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Molecules and Cells

Inhibition of Biphasic Ethylene Production Enhances Tolerance to Abiotic Stress by Reducing the Accumulation of Reactive Oxygen Species in *Nicotiana tabacum*

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Reactive oxygen species (ROS), such as H_2O_2, are important plant cell signaling molecules involved in responses to biotic and abiotic stresses and in developmental and physiological processes. Despite the well-known physiological functions of ethylene production and stress signaling via ROS during stresses, whether ethylene acts alone or in conjunction with ROS has not yet been fully elucidated. Therefore, we investigated the relationship between ethylene production and ROS accumulation during the response to abiotic stress. We used three independent transgenic tobacco lines, *CAS-AS-2*, *-3* and *-4*, in which an antisense transcript of the senescence-related ACC synthase (*ACS*) gene from carnation flower (*CARACC*, GenBank accession No. M66619) was expressed heterologously. Biphasic ethylene biosynthesis was reduced significantly in these transgenic plants, with or without H_2O_2 treatment. These plants exhibited significantly reduced H_2O_2-induced gene-specific expression of *ACS* members, which were regulated in a time-dependent manner. The higher levels of *NtACS1* expression in wild-type plants led to a second peak in ethylene production, which resulted in a more severe level of necrosis and cell death, as determined by trypan blue staining. In the transgenic lines, upregulated transcription of *CAB*, *POR1* and *RbcS* resulted in increased photosynthetic performance following salt stress. This stress tolerance of H_2O_2-treated transgenic plants resulted from reduced ethylene biosynthesis, which decreased ROS accumulation via increased gene expression and activity of ROS-detoxifying enzymes, including MnSOD, CuZnSOD, and catalase. Therefore, it is suggested that ethylene plays a potentially critical role as an amplifier for ROS accumulation, implying a synergistic effect between biosynthesis of ROS and ethylene.

INTRODUCTION

Various biotic and abiotic stresses, including pathogens, drought, excessive low/high temperatures, light and salinity, continuously threaten plants and are responsible for substantial losses in crop production. Under such conditions, survival is dependent upon the ability to perceive and respond to external stimuli in a timely manner (Anderson et al., 2004). Ethylene is an important signaling response hormone for many abiotic stresses and pathogen interactions (Chung et al., 2008; Díaz et al., 2002). The two steps of ethylene biosynthesis, conversion of S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) and its subsequent oxidation to ethylene, are regulated by ACC synthase (ACS) and ACC oxidase (ACO), respectively (Bleeker and Kende, 2000; Nehring and Ecker, 2004). These enzymes are encoded by multigene families present in all plant species, and consist of at least nine and four members, respectively, in *Arabidopsis* and tomato (Chae et al., 2003; Ralph et al., 2007), and nine and five members, respectively, in *Nicotiana tabacum* (Von Dahl et al., 2007).

In plants, various reactive oxygen species (ROS) are produced continuously as by-products of aerobic metabolism. Under physiological steady-state conditions, these molecules are scavenged by several antioxidative defense components, thereby maintaining a balance between ROS production and scavenging (Dat et al., 2003). This equilibrium may be perturbed by a number of adverse environmental factors that generate ROS by activating various oxidases and peroxidases, or lead to the rapid accumulation of ROS known as an oxidative burst (Apostol et al., 1989). Although exhibiting a surfeit of mechanisms to combat increased ROS levels during abiotic stress conditions, in some circumstances plants purposefully generate ROS as signaling molecules. Therefore, ROS are important for several processes in plants, including stomatal closure, root growth and gravitropism, as well as responses to abiotic stresses and pathogen challenge (Desikan et al., 2005). Considerable research has focused on the mechanisms of ROS generation and removal in plants, as well as its functions during development (Kim et al., 2007) and during conditions of biotic or abiotic stress (Apel and Hirt, 2004).

Despite the well-known physiological functions of ethylene

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production and stress signaling via ROS during abiotic and biotic stresses, whether ethylene acts alone or in conjunction with ROS has not yet been fully elucidated. There are several reports that demonstrate a functional link between ethylene and H_2O_2 synthesis, signaling in ozone-exposed tomato leaves (Moeder et al., 2002) and H_2O_2- or ethylene-treated stomatal guard cells (Desikan et al., 2005; 2006). The *Arabidopsis* ethylene receptor (ETR1) could act as a central node mediating cross-talk between ethylene and H_2O_2 signaling in stomatal guard cells (Desikan et al., 2005), and *Arabidopsis* NADPH oxidase (AtrbohF) was identified as a key mediator of the stomatal response to ethylene (Desikan et al., 2006). Ethylene synthesis and recognition are required for the burst of H_2O_2 production that regulates the spread of cell death (Moeder et al., 2002). Although correlations between ethylene synthesis, ROS accumulation and tissue damage have been reported previously, the functional interaction between ethylene biosynthesis and ROS detoxification, which might be important for alleviating cell damage and subsequent stress tolerance, has not yet been explored.

Here, we have used transgenic plants with impaired ethylene biosynthesis to investigate the complexity of ROS in response to abiotic stresses, and we consider the involvement of ethylene in ROS-detoxifying systems. At this time, our results suggest that ethylene and H_2O_2 act as mutual and self-amplifying signaling molecules in feed-forward loop regulation. If ethylene were to exceed a survivable threshold, subsequent endogenous ROS levels would determine the severity of tissue damage by increasing further ethylene biosynthesis.

MATERIALS AND METHODS

Transgenic plants and stress treatments

Transgenic tobacco (*Nicotiana tabacum* L. Wisconsin 38) plants expressing antisense RNA for the carnation *ACS* gene (*CARACC*, GenBank accession No. M66619) was produced by *Agrobacterium tumefaciens* (strain LBA 4404)-mediated transformation, as described previously (Wi et al., 2002). Transformation was confirmed by Southern blot analysis (Supplementary Fig. 1). Following kanamycin selection, we used plants of the T_1 or T_2 generation of *CAS-AS-2*, *CAS-AS-3*, and *CAS-AS-4* for further analyses. Discs from fully-matured leaves floating in a 20 mM H^+ 2-[N-morpholino]-ethanesulfonic acid (MES) buffer (pH 6.1) were subjected to oxidative stress (20 mM H_2O_2), salt stress (200 mM NaCl), or hormonal treatment (100 μM ABA), under light (50 μmol photons m^{-1} s^{-1}) at room temperature (25 ± 5°C).

RNA isolation, Northern blot analysis and semiquantitative RT-PCR

Total RNA isolation and Northern blot analyses were performed as described previously (Wi et al., 2002). Each specific primer for RT-PCR was designed using the GenBank database (Supplementary Table 1). The templates for all Northern blot probes were cloned by RT-PCR. For semiquantitative RT-PCR analysis of the *ACS* gene family, the first strand cDNA was obtained from different tissues and 1 μl of the RT mixture was added as a template for PCR amplification; specific primers were used for *NtACS1-2*, and *NtACS4-9*.

Ethylene measurement

Ethylene production by abiotic-stressed or non-stressed leaf discs (~1.2 cm in diameter) was measured by enclosing the leaf discs in 20 ml vials for 1 h. The ethylene content was determined from 1 ml of headspace gas using gas chromatography (Hewlett Packard 5890 Series II, USA), with an activated alumina column at 250°C and a flame ionization detector.

Quantitative real-time RT-PCR gene expression

To analyze the relative abundance of transcripts by quantitative real-time RT-PCR (qRT-PCR), 1 μg of total RNA from the leaf discs was reverse-transcribed for 30 min at 42°C in a 20 μl reaction volume using the High Fidelity PrimeScript™ RT-PCR kit (Takara, Japan), according to the manufacturer's instructions. Gene-specific PCR primers were designed using a stringent set of criteria, including a predicted melting temperature of 60°C ± 5°C, primer lengths of 20 to 24 nucleotides, a guanine-cytosine content of 50 to 60%, and PCR amplicon lengths of 100 to 250 bp (Supplementary Table 1). qRT-PCR was performed in optical 96-well plates with a Chromo 4™ Continuous Fluorescence Detector (Bio-Rad, USA). Reactions (20 μl) contained 10 μl of 2× SYBR Green Master Mix, 0.5 μM of each primer and 10 ng of cDNA. PCR conditions were as follows: 95°C for 15 min; followed by 45 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 30 s; and then 72°C for 10 min. Fluorescence threshold data (Ct) was analyzed using the MJ Opticon Monitor Software version 3.1 (Bio-Rad, USA), and then exported to Microsoft Excel for further analysis. Relative expression levels in each cDNA sample were normalized to the reference gene *β-actin*. PCR efficiencies (90 to 95%) for all primers were determined by serial dilution of cDNA from RNA samples.

Analyses of chlorophyll content and photosynthetic activity

Chlorophyll content was quantified photometrically, as reported previously (Wi et al., 2002). Chlorophyll was extracted from leaf discs with acetone:water (3:1, v/v), after which the absorbances at A_{660} and A_{642} were determined with a spectrophotometer (UV-1601; Shimadzu, Japan). Eight-week-old whole plants were transferred to a growth chamber and then steady-state net photosynthesis was determined with a Gas Exchange Measuring Station (Walz, Germany) using the built-in light source (210 μmol photons m^{-2} s^{-1}). A continuous gas stream (60 L h^{-1}, 21% O_2, 430 μl^{-1} CO_2) was provided to the photosynthesis unit by a mass-flow control system. The leaf temperature and humidity in the chamber were maintained at 25°C and 70 ± 1%, respectively.

