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Exploiting the Triple Response of *Arabidopsis* To Identify Ethylene-Related Mutants

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Alterations in the response of dark-grown seedlings to ethylene (the "triple response") were used to isolate a collection of ethylene-related mutants in *Arabidopsis thaliana*. Mutants displaying a constitutive response (*eto1*) were found to produce at least 40 times more ethylene than the wild type. The morphological defects in etiolated *eto1-1* seedlings reverted to wild type under conditions in which ethylene biosynthesis or ethylene action were inhibited. Mutants that failed to display the apical hook in the absence of ethylene (*hls1*) exhibited reduced ethylene production. In the presence of exogenous ethylene, hypocotyl and root of etiolated *hls1-1* seedlings were inhibited in elongation but no apical hook was observed. Mutants that were insensitive to ethylene (*ein1* and *ein2*) produced increased amounts of ethylene, displayed hormone insensitivity in both hypocotyl and root responses, and showed an apical hook. Each of the "triple response" mutants has an effect on the shape of the seedling and on the production of the hormone. These mutants should prove to be useful tools for dissecting the mode of ethylene action in plants.

INTRODUCTION

Ethylene is one of five known naturally occurring plant hormones. This chemically simple molecule participates in a diverse array of processes. The physiological basis of these processes has been studied extensively (reviewed by Abeles, 1973; Lieberman, 1979). In addition, the biosynthetic pathway of ethylene has been established (reviewed by Yang and Hoffman, 1984). Figure 1 depicts the ethylene biosynthesis pathway along with a hypothetical transduction pathway. Methionine is converted to ethylene with *S*-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) as intermediates. These two reactions are catalyzed by ACC synthase and ethylene-forming enzyme (EFE), respectively. Both reactions are known to increase dramatically during certain developmental processes of plant growth. Ripening in climacteric fruits (reviewed by Brady, 1987) as well as petal wilting in some flowers (Bufler et al., 1980) are accompanied by a rise in ethylene production. In addition, a variety of biotic and abiotic stimuli can also elicit ethylene production (Yang and Hoffman, 1984).

Little is known about the enzymes catalyzing these reactions and their regulation at the molecular level. Physiological analysis has suggested that the key regulatory step in ethylene biosynthesis is the formation of ACC and that the conversion of ACC to ethylene is mainly constitutive (Yang and Hoffman, 1984). A complementary DNA sequence encoding ACC synthase mRNA has recently been cloned from zucchini fruits (*Cucurbita pepo*) (Sato and Theologis, 1989). The ACC synthase activity is associated with a 46-kD polypeptide whose level varies as a result of the action of well-known inducers of the enzyme (Sato and Theologis, 1989). The characterization of EFE has been less rewarding. ACC is converted to ethylene in almost any system that produces oxidants (Apelbaum et al., 1981). Most of the EFE activity has been localized to vacuoles and is thought to depend on membrane integrity (Guy and Kende, 1984).

High-affinity binding sites for ethylene have been purified from plants (Jerie et al., 1979). However, the biochemical characterization of a physiological receptor has not yet been accomplished. Models that attempt to explain the interaction of ethylene with a receptor propose the presence of a metal ion at the receptor site (Burg and Burg, 1967) and suggest that ethylene action involves a *trans* effect on ligand-metal coordination (Sisler, 1977). It has been suggested that ethylene glycol, a metabolite of ethylene, may be the actual molecule responsible for ethylene effects (Blomstrom and Beyer, 1980). However, it has been shown recently that ethylene metabolism and ethylene action are independent (Sanders et al., 1989).

