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Influence of ozone on ribonuclease activity in wheat (*Triticum aestivum*) leaves

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Ribonucleases (RNases) degrade RNA and exert a major influence on gene expression during development and in response to biotic and abiotic stresses. RNase activity typically increases in response to pathogen attack, wounding and phosphate (P_i) deficiency. Activity also increases during senescence and other programmed cell death processes. The air pollutant ozone (O_3) often induces injury and accelerated senescence in many plants, but the biochemical mechanisms involved in these responses remain unclear. The objective of this study was to determine whether RNase activity and isozyme expression was stimulated in wheat (*Triticum aestivum* L.) flag leaves following treatment with O_3. Plants were treated in open-top chambers with charcoal-filtered air (27 nmol O_3 mol^{-1}) (control) or non-filtered air plus O_3 (90 nmol O_3 mol^{-1}) (O_3) from seedling to reproductive stage. After exposure for 56 days, RNase activity was 2.1 times higher in flag leaf tissues from an O_3-sensitive cultivar in the O_3 treatment compared with the control, which generally coincided with foliar injury and lower soluble protein concentration, but not soluble leaf [P_i]. Soluble [P_i] in leaf tissue extracts from the O_3 and control treatments was not significantly different. RNase activity gels indicated the presence of three major RNases and two nucleases, and their expression was enhanced by the O_3 treatment. Isozymes stimulated in the O_3 treatment were also stimulated in naturally senescent flag leaf tissues from plants in the control. However, soluble [P_i] in extracts from naturally senescent flag leaves was 50% lower than that found in green flag leaves in the control treatment. Thus, senescence-like pathological responses induced by O_3 were accompanied by increased RNase and nuclease activities that also were observed in naturally senescent leaves. However, [P_i] in the leaf tissue samples suggested that O_3-induced injury and accelerated senescence was atypical of normal senescence processes in that P_i export was not observed in O_3-treated plants.

Introduction

Ribonucleases (EC 3.1) are a superfamily of exo- and endonuclease enzymes that degrade RNA through hydrolysis of the phosphodiester bonds of polynucleotides, primarily rRNA (Green 1994). Most RNase activity in plant cells is located in the vacuole, although activity is also found in the extracellular space, endoplasmic reticulum and plastids (Green 1994). RNases are modulated during developmental and nutritional changes as well as in response to pathogens and environmental stresses. RNases are involved with general RNA turnover, gametophytic self-incompatibility, fruit development, phosphate (P_i) remobilization, pathogen defence, wounding responses, senescence, and other programmed cell death processes (Green 1994, Dangl et al. 2000). It is presumed that during cell degradation processes, RNases and other nucleases work together with phosphatases, primarily to release P_i from RNA for remobilization (Abel et al. 2000, Pérez-Amador et al. 2000). In addition, a recent study with yeast (*Saccharomyces cerevisiae*) suggested that during conditions of environmental stress, such as osmotic stress and heat shock, extracellular RNases have a role in regulating cell membrane permeability by degrading membrane-bound RNAs that affect ion permeability, thus allowing

Abbreviations – O_3, ozone; P_i, inorganic phosphate; RNase, ribonuclease.

membrane permeability or stability to change to maintain cell homeostasis (MacIntosh et al. 2001). The air pollutant O_3 is known to cause premature senescence in plants, induce pathogen defence and wound responses, and alter membrane permeability (Pell et al. 1997, Sandermann 1998), all of which might involve changes in RNase expression.

Three RNases and several nucleases have been characterized in senescing wheat leaves (Blank and McKeon 1989, 1991). The RNases consisted of an acidic RNase approximately 20 kDa in size, and two neutral RNases approximately 26 and 27 kDa in size that differed in their sensitivity to KCl and $MgCl_2$ (Blank and McKeon 1991, Chang and Gallie 1997). The neutral RNases and nucleases, 32–38 kDa in size, were present in green wheat leaf tissues but were stimulated during senescence and in darkened leaf tissues (Blank and McKeon 1989, 1991).

