

Recessive and dominant mutations in the ethylene biosynthetic gene *ACS5* of *Arabidopsis* confer cytokinin insensitivity and ethylene overproduction, respectively

(hormone biosynthesis/plant hormones)

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ABSTRACT We identified a set of cytokinin-insensitive mutants by using a screen based on the ethylene-mediated triple response observed after treatment with low levels of cytokinins. One group of these mutants disrupts *ACS5*, a member of the *Arabidopsis* gene family that encodes 1-aminocyclopropane-1-carboxylate synthase, the first enzyme in ethylene biosynthesis. The *ACS5* isoform is mainly responsible for the sustained rise in ethylene biosynthesis observed in response to low levels of cytokinin and appears to be regulated primarily by a posttranscriptional mechanism. Furthermore, the dominant ethylene-overproducing mutant *eto2* was found to be the result of an alteration of the carboxy terminus of *ACS5*, suggesting that this domain acts as a negative regulator of *ACS5* function.

Plant development is modulated by interactions among multiple hormones. An illustration of such interactions is the induction of biosynthesis of one hormone by another. Cytokinins, *N*⁶-substituted adenine derivatives, are one of the many factors that modulate the biosynthesis of the gaseous hormone ethylene (1–3). We are characterizing the induction of ethylene biosynthesis by cytokinin to understand how cytokinins and ethylene interact to regulate plant development.

Cytokinins were first identified as factors that acted synergistically with auxin to promote cell division *in vitro* and acted antagonistically to auxin in shoot and root initiation from callus cultures (4–6). Cytokinins have been implicated in many aspects of plant growth and development, including cell division, shoot initiation and growth, senescence, and photomorphogenesis (7–9). In contrast to the wealth of knowledge concerning the physiological effects of cytokinins, the molecular mechanisms underlying cytokinin perception and action remain largely unknown.

Ethylene is involved in a variety of plant growth and developmental processes, including senescence, leaf abscission, epinasty, and fruit ripening (2, 3). Recent studies have begun to shed light on the molecular mechanisms underlying ethylene signaling, including the identification of the genes encoding the ethylene receptor and several downstream signaling elements (10–14). The ethylene biosynthetic pathway has been worked out in great detail, and genes encoding the biosynthetic enzymes have been isolated from many plant species (1, 15, 16). The conversion of *S*-adenosyl methionine to 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase is the first committed step in ethylene biosynthesis and generally is considered to be the rate-limiting step. This enzyme is encoded by a divergent gene family in all plant

species analyzed so far. The expression of ACC synthase has been best studied in tomato, in which the gene family consists of at least nine members (17–21). The emerging picture is that different subsets of ACC synthase genes are expressed in response to various developmental, environmental, and hormonal factors. In *Arabidopsis*, five ACC synthase genes have been identified (*ACS1–ACS5*), three of which are expressed and encode active ACC synthase enzymes (22–24). The final step of ethylene biosynthesis is the conversion of ACC to ethylene by the enzyme ACC oxidase, which also is encoded by a multigene family in many plant species and whose members also are differentially regulated (25–30).

Ethylene biosynthesis is regulated by other hormones in many plant tissues, the best-studied example of which is auxin (1, 15). The steady-state level of mRNA from specific members of the *ACS* gene family become elevated in response to exogenous auxin in various plant species (19, 31, 32). In *Arabidopsis*, auxin specifically induces expression of *ACS4*, and four putative auxin response elements have been identified in the 5' regulatory sequences of this gene (32). Cytokinins also induce ethylene biosynthesis in many plant species, including *Arabidopsis* (33), but the mechanism for this induction is unknown. In at least one tissue, mung bean hypocotyls, the cytokinin benzyladenine (BA) has been shown to increase ACC synthase activity (34). To further understand how cytokinins regulate ethylene biosynthesis, we isolated a collection of *Arabidopsis* mutants that did not respond to cytokinin by increased ethylene biosynthesis. Here, we present the analysis of one such complementation group, *cin5* (cytokinin insensitive), which disrupts a member of the *ACS* gene family. Furthermore, we demonstrate that perturbation of the carboxy terminus of this same *ACS5* isoform results in ethylene overproduction.

MATERIALS AND METHODS

Plant Material. Columbia (Col) and Wassilewskija (WS) were the two *A. thaliana* ecotypes used in this study. The T-DNA mutagenized population (35) was obtained from the *Arabidopsis* Biological Resource Center (Ohio State University). Plants were grown in Metro-Mix 200 (Grace, Boca Raton, FL) under continuous illumination with fluorescent light at 23°C. Ethyl methanesulfonate (EMS)-mutagenized seeds were obtained by treating hydrated seeds (soaked overnight in

This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: BA, benzyladenine; ACC, 1-aminocyclopropane-1-carboxylic acid; Col, Columbia; MS, Murashige and Skoog salts; WS, Wassilewskija.

