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Two Arabidopsis Mutants That Overproduce Ethylene Are Affected in the Posttranscriptional Regulation of 1-Aminocyclopropane-1-Carboxylic Acid Synthase[1]

Keith E. Woeste, Chen Ye, and Joseph J. Kieber*

Department of Biological Sciences, Laboratory for Molecular Biology, University of Illinois at Chicago, Chicago, Illinois 60607

The Arabidopsis mutants *eto1* (ethylene overproducer) and *eto3* produce elevated levels of ethylene as etiolated seedlings. Ethylene production in these seedlings peaks at 60 to 96 h, and then declines back to almost wild-type levels. Ethylene overproduction in *eto1* and *eto3* is limited mainly to etiolated seedlings; light-grown seedlings and various adult tissues produce close to wild-type amounts of ethylene. Several compounds that induce ethylene biosynthesis in wild-type, etiolated seedlings through distinct 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) isoforms were found to act synergistically with *eto1* and *eto3*, as did the ethylene-insensitive mutation *etr1* (ethylene resistant), which blocks feedback inhibition of biosynthesis. ACS activity, the rate-limiting step of ethylene biosynthesis, was highly elevated in both *eto1* and *eto3* mutant seedlings, even though RNA gel-blot analysis demonstrated that the steady-state level of *ACS* mRNA was not increased, including that of a novel Arabidopsis *ACS* gene that was identified. Measurements of the conversion of ACC to ethylene by intact seedlings indicated that the mutations did not affect conjugation of ACC or the activity of ACC oxidase, the final step of ethylene biosynthesis. Taken together, these data suggest that the *eto1* and *eto3* mutations elevate ethylene biosynthesis by affecting the posttranscriptional regulation of ACS.

The gaseous hormone ethylene has been shown to influence numerous plant growth and developmental processes, including germination, root-hair initiation, leaf and flower senescence and abscission, fruit ripening, nodulation, and the response to a wide variety of stresses (Mattoo and Suttle, 1991; Abeles et al., 1992). Much progress has been made in elucidating the mechanisms of ethylene perception and signal transduction (Ecker, 1995; Kieber, 1997a, 1997b), as well as the ethylene-biosynthetic pathway (Kende, 1993). However, to fully understand the mechanism of ethylene action, it is important to delineate how its biosynthesis is regulated. We have chosen 3-d-old etiolated Arabidopsis seedlings as a model system to unravel this circuitry. Here we describe the characterization of two mutants that affect the regulation of ethylene biosynthesis in etiolated seedlings.

Almost all plant tissues have the ability to make ethylene, although in most cases the amount made is very low. Ethylene production increases dramatically during a number of developmental events such as germination, leaf and flower senescence and abscission, and fruit ripening (Yang and Hoffman, 1984; Mattoo and Suttle, 1991; Abeles et al., 1992). A diverse group of factors modify the level of ethylene biosynthesis, and a major effect of most of these is to increase the steady-state level of *ACS* mRNA (see Olson et al., 1991, 1995; Rottmann et al., 1991; Botella et al., 1993, 1995), although there is accumulating evidence to suggest that this enzyme is also posttranslationally regulated (Nakajima et al., 1990; Spanu et al., 1994; Oetiker et al., 1997; Vogel et al., 1998b).

The ethylene-biosynthetic pathway (for review, see Yang and Hoffman, 1984; Kende, 1993) starts with the conversion of Met to AdoMet by the enzyme Met adenosyltransferase. ACS, which converts AdoMet to ACC (Adams and Yang, 1979), is the first committed and generally rate-limiting step in ethylene biosynthesis. ACS is encoded by a small gene family comprising at least three to six members in the species that have been closely examined. Distinct subsets of *ACS* genes are expressed in response to various developmental, environmental, and hormonal factors.

In Arabidopsis six *ACS* genes have been identified (*ACS1–ACS6*), two of which are nonfunctional (Liang et al., 1992, 1995; Van der Straeten et al., 1992; Vahala et al., 1998). Inhibition of protein synthesis by cycloheximide treatment induces expression of the functional genes, suggesting that they are under negative control (Liang et al., 1992). Wounding, auxin, LiCl, and anaerobiosis differentially induce these genes (Liang et al., 1992, 1996; Van der Straeten et al., 1992). *ACS2* expression is higher in young, developing leaves and flowers compared with more mature tissues from these organs, and its expression is also correlated with the initial stages of lateral root formation (Rodrigues-Pousada et al., 1993). *ACS5* is the major isoform involved in the production of ethylene in response to low doses of cytokinin in etiolated seedlings, and this regulation is primarily via a posttranscriptional mechanism (Vogel et al., 1998b). *ACS4* transcription is induced by auxin, and several auxin-responsive elements have been identified upstream of the *ACS4* coding region (Abel et al., 1995). The steady-state level of *ACS6* transcript is increased by treatment with ozone (Vahala et al., 1998). The *ACS3* gene is most likely a

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* Corresponding author; e-mail jkieber@uic.edu; fax 1–312–413–2691.

Abbreviations: ACS, ACC synthase; AdoMet, *S*-adenosyl-Met.

pseudogene and *ACS1* encodes a nonfunctional ACS (Liang et al., 1995).

