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RNase Activities Are Reduced Concomitantly with Conservation of Total Cellular RNA and Ribosomes in O_2-Deprived Seedling Roots of Maize[1]

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The effect of O_2 deprivation on the activities of RNases and levels of total cellular RNA and ribosomes in seedling roots of maize (*Zea mays* L.) was investigated. Sodium dodecyl sulfate-polyacrylamide gels containing RNA were used to distinguish RNase isoenzymes by apparent molecular mass. Since O_2 deprivation causes a decrease in cytosolic pH from approximately pH 7.4 to 6.4 and an elevation in cytosolic Ca^{2+}, RNase levels were examined in the physiological range of cytosolic pH and in the presence of Ca^{2+}, Mg^{2+}, Zn^{2+}, ethylenediaminetetracetate, or ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid. The activity of a number of RNases present in aerobic roots was reduced in response to O_2 deprivation. Several RNases with a pH optimum of 6.4 were rapidly down-regulated by O_2 deprivation. Spectrophotometric assay of extracts revealed that RNase activity was higher at pH 6.4 than at 7.2, and ethylenediaminetetracetate-insensitive RNase activity decreased in response to O_2 deprivation. The decrease in RNase activity was correlated with no loss of total cellular RNA or ribosomes, despite a 4-fold decrease in run-on transcription of rRNA in isolated nuclei. Regulation of RNase activity may facilitate the conservation of nontranslating ribosomes and poorly translated mRNAs during O_2 deprivation.

RNase activities are modulated during plant development and in response to certain abiotic and biotic cues and are expected to play a role in the regulation of RNA levels and turnover (for reviews, see Wilson, 1975; Farkas, 1982; Green, 1994). The electrophoretic and biochemical nature of a number of individual plant RNases and nucleases have been described, and knowledge of the function of specific RNases is slowly accumulating. The best-characterized plant RNases are the extracellular S-RNases and S-like RNases. The S-RNases are glycoproteins of the floral stigma that play a role in the gametophytic self-incompatibility mechanism in several solanaceous species. Two RNases with biochemical characteristics of S-RNases are active in developing tomato (*Lycopersicon esculentum* L.) fruit (McKeon et al., 1991). Phylogenetic analysis confirmed that the S-like RNases of Arabidopsis (RNS1, RNS2, and RNS3) are evolutionarily related to S-RNases, but that these enzymes do not play a role in self-incompatibility (Taylor et al., 1993; Bariola et al., 1994). The expression of RNS1 and RNS2 is induced by phosphate starvation and that of RNS2 and RNS3 is induced by senescence. Phosphate starvation also induces the activity of an extracellular S-like RNase, several vacuolar RNases, and a putative ER-associated RNase in tomato (Jost et al., 1991; Loffler et al., 1992, 1993).

In wheat (*Triticum aestivum* L.) leaves the activity of a single-strand-preferring nuclease increases at the onset of dark-induced senescence and decreases when leaves are reilluminated (Blank and McKeon, 1989). The activities of three additional wheat leaf RNases are modulated by senescence and dark-induced senescence (Blank and McKeon, 1991a, 1991b). The induction of RNase activity in response to phosphate starvation and senescence may allow for remobilization of phosphate to other tissues. There is also evidence that increased activity of certain RNases is involved in programmed cell death processes, such as xylem formation and endosperm utilization, during germination (Farkas, 1982; Green, 1994). In contrast, a reduction in activity of a 27-kD RNase of wheat leaves occurs in response to heat shock, concomitantly with a change in electrophoretic mobility that is possibly due to dephosphorylation (Chang and Gallie, 1997). These authors suggest that reduced RNase activity in response to heat shock may be involved in stabilization of non-heat-shock protein mRNAs.

Progress has been made in the identification of nucleotide sequences that target mRNAs for rapid degradation (for reviews, see Green, 1993, 1994; Abler and Green, 1996), and the description of the processes of decay of specific mRNAs in vivo (Thompson et al., 1992; Higgs and Colbert, 1994) and in vitro in plants (Byrne et al., 1993; Tanzer and Meagher, 1994). Specific mRNAs are destabilized by plant growth regulators and external stimuli such as heat shock, fungal elicitors, Suc starvation, and light (for review, see Abler and Green, 1996). However, the enzymes involved in degradation of specific mRNAs are not known. Selective degradation of mRNAs may be accomplished through endo- and exonucleases that are solely responsible for mRNA degradation or by general RNases. In addition, the subcellular location of RNase activities is poorly understood. About 70 to 80% of plant RNases but only 3% of RNA are located in the vacuole (Boller and Kende, 1979;

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Abbreviations: ADH1, alcohol dehydrogenase-1; ANT, adenine nucleotide translocator; ssDNA, single-stranded DNA.

Abel and Glund, 1986; Abel et al., 1990). Ribonucleoprotein particles may be transported to the vacuole for degradation, and/or changes in the tonoplast membrane integrity may result in release of RNases into the cytosol.

RNases and RNase inhibitors are presumed to be involved in the modulation of levels of rRNA and ribosomes during development and in response to environmental signals. Early work by Trewavas (1970) provided direct evidence that cytoplasmic rRNA levels in *Lemna minor* fronds are reduced in response to nutrient deficiency, as a result of decreased rRNA synthesis and increased rRNA degradation. However, numerous biochemical analyses of RNases have failed to reveal a relationship between total cellular RNase activity and RNA content (for review, see Farkas, 1982). At this time, the identities of RNases specifically involved in degradation of rRNA and ribosomes are unknown.

