Identification of Novel Inhibitors of 1-Aminocyclopropane-1-carboxylic Acid Synthase by Chemical Screening in Arabidopsis thaliana

Received for publication, April 9, 2010, and in revised form, August 2, 2010 Published, JBC Papers in Press, August 3, 2010, DOI 10.1074/jbc.M110.132498

Lee-Chung Lin*,†, Jen-Hung Hsu*, and Long-Chi Wang*§

From the †Graduate Institute of Life Science, National Defense Medical Center, Taipei 114 and the §Institute of Plant and Microbial Biology, Academia Sinica, Taipei 11529, Taiwan

Ethylene is a gaseous hormone important for adaptation and survival in plants. To further understand the signaling and regulatory network of ethylene, we used a phenotype-based screening strategy to identify chemical compounds interfering with the ethylene response in Arabidopsis thaliana. By screening a collection of 10,000 structurally diverse small molecules, we identified compounds suppressing the constitutive triple response phenotype in the ethylene overproducer mutant eto1-4. The compounds reduced the expression of a reporter gene responsive to ethylene and the otherwise elevated level of ethylene in eto1-4. Structure and function analysis revealed that the compounds contained a quinazolinone backbone. Further studies with genetic mutants and transgenic plants involved in the ethylene pathway showed that the compounds inhibited ethylene biosynthesis at the step of converting S-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. Biochemical studies with in vitro activity assay and enzyme kinetics analysis indicated that a representative compound was an uncompetitive inhibitor of ACC synthase. Finally, global gene expression profiling uncovered a significant number of genes that were co-regulated by the compounds and aminothoxyvinylglycine, a potent inhibitor of ACC synthase. The use of chemical screening is feasible in identifying small molecules regulating the ethylene response in Arabidopsis seedlings. The discovery of such chemical compounds will be useful in ethylene research and can offer potentially useful agrochemicals for quality improvement in post-harvest agriculture.

Ethylene is an important gaseous phytohormone regulating plant growth and development in processes such as seed germination, root development, leaf and flower senescence, and fruit ripening and responds to a variety of stresses (1–4). Because of its versatile functions, ethylene has a critical role in adaptation and survival in plants. In the presence of ethylene, etiolated seedlings display photomorphogenesis, called the triple response phenotype, an exaggerated curvature of the apical hook, radial swelling of the hypocotyl, and shortening of the hypocotyl and root (5). The triple response phenotype has been successfully used to identify mutants defective in ethylene biosynthesis or response in Arabidopsis thaliana (5–8). Further studies of the ethylene mutants revealed the genetic hierarchy of key components in ethylene biosynthesis and signal transduction in Arabidopsis (3, 9). Ethylene signaling is initiated by the interaction between the ethylene ligand and its receptors localized in the endoplasmic reticulum (ER) membrane (10, 11). Binding of ethylene to the receptors inactivates a negative regulator, CTR1, that constitutively represses a positive regulator, EIN2 (12, 13). Ethylene receptors activate CTR1 to suppress EIN2 in the absence of ethylene and therefore function as negative regulators of the ethylene response (14, 15). A functional interaction among the ethylene receptors, CTR1 and EIN2, was postulated to take place in or near the ER membrane (10, 16, 17). De-repressed EIN2 stabilizes the otherwise labile transcription factor EIN3 by a yet unknown mechanism (14, 18–20). As a consequence, EIN3 activates an array of genes responsible for the ethylene response (21, 22). Although the ethylene signaling pathway has been elucidated by mainly studying genetic mutants in Arabidopsis, additional factors regulating the key components have been revealed by new approaches (18, 19, 23), which suggest the use of a new methodology to study ethylene function.

Ethylene gas is synthesized in almost all tissues of plants in the presence of oxygen (24). Ethylene biosynthesis involves three steps in plants. Methionine is catalyzed to form S-adenosylmethionine (AdoMet) by AdoMet synthetase. Biosynthesis of ethylene is committed by the conversion of AdoMet to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS) (24). ACC is subsequently oxidized to ethylene by ACC oxidase. Although ACC oxidase is constitutively expressed and can be further induced by wounding and ethylene (25, 26), the basal activity of ACS is extremely low unless induced by stress signals or at certain developmental stages (27). Therefore, ACS appears to catalyze the rate-limiting step in ethylene biosynthesis, which is a highly regulated process in higher plant species (24). All of the enzymes involved in ethylene biosynthesis, including AdoMet synthetase, ACC synthase,

* This work was supported by National Science and Technology Program Grant 9450201 for initial chemical screening, National Science Council Grant NSC9723311B001003, and Development Program of Industrialization for Agricultural Biotechnology Grant 995003008 for subsequent functional characterization (to L.-C.W.).
† The on-line version of this article (available at http://www.jbc.org) contains Tables S1–S4 and Figs. S1 and S2.
‡ To whom correspondence should be addressed. Tel.: 886-2-2787-1181; Fax: 886-2-2782-7954; E-mail: lwang@gate.sinica.edu.tw.
Chemical Compounds Inhibit Ethylene Biosynthesis

and ACC oxidase, are encoded by gene families, which suggests a complex and multilayered regulation of ethylene emanation (3).

Genetic mutants defective in the regulation of ethylene biosynthesis have been identified in Arabidopsis (7, 8). In etiolated seedlings, three ethylene overproducer (eto) mutants, eto1, eto2, and eto3, produce ethylene from 5- to 50-fold higher than that in wild-type Arabidopsis (7, 28). Arabidopsis ETO2 and ETO3 encode ACS5 and ACS9, respectively, two isoforms of type 2 ACS in the gene family (28–30). ETO1 binds type 2 ACS proteins and interacts with CUL3 in the SCF ubiquitin E3 ligase (31, 33). Hypermorphic mutations in eto2-1 and eto3-1 disrupt the protein interactions of ACS5 and ACS9, respectively, with ETO1 resulting in elevated ACS activity and subsequent ethylene overproduction, which phenocopies the loss-of-function mutations in ETO1 (7, 28, 29). How the protein-protein interaction between ETO1 and type 2 ACS is regulated by internal and external signals to mediate ethylene production remains largely unclear.

Chemical genetics, combining chemical screening and genetics approaches, has recently been appreciated as a novel methodology to probe plant physiology in Arabidopsis (34, 35). Small molecules offer advantages of reversible, conditional, and rapid effects for functional studies in organisms in which lethality is a critical issue in genetic mutants. In addition, small molecules can be agonists or antagonists to a group of proteins sharing conserved functions. Thus, use of small molecules may provide a solution to the issue of gene redundancy. Here, we report on the identification and characterization of chemical compounds acting as antagonists in the ethylene response by screening a collection of 10,000 small molecules. Using a phenotype-based strategy, we identified small molecules suppressing the constitutive triple response phenotype in etiolated eto1 seedlings by interfering with the biosynthesis but not the signal transduction of ethylene. Using an in vitro activity assay, we demonstrated that the compounds were inhibitors of ACS enzymes. Further enzyme kinetic analysis revealed that the compounds were novel ACS inhibitors different from the well known aminoethoxyvinylglycine (AVG). Finally, results of global gene expression analysis supported the physiological role of the compounds in the ethylene response by reverting the expression of numerous differentially expressed genes in eto1-4 to the levels of wild-type plants and revealed that more than 40% of genes in eto1-4 regulated by AVG are co-regulated by the compounds. Thus, our results demonstrate the feasibility of chemical screening in identifying small molecules modulating the ethylene response. Physiological and biochemical studies to analyze the role of these small molecules in the ethylene pathway are discussed.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions—All mutants and transgenic plants were derived from the wild-type A. thaliana Columbia ecotype (Col-0) and cultivated under a long day condition (16 h light/8 h dark at 22 °C) under white light (100–150 microeinstein m–2 s–1). A reporter construct, 5′X:EBS::LUC (a generous gift from Drs. Hai Li and Anna N. Stepanova, Salk Institute), containing five copies of the EIN3-binding sequence (EBS) fused with the luciferase gene (LLUC) was transformed into eto1-4 and subsequently used for screening the chemical library. Ethylene mutants eto1-4, eto2-1, ctr1-1, and the EIN3 overexpression line (35S::EIN3) were described previously (22). Seeds were sterilized with 30% bleach for 6 min and sown in half-strength Murashige and Skoog (0.5× MS) medium supplemented with 0.8% agar and stratified in the dark at 4 °C for 3–4 days before germination. For analysis of the triple response phenotype, stratified seeds were grown in the dark at 22 °C for 3 days before scoring the phenotype.

