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Senescence-Associated Gene Expression during Ozone-Induced Leaf Senescence in Arabidopsis[1]

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The expression patterns of senescence-related genes were determined during ozone (O_3) exposure in Arabidopsis. Rosettes were treated with 0.15 μL L^{-1} O_3 for 6 h d^{-1} for 14 d. O_3-treated leaves began to yellow after 10 d of exposure, whereas yellowing was not apparent in control leaves until d 14. Transcript levels for eight of 12 senescence related genes characterized showed induction by O_3. *SAG13* (senescence-associated gene), *SAG21*, *ERD1* (early responsive to dehydration), and *BCB* (blue copper-binding protein) were induced within 2 to 4 d of O_3 treatment; *SAG18*, *SAG20*, and *ACS6* (ACC synthase) were induced within 4 to 6 d; and *CCH* (copper chaperone) was induced within 6 to 8 d. In contrast, levels of photosynthetic gene transcripts, *rbc*S (small subunit of Rubisco) and *cab* (chlorophyll *a/b*-binding protein), declined after 6 d. Other markers of natural senescence, *SAG12*, *SAG19*, *MT1* (metallothionein), and *Atgsr2* (glutamine synthetase), did not show enhanced transcript accumulation. When *SAG12* promoter-*GUS* (β-glucuronidase) and *SAG13* promoter-*GUS* transgenic plants were treated with O_3, GUS activity was induced in SAG13-GUS plants after 2 d but was not detected in SAG12-GUS plants. *SAG13* promoter-driven GUS activity was located throughout O_3-treated leaves, whereas control leaves generally showed activity along the margins. The acceleration of leaf senescence induced by O_3 is a regulated event involving many genes associated with natural senescence.

Leaf senescence is the sequence of degradative processes leading to the remobilization of nutrients and eventual leaf death. The senescence process is highly regulated, involving photosynthetic decline, protein degradation, lipid peroxidation, and chlorophyll degradation (Smart, 1994). Total RNA levels decline during senescence as RNase activity increases (Blank and McKeon, 1991). Chloroplasts are one of the earliest sites of catabolism, while mitochondria remain intact until late in the senescence process in order for respiration to continue (Smart, 1994). Plant hormones are involved in regulating the senescence process, with cytokinins delaying senescence, ethylene modulating the timing of senescence, and the other hormones playing less prominent roles (Smart, 1994). Leaf senescence, like other developmental processes, is actively regulated by differential gene expression. Transcript levels for photosynthetic genes such as *rbc*S (small subunit of Rubisco) and *cab* (chlorophyll *a/b*-binding protein) decline (Bate et al., 1991), while other genes become activated (Buchanan-Wollaston, 1997; Weaver et al., 1997).

Using differential screening and subtractive hybridization techniques, researchers have identified genes with increased expression during senescence. These genes have been identified in Arabidopsis, oilseed rape, tomato, barley, potato, cucumber, rice, wheat, and maize (for reviews, see Buchanan-Wollaston, 1997; Weaver et al., 1997). Such genes are often referred to as SAGs or senescence-upregulated genes. Among the identified senescence-induced genes are genes encoding proteases, RNases, Gln synthetase, metallothioneins, protease regulators, ACC oxidase, lipases, glyoxylate cycle enzymes, catalase, endoxyloglucan transferase, pathogenesis-related proteins, ATP sulfurylase, glutathione *S*-transferase, Cyt P450, and polyubiquitin (Buchanan-Wollaston, 1997; Weaver et al., 1997). Some identified cDNA clones have no obvious senescence-related function and other senescence-induced clones remain unidentified.

While the initiation of leaf senescence depends upon the age of the leaf and the reproductive phase of the plant, external factors such as nutrient deficiency, pathogenic attack, drought, light limitation, and temperature can induce premature senescence (Smart, 1994). Researchers have begun to examine the similarities and differences in gene expression during natural senescence, hormone treatment, and stress by measuring the induction of senescence-related genes (Becker and Apel, 1993; Oh et al., 1996; Chung et al., 1997; Park et al., 1998; Weaver et al., 1998). Studies with ABA, ethylene, cytokinin, methyl jasmonate, wounding, dehydration, and dark treatment have shown that these genes are differentially regulated, suggesting that there are multiple signaling pathways leading to their induction (Gan and Amasino, 1997; Park et al., 1998; Weaver et al., 1998). Expression of some senescence-related genes appears to be quite specific to natural senescence,

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Abbreviations: PAG, photosynthesis-associated gene; SAG, senescence-associated gene.

whereas other transcripts are induced by treatments in addition to natural senescence (Weaver et al., 1998).

Ozone (O_3) is a stress known to induce accelerated foliar senescence in many plant species including potato, radish, alfalfa, wheat, and hybrid poplar (Pell and Pearson, 1983; Reich, 1983; Held et al., 1991; Nie et al., 1993; Pell et al., 1997). O_3 exposure accelerates chlorophyll and protein loss and reduces photosynthetic capacity and efficiency in older leaves (Reich, 1983; Held et al., 1991; Nie et al., 1993). Accelerated loss of Rubisco protein is also closely associated with O_3-induced senescence (Pell and Pearson, 1983; Nie et al., 1993; Pell et al., 1997). O_3 exposure reduced transcript levels for *cab*, *rbc*S, and *rbc*L (large subunit of Rubisco) in potato (Glick et al., 1995) and *cab* and *rbc*S in Arabidopsis (Conklin and Last, 1995) and tobacco (Bahl and Kahl, 1995). Accelerated yellowing of older leaves occurred in Arabidopsis plants following exposure to 0.10 to 0.15 μL L^{-1} O_3 given continuously for 2 d (Kubo et al., 1995). Exposure to 0.15 μL L^{-1} O_3 for 8 d reduced Arabidopsis rosette dry weight by 44% and reduced total chlorophyll, carotenoids, Rubisco activity, and levels of Rubisco large and small subunits (Rao et al., 1995). These results demonstrate that O_3 induces changes associated with natural senescence in many species including Arabidopsis. While a decline in message level for photosynthetic genes has been observed during O_3-induced accelerated leaf senescence, other molecular changes known to occur during natural senescence have not, to our knowledge, been reported.