The final step of ethylene biosynthesis, the conversion of ACC to ethylene, is catalyzed by the enzyme ACC oxidase, which can also play an important role in regulating ethylene biosynthesis, especially under conditions of high ethylene production (Nadeau et al., 1993; Kim and Yang, 1994; Tang et al., 1994; Barry et al., 1996; Lasserre, 1996; Mekhedov and Kende, 1996). ACC oxidase, like ACS, appears to be encoded by a gene family whose members are differentially regulated in a number of plants. There are several *ACO* genes in Arabidopsis (Newman et al., 1994), at least one of which is induced by ethylene itself (Gomez-Lim et al., 1993).

ACC can be conjugated to an inactive form, malonyl-ACC, by the enzyme ACC malonyltransferase (Amrhein et al., 1981; Hoffman et al., 1982; Kionka and Amrhein, 1984). A second ACC conjugate, 1-(γ-L-glutamylamino) cyclopropane-1-carboxylic acid, has also been identified (Martin et al., 1995), although recent evidence suggests that this is a much less abundant conjugate (Peiser and Yang, 1998). There is some evidence that the level of ACC conjugation is regulated, which may contribute to control of ethylene production (Jiao et al., 1986).

Treatment of etiolated seedlings with ethylene results in a morphology known as the triple response. In Arabidopsis the triple response consists of shortening and radial swelling of the hypocotyl, inhibition of root elongation, and exaggeration of the curvature of the apical hook (see Fig. 1). This response has been used to identify mutants disrupted in ethylene perception and signaling (Bleecker et al., 1988; Guzman and Ecker, 1990; Kieber et al., 1993), as well as mutants affected in the regulation of ethylene biosynthesis. Mutants in the latter class fall into two categories: (a) those that fail to induce ethylene in response to a particular inducer (cytokinin-insensitive mutants, Cin; Vogel et al., 1998a, 1998b); and (b) those that overproduce ethylene (ethylene overproducer, Eto; Guzman and Ecker, 1990; Kieber et al., 1993). Three Eto loci have been identified: *eto1* is inherited as a recessive mutation, and *eto2* and *eto3* are dominant. *eto2* was recently found to be the result of a disruption of the carboxy-terminal 11 amino acids of ACS5 (Vogel et al., 1998b). Here we describe the physiological characterization of the *eto1* and *eto3* mutants. This analysis suggests that these mutations are affected in the posttranscriptional regulation of ACS.

MATERIALS AND METHODS

Plant Lines and Growth Conditions

The Columbia ecotype of Arabidopsis was used in this study. Seeds were surface-sterilized as described previously (Vogel et al., 1998b), resuspended in a suitable volume of top agar (0.8% low-melt agarose), and spread onto Murashige and Skoog agar (Murashige and Skoog salts [GIBCO-BRL], 2% Suc, and 0.8% agar, pH 5.7). Seeds were cold treated for 4 d (4°C), exposed to light for 2 h, and then moved to a dark incubator at 23°C. The time at which the vials were moved to 23°C was designated as time 0. Adult plants were grown in potting soil (Metro Mix 250, Grace-Sierra, Boca Raton, FL) under continuous illumination at 23°C. The *eto1-1* and *eto3* mutants and the *eto1-1/etr1-3* double mutant were identified previously (Guzman and Ecker, 1990; Kieber et al., 1993; Roman et al., 1995). The *eto1-3* allele was isolated from an x-ray-mutagenized population (ecotype Columbia) (Kieber et al., 1993). This allele was used for all of the experiments, with the exception of the *eto1/etr1* double-mutant analysis, which used the *eto1-1* allele. The *eto1-3/ein2* double mutant was obtained by crossing an *eto1-3* homozygote to a *ein2-1* homozygote. The F_1 population was allowed to self, and seedlings that were phenotypically Eto^- were then selected in the F_2 line. Tall progeny from these individuals were then selected and self-set seed was collected. A line that retested as being ethylene insensitive and that overproduced ethylene was identified and used in further experiments.

Ethylene Measurement

Hormones and $CuSO_4$ were added to about 20 2-d-old etiolated seedlings that were grown in 22-mL GC vials containing 3 mL of Murashige and Skoog agar by pipetting 200 μL of solution on top of the seedlings. An equal volume of water plus solvent was added to the control treatments. These vials were then flushed with hydrocarbon-free air and capped, and the accumulated ethylene measured 24 to 48 h later, as described previously (Vogel et al., 1998b). Ethylene production was normalized to the number of seedlings in each vial and the time between capping and sampling. All observations are from at least three replicates, and each experiment was repeated at least once with comparable results. To measure ethylene from adult plants, tissues were detached, weighed, and then placed in 22-mL vials containing 3 mL of Murashige and Skoog agar. The vials were flushed with hydrocarbon-free air, sealed, and incubated in the light for the indicated times. The amount of ethylene produced by light-grown seedlings was determined by putting capped vials in a lighted growth chamber for 72 h.