Chemicals and Screening Procedure—A DIVERSet library (ChemBridge Inc.) containing 10,000 small molecules (in DMSO) was used for chemical screening. Three rounds of chemical screenings were carried out in 0.5× MS agar medium containing individual chemicals in each well of 96- and 24-well microtiter plates. The initial screening was performed by sowing 10–15 seeds in the wells of microtiter plates containing small molecules of 50 μM to score the long hypocotyl phenotype. For the second and third screenings, we used 25 μM small molecules selected from the first round of screening to score and confirm the phenotype. The seedling phenotype was scored by use of a digital camera attached to a Zeiss stereo microscope (Stereo V8), and hypocotyl length was quantitated by use of National Institutes of Health ImageJ software. AVG and silver thiosulfate (STS, by mixing silver nitrate and sodium thiosulfate at a 1:4 molar ratio immediately before use) were from Sigma and used as controls to suppress ethylene biosynthesis and perception, respectively. Agar medium was supplemented with ACC (10 μM, Merck) to induce the triple response in etiolated seedlings.

For live imaging of luciferase activity, plants were first grown in the dark at 22 °C for 3 days and then transferred to white light for 3 more days before the luminescence of seedlings was imaged. Six-day-old seedlings were sprayed with luciferin (2 μM, Biosynth International Inc.) and kept in the dark for 5 min before images were collected by use of the Xenogen IVIS System (Caliper Life Sciences, Inc.). For quantitative assay of luciferase activity, the etiolated seedlings mentioned above were transferred to white microtiter plates (Packard Optiplate-96, PerkinElmer Life Sciences) for an additional 3 days under white light in 0.5× MS solution. The luciferase activity of seedlings was quantitated by use of a microplate reader (CHAMELEON, Hidex Inc.) in the presence of 2 μM luciferin.

Protein Expression and Purification—The full-length cDNA of Arabidopsis ACS5 (At5g65800) was cloned into pETDuet (Novagen) to generate pETDuet-6His-ACS5 for expression in Escherichia coli (BL21-CodonPlus, Stratagene) and subsequent purification of recombinant ACS5 protein. Protein expression was induced at A600 0.6 by adding 0.4 mM isopropyl β-D-thio-galactoside, and cells were cultured at 16 °C for 18 h. Cells were harvested by centrifugation at 6000 × g for 10 min at 4 °C. The cell pellet was washed and suspended in 50 ml of phosphate buffer (300 mM NaCl, 20 mM phosphate buffer, pH 7.4) containing 20 mM imidazole and protease inhibitor mixture (Sigma). Cell lysis was achieved by a continuous high pressure cell disrupter (TS 2.2 KW, Constant System) with 30,000 p.s.i. at 4 °C.
After washing the cell disrupter twice with 50 ml of phosphate buffer containing 20 mM imidazole, the final 150-ml suspension was centrifuged at 10,000 \( \times g \) for 30 min at 4 \( ^\circ \)C. The supernatant was applied to a 5-ml HisTrap FF column in an AKTAprime system (GE Healthcare) and was subsequently washed stepwise with 100 ml of buffer A (5 mM DTT, 100 mM HEPES buffer, pH 8.0) containing 20 mM imidazole and then 100 ml of buffer A containing 100 mM imidazole. The bound protein was eluted by a 0.1–1 M gradient of imidazole in 25 ml of buffer A and stored at \(-80^\circ\)C until further analysis.

**Enzyme Activity and Kinetic Assays—** *In vitro* ACS activity assay was performed as described previously (36, 37), with minor modifications. Purified recombinant ACS5 protein (1 \( \mu \)g) in 2 ml of buffer A containing 10 \( \mu \)M pyridoxal 5’-phosphate (PLP) and AdoMet (250 \( \mu \)M) was mixed with different concentrations of the hit compounds or DMSO (as control) in 20-ml GC vials for enzymatic reaction for 30 min at 25 \( ^\circ \)C. The strong oxidant HgCl2 (100 \( \mu \)l, 20 mM) and NaOH/bleach (1:1, 100 \( \mu \)l) was added to the vials to stop reactions and to oxidize ACC to ethylene, which continued for 10 min on ice. Ethylene level was measured by use of a gas chromatograph (HP 6890, Hewlett Packard) equipped with a capillary column (19095P-U04, Agilent Technologies) and an autosampler (HP 7694, Agilent Technologies). A standard curve was prepared for the enzyme activity assay by replacing the enzyme and substrate with different concentrations of ACC (supplemental Fig. S1).

All of the ACS activity assays were performed in duplicate \((n = 3)\) and repeated at least three times.

For the enzyme kinetic assay, different concentrations of AdoMet (80, 100, 150, 200, 300, and 400 \( \mu \)M) were used to determine the \( K_m \) and \( V_{max} \) values of recombinant ACS5. In addition, we tested two concentrations, 0.01 and 0.05 \( \mu \)M, of AVG and compound 7303 to determine the inhibition constant \((K_i)\) and apparent kinetic parameters \((K_m \text{ and } V_{max})\). Reactions of enzyme kinetics assay were different from that for *in vitro* ACS activity assay by first adding different concentrations of AdoMet in 20-ml GC vials containing 10 \( \mu \)l PLP in 2 ml of buffer A, and chemical inhibitors or DMSO and recombinant ACS5 (1.6 \( \mu \)g) were then added concurrently to initiate the enzyme reaction for 30 min at 25 \( ^\circ \)C. Gas chromatography was used to quantitate the levels of ethylene chemically converted from ACC as described previously. Statistical analysis of data from enzyme kinetic assays and preparation of Lineweaver-Burk plots involved use of the add-on Enzyme Kinetic module of SigmaPlot (Systat Software Inc.).

**Transcriptional Profiling Data and Analysis—** For chemical treatments, seeds were sown on 0.5 \( \times \) MS agar medium supplemented with 10 \( \mu \)M chemicals (AVG, 9393, 9370, and 7303) or DMSO (as control). *Arabidopsis* seeds were stratified in the dark at 4 \( ^\circ \)C for 4 days and then germinated in the dark for 3 days at 22 \( ^\circ \)C. Approximately 2,000 etiolated seedlings of the wild-type or *eto1-4* were used to collect tissues for preparation of total RNA in each microarray experiment with *Arabidopsis* ATH1 GeneChip (Affymetrix). Extraction of RNA followed an established protocol (38). Total RNA (10 \( \mu \)g) was used to prepare biotinylated cRNA for hybridization to ATH1 GeneChip, and the subsequent experimental procedures followed the instructions of an in-house core facility.