Ribonucleases were also stimulated during senescence in other plants. For example, expression of three RNase genes was induced during leaf senescence in *Arabidopsis* (Green 1994). In addition, a bifunctional nuclease I gene and enzyme were induced specifically during leaf and stem senescence in *Arabidopsis* (Pérez-Amador et al. 2000). Two senescence-induced RNase genes were identified in tomato (*Lycopersicon esculentum* L.) leaves (Lers et al. 1998). A nuclease induced during tomato leaf senescence has also been identified (Lers et al. 2001).

RNase gene expression and activity often increase in response to pathogen attack and wounding. Several studies found higher RNase activities in diseased plants (Green 1994, Lusso and Kuc 1995, Galiana et al. 1997). An RNase gene in *Zinnia elegans* and tomato leaves was induced by wounding (Ye and Droste 1996, Lers et al. 1998). Wounding resulted in the induction of an RNS1 gene and enzyme as well as several nucleases in *Arabidopsis* leaves (LeBrasseur et al. 2002). The identification of multiple RNases suggests that different RNase genes are involved in different processes, although some RNases might have overlapping functions (Green 1994, Ye and Droste 1996).

Many symptoms of O_3 injury resemble senescence in plants, such as suppressed photosynthesis, chlorosis, chloroplast degeneration, protein loss, ethylene emission, necrotic lesions, and leaf abscission (Pell et al. 1997). One of the hallmarks of O_3 injury is a decrease in Rubisco content along with decreased mRNA transcript levels for the small subunit of Rubisco (*rbc*S) and chlorophyll *a*/*b* protein (*cab*) (Pell et al. 1997). This suggests that a decrease in levels of mRNA in response to O_3 is a reflection of regulated decreases in transcription or an increase in specific RNase activity (Pell et al. 1997). A number of genes show increased expression during senescence and are referred to as senescence associated genes or SAGs. Miller et al. (1999) found that eight SAGs were expressed in *Arabidopsis* plants following treatment with 150 nmol $O_3\,mol^{-1}$ 6 h daily for 14 days. Ozone treatment thus induces the expression of many physiological, biochemical and molecular markers of injury and senescence. The objective of this study was to determine whether chronic O_3 exposure affected RNase and nuclease activity in wheat flag leaf tissue.

Materials and methods

Plant growth conditions

The experiments were performed at a site 5 km south of Raleigh, NC, USA (36°N, 79°W). Soft red winter wheat (*Triticum aestivum* L., cv. Coker 9835 and Coker 9904, along with six other cultivars) was planted on 18 November 1997 and grown in pots containing 14 l of a 2:1:1 mixture of sandy loam soil:sand:Metro Mix 220 (Scotts-Sierra Horticultural Products Co., Marysville, OH, USA) (pH 6.2) (Heagle et al. 2000). Plants were irrigated with drip tubes as needed to prevent visible signs of water stress and fertilized biweekly with an aqueous solution of soluble fertilizer (Peters Professional, Scotts-Sierra Horticultural Products Co.) and micronutrients (STEM, Scotts-Sierra Horticultural Products Co.). Insects and mites were controlled with applications of acephate (Valent USA Corp., Walnut Creek, CA, USA), bifenthrin (Whitmire Micro-General Research Laboratories, Inc., St. Louis, MO, USA) and abamectin (Syngenta Crop Protection, Inc., Greensboro, NC, USA).

Plants were treated in 2.4 m tall × 3 m diameter open-top field chambers starting at 102 days after germination following an overwintering period outside the chambers. Ozone was produced by electrostatic discharge in dry O_2 (model GTC-1A, Ozonia North America, Elmwood Park, NJ, USA) and monitored at canopy height using UV photometric O_3 analysers (model 49, Thermo Environmental Instruments Co., Franklin, MA, USA). Plants were treated with charcoal-filtered air (control) or non-filtered air with O_3 added proportionally to ambient O_3 to provide approximately 1.86 times ambient [O_3] 12 h daily (O_3) (Heagle et al. 2000). Average seasonal 12 h (0800–2000 h EST) daily [O_3] in the control and O_3 treatments were 27 ± 1 and 90 ± 1 nmol mol^{-1}, respectively.