ACS Assays

ACS was assayed from 3-d-old etiolated seedlings as described previously (Peck and Kende, 1995), with some modifications. Sterilized seeds were plated on filter paper on Murashige and Skoog agar (10,000 seeds per 150-mm plate), cold incubated (4°C) for 4 d, and then moved to a dark chamber for 3 d at 23°C. Ten grams of tissue was added to 15 mL of buffer A (250 mM phosphate buffer, pH 8.0, 10 μM pyridoxal phosphate, 1 mM EDTA, 2 mM PMSF, and 5 mM DTT) and the sample was homogenized on ice for 4 min (maximum speed with a PowerGen 700 homogenizer [Fisher Scientific]). The sample was centrifuged at 15,000*g* for 15 min and the supernatant was respun at 15,000*g* for 15 min. One milliliter of the supernatant was placed into a 22-mL GC tube and 100 μL of 5 mM AdoMet was added. This was incubated for 1 h at 22°C. The ACC formed was converted to ethylene by addition of 100 μL of 20 mM HgCl, followed by 100 μL of a 1:1 mixture of

saturated NaOH:bleach (Lizada and Yang, 1979). The tubes were capped immediately after addition of the NaOH/bleach and incubated on ice for 10 min. Five milliliters of headspace was removed with a syringe and injected into a new vial, and the ethylene was measured as described previously (Vogel et al., 1998b). All reactions were done in triplicate and compared with controls, to which AdoMet was not added. Protein concentration was determined using the Bradford assay as described by the manufacturer (Bio-Rad).

RNA-Blot Analysis

Total RNA was prepared as described previously (Ausubel et al., 1994) and poly(A^+) RNA was isolated using Oligotex-dT resin, as described by the manufacturer (Qiagen, Chatsworth, CA). Five micrograms of poly(A^+) mRNA was separated on an agarose gel, blotted to a nylon membrane, and hybridized to radiolabeled probes as described previously (Ausubel et al., 1994). Fragments corresponding to each *ACS* gene and *β-tubulin* were obtained by amplifying each from Arabidopsis genomic DNA using PCR with oligonucleotide primers specific for each gene, or in the case of *ACS6*, the insert from the expressed sequence tag clone FAI88 (see below) was used. The signals were quantified with a phosphor imager and normalized to the level of the *β-tubulin* control. This analysis was repeated once with comparable results.

Isolation of *ACS7*

We searched the Arabidopsis expressed sequence tag database (Newman et al., 1994) for sequences similar to those of *ACS1* to *ACS6*. Three clones (FAI88, 288D2T7, and 240L12T7), corresponding to an identical gene, were identified that were similar to the previously identified Arabidopsis *ACS* genes, but encoded a novel ACS isoform. Sequence analysis revealed that these *ACS7* cDNA clones were missing the 5′ portion of the coding region (see "Results").

RESULTS

Ethylene Biosynthesis in the *eto1* and *eto3* Mutants Is Developmentally Regulated

The *eto1* and *eto3* mutants display a constitutive triple-response phenotype as etiolated seedlings (Fig. 1) caused by an overproduction of ethylene. We analyzed ethylene biosynthesis from wild-type and Eto mutant etiolated seedlings at various intervals during the first several days of growth (Fig. 2). There was almost no detectable ethylene produced by wild-type seedlings during the early stages of germination (<24 h), followed by a low, stable level during the next 6 d. Ethylene biosynthesis in *eto1-3* seedlings rose quickly between 48 and 60 h, and remained at a steady, elevated level for the next 60 h, after which it gradually declined to close to wild-type levels. *eto3* mutant seedlings displayed a similar pattern, although their peak production was about 2.5-fold higher and the rate of decline of production was increased compared with that observed in *eto1-3* seedlings. By d 9, both mutants produced close to wild-type levels of ethylene.

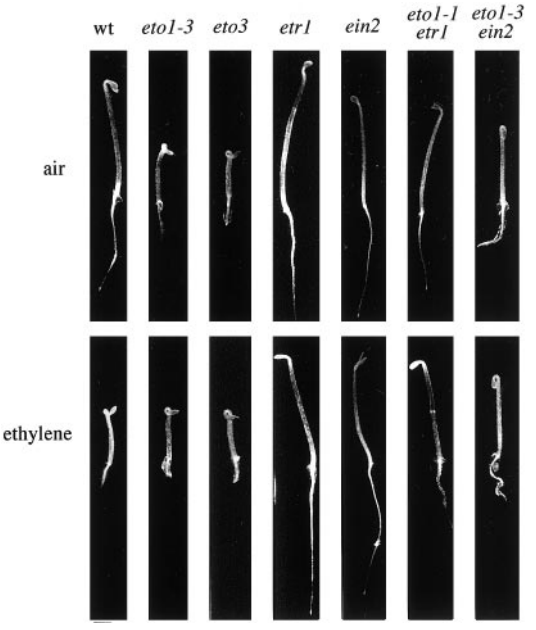

Figure 1. Phenotypes of 3-d-old etiolated wild-type (wt), single-mutant, and double-mutant Arabidopsis seedlings (as indicated) grown in air (top panels) or 10 μL L^{-1} ethylene (bottom panels). Representative seedlings were picked and photographed.