Microarray experiments were repeated in two independent biological duplicates, and the genes selected for further analysis were filtered by the following criteria using built-in programs in the Agilent GeneSpring GX. The MASS method was used for probe summarization, and normalization was by the all-sample median. Genes with expression > 100 and with present or marginal calls in both experiments were selected. To identify genes co-regulated by AVG and the three-hit compounds, we compared the genes with differential expression (2-fold cutoff) in *eto1-4* in the absence and presence of AVG or the compounds. One-way ANOVA was used to analyze the statistical significance \((p < 0.05)\) of differential gene expression co-regulated by AVG and the compounds. For analysis of genes co-regulated by the compounds but not by AVG, we first excluded the genes with more than 1.5-fold differential expression in *eto1-4* in the absence and presence of AVG (AVG-dependent). The remaining genes (AVG-independent) were analyzed by one-way ANOVA to select statistically significant expressed loci \((p < 0.05)\). Finally, genes with at least 2-fold differential expression in *eto1-4* with or without chemical treatment were selected to identify genes co-regulated by the compounds. Data presented in hierarchical clustering analysis were generated by Bioconductor software and in Venn diagrams by GeneSpring GX. Gene Ontology (GO) descriptions were generated by GeneSpring GX and further referred to the TAIR GO data base. Raw data are available in the GEO data base (www.ncbi.nlm.nih.gov) with accession number GSE20897.

**RESULTS**

**Phenotype-based Screening for Chemical Compounds Interfering with Ethylene Response—** In this study, our chemical screening aimed to identify small molecules that interfered with the ethylene response in *A. thaliana*. We devised a phenotype-based screening strategy whereby *Arabidopsis* seedlings were germinated in microtiter plates containing small molecules in individual wells. Several genetic mutants available for the proposed screening are *eto1-4*, *ctr1-1*, and multiple ethylene receptor mutants that show the constitutive triple response in etiolated seedlings (7, 15, 39). We used *eto1-4* for chemical screening because its site of action is at the early step of the ethylene response. Therefore, we could screen small molecules interfering with any step downstream of ACC formation. After screening 10,000 small molecules in DIVERSet by three consecutive cycles, we identified 74 chemical compounds affecting differential degrees of the triple response phenotype in *eto1-4* (Fig. 1A). We found two chemical compounds, designated 9393 and 9370 (for ID numbers 7659393 and 7669370 in DIVERSet, Table 1), that demonstrated effectiveness comparable with silver nitrate (in the form of STS; see under “Experimental Procedures”) in suppressing the *eto1-4* phenotype (Fig. 1B). Silver nitrate is an antagonist of ethylene receptors in blocking ethylene binding to suppress ethylene response (40). One additional chemical compound, 7303 (for ID number 9127303, Table 1), was uncovered later by searching structural analogs; the effect of the compound was comparable with that of 9393 and 9370 in suppressing the *eto1-4* phenotype (Fig. 1, A and B). Because of their effectiveness in phenotype assay, the small molecules 9393, 9370, and 7303 are hereafter named hit compounds.
The luciferase activity of $5 \times EBS::LUC$ reporter was constitutively activated in eto1-4 but suppressed in the presence of STS, which indicates the expected ethylene responsiveness (Fig. 1C). AVG is an inhibitor of ACC synthase and many other pyridoxal enzymes that require PLP as the cofactor (42, 43). AVG also suppressed the luciferase activity similar to STS. In the presence of the hit compounds, the otherwise activated luciferase activity in eto1-4 was suppressed, which suggests that the hit compounds disrupted the ethylene response (Fig. 1C). The second assay determined the ethylene level in the presence of chemical compounds. Results in Fig. 1D show that the excessive level of ethylene in the eto1-4 mutant was reduced to that of the wild type (WT = Col-0) by the hit compounds, which indicates that the ethylene biosynthesis was inhibited. Our results demonstrate that the phenotype-based strategy for chemical screening successfully identified small molecules suppressing the ethylene response, most likely by inhibiting ethylene biosynthesis in Arabidopsis.

Hit Compounds Suppressed the eto1-4 Phenotype in a Concentration-dependent Manner—To determine whether the hit compounds have different effectiveness in suppressing the ethylene response, we measured the hypocotyl length of etiolated eto1-4 seedlings for a quantitative assay of the ethylene response. A shortened hypocotyl in etiolated seedlings is one of the triple response phenotypes (7). Two chemical compounds negating the ethylene response, AVG and STS, were included as a control for comparison. Both AVG and STS effectively suppressed the ethylene response, as reflected by elongated hypocotyls in etiolated eto1-4 seedlings (Fig. 2). AVG promoted a visible hypocotyl elongation as low as 0.1 mm and reached maximal effect at 5 mM. However, STS became effective only at >1 mM. Compound 9393 resembled STS by showing a visible effect only at >1 mM. However, both 9370 and 7303 were effective at suppressing the ethylene response in hypocotyls between 0.1 and 1 mM and reached a nearly saturated effect at >5 mM similar to that of AVG. Thus, the hit compounds suppressed the hypocotyl phenotype of etiolated eto1-4, which resembled the effect of AVG or STS at comparable concentrations.

Structure and Function Analysis of the Hit Compounds and Structural Analogs—We identified 74 chemical compounds with differential degrees of suppression of the eto1-4 phenotype from screening the DIVERSet library (Fig. 1A). Among these compounds, two of the effective compounds, 9393 and 9370, contain a quinazolinone backbone (Table 1). A third hit compound, 7303, was subsequently identified by structural similarity and was the most effective (Table 1 and Fig. 3). Because the hit compounds 9370, 9393, and 7303 share an identical skeleton structure with different moieties at the side chains, we performed a structural and functional analysis of compounds with a quinazolinone backbone. From additional DIVERSet collections, we selected 26 structurally analogous small molecules based on 85% similarity to 9393 or 9370. Among the 29 compounds, including the hit compounds, only 14 resulted in hypocotyls in etiolated eto1-4 seedlings with relatively greater elongation than that with the control (DMSO only), which suggests that the quinazolinone structure is required but not sufficient for inhibition (Fig. 3A). For seven compounds, including 9393, 9370, and 7303, the antagonizing ethylene response to elongate...
hypocotyls ranged from 28 to 77% of that with AVG treatment (defined as 100%). To determine whether the ethylene level is the key factor in regulating the hypocotyl length in etiolated eto1-4 seedlings, we quantitatively analyzed ethylene emanation in the presence of chemical compounds by gas chromatography. We selected 14 compounds showing differential degrees in suppressing ethylene response in Fig. 3A for ethylene measurement. All of the 14 compounds at 10 μM were able to reduce ethylene levels in etiolated eto1-4 seedlings (Fig. 3B). Nine of the 14 compounds were able to suppress ethylene levels greater than 60% of that in etiolated eto1-4 as compared with AVG treatment (defined as 100%). Results of quantification of hypocotyl length and ethylene level are in good agreement in that the most effective compounds in both assays include the same group of seven small molecules as follows: 9028, 2305, 9393, 9370, 8107, 2616, and 7303 (Fig. 3).

The chemical structures of 29 compounds analyzed in Fig. 3A are shown in Table 1. On the basis of the side chains at the C7 position of the quinazolinone skeleton, we classified the tested compounds into two groups (Table 1). The compounds in type I contain phenyl moieties at position C7, whereas type II compounds have methyl groups at the same position. Four of the seven most effective small molecules belong to type I (7303, 9370, 8107, and 2616), and the remaining three compounds are type II (9393, 9028, and 2305). Except for the linear butylamino structure in 2616, six effective compounds have cyclic moieties.