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distilled water) with 0.4% EMS for 7 h. The seeds were washed thoroughly with water and then sown in flats (52 × 26 cm) at a density of 3,000 seeds per flat. Etiolated seedlings were grown as follows: seeds were surfaced sterilized for 8 min in 5% sodium hypochlorite (Clorox)/0.01% Tween 20 and rinsed five times with sterile water. The sterile seeds then were resuspended in 0.8% low-melting-point agarose and plated on 0.8% agar containing Murashige and Skoog salts (MS; GIBCO) supplemented with 2% sucrose. Seeds were incubated at 4°C for 4 days and then moved to 23°C in the dark for 3 days.

Ethylene Measurements. Seedlings (about 15 per vial) were grown in 22-ml gas chromatography vials containing 3 ml of MS medium in the dark at 23°C. Appropriate supplements as indicated were either included in the media or added just before capping. Added compounds were in a volume of 200 μ l (controls were 200 μ l of water). The vials were flushed with hydrocarbon-free air and then capped for the indicated times. The accumulated ethylene was measured by using a gas chromatograph (Perkin-Elmer) fitted with a PoraPLOT U column, a cryofocusing attachment, and a flame ionization detector. A sample of headspace from each sample was injected onto the column at -50°C via an autosampler, and the column was then warmed to 30°C. The ethylene peaks were quantified by using Turbochrome 4 software (Perkin-Elmer) based on comparison to a 1 μ l/liter ethylene standard. Ethylene production was normalized for the number of seedlings in each vial and the time between capping and sampling. All observations were from at least three replicates, and each experiment was repeated at least once with comparable results. The measurements of the basal level of ethylene from WS and *cin5* are from seven independent experiments with at least three replicates each.

Hypocotyl Measurements. Sterilized seeds were plated on MS medium and grown for 3 days at 23°C in the dark. The length of the hypocotyl was measured under a dissecting microscope, and the data presented as the mean \pm the SD. At least 80 seedlings were measured in each case.

Mapping. *cin5-3* (ecotype Col) was backcrossed to the WS ecotype to generate a mapping population. F₂ seeds were plated on MS medium supplemented with 0.5 μ M BA, and *cin5* mutant seedlings (tall) were transferred to MS medium without BA and allowed to grow for about 1 week. DNA was extracted (36), and minisatellite markers polymorphic between ecotypes were amplified by using PCR (37). The products were separated by gel electrophoresis in 4% agarose and visualized by ethidium bromide staining.

Cloning of CIN5. Plant DNA flanking the left border of the T-DNA insertion in *cin5-1* was isolated by plasmid rescue as follows: 5 μ g of genomic DNA from a plant homozygous for the *cin5-1* allele was digested with *Sa*I restriction enzyme. The reaction mixture was extracted once with an equal volume of phenol/CHCl₃/isoamyl alcohol (25:24:1), once with CHCl₃/isoamyl alcohol, and then ethanol precipitated. The DNA was ligated in a 500- μ l reaction according to the manufacturer's instructions (Boehringer Mannheim). The ligation mixture was precipitated with ethanol, transformed into XL1 blue, and plated on Luria-Bertani agar containing 100 μ g/ml of ampicillin (LB Amp). Colonies (300) were patched onto LB Amp plates, and colony lifts were prepared by using Hybond N⁺ (Amersham) as described (38). The filters were hybridized to a left border probe (obtained from the Arabidopsis Biological Resource Center) as described (38). Sixty-nine positive colonies were obtained, the most common class of which displayed a restriction pattern expected for a direct repeat of T-DNA. The second most common class consisted of six clones, and one of these (pTK2) was used to probe a Southern blot containing wild-type (ecotype WS) and *cin5-1* genomic DNA cleaved with various restriction enzymes. This analysis showed that the mobilities of the hybridizing bands were distinct in the *cin5-1* allele, indicating that pTK2 corresponded to the DNA flanking

the site of the T-DNA insertion. DNA sequence analysis showed that pTK2 contained a fusion of T-DNA and the *ACS5* gene.

