The Pennsylvania State University

The Graduate School

Intercollege Graduate Program in Plant Physiology

SENESCENCE-ASSOCIATED GENE EXPRESSION IN OZONE-STRESSED ARABIDOPSIS LEAVES

A Thesis in

Plant Physiology

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2000

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ABSTRACT

The similarities and differences between natural leaf senescence and O_3-induced accelerated leaf senescence were assessed by comparing the expression levels of senescence-associated genes (SAGs) and photosynthesis-associated genes (PAGs) in *Arabidopsis thaliana*. During natural leaf senescence SAG transcript levels increase while PAG transcript levels decline. SAG and PAG expression levels were determined by northern analysis in Arabidopsis ecotype Landsberg *erecta* plants treated with 0.15 μL L^{-1} O_3 for 6 hours per day for 14 days. Ozone treatment caused the early expression of *BCB*, *ERD1*, *SAG13*, *SAG18*, *SAG20*, *SAG21* and *CCH* and an early decline in *rbc*S and *cab* transcript levels. No induction of the following senescence-related genes was found: *SAG12*, *SAG19*, *MT1* and *Atgsr2*. The spatial distribution of SAG expression in leaves was investigated by treating transgenic plants carrying the *SAG13* promoter-GUS construct with O_3. *SAG13* promoter-driven GUS activity was located throughout O_3-treated leaves. This expression pattern differed from that found in naturally senescing leaves. While differences do exist between natural leaf senescence and O_3-induced accelerated leaf senescence, the process in O_3-treated leaves is highly regulated and involves the expression of many genes associated with natural senescence.

Protein levels for two SAGs were investigated during O_3 treatment to determine whether the increases in SAG transcript levels were accompanied by corresponding increases in protein levels. ERD1 and BCB protein levels were determined by western analysis in plants treated with 0.15 μL L^{-1} O_3 for 6 hours per day for 8 and 14 days.

ERD1 transcript levels were strongly induced by O_3 treatment, whereas ERD1 protein levels declined with increasing O_3 exposure. *BCB* transcript levels also increased during O_3 treatment, while no BCB protein accumulation was detected during the exposure. The reason for the disparity between transcript and protein levels is not known, causing the functional significance of O_3-induced increases in *ERD1* and *BCB* transcript levels to be questioned.

The plant hormone ethylene modulates the timing of leaf senescence and is produced during exposures to high doses of O_3, so the role of ethylene in inducing SAG expression during O_3 treatment was studied. SAG and PAG transcript levels were measured by northern analysis in wild-type Arabidopsis, ecotype Columbia, plants and the ethylene-insensitive mutant, *etr1-3*. During exposure to 0.15 μL L^{-1} O_3 for 6 hours per day for 14 days, equivalent changes in SAG and PAG expression levels were found in wild-type and *etr1-3* plants. The increase in SAG transcripts and decline in PAG transcripts occurred without any increase in ethylene production, ACC levels or *ACS6* transcript levels. During exposure to a higher O_3 concentration, 0.35 μL L^{-1} O_3 for 6 hours, ethylene production was induced, but once again the level of SAG induction and PAG transcript decline were similar in wild-type plants and *etr1-3* mutants. This work suggested that ethylene perception was not necessary for the induction of SAG transcripts and decline in PAG transcripts during O_3 exposure. The *etr1-3* mutant retains some partial sensitivity to ethylene, so the role of ethylene in O_3-induced SAG expression was investigated in three additional ethylene-insensitive mutants.

Wild-type plants and ethylene-insensitive mutants *etr1-3*, *ein2-1*, *ein3-1* and *ein4-1* were treated with 100 μL L^{-1} ethylene for 6 hours and expression of the ethylene-responsive gene, *HEL*, was measured by northern analysis to determine the ethylene sensitivity of leaves. Transcript for *HEL* was induced in ethylene-treated wild-type plants and some induction was also found in ethylene-insensitive mutants, *etr1-3* and *ein3-1*. No ethylene induction of *HEL* was found in *ein2-1* or *ein4-1* mutants.

The ethylene-insensitive mutants were treated with O_3 to see if SAGs could be induced in the absence of ethylene perception, as in *ein2-1* and *ein4-1* leaves, or under reduced ethylene perception, as in *etr1-3* and *ein3-1* leaves. Wild-type plants and *etr1-3*, *ein2-1*, *ein3-1* and *ein4-1* mutants were treated with 0.15 μL L^{-1} O_3 for 3.5 hours and SAG transcript levels were determined. SAGs were induced in wild-type plants and all ethylene-insensitive mutants at similar levels. Wild-type plants and mutants were treated with 0.30 μL L^{-1} O_3 for 3.5 hours to determine SAG expression levels when the O_3 treatment caused a sharp increase in ethylene production. In the presence of O_3-induced ethylene production, SAGs were once again induced at similar levels in wild-type plants and ethylene-insensitive mutants. This work demonstrates that ethylene is not the primary signal for SAG expression during O_3 treatment.

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ACKNOWLEDGMENTS

I acknowledge and thank my parents, Dean and Connie Puffett, for all of their encouragement and support throughout the years. My interest in the natural world was sparked by my parents during time spent in the cow barn with my father and wildflower collecting expeditions with my mother. While at Penn State, I have had the opportunity to work with several wonderful people who constantly supported my work and shared in many scientific discussions, to them I would like to offer my sincere thanks: Cosima Wiese, Gro Torsethaugen and Douglas Bielenberg. I would also like to thank Judy Sinn and Christian Vinten-Johansen for their technical assistance and emotional support. I extend my deepest gratitude to Dr. Eva Pell for providing this opportunity to explore my scientific interests and for her guidance along the way. I would also like to thank Dr. Richard Arteca for his many words of support and encouragement.

I would like to acknowledge the granting agencies that made this work possible. Funding was provided by the Environmental Protection Agency (grant no. U915212-01-1) and the U.S. Department of Agriculture (grant no. 93-38420-8742).

To my husband, Matthew Miller, thank you for your constant love and acceptance. You are my true inspiration and friend who always comforts me and reminds me of "oh the places you will go."

Chapter 1

Introduction to Ozone-Induced Leaf Senescence

Tropospheric ozone (O_3) is formed in the presence of UV light when nitrous oxides react with hydrocarbons during incomplete fossil fuel combustion (Comrie, 1990). Visual symptoms of O_3 pollution were first noted on grape vines in the Los Angeles basin (Richards et al., 1958). The characteristic small brown spots, termed stipple, were followed by accelerated leaf yellowing and leaf fall. Ozone is a gaseous pollutant and enters the leaf through open stomata where it quickly reacts with water and other cellular components to generate highly reactive oxygen species (ROS), including superoxide, hydrogen peroxide and hydroxyl radicals (Kangasjärvi et al., 1994). ROS have been associated with aging since the proposal of the free-radical theory of aging by Harman in 1956 and have been proposed as an explanation for accelerated leaf senescence during O_3 stress (Pell et al., 1997).

Natural leaf senescence is characterized by the breakdown of cellular components and remobilization of nutrients to other plant parts. Chloroplasts are one of the first organelles to exhibit changes during senescence, while mitochondria and the nucleus remain intact until later stages of this process (review in Smart, 1994). Protein, RNA, chlorophyll and lipids are gradually broken down throughout senescence (Hensel et al.,

1993; Lohman et al, 1994; Smart, 1994). Similar changes have been found in plants exposed to O_3 (Nie et al., 1993).

Oxidative conditions exist within leaves undergoing senescence. Lipid peroxidation is a source of ROS during leaf senescence and higher superoxide levels have been found in senescing tissues (Thompson et al., 1987). The oxidative conditions are exacerbated due to the reduced quantity and activity of superoxide dismutase and other antioxidant enzymes to scavenge ROS in mature, senescing leaves (Thompson et al., 1987; Casano et al., 1994). Increased superoxide production in senescing chloroplasts occurs as thylakoid membranes deteriorate, exposing chlorophyll molecules to photochemical reactions (McRae and Thompson, 1983). The chloroplasts are unlikely to scavenge increasing levels of superoxide production due to reduced antioxidant enzyme activity (McRae and Thompson, 1983). In peroxisomes, the activity of superoxide- and hydrogen peroxide-producing enzymes increases during senescence while catalase activity decreases, resulting in the accumulation of ROS (Pastori and del Rio, 1997). Hydrogen peroxide can diffuse out of the peroxisomes and enter the cytosol, where it can create additional oxidative stress (Pastori and del Rio, 1997).

Senescence is a highly regulated process demonstrated by the characterization of the hallmarks of programmed cell death, including DNA laddering and TUNEL positive cells, in naturally senescing leaves of five different plant species (Yen and Yang, 1998). The degradative processes occurring during natural senescence are under genetic control of the nucleus (Thomas et al., 1992). Differential gene expression occurs in senescing leaves during the conversion from active photosynthesis to senescence (Hensel el al.,

1993: Lohman et al., 1994). Message levels for a number of photosynthesis-associated genes (PAGs), including chlorophyll a/b binding protein (*cab*) and the small subunit of ribulose 1,5-bisphophate/carboxylase (*rbc*S), decline during natural senescence (Bate et al., 1991). In contrast to the drop in PAG mRNAs, several genes are induced at different times throughout senescence. Gene expression during natural senescence has been analyzed in several species and numerous upregulated genes have been identified and are termed senescence-related genes or senescence-associated genes (SAGs). These genes have been identified in Arabidopsis (Hensel et al., 1993; Taylor et al., 1993; Lohman et al., 1994; Oh et al., 1996; Thomas and de Villiers, 1996; Park et al., 1998, Weaver et al., 1998), oilseed rape (Buchanan-Wollaston, 1994: Buchanan-Wollaston and Ainsworth, 1997), tomato (Davies and Grierson, 1989; Drake et al., 1996), barley (Becker and Apel, 1993; Kleber-Janke and Krupinska, 1997) and maize (Smart et al., 1995). Some SAGs have been identified as encoding proteins involved in all aspects of leaf senescence from protein, lipid and chlorophyll degradation to nutrient remobilization, while the function of other SAGs is still unknown (reviewed in Buchanan-Wollaston, 1997 and Weaver et al., 1997).

Ozone-induced accelerated leaf senescence and natural senescence share many similarities, including altered PAG expression. Ozone has been shown to reduce PAG message levels for *rbc*S, *cab* and glyceraldehyde-3-phosphate dehydrogenase (*gap*A and *gap*B) (Reddy et al., 1993; Bahl and Kahl, 1995; Conklin and Last, 1995; Glick et al., 1995). While PAG transcript levels are known to decline during O_3 exposure, nothing is known about the expression of SAGs during O_3-induced accelerated leaf senescence.

The first major objective of this research was to determine whether SAGs were expressed during O_3-induced accelerated leaf senescence. The pattern of SAG and PAG expression was analyzed in Arabidopsis plants during a chronic O_3 exposure. This species was selected for studying the effects of O_3 on SAG and PAG expression in plants due to the identification of numerous SAGs in Arabidopsis.

Another important area of research in the senescence field is the identification of the signaling molecules responsible for SAG expression. Hormone and stress treatments have shown that there are multiple signaling pathways leading to the induction of any particular senescence-related gene (Gan and Amasino, 1997; Park et al., 1998; Weaver et al., 1998). Ethylene is an important regulator of leaf senescence. Leaf longevity in the Arabidopsis ethylene-insensitive mutant, *etr1-1*, was extended by 30% compared to wild-type plants (Grbic and Bleecker, 1995). Visual signs of leaf senescence, chlorophyll loss and decline in photosynthetic enzymes and mRNAs were delayed by one week in *etr1-1* (Grbic and Bleecker, 1995). Exogenous ethylene treatment accelerated the decline in PAG expression and induction of SAG expression in the oldest leaves of wild-type Arabidopsis plants (Grbic and Bleecker, 1995). Additional evidence for the role of ethylene in senescence has been demonstrated in ethylene-treated mature tomato leaves. Ethylene treatment causes chlorophyll loss and senescence-related gene expression and use of the ethylene inhibitor silver thiosulphate prevents these changes (Davies and Grierson, 1989). Transgenic tomato plants antisense for ACC oxidase are ethylene deficient and exhibit delayed leaf senescence (John et al., 1995). Leaf senescence still

occurs when ethylene perception or biosynthesis is altered, suggesting that ethylene modulates the timing of leaf senescence (Grbic and Bleecker, 1995; John et al., 1995).

Ethylene emission has been associated with the response to O_3. Ethylene is produced by plants treated with O_3, and O_3-induced ACC synthase genes have been identified in potato and Arabidopsis plants (Schlagnhaufer et al., 1995; Vahala et al., 1998). Tingey et al. (1976), Melhorn and Wellburn (1987) and Melhorn et al. (1991) determined that the degree of foliar injury during O_3 treatment was related to the rate of ethylene emission. Sensitivity to ozone could be reduced by treating plants with an inhibitor of ethylene biosynthesis, aminoethoxyvinylglycine (Melhorn et al., 1991). Various biochemical events have also been correlated with the increase in ethylene emission. Reddy et al. (1993) found a rapid drop in mRNA levels of both *rbc*L and *rbc*S associated with higher ethylene emissions after O_3 exposure in potato. The drop in *rbc*S transcript could be reduced by treating plants with the ethylene biosynthesis inhibitor, aminooxyacetic acid (Schlagnhaufer et al., 1995). Glick et al. (1995) showed that the timing of maximum ethylene emission also coincided with the decline in *rbc*S mRNA and most rapid reduction of net photosynthesis. The evidence suggests that ethylene may be an important signaling molecule in the response to O_3 treatment.

Due to the role of ethylene in many responses to O_3 and the ability of ethylene to modulate the timing of leaf senescence, the second objective of this work was to determine whether ethylene emission during O_3 exposure is responsible for increased SAG expression. To meet this objective, SAG expression was examined in several ethylene-insensitive mutants of Arabidopsis compared to wild-type Arabidopsis plants.

Chapter 2

Senescence-Associated Gene Expression during Ozone-Induced Leaf Senescence in Arabidopsis

(See reprint in pocket)

Chapter 3

ERD1 and BCB Protein Levels during Ozone-Induced Accelerated Leaf Senescence in Arabidopsis

Studies of gene expression during natural leaf senescence have shown that numerous genes are induced (for reviews, see Buchanan-Wollaston, 1997; Weaver et al., 1997). While this research has provided new information on the transcriptional changes occurring during senescence, there is less research demonstrating that protein levels are altered in concert. The functional importance of increased senescence-associated gene (SAG) transcripts depends upon successful translation of these genes into protein.

Ozone stress induces the early expression of *SAG13*, *SAG18*, *SAG20*, *SAG21*, *BCB*, *ERD1* and *CCH* (Miller et al., 1999). In order to determine if the accelerated induction of SAGs may be functionally important, the accumulation of selected SAG proteins was studied. Of the O_3-induced SAGs, *ERD1* and *BCB* were selected for this study due to the availability of antibodies against the ERD1 and BCB proteins.

Based on sequence similarity, ERD1 has been classified as a member of the Clp ATPase family sharing the strongest similarity with plant ClpCs (Weaver et al., 1999). Clp ATPase genes are characterized by two nucleotide-binding domains separated by a spacer region and have been divided into three subfamilies based on the length of the spacer, ClpA, ClpB, and ClpC (Squires and Squires, 1992). Depending on the type of

Clp ATPase and the source organism, Clp ATPases work independently as chaperonins or as subunits of a Clp protease (Squires and Squires, 1992; Weaver et al., 1999). There is evidence that ClpCs in plants serve both functions (Weaver et al., 1999). ERD1 is chloroplast targeted and present in the stromal fraction of chloroplasts (Weaver et al., 1999). The chloroplast localized Clp system (ClpP and ERD1) could potentially be involved in degrading chloroplast proteins during natural senescence and it might play a similar role during O_3-induced senescence. Recent work, however, suggests that ERD1 may not be involved in protein degradation during senescence. Weaver et al. (1999) found that *ERD1* mRNA accumulates during senescence, while its protein declines below the level of detection around the time of visible senescence. The ClpP protein showed a similar pattern of decline in senescing leaves (Weaver et al., 1999).

The other SAG chosen for study, *BCB*, encodes a membrane bound blue copper-binding protein similar to plastocyanin and stellacyanin (Van Gysel et al., 1993). Based on its similarity to low-molecular mass copper-binding proteins, BCB likely functions as an electron carrier (Van Gysel et al., 1993). An exact role for BCB is currently not known. The BCB protein (Weaver et al., 1999) and mRNA (Weaver et al., 1998) were induced in fully expanded leaves of plants grown under continuous light, and increased in abundance throughout senescence. The appearance of the BCB protein depends upon growth conditions. In plants grown under 16 hours of light, the BCB protein remained undetectable until late senescence (Weaver et al., 1999).