**TABLE 1**

Chemical structure of small molecules used in this study

| ID* | Structure | ID | Structure | ID | Structure |
|-----|-----------|----|-----------|----|-----------|
| 9127303 | ![Structure](image1) | 7659393 | ![Structure](image2) | 9040093 | ![Structure](image3) |
| 7992616 | ![Structure](image4) | 7972305 | ![Structure](image5) | 7995435 | ![Structure](image6) |
| 9118107 | ![Structure](image7) | 9119028 | ![Structure](image8) | 7646405 | ![Structure](image9) |
| 7669370 | ![Structure](image10) | 7879362 | ![Structure](image11) | 9139163 | ![Structure](image12) |
| 9038530 | ![Structure](image13) | 9125203 | ![Structure](image14) | 9002823 | ![Structure](image15) |
| 6100932 | ![Structure](image16) | 9115873 | ![Structure](image17) | 7645247 | ![Structure](image18) |
| 9131560 | ![Structure](image19) | 7674120 | ![Structure](image20) | 5219871 | ![Structure](image21) |
| 6101578 | ![Structure](image22) | 9020414 | ![Structure](image23) | ![Image](image24) | quinazolinone |
| 6101682 | ![Structure](image25) | 5547336 | ![Structure](image26) | ![Image](image27) | Aminoethoxyvinylglycine |
| 6101713 | ![Structure](image28) | 9118359 | ![Structure](image29) | |

* The underlined digits of chemical identification were used as abbreviations for small molecules described in the text.
Chemical Compounds Inhibit Ethylene Biosynthesis

at the C2 position; examples are anilino (in 7303, 9028, and 2305) and cyclopentylamino (9370, 8107, and 9393) structures, which indicate a preference for a ring structure attached at the C2 position. The cyclopentylamino structure (C2) missing from 1682 results in a loss of effectiveness as compared with 9370. Interestingly, the cyclopropyl ring (C2) in 5247 is not a substitute for the cyclopentyl structure in 9393. Furthermore, modifications of the ring structures (8530, 3827, 8359, 0414, 0093, 5435, and 2823) and an extra length of side chain (9362, 5873, 4120, 6405, and 9163) at the C2 position of quinazolinone reduced the effectiveness of compounds to different degrees. However, modifications in the benzene ring at position C7 of quinazolinone did not significantly affect the potency of type I compounds (7303, 9370, 8107, and 2616) (Fig. 3B). Finally, several type I compounds (0932, 1578, 1713, and 4752) missing a ring structure at C2 position were not effective compounds, regardless of benzene moieties being present at the C7 position. From structure and function analysis, we hypothesize that the quinazolinone is an essential core structure, and the cyclic ring moieties at C2 and C7 positions may enhance the potency of hit compounds to regulate ethylene biosynthesis.

Chemical Compounds Specifically Suppress Ethylene Biosynthesis but Not the Signaling Pathway—We demonstrated that the hit compounds and several structural analogs reduced ethylene emanation in etiolated eto1-4 seedlings. However, we do not know which step in the ethylene biosynthetic pathway is inhibited and whether the compounds affect the signaling relay downstream of the ethylene receptors. To clarify this issue, we used a quantitative assay of hypocotyl length to analyze the effect of hit compounds in etiolated Arabidopsis seedlings. Two ethylene mutants (eto1-4 and ctr1-1) and a transgenic Arabidopsis overexpressing EIN3 by the cauliflower mosaic virus 3SS promoter (3SS::EIN3 or EIN3OX) (21) exhibiting a constitutive triple response phenotype were used for analysis. The eto1-4 and ctr1-1 mutants are representative mutants defective in the biosynthetic and signaling pathways, respectively, of ethylene and show a constitutive triple response phenotype. EIN3 and EIN3-like (EIL) proteins are transcription factors responding to ethylene to activate the expression of primary response genes in the nucleus (21). ACC is the immediate precursor of ethylene and is routinely used to induce the triple response in etiolated seedlings. In the absence of hit compounds, the etiolated seedlings of the WT treated with ACC, eto1-4, ctr1-1, and EIN3OX showed a typical triple response, and the length of hypocotyls was measured for quantitative analysis (Fig. 4). The shortened hypocotyl in etiolated wild-type seedlings induced by ACC remained on treatment with the hit compounds, which suggests that the ACC oxidase and ethylene receptors were likely not the targets and thus were not affected (Fig. 4A). However, the hypocotyls of only eto1-4 but not ctr1-1 or EIN3OX were elongated in the presence of the hit compounds, which indi-
cates that the compounds suppressed the constitutive triple response only in *eto1-4* but not the signaling pathway downstream of ethylene receptors (Fig. 4, B–D). We found the same results using increased concentrations up to 50 μM of the hit compounds (data not shown). Therefore, our data strongly indicate that the hit compounds are involved in inhibition in the conversion of AdoMet to ACC catalyzed by ACC synthase.

**Chemical Compounds Reduce Ethylene Levels by Inhibiting ACC Synthase Activity**—To determine whether the hit compounds are inhibitors of ACC synthase, we first validated the effects of the hit compounds on *eto2-1*, which bears a missense mutation at the 3′ terminus of the *ACS5* gene to generate a mutated yet functional protein, ACS5<sup>eto2-1</sup>. ACS5<sup>eto2-1</sup> results in a constitutive triple response in etiolated seedlings because of producing 10–20-fold higher ethylene levels than in the WT (28, 33). The reduced ethylene levels and concomitant hypocotyl elongation in etiolated *eto2-1* seedlings were observed in the presence of different concentrations of hit compounds and AVG, which suggests that the hyperactive ACS5<sup>eto2-1</sup> was inhibited by the compounds (Fig. 5). Next, we purified recombinant *Arabidopsis* ACS5 protein from bacterial cells for an in vitro enzyme activity assay. Compounds 9370 and 7303 produced an apparent inhibition of ACS5 enzyme activity, with estimated half-maximal inhibitory concentration (IC<sub>50</sub>) at 1.4 and 0.5 μM, respectively (Fig. 6A). Although compound 9393 showed a comparable effect in suppressing the ethylene level and hypocotyl phenotype in *eto1-4* (Fig. 1, B and D), it was less effective in the in vitro activity assay, with an estimated IC<sub>50</sub> at 7.9 μM. AVG gave an IC<sub>50</sub> at ~0.7 μM, which was nearly the same as that of compound 7303 (Fig. 6A), which indicates that 7303 is as effective as AVG in the in vitro activity assay. Results from these assays provide direct evidence that the hit compounds are inhibitors of ACS enzymes.

AVG and its analogs are competitive inhibitors of ACC synthase (44). Because the hit compounds and AVG have distinct

---

**FIGURE 5. Hit compounds affect phenotype suppression of hypocotyls and ethylene levels in two *eto* mutants.** Seeds of *eto1-4* and *eto2-1* were germinated in the dark in the absence (−) or presence (+) of different concentrations (10, 20, or 30 μM) of the hit compounds and AVG. The length of hypocotyls and ethylene levels were quantified in 3-day-old etiolated seedlings. Data represent means ± S.E. (n > 40), and the experiments were repeated three times with similar results.

