule out the possibility that compartmentalization or association with a regulatory subunit could be additional means by which an RNase activity is controlled.

Protein modification can also affect enzyme function directly. Positive regulation through phosphorylation has been demonstrated for RNase III in *Escherichia coli* (Mayer and Schweiger, 1983). RNase III processes the early and late mRNAs of T7 bacteriophage (Dunn and Studier, 1983) and it exists as a phosphoprotein in vivo (Robertson et al., 1994). Phosphorylation of RNase III by the T7 gpo.7 protein kinase results in an increase in RNase III processing activity (Mayer and Schweiger, 1983). Whether RNase activity in wheat leaves is regulated through changes in phosphorylation following a heat shock remains an intriguing possibility. Once cDNAs are isolated for the wheat RNases examined in this study and recombinant RNase protein of each is available, it will be possible to precisely determine what role phosphorylation may play in the regulation of RNase activity.

Received August 22, 1996; accepted December 21, 1996.

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