The various *cin5* alleles were sequenced as follows: PCR using oligonucleotide primers (JV9: ATCGGATCCCAACA-GACCCATGCTTCTC and JV10: CGAGGATCCATAGC-CACTGCAAATCCACA) flanking the *ACS5* coding region was used to amplify *ACS5* from wild type and the various *cin5* alleles. The products were sequenced directly after removal of excess primers by ultrafiltration (Ultrafree-MC, Millipore). At least two independent PCR products were sequenced for each allele. DNA was sequenced by using gene-specific primers (JV1: GGCTATAAACGACGTGGACTCG; JV4: CAT-GAGAGTCTCATTTCGAG; JV11: ACGACTCAAGTCCA-GACAGA; JV16: GCATGGATCCTAATTCATCGT-TCATCAGGTAC) by automated sequencing.

Northern Blot Analysis. Seedlings were grown (2,000 per 100-mm plate) on MS agar for 3 days as described above except sterile filter paper was placed on the top of the agar. For cytokinin-treatments, 10 ml of 5 μ M BA (in liquid MS) were added, and the seedlings were harvested at the indicated times. Total RNA was prepared by extraction with phenol/chloroform, and poly(A)⁺-RNA was isolated by oligo(dT)-cellulose affinity chromatography (38). Northern blot analysis was performed as described (38), and signals quantitated by using a PhosphorImager (Molecular Dynamics). The β -tubulin fragment was obtained by amplifying the gene from *Arabidopsis* genomic DNA using oligonucleotide primers (JVTF: GAG-ATTCTTCACATCCAGGG; and JVTR: CATCTCGTCCA-TTCCCTCAC).

Transformation of Arabidopsis with Wild-Type and *eto2 ACS5*. The *ACS5* gene was amplified from wild-type (ecotype Col) and *eto2* genomic DNA using oligonucleotide primers (*ACS5*ka: AAAAGGATCCGTACTIONTCAAAAAGTG-GATATACG and *ACS5*kb: AAAAGAGCTACAATACACACAAACGTTTC) with TaKaRa polymerase as described by the manufacturer (Panvera, Madison, WI). The resultant 4.15-kilobase PCR product was composed of the *ACS5* coding region, 2.1 kilobases of the 5' flanking region, and the *ACS5* 3' untranslated region. The PCR products were cleaved with *Sac*I and *Bam*HI and ligated into the plant transformation vector pBII101 (39). The resultant plasmids then were transformed into wild-type WS and the *cin5-2* mutant by vacuum infiltration (40). Transformants were selected on MS medium containing 50 μ g/ml of kanamycin.

RESULTS

Isolation of Mutants Defective in Cytokinin-Induced Ethylene Biosynthesis. Measurements of ethylene production by etiolated *Arabidopsis* seedlings grown in the presence of increasing concentrations of the cytokinin kinetin show a biphasic dose-response curve with a reproducible inflection at approximately 10 μ M (Fig. 1). This finding indicates that there may be two at least partially independent signaling pathways mediating cytokinin induction of ethylene biosynthesis, a possibility supported by the identification of mutations that affect only the low-dose pathway (Fig. 1 and see below). The ethylene produced in response to low doses of cytokinin (0.5-10 μ M) is sufficient to induce a triple response in etiolated *Arabidopsis* seedlings (Fig. 2). The triple response of etiolated dicotyledonous seedlings to ethylene was first described in peas by Neljubow (41) and consists in *Arabidopsis* of a shortening and radial swelling of the hypocotyl, inhibition of root elongation, and exaggerated curvature of the apical hook (Fig. 2). This triple response is not observed in the presence of cytokinin if inhibitors of ethylene action or biosynthesis are also present or when ethylene-insensitive mutants are grown on media supplemented with cytokinin (not shown), indicating that cytokinin exerts its effects on seedling morphology pri-

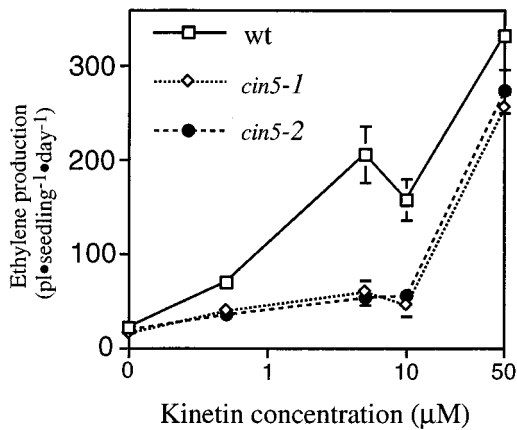


FIG. 1. Kinetin dose response of wild-type and *cin5* mutant seedlings. Approximately 15 seeds were sown in a 22-ml gas chromatography vial containing 3 ml of MS medium supplemented with the indicated concentration of kinetin, incubated at 4°C for 4 days, flushed with hydrocarbon-free air, and sealed. Accumulated ethylene was measured after 72 h of incubation in the dark at 23°C. The means \pm the SD based on three replicates are plotted. Very similar dose-response curves were obtained with the cytokinin benzyladenine (not shown). Note that the *cin5* mutation only disrupts ethylene biosynthesis at lower concentrations of kinetin.

marily by increasing ethylene biosynthesis, as has been previously observed (33).