Tissue sampling

Flag leaf tissue was obtained from two Coker 9835 and two Coker 9904 plants in each chamber after treatment for 56 days. The wheat cultivars were selected for testing because preliminary observations of foliar symptoms indicated that Coker 9835 was relatively resistant to O_3, whereas Coker 9904 was relatively sensitive (Heagle et al. 2000). Naturally senescent flag leaf tissue was also obtained from plants in the control treatments (senescent). All tissue samples were frozen in liquid N_2 and stored at −80°C. Visible foliar injury (chlorosis and necrosis) was estimated in 5% increments.

Leaf tissues (0.5 g FW) were ground in liquid N_2 and extracted with 4 ml of 50 m*M* HEPES buffer (pH 7.5) containing 5 m*M* Mg acetate, 120 m*M* K acetate, and 50 mg of polyvinylpolypyrrolidone (Chang and Gallie

1997). Homogenates were centrifuged (15 000 *g*) for 10 min at 4°C. The supernatants were filtered through a 0.45-μm nylon filter, and the filtrates were used for enzyme activity assays. Prior to electrophoresis, filtered supernatants were concentrated using a microcentrifugal filtration device (Amicon Microcon Model YM-10, Millipore, Inc., Bedford, MA, USA). Protein was determined by the Bradford (1976) method using bovine serum albumin as the standard.

RNase activity assay

RNase activity was determined by measuring the loss of RNA following incubation with soluble plant extract (Galiana et al. 1997). Aliquots (25 μl) of plant extract were mixed with 250 μl of 50 m*M* Tris-HCl buffer (pH 7) containing 0.4 mg ml^{-1} Type VI RNA from Torula yeast (Sigma Chemical Co., St. Louis, MO, USA) and 0.1 mg ml^{-1} bovine serum albumin. Samples were incubated at 30°C for 1 h. Afterward, 25 μl of 3 *M* sodium acetate (pH 5.5) and 688 μl of cold absolute ethanol were added to each sample tube followed by incubation at −20°C for 1 h to precipitate the remaining RNA (Sambrook et al. 1989). Samples were then centrifuged (14 000 *g*) for 10 min at 4°C. The pellets were washed with 75% (v/v) aqueous ethanol, centrifuged (14 000 *g*) and partially dried. The pellets were dissolved in 1 ml of 0.5% (w/v) SDS. RNase activity was determined by the decrease in absorbance at 260 nm compared with a RNase-free preparation that lacked plant extract. Recovery of soluble RNA by precipitation in the RNase-free preparation averaged 83%. The A_{260} values for preparations containing plant extract were 59–88% lower than those of the RNase-free preparations. RNA concentration was determined using $E_{1\%\,260\,nm} = 250$ (Robyt and White 1987). Measurements of sample 260:280 nm absorbance ratios averaged 1.6, indicating acceptable purity of the RNA precipitations (Robyt and White 1987).

RNase and DNase activity gels

RNase and DNase isozymes were identified using a substrate-based gel assay following separation by SDS-polyacrylamide gel electrophoresis (Yen and Green 1991). An equal volume of 2× sample-loading buffer [2% (w/v) SDS, 10% (v/v) glycerol and 0.025% (w/v) bromophenol blue in 50 m*M* Tris-HCl buffer, pH 6.8)] was mixed with each sample before electrophoresis. Reducing agents were omitted because they impede RNase renaturation (Sambrook et al. 1989, Yen and Green 1991). Proteins (20 μg) were separated on a 12% SDS-polyacrylamide gel containing 2.5 mg ml^{-1} Type VI RNA from Torula yeast. Afterward, gels were washed twice for 10 min with 25% (v/v) isopropanol in one of three buffer solutions: 10 m*M* Tris-HCl (pH 7), 10 m*M* Na acetate (pH 5.5) or 10 m*M* imidazole (pH 8.0) buffer. Gels were then washed twice in 10 m*M* concentrations of the appropriate buffer, each containing 2 μ*M* $ZnCl_2$. Gels were incubated in 100 m*M* Tris-HCl, 100 m*M* Na acetate, or 100 m*M* imidazole buffer containing 200 m*M* KCl and 10 m*M* $MgCl_2$ at 50°C for 1 h. Gels were then rinsed and stained with 0.2% (w/v) Toluidine Blue for 10 min. Isozymes on destained gels appeared as colourless bands on a dark background. Molecular mass of the isozymes was estimated based on the mobility of prestained low molecular mass markers (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