The main objective of this study was to determine whether O_3 exposure regulates the expression of SAGs. The expression pattern of *SAG12* and *SAG13* was determined by fluorometric quantification of GUS activity in transgenic Arabidopsis carrying either the SAG12 promoter-GUS or the SAG13 promoter-GUS fusion. Expression levels for *SAG12* and *SAG13* were also characterized by northern analysis. The spatial distribution of *SAG13* expression was determined by staining for GUS activity in O_3-treated and control transgenic SAG13-GUS plants. The expression patterns of 10 additional senescence-related genes were characterized by northern analysis in relation to the decline in PAG expression. SAG transcript levels were also analyzed following removal of the O_3 treatment to determine whether transcript levels remained elevated or returned to control levels.

MATERIALS AND METHODS

Plant Growth and O_3 Exposure Experiments

Seeds of Arabidopsis ecotype Lansberg *erecta* transformed with the *SAG12* promoter-*GUS* fusion or *SAG13* promoter-*GUS* fusion, were provided by S. Gan and Richard Amasino (University of Wisconsin, Madison). SAG12-GUS, SAG13-GUS, and wild-type Lansberg *erecta* seeds were planted on a commercial soil mix (Redi-earth Plug and Seedling Mix, Scotts-Sierra, Marysville, OH) supplemented with 20:20:20 fertilizer (Peters Professional, Scotts-Sierra) and imbibed overnight at room temperature. Seeds were placed in 4°C for four nights and then transferred to growth chambers to ensure uniform timing of germination. The plants were grown in growth chambers (Environmental Growth Chambers, Chagrin Falls, OH) at 23°C and 60% RH under a 12-h light/dark cycle at 200 μmol m^{-2} s^{-1}. Seedlings germinated within 2 d and were thinned to a single plant per cell pack.

Plants were treated with O_3 at 15 d post germination, when the fifth leaf, as counted by order of emergence from the meristem (cotyledons were not counted), was 3 to 4 d old. Half of the plants were exposed to 0.15 μL L^{-1} O_3 for 6 h d^{-1} and the other half remained nontreated in another growth chamber. O_3 was generated by passing oxygen through an ozonator (OREC V1-0, Ozone Research and Equipment, Phoenix), and O_3 concentrations in the growth chamber were monitored continuously with a UV photometric O_3 analyzer (model 49, Thermo Environmental Instruments, Franklin, MA). In experiments 1 and 2, plants were exposed to O_3 for 8 and 14 consecutive d, respectively. GUS activity was analyzed every 2 d in the fifth and sixth leaves harvested from four replicate SAG12-GUS and SAG13-GUS transgenic plants per treatment. For staining of GUS activity, the fifth and sixth leaves were collected from three replicate SAG13-GUS transgenic plants per treatment per sampling time. Leaves for GUS staining were harvested on d 3, 6, and 8 in experiment 1, and on d 4, 8, 12, and 14 in experiment 2. For northern analysis, three replicate samples of wild-type plants were collected per treatment every 2 d; each sample consisted of the fifth and sixth leaves pooled from six plants. In addition, one wild-type rosette was collected at each sampling time per treatment in experiment 2.

In a third experiment, wild-type plants were exposed to O_3 for 10 consecutive d. Two replicate samples of the fifth and sixth leaves pooled from six plants were collected per treatment at the end of the 6-h exposure and 18 h later, on d 6, 8, and 10 of the O_3 exposure. The samples were analyzed for SAG transcript levels.

GUS Activity Assays

For fluorometric quantification of GUS activity, samples were ground in microcentrifuge tubes under liquid nitrogen. Leaf tissue was lysed in 150 to 200 μL of extraction buffer (50 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% [v/v] Triton X-100, 0.1% [w/v] *N*-lauroylsarcosine, and 10 mM β-mercaptoethanol) and stored at −80°C for later analysis (Jefferson et al., 1987). Following centrifugation of the crude extract, 50 μL was incubated at 37°C in 500 μL of assay buffer (2 mM 4-methylumbelliferyl β-D-glucuronide in extraction buffer). At 1-h intervals, 100-μL aliquots were removed and the reaction was stopped with 900 μL of 0.2 M Na_2CO_3. Fluorescence of the methyl umbelliferone product was quantified with a fluorometer (CytoFluor II multi-well plate reader, PE Biosystems). Protein concentrations were measured with the protein-dye-binding assay (Bradford, 1976) using Coomassie Plus protein assay reagent (Pierce) with BSA as a standard.

For staining of GUS activity, leaves were vacuum infiltrated with 50 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 0.01% (v/v) Triton X-100, and 1 mM 5-bromo-4-

chloro-3-indolyl β-D-glucuronide (Gold BioTechnology, St. Louis) (Jefferson et al., 1987; Thoma et al., 1996). Leaves were incubated at 37°C until blue staining became evident, 72 h after infiltration. Following staining, leaves were cleared of chlorophyll with 70% (v/v) ethanol.

RNA Extraction and Analysis

Northern analysis was conducted with the probes listed in Table I. Leaf tissue was ground under liquid nitrogen and total RNA was extracted from 100 mg of tissue (RNeasy, Qiagen, Chatsworth, CA). Total RNA was fractionated in a 1% (w/v) agarose-formaldehyde gel, transferred to a membrane (Hybond-N, Amersham), and fixed to the membrane by baking for 2 h at 80°C. The membranes were prehybridized in 0.5 M sodium phosphate buffer and 7% (w/v) SDS at 65°C for 1 h (Church and Gilbert, 1984). Probes were random-primed labeled with [α-^{32}P]dCTP and unincorporated nucleotides were removed with spin columns (Quick Spin, Boehringer Mannheim). The membranes were hybridized overnight at 65°C. Following hybridization, the membranes were washed at 65°C twice in 40 mM sodium phosphate buffer, 5% SDS, and 1 mM EDTA for 20 min, and twice in 40 mM sodium phosphate buffer, 1% SDS, and 1 mM EDTA for 20 min. Membranes were exposed to film (X-Omat, Kodak) at −80°C with two intensifying screens. Membranes were stripped with boiling 0.1% SDS for rehybridizing with other probes. The final hybridization on each membrane was performed with cDNA for pea rRNA as a loading check (Jorgenson et al., 1982).