**FIGURE 6. Hit compounds are novel inhibitors of ACC synthase.** A, hit compounds reduce the enzyme activity of ACC synthase in vitro. Purified recombinant ACS5 was incubated with different concentrations of hit compounds and AVG in 20-ml GC vials for *in vitro* activity assay ("Experimental Procedures"). Activity unit (U) was defined as 1 μmol ACC converted by 1 μg of recombinant ACS5 in 30 min at 25 °C. B, Lineweaver-Burk plot of ACS5 kinetic data in the presence of AVG and compound 7303 as inhibitors. Different concentrations of AdoMet were incubated with recombinant ACS5 and AVG or compound 7303 at 0.01 and 0.05 μM for *in vitro* activity assay ("Experimental Procedures"). Data are means from at least three duplicates (n = 3–6). The best fit lines were plotted by using the enzyme kinetics module in SigmaPlot. Experiments were repeated twice with similar results. A representative result is shown in A and B. Error bars indicate S.E.

---

**Chemical Compounds Inhibit Ethylene Biosynthesis**

---

**FIGURE 4. Hit compounds specifically suppress the triple response phenotype in *eto1-4*.** Seeds of wild type (WT + ACC at 10 μM) (A), *eto1-4* (B), *EIN3OX* (C), and *ctr1-1* (D) were germinated with the hit compounds (bars 2–4; 10 μM) or DMSO (bar 1; control). Wild type without any chemical treatment is indicated by a black bar. Hypocotyl length of etiolated seedlings was quantified, and values are means ± S.E. (n > 40). A schematic representation of a simplified ethylene pathway is indicated. AVG, aminoethoxyvinylglycine.

---

**FIGURE 3. Chemical Compounds Reduce Ethylene Levels by Inhibiting ACC Synthase Activity.** To determine whether the hit compounds are inhibitors of ACC synthase, we first validated the effects of the hit compounds on *eto2-1*, which bears a missense mutation at the 3′ terminus of the *ACS5* gene to generate a mutated yet functional protein, ACS5<sup>eto2-1</sup>. ACS5<sup>eto2-1</sup> is as effective as AVG in the inhibition in the conversion of AdoMet to ACC catalyzed by ACC synthase.

---

**TABLE 1. Purified recombinant ACS5 was incubated with different concentrations of hit compounds and AVG in 20-ml GC vials for *in vitro* activity assay ("Experimental Procedures").** Activity unit (U) was defined as 1 μmol ACC converted by 1 μg of recombinant ACS5 in 30 min at 25 °C. B, Lineweaver-Burk plot of ACS5 kinetic data in the presence of AVG and compound 7303 as inhibitors (I). Different concentrations of AdoMet were incubated with recombinant ACS5 and AVG or compound 7303 at 0.01 and 0.05 μM for *in vitro* activity assay ("Experimental Procedures"). Data are means from at least three duplicates (n = 3–6). The best fit lines were plotted by using the enzyme kinetics module in SigmaPlot. Experiments were repeated twice with similar results. A representative result is shown in A and B. Error bars indicate S.E.
Chemical Compounds Inhibit Ethylene Biosynthesis

chemical structures (Table 1), they may use different mechanisms to inhibit ACC synthase. To clarify this issue, we selected 7303 as a representative of the hit compounds for enzyme kinetic assay to determine the inhibitory mechanism. Fig. 6B shows the Lineweaver-Burk plot of recombinant ACS5 treated with different concentrations of AVG or compound 7303. The plots were generated by an add-on module (Enzyme Kinetics) of the SigmaPlot software, and statistical analysis was used to determine the best fit inhibition model of AVG and compound 7303 (supplemental Table S1). Ranked by three statistical methods, the results show that AVG is indeed a competitive inhibitor, and compound 7303 is an uncompetitive inhibitor. Furthermore, the apparent kinetic parameters ($K_m$ and $V_{max}$) for recombinant ACS5 enzyme in the presence of inhibitors at 0.01 and 0.05 μM support the prediction of the inhibition model. The apparent $V_{max}$ values of ACS5 remained unchanged, whereas values of $K_m$ were increased by the increasing concentrations of AVG, a kinetic characteristic of competitive inhibitors. However, the apparent $K_m$ and $V_{max}$ values of ACS5 were both reduced with increasing concentrations of compound 7303, which indicates an uncompetitive inhibition (supplemental Table S2). The calculated inhibition constant ($K_i$) obtained from enzyme kinetic assays was 15 ± 3.5 and 23.5 ± 1.5 nM for AVG and 7303, respectively (Fig. 6B), which suggests that AVG is a more effective inhibitor of ACS5 than is compound 7303. Data from the enzyme kinetic assay support that the hit compounds are novel inhibitors of ACC synthase different from AVG and show uncompetitive inhibition of ACS activity.

Global Analysis of Gene Expression Profiles by the Hit Compounds—We showed that the hit compounds had different potencies as follows: 9393 was the least effective, with 5–15-fold higher $IC_{50}$ than 9370 and 7303 (Fig. 6A). However, the hit compounds at 10 μM did not differ in suppression of the triple response and ethylene emanation of etiolated $eto1-4$ seedlings (Figs. 4 and 5). The hit compounds may be metabolized to an identical active product after entering the plant cells, thus modulating the same biological process and leading to a similar phenotype. Alternatively, the concentration may be already satu- rated for phenotype analyses, regardless of the potential issues of permeability and structural modifications in the cells. To address this issue at the gene expression level, we extracted total RNA from 3-day-old etiolated seedlings in the absence (for WT and $eto1-4$) and presence (for $eto1-4$ only) of the hit compounds or AVG for transcriptome analysis by microarray experiments with Arabidopsis ATH1 GeneChip. The filtered and normalized data sets from 22810 probes on the ATH1 GeneChip were used to compare the global gene expression profiles in $eto1-4$ treated with AVG and the hit compounds (see under “Experimental Procedures” for details of data processing). One-way ANOVA was used to select genes with statistically significant expression ($p < 0.05$) in two independent experiments. We used a 2-fold cutoff to identify genes with differential expression in $eto1-4$ in the absence and presence of AVG and the hit compounds. In total, 264, 406, 392, and 454 genes in $eto1-4$ were regulated by compounds 9393, 9370, 7303, and AVG, respectively (Fig. 7A). Among these genes, 184, 245, and 289 loci were co-regulated by AVG and compounds 9393, 9370, and 7303, respectively. Clustering analysis with Venn diagrams indicated that more than 40% of the genes affected by AVG were also modulated by the individual hit compounds (Fig. 7A), and 166 genes were co-regulated by AVG and by all of the hit compounds (Fig. 7B and supplemental Table S3). Compound 7303, the most effective inhibitor of the three chemicals, co-regulated more than 60% of genes with AVG. These co-regulated genes likely contribute to the etiolated phenotype of $eto1-4$ by responding to elevated ethylene.