We used this seedling response to screen approximately one million mutagenized *Arabidopsis* seedlings. After separating the ethylene-insensitive mutants and performing complementation tests, we were left with five complementation groups called *cin1-cin5* (cytokinin insensitive). These *cin* mutants did not display the triple response in the presence of low concentrations of cytokinin but were indistinguishable from wild-type seedlings in their response to ethylene (Fig. 2). One complementation group, *cin5*, was comprised of three independent alleles and is the focus of this paper.

Characterization of *CIN5*. *cin5* segregates as single-gene, recessive mutation (e.g. *cin5-1*: 388 wild-type seedlings: 138 mutant seedlings in an F₂ from a backcross to wild type; $\chi^2 = 0.43$, $P > 0.4$), suggesting that it is a loss-of-function allele. This hypothesis was confirmed by molecular analysis (see below). *cin5* mutations have no detectable effect on the morphology of adult plants and do not significantly affect ethylene production in adult tissues (Table 1). The *cin5* mutation was mapped to the bottom of chromosome 5, 32 map units from the microsatellite marker nga129 (37), close to the position of the *eto2* mutation (11) as well as the *ACC synthase 5 (ACS5)* gene (24). Subsequent molecular analysis showed that these are, in fact, all a single gene (see below).

To learn more about the function of *CIN5*, the gene corresponding to the *cin5* mutations was cloned by T-DNA tagging. A T-DNA population (35) was screened for mutants that lacked a triple response in the presence of cytokinin, and a single line segregating for a mutation (*cin5-1*) that failed to complement *cin5-2* was identified. Analysis of the segregation of the kanamycin resistance marker carried on the T-DNA in this line suggested that it contained insertions at two unlinked loci (F₂ of a backcross segregated 363 kanamycin resistant/22 kanamycin sensitive, which fits a 15:1 ratio, $\chi^2 = 0.18$, $P > 0.05$). One of the two T-DNA insertions was found to be closely linked to the *cin5-1* mutation, and the DNA flanking the site of this insertion was isolated by plasmid rescue (see *Materials and Methods*). Southern blot analysis using this rescued DNA as a probe showed DNA polymorphisms between genomic DNA from wild-type and *cin5-1* plants, confirming that the DNA flanking the T-DNA insertion had been cloned (not shown). The cloned DNA was sequenced, and a database

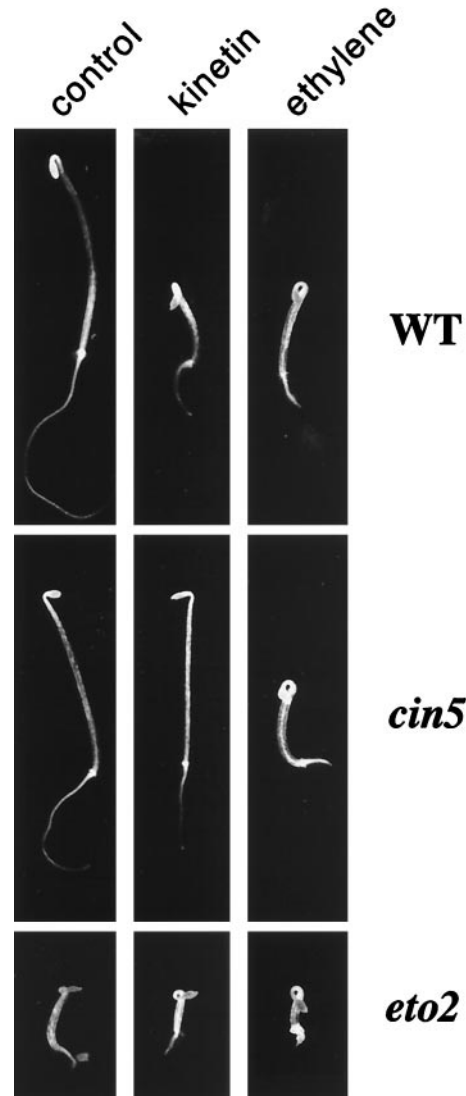


FIG. 2. Phenotype of *cin5* and *eto2* mutants. Wild-type (ecotype WS), *cin5-2*, or *eto2* seedlings grown in the dark for 3 days on MS medium supplemented with no hormones, 0.5 μ M kinetin, or in the presence of 10 μ l/liter ethylene as indicated. Representative seedlings were picked and photographed.