DNase activity was detected using a 12% SDS-polyacrylamide gel containing 0.5 mg ml^{-1} Type I predominantly double-stranded DNA from calf thymus (Sigma Chemical Co.). Samples containing 15 μg of protein were mixed with an equal volume of 2× loading buffer before electrophoresis. The gel was washed, incubated and stained in the Tris-HCl buffer system as described for the RNase activity gels.

Phosphate assay

Duplicate freeze-dried tissue samples (50 mg) were each mixed twice with 1 ml of 10% (w/v) trichloroacetic acid (TCA), incubated at 4°C for 30 min, centrifuged (12 000 *g*) for 10 min at 4°C, and the supernatants recovered. The supernatants were pooled by sample. Soluble extracts were assayed in duplicate for P_i as previously described (Taussky and Shorr 1953), with modifications. To determine total soluble [P_i], 0.6 ml of plant extract was mixed with 0.6 ml of 2 *M* HCl, incubated at 100°C for 10 min, and 0.5 ml samples were assayed for [P_i]. To assay for [P_i], 0.5 ml of extract was mixed with 0.5 ml of 0.5% TCA and 0.5 ml of acid molybdate reagent (16.2 m*M* $(NH_4)_6Mo_7O_{24}.4H_2O$, 2.5 *M* H_2SO_4, 180 m*M* $FeSO_4.7H_2O$), followed by incubation at 30°C for 15 min. Absorbance of the mixtures was read at 740 nm, and [P_i] was calculated from a standard curve. Background interference due to the plant extract, as measured in assays without acid molybdate reagent, was deducted from the absorbance measurements obtained in the P_i assays.

Statistics

The treatments consisted of two O_3 levels that were assigned to chambers in a completely randomized design. There were three replicate chambers per treatment. A complete set of wheat cultivars was randomly arranged within the northern and southern half of each chamber. Flag leaf tissue samples were obtained from one Coker 9835 and one Coker 9904 plant in each half of the chamber, and assay results were averaged by cultivar for each chamber replicate. Treatment effects and means for visible injury, soluble protein, RNase activity and [P_i] were estimated using a split-plot model in which chambers constituted the whole-plot and cultivar was the within-plot factor (SAS Proc Mixed) (Littell et al. 1996). Data were checked for homogeneity of variance and normality of distribution. Means were considered significantly different if $P \leq 0.05$, and marginally significantly different if $P \leq 0.1$.

Results

Foliar injury and soluble protein concentrations

Flag leaves were visibly injured in the O_3 treatments ($P \leq 0.001$), and foliar injury was more severe in Coker 9904 than in the Coker 9835 cultivar (significant O_3 treatment–cultivar interaction ($P \leq 0.001$) (Table 1). Soluble protein concentrations were up to 36% lower in flag leaf tissue from the O_3 treatment (Table 1). The difference was statistically significant for Coker 9904 ($P \leq 0.05$) and marginally significant for Coker 9835 ($P \leq 0.1$).

RNase activities

RNase activity was higher in extracts of flag leaf tissue from the O_3 treatment compared with controls ($P \leq 0.001$) (Fig. 1). However, the magnitude of the response differed between cultivars (significant O_3 treatment–cultivar interaction, $P \leq 0.001$). In Coker 9904, the O_3-sensitive cultivar, RNase activity was 2.1 times greater in leaf tissue extracts from the O_3 treatment compared with that in extracts from the control treatment ($P \leq 0.001$). In contrast, RNase activity was only 27% higher in Coker 9835 leaf tissue extracts from the O_3 treatment compared with the control treatment ($P \leq 0.1$).