Trypan blue staining

Trypan blue staining of the necrotic area was performed as described by Belenghi et al. (2003). Leaf discs were immersed for 2 min in a boiling solution of 10 ml lactic acid, 10 ml glycerol, 10 ml phenol and 0.4% (w/v) trypan blue. The solution was decanted and the leaf discs were destained with 70% (w/v) chloral hydrate, and then photographed with a digital camera.

Detection of ROS generation *in vivo*

Fully-expanded leaf discs were treated with abiotic stresses or chemicals for 1 h, and then immersed in 10 mM Tris-HCl (pH 7.2) containing 50 mM KCl and 50 μM a 2′,7′-dichlorofluorescein diacetate (DCFH-DA) for 10 min. DCFH-DA forms fluorescent 2′,7′-dichlorofluorescein (DCF) when oxidized by H_2O_2 (Leshem et al., 2007). Fluorescence was visualized by confocal microscopy with an excitation wavelength from an argon laser at 488 nm and an emission wavelength of 515 to 560 nm. *In vivo* H_2O_2 generation in plants was also detected by an endogenous peroxidase-dependent *in situ* histochemical staining procedure using 3,3′-diaminobenzidine (DAB) (Liu et al., 2007). Four-week-old whole tobacco plants were treated with abiotic stresses or chemicals for 30 min and then placed in a solution containing 1 mg ml^{-1} DAB (pH 3.8) for 8 h, after a brief vacuum

filtration.

Activity measurements of ROS-detoxifying enzymes

Superoxide dismutase (SOD) activity was determined spectrophotochemically by the xanthine oxidase/cytochrome c method (McCord et al., 1969). The amount of SOD required to inhibiting the rate of reduction of Cyt c by 50% was defined as 1 unit of activity. Ascorbate peroxidase (APX) activity was determined as described by Nakano and Asada (1987), and was calculated by the decrease in A_{290} (extinction coefficient 2.8 mM^{-1} cm^{-1}) over 2 min. One unit of APX activity was defined as the amount of enzyme required for oxidation of 1 μmol ascorbate per 1 min. Catalase (CAT) activity was determined based on the amount of decomposed H_2O_2, determined from the absorbance change at 240 nm (extinction coefficient 0.0644 mM^{-1} cm^{-1}) (Zelitch, 1990). Glutathione-S-transferase (GST) activity was spectrophotochemically assayed using 1-chloro-2,4-dinotrobenzene (CDNB) as a substrate (Habig, 1974). The reaction was followed for 5 min at 340 nm with a molar extinction coefficient of 9.6 mM^{-1} cm^{-1} for CDNB. The soluble proteins were quantified according to Bradford (Bradford, 1976) using bovine serum albumin as a standard.

Statistical analyses

All experiments were repeated at least three times with three replicates, and the data from one representative experiment was presented. Statistically significant differences according to the *t*-test between transgenic lines and the respective controls at each-time point are indicated with one asterisk (*) ($P < 0.05$) or two asterisk (**) ($P < 0.01$). Two-way ANOVA was also performed to investigate statistical differences between the responses of wild-type and transgenic lines in Figs. 2A, 2B, 4A, and 5B.

RESULTS

ACC synthase expression patterns

To isolate *ACS* multigene members from *N. tabacum*, a TBLASTN search of the GenBank database was performed using the tobacco *ACS* sequence (GenBank accession No. X65982) and GenBank sequence information from Nakajima et al. (2002) and Chen (2003). We obtained *ACS* cDNA sequences by RT-PCR using gene-specific primers (Supplementary Table 1), and identified eight *ACS* genes from *N. tabacum*. After phylogenetic analyses comparing these cDNA fragments to *ACS* genes from tobacco, tomato (Oetiker et al., 1997) and *Arabidopsis*, the eight *ACS* genes were designated *NtACS1, 2*, and *4* to *9*. Only the four sequences *NtACS1, 2*, *4*, and *5* contained an active site for ACC synthase activity. The nucleotide sequence of the carnation petal senescence-related *ACS* gene (*CARACC*) (Park et al., 1992), used here for heterologous expression of an antisense sequence in transgenic tobacco plants, exhibited 50 to 73% identity in amino acid sequence and 43 to 67% identity in nucleotide sequence to all *NtACS*. Several regions of high sequence conservation, including the active site, are apparent in the alignment between all of the *NtACS* sequences and *CARACC*. A phylogenetic tree was compiled using the predicted amino acid sequences encoded by the four tobacco *ACS* genes, containing conserved regions such as the active site, and seven tomato *ACS* gene members (Oetiker et al., 1997). The phylogenetic tree was constructed using MEGA 4 version with the Neighbor-Joining method (http://www.megasoftware.net/), based on the genetic distance between the protein sequences (Fig. 1A). NtACS1 belonged to the same phylogenetic class as LE-ACS2 and CARACC, which contain major peptides that respond to fruit-ripening and senescence. NtACS4 is phylogenetically close to LE-ACS3 and LE-ACS7, which are early-responsive (1 h) proteins to flooding and wounding (Shiu et al., 1998).

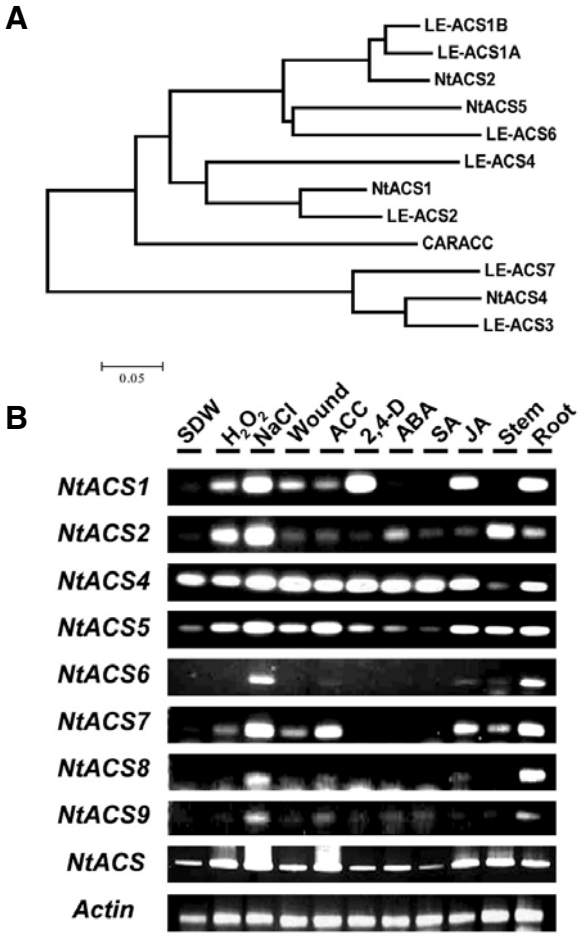

Fig. 1. Phylogenetic analysis and expression of 8 members of the *ACS* gene family. (A) Alignment of amino acid sequences encoded by 4 *ACS* members from tobacco, 7 *ACS* members from tomato and *CARACC*. Alignments were performed using MEGA 4 version with the Neighbor-Joining method (http://www.megasoftware.net/), based on the genetic distance. GenBank accession numbers are as follows: *LE-ACS1A* (AAF97614); *LE-ACS1B* (AAF97615); *LE-ACS2* (AAP96918); *LE-ACS4* (P29535); *LE-ACS5* (AAC49685); *LE-ACS6* (AB013100); *LE-ACS7* (AF179248); *NtACS1* (X65982); *NtACS2* (EU123522); *NtACS4* (EU123523); *NtACS5* (EU123524); and *CARACC* (M66619). (B) Semiquantitative RT-PCR analysis of cDNA from leaf discs treated with abiotic stresses (20 mM H_2O_2, 200 mM NaCl, or wounding with an iron brush), hormonal treatments (50 μM 2,4-D, 100 μM ABA, 300 μM SA, or 200 μM JA), or non-treated control stems and roots of wild-type tobacco plants. Non-treated control leaf discs were floated in Petri dishes containing sterile distilled water (SDW). Analyses were performed individually using gene-specific primers for the 8 members of the *ACS* gene family. RT-PCR was performed using primers for *NtACS* designed against conserved regions of *ACS* genes. To analyze the relative abundance of mRNA transcripts with RT-PCR, 20 ng of cDNA was amplified under the following PCR conditions: 95°C for 10 min; followed by 25 cycles of 94°C for 30 s, 60°C for 1 min and 72°C for 1 min 30 s; and then 72°C for 10 min.