To determine if the decline in ethylene biosynthesis was caused by feedback regulation, we examined the time course of ethylene biosynthesis in the double-mutant seedlings of *eto1-3* and the mutation *ein2* ethylene insensitive (Guzman and Ecker, 1990). The initial rate of increase in ethylene biosynthesis was the same for both *eto1-3* and *eto1-3/ein2* seedlings (Fig. 2B). However, the double mutant continued to increase its rate of ethylene biosynthesis beyond 60 h, the point at which the rate in *eto1-3* seedlings peaked. The peak of biosynthesis in the double mutants was close to what one would predict from an additive interaction, suggesting that these mutations act independently to regulate ethylene biosynthesis. The rate of ethylene biosynthesis in double-mutant seedlings also returned to close to that observed in wild-type seedlings, although the rate of decline was somewhat slower than in the *eto1-3* single mutant. This suggests that the decline in biosynthesis is not the result of negative-feedback regulation from the elevated ethylene levels but, rather, may reflect a developmental change in the regulation of ethylene biosynthesis (although feedback regulation may alter the rate at which this occurs).

We examined ethylene production from various adult tissues to determine if any were affected by the Eto mutations. Both *eto1-3* and *eto3* affected ethylene biosynthesis almost exclusively in etiolated seedlings: light-grown seedlings (not shown), adult leaves, flowers, and siliques from *eto1-3* and *eto3* mutants produced close to wild-type levels of ethylene (Fig. 3). Thus, these mutants are either specific

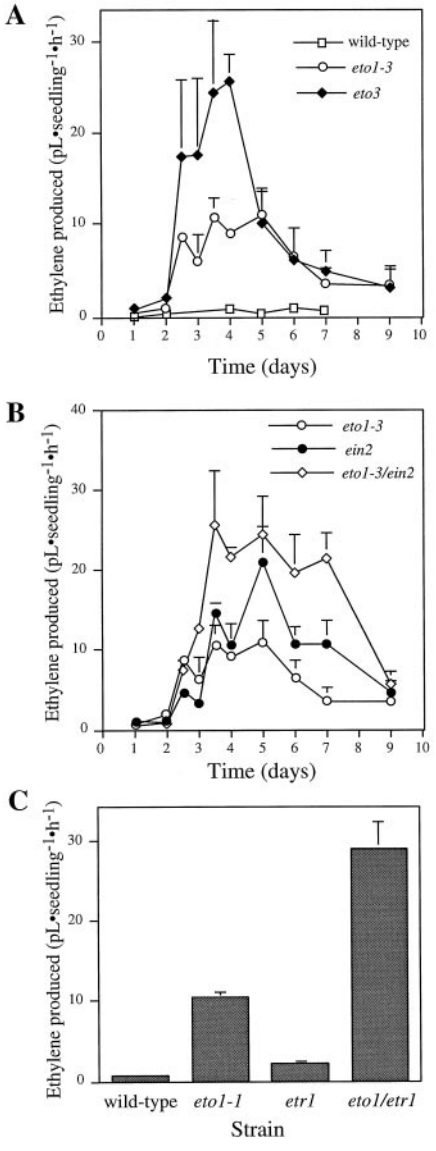

Figure 2. Ethylene biosynthesis by wild-type, single-mutant, and double-mutant etiolated Arabidopsis seedlings. Seedlings were grown for various times on Murashige and Skoog agar in GC vials and the accumulated ethylene was measured. A, Time course of ethylene production by etiolated wild-type, *eto1-3*, and *eto3* mutant seedlings. B, Time course of ethylene production by etiolated *eto1-3*, *ein2*, and *eto1-3*/*ein2* double-mutant seedlings. The data points in A and B represent the ethylene accumulated during the time intervals 0 to 24, 24 to 48, 48 to 60, 60 to 72, 72 to 84, 84 to 96, 120 to 144, 144 to 168, and 168 to 216 h. The second time point in each interval was plotted as the value for the *x* axis. C, Ethylene accumulated by etiolated wild-type *eto1-1*, *etr1*, and *eto1-1*/*etr1* double-mutant seedlings from 72 to 96 h. All values are means (±SD) of three replicates.

for etiolated seedlings or are involved in regulating ethylene biosynthesis only in the dark.

Interaction of the Eto Mutants and Various Inducers of Ethylene Biosynthesis

Ethylene biosynthesis in etiolated Arabidopsis seedlings is strongly induced by a number of plant hormones, as well as by the cupric ion. In some cases the target of these factors has been demonstrated to be distinct *ACS* genes (Liang et al., 1992; Abel et al., 1995; Vogel et al., 1998b). We examined the interaction of these factors and the *eto1-3* and *eto3* mutations to begin to address how these signaling pathways are related. The concentration of each inducer was chosen as the concentration that gave the peak of induction in wild-type etiolated seedlings (Woeste et al., 1999).

Both *eto1-3* and *eto3* displayed a synergistic interaction with auxin, cytokinin, cupric ion, and 24-epibrassinolide (Fig. 4). The level of ethylene produced was generally close to multiplicative. For example, the *eto1-3* mutation elevated ethylene biosynthesis approximately 15-fold above wild-type levels, 5 μM BA increased ethylene approximately 4-fold in etiolated, wild-type seedlings, and BA-treated *eto1-3* seedlings made close to 60-fold more ethylene than untreated, wild-type seedlings. In general, the synergism of these inducers with the *eto3* mutation was slightly less than that observed with *eto1-3*. This synergism indicates that the *eto1-3* and *eto3* mutations may act interdependently with these factors in regulating ethylene biosynthesis. Interestingly, exogenous ABA strikingly dampened the ethylene overproduction observed in the Eto mutants, although it did not appear to affect ethylene production in wild-type seedlings.