Although every tissue of the plant has the capacity to synthesize ethylene, it is normally produced only in re-

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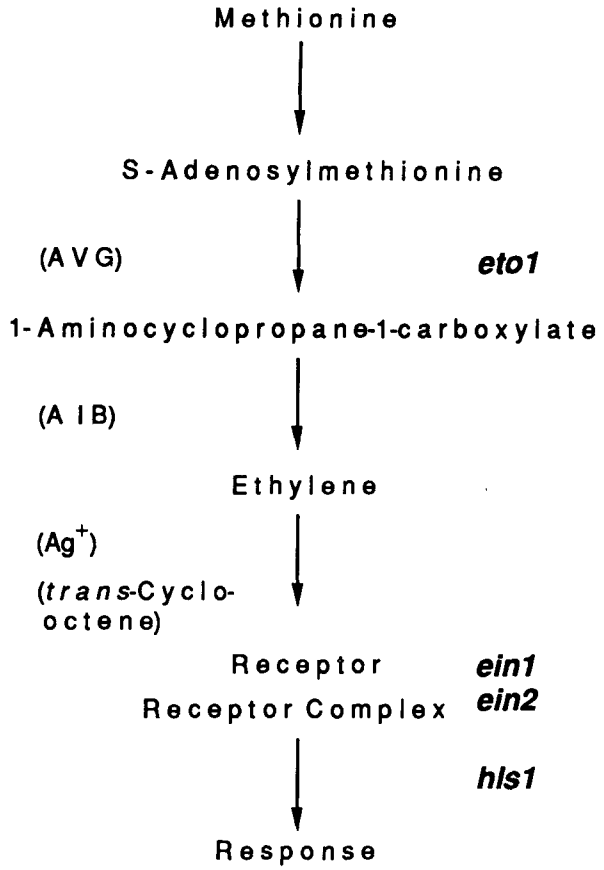

Figure 1. The Ethylene Biosynthesis Pathway and the Hypothetical Ethylene Signal Transduction Pathway in Plants.

The intermediates of the biosynthetic pathway are diagrammed; receptor and receptor complex are hypothetical (Yang and Hoffman, 1984). Ethylene biosynthesis and action inhibitors are positioned to the left. The presumed step in which the mutants are defective appears to the right in boldface letters. AVG, aminoethoxyvinylglycine; AIB, α-aminoisobutyric acid.

sponse to certain developmental and environmental stimuli. During seed germination and emergence from the soil, the terminal part of the shoot axis of certain dicotyledonous plants exhibits an apical arch-shaped structure, the apical hook. As first suggested by Haberlandt (quoted in Darwin, 1896), this curved structure may protect the delicate apical tissues of the growing meristem from injury while the stem is emerging from the soil into the atmosphere. Ethylene is involved in determining the shape of etiolated seedlings (dark-grown seedlings) (Goeschl et al., 1966). In germinating seedlings, ethylene production is localized in the apical hook region (Goeschl et al., 1967; Taylor et al., 1988). A transient decrease in ethylene production occurs when seedlings are exposed to white light. Furthermore, as the etiolated tissue becomes photosynthetically competent, it loses sensitivity to ethylene (Goeschl et al., 1967). The application of low levels of ethylene to etiolated seedlings results in seedlings with an exaggerated curvature of the apical hook. This effect, which was first described by Neljubow (1901), is known as the "triple response" of etiolated seedlings. It consists of three distinct morphological changes in the shape of the seedling: inhibition of stem elongation, radial swelling of the stem, and absence of normal geotropic response (diageotropism) (Knight and Crocker, 1913). Herein we describe the identification and characterization of three different mutants that affect the triple response in *Arabidopsis*. This is the first step toward understanding the control of ethylene biosynthesis and signal transduction pathways at the molecular level.

RESULTS

Features of the Triple Response in *Arabidopsis*

The morphological changes that occur during the growth of the etiolated *Arabidopsis* seedlings are easily detected by visual inspection or with the aid of a dissecting microscope. After 3 days of incubation at 23°C in the dark, seedlings germinated in air are readily distinguished from seedlings germinated in 10 μL/L ethylene. Air-treated *Arabidopsis* seedlings are highly elongated and exhibit an apical hook at the terminal part of the shoot axis (Figure 2B). Conversely, ethylene-treated seedlings show inhibition of root and hypocotyl elongation, exaggerated tightening of the apical hook, and swelling of the hypocotyl (i.e., a triple response). The visual features of this response in *Arabidopsis* are shown in Figure 2A. In our search for mutants affected in ethylene-mediated processes, three alterations in the triple response were sought: (1) a constitutive triple response (i.e., inhibition of hypocotyl and root elongation with exaggerated tightening of the apical hook in the absence of exogenous ethylene), (2) the absence of an apical hook (i.e., presence of a straight hypocotyl in the absence of exogenous ethylene), and (3) insensitivity to ethylene (i.e., root and hypocotyl elongation in the presence of high concentrations of ethylene).