FIGURE 7. Global analysis of gene expression profiles in $eto1-4$ in the presence of hit compounds and AVG. A, Venn diagrams show the genes co-regulated by AVG and individual hit compounds (9393, 9370, and 7303). The percentages of co-regulated genes in AVG-treated $eto1-4$ seedlings are indicated. B, 166 genes are co-regulated by AVG and all of the hit compounds (supplemental Table S1). C, hierarchical clustering of co-regulated genes shown in A and in the wild type. The heat maps were generated by use of BioConductor, and colors represent relative expression levels as indicated in the color key. 1, $eto1-4$; 2, wild type; 3, $eto1-4$ treated with hit compounds (10 μM); 4, $eto1-4$ treated with AVG (10 μM). Bars on the right margin indicate the genes with similar expression patterns in the wild type and in $eto1-4$ treated with inhibitors. D, Venn diagram shows the genes co-regulated in $eto1-4$ by all of the hit compounds but not by AVG (supplemental Table S2). Co-regulated genes were identified by statistical analysis to show significant gene expression ($p < 0.05$, one-way ANOVA) and by a 2-fold cutoff with differential expression in the absence and presence of chemicals.
groups related to various biological functions (supplemental Table S3). Several genes are involved in the biosynthetic pathway or response to phytohormones, including auxin, ethylene, cytokinin, gibberellic, and brassinosteroid, which suggest a cross-talk among phytohormones to contribute to the triple response phenotype in eto1-4. We also uncovered different types of transcription factor effects, such as those in AP2/ERF, bHLH, bZIP, C2H2-type zinc finger, MYB, and WRKY gene families, of which some are involved in response to light and phytohormones. In addition, genes responsive to abiotic stress and pathogen infection were among the co-regulated genes, which was expected because ethylene is known to be involved in both biotic and abiotic stresses. To investigate the potential role of the 166 co-regulated genes in mediating the eto1-4 phenotype, we compared the expression levels of these genes in the WT and eto1-4 treated with inhibitors. By hierarchical clustering analysis, the expression profiles of 166 co-regulated genes are as expected, showing reversed trends (up-regulated or down-regulated) in eto1-4 in the absence or presence of AVG or the hit compounds (Fig. 7C; compare columns 1 and 4 for AVG, 1 and 3 for the hit compounds). However, the same group of genes does not completely show identical profiles in the WT as would be expected for the eto1-4 seedlings treated with inhibitors, which suggests that not all of the co-regulated genes contribute to the different phenotypes between WT and eto1-4 (Fig. 7C). The genes that showed a similar trend (i.e. up-regulation or down-regulation) in both WT and eto1-4 treated with inhibitors are indicated by solid bars on the right margin of heat maps in Fig. 7C.

The hit compounds may have additional roles in planta other than regulating ACS activity. To test this possibility, we analyzed the genes regulated only by the hit compounds but not by AVG. In total, 1399 genes (one-way ANOVA, p < 0.05) with less than 1.5-fold differential expression in eto1-4 in the absence and presence of AVG were selected for identification of genes co-regulated by all of the hit compounds with a 2-fold cutoff. We identified only 11 genes that were co-regulated by the hit compounds but not by AVG (Fig. 7D and supplemental Table S4). Considering the low number of genes (11 in Fig. 7D) as compared with the number of genes co-regulated by AVG and all of the hit compounds (166 in Fig. 7B), we concluded that regulation of ACS activity and ethylene biosynthesis is likely the primary function of the hit compounds.

**DISCUSSION**

This study demonstrates the utility of chemical screening to identify small molecules that interfere with the ethylene response in Arabidopsis. Conventional genetic studies of primarily Arabidopsis mutants to uncover the key components establishing the ethylene pathway in a hierarchical manner have laid the foundation to understand how the ethylene response is initiated and transduced. Here, we demonstrate an alternative approach by using a combination of ethylene mutants and chemical screening to further explore the ethylene pathway in plants. Our work identified three structurally related quinazolinones as the hit compounds 9393, 9370, and 7303 (Table 1) that function as ethylene antagonists from phenotype-based screening. Further characterization of the hit compounds revealed that these small molecules are novel inhibitors of ACC synthase in suppressing ethylene biosynthesis in an uncompetitive fashion. Identification of such small molecules not only provides new material for ethylene research but also offers potential applications for post-harvest management.

Our chemical screening based on suppression of the triple response phenotype in etiolated eto1-4 successfully identified hit compounds that are novel inhibitors of ACC synthase. The hit compounds have a quinazolinone skeleton that is unrelated to AVG. Quinazolinones and their derivatives have a wide range of important pharmacological properties in clinical studies (45). Naturally occurring quinazolinones are alkaloids isolated from plants and microorganisms, and subsequently, bioactive derivatives can be chemically synthesized for clinical treatments. However, the hit compounds we identified have two major distinct features unlike typical, natural, or synthetic quinazolinones. First, the ketone in the hit compounds is located at the C5 position to form quinazolin-5-ones, whereas the typical quinazolinones are quinazolin-4-ones with the ketone at the C4 position (Table 1). Second, substitutions in the hit compounds are at positions C2 and C7, with no modification at the N3 position, which is commonly substituted in both synthetic and natural quinazolinones of therapeutic importance (45). Because of the diverse structures and pharmacological properties, the detailed mechanisms of quinazolinones affecting biological processes, as well as identification of cellular targets, remain to be explored. Our studies have uncovered a group of synthetic quinazolinones that are inhibitors of ACC synthase. Because ACC synthase belongs to PLP-dependent enzymes, some of the clinically important quinazolinones may target PLP-containing proteins. However, the hit compounds and AVG have distinct structures and are different types of inhibitors, so this possibility seems unlikely and awaits further studies to determine additional targets of the synthetic quinazolinones we identified.

We used eto1-4 for phenotype-based chemical screening to identify small molecules that interfere with the ethylene response, which may take place at any step downstream of the conversion of AdoMet to ACC by ACC synthase in the ethylene biosynthetic pathway. However, the hit compounds we identified affected only the ethylene biosynthesis but not the signaling pathway. We may have failed to uncover additional compounds effectively modulating the ethylene signaling pathway because we focused on the full suppression of eto1-4 and overlooked subtle changes in the triple response phenotype. This possibility is supported by our results shown in Fig. 5. We found no significant difference between eto2-1 and eto1-4 in hypocotyl phenotypes, despite eto2-1 producing nearly five times the ethylene of eto1-4. Although 10 μM of the hit compounds completely suppressed the ethylene emanation and hypocotyl phenotype in eto1-4, it suppressed only 25% of the hypocotyl length in eto2-1 even with 30 μM of the most effective compound 7303. This finding indicates that the severity of the mutant phenotype and the potency of compounds determine the outcome of phenotype-based chemical screening. Despite the potency of AVG in inhibiting ethylene biosynthesis, increasing concentrations of AVG still did not completely suppress the hypocotyl pheno-
type in eto2-1 as compared with eto1-4; the ethylene production in both mutants was reduced to a comparable level (Fig. 5). The hypocotyl phenotype in eto2-1 may not entirely depend on overproduced ethylene and thus not be completely affected by the hit compounds. Therefore, uncovering single molecules that substantially modulate phenotypes resulting from different effectors would be difficult. Alternative approaches, including reporter gene- and activity-based screening strategies, can provide better resolution of the analysis for chemical screening.

A structure-based design in chemical synthesis may be an alternative way to identify compounds targeting specific components in the ethylene pathway, such as the ethylene receptors, Raf-like kinase CTR1, transcription factor EIN3, and ethylene-forming enzyme ACC oxidase.

On the basis of the $K_i$ values from enzyme kinetic assays, AVG is only 1.5-fold more potent than compound 7303 in inhibiting recombinant ACS5 activity in vitro. The chemical structure of AVG and the hit compounds differ, which suggests distinct biochemical properties. Indeed, our results indicated that AVG and the hit compounds inhibit ACC synthase by different mechanisms. Quinazolinone alkaloids and synthetic derivatives have a broad range of biological properties of clinical importance (45–47). However, information is limited on the effects of ~150 naturally occurring quinazolinones in plant physiology. The hit compounds from our screening were generated by total synthesis and have a structural variation to the known quinazolinones and their synthetic derivatives. Nevertheless, we have demonstrated that the hit compounds are inhibitors of ACS activity in ethylene hormone biosynthesis. Testing whether the hit compounds have any pharmacological activities similar to natural quinazolinones and vice versa for the ethylene hormone response is of interest.