We were interested in the possible involvement of RNases in the posttranscriptional regulation of gene expression in seedling roots of maize (*Zea mays* L.) deprived of O_2 by flooding. O_2 deprivation, by flooding or exposure to an Ar atmosphere, results in a rapid and dramatic reduction in protein synthesis and a progressive induction of synthesis of a group of proteins known as the anaerobic polypeptides (Sachs et al., 1980). This response is regulated at both transcriptional and posttranscriptional levels (Rowland and Strommer, 1986; Fennoy and Bailey-Serres, 1995). Increased synthesis of the anaerobic polypeptide ADH1 is accompanied by induction of transcription, increased transcript stability, and efficient loading of ribosomes onto the ADH1 transcript (Fennoy and Bailey-Serres, 1995). Sequences in the 5′ and 3′ regions of ADH1 mRNA are necessary to confer high levels of translation of a chimeric mRNA in maize cell protoplasts (Bailey-Serres and Dawe, 1996). By contrast, the decrease in synthesis of a normal cellular protein, ANT, is posttranscriptionally regulated (Fennoy and Bailey-Serres, 1995).

In O_2-deprived roots ANT gene transcription is constitutive and mRNA accumulation is slightly reduced; poor synthesis of ANT results from impairment of ANT mRNA translation. Further studies have confirmed that synthesis of a number of other normal cellular proteins is similarly regulated (S. Fennoy, T. Nong, and J. Bailey-Serres, unpublished data). The reduction in protein synthesis in O_2-deprived roots is evidenced by the loss of large polyribosomes and an increase in monoribosomes and ribosomal subunits. Treatment of polyribosomes with a high concentration of salt revealed that the increase in monoribosomes is due to an increase in 80S ribosomes that are not associated with mRNA (Fennoy and Bailey-Serres, 1995). These nontranslating ribosomes appear to be rapidly recruited for the initiation of translation when seedlings are returned to aerobic conditions (Bailey-Serres and Freeling, 1990).

We considered that RNases may be involved in the regulation of RNA levels and turnover in O_2-deprived roots. As a first step, we have examined whether changes in specific RNases or total RNase activity are correlated with the conservation of RNA and ribosomes in response to O_2 deprivation.

MATERIALS AND METHODS

Plant Material and O_2-Deprivation Treatment

Maize (*Zea mays* L.; inbred line B73, Pioneer Hi-Bred International, Johnston, IA) was grown for 5 to 7 d and deprived of O_2 by submergence exactly as described by Fennoy and Bailey-Serres (1995).

Preparation of Protein Extracts

One gram of frozen root tissue was homogenized in liquid nitrogen in a mortar and pestle, thawed in 1.5 mL of protein extraction buffer (1 M Tris-HCl, pH 7.5, and 0.1 mM PMSF), and further homogenized at 4°C. Insoluble material was removed from the extract by centrifugation for 20 min at 10,000*g* at 4°C (JA13.1 rotor, JS-21 centrifuge, Beckman). The soluble protein extract was aliquoted and stored at −80°C until use. Protein concentration was determined by the Bradford method using the Protein Determination Reagent (United States Biochemical-Amersham) and BSA as the standard.

SDS-Activity Gel Electrophoresis

Protein extracts (20–100 μg) were diluted with SDS-sample buffer to a final concentration of 1% (w/v) SDS, 5% (v/v) glycerol, 0.125% (w/v) bromphenol blue, and 25 mM Tris-HCl, pH 6.8. Proteins were fractionated in resolving gels that contained 12 or 14% (w/v) acrylamide, 0.3 or 0.38 (w/v) *N′,N′*-methylene-bis-acrylamide, 0.37 M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.01% (w/v) fibrinogen, 2.5 mg/mL Torula yeast (*Torulopsis utilis*) RNA (Sigma), 0.08% (w/v) ammonium persulfate, and 0.08% (v/v) *N,N,N′,N′*-tetramethylethylenediamine, and 0.075- × 5.5- × 8-cm slab gel (Laemmli, 1970; Yen and Green, 1991). Gels were run at a constant voltage (200 V) in running buffer containing 1.4% (w/v) Gly, 27.5 mM Tris-base, and 0.1% (w/v) SDS. The following steps were performed at room temperature. After electrophoresis, gels were fixed by incubation for 20 min in 125 mL of 20% (v/v) 2-propanol, 0.05 M Tris-HCl, pH 6.4 to 8.0, or 0.025 M Mes-HCl, pH 5.0 to 6.8 (as indicated in the figure legends), with two changes of solution.

Proteins were renatured by removal of SDS with two 10-min washes in preincubation buffer (0.05 M Tris-HCl or Mes-HCl [at the pH indicated in the text or figure legends]). When sensitivity to cations and chelators was tested, these were added to the preincubation buffer at the concentrations indicated. Gels were incubated at room temperature in incubation buffer (0.05 M Tris-HCl or Mes-HCl [at the pH indicated]) for 16 h to allow for enzymatic degradation of embedded RNA, and stained with toluidine blue (0.1% [w/v] toluidine blue O and 0.01 M Mes-HCl, pH 6.7). Gels were destained in 0.01 M Tris-HCl (pH 6.8) until a good contrast between bands and background was obtained. Nuclease activity was assayed on gels in the same manner as RNase activity, except that gels contained 0.4 mg/mL of ssDNA from calf thymus (Sigma) in the absence of RNA or contained a combination of 2.5 mg/mL Torula

yeast RNA and 0.4 mg/mL ssDNA in the gel matrix. In the nuclease assay the reincubation and incubation buffers were 0.01 M Tris-HCl, pH 6.8.