The purpose of this work was to determine whether the large increases in *ERD1* and *BCB* transcript levels during O_3 treatment were accompanied by increases in ERD1

and BCB protein levels. Protein and transcript levels were measured by western and northern analysis, respectively, in leaf tissue treated with 8 and 14 days of O_3.

Material and Methods

Arabidopsis ecotype Landsberg *erecta* were grown as previously described (Miller et al., 1999). Plants were treated with O_3 at 15 days post germination when the fifth leaf, as counted by order of emergence from the meristem (cotyledons were not counted), was 3 to 4 days old. Half of the plants were treated with 0.15 μL L^{-1} O_3 for 6 hours per day as described in Miller et al. (1999). The other half were not treated and remained in another growth chamber. Leaves five and six were pooled from six plants and harvested on day 8 in experiment 1 and days 8, 10, 12 and 14 in experiment 2. Three replicate samples per treatment were collected in experiment 1 and one sample per treatment per sampling time was collected in experiment 2.

Leaf soluble protein was extracted by grinding tissue under liquid N_2 and adding lysis buffer (50 mM Tris-HCl, pH 7.5, 1mM EDTA, 100 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.1 % Triton X-100, 0.7% 2-mercaptoethanol, and 1 mM PMSF) as described by Weaver et al. (1999). Following addition of 5 μL lysis buffer mg^{-1} tissue, samples were vortexed and centrifuged. The supernatant was removed and total soluble protein was quantified with the Bradford dye-binding assay (Bradford, 1976) using a protein assay reagent (Coomassie blue, Bio-Rad) and bovine serum albumin as the standard. An equal volume of 2X loading buffer (125 mM Tris-HCl, pH 7.5, 1% 2-mercaptoethanol,

4% SDS, 20% glycerol, and 0.01% bromophenol blue) was added to the supernatant and proteins were separated by SDS-PAGE. For electrophoresis, samples were applied on an equal volume or equal total protein basis to 9% polyacrylamide separating gels (pH 8.8) with 4.55% polyacrylamide stacking gels (pH 6.8). *Escherichia coli* expressed partial ERD1 and BCB proteins, obtained from L.M. Weaver and R.M. Amasino (University of Wisconsin, Madison), were applied to gels as positive controls during immunodetection.

Proteins were electroblotted onto Zeta-Probe blotting membranes (Bio-Rad) according to Landry and Pell (1993). Immunodetection was performed with ERD1 and BCB polyclonal antibodies raised to *E. coli* expressed ERD1 and BCB peptides obtained from L.M. Weaver and R.M. Amasino (University of Wisconsin, Madison); antibody preparation was described in Weaver et al. (1999). Following transfer, membranes were incubated overnight with rabbit anti-ERD1 or rabbit anti-BCB immunoglobulins. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) was used as a secondary antibody at 1/3000 dilution. Immunoreactive proteins were visualized by chemical development for 5-10 min with a developer containing 4-chloro-1-naphthol and hydrogen peroxide (Bio-Rad).

Northern analysis was conducted as described in Miller et al. (1999).

Results

Protein and total RNA were extracted to determine if the level of protein and transcripts of two senescence-associated genes, *ERD1* and *BCB*, increased in leaves

exposed to O_3. Leaves five and six were harvested on day 8 of the O_3 treatment when there were no visible signs of leaf yellowing. Equivalent levels of ERD1 protein were immunodetected in O_3-treated and nontreated samples, whereas no BCB protein was found (*Figure 3–1A*). These samples were applied to gels on an equal volume basis, which represented an equal leaf volume, according to the method of Weaver et al. (1999). ERD1 protein was detected in green leaf tissue, but not in partially yellow, senescing tissue. The ERD1 antibody detected a 95-kDa protein in plant leaves and a 62-kDa band in *E. coli* cells. An immunoreactive band below the 95-kDa ERD1 protein was detected in some plant samples as previously found by L.M. Weaver (personal communication). The *E. coli* expressed partial BCB protein was successfully detected by immunoblotting, indicating that BCB protein levels in O_3-treated and nontreated samples were below detectable limits. The BCB antibody detected an approximately 30-kDa protein in the *E. coli* sample. A 33-kDa band was expected for the native BCB protein in leaf samples (L.M. Weaver, personal communication).

In contrast to the lack of induction of ERD1 and BCB protein levels, O_3 treatment strongly induced *ERD1* and *BCB* transcript levels by day 8 of the exposure (*Figure 3-1B*). Only weak signals were detected for *ERD1* and *BCB* in nontreated samples.

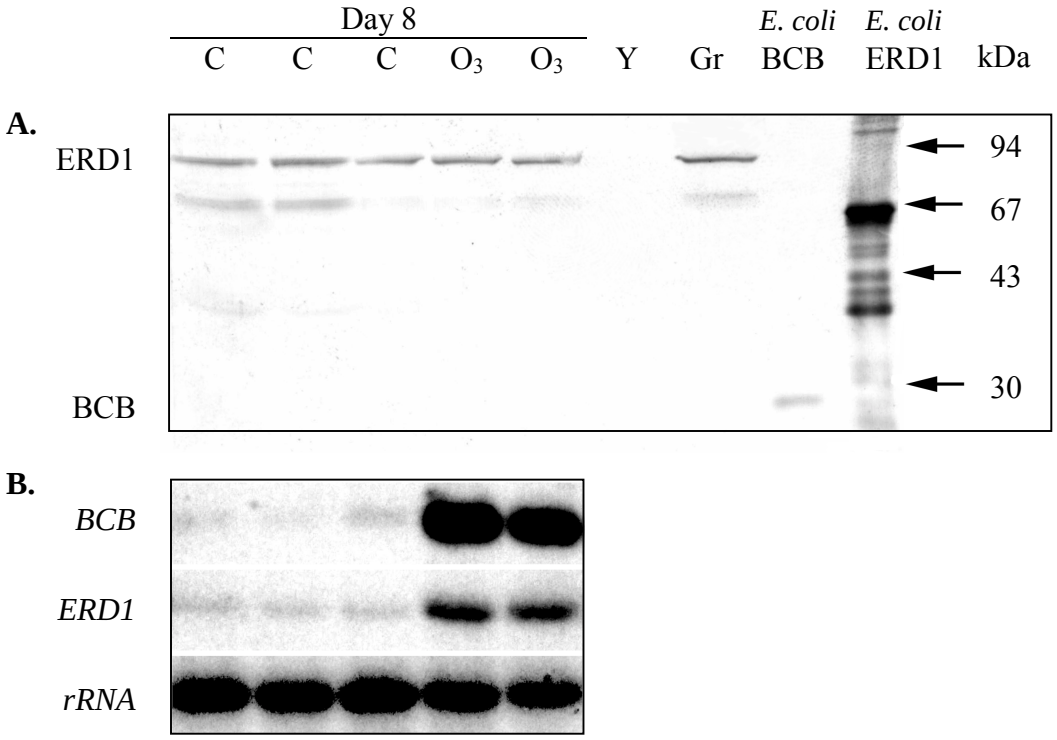

Figure 3–1: ERD1 and BCB protein levels do not increase during 8 days of O_3 treatment, whereas *ERD1* and *BCB* transcripts are strongly induced. Plants were treated with 0.15 μL L^{-1} O_3 for 6 hours per day for 8 days and leaves five and six were harvested and pooled from six plants. (A) Total soluble protein was extracted and applied to gels on an equal volume basis (20 μL). Proteins were separated by SDS-PAGE, electroblotted onto membranes, and incubated with polyclonal antibodies against ERD1 (1/1000 dilution) and BCB (1/2000 dilution). Molecular mass standards are indicated on the right in kDa. (B) 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. C, Control, nontreated plants; O_3, O_3-treated plants; Y, partially yellow plants; Gr, green plants; *E. coli* BCB, *E. coli* expressed partial BCB protein; *E. coli* ERD1, *E. coli* expressed partial ERD1 protein. Three replicate control samples and two of three replicate O_3 samples are shown.

When protein samples from leaves treated with 8 to 14 days of O_3 were analyzed and loaded on an equal protein basis (10 μg), less ERD1 protein was found in O_3-treated

samples than nontreated samples (*Figure 3–2*). ERD1 protein was detected on day 8 of the O_3 treatment, but levels declined during subsequent O_3 exposure until day 14 when ERD1 was no longer detected in O_3 samples. ERD1 protein remained detectable in all nontreated samples measured; however, levels declined as the leaves aged.

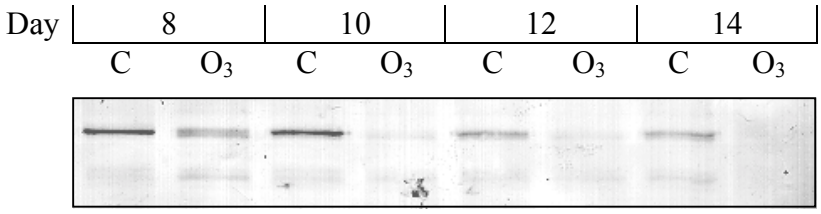

Figure 3–2: ERD1 protein levels decline during O_3-induced accelerated leaf senescence. Fifteen-day-old Arabidopsis plants were treated with 0.15 μL L^{-1} O_3 for 6 hours per day for 14 days. Total soluble protein was extracted from leaves five and six pooled from six plants and 10 μg of total protein were applied to gels. Proteins were separated by SDS-PAGE, electroblotted onto membranes, and incubated with a polyclonal antibody against ERD1 (1/1000 dilution). Only the region of the blot surrounding the 95-kDa ERD1 protein is shown. C, Control, nontreated plants; O_3, O_3-treated plants. Only one sample per treatment per sampling time was analyzed.

On day 14 of the O_3 treatment, faint BCB immunoreactive bands were detected in both O_3-treated and nontreated leaves (*Figure 3–3*). These leaves were harvested from the oldest plants measured at 29 days post germination and were beginning to show signs of senescence at the leaf tip. There was no discernible difference in BCB protein abundance between nontreated and O_3-treated samples. The BCB protein remained below the limits of detection in samples collected from younger leaf tissue, corresponding to days 10 and 12 of the O_3 exposure.

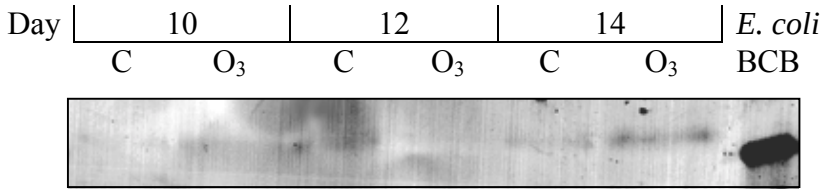

Figure 3–3: Immunoblot analysis of BCB protein in O_3-treated and nontreated leaves on days 10, 12 and 14 of the O_3 experiment. Fifteen-day-old Arabidopsis plants were treated with 0.15 μL L^{-1} O_3 for 6 hours per day for 14 days. Total soluble protein was extracted from leaves five and six pooled from six plants. Only one sample per treatment per sampling time was analyzed. Protein samples were applied to gels on an equal volume basis (20 μL), which corresponded to 34, 37, 34, 33, 32 and 31 μg in lanes 1–6, respectively. Proteins were separated by SDS-PAGE, electroblotted onto membranes, and incubated with polyclonal antibodies against BCB (1/500 dilution). Only the region of the blot surrounding the 33-kDa BCB protein is shown. C, Control, nontreated plants; O_3, O_3-treated plants; *E. coli* BCB, *E. coli* expressed partial BCB protein.

Discussion

Ozone stress in Arabidopsis induces a strong increase in transcript level of many genes associated with natural senescence (Miller et al., 1999). This work suggests that O_3 treatment induces the early onset of leaf senescence, due to the upregulation of these SAGs. This conclusion is based upon the assumption that increased transcription is followed by successful translation and protein activation. Study of transcript levels alone does not provide a complete picture of all cellular changes occurring during leaf senescence. Measured steady state transcript levels may not adequately reflect protein levels. In order to get an indication of whether the observed changes in transcript levels reflect changes in protein levels, the synthesis of two proteins was studied by western

analysis. Of the SAG transcripts induced during O_3 treatment (Miller et al., 1999), *ERD1* and *BCB* were chosen for study based upon the current availability of antibodies against the ERD1 and BCB proteins.

ERD1 protein was detectable in O_3-treated and nontreated tissue on day 8 at roughly equivalent levels, whereas *ERD1* transcript levels differed greatly in nontreated and O_3-treated samples. Further study showed that ERD1 protein levels declined during prolonged exposure to O_3. In contrast, *ERD1* mRNA in these same samples was first induced between day 2 and 4 of O_3 treatment and continued to show strong induction through day 14 (Miller et al., 1999).

This disparity between protein and transcript levels of ERD1 was found in naturally senescing leaf tissue (Weaver et al., 1999). The highest ERD1 protein levels were found in green, fully expanded leaves, while protein levels declined after the leaves reached full maturation and became undetectable in yellowing leaves (Weaver et al., 1999). Weaver et al. (1999) provided several possible explanations for the difference in ERD1 protein and mRNA levels: lack of ERD1 translation and/or highly unstable protein during senescence; fast translation and/or protein stability in young tissue. If ERD1 is involved in senescence, the protein may be active but extremely unstable thus requiring massive transcription to produce adequate amounts. If ERD1 is not involved in senescence, then the stress-like conditions of senescence may induce the expression of ERD1 due to its function in the response to stress (Weaver et al., 1999). The example of stress studied in this experiment, O_3, did not lead to coordinated transcript and protein accumulation, just as during natural senescence. Thus based on protein levels, it is

unclear whether ERD1 is functionally important in the response to stress. Further study of protein activities during treatment with other stresses would be required to determine the functional importance of ERD1 in the stress response.

During natural leaf senescence, BCB protein (Weaver et al., 1999) and mRNA (Weaver et al., 1998) first appeared in fully expanded, early senescent leaves and increased in abundance as the leaves turned yellow. In contrast, BCB mRNA was quickly induced in O_3-treated leaves (Miller et al., 1999) while the BCB protein remained undetectable until day 14 when a faint immunoreactive band was detected. Leaves were beginning to show signs of visible yellowing by the end of the 14-day O_3 study, but were not as yellow as leaves with the largest increase in BCB protein in the study by Weaver et al. (1999). While the plants used in this O_3 study were of similar age to those showing BCB protein in the Weaver et al. (1999) study, the plants were grown under very different conditions preventing a direct comparison based on age. Plants used by Weaver et al. (1999) were grown under continuous light in contrast to the plants used in this O_3 study, which were grown under 12 hours of light. In a different study with more similar light conditions, BCB protein was first immunodetected 33 days after germination in plants grown under 16 hours of light (Weaver et al., 1999). Our last harvest occurred 29 days after germination. Further study of later time points during O_3 treatment may have shown an increase in BCB protein. Regardless of this possibility, strong induction of *BCB* transcript by O_3 was not accompanied by increases in BCB protein.

This study of ERD1 and BCB protein levels by immunological analysis yielded questions regarding the importance of these SAGs in the senescence process. Ozone

stress causes transcript levels to increase sharply, but without a corresponding alteration of protein level. The reason for this disparity is not known at this time. The study of these two proteins did not provide adequate information to determine whether O_3-induced increases in SAGs, in general, correspond with increases in SAG protein products. Further study of additional SAG protein levels and activity is necessary to provide a better understanding of the functional importance of O_3-induced SAG expression.

Chapter 4

Ozone-Induced Senescence-Associated Gene Expression in Wild-Type Arabidopsis and the Ethylene-Insensitive *etr1-3* Mutant

Changes occurring during O_3-induced accelerated leaf senescence closely resemble those found during natural senescence, including loss of total protein, Rubisco and chlorophyll; increased leaf abscission; diminishing photosynthesis-associated gene (PAG) transcript levels and increased senescence-associated gene (SAG) expression (Pell and Pearson, 1983; Reich, 1983; Held et al., 1991; Nie et al., 1993; Bahl and Kahl, 1995; Conklin and Last, 1995; Glick et al., 1995; Miller et al., 1999). The signaling molecules leading to these changes during natural senescence may also be elicited by O_3. Once O_3 enters leaves through open stomata, it is either scavenged in the apoplastic fluid or reacts with the wall or membrane to produce reactive oxygen species (Kangasjärvi et al., 1994; Ranieri et al., 1999). Oxidative stress is closely associated with aging (Thompson et al., 1987) and the oxidative load on cells increases during O_3 treatment. Treatment with O_3 also causes ethylene emission to increase (Tingey et al., 1976) and an increase in salicylic acid (Yalpani et al., 1994; Sharma et al., 1996; Rao and Davis, 1999) and calcium levels (Castillo and Heath, 1990; Clayton et al., 1999).