Distinct mechanisms of AVG and the hit compounds in inhibiting ACC synthase may contribute to their effectiveness in suppressing ethylene production. ACC synthase is a PLP-dependent enzyme and related to aminotransferases (24, 43). AVG is a competitive inhibitor that competes with AdoMet for binding to the catalytic site of ACC synthase (44, 48). In addition, crystal structure studies of ACC synthases from apple and tomato provided atomic details to propose the chemical interactions of PLP and AVG in the active site of ACS enzymes (49–51). A stable ketimine structure was formed by AVG interacting with PLP in the active site of ACC synthase and thus presented unfavorable catalytic sites to accommodate AdoMet as a substrate (49). The competitive inhibition by AVG should be partially reverted by increasing concentrations of PLP, and this is what we found by our in vitro activity assay (supplemental Fig. S2). However, such a competitive scheme does not seem to apply to compound 7303 because elevated PLP concentrations did not affect its inhibitory effect, which indicated that compound 7303 did not compete with PLP to regulate ACS activity (supplemental Fig. S2). Unlike AVG, compound 7303 displayed an uncompetitive inhibition, whereby such an inhibitor interacted with an enzyme-substrate (ES) complex instead of enzyme (E) alone to abrogate product formation. The apparent kinetic parameters of enzymes such as $K_m$ and $V_{max}$ are both reduced in uncompetitive inhibition (supplemental Table S3). Because $K_m$ is the measure of affinity between substrates and enzymes, the reduced $K_m$ value corresponds to a higher affinity in the presence of uncompetitive inhibitors. This intriguing phenomenon occurs because the equilibrium is shifted to form an ES complex due to binding of the inhibitor (I) to ES to form the unproductive ESI complex, which results in decreased concentration of the ES complex. Results from enzyme kinetic studies indicated that the $K_m$ and $V_{max}$ values of recombinant ACS5 enzyme were decreased, from 17.5 ± 6.7 to 5.4 ± 3.9 μM and from 85.9 ± 3.5 to 28.1 ± 0.8 μmol h$^{-1}$ mg$^{-1}$, respectively, in the presence of 0.05 μM of compound 7303 (Fig. 6B and supplemental Table S4), which supports that compound 7303 is an uncompetitive inhibitor. Because AVG can interact with PLP to form an inhibitory adduct, it is not surprising that AVG is also an inhibitor of PLP-dependent enzymes (52, 53). However, the hit compounds we identified are unlikely general inhibitors of PLP-dependent enzymes.

The uncompetitive inhibition mode of compound 7303 suggests that the hit compounds may function to lock the conformation of an ACS-AdoMet complex to prevent efficient formation of ACC. The hit compounds may interact with residues surrounding or affecting the active site of the ACS enzyme to alter the conformation of the ACS-AdoMet complex and abolish the enzymatic reaction and/or ACC release. The hypothesized inhibition model can be enlightened by the studies of brefeldin A, an uncompetitive inhibitor of the guanine nucleotide exchange factors (54). The small GTP-binding protein ADP-ribosylation factors, such as yeast ARF1, functions to mediate vesicle trafficking for protein transport from the ER to the Golgi apparatus. Active forms of ADP-ribosylation factors are GTP-bound and membrane-associated, whereas GDP-bound ARF1 is inactive and soluble. Guanine nucleotide exchange factor activity is required to activate ARF1 for dynamic vesicle trafficking by switching GDP-bound to GTP-bound ARF1. Brefeldin A inhibits the early steps in ER-Golgi protein transport in budding yeasts by acting like an uncompetitive inhibitor of guanine nucleotide exchange factors to lock the ARF1-GDP-guanine nucleotide exchange factor complex to block further nucleotide exchange (54). Alternatively, because ACS enzymes can form homo- or heterodimers with shared catalytic sites from two monomers for optimal activity (37, 55), the hit compounds may associate with the ACS enzymes that are in complex with the substrate to abort the enzymatic reaction by modifying ACS dimerization. Further structure-activity relationship analysis with structurally related compounds combined with computer-aided molecular modeling and, ultimately, structural studies by protein crystallography will reveal the mechanistic details of interaction between the quinazolinone inhibitors and ACC synthase.

The hit compounds we found effectively inhibited ACC synthase activity in vitro and suppressed ethylene production in eto1-4 seedlings. However, the potency of the hit compounds differs. To understand the physiological impact of hit compounds at the gene expression level, we used DNA microarray methodology to compare the global gene expression patterns regulated by the hit compounds and by AVG. More than 40% of genes with 2-fold differential expression by AVG treatment were co-regulated by the hit compounds, which indicates an overlap function on suppression of the triple response pheno-
type in eto1-4. Previous transcriptome studies of ethylene-responsive genes focused on those differentially regulated by ethylene treatment within a short period of time (56, 57), and the genes identified from those experiments would likely be the immediate targets specific to ethylene induction. However, the 166 genes we identified by microarray analysis resulted from differential expression in 3-day-old etiolated eto1-4 seedlings treated with ACS inhibitors and may not be the primary ethylene-responsive genes. Instead, these genes may represent a regulation network leading to the triple response phenotype in etiolated seedlings responding to elevated ethylene. Only 17 of the 166 genes were identical to those reported by Nemhauser et al. (57) upon transient ethylene induction, which suggests a major difference in sustained and early responses to ethylene.

Five of the 17 genes are likely involved in response to three phytohormones, ethylene, auxin, and brassinosteroid (supplemental Table S3), which suggests that a cross-talk among phytohormones contributes to the triple response phenotype. Because the 17 genes co-regulated by AVG and the hit compounds show differential expression in the wild type and in eto1-4 (Fig. 7C), which suggests that the hit compounds may have targets other than ACS. However, only 11 genes revealed by global gene expression profiles were co-regulated by the hit compounds but were independent of AVG regulation (Fig. 7D), which strongly implies that the primary function of the hit compounds involves ethylene biosynthesis by regulating ACS activity. Hierarchical clustering analysis to interpret the expression patterns of the 116 genes co-regulated by the ACS inhibitors in the wild type can uncover a minimal set of genes required to establish the triple response phenotype during etiolated growth. In conclusion, our phenotype-based screening uncovers a minimal set of genes required to establish the triple response phenotype during etiolated growth.

Acknowledgments—We thank the Affymetrix Gene Expression Service Laboratory, the Institute of Plant and Microbial Biology, Academia Sinica, for transcriptome analysis, and Drs. Hai Li and Anna N. Stepanova (Salk Institute) for providing the 5×EBS:LLIC construct to generate transgenic lines. We thank the reviewers for exceptionally helpful comments on the manuscript.