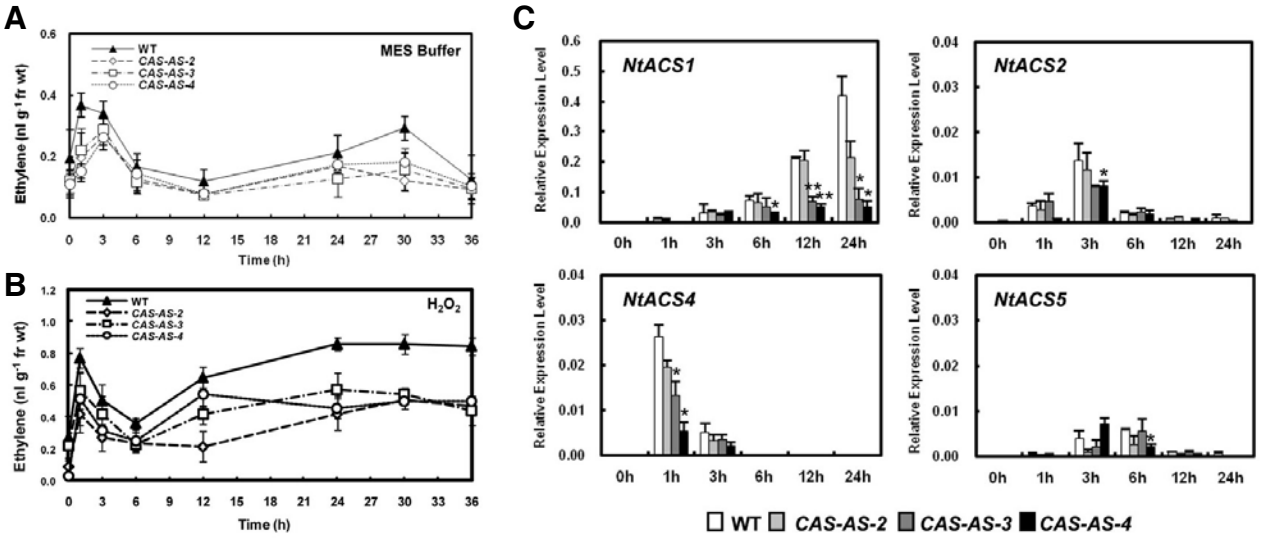

Fig. 2. Reduction of ethylene production and transcript levels of *NtACS* gene family members in transgenic tobacco plants. Leaf discs were prepared with a cork borer (1.2 cm diameter). Ethylene measurements were performed by floating the discs on 20 mM MES buffer without (A) or with (B) 20 mM H_2O_2 (final concentration), and then incubating them in air-tight glass vials for 1 h under illumination (photon flux density of 50 μmol m^{-1} s^{-1}). Wild-type plants (▲, WT) and three independent transgenic lines (◇, *CAS-AS-2*; □, *CAS-AS-3*; and ○, *CAS-AS-4*) were tested. When two-way ANOVA was used to identify statistically significant differences between H_2O_2-treated wild-type plants and all three transgenic lines ($P < 0.05$), the differences were found to be statistically significant. (C) The accumulation of endogenous *NtACS1*, *2*, *4* and *5* transcripts in leaf discs of wild-type and transgenic lines floated on 20 mM MES buffer with 20 mM H_2O_2 (starting at time 0). Transcript abundance is expressed relative to a reference gene (*β-actin*) following amplification by real-time qRT-PCR with gene-specific PCR primers. cDNA was prepared from 1 μg of total RNA from leaf discs harvested at the times indicated from wild-type plants and three transgenic lines. Expression levels are reported as means ± SD. Significant differences between H_2O_2-treated transgenic and wild-type plants based on two-tailed Student's *t*-test are indicated by one ($P < 0.05$) or two asterisks ($P < 0.01$).

To investigate possible differences in RNA expression among these *ACS* genes, the relative abundances of the eight different *ACS* transcripts were quantified. Semiquantitative RT-PCR was performed on total RNA, which was isolated from wild-type leaf discs treated with various stresses or hormones for 3 h, or other untreated wild-type tissues such as stems and roots (Fig. 1B). The *NtACS4* gene exhibited relatively high levels of basal expression in all treatments and tissues, with the exception of the stem. All *ACS* transcripts were strongly inducible at high salinity and were basally expressed in roots. H_2O_2 treatment induced transcription of *NtACS1*, *NtACS2*, *NtACS4*, *NtACS5* and *NtACS7*. *NtACS1* transcription was strongly induced in leaf discs treated with 200 mM NaCl, 50 μM 2,4-D, or 200 μM JA. Wounding stress specifically induced high levels of *NtACS4* transcript and lower but elevated levels of the *NtACS1*, *NtACS5* and *NtACS7* transcripts.

Ethylene production and induced transcript profiles of *NtACS* genes

Transgenic tobacco plants that expressed antisense RNA of the senescence-related gene *CARACC,* which exhibited a significant homology to ripening-dependent *LE-ACS2* (Oetiker et al., 1997), were produced by *Agrobacterium*-mediated transformation, and transgene incorporation was verified by Southern blot analysis (Supplementary Fig. 1). Phenotypic changes were observed in the transgenic tobacco plants *CAS-AS-2*, *CAS-AS-3* and *CAS-AS-4*, including slightly larger and more abundant green leaf production, as well as taller stems than wild-type. Interestingly, each plant demonstrated a significant stress-tolerant phenotype not only against abiotic stresses, such as high salinity, oxidative stress and acidic stress, but also in response to ABA treatment (Wi et al., 2002). These findings suggest that this stress-tolerant phenotypic change may be due to stress-inducible defense machinery in the transgenic tobacco lines.

In transgenic lines treated with or without H_2O_2, ethylene production could be recovered completely by complementation with 0.5 mM of exogenous ACC (Supplementary Fig. 2). ACC treatment induced a dramatic biphasic increase in ethylene production, with no significant differences in production between transgenic and wild-type lines. These results suggest that the exogenous application of ACC completely rescued impaired ethylene production in the transgenic lines. Therefore, the ACC synthase step might only be impaired in the transgenic lines. Furthermore, they imply that biphasic ethylene emission was also responsible for the biphasic regulation of ACO expression or activity. We performed ethylene measurements by cutting the leaf discs with a cork borer (1.2 cm diameter) and then immediately incubating them in MES buffer. Therefore, biphasic emission of ethylene following ACC treatment may have been partially resulted from a weak wounding stress and production of ROS in both wild-type and transgenic lines.

In the absence of treatment, and following incubation in MES buffer, ethylene biosynthesis was inhibited significantly in leaf discs from all transgenic lines compared to wild-type (Fig. 2A). After 30 h, ethylene production was reduced markedly in wild-type and all transgenic lines; by 36 h, it had almost returned to basal levels. In contrast, ethylene production did not return to basal levels in H_2O_2-treated wild-type and transgenic plants (Fig. 2B). Also, ethylene levels in wild type plants were significantly higher than those in all transgenic lines. This pattern of ethylene production corresponded with our previous report that indicated stress-induced senescence was attenuated in these transgenic plants.

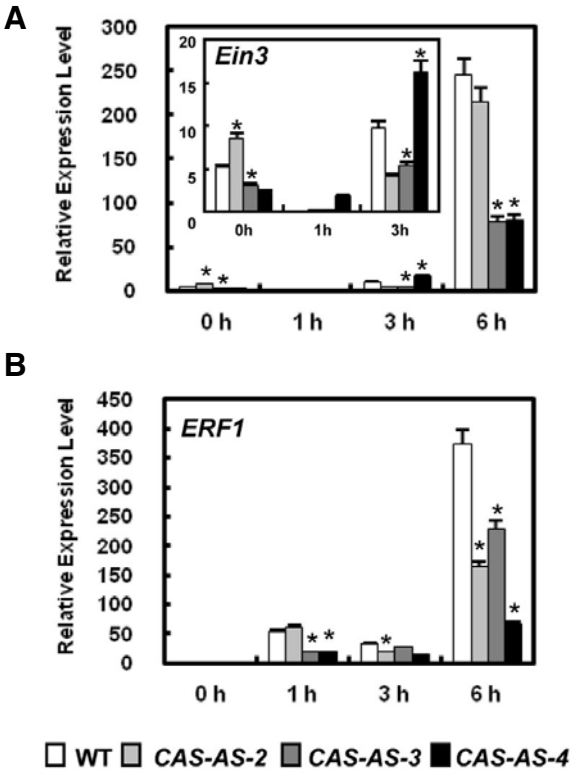

Fig. 3. Accumulation of gene transcripts related to ethylene signaling pathways. Transcript abundances of *Ein3* or *ERF1* are expressed relative to a reference gene (*β-actin*) following qRT-PCR with gene-specific PCR primers. At time 0, leaf discs were treated with 20 mM H_2O_2 in 20 mM MES buffer. Expression levels are reported as means ± SD. Significant differences between H_2O_2-treated transgenic and wild-type plants based on two-tailed Student's *t*-test are indicated by one ($P < 0.05$) or two asterisks ($P < 0.01$).