RESULTS

Arabidopsis plants exhibited downward leaf rolling after 4 d of treatment with 0.15 μL L^{-1} O_3. O_3 treatment reduced rosette leaf growth and accelerated the yellowing of older leaves. The fifth leaf began to show signs of senescence after 10 d of O_3 exposure, whereas control leaves did not begin to show signs of senescence until d 14, the last day of the experiment. In an independent experiment, chlorophyll levels per unit area declined more rapidly in O_3-treated leaves (data not shown). These changes in growth and development occurred without any visible signs of hypersensitive-response-like necrosis.

Effects of O_3 Exposure on *SAG12* and *SAG13* Expression

In experiment 1, the O_3 exposure was 8 d in duration, and in experiment 2 the exposure was for 14 d. As the results in experiment 1 were supported in experiment 2, only the more extensive data of the latter experiment are presented here. *SAG12* promoter-driven GUS activity was not detected in control or O_3-treated plants on any sampling day throughout the 14 d of the experiment (data not shown), while O_3 exposure did accelerate the onset of *SAG13* promoter-driven GUS activity (Fig. 1). O_3-induced, *SAG13* promoter-driven GUS activity was first detected on

Table I. *SAGs used in the study of O_3-induced accelerated leaf senescence*

Selected references include information on clone identification and expression patterns.

Gene	Identity/Similarity	Reference	Time of Induction
			d of O_3 exposure
SAG12	Cys protease	Lohman et al. (1994) Gan and Amasino (1997)	NI[a]
SAG13	Short-chain alcohol dehydrogenase	Lohman et al. (1994) Weaver et al. (1997)	2–4
BCB (SAG14)	Blue copper-binding protein (membrane)	Van Gysel et al. (1993) Lohman et al. (1994) Weaver et al. (1997)	2–4
ERD1 (SAG15)	ClpC-like gene (chloroplast)	Kiyosue et al. (1993) Lohman et al. (1994) Weaver et al. (1998)	2–4
MT1 (SAG17)	Metallothionein	Zhou and Goldsbrough (1994) Lohman et al. (1994) Weaver et al. (1997)	NI
SAG18	Novel gene	Weaver et al. (1998)	4–6
SAG19	Unidentified	L.M. Weaver and R.M. Amasino (personal communication)	NI
SAG20	Novel gene	Weaver et al. (1998)	4–6
SAG21	Late embryogenesis-abundant gene	Weaver et al. (1998)	2–4
CCH	Copper chaperone	Himelblau et al. (1998)	6–8
Atgsr2	Glutamine synthetase (cytosol)	Peterman and Goodman (1991) Bernhard and Matile (1994)	NI
ACS6	ACC synthase	Vahala et al. (1998) Arteca and Arteca (1999)	4–6

[a] NI, Not induced by 14-d O_3 exposure.

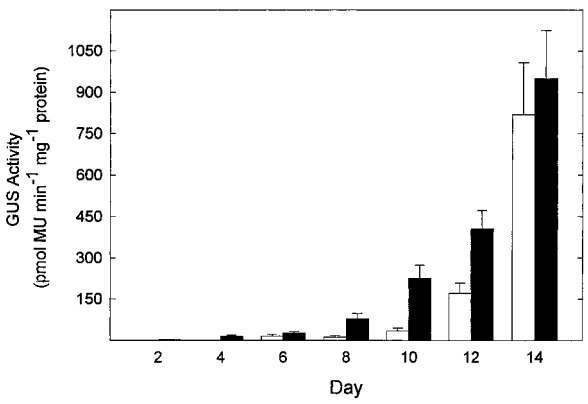

Figure 1. *SAG13* promoter-driven GUS activity was induced by O_3 treatment. Fifteen-day-old Arabidopsis ecotype Landsberg *erecta* plants transformed with the *SAG13* promoter-*GUS* fusion were exposed to 0.15 μL L^{-1} O_3 for 6 h d^{-1} for 14 d. The fifth and sixth leaves were harvested from a single plant and GUS activity was measured by fluorometric quantification of 4-methyl umbelliferone (MU). Black bars, O_3-Treated leaves; white bars, nontreated leaves. Each bar represents the mean of four samples ± SE, except control bars on d 2 and 10, where the mean of three samples was taken. No GUS activity was detected in nontransformed plants (data not shown).

d 2, whereas GUS activity was not detected until d 6 in control leaves. GUS activity gradually increased in O_3-treated and control leaves through the remainder of the experiment. *SAG13* promoter-driven GUS activity in O_3-treated leaves always exceeded the level found in control leaves, except on d 14, when the difference between treatments was no longer detected (Fig. 1). *SAG13* promoter-driven GUS activity appeared after 2 d in O_3-treated leaves, while yellowing did not occur on the fifth leaf until d 10. No *SAG12* or *SAG13* promoter-driven GUS activity was detected in treated or control nontransformed plants.

The localization of *SAG13* expression was determined by staining for GUS activity (Fig. 2). The staining pattern was altered spatially and temporally by O_3 treatment. GUS staining was diffusely distributed in the interior of O_3-treated leaves on d 4, while no staining could be detected in control leaves. By d 8 of the experiment, intense GUS

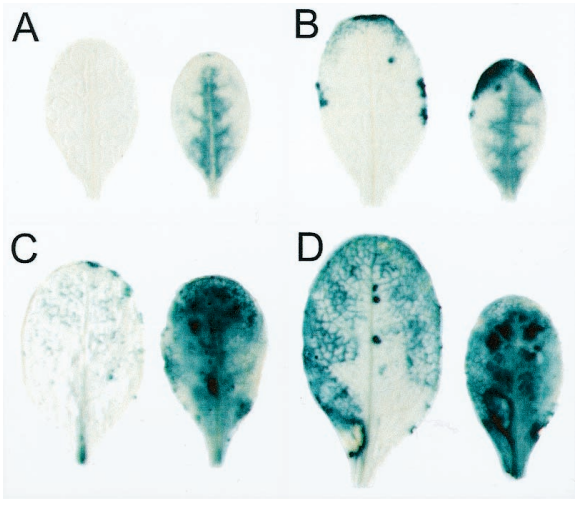

Figure 2. Photographs showing O_3-induced GUS staining in the fifth leaf of transgenic SAG13-GUS plants. Fifteen-day-old SAG13-GUS plants were exposed to 0.15 μL L^{-1} O_3 for 6 h d^{-1} for 14 d. Leaves were vacuum infiltrated with 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide, incubated at 37°C for 72 h, and cleared of chlorophyll with 70% ethanol. Nontreated leaves are shown on the left and O_3-treated leaves on the right from samples harvested 4, 8, 12, and 14 d after exposure in A through D, respectively. A similar pattern of expression was found in the sixth leaf (data not shown). The leaves shown are representative of three leaves per treatment per day.