Expression studies of senescence-related genes during natural senescence, hormone treatment and stress suggest that multiple signaling pathways are involved and

that the gene(s) invoked depends upon the particular conditions of each treatment (Gan and Amasino, 1997; Park et al., 1998; Weaver et al., 1998). Some SAGs induced by O_3 have also been induced by darkness, dehydration, and treatment with ethylene or ABA (Kiyosue et al., 1993; Nakashima et al., 1997; Weaver et al., 1998). Of the signaling molecules produced during O_3 stress capable of inducing SAG expression, ethylene is a likely candidate. Ozone treatment causes plants to produce ethylene and O_3-induced ACC synthase genes have been identified in Arabidopsis and potato plants (Schlagnhaufer et al., 1995; Vahala et al., 1998). The amount of ethylene produced during O_3 exposure can be correlated with the extent of leaf damage (Tingey et al., 1976; Mehlhorn and Wellburn, 1987; Mehlhorn et al., 1991; Tuomainen et al., 1997). Ozone-induced injury and drop in net photosynthesis can be reduced by treating plants with an inhibitor of ethylene biosynthesis, aminoethoxyvinylglycine, or an inhibitor of ethylene action, 2,5-norbonadiene (Taylor et al., 1988; Mehlhorn et al., 1991; Bae et al., 1996; Tuomainen et al., 1997). Treatment with ethylene inhibitors can also prevent O_3-induced changes in gene expression. In potato plants pretreated with the ethylene biosynthesis inhibitor aminooxyacetic acid, O_3-induced declines in *rbc*S message levels were diminished (Schlagnhaufer et al., 1995). Ethylene is also associated with senescence and has been shown to regulate the timing of leaf senescence in Arabidopsis (Grbic and Bleecker, 1995).

The purpose of this study was to determine whether SAGs are induced in an ethylene dependent manner during O_3 stress. Ethylene emission, *ACS6* transcript levels and SAG expression were measured in plants treated with two different O_3

concentrations. In addition, the requirement for ethylene as a signaling molecule during O_3 stress was determined by comparing SAG and PAG expression levels in wild-type and ethylene-insensitive Arabidopsis plants.

The ethylene-insensitive mutant, *etr1-3* (formerly *ein1-1*; Guzman and Ecker, 1990), was selected for study. The *etr1-3* mutant contains a dominant mutation in the *ETR1* gene due to a substitution of valine for alanine-31 (Hall et al., 1999). This mutation in the ethylene receptor reduces ethylene binding and prevents derepression of the signal transduction cascade, resulting in the ethylene-insensitive phenotype (Hall et al., 1999). The *etr1-3* mutant was identified in a screen of mutagenized seed for seedlings lacking the triple response to ethylene (Guzman and Ecker, 1990). In the presence of ethylene, dark-grown wild-type seedlings exhibit short hypocotyls and roots, tight apical hooks and thick hypocotyls. The root and hypocotyls of the *etr1-3* mutant continued to elongate and did not form an apical hook in the presence of 10 $\mu L\ L^{-1}$ ethylene for 3 days (Guzman and Ecker, 1990).

Material and Methods

Seeds of wild-type Arabidopsis ecotype Columbia (Col-0) and the *etr1-3* mutant (Guzman and Ecker, 1990) were grown as previously described (Miller et al., 1999). Half of the plants were treated with O_3 as described in Miller et al. (1999). Three different acute O_3 exposures were conducted: plants 23 days after germination (DAG) were treated with 0.15 $\mu L\ L^{-1}$ O_3 for 4 hours; plants 22 DAG were treated with 0.25 μL

L^{-1} O_3 for 3 hours and plants 19 DAG were treated with 0.35 μL L^{-1} O_3 for 6 hours. During a chronic O_3 exposure, plants 16 DAG were treated with 0.15 μL L^{-1} O_3 for 6 hours per day for 14 days.

Ethylene emission was measured from rosettes sealed in 5.5 mL or 19 mL vials with 0.5-1.0 mL water for 2-3 hours. The ethylene concentration in the headspace was measured according to Arteca (1982) with a Hewlett Packard 5830 dual column gas chromatograph. Aminocyclopropane-1-carboxylic acid (ACC) was extracted from 0.05-0.1 g of leaf tissue and analyzed according to Arteca and Arteca (1999).

Transcript levels were measured by northern analysis as described in Miller et al. (1999) with the following exceptions. Following hybridization with radiolabeled probes, membranes were exposed to phosphor screens (Molecular Dynamic, Sunnyvale, CA). To obtain quantitative data, the screens were scanned with Molecular Dynamics PhosphorImager (Sunnyvale, CA) and images were analyzed with Molecular Dynamics ImageQuant software (Sunnyvale, CA). The total pixel values (counts) in equal-sized areas were calculated and corrected for loading differences by dividing with the counts obtained with the rRNA probe (Jorgenson et al., 1982). Transcript levels for *ACS6*, *BCB*, *ERD1*, *CCH*, *SAG13*, *SAG18*, *SAG20*, *SAG21*, *rbc*S and *cab* were measured.

Results

In studying the relationship between ethylene and SAG expression, wild-type and *etr1-3* plants were treated with three different O_3 concentrations for 3-4 hours to

determine the concentration required to cause measurable ethylene production. Ethylene emission was measured by gas chromatography in wild-type and *etr1-3* rosettes treated with 0.15 μL L^{-1} O_3, 0.25 μL L^{-1} O_3 and 0.35 μL L^{-1} O_3 (*Figure 4–1*). Ethylene emission increased with increasing O_3 concentration in both wild-type and *etr1-3* plants.

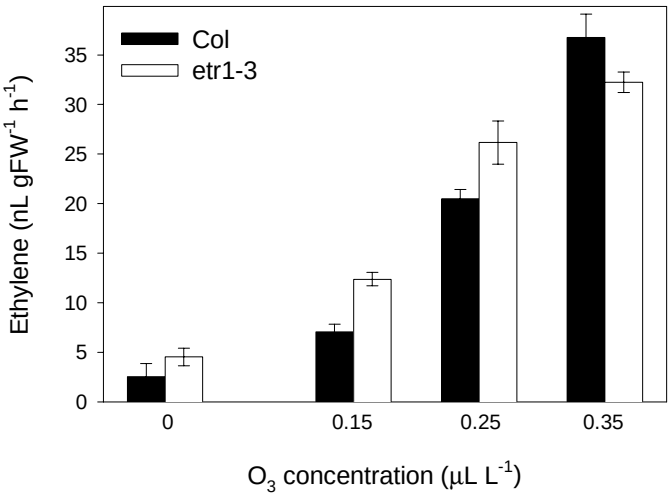

Figure 4–1: Ozone-induced ethylene production in wild-type and *etr1-3* plants. At 23, 22 and 19 days after germination, plants were treated with 0.15 μL L^{-1} O_3 for 4 hours, 0.25 μL L^{-1} O_3 for 3 hours and 0.35 μL L^{-1} O_3 for 3 hours, respectively. Ethylene production from rosettes was measured by gas chromatography. Each point represents the mean of three samples ± SE.

In order to determine whether ethylene production and perception are required for O_3-induced SAG expression, wild-type and *etr1-3* plants were treated with 0.15 μL L^{-1} O_3 for 6 hours per day for 14 days. This treatment caused all leaves to roll downward

and the oldest leaves to yellow in both wild-type and *etr1-3* (*Figure 4–2*). These responses were previously seen in Arabidopsis ecotype Landsberg *erecta* (Ler) treated with the same O_3 exposure conditions (Miller et al., 1999). The O_3 treated wild-type and *etr1-3* plants in this experiment, however, did not show the same degree of leaf yellowing as was observed in Ler.

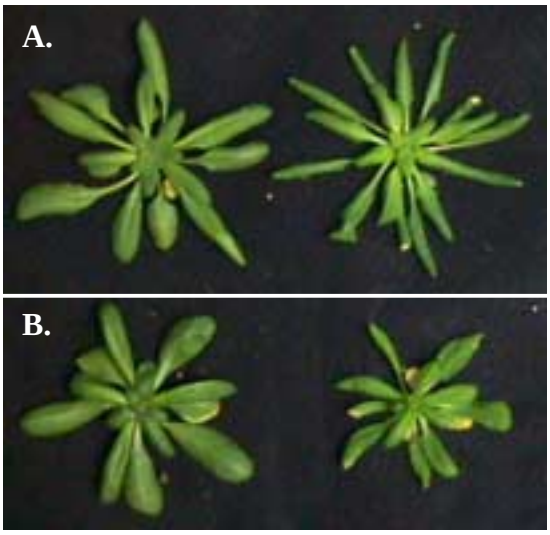

Figure 4–2: Photograph showing O_3-induced leaf curling and yellowing in wild-type and *etr1-3* plants. Sixteen-day-old plants were treated with 0.15 μL L^{-1} O_3 for 6 hours per day for 14 days. Nontreated plants are shown on the left and O_3-treated plants on the right. (A) Wild-type plants. (B) *etr1-3* plants.

Ethylene production was measured in rosettes excised at the end of the 6-hour exposure every 2 days during the 14-day treatment. This O_3 exposure did not cause an increase in ethylene emission in O_3-treated plants as compared to nontreated plants (*Figure 4–3A*). Ethylene emission was higher in control and O_3-treated *etr1-3* plants compared to control and O_3-treated wild-type plants. When levels of the ethylene precursor ACC were measured, no differences were detected between nontreated and O_3-

treated plants (*Figure 4–3B*). Some sampling days show higher ACC levels in O_3-treated plants, but the variability was high and no clear trends emerged. Message level for one isoform of ACC synthase, *ACS6*, responsible for the conversion of ACC to ethylene did not show any appreciable difference between nontreated and O_3-treated plants (*Figure 4–3C*).

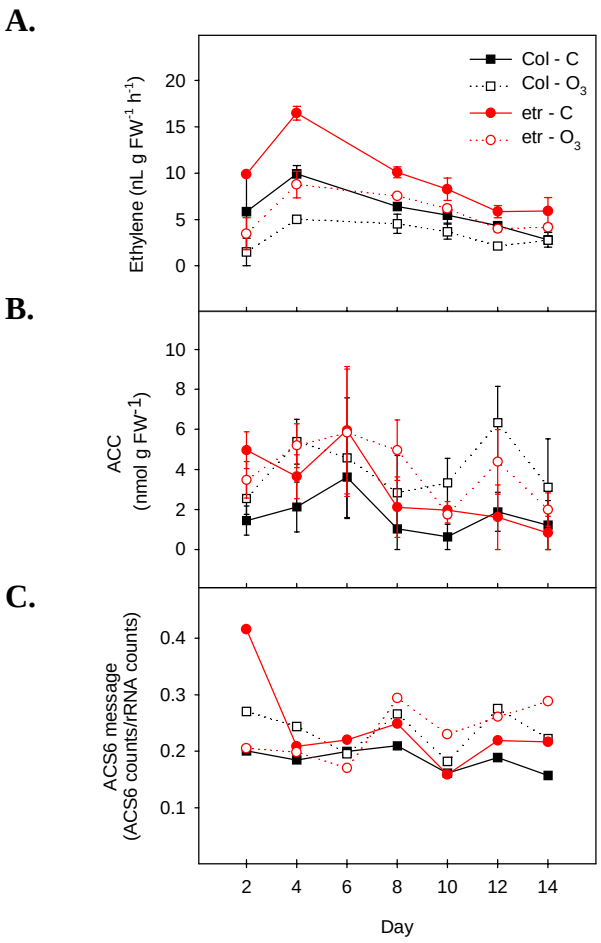

Figure 4–3: Ethylene emission, ACC levels and *ACS6* mRNA levels in O_3-treated wild-type and *etr1-3* plants. Plants were exposed to 0.15 µL L^{-1} O_3 for 6 hours per day for 14 days. (A) Ethylene emission from rosettes. Each point represents the mean of three samples ± SE. (B) ACC levels in the fifth and sixth leaves pooled from five plants. Each point represents the mean of three samples ± SE. (C) *ACS6* transcript levels in the fifth and sixth leaves pooled from five plants. Total RNA was extracted and 6 µg of RNA was separated on 1% formaldehyde-agarose gels, transferred to membranes, and hybridized with radiolabeled ACS6 probe. The radioactive bands were quantified using a PhosphorImager and the data shown were corrected for loading differences using the counts obtained with the rRNA probe. The samples shown are one representative replicate of two. C, Control, nontreated plants; O_3, O_3-treated plants.

Despite a lack of change in ethylene production, ACC levels and *ACS6* message, the majority of SAGs studied were induced by O_3. The degree of gene induction varied depending upon the particular SAG (*Figure 4–4*). Transcript levels of *SAG13* and *SAG20* gradually increased in O_3-treated wild-type plants, while only background levels were detected in nontreated plants. By day 14, *SAG13* and *SAG20* message levels were 3 to 4-fold higher in O_3-treated wild-type leaves. *BCB* transcript levels were higher in O_3-treated plants compared to nontreated plants. While *ERD1* message levels increased during the experiment in both O_3-treated and nontreated wild-type plants, levels were generally higher in O_3-treated leaves. *SAG21* transcript levels in O_3-treated wild-type leaves were approximately twice the levels found in nontreated leaves throughout the exposure. The expression of two SAGs was not induced by this O_3 treatment in wild-type plants (*Figure 4–5*); *SAG18* and *CCH* transcript levels gradually increased in abundance through the 14-day exposure in nontreated and O_3-treated leaves.

A similar pattern of SAG induction occurred in O_3-treated *etr1-3* plants. *BCB*, *ERD1*, *SAG13*, *SAG20* and *SAG21* were all induced by O_3 in *etr1-3* leaves. A slight delay in *SAG13* and *SAG21* induction was found in the replicate shown; however, the other replicate shows less difference between *SAG13* and *SAG21* transcript levels in O_3-treated wild-type and O_3-treated *etr1-3* plants. In addition, *SAG18* and *CCH* were not induced by O_3 in *etr1-3* plants.

Ozone stress causes a decrease in *rbc*S and *cab* message levels (Miller et al., 1999). Transcript levels for these PAGs declined with age in both O_3-treated and nontreated wild-type and *etr1-3* leaves (*Figure 4–6*). Ozone exposure caused a

pronounced decrease in *rbc*S transcript levels in both wild-type and *etr1-3*. Less *cab* transcript was found in O_3-treated wild-type and *etr1-3* plants; however, the decline was not as great as was found for *rbc*S transcript levels.

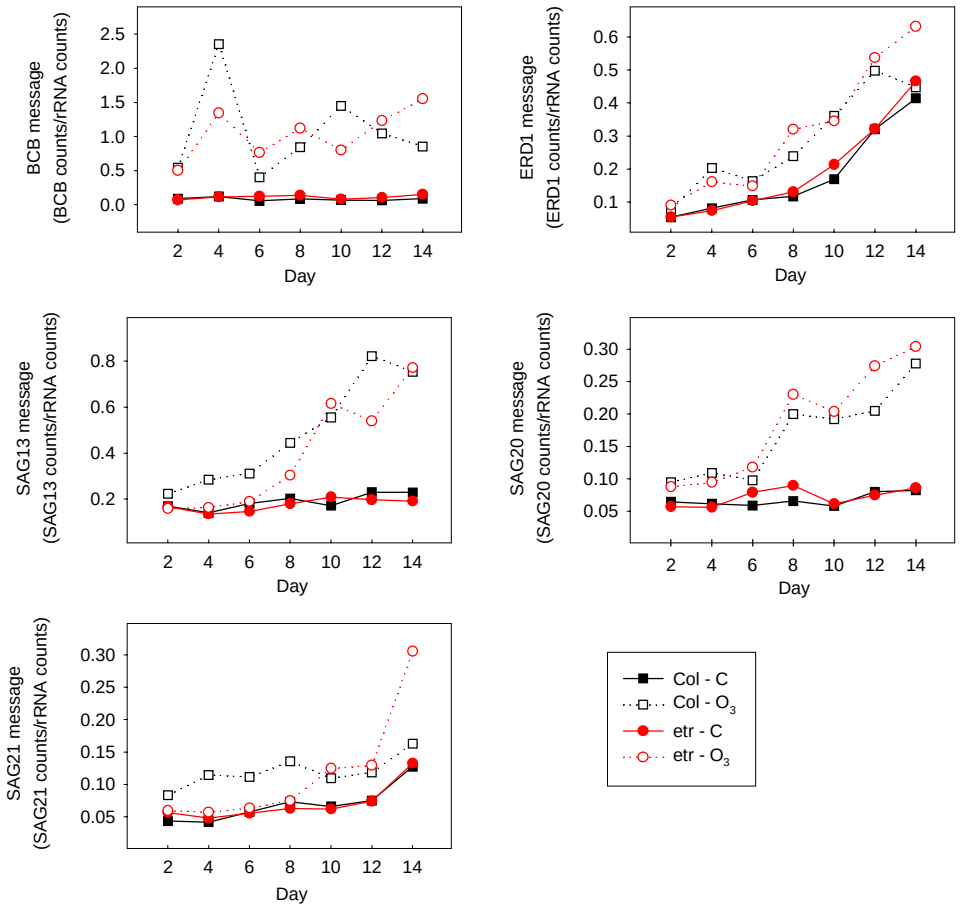

Figure 4–4: Ozone-induced *BCB*, *ERD1*, *SAG13*, *SAG20* and *SAG21* expression in wild-type and *etr1-3* plants. Sixteen-day-old plants were treated with 0.15 µL L^{-1} O_3 for 6 hours per day for 14 days. Total RNA was extracted from the fifth and sixth leaves pooled from five plants. Samples were prepared as in *Figure 4-3*. The samples shown are one representative replicate of two. C, Control, nontreated plants; O_3, O_3-treated plants.