In this experiment, we were especially concerned with biphasic ethylene biosynthesis in response to abiotic stress. Ethylene synthesis was induced in a biphasic manner, regardless of oxidative stress. In all leaf discs, ethylene production peaked rapidly, i.e., within 1 h post-H_2O_2 treatment, and then declined to a level similar to that before treatment (phase I) (Fig. 2B). At 6 h post-treatment, ethylene production again began to increase gradually, reaching a second, higher peak at 24 h (phase II). In wild-type plants, this second peak led to ethylene levels that remained significantly higher than those in transgenic plants. Although ethylene levels decreased somewhat after the second peak, all transgenic lines exhibited a greater concentration and total abundance of ethylene than observed in the first peak for up to 36 h. Moreover, following treatment with H_2O_2, ethylene levels in wild-type plants were more than double those of the transgenic lines (Fig. 2B). It is possible that slight biphasic ethylene production occurs in leaf discs in the absence of oxidative stress, resulting in a first and second peak that are much smaller than those observed following H_2O_2 treatment, and representing a response to wounding stress during cutting and manipulation.

We examined the expression profiles of four *NtACS* transcripts (*NtACS 1, 2, 4,* and *5*) with respect to oxidative stress, because those transcripts were found to be strongly inducible by H_2O_2 treatment in Fig. 1B. Mature leaf discs from wild-type and T_2-generation transgenic tobacco plants were subjected to 20 mM H_2O_2 for 24 h, and the transcript profiles, monitored by real-time qRT-PCR at specific time points after treatment onset, were compared with untreated control tissues (Fig. 2C). We were unable to detect induction of any transcripts in the untreated control tissues of wild-type or transgenic plants. Compared to the 0 h baseline, the *NtACS4* transcript levels peaked by 1 h post-treatment, with the largest increase observed in wild-type plants and more moderate increases observed in the transgenic lines. *NtACS4* expression was suppressed by 79.9% in *CAS-AS-4*, 49.9% in *CAS-AS-3*, and 26.1% in *CAS-AS-2* at 1 h post-treatment with H_2O_2. The reductions in *NtACS4* expression were due to the specific properties of highly conserved nucleotide sequences in the active sites of members of *ACC* synthase gene family. Therefore, these result confirmed that the *CARACC* transgene effectively suppressed oxidative stress-induced ethylene production in transgenic tobacco systems, although the degree of suppression was different depending on the lines.

The *NtACS2* transcript was induced transiently and peaked at 3 h, which indicated a strong expression in response to H_2O_2 treatment. In all plants, *NtACS1* transcript induction occurred last, remaining at relatively basal levels until 3 h post-treatment. *NtACS1* levels increased markedly in wild-type plants at 6 to 24 h. H_2O_2-induced *NtACS1* expression was suppressed by 87.6% in *CAS-AS-4*, and by 48.8% in *CAS-AS-2* at 24 h. Inhibition of both ethylene production and *ACS* transcript generation was most obvious in the *CAS-AS-4* line, which was previously reported to have the strongest tolerance to oxidative stress of the three transgenic lines, which may explain the enhanced agronomic traits (e.g., biomass) (Wi et al., 2002). In all transgenic plants, ethylene production was blocked more effectively in the second peak than in the first peak, with or without induction by exogenous H_2O_2 (Figs. 2A and 2B).

Transcript profiles of *EIN3* and *ERF1* in response to oxidative stress

Ethylene is reportedly the major endogenous low-molecular-weight signaling molecule that regulates the plant defense response to abiotic and biotic stresses (Anderson et al., 2004). Ethylene fine-tunes the kinetics and magnitude of lesion formation in O_3-sensitive plants (Rao et al., 2002). We hypothesized that changes in ethylene-responsive gene expression could be attributed to modulation of endogenous ethylene production by antisense expression of *ACS* in tobacco plants. To test this hypothesis, we examined expression of the ethylene signaling genes, ethylene insensitive 3 (*EIN3*) and ethylene response factor 1 (*ERF1*) (Solano et al., 1998). Using qRT-PCR, we found that H_2O_2 treatment significantly induced both transcripts in all plants (Fig. 3). Expression of *EIN3* and *ERF1* in wild-type and transgenic leaves increased markedly at 6 h post-treatment with H_2O_2, compared to the 0 h control. A 6 h H_2O_2 treatment only induced moderate expression of *EIN3* and *ERF1* in *CAS-AS-4* plants, i.e., 32.9% and 17.8%, respectively, compared to wild-type plants. These results suggest that reduced ethylene production might be responsible for the reduced expression of *EIN3* and *ERF1* in *CAS-AS-4* plants.

Chlorophyll degradation and cell damage in response to abiotic stresses

H_2O_2 treatment of wild-type or transgenic plants resulted in a rapid decrease in chlorophyll content, with chlorophyll levels in wild-type leaves being much lower than those in transgenic leaves (Fig. 4A). Although each of the transgenic plants exhibited a significant loss of chlorophyll in response to oxidative

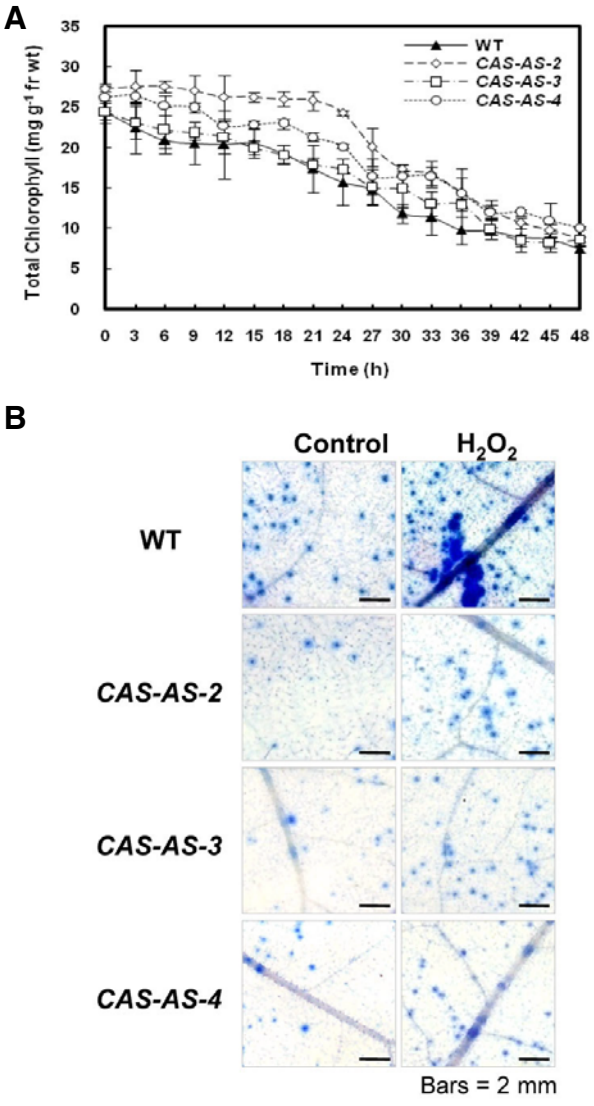

Fig. 4. Total chlorophyll content and cell damage in leaf discs from wild-type and transgenic lines (T_2 progeny for *CAS-AS* lines), treated with 20 mM H_2O_2 in 20 mM MES buffer under high light (200 μmol photons m^{-2} s^{-1}) at 25 ± 5°C. (A) Means ± SDs of the total chlorophyll content in the leaf discs. When a two-way ANOVA was used to identify statistically significant differences between H_2O_2-treated wild-type plants and all three transgenic lines ($P < 0.05$), the differences were found to be statistically significant. (B) Necrotic areas in leaf discs were stained with trypan blue, and then photographed with a digital camera.

stress, the total amount of chlorophyll in *CAS-AS-2* remained relatively constant until 21 h, after which point rapid degradation occurred. Similarly, chlorophyll degradation in *CAS-AS-4*, and to a lesser extent in *CAS-AS-2*, was considerably retarded until 18 h. Necrotic areas were observed as blue spots, which indicated accumulation of trypan blue. After 24 h of H_2O_2 treatment, necrosis appeared less severe in the transgenic plants compared to wild type (Fig. 4B). The more frequent and larger areas of necrosis on wild-type leaf discs reflected the more severe cell damage that occurred in these plants following abiotic stress. In all tobacco lines, the chlorophyll degradation rates accelerated after the second peak of ethylene biosynthesis, implying that ethylene was directly responsible for the cell damage.

Photosynthesis rates and gene expression in salt-stressed *CAS* transgenic plants

To validate stress tolerance in the *CAS* transgenic plants, we determined constitutive transcript levels for photosynthesis-related genes using qRT-PCR in untreated plants. These analyses included genes encoding the chlorophyll a/b binding proteins CAB, CAB21 and CAB36, and NADPH:protochlorophyllide oxidoreductase (POR). A key enzyme in chlorophyll biosynthesis and subsequent assembly of the photosynthetic apparatus, POR catalyzes the *trans* addition of hydrogen to chlorophyll, which produces chlorophyllide (Heyes et al., 2007). *CAB* gene expression was higher in *CAS-AS-4* than in wild-type plants, i.e., the levels of *CAB21* and *CAB36* were 2.3- and 6.2-fold greater, respectively (Fig. 5A). *POR* transcripts were also expressed more abundantly in *CAS-AS-2* and *CAS-AS-4* than in wild-type plants. Photosynthetic gas exchange parameters indicated that *CAS-AS-4* plants maintained a significantly higher rate of photosynthesis than wild type, under both control and salt-stressed conditions (watered with 200 mM NaCl daily for 30 days; Fig. 5B). Following high salt treatment with floating of leaf discs in 200 mM NaCl for 1 day, the transcript level of the ribulose bisphosphate small subunit (*RbcS*), which was light-responsive and greatly reduced as afternoon approached, decreased more rapidly in wild-type leaf discs than in transgenic plants (Fig. 5C). *CAB* transcripts were also more abundant in *CAS-AS-4* than wild-type plants. The tolerance of the transgenic plants to oxidative stress (Figs. 4A and 4B) was reinforced by photosynthetic activity via increased expression of *POR1* and *RbcS*, as well as the *CAB* genes, *CAB21* and *CAB36* (Figs. 5A and 5C). These results may explain previously observed enhanced agronomic traits of *CAS-AS-4* plants (e.g., biomass).