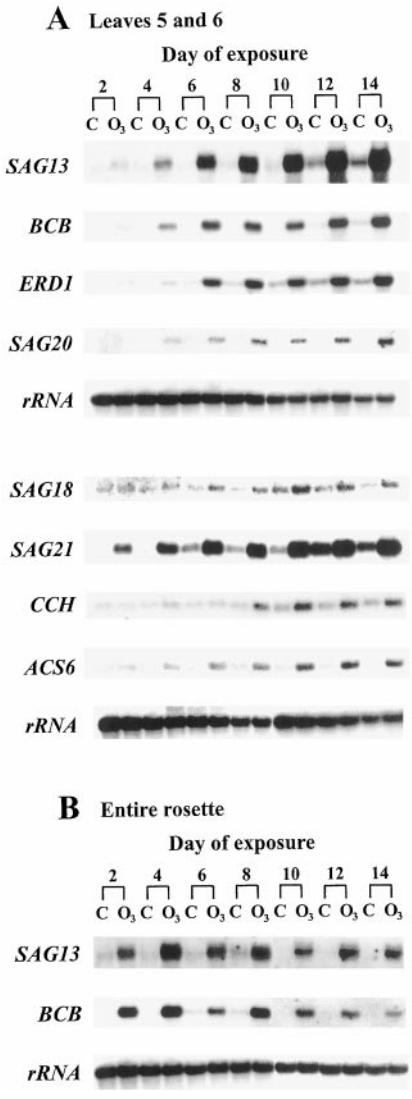

Figure 3. Induction of senescence-related transcripts in O_3-treated Arabidopsis plants. Fifteen-day-old plants were exposed to 0.15 μL L^{-1} O_3 for 6 h d^{-1} or remained nontreated. Total RNA was extracted and 3 μg of RNA was separated on 1% formaldehyde-agarose gels, transferred to membranes, and hybridized with the radiolabeled probes indicated. A, Each lane contains RNA extracted from the fifth and sixth leaves pooled from six plants. The samples shown are one representative replicate from a total of three. B, Each lane contains RNA extracted from one rosette and only one replicate was analyzed. C, Control, nontreated plants; O_3, O_3-treated plants.

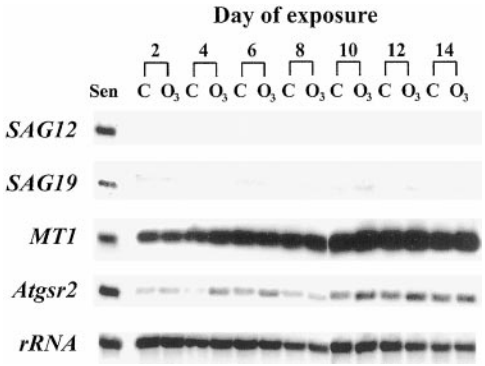

Figure 4. *SAG12*, *SAG19*, *MT1*, and *Atgsr2* transcript levels were not altered by O_3 treatment. Fifteen-day-old Arabidopsis plants were exposed to 0.15 μL L^{-1} O_3 for 6 h d^{-1} or remained nontreated. Samples were prepared as in Figure 3. Each lane contains RNA extracted from the fifth and sixth leaves pooled from six plants. The samples shown are one representative replicate from a total of three. Sen, RNA sample extracted from yellowing (senescent) leaves older than 30 d; C, control, nontreated plants; O_3, O_3-treated plants.

staining was present at the leaf margin and interior of O_3-treated leaves. In control leaves, staining was localized to discrete areas along the margins, with some faint and variable staining at the leaf tip. Following d 12 and 14, O_3-treated leaves showed intense blue staining throughout the entire leaf, and control leaves began to show stronger staining in the leaf interior as senescence progressed from the margins inward.

The effect of O_3 exposure on *SAG12* and *SAG13* expression was also determined by northern analysis. Increased abundance of the *SAG13* transcript was detected after 2 to 4 d of O_3 exposure (Fig. 3), whereas the *SAG12* transcript remained undetectable in O_3-treated and nontreated leaves and rosettes on all sampling days (Fig. 4). These results support the GUS activity data obtained from SAG12-GUS and SAG13-GUS transgenic leaves (Fig. 1). *SAG13* transcript levels gradually increased in O_3-treated leaves five and six at later time points and did not appear in control leaves until d 10 to 12 (Fig. 3A). *SAG13* transcript levels in entire rosettes did not show this gradual increase in abundance, yet levels did remain elevated in O_3-treated rosettes compared with nontreated rosettes (Fig. 3B). The *SAG13* transcript was always more abundant in O_3-treated leaves than in control leaves (Fig. 3). In contrast, *SAG13* promoter-driven GUS activity was similar in O_3-treated and control leaves on d 14 (Fig. 1). This discrepancy may be due to the long half-life of GUS, which is approximately 50 h in living mesophyll protoplasts (Jefferson et al., 1987).