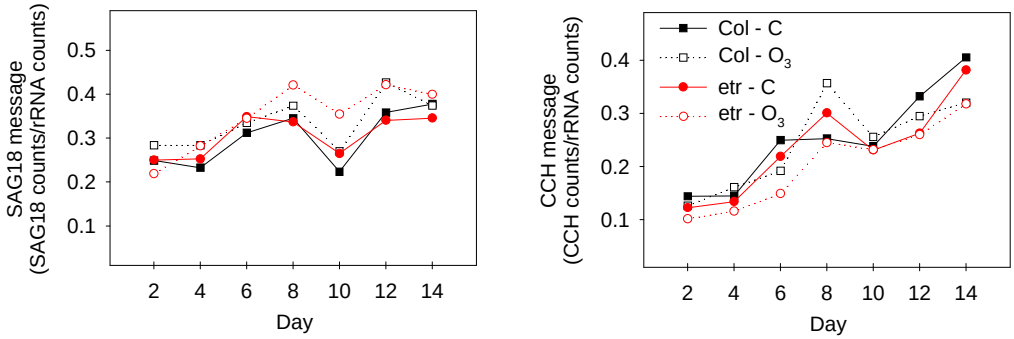

Figure 4–5: *SAG18* and *CCH* transcript levels in wild-type and *etr1-3* plants were not altered by treatment with O_3. Sixteen-day-old plants were treated with 0.15 µL L^{-1} O_3 for 6 hours per day for 14 days. Total RNA was extracted from the fifth and sixth leaves pooled from five plants. Samples were prepared as in *Figure 4-3*. The samples shown are one representative replicate of two. C, Control, nontreated plants; O_3, O_3-treated plants.

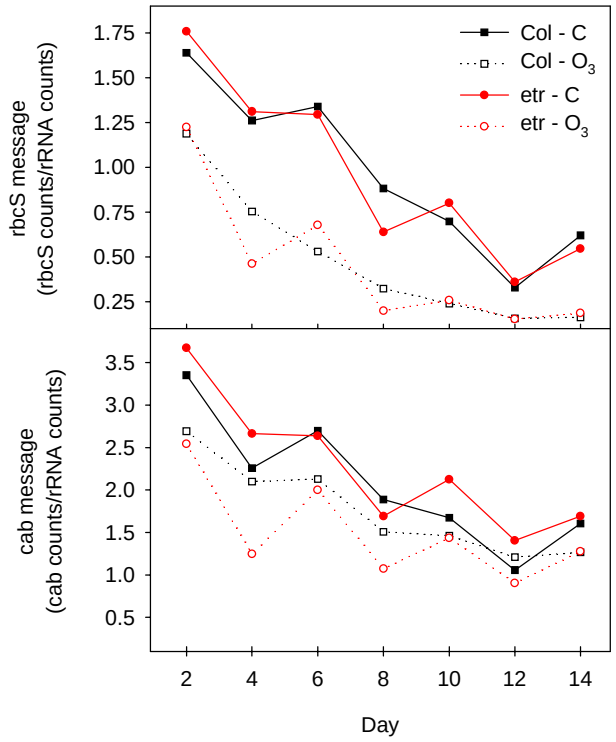

Figure 4–6: Decline in *rbc*S and *cab* transcript levels in wild-type and *etr1-3* plants treated with O_3. Sixteen-day-old plants were treated with 0.15 µL L^{-1} O_3 for 6 hours per day for 14 days. Total RNA was extracted from the fifth and sixth leaves pooled from five plants. Samples were prepared as in *Figure 4-3*. The samples shown are one representative replicate of two. C, Control, nontreated plants; O_3, O_3-treated plants.

Due to the lack of measurable ethylene production in the previous experiment, SAG and PAG expression were measured after an acute exposure to 0.35 µL L^{-1} O_3, a concentration great enough to induce ethylene production in wild-type and *etr1-3* plants. This high level of O_3 stress caused some immediate cell collapse (*Figure 4–7*), which resulted in dead, necrotic regions days later. This acute treatment caused measurable

ethylene emission in both wild-type and *etr1-3* plants (*Figure 4–8*). Ethylene emission peaked between 2-3 hours in wild-type plants and *etr1-3* mutants. The greatest *ACS6* message levels in wild-type plants and *etr1-3* mutants occurred after 1 hour of O_3 treatment. Transcript levels were lower at 3 and 6 hours, but still remained elevated compared to nontreated leaves.

Figure 4–7: Photograph showing some cell collapse in wild-type and *etr1-3* plants following acute O_3 treatment. Nineteen-day-old plants were treated with 0.35 μL L^{-1} O_3 for 6 hours. Nontreated plants are shown on the left and O_3-treated plants on the right. (A) Wild-type plants. (B) *etr1-3* plants.

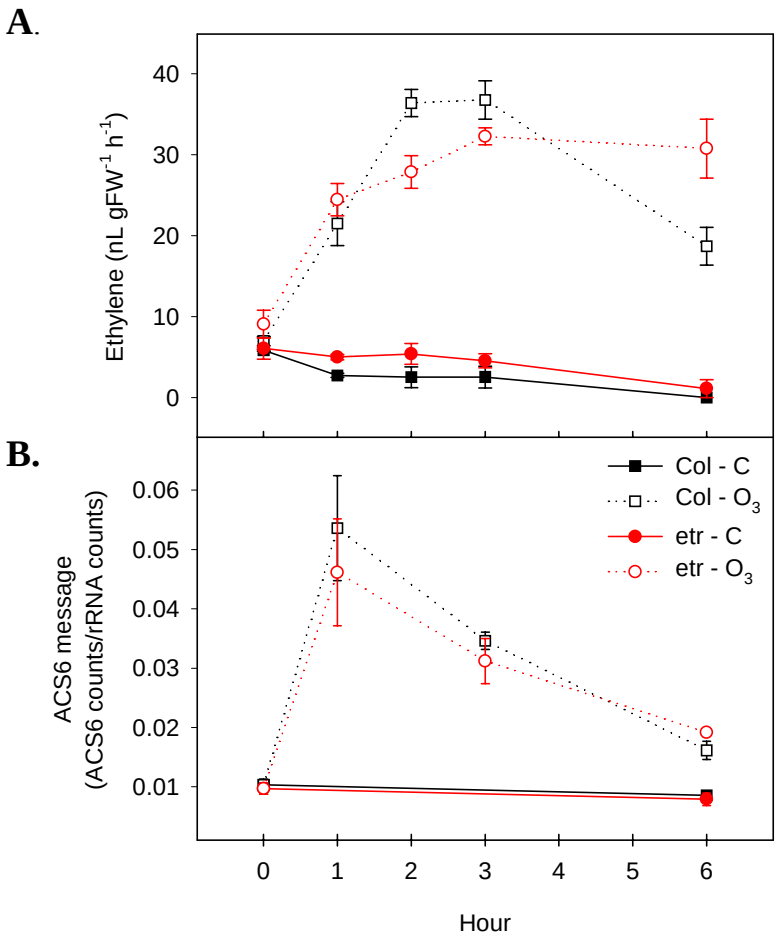

Figure 4–8: Ethylene emission and *ACS6* transcript levels in wild-type and *etr1-3* plants during acute O_3 exposure. Nineteen-day-old plants were treated with 0.35 µL L^{-1} O_3 for 6 hours. (A) Ethylene emission from rosettes. Each point represents the mean of three samples ± SE. (B) *ACS6* transcript levels in the fifth and sixth leaves pooled from six plants. Samples were prepared as in *Figure 4-3*. Each point represents the mean of three samples ± SE. C, Control, nontreated plants; O_3, O_3-treated plants.

SAG and PAG expression levels were determined during the acute O_3 exposure as well. Of the seven SAGs examined, *ERD1*, *BCB*, *SAG13*, *SAG20* and *SAG21* were strongly induced by O_3 in wild-type plants (*Figure 4–9*). *SAG18* showed some induction, while *CCH* showed no O_3 induction (*Figure 4–9*). Of the SAGs showing strong induction, the pattern of induction varied. The highest *SAG20* and *SAG21* transcript levels occurred within 1 hour of O_3 treatment and then levels declined. *ERD1* and *BCB* transcript levels did not peak until 3 hours into the treatment and declined after 6 hours. In contrast, *SAG13* gradually increased throughout the 6-hour exposure. SAG expression levels in O_3-treated *etr1-3* plants closely resembled the pattern found in O_3-treated wild-type plants.

A sharp decline in *rbc*S and *cab* transcript levels were found in O_3-treated wild-type plants (*Figure 4–10*). The largest decrease in transcript level occurred between 1 and 3 hours of O_3 treatment. A similar pattern was found in O_3-treated *etr1-3* leaves.

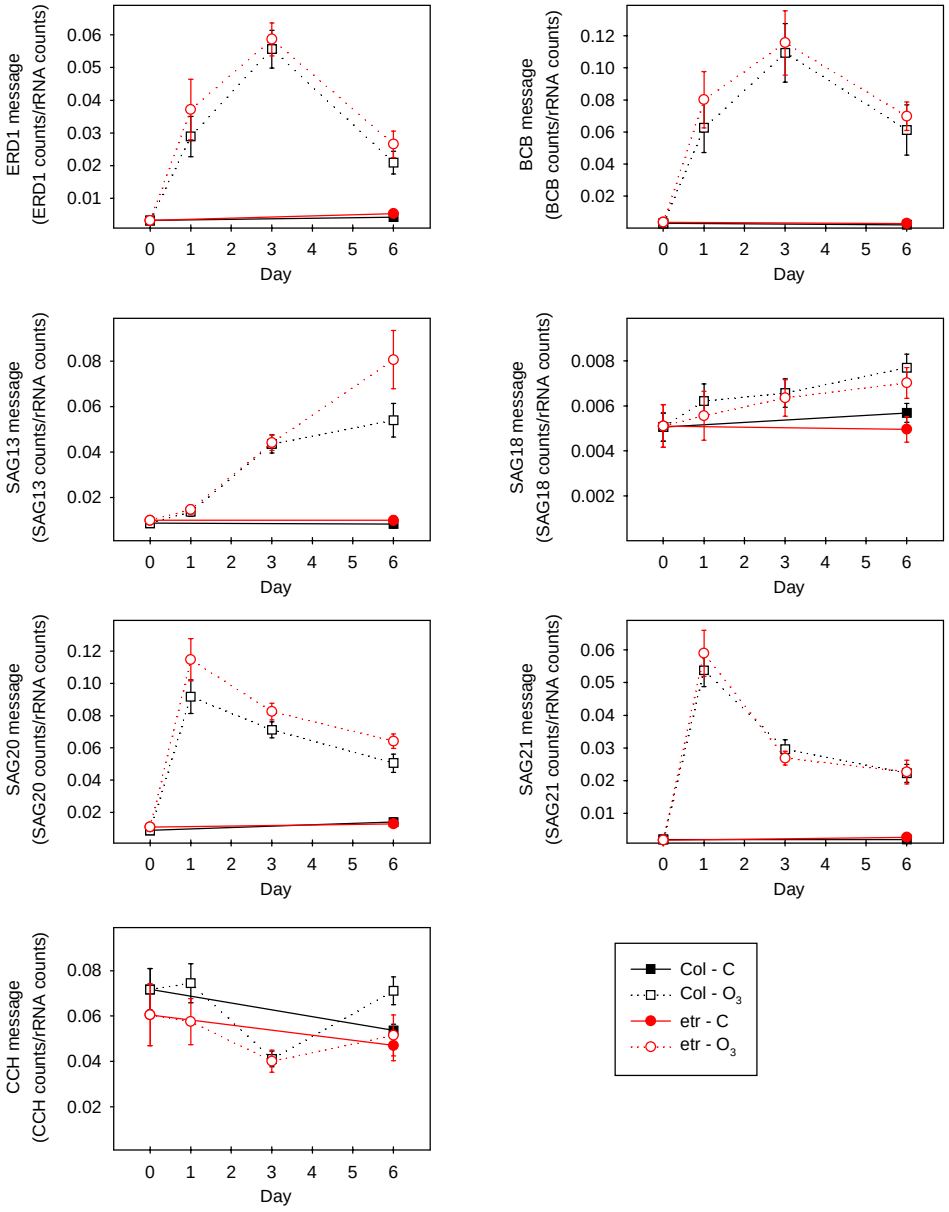

Figure 4–9: *ERD1*, *BCB*, *SAG13*, *SAG18*, *SAG20*, *SAG21* and *CCH* transcript levels in wild-type and *etr1-3* plants during acute O_3 exposure. Nineteen-day-old plants were treated with 0.35 µL L^{-1} O_3 for 6 hours. Total RNA was extracted from the fifth and sixth leaves pooled from six plants. Samples were prepared as in *Figure 4-3*. Each point represents the mean of three samples $\pm$ SE. C, Control, nontreated plants; O_3, O_3-treated plants.

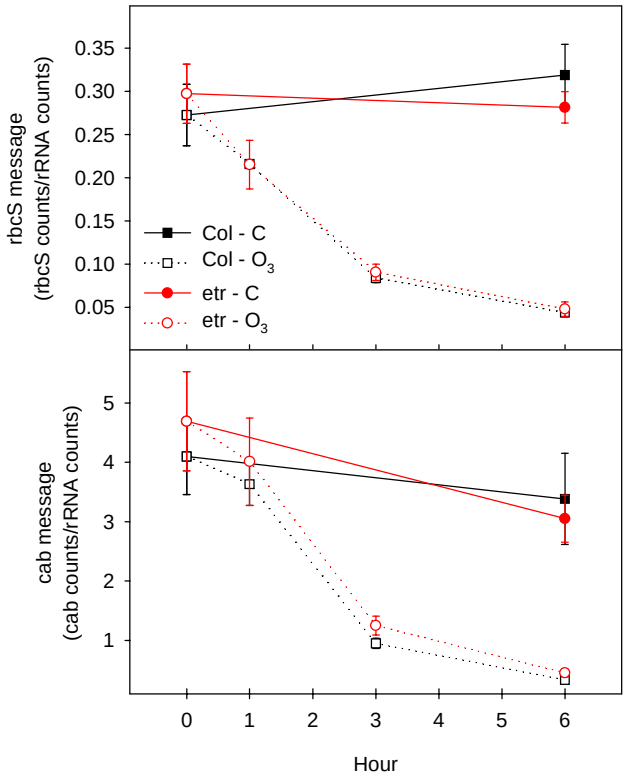

Figure 4–10: Decline in *rbc*S and *cab* transcript levels in wild-type and *etr1-3* plants during acute O_3 exposure. Nineteen-day-old plants were treated with 0.35 µL L^{-1} O_3 for 6 hours. Total RNA was extracted from the fifth and sixth leaves pooled from six plants. Samples were prepared as in *Figure 4-3*. Each point represents the mean of three samples $\pm$ SE. C, Control, nontreated plants; O_3, O_3-treated plants.

Discussion

A wide variation in ecotype sensitivity to O_3 exists in Arabidopsis and in a survey of 36 ecotypes three were identified as extremely sensitive to O_3 (Sharma and Davis,

1997). The work presented in this chapter revealed that Col-0 plants were less sensitive to O_3 as compared to Ler plants. In a previous study, eight of twelve SAGs were induced by chronic O_3 treatment of Ler (Miller et al., 1999). The chronic O_3 exposure of Col-0 induced only five of these SAGs. In addition, older Col-0 leaves did not exhibit as much yellowing as was observed in Ler plants on day 14 of the O_3 treatment.

The induction of SAGs had previously only been studied during chronic O_3 exposure (Miller et al., 1999), except for *BCB*, which was induced within a 3-hour exposure to 0.30 μL L^{-1} O_3 (Richards et al., 1998). The work presented here shows that short-term O_3 exposures of very high concentrations can cause the induction of *ERD1*, *BCB*, *SAG13*, *SAG20* and *SAG21*. The significance of SAG induction during acute exposure and the early onset of leaf senescence is not known.

The goal of this work was to determine whether the hormone ethylene acts as a signaling molecule to increase SAG expression during O_3 stress. Wild-type and ethylene-insensitive Arabidopsis plants were treated with O_3, and SAG transcript levels were determined. It was hypothesized that O_3-induced SAG expression would not occur in ethylene-insensitive plants, or would be greatly reduced in ethylene-insensitive plants as compared to wild-type plants. Two different O_3 exposures were conducted; the first was a low-level chronic exposure for 14 days and the second was a high-level acute exposure for 6 hours.