Interaction with Mutants Affected in Ethylene Biosynthesis

Ethylene-insensitive mutations produce elevated levels of ethylene, most likely because they block the feedback inhibition of ethylene biosynthesis in vegetative Arabidopsis tissue (Guzman and Ecker, 1990). To determine the effect of ethylene feedback on the Eto mutants, we constructed double mutants of *eto1* and the ethylene-insensitive mutants *ein2* and *etr1-3* (ethylene resistant) (Fig. 1). The average length of hypocotyls from the double mutants was not significantly different from that of each ethylene-insensitive parent (not shown), which indicates that the *etr1-3* and *ein2* mutations are epistatic to *eto1*. This

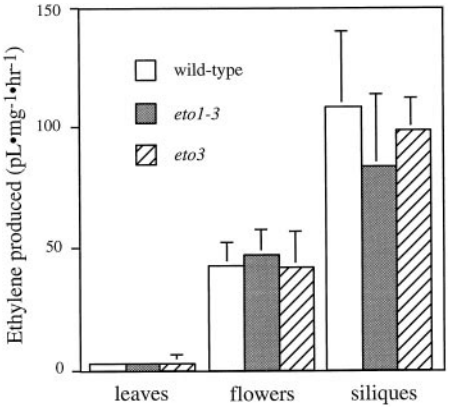

Figure 3. Ethylene produced by adult tissues of wild-type, *eto1-3*, and *eto3* mutant plants growing in soil at 23°C under continuous illumination. Ethylene measurements were as described in "Materials and Methods." Values are means (±SD) of three replicates.

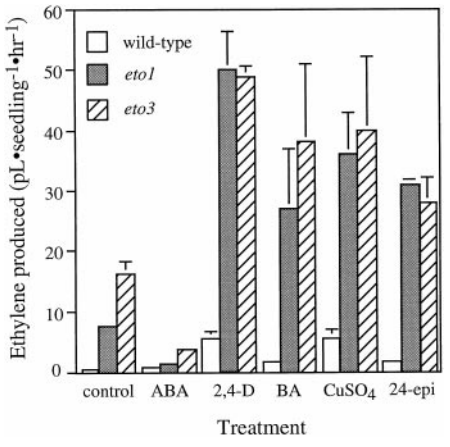

Figure 4. Ethylene produced by wild-type and mutant etiolated Arabidopsis seedlings in response to treatment with ABA (75 μM), 2,4-D (160 μM), BA (5 μM), $CuSO_4$ (20 mM), and 1 μM 24-epibrassinolide (24-epi). For all treatments except BA, seedlings were grown in GC vials, and 200 μL of the solutions of the indicated concentrations was added at 48 h. The vials were flushed with hydrocarbon-free air, sealed, and returned to the dark at 23°C. The ethylene that accumulated during the next 24 h was measured. For BA treatment, the seedlings were germinated on Murashige and Skoog medium supplemented with 5 μM BA and the ethylene production during the first 72 h of germination was measured. Values are means (±SD) of three replicates.

confirms the expectation that mutations defective in the perception of ethylene act downstream of those affecting ethylene biosynthesis (Roman et al., 1995). However, under the conditions that we used, the hypocotyl lengths of *ein2* etiolated seedlings were much more variable than those of either the wild-type or *eto1-3/ein2* etiolated seedlings (not shown). It is interesting that the apical hooks of the *eto1-3/ein2* double-mutant etiolated seedlings generally appeared to be more closed that those of the *ein2* single parents (Fig. 1), which have a hook that is less angled than that of wild-type etiolated seedlings.

When combined with the *eto1-1* mutation, the *etr1-3* mutation displayed a synergistic interaction in terms of the amount of ethylene produced (Fig. 2C). However, the amount of ethylene produced by the *eto1-3/ein2* double mutant appeared to be close to additive relative to the parental seedlings (Fig. 2B). This difference may reflect a branch in the ethylene-response pathway or perhaps subtle differences between the *eto1-1* and *eto1-3* alleles.

eto1 and *eto3* Etiolated Seedlings Have Elevated Levels of ACS Activity

One likely target of these ethylene-overproducing mutants is ACS, the rate-limiting step of ethylene biosynthesis. Previous work demonstrated that ACS is the target of various inducers of ethylene biosynthesis in etiolated Arabidopsis seedlings by both transcriptional and posttranscriptional mechanisms (Liang et al., 1992, 1996; Van der Straeten et al., 1992; Abel et al., 1995; Vogel et al., 1998b). We assayed the level of ACS in crude extracts from wild-type and *eto1-3* and *eto3* etiolated seedlings (Fig. 5). Both mutants showed high elevated levels of ACS activity compared with wild-type etiolated seedlings, which had barely detectable levels of ACS activity. This indicates that increases in ACS activity may be responsible for the elevated ethylene biosynthesis observed in the mutant seedlings.