search showed that the T-DNA had inserted 608 bp upstream of the start of transcription of the *ACS5* gene (42). This position suggests that the T-DNA insertion disrupts expression of *ACS5* mRNA, which is consistent with the finding that no

Table 1. Ethylene production by various wild-type and mutant tissues

Tissue	Ethylene production, pl·mg ⁻¹ ·h ⁻¹		
	Wild type (WS)	<i>cin5-1</i>	<i>eto2-1</i>
Seedlings			
Etiolated	1.8 \pm 3.5	1.2 \pm 2.0	33.2 \pm 3.2
Light-grown	11.6 \pm 0.7	11.2 \pm 1.2	15.7 \pm 0.4
Adult tissues			
Flowers	39.0 \pm 5.8	44.0 \pm 4.3	47.5 \pm 8.0
Siliques	19.7 \pm 2.4	19.8 \pm 5.0	13.4 \pm 3.0
Leaves	1.6 \pm 0.4	1.4 \pm 0.2	2.0 \pm 0.2

The mean of at least three independent replicates \pm SD. Seedlings were grown or excised tissue placed in vials containing 3 ml of MS agar. The vials were flushed with hydrocarbon-free air, sealed, and incubated at 23°C (3 days for seedlings, 7 h for tissue), and the accumulated ethylene was measured.

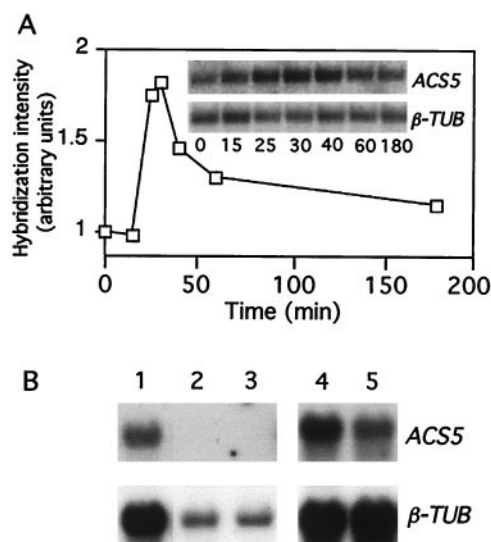


FIG. 3. Expression of *ACS5* mRNA in wild-type and mutant lines. (A) Time course of *ACS5* mRNA accumulation. Intact 3-day-old etiolated seedlings were incubated in 5 μ M benzyladenine for the times indicated; total RNA was isolated and analyzed by Northern blotting. The blot was hybridized with an *ACS5* probe, stripped, and rehybridized with a β -tubulin probe as a loading control. Hybridization intensities were quantified with a PhosphorImager (Molecular Dynamics). The level of *ACS5* expression was normalized to that of β -tubulin and plotted. (Inset) The original images of the blot. (B) Northern analysis of RNA from variously treated seedlings. Poly(A)⁺ RNA was prepared from 3-day-old etiolated seedlings grown as follows: lane 1, *acs5-2* plants grown on MS medium; lane 2, *acs5-1* plants grown on MS medium or lane 3, MS medium plus 5 μ M benzyladenine; lane 4, wild-type (Col) grown on MS medium; and lane 5, *eto2* grown on MS medium. Note that lanes 4 and 5 are from an independent experiment.

ACS5 transcript is detected in RNA isolated from *cin5-1* seedlings (Fig. 3B).

To verify that the *cin5* phenotypes were caused by mutations in *ACS5*, we transformed *cin5-2* with a 4.15-kilobase genomic clone containing the wild-type *ACS5* gene. In five independent transformants, *ACS5* was able to complement the seedling phenotype of *cin5-2* (Fig. 4). To further confirm that *cin5* disrupts *ACS5*, two ethyl methanesulfonate-induced *cin5* alleles were sequenced (Fig. 5). *cin5-2* was found to have a G to