ROS accumulation monitored by fluorescence imaging and DAB staining

To investigate the physiological function of stress ethylene in ROS accumulation, we measured ROS levels in H_2O_2-treated leaf discs and whole plants using confocal microscopy with DCFH-DA fluorescence (Fig. 6A) and DAB staining (Fig. 6B), respectively. DCF, a sensitive reporter of intracellular ROS levels, enters epidermal cells in a DCFH-DA form, where it is hydrolyzed and trapped as non-fluorescent DCFH. Subsequent oxidation of DCFH by cytosolic H_2O_2 yields the strongly fluorescent DCF (Allan et al., 2001). We used confocal microscopy of DCF to monitor ROS accumulation in tobacco plants. Prior to oxidative treatment, ROS production was observed at very low levels in most of the loaded guard cells within the confocal slice from all plants (Fig. 6A). Following H_2O_2 treatment, guard cell fluorescence increased rapidly in both wild-type and *CAS-AS-4* plant leaves. After 1 h of treatment, ROS-mediated fluorescence increased significantly in the guard cells of wild-type leaf discs and was considerably greater than that observed in *CAS-AS-4*. This line also demonstrated lower accumulation of ROS than wild-type plants following treatments with high salt (200 mM NaCl) or 100 μM ABA. The results were very highly similar in all experiments which were repeated three times in duplicate. In response to ABA, NaCl and H_2O_2, the stomata of wild-type tobacco plants were found to close within a few minutes, whereas the stomata of all the transgenic plant lines showed a similar but less sensitive abiotic response (Supplementary Fig. 3). The reduced sensitivity of transgenic plants with respect to closure of the stomatal aperture corresponded to the lower level

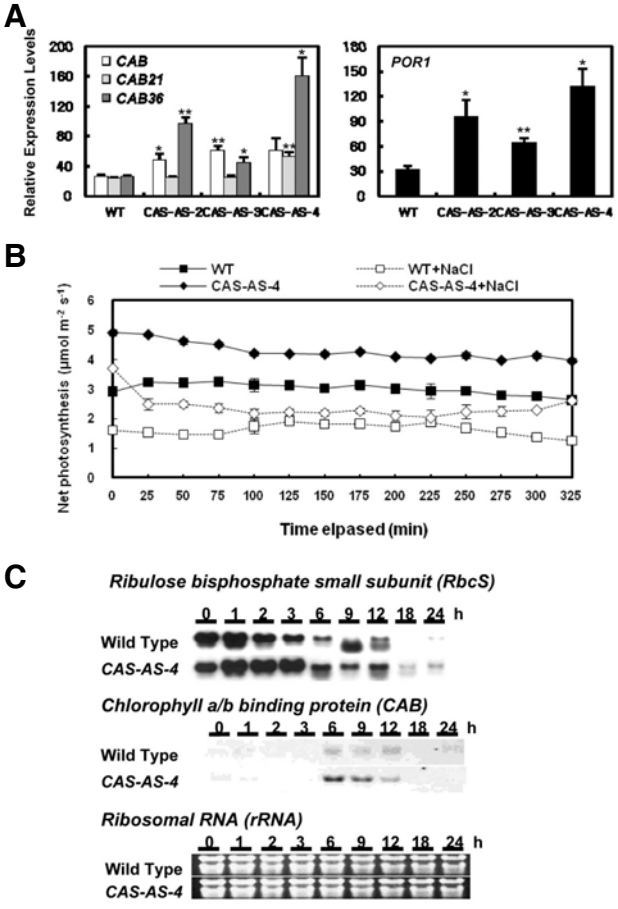

Fig. 5. Expression of photosynthesis-related genes and measurement of net photosynthetic rates after treatment with NaCl. (A) Accumulation of endogenous *CAB* and *POR1* transcripts in untreated plants. Transcript abundances are expressed relative to a reference gene (*β-actin*) after qRT-PCR with gene-specific primers. Expression levels are reported as means ± SD. Significant differences between H_2O_2-treated transgenic and wild-type plants based on two-tailed Student's *t*-test are indicated by one ($P < 0.05$) or two asterisks ($P < 0.01$). (B) Photosynthetic activity of salt-stressed whole plants of wild-type and transgenic lines (T_1 progeny). For stress treatment, whole 8-week-old tobacco plants were watered for 30 days with 20 mM MES buffer containing 200 mM NaCl under light (100 μmol photons m^{-2} s^{-1}) at 25 ± 5°C. After moving the whole plants to a Gas Exchange Measuring Station (Walz, Germany), the net photosynthetic rates were measured in triplicate. Values are means ± SD. When a two-way ANOVA was used to identify statistically significant differences between H_2O_2-treated wild-type plants and all three transgenic lines ($P < 0.05$), the differences were found to be statistically significant. (C) Expression of *RbcS* and *CAB* genes in salt-stressed leaf discs of wild-type and transgenic plants (T_2 progeny). Total RNA was extracted at the times indicated from leaf discs treated with 200 mM NaCl for 24 h, and then subjected to Northern blot analysis with the probes. Ethidium bromide staining was included to verify equal RNA loading.

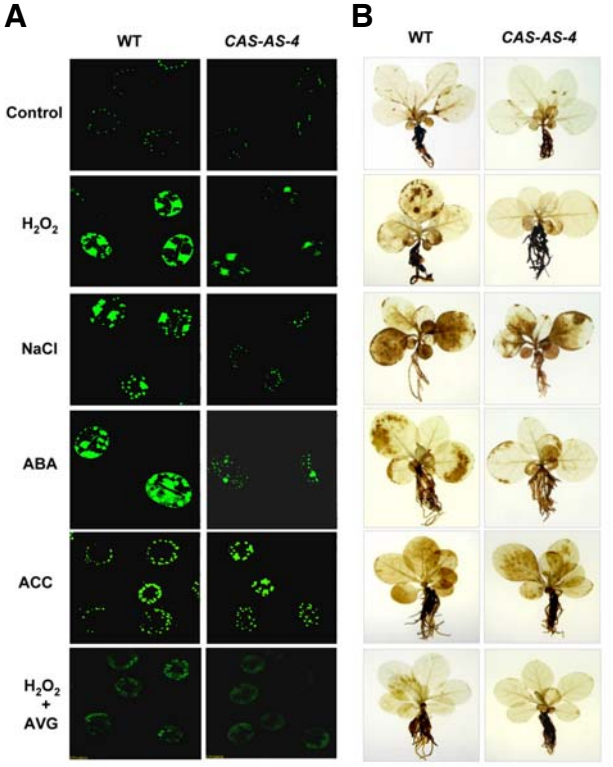

Fig. 6. Accumulation of intracellular ROS in response to abiotic stresses in whole plants of wild-type and transgenic tobaccos (*CAS-AS-4*) was determined using fluorescent imaging (A) and DAB staining (B). Confocal images from leaf discs were obtained after incubation with DCFH-DA for 10 min. Leaf discs (A) or four-week-old whole plants (B) of wild-type and *CAS-AS-4* were pretreated with MES buffer containing 20 mM H_2O_2 with or without 5 μM AVG, 100 μM ABA, 200 mM NaCl, or 0.5 mM ACC, for 30 min (B) or 1 h (A).

of ROS accumulation in guard cells after oxidative stress. It has been suggested that the rapid accumulation of ROS in guard cells that occurs at an early stage of the stress response might be responsible for the rapid adaptation to stress. ROS accumulation after ACC treatment in *CAS-AS-4* plants was very similar to that in wild-type plants. Also, treatment with aminoethoxyvinylglycine (AVG), an inhibitor of ACC synthase, almost completely suppressed H_2O_2-induced ROS accumulation in both wild-type and *CAS-AS-4* plants. These results imply that H_2O_2-induced ROS accumulation was entirely attributed to the expression of ACC synthase. Therefore, ethylene might be a factor that determines the endogenous levels of ROS during abiotic stress in plants.