Table 1. Visible foliar injury and soluble protein concentration in wheat flag leaf tissues following chronic treatment with O_3. Wheat (cv. Coker 9835 and Coker 9904) was treated with charcoal-filtered air (control) or non-filtered air plus 1.86 times ambient [O_3] (O_3). Visible injury ratings were made after treatment for 56 days. Values are means ± SE of three replicates. Statistics: †, $P \leq 0.1$; *, $P \leq 0.05$; ***, $P = 0.001$.

Cultivar	Treatment	Visible injury (%)	Protein (mg g FW^{-1})
9835	Control	8 ± 3	3.53 ± 0.30
	O_3	12 ± 3	2.66 ± 0.30†
9904	Control	19 ± 3	3.32 ± 0.30
	O_3	87 ± 3***	2.12 ± 0.30*

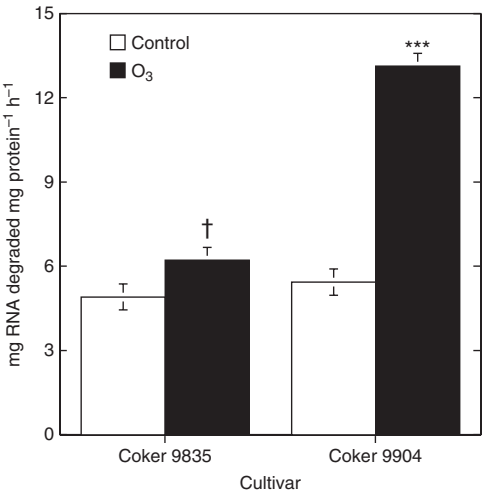

Fig. 1. RNase activities in leaf tissue extracts from wheat cultivars (Coker 9835 [O_3 tolerant] and Coker 9904 [O_3 sensitive]) treated for 56 days with charcoal-filtered air (control) or non-filtered air plus O_3 (O_3). Values are means ± SE of three replicates. Statistics: †, $P \leq 0.1$; ***, $P = 0.001$.

RNase and DNase activity gels

Activity gels showed that leaf extracts contained three major RNases and two nucleases with distinctive electrophoretic and catalytic properties (Figs 2, 3 and 4). RNase isozymes 20, 23 and 25 kDa in size were evident in activity gels (Fig. 2). Activity of the 20 kDa isozyme was