REFERENCES

1. Bleecker, A. B., and Kende, H. (2000) *Annu. Rev. Cell Dev. Biol.* 16, 1–18
2. Johnson, P. R., and Ecker, J. R. (1998) *Annu. Rev. Genet.* 32, 227–254
3. Lin, Z., Zhong, S., and Grierison, D. (2009) *J. Exp. Bot.* 60, 3311–3336
4. Wang, K. L., Li, H., and Ecker, J. R. (2002) *Plant Cell* 14, S131–S151
5. Ecker, J. R. (1995) *Science* 268, 667–675
6. Chang, C., Kwok, S. F., Bleecker, A. B., and Meyerowitz, E. M. (1993) *Science* 262, 539–544
7. Gúzmán, P., and Ecker, J. R. (1990) *Plant Cell* 2, 513–523
8. Roman, G., Lubarsky, B., Kieber, J. J., Rothenberg, M., and Ecker, J. R. (1995) *Genetics* 139, 1393–1409
9. Yoo, S. D., Cho, Y., and Sheen, J. (2009) *Trends Plant Sci.* 14, 270–279
10. Chen, Y. F., Randlesett, M. D., Findell, J. L., and Schaller, G. E. (2002) *J. Biol. Chem.* 277, 19861–19866
11. Grefen, C., Stadéle, K., Rößicka, K., Obrdlik, P., Harter, K., and Horák, J. (2008) *Mol. Plant* 1, 308–320
12. Bleecker, A. B., Esch, J. J., Hall, A. E., Rodriguez, F. I., and Binder, B. M. (1998) *Philos. Trans. R Soc. Lond. B Biol. Sci.* 353, 1405–1412
13. Hua, J., and Meyerowitz, E. M. (1998) *Cell* 94, 261–271
14. Alonso, J. M., Hirayama, T., Roman, G., Nourizadeh, S., and Ecker, J. R. (1999) *Science* 284, 2148–2152
15. Kieber, J. J., Rothenberg, M., Roman, G., Feldmann, K. A., and Ecker, J. R. (1993) *Cell* 72, 427–441
16. Bissone, M. M., Bleckmann, A., Allekotte, S., and Groth, G. (2009) *Biochem. J.* 424, 1–6
17. Gao, Z., Chen, Y. F., Randlesett, M. D., Zhao, X. C., Findell, J. L., Kieber, J. J., and Schaller, G. E. (2003) *J. Biol. Chem.* 278, 34725–34732
18. Guo, H., and Ecker, J. R. (2003) *Cell* 115, 667–677
19. Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, C., and Genschik, P. (2003) *Cell* 115, 679–689
20. Qiao, H., Chang, K. N., Yazaki, J., and Ecker, J. R. (2009) *Genes Dev.* 23, 512–521
21. Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J. R. (1997) *Cell* 89, 1133–1144
22. Solano, R., Stepanova, A., Chao, Q., and Ecker, J. R. (1998) *Genes Dev.* 12, 3703–3714
23. Yoo, S. D., Cho, Y. H., Tena, G., Xiong, Y., and Sheen, J. (2008) *Nature* 451, 789–795
24. Yang, S. F., and Hoffman, N. E. (1984) *Annu. Rev. Plant Physiol.* 35, 155–189
25. Barry, C. S., Blume, B., Bouzayen, M., Cooper, W., Hamilton, A. J., and Grierison, D. (1996) *Plant J.* 9, 525–535
26. English, P. J., Lyckett, G. W., Roberts, J. A., and Jackson, M. B. (1995) *Plant Physiol.* 109, 1435–1440
27. Tsuchisaka, A., and Theologis, A. (2004) *Plant Physiol.* 136, 2982–3000
28. Chae, H. S., Faure, F., and Kieber, J. J. (2003) *Plant Cell* 15, 545–559
29. Vogel, J. P., Woeste, K. E., Theologis, A., and Kieber, J. J. (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95, 4766–4771
30. Yoshida, H., Nagata, M., Saito, K., Wang, K. L., and Ecker, J. R. (2005) *BMC Plant Biol.* 5, 14
31. Christians, M. J., Gingerich, D. J., Hansen, M., Binder, B. M., Kieber, J. J., and Vierstra, R. D. (2009) *Plant J.* 57, 332–345
32. Thonmann, A., Dieterle, M., and Genschik, P. (2005) *FEBS Lett.* 579, 3239–3245
33. Wang, K. L., Yoshida, H., Lurin, C., and Ecker, J. R. (2004) *Nature* 428, 945–950
34. Blackwell, H. E., and Zhao, Y. (2003) *Plant Physiol.* 133, 448–455
35. Töth, R., and van der Hoorn, R. A. (2010) *Trends Plant Sci.* 15, 81–88
36. Lizada, M. C., and Yang, S. F. (1979) *Anal. Biochem.* 100, 140–145
37. Yanagamini, T., Tsuchisaka, A., Yamada, K., Haddon, W. F., Harden, L. A., and Theologis, A. (2003) *J. Biol. Chem.* 278, 49102–49112
38. Chang, S., Puryear, J., and Caimrey, J. (1993) *Plant Mol. Biol. Rep.* 11, 113–117
39. Xu, Q., Hall, B. P., Gao, Z., and Schaller, G. E. (2007) *BMC Plant Biol.* 7, 3
40. Beyer, E. M. (1976) *Plant Physiol.* 58, 268–271
41. Yanagisawa, S., Yoo, S. D., and Sheen, J. (2003) *Nature* 425, 521–525
42. Adams, D. O., and Yang, S. F. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 113–117
Chemical Compounds Inhibit Ethylene Biosynthesis

170–174
43. Eliot, A. C., and Kirsch, J. F. (2004) Annu. Rev. Biochem. 73, 383–415
44. Boller, T., Herner, R. C., and Kende, H. (1979) Planta 145, 293–303
45. Mhaske, S. B., and Argade, N. P. (2006) Tetrahedron 62, 9787–9826
46. Ozaki, K., Yamada, Y., Oine, T., Ishizuka, T., and Iwasawa, Y. (1985) J. Med. Chem. 28, 568–576
47. Xia, Y., Yang, Z. Y., Hour, M. J., Kuo, S. C., Xia, P., Bastow, K. F., Nakanishi, Y., Namrpothiri, P., Hackl, T., Hamel, E., and Lee, H. K. (2001) Bioorg. Med. Chem. Lett. 11, 1193–1196
48. Hyodo, H., and Tanaka, K. (1986) Plant Cell Physiol. 27, 391–398
49. Capitani, G., McCarthy, D. L., Gut, H., Grütter, M. G., and Kirsch, J. F. (2002) J. Biol. Chem. 277, 49735–49742
50. Capitani, G., Tschopp, M., Eliot, A. C., Kirsch, J. F., and Grütter, M. G. (2005) FEBS Lett. 579, 2458–2462
51. Huai, Q., Xia, Y., Chen, Y., Callahan, B., Li, N., and Ke, H. (2001) J. Biol. Chem. 276, 38210–38216
52. Clausen, T., Huber, R., Messerschmidt, A., Pohlenz, H. D., and Laber, B. (1997) Biochemistry 36, 12633–12643
53. Krupka, H. I., Huber, R., Holt, S. C., and Clausen, T. (2000) EMBO J. 19, 3168–3178
54. Chardin, P., and McCormick, F. (1999) Cell 97, 153–155
55. Tsuchisaka, A., Yu, G., Jin, H., Alonso, J. M., Ecker, J. R., Zhang, X., Gao, S., and Theologis, A. (2009) Genetics 183, 979–1003
56. Goda, H., Sasaki, E., Akiyama, K., Maruyama-Nakashita, A., Nakabayashi, K., Li, W., Ogawa, M., Yamauchi, Y., Preston, J., Aoki, K., Kiba, T., Takatsuto, S., Fujioka, S., Asami, T., Nakano, T., Kato, H., Mizuno, T., Sakakibara, H., Yamaguchi, S., Nambara, E., Kamiya, Y., Takahashi, H., Hirai, M. Y., Sakurai, T., Shinozaki, K., Saito, K., Yoshida, S., and Shimada, Y. (2008) Plant J. 55, 526–542
57. Nemhauser, J. L., Hong, F., and Chory, J. (2006) Cell 126, 467–475