Assay of RNase Specific Activity

Total RNase activity was assayed by a modification of the methods of Blank and McKeon (1991a) and Brown and Ho (1986). Forty-three micrograms of total protein was added to a reaction cocktail (0.05 M imidazole, pH 7.0, and 0.5% [w/v] BSA with or without 10 mM EDTA). The reaction was started by addition of Torula yeast RNA to a final concentration of 2.5 mg/mL and transferred to 31°C. Aliquots of 333 μL were removed every 10 min for 30 min and immediately added to a stop solution (25% [v/v] perchloric acid and 0.75% [w/v] uranyl acetate [radioactively depleted, Mallinckroft Analytical Reagents, St. Louis, MO]). Stopped reactions were held on ice for 5 min and then centrifuged at 14,000*g* at 4°C for 5 min. The increased A_{260} of acid-soluble oligonucleotides in the supernatant was determined following a 40-fold dilution with water. One unit of RNase activity is defined as that amount that yields a 1 optical density unit change in A_{260} min^{-1} mL^{-1}. The specific activity is the units of enzyme per milligram of protein.

RNA Isolation and Quantitation

Total RNA was isolated from a 0.1-g aliquot from 5 g of pulverized, frozen root tissue using the guanidinium thiocyanate (Chomczynski and Sacchi, 1987) or CsCl purification procedure (Glisin et al., 1974). RNA yield (in micrograms) was determined spectrophotometrically.

Polyribosome Analysis

Roots (0.2 g) were homogenized with a mortar and pestle in 0.8 mL of buffer that contained 0.2 M Tris-HCl, pH 8.5, 0.05 M KCl, 0.03 M $MgCl_2$, 100 mM β-mercaptoethanol, 2 mM EGTA, 50 μg/mL cyclohexamide, 50 μg/mL chloramphenicol, 5 mM DTT, 1 mM PMSF, 1% (v/v) Triton X-100, 1% (v/v) Brij-35, 1% (v/v) Tween-40, and 1% (v/v) Nonidet P-40. The homogenate was clarified by centrifugation in a microcentrifuge (16,000*g*) at 4°C for 2 min. Suc-density gradient centrifugation was as previously described (Fennoy and Bailey-Serres, 1995). Briefly, 300 μL of the homogenate supernatant was layered over a 5-mL 20 to 60% (w/v) Suc-density gradient (0.2 M KCl) and centrifuged (115,000*g*, 1.45 h; L8-M centrifuge, SW-55 rotor, Beckman). Gradients were analyzed at A_{254} with a UV detector (model UA5, ISCO, Lincoln, NE) and fractionator (model 185, ISCO). The quantities of 40S and 60S ribosomal subunits, 80S monoribosomes, and >80S polyribosomes were determined using weighed area averages of three scanned profiles with a scan of a blank Suc gradient as the baseline (Bailey-Serres and Freeling, 1990).

Run-on Transcription Assays in Isolated Nuclei

Slot blots were prepared of plasmid (pGEM11 [Promega]) DNA that contained a tomato 18S rDNA insert (DB292, D. Bird, personal communication) or that lacked the insert (plasmid DNA control). The amount of 13.2 μg of DB292 (equivalent to 5 μg of cDNA insert and 8.2 μg of plasmid DNA) or 8.2 μg of pGEM11 was dissolved in 0.1× SSC and 0.3 M NaOH, boiled for 1 min, and diluted with 2 M NaCl to a final concentration of 1.8 M. Samples were applied to a nylon membrane (MSI-Magna-NT, Westburo, VA) as previously described (Fennoy and Bailey-Serres, 1995). Run-on transcription in isolated nuclei was performed essentially as described by Fennoy and Bailey-Serres (1995). Labeled transcripts (2.5×10^6 cpm) were hybridized to slot blots in 5 mL of 7% (v/v) SDS and 50 mM sodium phosphate buffer, pH 7.0 (Church and Gilbert, 1984), at 65°C for 72 h. Filters were washed twice for 15 min at 65°C in 2× SSC and 0.1% (w/v) SDS and then 0.2× SSC and 0.1% (w/v) SDS. Radioactive signals were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The signal of the plasmid DNA control was subtracted from that of the rDNA plasmid. For calculation of relative level of rDNA transcription, the level in aerobic roots was set at 1.

RESULTS

Activities of Specific RNases Decrease in Response to O_2 Deprivation

The activity of RNases in roots of maize seedlings that were deprived of O_2 for 0, 6, 12, or 24 h was examined using an RNA-embedded SDS-polyacrylamide gel assay. Whole seedlings were submerged in Ar-saturated buffer for the set time, harvested, and used to prepare protein extracts that were fractionated on a polyacrylamide gel containing Torula yeast RNA. Gels were stained with toluidine blue and proteins with RNase activity were detected as unstained bands. Twelve bands of RNase activity were observed in control (aerobic) roots that were not deprived of O_2, whereas eight bands were detected in roots deprived of O_2 for 6, 12, or 24 h (Fig. 1; Table I). RNases with an apparent molecular mass of 16.2, 11, and 6 kD were present in aerobic roots but not detected in O_2-deprived roots. In addition, activities of 38- and 28-kD proteins were detected at reduced levels in O_2-deprived roots. No bands of RNase activity were unique to O_2-deprived roots. These data demonstrate that low-O_2 stress results in the reduction in the activity of a number of root RNases.