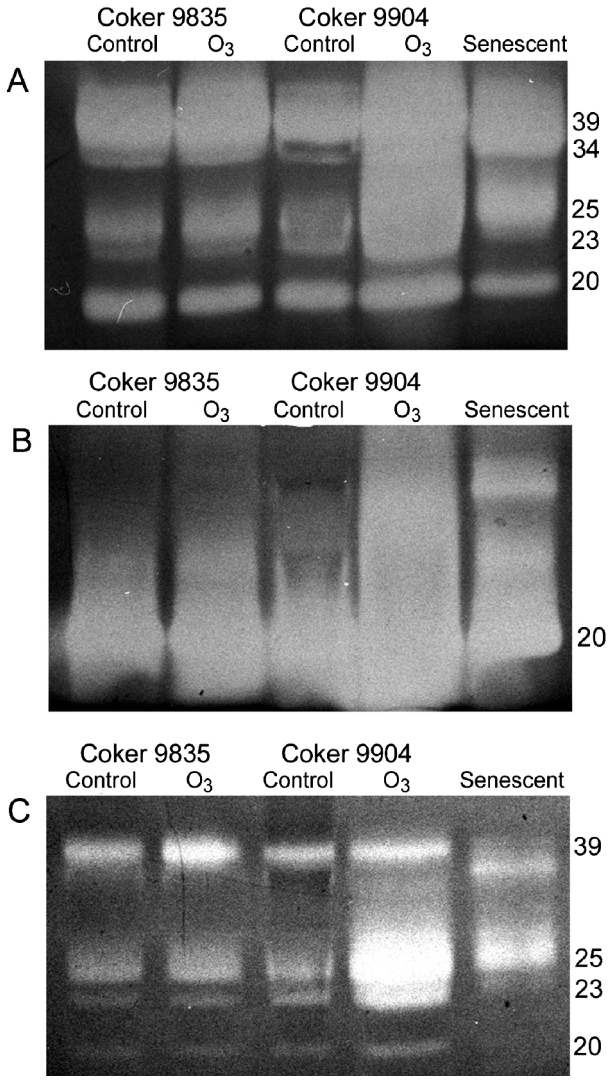

Fig. 2. RNase activity gel of wheat leaf extracts (20 µg protein) of Coker 9835 (O_3 tolerant) and Coker 9904 (O_3 sensitive) plants treated for 56 days with charcoal-filtered air (control) or non-filtered air plus O_3 (O_3). RNase isozyme expression in a tissue extract from senescent Coker 9904 leaves in the control treatment (senescent) is also shown. (A) Tris-HCl buffer (pH 7.0); (B) Na acetate buffer (pH 5.5); (C) imidazole buffer (pH 8.0) plus 200 m*M* KCl and 10 m*M* $MgCl_2$. Putative molecular masses of RNase and nuclease isozymes are indicated numerically in the figure.

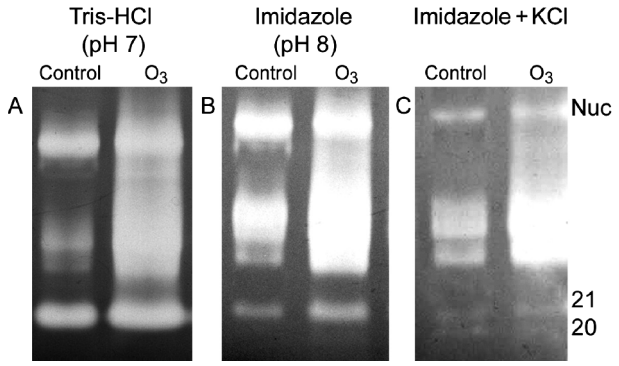

Fig. 3. Triplicate RNase activity gels of wheat flag (Coker 9904, O_3 sensitive) leaf extracts (15 μg protein) following incubation without $ZnCl_2$ in the RNase activity gel incubation procedure. (A) Tris-HCl (pH 7.0) buffer; (B) imidazole buffer (pH 8.0); (C) imidazole buffer (pH 8.0) plus 200 m*M* KCl. Putative molecular masses of two RNase isozymes are indicated numerically in the figure.

stimulated by incubation in Na acetate buffer while all three isozymes were partially inhibited by incubation in the salt-containing imidazole buffer (Fig. 2).

RNase isozyme activities on the basis of total protein loading were all higher in leaf extracts from O_3-treated Coker 9904 wheat plants compared with either the control treatment or the Coker 9835 samples (Figs 2 and 3). Patterns of RNase isozyme expression were consistent with results from the RNase activity assays. In addition, the activity gels showed that the RNase isozymes stimulated in the O_3 treatment also were stimulated in extracts from naturally senescent leaves from the control treatment (Fig. 2).

To determine the effects of buffers and KCl on RNase isozyme activities, triplicate gels loaded with identical protein levels of leaf extracts were washed after electrophoresis in either 10 m*M* Tris-HCl (pH 7.0) or 10 m*M* imidazole buffer (pH 8.0) solutions as described previously except that $ZnCl_2$ was omitted. Gels were then incubated at 50°C for 1 h in 100 m*M* Tris-HCl (pH 7.0) buffer, 100 m*M* imidazole buffer (pH 8.0), or 100 m*M* imidazole buffer plus 200 m*M* KCl. Destained gels indicated that incubation in imidazole buffer (pH 8.0), especially in the presence of KCl, inhibited expression of the low MW RNases (Fig. 3). Activities of the neutral RNases were essentially unchanged.