BCB, *ERD1*, *SAG13*, *SAG20* and *SAG21* were induced by 0.15 μL L^{-1} O_3 for 6 hours per day for 14 days in wild-type plants without any measurable ethylene production, increase in ACC levels or *ACS6* transcript. These findings suggest that

ethylene production was not required for O_3-induced SAG expression to occur. *BCB*, *ERD1*, *SAG13*, *SAG20* and *SAG21* were also induced by the chronic O_3 treatment of the ethylene-insensitive mutant, *etr1-3*, providing further evidence that ethylene is not required for O_3-induced SAG expression. The results of this experiment do not support the hypothesis that ethylene is a primary signaling molecule during O_3-induced SAG expression.

While ethylene production is not necessary for O_3-induced SAG expression to occur, the possibility exists that ethylene may alter the degree of SAG induction during O_3 stress. The hypothesis that SAG expression levels would be lower in *etr1-3* mutants compared to wild-type plants in the presence of O_3-induced ethylene emission was tested. In order to determine whether ethylene might act as a secondary signal to further modulate the level of SAG induction, the experiment was repeated at a higher O_3 concentration known to cause a large increase in ethylene emission. Wild-type plants and *etr1-3* mutants were treated with 0.35 $\mu L\ L^{-1}$ O_3 for 6 hours and SAG and PAG transcript levels were measured at 1, 3 and 6 hours. This acute O_3 exposure caused a sharp increase in *ERD1*, *BCB*, *SAG13*, *SAG20* and *SAG21* transcript levels and a dramatic decline in *rbc*S and *cab* message levels in wild-type plants. These changes in SAG and PAG expression were also induced by O_3 in *etr1-3* plants with nearly identical expression patterns. SAG expression was never lower in O_3-treated *etr1-3* plants compared to O_3-treated wild-type plants. Since the lack of ethylene perception did not prevent the induction of SAGs or decline in PAGs, ethylene does not appear to have an important role as a secondary signal in O_3-induced SAG expression.

These experiments cannot completely rule out a role for ethylene during O_3-induced SAG expression, since the *etr1-3* mutant has been shown to be partially responsive to ethylene (Hall et al., 1999). Ethylene binding in yeast expressing the *etr1-3* protein was not completely abolished, but equal to 23% of the binding activity measured in yeast expressing wild-type *ETR1* (Hall et al., 1999). In an ethylene dose-response curve, seedling growth was 10% of the wild type in the hypocotyl and 23% of the wild type in the root (Hall et al., 1999). Mature leaves of the *etr1-3* mutant might also retain some sensitivity to ethylene. ETR1 is one member of a small family of ethylene receptors. It is possible that other ethylene receptors allow for ethylene responsiveness in mature Arabidopsis leaves. For example, ethylene responsiveness in the *etr1-1* mutant was restored by increasing the wild-type allele copy number (Hall et al., 1999). In a study of the ethylene-insensitive *Never-ripe* (*Nr*) mutant of tomato, the fruits were shown to be ethylene insensitive while leaf tissue remained responsive (Appendix A). When *Nr* plants were treated with O_3, the leaves produced similar amounts of ethylene and showed the same signs of leaf injury as the wild-type tomato plants (Appendix A). There are several different ethylene receptors in tomato and they are not expressed at the same levels under all conditions (Lashbrook et al., 1998).

Ethylene is not required for O_3-induced SAG expression, but due to the partial ethylene response in *etr1-3* mutants further study is needed to declare that ethylene does not contribute to the increased expression of SAGs during O_3 treatment. SAG transcript levels need to be measured in additional ethylene-insensitive mutants treated with O_3.

Ozone exposures of *etr1-3* and three other ethylene-insensitive mutants were conducted and SAG transcript levels were measured and the results are presented in chapter 5.

Chapter 5

Ozone-Induced Senescence-Associated Gene Expression Does Not Require Ethylene Perception in Arabidopsis

Ozone induces accelerated leaf senescence as discussed in chapter 2. Ethylene is a plant hormone frequently associated with senescence (Matoo and Aharoni, 1988) and has been shown to regulate the timing of leaf senescence by the delayed leaf senescence observed in the ethylene-insensitive mutant *etr1-1* (Grbic and Bleecker, 1995). Plants producing the largest quantities of ethylene during exposure to O_3 exhibit the greatest leaf injury (Tingey et al., 1976; Mehlhorn and Wellburn, 1987; Mehlhorn et al., 1991; Tuomainen et al., 1997). Whether ethylene emission has any regulatory function in O_3-induced leaf senescence remains an unanswered question.

The results presented in chapter 4 suggest that ethylene perception is not required for O_3-induced senescence-associated gene (SAG) expression. Equivalent levels of SAG message were found in O_3-treated wild-type plants and ethylene-insensitive *etr1-3* mutants. Since SAG expression was induced without the perception of ethylene in *etr1-3* mutants, ethylene does not appear to be the signaling molecule causing O_3-induced SAG expression. This conclusion was based upon the assumption that leaves of the *etr1-3* mutant are completely ethylene insensitive. The *etr1-3* mutant was identified at the seedling stage by its lack of the triple response to ethylene (Guzman and Ecker, 1990).

Ethylene binding is severely reduced in yeast expressing the mutant etr1-3 protein, but not completely abolished resulting in only partial ethylene insensitivity in *etr1-3* seedlings (Hall et al., 1999). Consequently, the possibility that ethylene was involved in the regulation of O_3-induced SAG expression could not be eliminated. In addition, the ethylene receptor system in Arabidopsis is composed of a family of genes with differing expression levels during development, in different tissues and with ethylene treatment (Hua and Meyerowitz, 1998; Hua et al., 1998).

Because *etr1-3* does not completely eliminate perception of ethylene there is still the possibility that this hormone plays a part in O_3-induced accelerated leaf senescence. Additional evidence that ethylene regulates responses to O_3 came from experiments with ethylene inhibitors in which pretreatment with inhibitors prevented some of the detrimental effects of O_3 (Taylor et al., 1988; Mehlhorn et al., 1991; Schlagnhaufer et al., 1995; Bae et al., 1996; Tuomainen et al., 1997).

To further determine whether ethylene perception was required for O_3-induced SAG expression, SAG transcript levels were analyzed in wild-type plants and several ethylene-insensitive mutants following treatment with O_3. Of the available ethylene-insensitive mutants in Arabidopsis, four were selected for this study based upon their delay in dark-induced yellowing of O_3-treated leaves (Appendix B). The mutations occur in different components of the ethylene signal transduction pathway: two ethylene receptor mutants, *etr1-3* and *ein4-1*, and two down-stream signaling component mutants, *ein2-1* and *ein3-1*. The *etr1-3* mutant was included in this study to allow for comparisons to previous experiments presented in chapter 4. The ethylene responsiveness of leaves in

these mutants was confirmed by measuring transcript levels for an ethylene-inducible gene following ethylene treatment. To further understand the relationship between ethylene and SAG expression during O_3 exposure, the ability of ethylene to independently induce SAG expression was also studied.

Material and Methods

Seeds of wild-type Arabidopsis ecotype Columbia (Col-0) and ethylene-insensitive mutants *etr1-3*, *ein2-1*, *ein3-1* and *ein4-1* (Guzman and Ecker, 1990; Kieber et al., 1993; Roman et al., 1995) were grown as previously described (Miller et al., 1999). Plants were treated with 0.15 μL L^{-1} or 0.30 μL L^{-1} O_3 for 3.5 hours as described in Miller et al. (1999). Rosettes were harvested for analysis of ethylene emission and transcript levels at 1 and 3.5 hours.

Ethylene treatment was delivered to three-week-old plants enclosed in airtight chambers (Nalgene, Rochester, NY) modified for continuous gas flow-through. Plants were treated with certified standard mixture balanced air or 100 μL L^{-1}ethylene in balanced air (Messer MG Industries, Morrisville, PA) concurrently in separate chambers for 6 hours. Gas was delivered at a rate of one air exchange every 10-20 minutes in five replicate experiments. Fluorescent lights were placed above the two chambers, which provided 55-70 μmol m^{-2} s^{-1} inside the chambers. Rosettes were harvested at 3 and 6 hours for analysis of transcript levels. The ethylene responsiveness of wild-type and ethylene-insensitive mutants *etr1-3*, *ein2-1*, *ein3-1* and *ein4-1* was determined by treating

plants in flow-through chambers with 100 μL L^{-1} ethylene and analyzing *HEL* (hevein-like) transcript levels. Ethylene treatment has been shown to induce the expression of *HEL* in mature leaves (Potter et al., 1993), thus the induction of this transcript was selected as the marker for ethylene responsiveness in this study.

Ethylene emission was measure by gas chromatography from rosettes sealed in 19 mL vials with 0.5-1.0 mL water for 2-3 hours. The ethylene concentration in the headspace was measured with a Hewlett Packard 6890 Series Gas Chromatography System with a Hewlett Packard 3396 Series III Integrator (Palo Alto, CA).

Transcript levels of *ACS6*, *BCB*, *ERD1*, *SAG13*, *SAG20* and *SAG21* were measured by northern analysis as described in Miller et al. (1999) with the following exceptions. Following hybridization with radiolabeled probes, membranes were exposed to phosphor screens (Molecular Dynamic, Sunnyvale, CA). To obtain quantitative data, the screens were scanned with Molecular Dynamics PhosphorImager (Sunnyvale, CA) and images were analyzed with Molecular Dynamics ImageQuant software (Sunnyvale, CA). The total pixel values (counts) in equal-sized areas were calculated and corrected for loading differences by dividing with the counts obtained with the rRNA probe (Jorgenson et al., 1982).

Results

Ethylene treatment caused *HEL* transcript levels to increase in wild-type, *etr1-3* and *ein3-1* plants, while no induction was detected in *ein2-1* and *ein4-1* plants (*Figure 5–1*). After 6 hours, *HEL* message levels in ethylene-treated wild-type plants were double levels found in air-treated wild-type plants (*Figure 5–1B*). A less than twofold increase in *HEL* transcript levels was found in ethylene-treated *etr1-3* and *ein3-1* plants compared to air-treated plants after 6 hours (*Figure 5–1B*).

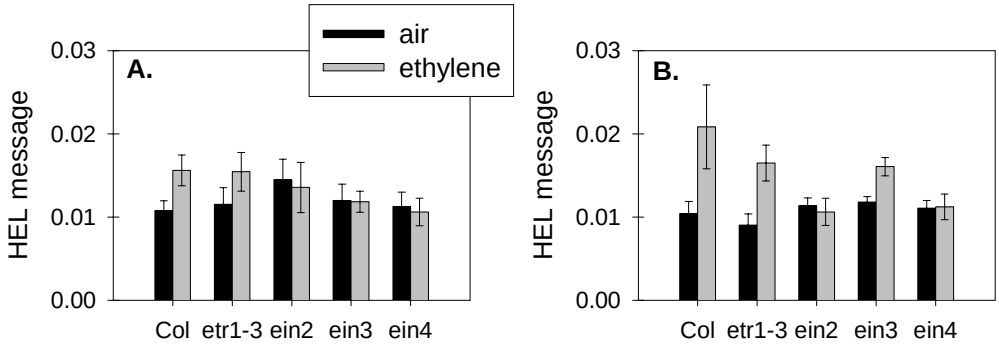

Figure 5–1: *HEL* transcript levels in ethylene- and air-treated wild-type, *etr1-3*, *ein2-1*, *ein3-1* and *ein4-1* plants. Three-week-old plants were treated with air or 100 μL L^{-1} ethylene for 3 or 6 hours. (A) *HEL* transcript levels after 3 hours of treatment. (B) *HEL* transcript levels after 6 hours of treatment. Total RNA was extracted from rosettes and 6 μg of RNA was separated on 1% formaldehyde-agarose gels, transferred to membranes, and hybridized with radiolabeled HEL probe. The radioactive bands were quantified using a PhosphorImager and the data shown were corrected for loading differences using the counts obtained with the rRNA probe. Each bar represents the mean of five samples ± SE.

To determine whether ethylene production and perception are required for O_3-induced SAG expression, wild-type and ethylene-insensitive mutants were treated with 0.15 μL L^{-1} O_3 for 3.5 hours. Rosettes were harvested and ethylene emission and SAG transcript levels were measured at 1 and 3.5 hours. Treatment with 0.15 μL L^{-1} O_3 for 3.5 hours caused ethylene emission to increase in wild-type and *etr1-3* plants (*Figure 5–2A-B*). A slight increase in ethylene emission was detected in O_3-treated *ein2-1* and *ein3-1* plants, while no change was found in O_3-treated *ein4-1* plants (*Figure 5–2A-B*). *ACS6* transcript levels were induced in all plants with 1 hour of O_3 treatment (*Figure 5–2C*). After 3.5 hours, *ACS6* transcript levels were lower in O_3-treated plants compared to the 1-hour time point (*Figure 5–2D*), yet levels in O_3-treated wild-type, *etr1-3* and *ein2-1* plants were still greater than levels in nontreated plants (*Figure 5–2D*). *ACS6* transcript levels and ethylene emission in O_3-treated and nontreated plants did not correlate at the 1-hour sampling time, but by 3.5 hours similar patterns were found.

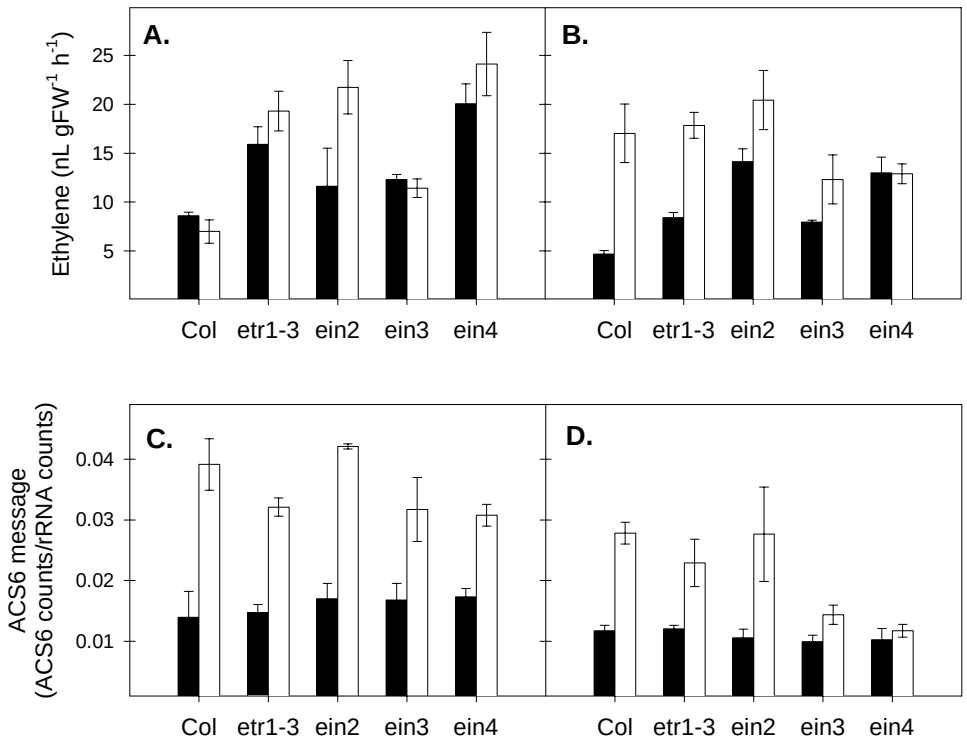

Figure 5–2: Ethylene emission and *ACS6* transcript levels in wild-type, *etr1-3*, *ein2-1*, *ein3-1* and *ein4-1* plants treated with 0.15 μL L^{-1} O_3. Twenty-two-day-old plants were treated with 0.15 μL L^{-1} O_3 for 3.5 hours. (A) Ethylene emission from rosettes at 1 hour. (B) Ethylene emission at 3.5 hours. (C) *ACS6* transcript levels at 1hour. (D) *ACS6* transcript levels at 3.5 hours. Total RNA was extracted from rosettes and prepared as in *Figure 5-1*. Black bars, nontreated plants; white bars, O_3-treated plants. Each bar represents the mean of three samples ± SE.

Treatment with 0.15 μL L^{-1} O_3 caused an increase in *BCB*, *ERD1*, *SAG20* and *SAG21* transcript levels in wild-type and all ethylene-insensitive mutants within 1 hour (*Figure 5–3A*). *SAG13* transcript levels increased in O_3-treated wild-type and *etr1-3*

plants, but only a slight induction was found in *ein2-1* and *ein3-1* plants and no induction was found in *ein4-1* plants (*Figure 5–3A*). The increase in SAG transcript levels occurred without an increase in ethylene emission in wild-type, *etr1-3*, *ein3-1* and *ein4-1* plants (*Figure 5–2A*). Ozone-induced ethylene emission was only found in *ein2-1* after 1 hour of treatment (*Figure 5–2A*). After 3.5 hours, transcript levels for *BCB*, *ERD1*, *SAG20* and *SAG21* were lower in O_3-treated plants than at the 1-hour time point (*Figure 5–3B*). While all SAGs, except *SAG13*, were induced in the ethylene-insensitive mutants, transcript levels for *BCB*, *ERD1* and *SAG21* were slightly lower in *ein4-1* mutants compared to wild-type plants (*Figure 5–3A*).