Oxidation of DAB leads to its polymerization and deposition at sites of ROS generation via an endogenous peroxidase-catalyzed reaction (Liu et al., 2007). Following 30 min of abiotic stress with H_2O_2, ABA, or NaCl, ROS accumulation was measured by DAB in whole plants. Dark-brown DAB precipitates were a little more abundant at 30 min in all stress treatments on 4-week-old wild-type and transgenic tobacco plant with the highest intensity observed in salt-stressed wild-type plants (Fig. 6B). In comparison to wild-type, exogenous ACC application substantially compensated for ROS accumulation in *CAS-AS-4* plants. Because only *ACS* gene expression was impaired in the *CAS-AS-4* line, ROS accumulation was almost completely compensated in this transgenic plant after ACC treatment. Furthermore, AVG almost completely prevented H_2O_2-induced ROS accumulation in wild-type and *CAS-AS-4* plants. These results suggest that ACC synthase is a crucial enzyme for ROS accumulation via the ethylene biosynthesis pathway, indicating

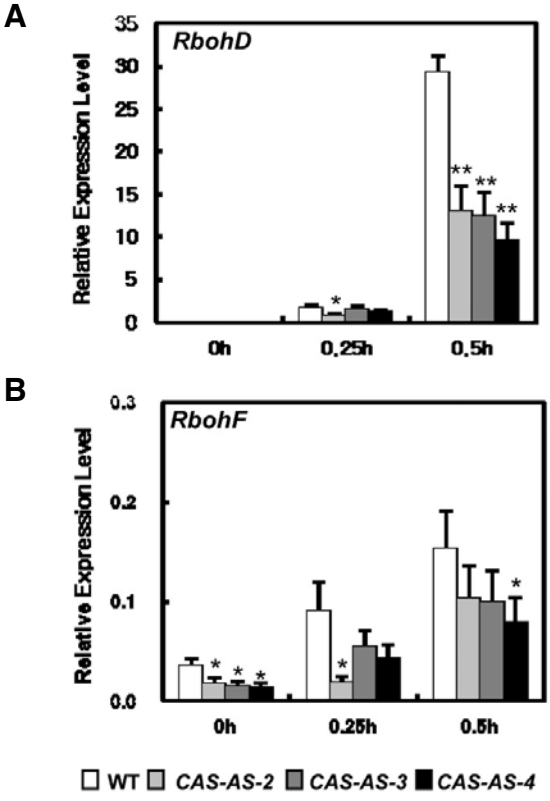

Fig. 7. Accumulation of endogenous *RbohD* and *RbohF* relative to the reference gene of β-actin after qRT-PCR with gene-specific primers. At time 0, leaf discs were treated with 20 mM H_2O_2. Expression levels are reported as means ± SD. Significant differences between H_2O_2-treated transgenic and wild-type plants based on two-tailed Student's *t*-test are indicated by one ($P < 0.05$) or two asterisks ($P < 0.01$).

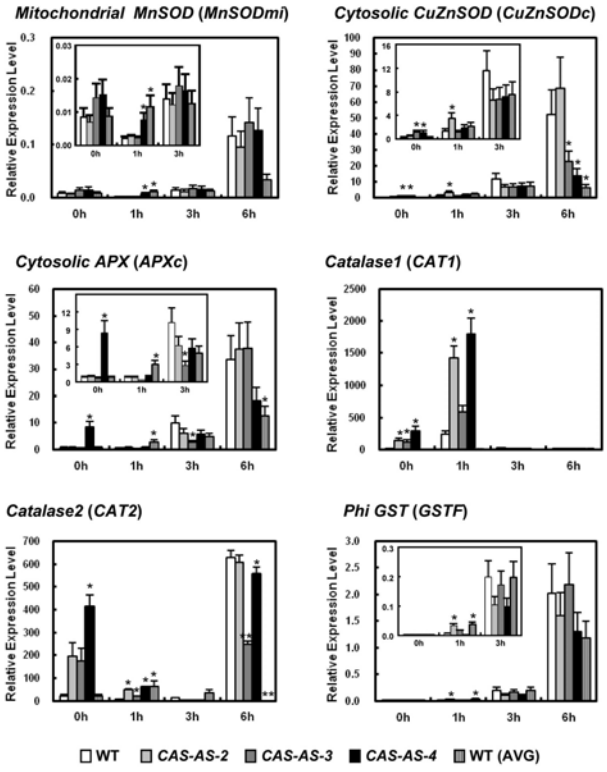

Fig. 8. Transcript accumulation of endogenous ROS-detoxifying enzymes, including *MnSODmi*, *CuZnSODc*, *APXc*, *CAT1*, *CAT2* and *GSTF*. Transcripts amounts were expressed relative to the reference gene of β-actin after qRT-PCR with gene-specific primers. At time 0, leaf discs were treated with 20 mM H_2O_2 in 20 mM MES buffer, and then incubated for 6 h. At the indicated times, leaf discs were displaced and mRNA was extracted. Wild-type plants were treated with 20 mM H_2O_2 with or without AVG, and each line of transgenic plants was treated with only 20 mM H_2O_2. Expression levels are reported as means ± SD. Significant differences between H_2O_2-treated transgenic and wild-type plants based on two-tailed Student's *t*-test are indicated by one ($P < 0.05$) or two asterisks ($P < 0.01$).

that ethylene synthesis may act upstream of ROS accumulation in response to oxidative stress following H_2O_2 treatment.

Transcript profiles of ROS-synthesizing enzymes

After H_2O_2 treatment, *RbohD* and *RbohF* transcript levels rapidly increased from 15 min, peaked at 30 min (Fig. 7), and then returned to a almost basal level at 1 h (data not shown). The ROS generation, which was determined by gene expression of *RbohD* and *RbohF*, occurred slightly earlier than *ACS* gene expression and ethylene production. The *RbohD* and *RbohF* transcripts were induced more significantly by H_2O_2 treatment in wild-type compared to impaired ethylene biosynthetic plants. Also, the relative expression levels of *RbohD* transcripts were significantly high compared to those of *RbohF*, implying *RbohD* expression might be considerably responsible for stress-induced ROS generation.

Expression profiles and activities of stress-induced ROS-detoxifying enzymes

The increased expression of antioxidative genes appears to be a general response to abiotic stresses. It has been observed in related studies, including those on oxidative stress; therefore, antioxidative proteins must play an important role in the adaptation process. It would be informative to determine the capacity for ROS-detoxification of ethylene-impaired transgenic plants that show stress-tolerant phenotypes. We investigated whether antisense expression of *ACS* could influence gene expression and activity of ROS-detoxifying enzymes in tobacco leaves.

We examined the gene expression levels and the activities of ROS-detoxifying enzymes, such as SOD, APX, GST, or CAT, during ethylene production after H_2O_2 treatment. MnSOD acts as the first line of defense against ROS, and is responsible for the dismutation of superoxide into oxygen and H_2O_2, after which H_2O_2 is subsequently detoxified by APX and CAT (Apel and Hirt, 2004). We focused on the transcripts for enzymes playing a major role in detoxifying ROS: mitochondrial manganese-superoxide dismutase (*MnSODmi*), cytosolic copper/zinc superoxide dismutase (*CuZnSODc*), cytosolic ascorbate peroxidase (*APXc*), catalase (*CAT1* and *CAT2*), and phi glutathione-S-transferase (*GSTF*), as identified in our preliminary microarray analysis (data not shown).

Before oxidative stress, the relative expressions of *MnSODmi*, *CuZnSODc*, *APXc*, *CAT1* and *CAT2* were up-regulated compared to wild-type plants (Fig. 8). The enzymatic activities of SOD, APX, catalase, and GST were also higher in the untreated transgenic lines than in the wild-type (Fig. 9). The relative constitutive expressions of *CAT1* and *CAT2,* normalized to

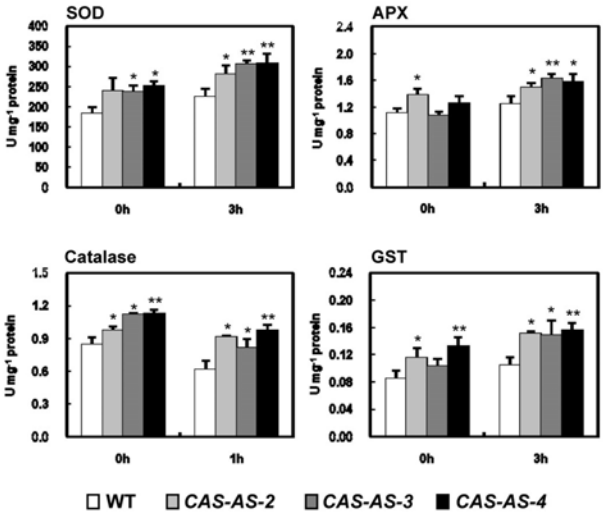

Fig. 9. Activities of ROS-detoxifying enzymes, including SOD, APX, catalase, and GST. At time 0, leaf discs were treated with 20 mM H_2O_2 in 20 mM MES buffer. Enzyme activities of SOD, APX and GST were determined from leaf discs treated with H_2O_2 for 3 h and catalase activity was measured after treatment with H_2O_2 for 1 h. Enzyme activity is reported as means ± SDs. Significant differences between H_2O_2-treated transgenic and wild-type plants based on two-tailed Student's *t*-test are indicated by one ($P < 0.05$) or two asterisks ($P < 0.01$).