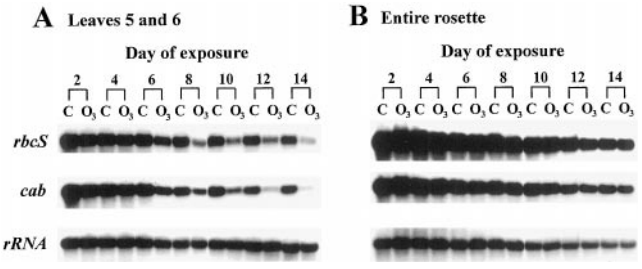

Figure 5. PAG transcript levels declined after treatment with O_3. Fifteen-day-old Arabidopsis plants were exposed to 0.15 μL L^{-1} O_3 for 6 h d^{-1} or remained nontreated. Samples were prepared as in Figure 3. A, Each lane contains RNA extracted from the fifth and sixth leaves pooled from six plants. The samples shown are one representative replicate from a total of three. B, Each lane contains RNA extracted from one rosette and only one replicate was analyzed. C, Control, nontreated plants; O_3, O_3-treated plants.

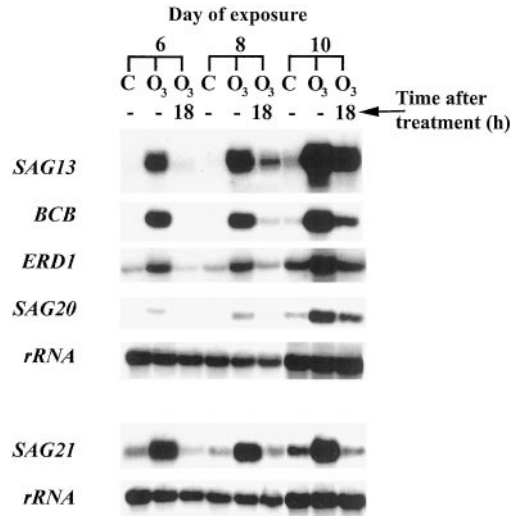

Figure 6. *SAG13*, *BCB*, *ERD1*, *SAG20*, and *SAG21* transcript levels declined following a recovery period in O_3-free air. Fifteen-day-old Arabidopsis plants were exposed to 0.15 μL L^{-1} O_3 for 6 h d^{-1} or remained nontreated. The fifth and sixth leaves were harvested from six plants immediately following 6 h of exposure to O_3 or 18 h after removal of O_3. Samples were prepared as in Figure 3. The samples shown are one of two replicates. C, Control, nontreated plants; O_3, O_3-treated plants.

Effects of O_3 Exposure on PAG and SAG Expression

Transcript levels for the PAGs *rbc*S and *cab* showed a strong reduction in the fifth and sixth leaves after 6 d of O_3 exposure (Fig. 5A). PAG transcript levels continued to decline gradually throughout the remainder of the experiment. Only a slight decline in PAG mRNA levels was found in control leaves (Fig. 5A). The O_3-induced decline in PAG expression, as found in the fifth and sixth leaves, was not readily detectable in RNA samples extracted from entire rosettes (Fig. 5B). PAG transcript levels declined with age in both O_3-treated and nontreated rosettes.

SAG expression levels were determined in three replicate samples, and the range of days given for the time of induction represents the variability within these samples. *SAG13*, *SAG21*, *BCB* (blue copper-binding protein), and *ERD1* (early responsive to dehydration) were induced in the fifth and sixth leaves between d 2 and 4 of O_3 treatment (Fig. 3A), prior to any detectable decline in PAG transcript levels. *SAG18*, *SAG20*, and *ACS6* (ACC synthase) were induced between d 4 and 6 and *CCH* (copper chaperone) was induced between d 6 and 8 of the O_3 treatment in the fifth and sixth leaves (Fig. 3A). Transcripts for all of these genes continued to accumulate throughout the 14 d of

exposure. Transcripts for most of these genes were detected in control leaves, but did not appear until later and levels remained below those found in O_3-treated samples. The *SAG21* transcript was detected in the fifth and sixth control leaves on d 6; *ERD1* between d 8 and 10; *SAG13*, *SAG18*, and *CCH* between d 10 and 12; and *BCB* between d 12 and 14 (Fig. 3A). *SAG20* and *ACS6* did not show any appreciable accumulation in the fifth and sixth control leaves during the experimental period (Fig. 3A). Transcript levels for *SAG13* and *BCB* were greater in O_3-treated rosettes compared with nontreated rosettes; however, transcript accumulation throughout the 14 d of exposure, as found for leaves five and six, was not detected in rosettes (Fig. 3B). Similar results were obtained for *SAG21* and *ERD1* transcript levels in O_3-treated rosettes and *SAG18*, *SAG20*, *CCH*, and *ACS6* transcript levels were more abundant in O_3-treated rosettes on only some of the harvest days (data not shown).

Not all of the characterized SAGs were induced by O_3 treatment. *SAG12*, *SAG19*, *MT1* (metallothionein), and *Atgsr2* (glutamine synthetase) were not induced by O_3 treatment during the 8-d exposure in experiment 1 (data not shown). The O_3 exposure period in experiment 2 was extended for a total of 14 d to determine if the expression of these SAGs could be induced with a longer O_3 treatment. *SAG12*, *SAG19*, *MT1*, and *Atgsr2* were not induced to any measurable degree during the 14-d exposure (Fig. 4). *MT1* and *Atgsr2* transcripts were present in all samples and transcript levels gradually increased in abundance equally in O_3-treated and control leaves. Slightly greater signals for the *MT1* and *Atgsr2* transcripts were found in a few of the O_3-treated samples, but this response was not consistent. *SAG12* transcript was not detected in any sample and *SAG19* transcript remained nearly undetectable (Fig. 4). RNA was extracted from partially yellow leaves harvested from nontreated plants older than 30 d post germination and was included on membranes to demonstrate that *SAG12* and *SAG19* transcripts could be detected (Fig. 4).

The ability of O_3-treated leaves to recover from the accelerated induction of SAGs was investigated by analyzing transcript levels following removal of O_3 on d 6, 8, and 10 of the exposure. The fifth and sixth leaves were harvested from plants immediately following the daily 6-h O_3 treatment and from another set of plants allowed to recover from the treatment for an additional 18 h in O_3-free air. Transcript levels for *SAG13*, *BCB*, *ERD1*, *SAG20*, and *SAG21* were greater in leaves treated with 6 h of O_3 on d 6, 8, and 10 than in nontreated leaves (Fig. 6). Transcript levels for these genes declined following 18 h in O_3-free air. On d 6, SAG transcripts were nearly undetectable in control leaves, but were induced in O_3-treated leaves. Following 18 h in O_3-free air, transcript levels were undetectable in O_3-treated samples. On d 8, transcript levels remained nearly undetectable in control leaves, but were induced in O_3-treated samples and once again declined in O_3-treated samples following the 18-h period. By d 10, SAG transcripts were detected in controls and O_3-treated samples. The decline in transcript level 18 h after the removal of O_3 was still apparent.