RNase Activities Vary over the Range of Cytosolic pH of Aerobic and O_2-Deprived Roots

When intact seedlings of maize are rapidly deprived of O_2, the cytosolic pH of the root tips decreases from 7.4 to 6.4 within 1 h (Roberts et al., 1984a, 1984b). Because of the decrease in cytosolic pH in response to O_2 deprivation, we examined the activity of root RNases at pH 6.4, 6.8, and 7.2 using the gel assay (Fig. 1). The activities of several RNases varied over this physiological range in pH. RNases of 36.3, 33, 30.9, and 28 kD were more active at a higher pH, whereas the activity of the 22.4-kD RNase was similar at pH 6.4, 6.8, and 7.2. An 18.6-kD RNase that is abundant in

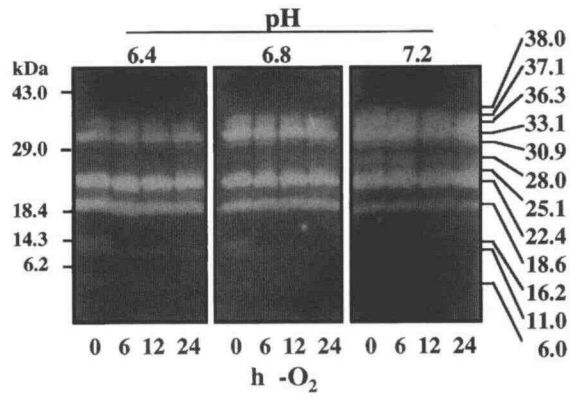

Figure 1. SDS-PAGE of protein extracts from seedling roots of maize deprived of O_2 for 0, 6, 12, or 24 h and gel assay of RNase activity. Protein (50 μg) was fractionated by electrophoresis through a 12% polyacrylamide gel containing 2.5 mg/mL Torula yeast RNA, washed to remove the SDS, incubated in 50 mM Tris-HCl at pH 6.4, 6.8, or 7.2, and stained with toluidine blue to visualize regions of nuclease activity, as described in "Materials and Methods." Positions of molecular mass markers are indicated on the left (in kD), and RNase activity bands are identified by apparent molecular mass on the right.

aerobic and O_2-deprived roots was more active at a lower pH. RNases of 16.2, 11.0, and 6.0 kD had the highest activity at pH 6.4 and were undetectable at pH 7.2; these RNases were undetectable in 6- and 12-h O_2-deprived roots.

The pH optima for plant RNases ranges from pH 5.0 to 9.0 (Wilson, 1975) and is often a basis of classification. To more accurately classify the maize RNases the activity of enzymes from aerobic (0-h O_2-deprived) roots were examined in gel strips incubated in solutions buffered at pH 5.0, 6.4, 6.8, 7.2, 7.4, or 8.0. The pH optima of the maize root RNases ranged from pH 6.4 to 8.0 (Table I). Only the 22.4- and 18.6-kD RNases had detectable activity at pH 5.0; these RNases were considerably less active at pH 5.0 than at pH 6.8.

DNase Activities Are Not Affected by O_2 Deprivation

To determine which activities detected in the SDS-PAGE assay were specific for RNA, we monitored the activities in gels containing ssDNA and/or RNA as substrates (Fig. 2). Nuclease activities were assayed for extracts prepared from 0- and 24-h O_2-deprived seedling roots at pH 6.8. In gels that contained ssDNA one major protein of 33.1 kD and four minor proteins of 38, 37.1, 30.9, and 29 kD were detected. No difference in activity pattern was observed when ssDNA or double-stranded DNA was used as the substrate, except that the activity detected with ssDNA was considerably higher (data not shown). To further examine whether any of the DNases were substrate specific, nuclease activities were compared in gels containing ssDNA and RNA or only RNA. It is apparent that the proteins of 33.1 to 38 kD resolved by this method can be classified as general nucleic acid-hydrolyzing enzymes or nucleases. Levels of resolved nucleases were similar in aerobic and O_2-deprived roots.

RNase Activities Are Sensitive to Divalent Cations and Chelators

To further classify the root RNases the effect of divalent cations and their chelators on activity was investigated using the gel assay. Extracts from aerobic roots were fractionated by SDS-PAGE, and proteins immobilized in the gel strips were renatured in the presence of $MgCl_2$, $CaCl_2$, or $ZnCl_2$ and then further incubated in the presence or absence of EDTA or EGTA. The activity of each RNase in the presence or absence of these compounds was distinct and reproducible; many RNases varied in sensitivity to the individual divalent cations and chelators.

A number of RNases showed altered activity in the presence of EDTA (Fig. 3; Table I). The activity of a 28-kD RNase was stimulated by the addition of 1 mM EDTA and further enhanced by 10 mM EDTA (Fig. 3A, lanes 1–3; Table

Table I. *Characteristics of maize root RNases*

−, Sensitive, reduced activity; o, insensitive, no change in activity; ND, not determined; +, sensitive, increased activity.