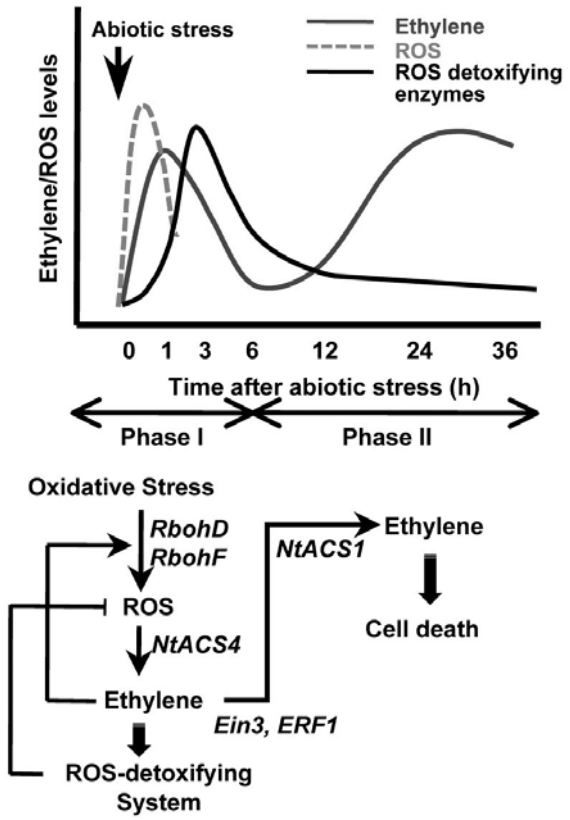

Fig. 10. Scheme of the ethylene/ROS signaling pathway in response to abiotic stress. Abiotic stress such as H_2O_2 treatment leads to a transient accumulation of ROS, which in turn results in elevated ethylene production during phase I, and subsequent autoactivation for further ethylene biosynthesis during phase II. Positive feedback loop exists between H_2O_2 and ethylene biosynthesis in a biphasic manner, resulting in a self-enhanced amplification of both ethylene and ROS signaling.

the reference gene *β*-actin, in untreated transgenic tobacco leaves were significantly higher than those of other transcripts. *CAT1* and *CAT2* were the most upregulated enzymes, displaying 19.3 to 18.9-fold higher expression in *CAS-AS-4* than in the wild-type (Fig. 8). The relative expression level of *APXc* in untreated *CAS-AS-4* leaves was upregulated by 11.9-fold compared to the wild-type.

We treated wild-type and transgenic tobacco plants with 20 mM H_2O_2, and measured the gene expression profiles, as determined by real-time qRT-PCR (Fig. 8) and activities (Fig. 9) of these several enzymes. The expression of ROS-scavenging enzymes, except for *CAT1,* was markedly higher in stressed wild-type leaves than in the untreated control at 6 h post-H_2O_2 treatment. *MnSODmi* exhibited a 13.6-fold increase, *CuZn-SODc* a 46.4-fold increase, *APXc* a 14.4-fold increase, and *CAT1* a 28.5-fold increase over the levels in the untreated controls. Although *GSTF* transcripts were almost not detected in untreated wild-type tobacco leaves, *GSTF* expression was rapidly induced by H_2O_2 treatment after 3 h. In this study, the transcripts of all ROS-detoxifying genes, *MnSODmi, CuZn-SODc*, *APXc*, *CAT1*, and *CAT2*, were higher in transgenic lines than in the wild-type at 1 h post-H_2O_2treatment (see inserts in Fig. 8), implying these phenomena was responsible for more accumulation of ROS wild-type plants. GST catalyzes the nucleophilic attack of the tripeptide glutathione on lipophilic compounds with electrophilic centers, and it plays an important role in protecting cells from oxygen toxicity (Wagner et al., 2002). Even though the *GSTF* transcript levels increased steadily after 1 h of H_2O_2 treatment in both wild type and other transgenic lines, the level, which were reacted in biphasic manner of these transcripts were much low at 1 h in *CAS-AS*-4 than in *CAS-AS-2* and *CAS-AS-3*, indicating that *GSTF* had little effect on ROS detoxification in *CAS-AS-4* (Fig. 8). Thus, the lower levels of ROS in *CAS-AS-4* (Fig. 7) are due to the enhanced endogenous expression and activity of ROS-detoxifying enzymes in 0 h through 1 h after stress treatment. These lower levels might also be related to the suppression of ethylene production in the early phase (phase I). The lower ROS levels would have reduced further stress-induced ethylene production, subsequently resulting in the retardation of stress-induced cell damage, including chlorophyll degradation. However, these changes in transcripts of antioxidative enzymes in transgenic lines did not continue until 6 h post-H_2O_2treatment. Wild-type plants, in which H_2O_2-induced ethylene production was prevented by AVG treatment, showed a significant reduction in transcripts of antioxidative enzymes at 6 h, compared to wild-type treated with only H_2O_2.

In contrast to its expected detoxifying role in abiotic stress, *CAT1* expression decreased after 1 h of H_2O_2 treatment in both wild-type and transgenic plants, and was almost absent after 3 h of H_2O_2 treatment. However, *CAT2* expression was greatly reduced after 1 h of stress treatment and reached a basal level at 3 h. After this, its expression increased significantly after 6 h of stress treatment. The fluctuations in *CAT1* and *CAT2* gene expression were similar both in wild-type and transgenic plants. The findings may relate to the circadian rhythm, as suggested by fluctuations in catalase transcript abundance over the day/light cycle (Luna et al., 2005). *CAT1* mRNA levels are

highest early in the light period, while *CAT2* mRNA levels are highest later in the day. Since our experimental leaf discs were prepared at 10:00 AM, *CAT1* expression was at its highest level at 0 h (Fig. 8).

Enzyme activities of SOD, APX, catalase, and GST showed higher basal levels in the untreated transgenic leaf discs compared to wild-type discs (Fig. 9). Following H_2O_2 treatment for 1 h (CAT) or 3 h (SOD, APX and GST) in early stage (phase I), these activities increased and were present at significantly higher levels in the transgenic than in the wild-type plants. The activities of ROS-detoxifying enzymes increased most prominently in the *CAS-AS-4* line with or without stress treatment, compared to the wild-type and other transgenic lines. Lower ROS accumulation in *CAS-AS-4* might be due to the higher performance of these enzymes in this plant.

DISCUSSION

When plants are exposed to conditions that threaten their ability to survive, the same mechanism that produces ethylene for normal development functions to produce what is known as stress ethylene (Stearns and Glick, 2003). Fine-tuning of ethylene production is an important factor in developmental processes and plant responses to stresses. In plants exposed to various types of abiotic and biotic stresses, increased ethylene levels correspond to increased damage, implying that stress ethylene is deleterious to plants. However, it was also reported that transcriptional activation of ERF in ethylene signa-ling process enhances stress tolerance in tobacco seedlings by decreasing ROS accumulation in response to salt, drought and freezing (Wu et al., 2008). It may be that these discrepancies are due to differences in the amount of endogenous ethylene production, and also in the period of stress treatment used in each case, in addition to the plant tissue studied. Therefore, to get deeper insights into the action mechanism of ethylene involved in stress response, the analyses of ethylene and ROS production and their biosynthetic gene regulation were carried out using stress-tolerant tobacco plants.

ROS, such as singlet oxygen, superoxide anion radicals, hydroxyl radical and H_2O_2, are important plant cell signaling molecules involved in biotic/abiotic stress responses and in developmental and physiological processes (Shirasu et al., 1997). These ROS are also important components regulating cell death during the stress response and in developmental processes (Moeder et al., 2002), with similarity to the physiological roles of ethylene in some ways. Recent data indicate that H_2O_2-induced stomatal closure requires the ethylene receptor ETR1, and ethylene-induced stomatal closure is dependent upon H_2O_2 production in guard cells (Desikan et al., 2006). Therefore, we investigated first the amounts of ethylene production and the level of ROS accumulation during the stress response by comparing those between wild-type and stress-tolerant plants.

The transgenic tobacco lines used here as stress-tolerant plants expressed the antisense gene for *CARACC*, which shows the significant homology to ripening-dependent *LE-ACS2,* a gene that is strongly induced by elicitor, in tomato (Oetiker et al., 1997). Sterns and Glick (2003) suggested a biphasic model for stress ethylene that explains its seemingly paradoxical effects on plants, and which appears to be directly applicable to our experiments. Specifically, in stressed plant tissues there is an initial small peak (phase I) of ethylene close in time to the onset of stress and then a second, much larger, peak (phase II) at some later time point. In our transgenic plants, ethylene production in phase II was substantially reduced following oxidative stress, as a result of antisense *CARACC*-mediated disruption of stress-related *ACS* transcripts. It was reported that epidermal cell death in rice was induced by ethylene with a lag phase longer than 26 h (Steffens and Sauter, 2009). Therefore, the decrease in the second peak of ethylene production, which is so large that it initiates processes such as senescence, chlorosis and necrosis, may be responsible to stress tolerance in transgenic tobacco lines.