Isolation of Constitutive Triple Response Mutants

In an attempt to identify mutations in genes that regulate ethylene biosynthesis and signal transduction, we sought to identify a class of mutants that simulate an ethylene response in the absence of exogenously supplied hormone. In a population of ethylene-treated wild-type seedlings, a small fraction of plants that are inhibited in hypocotyl and root elongation do not exaggerate the curvature

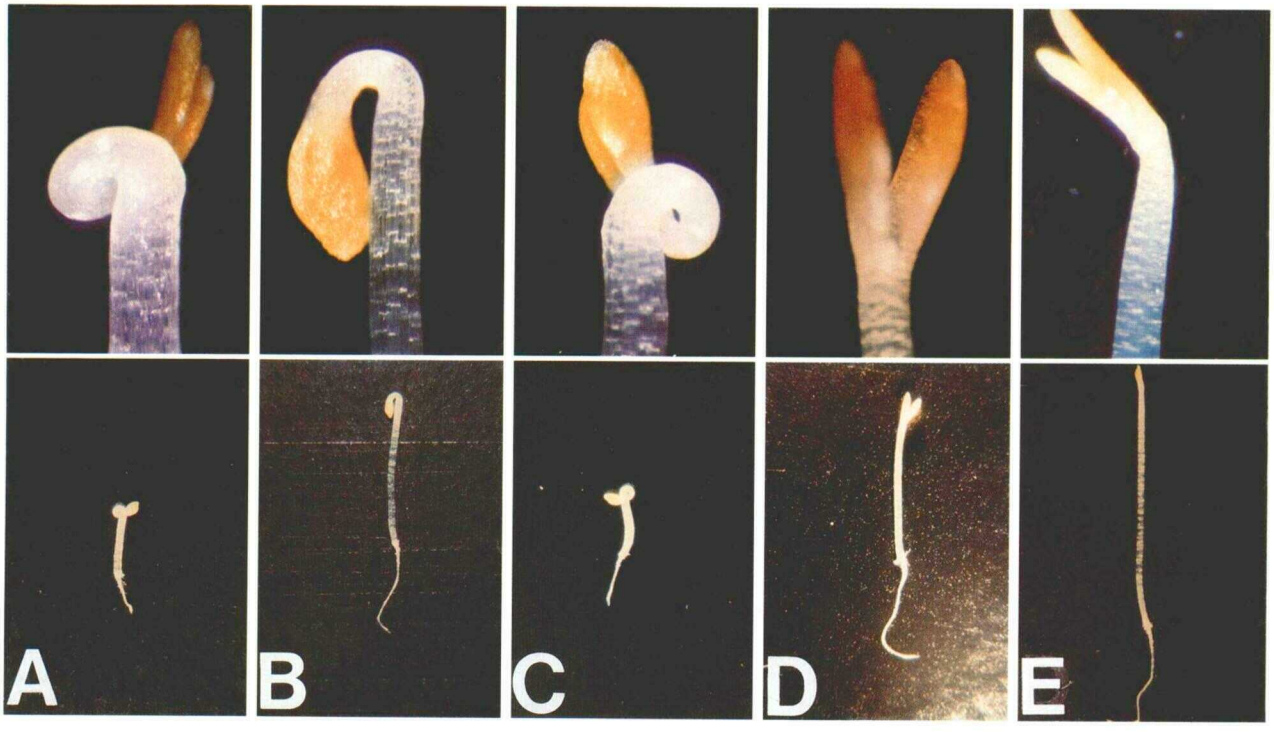

Figure 2. Morphological Features of the Triple Response in Wild-Type *Arabidopsis* and in the Triple Response Mutants.