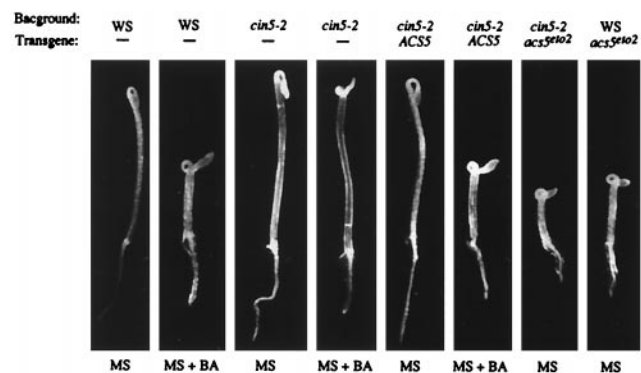


FIG. 4. Seedling phenotypes of *Arabidopsis* transformants containing a wild-type or *eto2* *ACS5* transgene. The transgene contained in each line is indicated above the seedling pictures, as is the background into which the transgene was transformed; - indicates that the line was not transformed. Seedlings were plated on either MS medium or MS medium supplemented with 5 μ M BA as indicated below each picture. The seedlings were grown for 3 days in the dark, and representative seedlings were picked and photographed. Note that transformation with the *eto2* version of *ACS5* results in a constitutive triple-response phenotype.

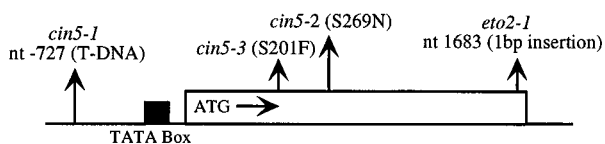


FIG. 5. Sequence alterations in the *cin5* and *eto2* alleles of the *ACS5* gene. The putative TATA box is indicated (from ref. 42). Both indicated Ser residues are highly conserved in the aminotransferase superfamily to which ACC synthase enzymes belong. The insertion in *eto2* is a C (on the coding strand) at the indicated position that is predicted to disrupt the carboxy-terminal 12 amino acids.

A transition that is predicted to result in a Ser to Asn substitution at amino acid 269. The *cin5-3* allele has a C to T transition that is predicted to result in a Ser to Phe substitution at amino acid 201. Both of these amino acid residues are highly conserved in other ACC synthases and the Ser at position 269 corresponds to a predicted active site residue of aminotransferases (43). This analysis suggests that these mutations disrupt the catalytic activity of *ACS5*.

Role of *ACS5* in Ethylene Biosynthesis. The loss-of-function *cin5* mutations allowed us to characterize the role of *ACS5* in ethylene biosynthesis under various conditions. The basal level of ethylene biosynthesis is lower in etiolated *cin5* mutant seedlings (13.1 ± 2.1 pl-seedling⁻¹day⁻¹ for wild-type seedlings and 7.5 ± 1.4 pl-seedling⁻¹day⁻¹ for *cin5-1* mutant seedlings); accordingly, the hypocotyls of etiolated *cin5* mutant seedlings are slightly longer than are those of wild-type seedlings ($X = 5.2 \pm 1.0$ mm for wild-type WS etiolated seedlings and $X = 8.2 \pm 1.4$ mm for *cin5-1* etiolated seedling; $P < 0.05$ with Student's *t* test). At low cytokinin concentrations, *cin5* seedlings produce much less ethylene than do wild-type seedlings, but there is no significant difference at higher concentrations (Fig. 1). This finding suggests that *ACS5* is responsible for almost all of the ethylene produced in response to low levels (<10 μ M) of cytokinin, but does not play a significant role in ethylene biosynthesis at higher cytokinin concentrations. The residual response of *cin5* mutants to low doses of cytokinin could be caused by a slight leakiness of our *cin5* alleles, partial induction of the high dose pathway, or a minor response of other ACS isoforms.

Wild-type, 2-day-old etiolated *Arabidopsis* seedlings also produce high levels of ethylene in response to CuSO₄, AgCl, and auxin (Table 2). Auxin has been found to increase the steady-state level of *ACS4* mRNA in *Arabidopsis* (32). Silver ions most likely increase ethylene production by blocking negative feedback regulation (44). *cin5* mutants produce wild-type levels of ethylene in response to the synthetic auxin 2,4-dichlorophenoxyacetic acid and AgCl (Table 2). *cin5-1* and *cin5-2*, which are in the WS ecotype of *Arabidopsis*, produce

Table 2. Ethylene production by etiolated seedlings

Treatment	Ethylene production, pl-seedling ⁻¹ day ⁻¹	
	Wild type (WS)	<i>cin5-1</i>
Control	18 \pm 7	12 \pm 2
H ₂ O	21 \pm 6	14 \pm 8
AgCl (17 μ g/ml)	46 \pm 8	48 \pm 7
2,4-D (50 μ M)	94 \pm 16	86 \pm 22
CuSO ₄ (20 mM)	114 \pm 18	68 \pm 10