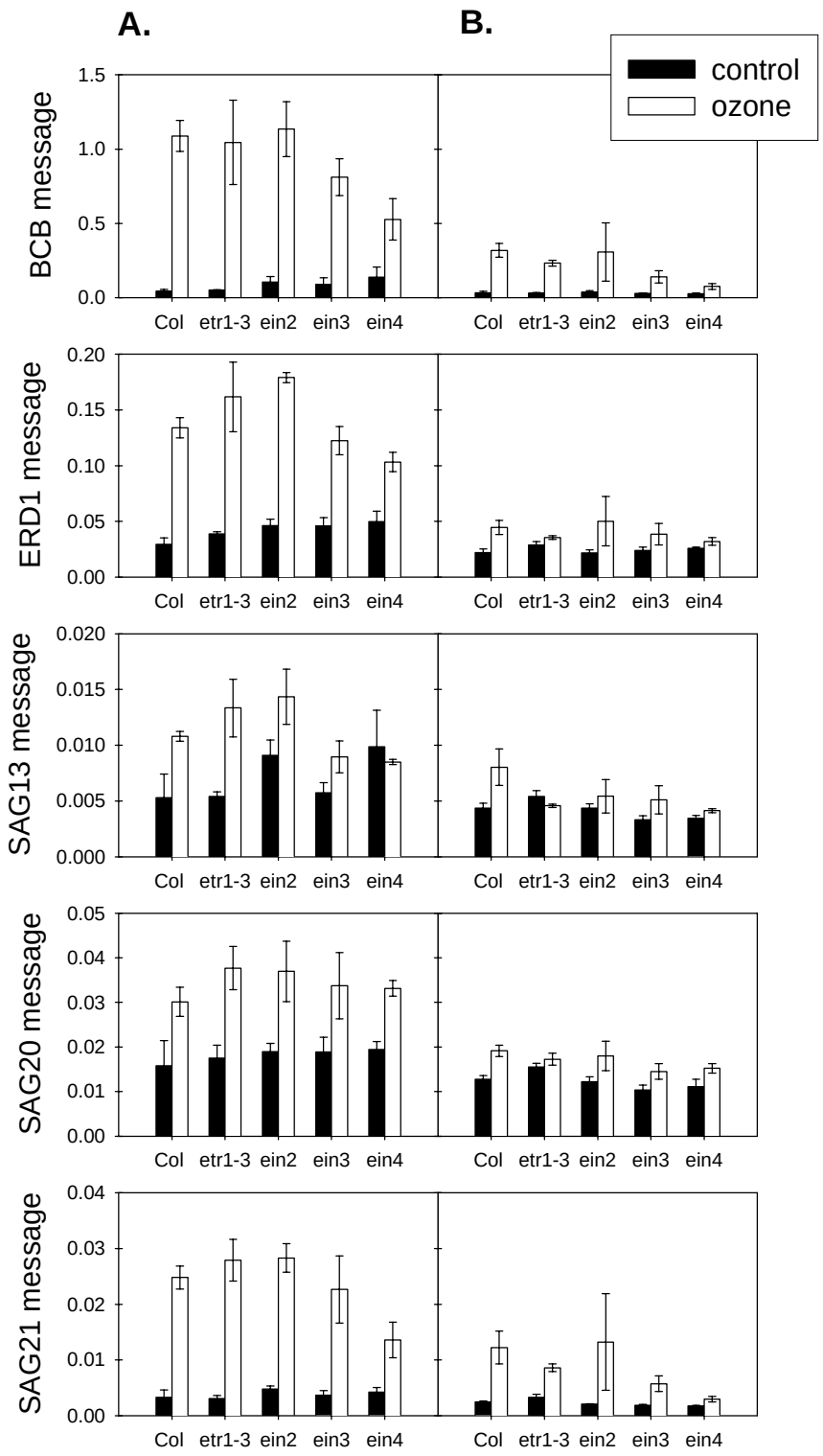
A.
B.
control
ozone
BCB message
ERD1 message
SAG13 message
SAG20 message
SAG21 message
Col
etr1-3
ein2
ein3
ein4

Figure 5–3: Ozone-induced SAG expression in wild-type, *etr1-3*, *ein2-1*, *ein3-1* and *ein4-1* plants treated with 0.15 μL L^{-1} O_3. Twenty-two-day-old plants were treated with 0.15 μL L^{-1} O_3 for 3.5 hours. (A) *BCB*, *ERD1*, *SAG13*, *SAG20* and *SAG21* message levels in rosettes at 1 hour. (B) *BCB*, *ERD1*, *SAG13*, *SAG20* and *SAG21* message levels in rosettes at 3.5 hours. Total RNA was extracted from rosettes and prepared as in *Figure 5-1*. Black bars, nontreated plants; white bars, O_3-treated plants. Each bar represents the mean of three samples ± SE.

Treatment with 0.30 μL L^{-1} O_3 caused an increase in ethylene emission from all plants (*Figure 5–4A-B*). Ethylene production was greater in O_3-treated plants than nontreated plants after 1 hour and was even higher after 3.5 hours of O_3 treatment (*Figure 5–4A-B*). *ACS6* transcript levels were also higher in O_3-treated wild-type, *etr1-3*, *ein2-1*, *ein3-1* and *ein4-1* plants at 1 and 3.5 hours (*Figure 5–4C-D*). At 3.5 hours, ethylene emission during treatment with 0.30 μL L^{-1} O_3 was more than double the ethylene production after 3.5 hours of treatment with 0.15 μL L^{-1} O_3 in wild-type plants (*Figures 5–2B* and *5–4B*).

BCB, *ERD1*, *SAG13*, *SAG20* and *SAG21* transcript levels were induced in all wild-type and ethylene-insensitive plants treated with 0.30 μL L^{-1} O_3 (*Figure 5–5*). Strong induction of *BCB*, *SAG20* and *SAG21* message levels was detected after only 1 hour of treatment, whereas *SAG13* message levels did not show an induction in O_3-treated plants until 3.5 hours (*Figure 5–5*). ERD1 transcript levels were slightly greater after 1 hour of O_3 treatment and showed a strong induction after 3.5 hours (*Figure 5–5*).

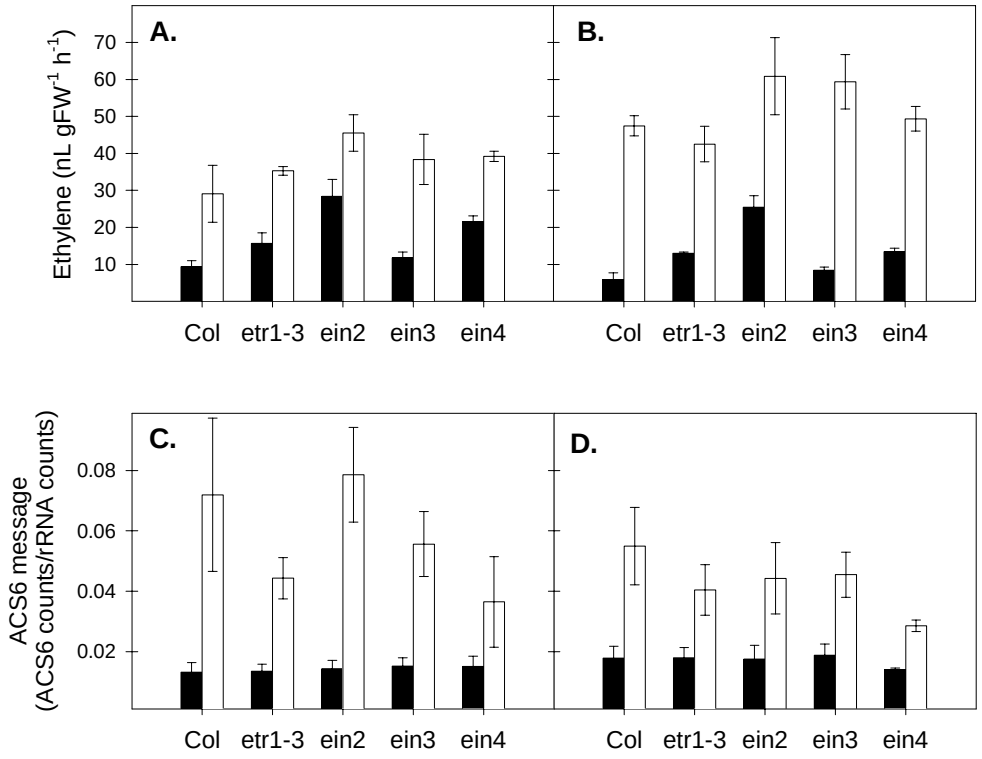

Figure 5–4: Ethylene emission and *ACS6* transcript levels in wild-type, *etr1-3*, *ein2-1*, *ein3-1* and *ein4-1* plants treated with 0.30 µL L^{-1} O_3. Twenty-one-day-old plants were treated with 0.30 µL L^{-1} O_3 for 3.5 hours. (A) Ethylene emission from rosettes at 1 hour. (B) Ethylene emission at 3.5 hours. (C) *ACS6* transcript levels at 1hour. (D) *ACS6* transcript levels at 3.5 hours. Total RNA was extracted from rosettes and prepared as in *Figure 5-1*. Black bars, nontreated plants; white bars, O_3-treated plants. Each bar represents the mean of three samples $\pm$ SE.

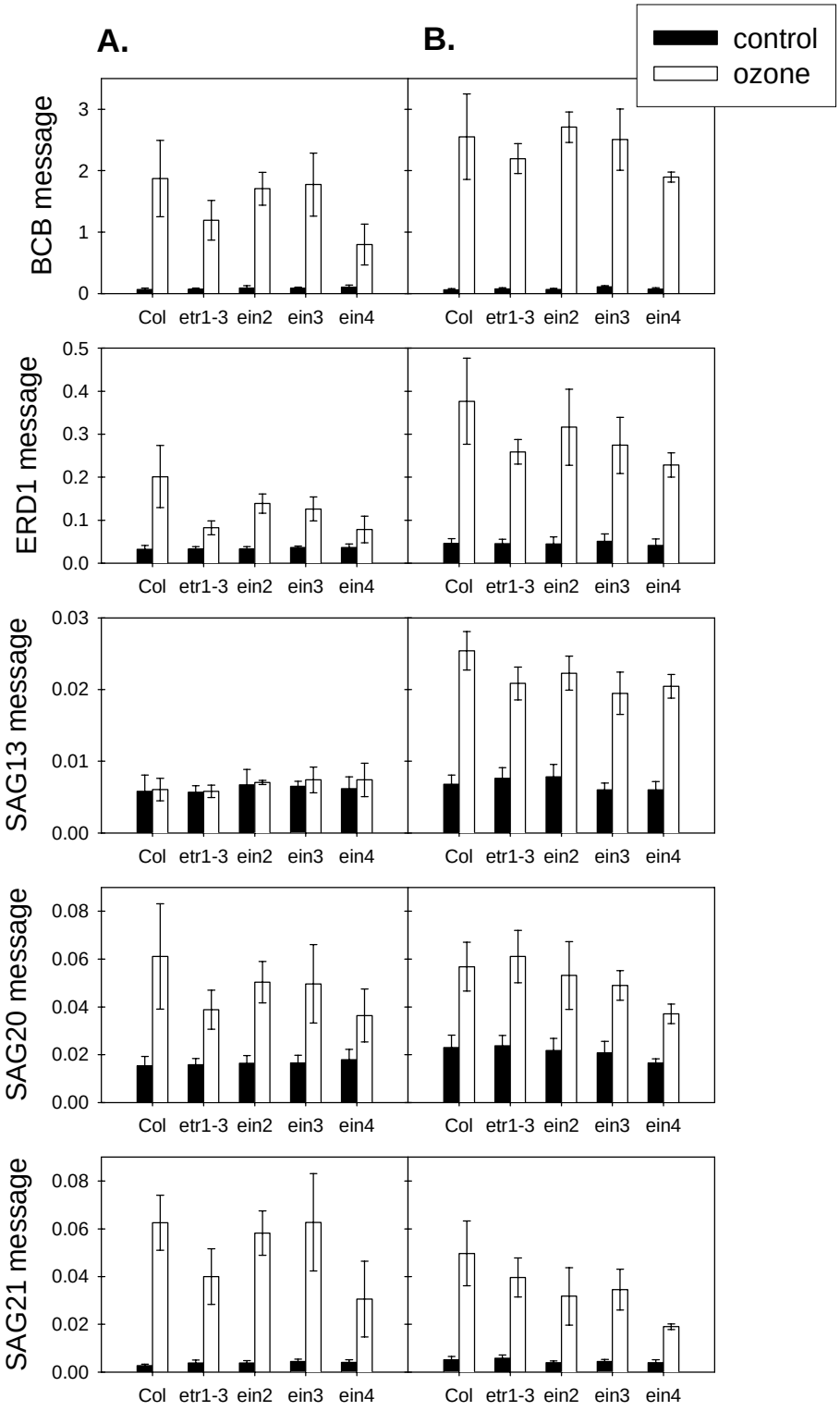
A.
B.
control
ozone
BCB message
ERD1 message
SAG13 message
SAG20 message
SAG21 message
Col
etr1-3
ein2
ein3
ein4

Figure 5–5: Ozone-induced SAG expression in wild-type, *etr1-3*, *ein2-1*, *ein3-1* and *ein4-1* plants treated with 0.30 μL L^{-1} O_3. Twenty-one-day-old plants were treated with 0.30 μL L^{-1} O_3 for 3.5 hours. (A) *BCB*, *ERD1*, *SAG13*, *SAG20* and *SAG21* message levels in rosettes at 1 hour. (B) *BCB*, *ERD1*, *SAG13*, *SAG20* and *SAG21* message levels in rosettes at 3.5 hours. Total RNA was extracted from rosettes and prepared as in *Figure 5-1*. Black bars, nontreated plants; white bars, O_3-treated plants. Each bar represents the mean of three samples ± SE.

The ability of ethylene to induce SAG expression directly was studied by treating wild-type and ethylene-insensitive mutants with 100 μL L^{-1} ethylene for 6 hours. Rosettes were harvested at 3 and 6 hours for the analysis of transcript levels. Total RNA from rosettes treated with O_3 was also included on the gels to compare the magnitude of SAG induction by ethylene versus O_3. Ethylene treatment caused *BCB*, *ERD1 SAG20* and *SAG21* transcript levels to increase in wild-type plants (*Figure 5–6A-B*). *ERD1*, *SAG20* and *SAG21* transcript levels were also induced by 6 hours of ethylene in *etr1-3* plants (*Figure 5–6B*). Ethylene treatment also caused a small increase in *SAG21* transcript levels in *ein3-1* plants at 6 hours (*Figure 5–6B*). No ethylene-induced SAG expression was found in *ein2-1* or *ein4-1* plants (*Figure 5–6A-B*). Treatment of wild-type plants with 1 μL L^{-1} ethylene only caused *SAG21* transcript levels to increase (data not shown). The level of ethylene-induced SAG expression in wild-type plants was minimal, though, compared to the increase induced by O_3 (*Figure 5–6C*). Ethylene treatment caused an approximately 2-fold increase in *BCB* transcript levels in wild-type plants, whereas 0.35 μL L^{-1} O_3 caused an approximately 23-fold increase in *BCB* levels. *SAG21*

message levels exhibited a strong ethylene induction of approximately 4-fold, but this was still lower than the 9-fold increase with 0.35 μL L^{-1} O_3 in wild-type plants.