The formation of ACC is generally the rate-limiting step of ethylene biosynthesis, but regulation may also be mediated by changes in ACC oxidase levels or by changes in the amount of ACC that is conjugated. To address this issue, we examined the ability of intact wild-type and mutant seedlings to convert exogenous ACC to ethylene, which should reflect the level of ACC oxidase activity minus any ACC that becomes conjugated (Fig. 6). Both *eto1-3* and *eto3* mutants were indistinguishable from wild-type seedlings in their conversion of ACC to ethylene over a wide range of exogenous ACC concentrations. Consistent with this, *eto1-3* and *eto3* seedlings contained wild-type or slightly elevated levels of ACC *N*-malonyltransferase and γ-glutamyltranspeptidase activity (not shown). These data suggest that these mutants elevate ethylene biosynthesis primarily by increasing ACS activity.

eto1 and *eto3* Etiolated Seedlings Have Wild-Type Levels of *ACS* mRNA

Elevation of ethylene biosynthesis has often been correlated with increases in the steady-state levels of *ACS* mRNAs, although in a few cases posttranscriptional control has also been demonstrated. To determine if elevated *ACS* mRNA contributes to the increased ethylene biosynthesis observed in *eto1* and *eto3* etiolated seedlings, we analyzed *ACS* mRNA levels by northern blotting (Fig. 7). We analyzed the expression of *ACS2*, *ACS4*, *ACS5*, and *ACS6*, the three previously identified, active *ACS* genes in Arabidopsis. In addition, we analyzed a novel *ACS* gene

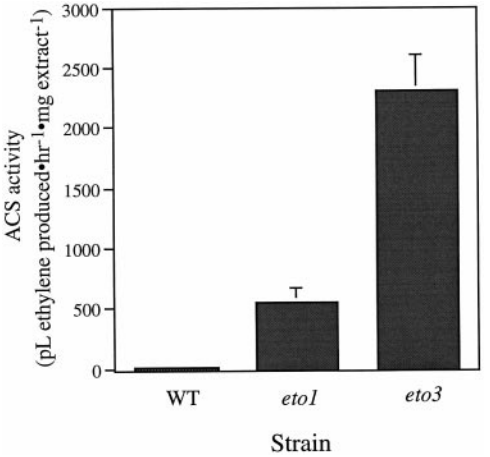

Figure 5. ACS activity in crude extracts from 3-d-old, etiolated wild-type (WT), *eto1*, and *eto3* mutant seedlings. The enzyme was assayed by incubating crude extracts in buffer with or without AdoMet, and then measuring the amount of ACC formed by converting it to ethylene (see "Materials and Methods"). The activity was calculated by subtracting the amount of ethylene produced in the absence of added AdoMet and then normalizing to the protein concentration of each sample. Values are means (±SD) of three replicates.

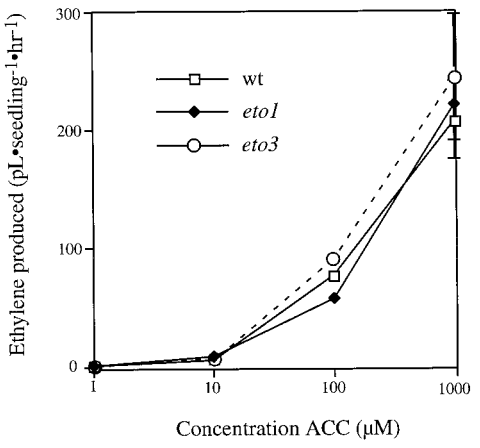

Figure 6. Ethylene produced by etiolated wild-type (wt), *eto1*, and *eto3* Arabidopsis seedlings in response to varying concentrations of supplemented ACC. Seedlings were grown on 3 mL of Murashige and Skoog agar in GC vials. ACC of the indicated concentrations, in a total volume of 200 μL, was added to the seedlings 48 h after moving to 23°C. The vials were then flushed with hydrocarbon-free air and sealed, and the ethylene produced during the next 24 h was measured. Values are means (±SD) of three replicates.

that we found by searching the Arabidopsis expressed sequence tag database (Newman et al., 1994), which we have named *ACS7* (Fig. 8).

The *ACS7* cDNA clones all appear to lack the 5′ end of the gene, because none was as large as the size predicted from RNA gel-blot analysis (approximately 1.6 kb) and the open reading frame continued to the 5′ end of the longest sequence. However, the 3′ end of the gene is intact and an in-frame stop codon is present, as is a short poly(A^+) tail. A comparison of the predicted amino acid sequence of *ACS7*, derived from the sequence of a non-full-length cDNA expressed sequence tag clone, with the other Arabidopsis *ACS* genes reveals that this clone lacks the variable carboxy-terminal extension present in other ACS proteins (Fig. 8). This carboxy-terminal domain has been demonstrated in at least two cases to negatively regulate the function of the ACS protein (Li and Mattoo, 1994; Vogel et al., 1998).

Overall, the level of expression of all five *ACS* genes was very low, in most cases just above the level of detection using 10 μg of poly(A^+) mRNA. The steady-state level of mRNA for *ACS5* in *eto1-3* and *eto3* etiolated seedlings was close to the level observed in wild-type seedlings. This was also confirmed by quantitative reverse-transcriptase PCR analysis (not shown). *ACS2* and *ACS4* steady-state levels were actually lower in the *Eto* mutants, perhaps reflecting negative feedback from the elevated ethylene levels. There was no detectable *ACS6* or *ACS7* expression in either wild-type or mutant etiolated seedlings, although treatment with the protein-synthesis inhibitor cycloheximide resulted in high levels of *ACS7* expression (not shown), as is the case with the other Arabidopsis *ACS* genes (Liang et al., 1992). These results indicate that the increased ACS activity is not caused by elevated gene expression, but is likely attributable to a posttranscriptional mechanism.