Seeds were grown on MS agar for 48 h as described in *Materials and Methods*, and 200 μ l of the indicated solution was added just before the vials were sealed. The control samples had nothing added to the vials. The vials then were incubated for an additional 24 h, and the accumulated ethylene was measured. For the silver ion treatment, seeds were sown directly on medium containing AgCl. Numbers represent the mean \pm the SD based on three replicates. 2,4-D, 2,4-dichlorophenoxyacetic acid.

slightly less ethylene than do wild-type seedlings in response to CuSO₄ (Table 2 and not shown). However, *acs5-3* (Col ecotype) produces wild-type levels of ethylene in response to CuSO₄ (not shown), indicating that these two ecotypes regulate ethylene in response to CuSO₄ slightly differently. An additional difference between these ecotypes is that Col seedlings produce only half as much ethylene as do WS seedlings in response to cytokinin (not shown).

Ethylene biosynthesis also increases sharply during flower and fruit development in many plant species (1-3, 15), including in *Arabidopsis* (Table 1). Light-grown *Arabidopsis* seedlings also produce more ethylene than do comparable etiolated seedlings. Flowers, siliques, and leaves from *cin5* mutant plants all produced wild-type levels of ethylene (Table 1), indicating that *ACS5* does not play a major role in the developmental regulation of ethylene biosynthesis in these tissues. Three-day-old light-grown *eto2* seedlings produce slightly more ethylene than do wild type, but not nearly the many-fold excess observed in etiolated seedlings.

Regulation of *ACS5* mRNA Levels by Cytokinin. Liang *et al.* (42) have found that *ACS5* transcription is induced by Li⁺ and cycloheximide. To determine whether *ACS5* gene expression is regulated by cytokinin, we examined *ACS5* mRNA levels at various times after exposure to cytokinin (Fig. 3A). The steady-state level of *ACS5* mRNA displayed a rapid, transient and modest (less than 2-fold) increase in response to exogenous cytokinin. Seedlings pretreated with the protein synthesis inhibitor cycloheximide displayed a similar response (not shown), indicating that protein synthesis is not required for this induction. Because the induction of *ACS5* by cytokinin is transient and modest, increased *ACS5* mRNA levels alone most likely do not account for the rise in ethylene production observed in response to cytokinin (the mRNA level increases less than 2-fold for 1 h, but ethylene biosynthesis increases more than 8-fold over 4 days), especially because ACC synthase proteins generally have a short half-life (45-47). Taken together, these observations indicate that increases in *ACS5* mRNA most likely do not contribute significantly to the sustained rise in ethylene biosynthesis observed in response to cytokinin. We conclude that posttranscriptional modulation of *ACS5* very likely plays the major role in the regulation of cytokinin-mediated ethylene biosynthesis.

The *eto2* Mutation Disrupts *ACS5*. Additional evidence that posttranscriptional modifications play a role in the regulation of *ACS5* was provided by the analysis of the *eto2* mutation. *eto2* was identified as a mutant that displays a triple response in the absence of exogenous ethylene (Fig. 2) and is inherited as a single gene, dominant mutation (11). Three-day-old etiolated *eto2* seedlings produce approximately 20-fold more ethylene than do wild-type seedlings (Table 1). Because *eto2* and *cin5* are closely linked, we sequenced the *ACS5* gene from an *eto2* mutant and found a single base-pair insertion 35 bp upstream from the stop codon. This frame-shift mutation is predicted to change the 12 terminal amino acids of *ACS5* from RVSYT-DRVPDER to PGFMDRST. To confirm that ethylene overproduction in the *eto2* mutant is the result of this disruption of *ACS5*, an *eto2* version of *ACS5* was transformed into wild-type *Arabidopsis* as well as the *cin5-2* mutant. Five of seven and 5/5 independent transformants with the *eto2* version of *ACS5* into wild type and *cin5-2*, respectively, displayed a constitutive triple response phenotype (Fig. 4). In contrast, 0/7 independent transformants of *cin5-2* with the wild-type *ACS5* displayed a constitutive triple response phenotype. Measurements of ethylene production from etiolated seedlings confirm that the WS::*ACS5*^{eto2} lines overproduce ethylene: two independent WS::*ACS5*^{eto2} lines produce 6-fold and 4.5-fold more ethylene than do even homozygous *eto2* mutants. These data confirm that the alteration of the *ACS5* carboxy-terminus results in an Eto phenotype.