We also observed that wild-type and all transgenic plants exhibited biphasic gene-specific regulation of *NtACS* expression in response to H_2O_2. It may be inferred that H_2O_2 treatment led to the expression of *NtACS4* for stress signaling during phase I and of *NtACS1* for necrosis during phase II. Many studies have demonstrated that the differential transcription of *ACS* gene family members is an important factor for regulating ethylene production in response to different stimuli (Pech et al., 2004). Our results were also implied that each transcript of *ACS* gene family was differentially controlled in a time-depen-dent induction after same stimulus. *LE-ACS2*, a homolog to *NtACS 1*, which was commonly known as fruit ripening-related *ACS* gene, was expressed 8 h after flooding stress (Shiu et al., 1998). However, *LE-ACS3* and *LE-ACS7*, homologues of *NtACS4*, played a role early during flooding stress. The induction pattern of *LE-ACS7* by flooding and wounding (Shiu et al., 1998) was almost identical to that of *NtACS4* (Fig. 2C), suggesting that those genes were relatively contributed to the initial burst (phase I) of biphasic ethylene production. In many plant tissues, ACS activity displays a short half-life (i.e., between 15 and 25 min in vegetative tissues) (Chae and Kieber, 2005), being post-transcriptionally (Woeste et al., 1999) or post-translationally regulated (Tatsuki et al., 2001). It is implied that its more rapid turnover is doing better driving gene-specific control of each *NtACS* transcript in different conditions and time.

Ethylene activates its own biosynthesis in plants. Autocatalytic ethylene production, which represents self-induced ethylene production required for its own biosynthesis, is attributed to the increases needed in fruit-ripening and leaf-senescent tissues (Zhang et al., 2009). We previously reported that *CARACC* provides autocatalytic regulation at the transcriptional level in carnation petals (Park et al., 1992). It may be that further biosynthesis is autocatalyzed by transient, stress-induced ethylene synthesis, mediated by increased *NtACS4* expression during phase I, leading to the marked increase in ethylene production observed in phase II, mediated by increased *NtACS1* expression (Fig. 2). *LEACS2* and *LEACO1* mRNA levels were regulated by ethylene through positive feedback activation. Expression of *LeERF2* in tomato was induced within 0.5 h of ethylene treatment, and the transcript levels peaked after 2 h (Zhang et al., 2009). Also, tomato that overexpressed *LeERF2* showed enhanced expression of ethylene biosynthesis genes such as *LEACS1*, *LEACS2*, *LEACS3*, *LEACS4*, and *LEACO3*, whereas tomato that expressed antisense *TERF2* showed significantly reduced expression of *LEACS2*, *LEACS4*, and *LEACO3* but not that of *LEACS1* and *LEACS3* (Zhang et al., 2009). Therefore, our results and their results would seem to indicate that one member of the *ACS* gene family was responsible for the activation of other members of this family, in a time-dependent manner, during stress-induced ethylene production. However, not all members of the *ACS* gene family were expressed following autocatalyzed activation. For example, in tomato fruit, mRNA levels of *LEACS6*, which has higher similarity with *NtACS5*, were regulated by ethylene accumulation through an autoinhibitory mechanism (Zhang et al., 2009).

Robinson et al. (2001) suggested that the first low level production of ethylene, which is observed 3 to 6 h after abiotic stress, consumes the existing pool of ACC and this then accu-

mulates to fuel the second wave of ethylene production. However, our experiment suggests that the first peak and second peak of ethylene production may have resulted from *de novo* synthesis of ACC by the stress-inducible ACS members, *NtACS1* and *NtACS4,* respectively. Therefore, ethylene evolution in biphasic manner, as characterized by its rapid activation and autocatalyzation, results in several subsequent hallmarks of programmed cell death (Castagna et al., 2007). Accumulation of *EIN3* and *ERF1* transcripts increased markedly at the beginning of phase II (6 h) after H_2O_2 treatment (Fig. 3), implying a transcriptional cascade initiated between Phase I and Phase II in stress-induced ethylene signaling. Therefore, we suggest a hypothesis in which autocatalytic induction resulted from endogenous ethylene levels represent a key component between the first and second peaks of ethylene production, in response to stresses.

Several reports have indicated that ethylene plays a crucial role in the production or maintenance of ROS levels (de Jong et al., 2002). Exogenous H_2O_2 or enhancement of endogenous H_2O_2 production promoted epidermal cell death in a dose-dependent manner (Castagna et al., 2009). Also, inhibition of NADPH oxidase lowered ethylene-induced cell death rates. In O_3-exposed plants, AVG-mediated inhibition of ACC synthase activity, Co^{2+}-inhibited ACC oxidase activity and 2,5-norbornadiene-inturrupted ethylene recognition, all reduce H_2O_2 accumulation at 6 to 8 h, as well as tissue damage at 24 h (Moeder et al., 2002). Therefore, it can be hypothesized that the suppressed level of ethylene production, which might result in decreased ROS accumulation, was responsible to a stress tolerance in transgenic tobacco lines.

Not only ACC treatment considerably induced ROS accumulation in untreated wild-type plants, but also the blockage of ethylene production with AVG treatment significantly prevented H_2O_2-induced ROS accumulation (Fig. 6). In addition to these facts, transcript increases of *RbohD* and *RbohF* in 30 min (Fig. 7) and ROS burst in 30 to 60 min (Fig. 6) after oxidative stress with H_2O_2 were consistent with a initial burst of biphasic ethylene production (Fig. 2). Therefore, it has been also suggested that ethylene and ROS act in a positive feedback cycle that resulted in mutual enhancement of ethylene and ROS production during stress-induced cell death. It can be imagined that the first peak of endogenous ROS accumulation following the onset of H_2O_2 treatment led to the expression of *NtACS4* for stress signaling ethylene production during phase I and then to a second phase, which was resulted from activation of *NtACS1* expression for a larger and more prolonged period of ethylene production that correlated with necrosis. These results implied that ROS could also act as a signal upstream of ethylene biosynthesis, in addition to the role of ethylene as an upstream signaling molecule in stress-induced ROS accumulation as mentioned earlier.

It is possible that a survivable threshold level of ROS accumulation/ethylene biosynthesis determines whether the stress-treated cells live or die via amplifying a burst of ROS production (Dat et al., 2003). It is thought that the rapid accumulation of ethylene-induced ROS represents a signal for stress metabolism during environmental stress (Mittler et al., 1999). This implies that ROS may play a role in the avoidance of cell death, possibly by inducing transcriptional activation of protective genes. Recent studies have further defined the sources and control of ROS production (Overmyer et al., 2003). We observed that the severity of necrosis was proportional to the synergistic accumulation of ROS and ethylene in comparing between wild-type and ethylene-impaired transgenic plants. It is possible that higher concentrations of ROS would result in cell death rather than in protective gene induction; thus, ROS-mediated cell death induction appears to be a threshold phenomenon (Dat et al., 2003; Tenhaken et al., 1995).

Increases in ROS-detoxifying machineries were very effective at increasing tolerance to oxidative and other abiotic stresses. Thus, appropriate ROS detoxification responses and the proper regulation of steady-state ROS levels are vital for mediating tolerance or cell death. Since the local antioxidant capacity determines the susceptibility of a cellular target to oxidative damage or H_2O_2 signaling, it is also important to determine the impact of antioxidant enzyme regulation on ROS-sensing/signaling events (Veal et al., 2007). The studies described here, particularly those concerning the complex relationships between regulation of ROS-detoxifying enzymes and ethylene production involved in stress tolerance, provide insights into how plants coordinate appropriate cellular responses to abiotic stress.

It is known that transcripts of *MnSODmi*, *CuZnSODc*, *APXc* and *GSTF,* which were studied in our experiments, are specifically expressed in response to abiotic stress such as O_3 fumigation (Castagna et al., 2007), light stress (Davletova et al., 2005), and water deficit (Gallé et al., 2009). The transcripts of those ROS-detoxifying genes were more abundant and their activities were higher in transgenic plants leaves than wild-type tobacco at untreated control and phase I after H_2O_2 treatment (Figs. 8 and 9). The transient ROS burst, which is greatly increased by less efficient ROS-detoxification, might be important as an integral part of the resulting long-term response to high levels of late ethylene production and subsequent cell damage. The lesser activation of antioxidative enzyme gene expression in wild-type plants brought about by the higher amounts of ROS accumulation/ethylene production at a later stage than in transgenic tobacco plants.

The stressed plants with a massive abiotic stress-induced ethylene could not be protected from deleterious damages by the oxidative burst, because antioxidant enzymes were not induced effectively at the transcriptional and/or activity levels. Ethylene levels raise cellular concentrations above a threshold level, resulting in increased ROS accumulation, which is responsible for cell death. Stress ethylene at high level specially potentiates the detrimental functions for defensive pathway. For tolerance to abiotic stress, the biosynthesis of a survivable level of stress-generated ethylene may be very important for adequate activation of the ROS-detoxifying system in the plant. Emerging evidence indicates that ROS signaling pathways play key roles in the crosstalk between biotic and abiotic stress signaling, in concert with hormone signaling pathways regulated by ABA, SA, JA and ethylene (Fujita et al., 2009). In summary, it is proposed that ethylene and H_2O_2 act as mutually and self-amplifying signal molecules in feed-forward loop regulation. A model summarizing these findings is shown in Fig. 10.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

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