Molecular Mass	Response to O_2 Deprivation	pH Optimum	Enzyme Type	Response			
				EDTA	Mg^{2+}	Ca^{2+}	Zn^{2+}
kD							
38.0	Decrease	7.0	Nuclease	−	−	−	o
37.1	Decrease	7.0	Nuclease	−	−	−	o
36.3	Decrease	7.0	RNase	−	−	−	o
33.1	None	7.0	Nuclease	−	−	−	o
33.1[a]	Decrease	ND	RNase	o	ND	ND	ND
30.9	None	7.0	RNase	−	−	−	o
28.0	Decrease	8.0–7.2	RNase	+	o	o	−
25.1	None	8.0–7.2	RNase	o	o	o	−
22.4	None	6.4–8.0	RNase	−	o	o	−
18.6	None	6.4	RNase	+	o	o	−
16.2	Decrease	6.4	RNase	o	o	o	o
11.0	Decrease	6.4	RNase	o	−	−	−
10.0	ND	ND	RNase	+	o	−	o
6.0	Decrease	6.4	RNase	o	o	−	o

[a] RNase (33.1 kDa) was detected only when assayed in the presence of EDTA.

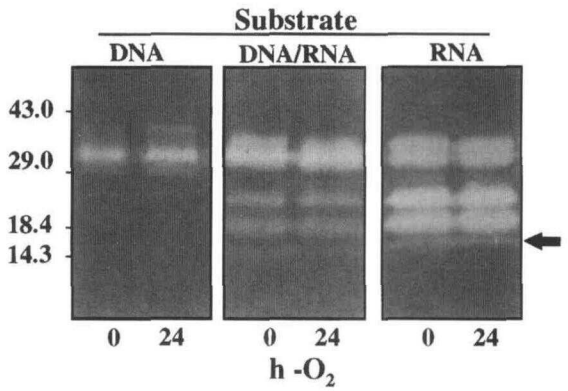

Figure 2. SDS-PAGE of protein extracts from seedling roots of maize deprived of O_2 for 0 or 24 h and gel assay of RNase and DNase activity. Proteins (50 μg) were fractionated through 14% polyacrylamide gels that contained 0.4 mg/mL calf thymus ssDNA and/or 2.5 mg/mL Torula yeast RNA. Nuclease activity was detected as described for Figure 1 using 50 mM Tris-HCl, pH 6.8. Positions of molecular mass markers are indicated to the left. The position of a contaminating RNase activity present in the gel system is indicated by an arrow.

I). The presence of 1 mM EDTA dramatically reduced the activity of RNases ranging from 29 to 38 kD.

The activity of a number of RNases was affected by renaturation in the presence of divalent cations followed by incubation in the absence or presence of EDTA (Fig. 3A, lanes 4–9; Table I). For example, a modified profile of RNase activities was observed when $MgCl_2$ or $CaCl_2$ was included in the preincubation buffer. The presence of 2 mM $MgCl_2$ or $CaCl_2$ resulted in a slight reduction in the activity of the 33.1- to 37.1-kD enzymes. The presence of $MgCl_2$ reduced the activity of the 11-kD RNase, whereas the presence of $CaCl_2$ reduced the 16.2-, 11-, 10-, and 6-kD RNases. Physiological levels of Ca^{2+} (0.1–1 μM) had no detectable effect on the profile of RNase activities (data not shown). When RNases were renatured in the presence of 2.0 mM $MgCl_2$ or $CaCl_2$ and further incubated in buffer containing 10.0 mM EDTA, the activity of the 36.3- to 38-kD RNases decreased, whereas that of the 28-kD RNase increased, as when EDTA was present alone (Fig. 3A, compare lanes 3, 5, and 7). In addition, the presence of EDTA in the incubation buffer was sufficient to recover the activity of the Mg^{2+}- and Ca^{2+}-sensitive 6- to 16.2-kD RNases. The effect of the renaturation of the RNases in the presence of Zn^{2+} was also examined. We observed that 20 μM Zn^{2+} inhibited the activity of the 28-, 25.1-, 18.6-, and 11-kD RNases (Fig. 3A, lane 8). When RNases were renatured in the presence of 2 μM Zn^{2+}, a similar profile of activity was observed (data not shown). The effect of Zn^{2+} on RNase activity was reversible upon addition of EDTA (Fig. 3A, lane 9).

Activity of EDTA-Insensitive RNases Decreases in Response to O_2 Deprivation

Notable differences between RNases of aerobic and 24-h O_2-deprived roots were detected when activities were examined by the gel assay in the presence or absence of 1 mM EDTA. The activity of the 33.1-, 30.9-, and 28-kD RNases were markedly reduced in the O_2-deprived roots (Fig. 3B). Similar profiles of RNase activity were observed when EGTA was substituted for EDTA in the incubation buffer (data not shown). The group of RNases with clear sensitivity to the presence of EDTA included the nucleases. This variation in sensitivity to EDTA provided a means to further demonstrate modulation of RNase activities of aerobic and O_2-deprived roots (Table I).