Each panel is composed of two photomicrographs of an etiolated *Arabidopsis* seedling; the upper part shows the apical region of the hypocotyl and the lower part shows the complete seedling. Surface-sterilized seeds were planted in growth medium and cold treated at 4°C for 4 days before germination and growth in the dark at 23°C for 66 hr to 72 hr.

(A) Wild type displaying the triple response in the presence of 10 μL/L ethylene.

(B) Wild type incubated without ethylene.

(C) *eto1-1* displaying the triple response in the absence of ethylene.

(D) *hls1-1* incubated without ethylene.

(E) *ein2-1* incubated in the presence of 10 μL/L ethylene.

of the apical hook. Thus, in the initial screen for constitutive triple response mutants (see Methods), we chose seedlings that exhibited inhibition of hypocotyl and root elongation regardless of extent of apical hook curvature. Two seedlings were found to be phenotypically indistinguishable from ethylene-treated wild-type seedlings. These putative mutant seedlings exhibited inhibition of hypocotyl and root elongation as well as swelling of the hypocotyl and exaggerated tightening of the apical hook. One of these mutants is shown in Figure 2C.

The genetic basis of these mutations was investigated by Mendelian segregation analysis. A summary of the genetic characterization of the mutants is shown in Table 1. The patterns of segregation indicated that the constitutive triple response phenotype was caused by a single recessive mutation (Table 1A, lines 1 and 2). We designated this previously unidentified mutant phenotype Eto for *et*hylene *o*verproducer (see below). In addition, complementation analysis indicated that the two *eto* mutations, *eto1-1* and *eto1-2*, were allelic (Table 1A, line 3).

Analysis of Ethylene Production in Constitutive Triple Response Mutants

Because it seemed likely that the constitutive triple response phenotype of the *eto1-1* mutant was the result of high-level ethylene production, we compared ethylene levels in *eto1-1* and wild-type plants. These data are presented in Table 2. A dramatic difference was detected in etiolated seedlings; *eto1-1* seedlings produced at least 40 times more ethylene than wild-type seedlings (Table 2, lines 1 and 6). Light-grown *eto1-1* seedlings and tissues from adult plants also showed a twofold to fivefold increase in ethylene production relative to wild type (Table 2).

ACC synthase, the key regulatory enzyme in ethylene

Table 1. Genetic Analysis of Triple Response Mutants[a]

Crosses	Type	Total			χ^{2b}
(A) *eto* mutants[c]			Constitutive Triple Response		
			+	−	
eto1-1/eto1-1 × *ETO1/ETO1*	F1	25	0	25	
ETO1/eto1-1 × *ETO1/eto1-1*	F2	397	96	301	0.14[d]
eto1-1/eto1-1 × *eto1-2/eto1-2*	F1	12	12	0	
(B) *hls* mutants[e]			Apical Hook		
			+	−	
hls1-1/hls1-1 × *HLS1/HLS1*	F1	9	9	0	
HLS1/hls1-1 × *HLS1/hls1-1*	F2	284	224	60	2.26[d]
hls1-1/hls1-1 × *hls2-1/hls2-1*	F1	33	33	0	
(C) *ein* mutants[f]			Ethylene Sensitivity		
			+	−	
ein1-1/ein1-1 × *EIN1/EIN1*	F1	26	0	26	
EIN1/ein1-1 × *EIN1/ein1-1*	F2	106	24	82	0.30[g]
ein2-2/ein2-2 × *EIN2/EIN2*	F1	18	18	0	
EIN2/ein2-2 × *EIN2/ein2-2*	F2	74	60	14	1.46[d]
ein2-1/ein2-1 × *ein2-2/ein2-2*	F1	20	0	20	