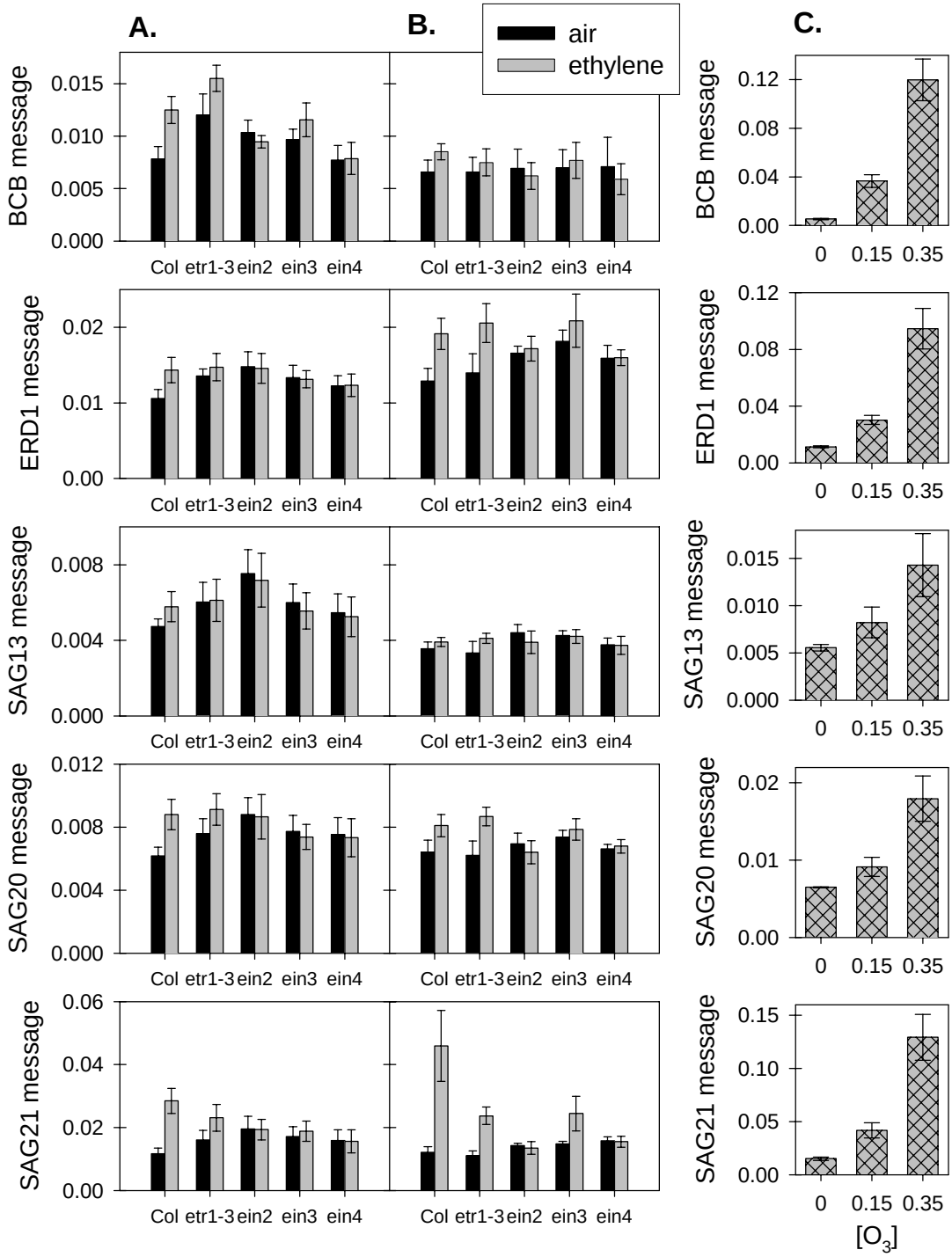
A.
B.
C.
air
ethylene
BCB message
ERD1 message
SAG13 message
SAG20 message
SAG21 message
Col etr1-3 ein2 ein3 ein4
0 0.15 0.35
[O3]

Figure 5–6: SAG expression in wild-type, *etr1-3*, *ein2-1*, *ein3-1* and *ein4-1* plants treated with 100 μL L^{-1} ethylene. Three-week-old plants were treated with 100 μL L^{-1} ethylene for 6 hours. (A) SAG message levels in rosettes after 3 hours of ethylene. (B) SAG message levels in rosettes after 6 hours of ethylene. (C) SAG message levels in wild-type rosettes treated with 0, 0.15 or 0.35 μL L^{-1} O_3 for 3 hours. Total RNA was extracted from rosettes and prepared as in *Figure 5-1*. Each bar represents the mean of five samples $\pm$ SE in A and B and the mean of three samples $\pm$ SE in C.

Discussion

To determine whether ethylene regulated the induction of SAG expression during O_3 stress, several ethylene-insensitive mutants were selected for study. The mutants *etr1-3*, *ein2-1*, *ein3-1* and *ein4-1* are unable to perceive or respond to ethylene. Ethylene perception is altered in *etr1-3* and *ein4-1* mutants due to mutations in ethylene receptor genes *ETR1* and *EIN4*, respectively (Hua et al., 1998; Hall et al., 1999). The *etr1-3* and *ein4-1* mutants are unable to inactivate the CTR1 protein, which when active represses transduction of the ethylene signal (Kieber et al., 1993). The *ein2-1* mutant is unable to transmit the ethylene signal between CTR1 and EIN3/EIL (Roman et al., 1995; Chao et al., 1997) due to a mutation in the transmembrane EIN2 protein (Alonso et al., 1999). The fourth ethylene-insensitive mutant selected for this study, *ein3-1*, does not respond to ethylene due to a mutation in the nuclear-localized EIN3 protein, a transcription factor according to sequence similarities (Chao et al., 1997).

Mature leaves of *ein2-1* and *ein4-1* mutants were completely insensitive to ethylene as shown by the lack of *HEL* induction during ethylene treatment (*Figure 5–1*). In contrast, some ethylene-induced increase in *HEL* transcript levels was found in *etr1-3* and *ein3-1* mutants. Similar results were found when *ein2-1* and *ein3-1* mutants were treated with ethylene for 24 hours; no *HEL* induction was observed in ethylene-treated *ein2-1* mutants, while some *HEL* transcript accumulation was found in ethylene-treated *ein3-1* mutants (Lawton et al., 1994). In this study and the Lawton et al. (1994) study, the increase in *HEL* transcript levels in ethylene-treated *ein3-1* mutants was less than the increase found in ethylene-treated wild-type plants. Characterizations of the ethylene response in *etr1-3* and *ein3-1* mutants have also revealed that these mutants are partially leaky (Roman et al., 1995; Hall et al., 1999).

When wild-type plants and ethylene-insensitive mutants were treated with O_3, similar patterns of SAG expression were found in wild-type and mutant plants during the 0.15 and 0.30 μL L^{-1} O_3 exposures. SAG expression was induced in O_3-treated wild-type plants and O_3-treated *etr1-3*, *ein2-1*, *ein3-1* and *ein4-1* mutants at similar levels. While no major differences were observed between wild-type and mutant plants, slightly less transcript was found for some SAGs in O_3-treated *ein4-1* mutants compared to O_3-treated wild-type plants. The *ein4-1* mutant may be less sensitive to O_3. One explanation might be lower gas exchange rates in *ein4-1* mutants, but no major differences in rosette size were noticed indicating that gas exchange rates quite possibly were similar. Based on the experiments presented in this chapter and in chapter 4, it appears that ethylene perception is not required for O_3-induced SAG expression. This conclusion is supported by the

findings that SAG expression was induced to similar levels in wild-type plants and ethylene-insensitive mutants and that SAG expression could be induced by O_3 in the absence of a large increase in ethylene emission.

The less than twofold induction of SAGs by ethylene is an indication that ethylene is not a strong regulator of SAG expression. Weaver et al. (1998) previously reported a strong increase in *BCB*, *ERD1*, *SAG13*, *SAG20* and *SAG21* transcript levels after treatment with ethylene for 10 and 24 hours. Weaver et al. (1998) injected ethylene at the beginning of the exposure into Plexiglas chambers containing plants, while control plants were left in growth chambers. The differing environmental conditions between ethylene-treated and nontreated plants may have contributed to the changes in gene expression found in the Weaver et al. (1998) study. In this study SAG transcript levels were measured after 3 and 6 hours of treatment, which coincided with the timing of ethylene emission in response to an O_3 exposure. The ethylene-induced change in SAG transcript levels observed in this study was minimal compared to the induction found in O_3-treated plants.

This work demonstrates that ethylene production and perception are not required for O_3-induced SAG expression. Other signaling molecules must be involved in this response; candidates might include calcium, salicylic acid or reactive oxygen species (ROS). During exposures to high concentrations of O_3, calcium and salicylic acid levels increase (Yalpani et al., 1994, Sharma and Davis, 1997; Clayton et al., 1999); however, the connection with senescence is not known. Ozone treatment causes ROS to increase as O_3 degrades during reactions with cellular components and from endogenous

production by cells possibly through the activity of plasma membrane NADPH oxidase and cell wall peroxidases (Schraudner et al., 1998; Pellinen et al., 1999; Rao and Davis, 1999). ROS have been associated with aging since the proposal of the free radical theory of aging by Harman in 1956 and this theory continues to shape studies in the field of aging (Sohal and Allen, 1990). The increase in ROS through enzyme activity during O_3 stress closely resembles the oxidative burst in plant pathogen interactions (Schraudner et al., 1998; Pellinen et al., 1999; Rao and Davis, 1999). If the scavenging capacity of the antioxidant system is exceeded, ROS can react with cell components causing damage to nucleic acids, proteins and lipids (reviewed in Alscher et al., 1997 and Foyer et al., 1997). In addition, ROS can alter gene expression and are involved in programmed cell death (reviewed in Foyer et al., 1997 and Pennell and Lamb, 1997).

That ROS may be the signal leading to the expression of SAGs during O_3 exposure is supported by a report of a hydrogen peroxide inducible clone, which shows strong similarity to the late embryogenesis-abundant gene *SAG21* (Desikan et al., 2000). *SAG21* expression showed strong induction during acute O_3 exposures presented in this chapter and during chronic O_3 exposure (Miller et al., 1999).

This work has demonstrated that ethylene is not required for O_3-induced SAG expression. Future attempts to identify the primary signal responsible for SAG expression during O_3 stress should focus on ROS.

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Appendix A

Ethylene Sensitivity in Leaves and Fruits of the *Never-ripe* Mutant of *Lycopersicon esculentum* cv. Alisa Craig

The ethylene insensitivity of the tomato *Never-ripe* (*Nr*) mutant was assessed to determine if this mutant could be used to study the role of ethylene in O_3-induced SAG induction. The *Nr* mutation was originally identified by its fruit, which did not completely ripen (Rick and Butler, 1956). The *Nr* mutant lacks the triple response of shortened, thickened hypocotyls and roots and a pronounced apical hook when grown in the presence of ethylene (Lanahan et al., 1994). The mutation is a single-base change in the *NR* gene, a homolog of the ethylene receptor *ETR1* in Arabidopsis (Wilkinson et al., 1995). *NR* transcript increases in ripening fruit and senescing flowers and is found in abscission zones, while no *NR* transcript was detected in unripe fruit or pre-senescent flowers (Payton et al., 1996). The *Nr* mutation is present in the Alisa Craig and Pearson cultivars. *Nr* fruit of Alisa Craig show more ripening than *Nr* fruit of Pearson background, indicating that the *Nr* mutant in Alisa Craig retains residual ethylene responsiveness (Lanahan et al., 1994). *Nr* hypocotyls of Alisa Craig also retain residual sensitivity to 1 $\mu L\ L^{-1}$ ethylene (Yen et al., 1995). Mature green fruit of the *Nr* mutant treated with 20 $\mu L\ L^{-1}$ ethylene did not express the ethylene-responsive genes *E4* and *E8*; however, another ethylene-responsive gene was partially induced (Yen et al., 1995).

Wild-type and *Nr* mutant tomato plants cv. Alisa Craig were grown in a greenhouse and treated with 0.20 µL L^{-1} O_3 for 4.5 hours. This treatment caused a sharp increase in ethylene emission in both wild-type and *Nr* mutant plants (*Figure A–1*). *Nr* tomato plants responded to O_3 treatment by producing ethylene at levels equivalent to that of wild-type plants.

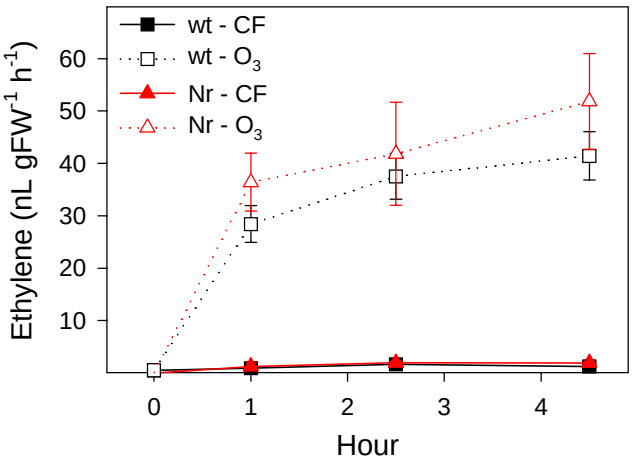

Figure A–1: Ozone-induced ethylene emission in wild-type and *Nr* mutant plants. Tomato plants were treated with 0.20 µL L^{-1} O_3 for 4.5 hours and ethylene emission from leaves was measured by gas chromatography. Each point represents the mean of three sample ± SE.

In order to determine the insensitivity of the *Nr* mutant, fruits and leaves were treated with 10 µL L^{-1} ethylene. The molecular marker used to indicate an ethylene response was the induction of the ethylene-responsive gene *E4*. The *E4* gene was identified when it was induced during the treatment of unripe mature green (MG) stage 1

tomato fruit with ethylene (Lincoln et al., 1987). In the absence of ethylene, ripening tomato fruits express *E4* at the MG3 and MG4 stages (Lincoln and Fischer, 1988). *E4* expression can also be induced in leaves treated with 10 μL L^{-1} ethylene (Lincoln and Fischer, 1988).

Fruit at the MG1 stage were treated with 10 μL L^{-1} ethylene for 8 hours. The ethylene responsive *E4* gene was induced in wild-type fruit treated with ethylene (*Figure A–2A*). Transcript for *E4* remained nearly undetectable in ethylene-treated *Nr* fruit. The level of *E4* transcript was also determined in ripe fruit and was much more abundant in wild-type tomatoes than *Nr* tomatoes (*Figure A–2B*), confirming that the fruit of *Nr* was ethylene insensitive.

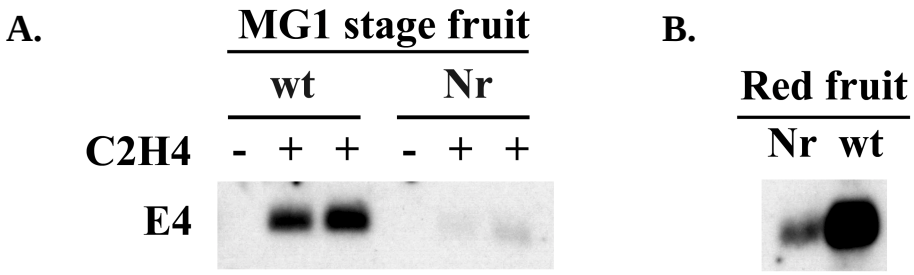

Figure A–2: *E4* expression in ripe and ethylene-treated wild-type and *Nr* fruit. (A) MG1 fruit from wild-type and *Nr* plants were treated with 10 μL L^{-1} ethylene for 8 hours. Total RNA was extracted from fruits and separated on 1% formaldehyde-agarose gels, transferred to membranes, and hybridized with radiolabeled E4 probe. (B) *E4* expression in ripened wild-type and *Nr* fruit. Wild-type fruit were red and fully ripened, while *Nr* fruit of the same age were orange in color.

The ethylene responsiveness of mature leaves was also tested to determine if the *Nr* mutant could be used in the study of O_3-induced leaf senescence. Tomato plants were

treated with 10 μL L^{-1} ethylene for 2.5 hours. *E4* transcript levels were determined by northern analysis. *E4* transcript was detected in wild-type and *Nr* leaves treated with ethylene (*Figure A–3*). Plants were treated in humidified chambers, so the chamber effect was determined by analyzing *E4* transcript levels in leaves placed in the humidified chamber without ethylene (*Figure A–3B)*. A strong induction of *E4* was only seen when ethylene was present. Levels of *E4* induction were similar in wild-type and *Nr* leaves (*Figure A–3*). Contrary to the results with *Nr* fruit, this experiment indicated that *Nr* leaves remained ethylene responsive. While the *Nr* mutation in the Alisa Craig background is known to be partially responsive to ethylene, the ethylene responses have all been attenuated compared to wild-type plants (Yen et al., 1995). In this study, there was no discernable difference in *E4* transcript levels in ethylene-treated wild-type and *Nr* leaves.

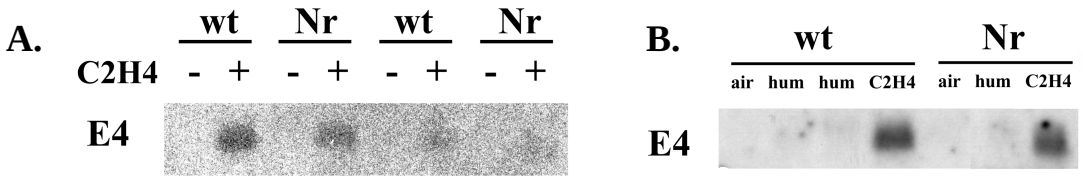

Figure A–3: Ethylene-induced *E4* expression in leaves of wild-type and *Nr* tomato plants. (A) Wild-type and *Nr* plants were treated with 10 μL L^{-1} ethylene in a humidified chamber for 2.5 hours or remained in ethylene-free air. Total RNA was extracted from leaves and separated on 1% formaldehyde-agarose gels, transferred to membranes, and hybridized with radiolabeled E4 probe. (B) Wild-type and *Nr* plants were treated with 10 μL L^{-1} ethylene in a humidified chamber for 2.5 hours, with ethylene-free air in a humidified chamber or remained in ethylene-free air. Air, air-treated; hum, humidified air

This study showed that fruits of the *Nr* mutant are ethylene insensitive, while the leaves remain responsive. Ethylene perception is complex with five different ethylene receptors in tomato showing differential regulation (Lashbrook et al., 1998; Tieman and Klee, 1999). The *Nr* mutant is useful in experiments on the ethylene response in fruit, but is not suitable as an ethylene-insensitive mutant when studying leaf processes.