DISCUSSION

The *eto1* and *eto3* mutants were isolated as seedlings that displayed a constitutive triple-response phenotype caused by an elevation of ethylene biosynthesis. We demonstrate that this ethylene overproduction is brought about by increased ACS activity, and that this is most likely the result of posttranscriptional regulation. This conclusion is based on measurements of ACS activity and mRNA levels, as well as the interactions with various inducers of ethylene biosynthesis. This is consistent with previous reports of posttranscriptional regulation of ACS in etiolated Arabidopsis seedlings (Vogel et al., 1998b), and suggests that this may be a major mechanism for modulating the level of ethylene biosynthesis in this tissue. Alternatively, it is possible that an additional *ACS* gene(s) is present in Arabidopsis and that the transcription of this gene is elevated in the Eto mutants. However, if this were the case, one would predict an additive interaction of these mutations with inducers that act through independent *ACS* genes, which is clearly not the case with either auxin or cytokinin. Thus, the model that is most consistent with the data presented here is that the Eto mutations affect the posttranscriptional regulation of ACS.

One possible mechanism for this posttranscriptional regulation is that ACS protein could be modified to increase its activity or stability, and the *Eto* mutations affect this modification. Protein phosphorylation has been found to play a

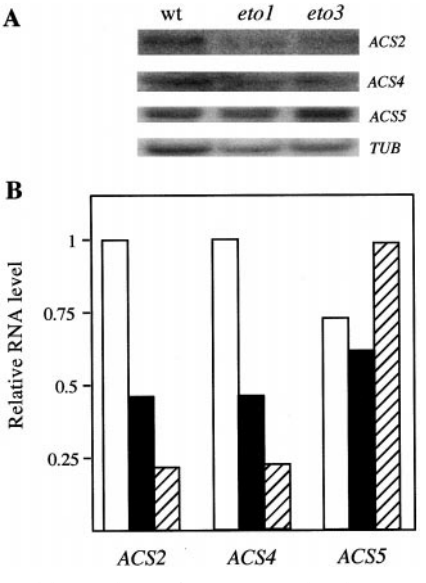

Figure 7. RNA-blot analysis of *ACS* mRNA levels in etiolated seedlings. Five micrograms of poly(A^+) RNA from 3-d-old wild-type (open bars), *eto1* (closed bars), and *eto3* (hatched bars) seedlings was separated by agarose-gel electrophoresis, blotted to a nylon membrane, and hybridized to an *ACS2*, *ACS4*, *ACS5*, or *β-tubulin* (*TUB*) probe. A, The original images of the blots. B, The quantification of the blots in A. The signal from each band was quantified using a phosphor imager. The value for each *ACS* band was divided by its *β-tubulin* loading control. The highest level in each set was assigned a value of 1, and the other values are expressed relative to this. The *ACS5* blot was from an independent RNA blot and was normalized to its own *β-tubulin* loading control (not shown).

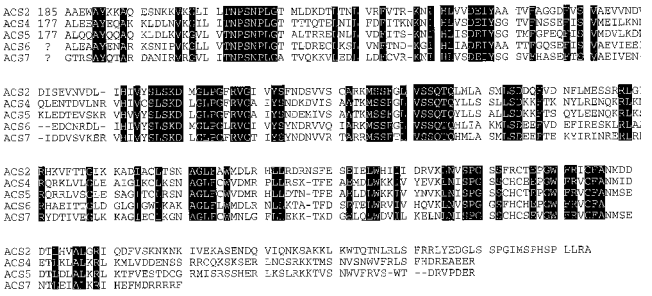

Figure 8. A comparison of the protein sequences deduced from the cDNA clones for the Arabidopsis *ACS* genes. Regions of amino acid identity are shaded. The alignment was produced using Clustal software. The sequences for ACS2, ACS4, and ACS5 were from Liang et al. (1992). The sequence for ACS6 was from Vahala et al. (1998). The ACS7 protein sequence was deduced from the DNA sequence of the expressed sequence tag FAI88 (Newman et al., 1994). The amino acid position of the first residue shown for each protein is indicated in the first line.

role in regulating the function of ACS in tomato (Spanu et al., 1994), and perhaps *eto1* and *eto3* affect the phosphorylation state of ACS. Alternatively, the translation of *ACS* mRNA could be enhanced by the *eto1* and *eto3* mutations, leading to increased levels of ACS enzyme.

Arabidopsis does not display a detectable burst of ethylene in the first 24 h of germination, as is observed in some other plant species (Yang and Hoffman, 1984; Abeles et al., 1992), even though ethylene can stimulate germination in Arabidopsis (Bleecker et al., 1988). In contrast to the wild-type, *eto1*, *eto3*, and *ein2* mutant seedlings display a sharp increase in ethylene production starting at about 48 h. This increase is then followed by a gradual decline to close to wild-type levels. The constitutive triple-response phenotype of *eto1* and *eto3* seedlings becomes decreasingly distinct as etiolation continues beyond 4 d (K.E. Woeste and J.J. Kieber, unpublished data), which reflects this diminution of ethylene biosynthesis. One model for this decrease in ethylene biosynthesis in the *Eto* mutants is that there is a developmental change in the regulation of ethylene biosynthesis. Alternatively, it may reflect an exhaustion of some metabolite required for ethylene biosynthesis, although the observation that *eto1/ein2* double mutants decrease ethylene production more slowly suggests that this is not the case, because they presumably have similar metabolic limitations. The hypothesis that developmental changes affect the function of *ETO1* and *ETO3* is also supported by the observation that mutations in these genes affect only etiolated seedlings. It is possible that the *ETO1* and *ETO3* gene products are only required in etiolated seedlings, or, alternatively, that they affect the regulation of ethylene in multiple tissues but only in the dark, perhaps playing a role in the circadian regulation of ethylene biosynthesis (Finlayson et al., 1998).