DISCUSSION

In the present study, chronic O_3 treatment accelerated the normal rate of foliar senescence in Arabidopsis plants. This response occurred in the absence of the necrosis observed in response to higher O_3 concentrations reported previously for other species (Pell et al., 1997). O_3-induced leaf yellowing in Arabidopsis was previously observed in older leaves exposed to O_3 continuously for 2 d (Kubo et al., 1995). Rosette growth was reduced and downward leaf curling was evident within 4 d of O_3 exposure, similar to results obtained by Sharma and Davis (1994) and Rao et al. (1995). Leaf curling appeared to be an altered growth response and was not the result of dehydration, since the percent dry matter did not vary between treatments in an independent experiment (data not shown). A suite of O_3-induced changes in transcript levels were observed, including reductions in levels of PAGs and increased levels of many but not all SAGs measured (Table I; Figs. 3–5). These changes were only expressed in leaves of a discrete developmental age. Hence, observations of O_3-induced decline in PAG transcript levels, for example, were observed in the fifth and sixth leaves but were not detected when whole rosettes were analyzed (Fig. 5).

Similarities and Contrasts to Natural Senescence

O_3 induces many changes common to natural senescence, but at an accelerated rate: for example, loss of total protein, Rubisco, chlorophyll, and increased leaf abscission (Pell and Pearson, 1983; Reich, 1983; Held et al., 1991; Nie et al., 1993). Diminishing *rbc*S and *cab* transcript levels are indicators of declining photosynthetic activity during natural senescence; the observation in this experiment that O_3 treatment reduced the level of these transcripts was supported by previous investigations (Bahl and Kahl, 1995; Conklin and Last, 1995; Glick et al., 1995). Transcript levels for two other genes, *SDG1* (senescence-down-regulated gene) and *SDG2*, declined during the O_3 exposure with an expression pattern similar to *rbc*S and *cab* (data not shown). *SDG1* and *SDG2* showed reduced transcript abundance in a differential screen of nonsenescent versus senescent leaves (Lohman et al., 1994).

O_3 treatment induced the early expression of many molecular markers of senescence, providing additional evidence that changes in gene expression during chronic O_3 treatment are similar to natural senescence. Two metal-binding proteins, CCH (copper chaperone) and BCB (blue copper-binding protein), are among the genes induced by O_3. These genes were previously shown to be induced by acute O_3 exposure; *BCB* was induced within a 3-h exposure to 0.30 μL L^{-1} O_3 (Richards et al., 1998) and *CCH* transcript levels increased by 30% after a 30-min exposure to 0.80 μL L^{-1} O_3 (Himelblau et al., 1998). Metal-binding proteins may play an important role in metal remobilization during senescence. O_3 treatment also induced transcript accumulation of a protease regulator, ERD1; proteases are involved in protein degradation during natural senescence and may be further required for degradation of oxidized proteins during O_3-induced accelerated senescence. Transcript ac-

cumulation of other genes, including *SAG13*, *SAG18*, *SAG20*, and *SAG21*, was also induced by O_3 treatment; the function of these genes in senescence remains unclear. While O_3 induced the buildup of SAG transcripts, it is not known how this translates into accumulation of the protein products.

Transcripts for *SAG12*, *SAG19*, *Atgsr2*, and *MT1* accumulate during natural senescence, but were not induced by chronic O_3 treatment. These genes may lack responsive elements able to recognize O_3-induced signaling compounds. Proteases other than the Cys protease SAG12 may have been available for proteolysis and adequate quantities of Gln synthetase, Atgsr2, and metallothionein, MT1, may have been present due to high basal transcript levels. If all senescence-related genes play critical roles in cellular degradation and nutrient remobilization during natural senescence, the lack of these gene products during O_3-induced accelerated senescence may reduce the efficiency of nutrient recovery.

Specific and perhaps premature induction of gene expression in response to O_3 is reminiscent of molecular changes in response to other stresses (Weaver et al., 1997). Genes induced during chronic O_3 exposure have also been shown to be induced by darkness, dehydration, and treatment with ethylene or ABA. Dark treatment induced the O_3-responsive genes, *ERD1*, *BCB*, and *SAG20*, dehydration induced *ERD1*, *BCB*, *SAG20*, and *SAG21*, ethylene treatment induced *ERD1*, *BCB*, *SAG13*, *SAG20*, and *SAG21*, and ABA treatment induced *ERD1* and *SAG13* (Kiyosue et al., 1993; Nakashima et al., 1997; Weaver et al., 1998). The overlap in gene expression suggests that O_3 treatment, darkness, and dehydration may induce similar signaling molecules. Ethylene and ABA may play a role as signals during O_3-induced accelerated leaf senescence, as discussed below.

In addition to affecting the timing of induction of some SAGs, O_3 also seems to influence the spatial distribution of that induction. *SAG13*-promoter driven GUS activity first appeared at the leaf margin in control leaves, which resembles the pattern of yellowing found in naturally senescing leaves (Weaver et al., 1998). In contrast, O_3 treatment induced *SAG13* expression throughout the leaves. This distribution of *SAG13* expression probably coincided with regions where O_3 entered through open stomata.

Potential Signals of Molecular Events

Elevated *SAG13*, *SAG20*, *SAG21*, *BCB*, and *ERD1* transcript levels in O_3-treated leaves were not sustained following the removal of O_3. Daily O_3 exposures were required to provide a signal to maintain enhanced SAG transcript levels, suggesting that the leaves may retain some ability to recover from exposure to O_3. A similar recovery was shown for *rbc*S and *cab* transcripts in Arabidopsis following a 24-h O_3-free period after treatment with 0.175 μL L^{-1} O_3 for 8 h d^{-1} for 4 d (Conklin and Last, 1995).