Nucleases that degrade both RNA and DNA were evident in RNase and DNase activity gels (Figs 2, 3 and 4). Activity gels showed a major nuclease isozyme 39 kDa in size and a minor one 34 kDa in size (Figs 2 and 4). Activity of the nucleases was partially inhibited by incubation in either Na acetate or imidazole buffer containing KCl and $MgCl_2$, although the 34 kDa isozyme appeared completely inhibited by incubation in the salt-containing imidazole buffer (Fig. 2). Nuclease activity was stimulated in leaf extracts from O_3-treated Coker 9904 wheat plants (Figs 2 and 4).

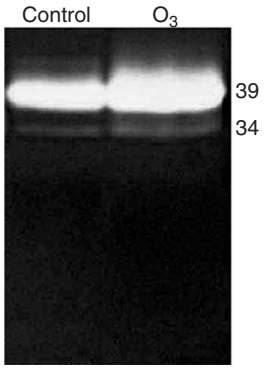

Fig. 4. DNase activity gel of extracts from Coker 9904 wheat leaves (15 μg protein) from the control and O_3 treatments. Putative molecular masses of DNase isozymes are indicated numerically in the figure.

Phosphate assays

Soluble [P_i] in extracts of flag leaves from the O_3 treatments was not significantly different from that in the controls for either cultivar ($P \geq 0.1$) (Fig. 5). Soluble [P_i] in extracts from naturally senescent Coker 9904 flag leaf tissues from the control treatment was 50% lower than that found in green flag leaf tissues obtained from the control treatment ($P = 0.1$) (Fig. 5).

Discussion

Increased RNase activity is a prominent feature of leaf senescence, pathogen defence and wounding responses (Green 1994, Dangl et al. 2000). It is thought that RNases and nucleases participate in the disassembly of cells during which leaves transition from a carbon source to a mineral source. The catabolysed nucleic acids become a mobilized source of P_i and N that is transported elsewhere in the plant (Lers et al. 1998). Ozone

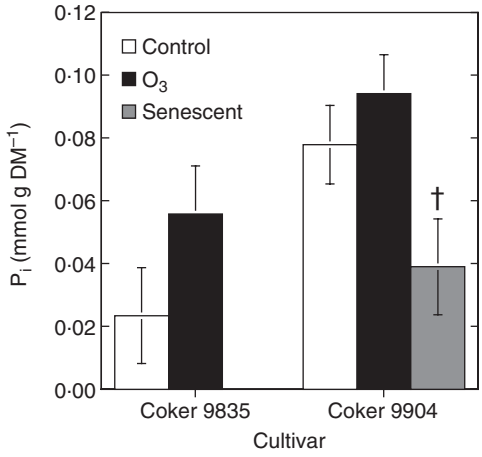

Fig. 5. Soluble [P_i] in wheat leaf extracts from plants in the control and O_3 treatments. Soluble [P_i] in extracts from senescent leaves in the control is also shown (senescent). Values are means ± SE of three replicates. Statistics: †, $P \leq 0.1$.

exposure causes injury in most plants and leads to accelerated senescence. Hence, enzymes involved in regulated cellular disassembly, such as RNases and nucleases, were suspected to be involved in plant responses to O_3. As shown in this study, RNase and nuclease activities were increased in flag leaf tissues from plants chronically exposed to O_3. Similar RNase and nuclease isozyme activities were elevated in naturally senescent flag wheat leaves.

Previous studies on the effects of O_3 on RNase activity have reported mixed results. Craker and Starbuck (1972) found that an O_3 exposure of 250 nmol mol^{-1} for 2 h doubled RNase activity, decreased quantities of RNA by 35% and produced severe visible injury in bean (*Phaseolus vulgaris* L., cv. Pinto) leaf tissues 24 h after exposure. In contrast, RNase activity was not stimulated in soybean (cv. Dare) leaf tissues following exposure of plants to 500 nmol O_3 mol^{-1} for 2 h, although visible injury was apparent after 24 h (Tingey et al. 1975). In the present study, RNase activity and isozyme expression were highest in extracts of severely injured flag leaf tissues from the O_3-sensitive cultivar (Coker 9904), but were much lower in extracts of moderately injured flag leaf tissue from the O_3-tolerant cultivar (Coker 9835). Increased RNase expression might thus constitute a relatively late event in O_3-induced injury and accelerated senescence due to chronic O_3 exposure.