In Arabidopsis vegetative tissue, ethylene biosynthesis appears to be autoinhibitory (Guzman and Ecker, 1990), although the mechanism for this negative-feedback regulation is unknown. Autoinhibition in other systems has been linked to decreased ACS, decreased ACC oxidase, and/or up-regulation of ACC conjugation (Abeles et al., 1992). The ethylene-insensitive mutants *etr1* and *ein2* block this negative-feedback regulation in etiolated seedlings, leading to an increase in ethylene production (Guzman and Ecker, 1990). The additive interaction of *eto1* and *ein2* suggests that these mutations act in parallel to regulate ethylene biosynthesis. *etr1*, unlike *ein2*, displays a synergistic interaction with *eto1*, which suggests that *eto1* and *etr1* act in an interdependent manner to regulate ethylene production. For example, *etr1* could elevate ethylene biosynthesis by increased *ACS* transcription or decreased ACC conjugation, either of which, when coupled with the effect of *eto1* on the posttranscriptional regulation of ACS, would lead to a synergistic interaction. The observation that *etr1* and *ein2* differ in their interaction with *eto1* suggests that feedback regulation may occur via multiple mechanisms, and that the feedback pathway may be complex.

We evaluated the effects of a number of inducers of ethylene biosynthesis on *eto1* and *eto3* mutants to determine how they interact. We found that both cytokinin, which in low doses acts almost exclusively through the *ACS5* isoform (Vogel et al., 1998b), and auxin, which acts through *ACS4* (Abel et al., 1995), appear to interact with the Eto mutations in a synergistic fashion. In addition, cupric ion and 24-epibrassinolide also act synergistically with *eto1* and *eto3*. This suggests that these factors, like *etr1*, act interdependently to regulate ethylene biosynthesis. This is readily explained in the case of auxin: auxin elevates *ACS4* mRNA levels, and *eto1* and *eto3* increase *ACS* gene function posttranscriptionally, which together would lead to a synergistic effect. Likewise, cupric ion has also been shown to elevate *ACS* mRNA levels in tobacco (Avni et al., 1994), and thus its synergism with the Eto mutants could occur by a similar mechanism. However, the synergism of cytokinin and the Eto mutations is somewhat surprising, because both appear to affect the posttranscriptional regulation of ACS. This interaction suggests that cytokinin and the *Eto* mutants affect distinct posttranscriptional mechanisms, which could include translational efficiency of *ACS* mRNA and protein stability or activity.

To evaluate the possibility that the *Eto* mutations affect the regulation of ACC oxidase, we provided wild-type and mutant plants with an excess of substrate (ACC) and determined that *Eto* mutants and wild-type plants convert ACC to ethylene at the same rate over a 3-log range of concentrations (Fig. 6). These results suggest that ACC oxidase is not the rate-limiting step of ethylene biosynthesis in etiolated Arabidopsis seedlings, as is also the case in many other plant tissues, and that the *Eto* mutations do not affect the metabolism of ACC. This, coupled with direct measurements of ACC malonyltransferase and glutamyltranspeptidase activities, which showed the *Eto* mutants did not decrease ACC conjugation, supports the hypothesis that *eto1* and *eto3* primarily affect ACS activity.

ABA treatment reduces ethylene production in *eto1* and *eto3* mutant etiolated seedlings. ABA has also been shown to reduce the induction of ethylene biosynthesis by a number of factors in other plant tissues, including IAA-stimulated and drought-stressed leaves (Wright, 1980; Yoshii and Imaseki, 1981; McKeon et al., 1982; Tan and Thimann, 1989). The reduction of ethylene biosynthesis by

ABA has been linked to both decreased ACC oxidase levels and increased ACC conjugation (McKeon et al., 1982; Corbineau et al., 1989; Tan and Thimann, 1989). The difference in the effect of ABA on *Eto* mutants versus its effect on wild-type etiolated seedlings can be explained if regulation by ABA is only required under conditions of high ethylene production, conditions not normally found in germinating wild-type Arabidopsis seedlings. The *Eto* mutations do not display any other obvious defects in ABA responses. ABA may act to suppress excess ethylene production downstream of the wild-type gene product of both *Eto* mutations.

The emerging picture from these and other studies is that posttranscriptional events play an important role in regulating ethylene biosynthesis. The interactions of the *Eto* mutations with other regulators of ethylene biosynthesis suggest that the pathways regulating this biosynthetic pathway are complex, reflecting the multitude of regulatory inputs that affect ethylene biosynthesis. Cloning of the genes corresponding to the *eto1* and *eto3* mutations should shed further light on the role that these genes play in regulating ethylene biosynthesis.

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