Since O_3 treatment induced premature changes in transcript levels of genes associated with natural senescence, O_3 may elicit some of the same signals involved in natural senescence. The common mechanism regulating O_3-induced accelerated leaf senescence and natural leaf senescence may involve reactive oxygen species. Oxidative stress has long been associated with senescence (Thompson et al., 1987), and recently this link was shown in the late-flowering (or extended longevity) Arabidopsis mutant *gigantea* (gi-3), which exhibited enhanced tolerance to methyl viologen-induced oxidative stress (Kurepa et al., 1998). Following stomatal uptake of O_3, internal O_3 concentrations rapidly drop (Laisk et al., 1989) as decomposition products, including reactive oxygen species, are formed. These reactive oxygen species can react with membrane lipids to produce more reactive oxygen intermediates. A second sustained peak of reactive oxygen species was found in the O_3-sensitive tobacco cv Bel W3 following O_3 exposure, and was not found in the O_3-tolerant cv Bel B (Schraudner et al., 1998). An O_3-responsive region in the stilbene synthase promoter has been identified (Schubert et al., 1997), and a comparison of this 150-bp region with the *SAG13* promoter (S. Gan and R.M. Amasino, personal communication) did not reveal any strong sequence similarity (data not shown). The presence of O_3 or reactive oxygen species responsive elements in SAGs is worthy of future investigation.

Alternatively, the O_3-induced changes in gene expression could have been induced through a secondary signal. Ethylene treatment induces many of the O_3-responsive SAGs (Weaver et al., 1998), and plants exposed to high doses of O_3 produce large quantities of ethylene (Pell et al., 1997). Ethylene has been shown to regulate the timing of leaf senescence in Arabidopsis (Grbic and Bleecker, 1995). In our experiments, *ACS6*, one member of the gene family encoding ACC synthase in Arabidopsis, was detected within 4 to 6 d of O_3 exposure. This gene is induced by many stresses, including O_3, while *ACS1*, *ACS2*, *ACS4*, and *ACS5* are not induced by O_3 treatment (Vahala et al., 1998; Arteca and Arteca, 1999). At high O_3 concentrations, ethylene emission is one of the first responses and is correlated with the degree of lesion formation (Tuomainen et al., 1997). The importance of ethylene in regulating the response to low O_3 concentrations in the induction of accelerated leaf senescence remains to be determined. We are currently investigating the need for ethylene perception in the induction of this suite of SAGs.

Other potential signaling molecules include ABA, salicylic acid, and calcium. ABA is another senescence-promoting hormone, and some of the O_3-responsive SAGs are inducible by ABA treatment (Weaver et al., 1998). Salicylic acid and calcium increase during exposure to high O_3 concentrations and are involved in the induction of antioxidant gene expression (Yalpani et al., 1994; Sharma and Davis, 1997; Clayton et al., 1999); however, it is not known whether they are involved in the response to chronic O_3.

In conclusion, chronic O_3 treatment induced SAG expression while suppressing PAG expression. An initial pattern of senescence-related gene induction by O_3 has emerged. Future experiments should focus on determining which genes are essential for the induction of O_3-induced accelerated leaf senescence and what, if any, interdependency exists between these genes. Further investigation will determine the identity of signals required for O_3-induced

accelerated leaf senescence and elucidate the role of oxidative stress in the progression of natural leaf senescence.

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LITERATURE CITED

Arteca JM, Arteca RN (1999) A multi-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase (*ACS6*) in mature *Arabidopsis* leaves. Plant Mol Biol **39:** 209–219

Bahl A, Kahl G (1995) Air pollutant stress changes the steady-state transcript levels of three photosynthesis genes. Environ Pollut **88:** 57–65

Bate NJ, Rothstein SJ, Thompson JE (1991) Expression of nuclear and chloroplast photosynthesis-specific genes during leaf senescence. J Exp Bot **42:** 801–811

Becker W, Apel K (1993) Differences in gene expression between natural and artificially induced leaf senescence. Planta **189:** 74–79

Bernhard WR, Matile P (1994) Differential expression of glutamine synthetase genes during the senescence of *Arabidopsis thaliana* rosette leaves. Plant Sci **98:** 7–14

Blank A, McKeon TA (1991) Expression of three RNase activities during natural and dark-induced senescence of wheat leaves. Plant Physiol **97:** 1409–1413

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Buchanan-Wollaston V (1997) The molecular biology of leaf senescence. J Exp Bot **48:** 181–199

Chung BC, Lee SY, Oh SA, Rhew TH, Nam HG, Lee CH (1997) The promoter activity of *sen1*, a senescence-associated gene of *Arabidopsis*, is repressed by sugars. J Plant Physiol **151:** 339–345

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA **81:** 1991–1995

Clayton H, Knight MR, Knight H, McAinsh MR, Hetherington AM (1999) Dissection of the ozone-induced calcium signature. Plant J **17:** 575–579

Conklin PL, Last RL (1995) Differential accumulation of antioxidant mRNAs in *Arabidopsis thaliana* exposed to ozone. Plant Physiol **109:** 203–212

Gan S, Amasino RM (1997) Making sense of senescence: molecular genetic regulation and manipulation of leaf senescence. Plant Physiol **113:** 313–319

Glick RE, Schlagnhaufer CD, Arteca RN, Pell EJ (1995) Ozone-induced ethylene emission accelerates the loss of ribulose-1,5-bisphosphate carboxylase/oxygenase and nuclear-encoded mRNAs in senescing potato leaves. Plant Physiol **109:** 891–898

Grbic V, Bleecker AB (1995) Ethylene regulates the timing of leaf senescence in Arabidopsis. Plant J **8:** 595–602

Held AA, Mooney HA, Gorham JN (1991) Acclimation to ozone stress in radish: leaf demography and photosynthesis. New Phytol **118:** 417–423

Himelblau E, Mira H, Lin SJ, Culotta VC, Peñarrubia L, Amasino RM (1998) Identification of a functional homolog of the yeast copper homeostasis gene *ATX1* from Arabidopsis. Plant Physiol **117:** 1227–1234