Appendix B

Dark-Induced Yellowing in Air and Ozone-Treated Leaves of Wild-Type Arabidopsis and Ethylene-Insensitive Mutants

A screen of ethylene-insensitive mutants was performed to identify useful mutants for studying the role of ethylene in O_3-induced senescence-associated gene (SAG) expression. Dark-induced yellowing following O_3 treatment was analyzed in wild-type plants and ethylene-insensitive mutants *etr1-3*, *ein2-1*, *ein3-1*, *ein4-1*, *ein5*, *ein7* and *hls1-1* (Guzman and Ecker, 1990; Kieber et al., 1993; Roman et al., 1995). At 22 days after germination, half of the plants were treated with 0.25 μL L^{-1} O_3 for 4 hours. The fifth and sixth leaves were detached and placed on moist paper towels in the dark. The yellow area of each leaf was determined over twelve days by scoring the leaves as 0, 10, 20, 40, 60, 80 or 100% yellow.

Ozone treatment caused wild-type leaves placed in the dark to senescence more quickly than air-treated leaves placed in the dark (*Figure B–1*). Leaves of O_3-treated ethylene-insensitive mutants also exhibited more yellowing than their air-treated counterparts. Four of the seven ethylene-insensitive mutants exhibited delayed leaf senescence compared to wild-type plants. Air- and O_3-treated *etr1-3*, *ein2-1*, *ein3-1* and *ein4-1* leaves did not senesce as quickly as air- and O_3-treated wild-type leaves. The largest delay in dark-induced yellowing of O_3-treated leaves was found in *ein2-1* leaves.

Dark-induced yellowing in O_3-treated *ein3-1* and *ein4-1* was also delayed, while yellowing in O_3-treated *etr1-3* exhibited the smallest delay from wild-type leaves.

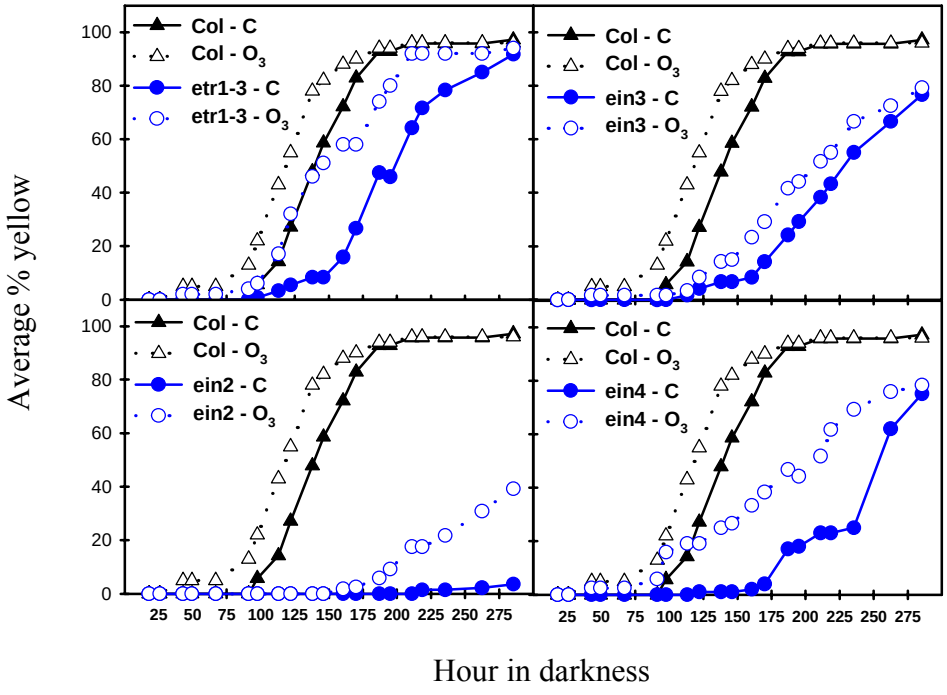

Figure B–1: Dark-induced senescence in air- and O_3-treated wild-type, *etr1-3*, *ein2-1*, *ein3-1* and *ein4-1* leaves. Following treatment with 0.25 µL L^{-1} O_3 for 4 hours, the fifth and sixth leaves were detached and placed on moist towels in the dark. The percent yellowing of air- and O_3-treated leaves was determined by visual inspection. (A) Average % yellow in wild type and *etr1-3*. (B) Average % yellow in wild type and *ein2-1*. (C) Average % yellow in wild type and *ein3-1*. (D) Average % yellow in wild type and *ein4-1*. C, Control, nontreated plants; O_3, O_3-treated plants.

The remaining ethylene-insensitive mutants *ein5*, *ein7* and *hls1-1* senesced more quickly or at the same rate as wild-type leaves (*Figure B–2*). Both air- and O_3-treated

hls1-1 mutants senesced rapidly after being placed in darkness. This mutant was previously found to bolt and senesce earlier than wild-type plants (Guzman and Ecker, 1990).

Based upon the delayed senescence of *etr1-3*, *ein2-1*, *ein3-1* and *ein4-1* compared to wild type, these mutants were selected for use in the study of ethylene in O_3-induced SAG expression.

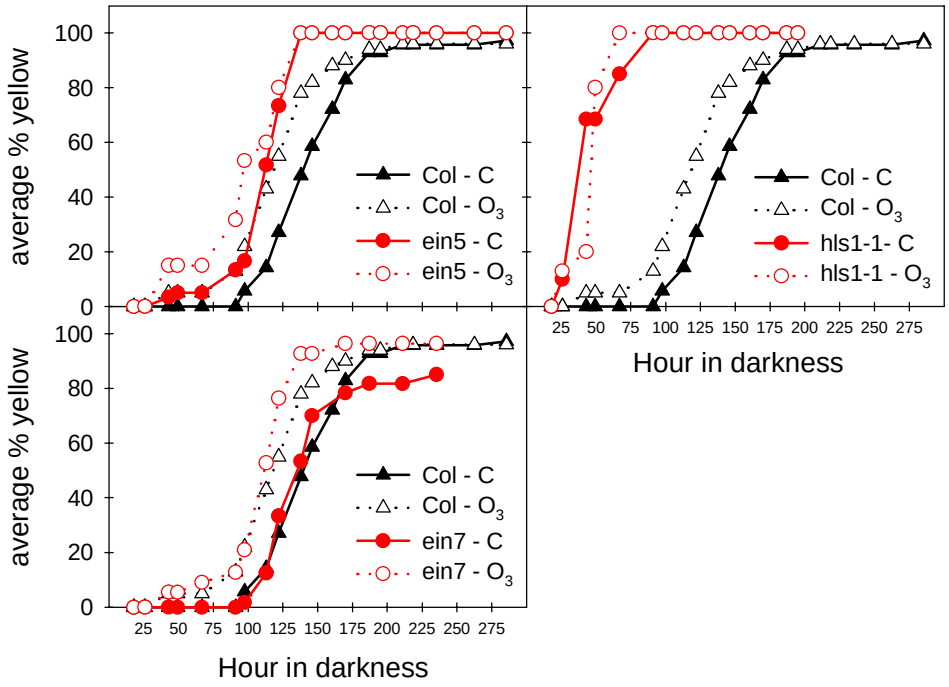

Figure B–2: Dark-induced senescence in air- and O_3-treated wild-type, *ein5*, *ein7* and *hls1-1* leaves. Following treatment with 0.25 µL L^{-1} O_3 for 4 hours, the fifth and sixth leaves were detached and placed on moist towels in the dark. The percent yellowing of air- and O_3-treated leaves was determined by visual inspection. (A) Average % yellow in wild type and *ein5*. (B) Average % yellow in wild type and *ein7*. (C) Average % yellow in wild type and *hls1-1*. C, Control, nontreated plants; O_3, O_3-treated plants.

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Plant Physiology, August 1999, Vol. 120, pp. 1015–1023, www.plantphysiol.org © 1999 American Society of Plant Physiologists

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,

[1] External funding for this research was provided by the Environmental Protection Agency (grant no. U915212–01–1) and by the U.S. Department of Agriculture (grant no. 93–38420–8742). This research was also supported in part by the Pennsylvania Agricultural Experiment Station and the Environmental Resources Research Institute. It is contribution no. 2064 from the Department of Plant Pathology, The Pennsylvania State University.

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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.

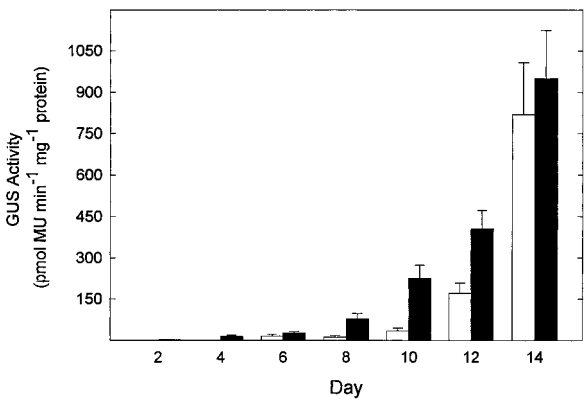

Figure 1. *SAG13* promoter-driven GUS activity was induced by O_3 treatment. Fifteen-day-old Arabidopsis ecotype Landsberg *erecta* plants transformed with the *SAG13* promoter-*GUS* fusion were exposed to 0.15 μL L^{-1} O_3 for 6 h d^{-1} for 14 d. The fifth and sixth leaves were harvested from a single 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

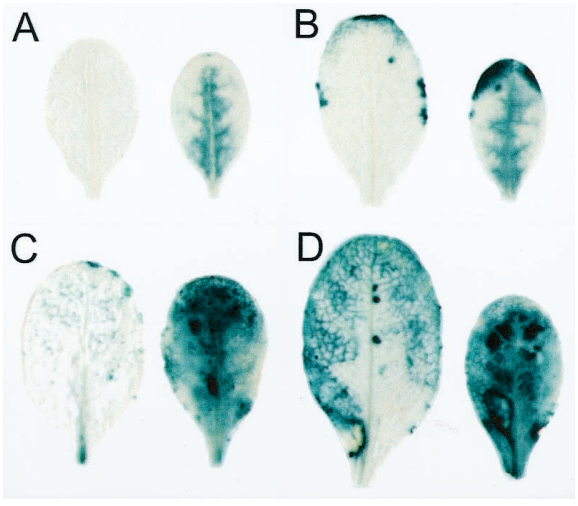

Figure 2. Photographs showing O_3-induced GUS staining in the fifth leaf of transgenic SAG13-GUS plants. Fifteen-day-old SAG13-GUS plants were exposed to 0.15 μL L^{-1} O_3 for 6 h d^{-1} for 14 d. Leaves were vacuum infiltrated with 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide, incubated at 37°C 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.

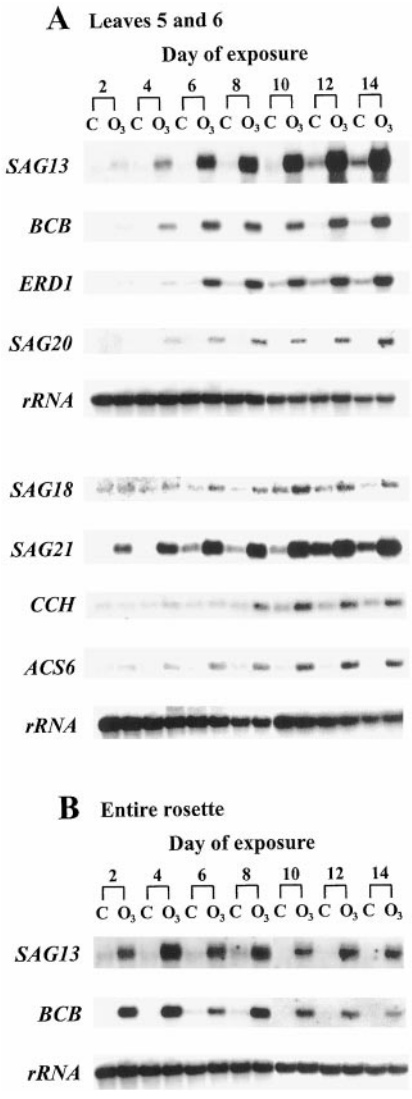

Figure 3. Induction of senescence-related transcripts in O_3-treated Arabidopsis plants. Fifteen-day-old plants were exposed to 0.15 μL L^{-1} O_3 for 6 h d^{-1} or remained nontreated. Total RNA was extracted and 3 μg of RNA was separated on 1% formaldehyde-agarose gels, transferred to membranes, and 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.

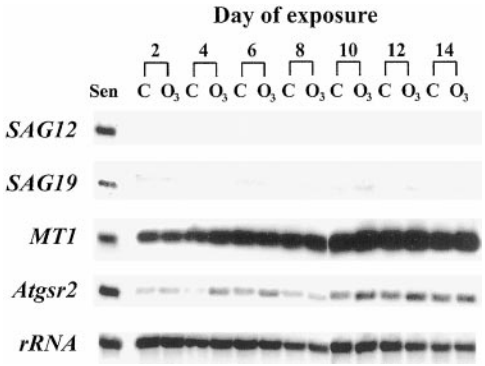

Figure 4. *SAG12*, *SAG19*, *MT1*, and *Atgsr2* transcript levels were not altered by O_3 treatment. Fifteen-day-old Arabidopsis plants were exposed to 0.15 μL L^{-1} O_3 for 6 h d^{-1} or remained nontreated. Samples were prepared as in Figure 3. Each lane contains RNA extracted from the fifth and sixth 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).

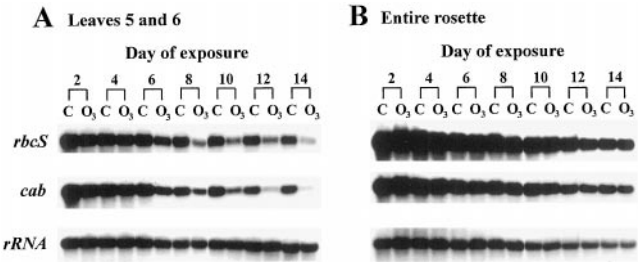

Figure 5. PAG transcript levels declined after treatment with O_3. Fifteen-day-old Arabidopsis plants were exposed to 0.15 μL L^{-1} O_3 for 6 h d^{-1} or remained nontreated. Samples were prepared as in Figure 3. A, Each lane contains RNA extracted from the fifth and sixth leaves pooled from six plants. 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.

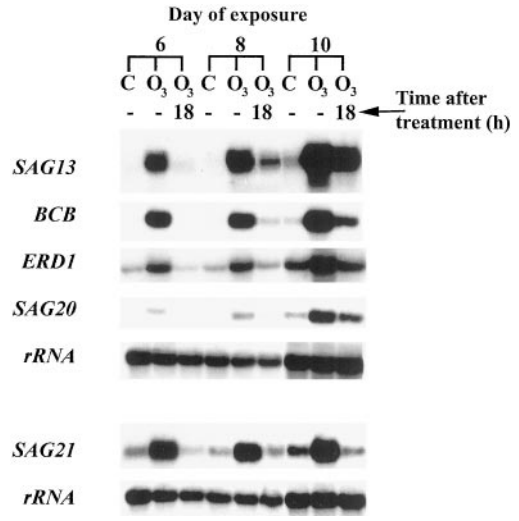

Figure 6. *SAG13*, *BCB*, *ERD1*, *SAG20*, and *SAG21* transcript levels declined following a recovery period in O_3-free air. Fifteen-day-old Arabidopsis plants were exposed to 0.15 μL L^{-1} O_3 for 6 h d^{-1} or remained nontreated. The fifth and sixth leaves were harvested from six plants immediately 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.

ACKNOWLEDGMENTS

The authors thank Richard Amasino for the generous gift of the SAG clones and the Arabidopsis Biological Resource Center (Columbus, OH) for the *Atgsr2* clone, stock no. CD3-195, donated by T.K. Peterman. We are also grateful to Ed Himelblau and Michael Weaver for helpful discussions and Nan Eckardt and Judy Sinn for critical reading of the manuscript.

Received January 27, 1999; accepted May 11, 1999.

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