Spectrophotometric assay of RNase activity in crude extracts was performed to examine the sensitivity of total cellular RNases to pH, EDTA, and O_2 deprivation. RNase activity in extracts from aerobic roots increased as the reaction pH was reduced from 7.2 to 6.4 (Fig. 4A). Determination of total RNase activity over a time course of O_2 deprivation revealed no significant effect on RNase activity (Fig. 4B, open bars). When 10 mM EDTA was included in

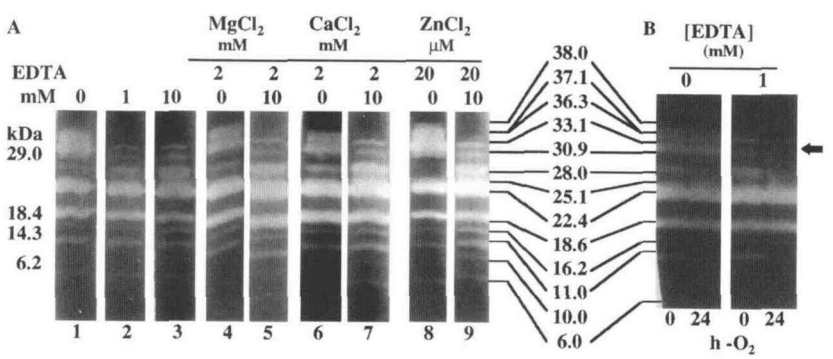

Figure 3. Effect of divalent cations and chelators on RNase activities. A, Fifty micrograms of protein extract from aerobic roots (0 h O_2-deprived) was electrophoresed in 12% polyacrylamide gels as described for Figure 1. Gel strips were preincubated in 50 mM Tris-HCl, pH 6.8, that contained 0 to 2 mM $MgCl_2$, 0 to 2 mM $CaCl_2$, or 0 to 20 μM $ZnCl_2$. Gel strips were incubated for 18 h in 5 mM Tris-HCl, pH 6.8, that contained 0, 1, or 10 mM EDTA. B, Fifty micrograms of protein extract from aerobic roots and roots deprived of O_2 for 24 h was electrophoresed as in A. Gel strips were incubated for 18 h in 50 mM Tris-HCl, pH 6.8, that contained 0 or 1 mM EDTA. Gel strips were stained and destained as described in "Materials and Methods." Positions of molecular mass markers are indicated to the left. The apparent molecular masses of RNase activities are indicated.

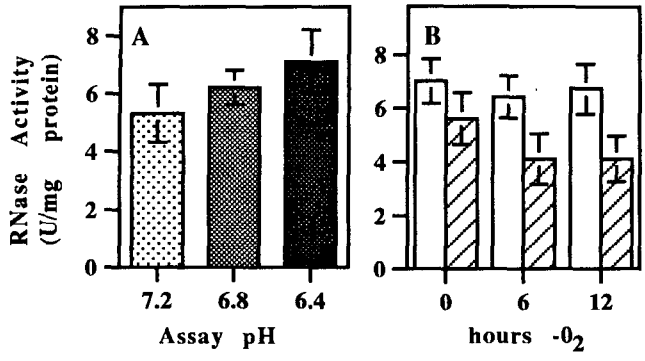

Figure 4. Effect of pH and O_2 deprivation on total RNase activity from root extracts. RNase activity per milligram of protein was measured for root extracts as described in "Materials and Methods." A, Assays performed on extracts from aerobic roots at pH 7.2, 6.8, or 6.4. B, Assays performed at pH 7.0 in the presence (striped bars) or absence (open bars) of 10 mM EDTA on extracts from 0-, 6-, or 12-h O_2-deprived roots. Measurements are the means ± SD of three independent experiments. U, Unit.

the reaction mixture to inhibit nuclease activity, a reproducible decrease in RNase activity was detected in response to O_2 deprivation (Fig. 4B, striped bars). This result is consistent with the observation that the activities of 33.1-, 30.9-, and 28-kD RNases, assayed in the presence of 1 mM EDTA, were highest in control roots (Fig. 3B). Results obtained with both gel-assay and spectrophotometric techniques support the conclusion that RNase activity is sensitive to physiological alterations in pH; however, only the gel assay clearly demonstrated that activity of EDTA-insensitive isozymes is reduced in response to low O_2.

O_2 Deprivation Reduces rRNA Transcription but Not Levels of Ribosomes

O_2 deprivation causes a rapid reduction in the quantity of polyribosomes and an increase in monoribosomes; polyribosome levels rapidly recover upon return to aerobic conditions (Bailey-Serres and Freeling, 1990). Thus, ribosome degradation may not occur during the stress response. We previously showed that the run-on transcription of rDNA in isolated nuclei is significantly reduced (Fennoy and Bailey-Serres, 1995), and the synthesis of the majority of ribosomal proteins is inhibited by O_2 deprivation (Bailey-Serres and Freeling, 1990). Thus, it is unlikely that ribosomes are synthesized during the stress response. We performed a comparative analysis of the effect of O_2 deprivation on synthesis of rRNA and levels of total cellular RNA and ribosomes to further investigate the effect of this stress on rRNA metabolism. Run-on transcription was performed in nuclei from aerobic and 6- and 12-h O_2-deprived roots, equal quantities were hybridized to 18S rDNA immobilized on nylon filters, and rRNA transcription levels were quantitated using a PhosphorImager. Three independent experiments confirmed that rDNA transcription is reduced 4-fold within 12 h of O_2 deprivation (Fig. 5A). To measure levels of ribosomes, crude cell homogenates were fractionated through Suc-density gradients, and the proportion of 40S and 60S ribosomal subunits, 80S monoribosomes, and ≥80S polyribosomes was quantitated from the UV absorbance (A_{254}) profiles of the gradients (Fig. 5B). These data were also used to determine levels of ribosomal components on a root fresh weight basis (Fig. 5C). This technique accurately measures the relative levels of the ribosomal components in the cell because ribosomes were not concentrated through a Suc