Based on the properties of the low molecular mass RNase isozymes observed in the activity gels, the 20 kDa wheat RNase is probably a RNase I-type enzyme (endonucleases with molecular masses from 20 to 25 kDa and pH optima between 5.0 and 6.0) (Bariola and Green 1997). In a previous study (Blank and McKeon 1991), the 20 kDa RNase stimulated in senescing wheat flag leaves also was an RNase I-type enzyme. In addition, KCl or $MgCl_2$ in imidazole buffer (pH 8.0) promoted activity of one RNase 26 kDa in size while KCl, $MgCl_2$ and Tris buffer (pH 7.0) suppressed activity of a 27-kDa isozyme. The opposite isozyme expression pattern was observed when gels were incubated without KCl or $MgCl_2$ in imidazole buffer (pH 8.0) (Blank and McKeon 1991). In the present study, isozymes 23 and 25 kDa in size were evident following incubation in Tris-HCl buffer (pH 7.0), imidazole buffer (pH 8.0), or imidazole buffer containing either KCl or KCl and $MgCl_2$ (Figs 2 and 3). These responses were observed when $ZnCl_2$ was included (Fig. 2) or omitted (Fig. 3) in the incubation procedure.

These results differ in some ways with previously published reports. Differences in RNase electrophoretic and catalytic characteristics between the current study and those of Blank and McKeon (1991) and Chang and Gallie (1997) might be related to the lack of complete enzyme denaturation prior to electrophoresis in the current study, which could affect migration of the isozymes and sensitivity to incubation buffer pH and salt composition, as well as to differences between wheat cultivars. The inclusion of $ZnCl_2$ in the incubation procedure, which was reported to provide the ZN_2^+ required for certain RNase and nuclease activities (Yen and Green 1991), was apparently not essential for wheat RNase and nuclease expression in activity gels.

Pérez-Amador et al. (2000) reported relatively high mRNA levels and activity of a nuclease I enzyme during *Arabidopsis* leaf and stem senescence, and suggested that the strong response of the nuclease gene to senescence indicated that it might be a useful tool with which to study the mechanisms of senescence induction. Nucleases also were identified in the present study, and since their activities were stimulated by O_3, they might also be useful for studying O_3-induced senescence.

The effect of O_3 on RNase activity was apparently not related to the P_i status of the leaf tissue. Soluble [P_i] in O_3-treated leaf tissues was not significantly different from that in the controls (Fig. 5). However, senescent flag leaf tissues from the control treatment had lower [P_i] compared with green flag tissue obtained from the control. In previous studies, RNase activity and gene expression were stimulated in response to P_i deficiency (Green 1994, Howard et al. 1998). In this study, however, the responses of RNases and nucleases to O_3 suggest that O_3-induced accelerated senescence was atypical of normal senescence processes in that P_i export was not observed in O_3-treated plants. Recent studies on the inhibitory effects of O_3 on phloem loading of carbohydrates (Grantz and Farrar 1999, Grantz and Yang 2000) suggest the possibility that phloem transport of P_i was suppressed in O_3-treated leaf tissues and that export of P_i was restricted.

Ozone invokes many symptoms associated with injury and accelerated senescence, including induction of several molecular markers of senescence (Miller et al. 1999). Increased nuclease and RNase isozyme activity in response to O_3 provides additional evidence that chronic O_3 exposure stimulates enzymes and presumably genes involved in injury and senescence. However, the RNases and nucleases appear to be composed of multiple isozymes that need to be specifically identified in order to determine whether they are modified versions of the same gene product or of different gene products (Green 1994). The characterization of multiple RNase genes in plants indicates that different RNases genes are involved in different processes, although some RNases have overlapping functions (Green 1994). Additional studies will be needed to identity signals responsible for O_3-induced responses and to clarify the role of oxidative stress in natural and accelerated leaf senescence. An in-depth understanding of how oxidative stress invoked by O_3 affects RNase and nuclease expression might prove useful in understanding how these processes are linked.

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