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J **6:** 3901–3907

Jorgenson RA, Cuellar RE, Thompson WF (1982) Modes and tempos in the evolution of nuclear encoded ribosomal RNA genes in legumes. Carnegie Inst Wash Year Book **81:** 98–101

Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K (1993) Characterization of cDNA for a dehydration-inducible gene that encodes a CLP A, B-like protein in *Arabidopsis thaliana* L. Biochem Biophys Res Commun **196:** 1214–1220

Kubo A, Saji H, Tanaka K, Kondo N (1995) Expression of *Arabidopsis* cytosolic ascorbate peroxidase gene in response to ozone or sulfur dioxide. Plant Mol Biol **29:** 479–489

Kurepa J, Smalle J, Van Montagu M, Inzé D (1998) Oxidative stress tolerance and longevity in *Arabidopsis*: the late-flowering mutant *gigantea* is tolerant to paraquat. Plant J **14:** 759–764

Laisk A, Kull O, Moldau H (1989) Ozone concentration in leaf intercellular air spaces is close to zero. Plant Physiol **90:** 1163–1167

Lohman KN, Gan S, John MC, Amasino RM (1994) Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*. Physiol Plant **92:** 322–328

Nakashima K, Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K (1997) A nuclear gene, *erd1*, encoding a chloroplast-targeted Clp protease regulatory subunit homolog is not only induced by water stress but also developmentally up-regulated during senescence in *Arabidopsis thaliana*. Plant J **12:** 851–861

Nie GY, Tomasevic M, Baker NR (1993) Effects of ozone on the photosynthetic apparatus and leaf proteins during leaf development in wheat. Plant Cell Environ **16:** 643–651

Oh SA, Lee SY, Chung IK, Lee CH, Nam HG (1996) A senescence-associated gene of *Arabidopsis thaliana* is distinctively regulated during natural and artificially induced leaf senescence. Plant Mol Biol **30:** 739–754

Park JH, Oh SA, Kim YH, Woo HR, Nam HG (1998) Differential expression of senescence-associated mRNAs during leaf senescence induced by different senescence-inducing factors in *Arabidopsis*. Plant Mol Biol **37:** 445–454

Pell EJ, Pearson NS (1983) Ozone-induced reduction in quantity of ribulose-1,5-bisphosphate carboxylase in alfalfa foliage. Plant Physiol **73:** 185–187

Pell EJ, Schlagnhaufer CD, Arteca RN (1997) Ozone-induced oxidative stress: mechanisms of action and reaction. Physiol Plant **100:** 264–273

Peterman TK, Goodman HM (1991) The glutamine synthetase gene family of *Arabidopsis thaliana*: light-regulation and differential expression in leaves, roots and seeds. Mol Gen Genet **230:** 145–154

Rao MV, Paliyath G, Ormrod DP (1995) Differential response of photosynthetic pigments, Rubisco activity and Rubisco protein of *Arabidopsis thaliana* exposed to UVB and ozone. Photochem Photobiol **62:** 727–735

Reich PB (1983) Effects of low concentrations of O_3 on net photosynthesis, dark respiration, and chlorophyll contents in aging hybrid poplar leaves. Plant Physiol **73:** 291–296

Richards KD, Schott EJ, Sharma YK, Davis KR, Gardner RC (1998) Aluminum induces oxidative stress genes in *Arabidopsis thaliana*. Plant Physiol **116:** 409–418

Schraudner M, Moeder W, Wiese C, Van Camp W, Inzé D, Langebartels C, Sandermann H (1998) Ozone-induced oxidative burst in the ozone biomonitor plant, tobacco Bel W3. Plant J **16:** 235–245

Schubert R, Fischer R, Hain R, Schreier PH, Bahnweg G, Ernst D, Sandermann H (1997) An ozone-responsive region of the grapevine resveratrol synthase promoter differs from the basal pathogen-responsive sequence. Plant Mol Biol **34:** 417–426

Sharma YK, Davis KR (1994) Ozone-induced expression of stress-related genes in *Arabidopsis thaliana*. Plant Physiol **105:** 1089–1096

Sharma YK, Davis KR (1997) The effects of ozone on antioxidant responses in plants. Free Radic Biol Med **23:** 470–488

Smart CM (1994) Gene expression during leaf senescence. New Phytol **126:** 419–448

Thoma S, Sullivan ML, Vierstra RD (1996) Members of two gene families encoding ubiquitin-conjugating enzymes, AtUBC1–3 and AtUBC4–6, from *Arabidopsis thaliana* are differentially expressed. Plant Mol Biol **31:** 493–505

Thompson JE, Legge RL, Barber RF (1987) The role of free radicals in senescence and wounding. New Phytol **105:** 317–344

Tuomainen J, Betz C, Kangasjärvi J, Ernst D, Yin ZH, Langebartels C, Sandermann H (1997) Ozone induction of ethylene emission in tomato plants: regulation by differential accumulation of transcripts for the biosynthetic enzymes. Plant J **12:** 1151–1162

Vahala J, Schlagnhaufer CD, Pell EJ (1998) Induction of an ACC synthase cDNA by ozone in light-grown *Arabidopsis thaliana* leaves. Physiol Plant **103:** 45–50

Van Gysel A, Van Montagu M, Inze D (1993) A negatively light-regulated gene from *Arabidopsis thaliana* encodes a protein showing high similarity to blue copper-binding proteins. Gene **136:** 79–85

Weaver LM, Gan S, Quirino B, Amasino RM (1998) A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatment. Plant Mol Biol **37:** 455–469

Weaver LM, Himelblau E, Amasino RM (1997) Leaf senescence: gene expression and regulation. *In* JK Setlow, ed, Genetic Engineering, Vol 19. Plenum Press, New York, pp 215–234

Yalpani N, Enyedi AJ, León J, Raskin I (1994) Ultraviolet light and ozone stimulate accumulation of salicylic acid, pathogenesis-related proteins and virus resistance in tobacco. Planta **193:** 372–376

Zhou J, Goldsbrough PB (1994) Functional homologs of fungal metallothionein genes from Arabidopsis. Plant Cell **6:** 875–884