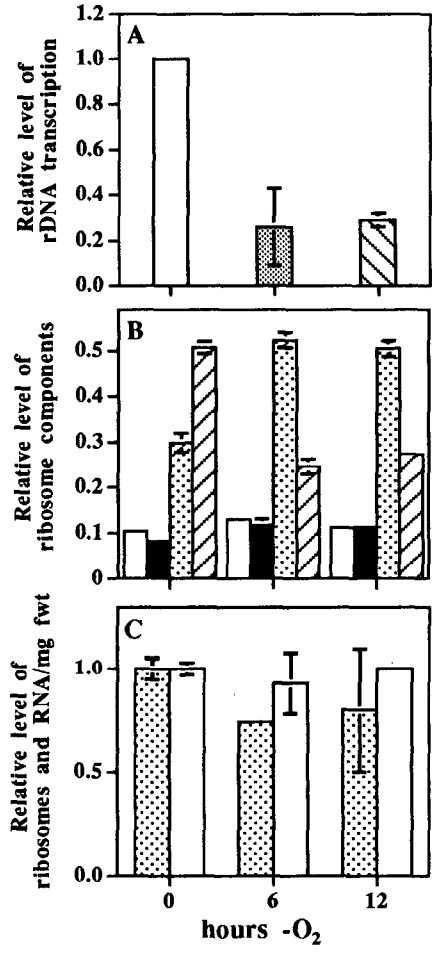

Figure 5. Quantification of run on transcription of rRNA, ribosome subunits, monoribosomes and polyribosomes, and total cellular RNA in extracts of roots deprived of O_2 for 0, 6, or 12h. A, rRNA transcription was quantitated by hybridization of 5 μg of an 18S rRNA cDNA to ^{32}P-labeled transcripts (2.5×10^6 cpm) synthesized in vitro in nuclei isolated from 0-, 6-, or 12-h O_2-deprived roots. B, Levels of 40S (white bars), 60S ribosomal (black bars) subunits, 80S monoribosomes (stippled bars), and >80S polyribosomes (striped bars) relative to the total ribosomal component were quantitated as described in "Materials and Methods." C, Total levels of ribosomal components (combined values of 40S and 60S ribosomal subunits, 80S monoribosomes, and >80S ribosomes) per milligram fresh weight (stippled bars) and levels of total cellular RNA per milligram fresh weight (open bars) of roots. For ease of comparison, values in A and C were adjusted relative to levels in aerobic (0 h, O_2-deprived) roots (given a value of 1). Measurements in A are the means ± SD of two independent experiments and B represents the data from one experiment. In C, levels of ribosomes and rRNA are the means ± SD of four and three independent experiments, respectively. fwt, Fresh weight.

cushion. O_2 deprivation resulted in a reduction of polyribosomes and an increase in monoribosomes (Fig. 5B) but had no significant effect on the quantity of ribosomes or total cellular RNA on a fresh weight basis (Fig. 5C). Since total cellular RNA is 95 to 99% rRNA, measurement of both ribosome and total cellular RNA reflects levels of rRNA.

DISCUSSION

By assessment of molecular mass, pH optimum, sensitivity to EDTA and divalent cations, and activity in O_2-deprived roots, we identified up to 14 RNases and nucleases in roots of maize seedlings with distinct biochemical characteristics (Table I). Wilson (1975) classified RNases of maize tissues into three types on the basis of biochemical characteristics. In roots 50% of the total RNase activity was an EDTA-sensitive, Zn^{2+}-requiring enzyme of an approximately 33,000 molecular weight that degraded both RNA and ssDNA (nuclease I), 30% was a 20,000 to 25,000 molecular weight RNase that was active at acidic pH (approximately pH 5.0; RNase I), and 10 to 20% was a 17,000 to 20,000 molecular weight RNase that was active at pH 6.0 (RNase II). A number of the maize root enzymes we observed can be classified as nuclease I types (38.0, 37.1, and 33.1 kD) but few can be unambiguously classified as RNase I or RNase II types. A similar observation was made for RNases of leaves and stems of Arabidopsis (Yen and Green, 1991). Hence, analysis of RNases by gel assay yields more detailed information than can be obtained by gel-filtration and spectrophotometric techniques. Examination of RNases in a variety of maize tissues (root, leaf, coleoptile, and silk) revealed organ-specific differences in RNase activities in maize (S.L. Fennoy and J. Bailey-Serres, unpublished data). Organ-specific differences in RNase activities were also detected in Arabidopsis (Yen and Green, 1991) and wheat (Chang and Gallie, 1997).

The activity levels of eight RNases of maize roots decreased in response to flooding (Table I). Two patterns of response were observed: the activity of the 38- and 37.1-kD nucleases and the 36.3- and 28-kD RNases slowly decreased during the time course, whereas the activity of the 16.2-, 11.0-, and 6.0-kD RNases decreased to undetectable levels within 6 h of O_2 deprivation. There were no unifying biochemical characteristics of these enzymes. The larger-molecular-mass RNases had neutral to alkaline pH optima and were sensitive to EDTA. By contrast, the 16.2-, 11-, and 6-kD RNases that were the most rapidly and dramatically affected by O_2 deprivation had pH optima of approximately 6.4 and were insensitive to EDTA. The biochemical characteristics of the low-molecular-mass RNases (pH optima, sensitivity to EDTA, and responses to divalent cations) were distinct from those of the higher-molecular-mass enzymes that decreased in activity in response to O_2 deprivation. Thus, it is unlikely that the small RNases are degradation products of the larger enzymes. O_2 deprivation clearly modulates the activity of several RNases and nucleases of roots.