In 3-day-old etiolated *eto2* seedlings, the steady-state level of *ACS5* mRNA is slightly lower than in wild-type seedlings (Fig. 3B). This result indicates that the increased ethylene biosynthesis observed in *eto2* is not caused by an increase in *ACS5* mRNA levels, but rather to an alteration in the carboxy domain of the enzyme. The decrease in the steady-state level of *ACS5* mRNA in *eto2* mutant seedlings may be caused by negative feedback regulation by the elevated ethylene levels in these seedlings.

Adult tissues from *eto2* mutants and light-grown *eto2* seedlings produce a level of ethylene that is indistinguishable from or only slightly higher than that produced by wild-type tissues (Table 1). This finding is consistent with the analysis of the loss-of-function *acs5* mutations that indicates that this gene does not contribute in a significant manner to ethylene production in light-grown seedlings or various adult tissues. This may be because of a very low level of *ACS5* expression in these tissues, or, alternatively, the *eto2* change in the carboxy-terminus of *ACS5* may not increase the function of the protein in these tissues.

DISCUSSION

By using the triple response as a genetic screen, we have identified recessive and dominant mutations in *ACS5* that lead to a disruption of cytokinin-induced ethylene biosynthesis and to ethylene overproduction, respectively. Our analysis indicates that cytokinin elevates ethylene biosynthesis primarily by a posttranscriptional modification of *ACS5*. Furthermore, ethylene overproduction in the dominant *eto2* mutation was found to be caused by a perturbation of the carboxy terminus of *ACS5*, indicating that this domain negatively affects *ACS5* function. It is plausible that cytokinin acts by relieving the negative influence of the carboxy terminus by a modification of the *ACS5* protein.

The isolation of loss-of-function *ACS5* alleles allowed us to characterize the role played by *ACS5* in the biosynthesis of ethylene under various conditions. *ACS5* seems to be responsible for almost all of the ethylene produced in response to low levels of cytokinin by etiolated seedlings and is not responsible for any significant portion of the ethylene produced under the other conditions tested, with the possible exception of CuSO₄ in WS ecotype plants. This finding, together with the studies of *ACS* message levels, suggests that different *ACS* isoforms respond to distinct regulatory inputs.

The analysis of *eto2* is consistent with the idea that *ACS5* is regulated posttranscriptionally. One model for the increased ethylene biosynthesis observed in *eto2* seedlings is that the alteration of the carboxy terminus of *ACS5* may create a hyperactive enzyme. The carboxy termini of ACC synthases are the least-conserved portions of the molecule (15, 16). This hypervariable carboxy-terminal domain has been shown to affect the activity and dimerization of tomato ACC synthase when expressed in *Escherichia coli*; truncation of the 46-52 carboxy-terminal amino acids resulted in a monomeric enzyme that had 4-fold higher specific activity (48). However, the magnitude of the increase in activity of *ACS5* would have to be much greater than 4-fold to account for the 20-fold increase in ethylene biosynthesis observed in *eto2* mutant seedlings. Additionally, we have found that the wild-type and *eto2* versions of *ACS5* have comparable specific activities when expressed in *E. coli* (Hui Li and J.J.K., unpublished observations). An alternative possibility is that the carboxy terminus of *ACS5* is the target of a negative modification *in vivo*, perhaps phosphorylation (49), or that the wild-type carboxy terminus of *ACS5* acts to destabilize the protein *in vivo*.

A simple model for the action of cytokinin in etiolated seedlings is that it increases the activity of the *ACS5* protein, possibly by relieving the negative effect of the carboxy terminus. This work suggests that modifications of ACC synthase

proteins, rather than simply altered transcription of these genes, play an important role in the regulation of ethylene biosynthesis. Consistent with this, Spanu *et al.* (49) found that perturbation of protein phosphorylation affects the regulation of tomato ACC synthase in response to elicitor treatment. Negative regulation of ACS function by the carboxy terminus may be a general feature of these enzymes; this domain may be a target of posttranslational modification in response to a variety of regulatory inputs.

We have demonstrated that the triple response can be used as a powerful screen to identify mutants disrupted in cytokinin signaling to ethylene biosynthesis. Cytokinin signaling has been recalcitrant to genetic analysis, possible because of its essential role in plant development or the complex nature of cytokinin responses. The genetic screen used here may overcome these difficulties, and analysis of the additional *cin* mutants may help to elucidate the mechanism of cytokinin perception.

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