The modulation of activity of the 16.2, 11-, and 6-kD RNases by O_2 deprivation and pH is striking. The activity of these RNases decreases in response to the stress, and yet activity was undetectable at pH 7.2 and detectable at pH 6.4 to 6.8. Roberts et al. (1984a, 1984b) showed that 15 min of anoxia is sufficient to cause a decrease in cytosolic pH from approximately 7.2 to 6.4 in root tips of maize seedlings. After 5 h of anoxia there is a significant increase in vacuolar pH from approximately 5.6 to 5.9 (Roberts et al., 1984a). Farkas (1982) suggested that small differences in pH optima of RNases are not of biological significance. Contrary to this view, we propose that slight differences in the pH optima of RNases located in the cytoplasm or vacuole may be of biological importance in response to O_2 deprivation. For example, the activities of the 16.2-, 11-, and 6-kD RNases would dramatically increase as the cytosolic pH decreases from 7.2 to 6.4, as in response to low O_2. The marked decrease in activity of these RNases in response to 6 h of O_2 deprivation may reflect the ability of the cell to eliminate or inactivate RNases that impair the conservation of RNA when the cytosolic pH decreases to less than 6.8. Confirmation of this model requires information about the subcellular storage and movement of RNases, about which extremely little is known.

The effect of cations and chelators on RNase activity was examined for two reasons. First, the inhibitory effect of cations and/or chelators on RNase activity provides information about ion requirements of specific enzymes. For example, EDTA, $MgCl_2$, or $CaCl_2$, but not $ZnCl_2$, reduced the activity of the 30.9- to 37.1-kD RNases and nucleases, indicating that Zn^{2+} is required for activity. Whereas the inhibitory effect of EDTA and $CaCl_2$, but not $MgCl_2$ or $ZnCl_2$, on the 6-kD RNase indicates that either Mg^{2+} or Zn^{2+} is required for activity. In contrast, the activity of the 28-, 18.6-, and 10-kD RNases was stimulated in the presence of EDTA and inhibited by certain ions, indicating no ion requirement. Second, we considered that changes in Ca^{2+} might influence RNase activity, since a measurable increase in cytosolic Ca^{2+} levels occurs within 15 min of O_2 deprivation of maize suspension-cultured-cells (Subbaiah et al., 1994). We found that physiological concentrations of Ca^{2+} (0.1–1 μM) had no effect on the activity of maize RNases (data not shown), although 2 mM Ca^{2+} inhibited the activity of the 16.2-, 11-, and 6-kD RNases, all with a pH 6.4 optimum. Thus, it seems unlikely that the increase in cytosolic Ca^{2+} would affect the activity of these RNases in vivo, although the local concentration of Ca^{2+} within the cell may be high enough to impact RNase activity. The sensitivity of these RNases to Ca^{2+} most likely indicates preference for another divalent cation for activity.

The loss of activity of specific RNases in response to low-O_2 stress could be due to a number of factors. Based on our knowledge of the O_2-deprivation response it is likely that a decrease in RNase synthesis may result from reduced gene transcription and/or mRNA translation. The decrease in abundance of certain RNases after 6 h of O_2 deprivation may indicate that these proteins have a short half-life. Finally, reduced RNase activity could result from the inhibition of a specific posttranscriptional modification, as postulated for a 27-kD RNase of wheat leaves following heat shock (Chang and Gallie, 1997).

The reduction in activity of RNases may be involved in reducing the overall turnover of RNA and preservation of ribosomes during O_2 deprivation. Consistent with this hypothesis is that mRNAs that encode normal cellular proteins are synthesized and maintained and yet poorly translated in O_2-deprived roots (Fennoy and Bailey-Serres, 1995; S. Fennoy, T. Nong, and J. Bailey-Serres, unpublished data). In addition, the physical half-lives of synthetic mRNAs, which are efficiently or poorly translated in hypoxic protoplasts, are similar under aerobic and hypoxic conditions (Bailey-Serres and Dawe, 1996). O_2 deprivation also causes a reduction in levels of translating ribosomes but allows for the maintenance of nontranslating ribosomes (Bailey-Serres and Freeling, 1990). Here we show that despite a 75% reduction in rRNA synthesis the levels of total cellular RNA and ribosomes are maintained in roots deprived of O_2. The newly synthesized rRNA may represent a small proportion of the total pool of rRNA, and consequently the reduction of transcription may not impact total rRNA levels. Pulse-chase labeling of de novo-synthesized RNA is required to determine if the half-life of rRNA is affected by O_2 deprivation.

The data presented here indicate that decreased activity of several RNases with specific biochemical characteristics is concomitant with conservation of total cellular RNA and ribosomes in O_2-deprived roots. Plant response to transient abiotic stresses such as O_2 deprivation and heat shock appears to include regulation of the RNA degradation machinery by changes in RNase activity. In the case of O_2 deprivation, modulation of cytosolic pH and possibly ion concentration may be involved in the regulation of RNase activity. The reduction in activity of specific RNases coincides with the conservation of poorly translated mRNAs and inactive monoribosomes. We hypothesize that the dampening of RNase activity enhances the ability of cells to recover normal patterns and levels of protein synthesis upon recovery from O_2 deprivation.

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