[a] Wild type are constitutive triple response (−), apical hook (+), and ethylene sensitivity (+).
[b] $P > 0.05$.
[c] Similar results were obtained from crosses of *eto1-1* or *eto1-2* to wild type; only the data for the cross with *eto1-1* are presented.
[d] χ^2 expected ratio, 3 wild type:1 mutant.
[e] Similar results were obtained from crosses of *hls1-1* or *hls1-2* to wild type; only the data for the cross with *hls1-1* are presented.
[f] Similar results were obtained from crosses of *ein2-1*, *ein2-2*, *ein2-3*, *ein2-4*, and *ein2-5* to wild type or from crosses of *ein2-2*, *ein2-3*, *ein2-4*, and *ein2-5* to *ein2-1*; only the data of the cross of *ein2-2* to wild type and *ein2-1* to *ein2-2* are presented.
[g] χ^2 expected ratio, 3 mutant:1 wild type.

biosynthesis, is a pyridoxal phosphate-requiring enzyme that catalyzes the conversion of SAM to ACC. Aminoethoxyvinylglycine (AVG), an effective inhibitor of pyridoxal phosphate-mediated enzymes reactions, inhibits ACC synthase activity in vivo as well as in vitro (Yang and Hoffman, 1984). α-Aminoisobutyric acid (AIB) is a structural analog of ACC that has been shown to inhibit competitively the formation of ethylene from ACC (Satoh and Esashi, 1980). To determine whether ethylene biosynthesis in *eto1-1* was dependent on the formation of ACC and on the conversion of ACC to ethylene, the in vivo effects of AVG and AIB on ethylene production were examined. The levels of ethylene production and the morphological characteristics of *eto1-1* and wild-type seedlings are presented in Table 3. Ethylene production was inhibited in *eto1-1* seedlings that were germinated in media supplemented with AVG or AIB. Furthermore, the constitutive triple response morphology of *eto1-1* seedlings was reverted to wild type under these conditions; the hypocotyl and root were highly elongated and curvature of the apical hook was not exaggerated. Thus, the morphology of etiolated *eto1-1* seedlings is likely to be caused by the high levels of ethylene production.

A number of chemical compounds have been identified that prevent ethylene action by interfering with the binding of ethylene to putative receptor sites. *cis*-Butene and certain cyclic olefins, such as 2,5-norbornadiene, are known to compete with ethylene for binding sites without eliciting an ethylene response (Sisler and Yang, 1984). Ag^+ ion is also a potent inhibitor of ethylene action (Beyer, 1976). Although the mechanism of action is unknown, Ag^+ blocks the effect of exogenously applied ethylene in several classical ethylene responses such as leaf abscission, petal senescence, and the triple response. Ag^+ may interfere with the binding of ethylene by substitution of the metal ion from the receptor (Beyer, 1979). *trans*-Cyclooctene, a highly strained aromatic hydrocarbon, has recently been shown to inhibit competitively ethylene action at a much lower concentration than any cyclic olefin, including 2,5-norbornadiene, the most effective antagonist of ethylene action (Sisler et al., 1990). Unlike 2,5-norbornadiene, which has deleterious effects on the growth of etiolated *Arabidopsis* seedlings at the effective concentrations (P. Guzmán, unpublished results), *trans*-cyclooctene and Ag^+ were effective in blocking ethylene action in *Arabidopsis*. This effect was detected by the ability of these antagonists to revert the triple response morphology of *eto1-1* seedlings to wild type (Table 3). *trans*-Cyclooctene and Ag^+

Table 2. Ethylene Production in Triple Response Mutants

Strain	Ethylene Accumulation
Wild type	
Etiolated seedlings	6.76 ± 0.68 nL
Light-grown seedlings	84.25 ± 13.96 nL
Leaves	73.01 ± 17.64 nL/g
Siliques	144.96 ± 28.99 nL/g
Inflorescence	234.53 ± 18.04 nL/g
eto1-1	
Etiolated seedlings	276.72 ± 53.70 nL
Light-grown seedlings	182.01 ± 24.84 nL
Leaves	174.39 ± 29.18 nL/g
Siliques	322.16 ± 38.66 nL/g
Inflorescence	1061.84 ± 72.16 nL/g
hls1-1	
Etiolated seedlings	5.81 ± 0.32 nL
Leaves	31.56 ± 8.98 nL/g
ein1-1	
Etiolated seedlings	12.73 ± 2.79 nL
Leaves	222.95 ± 70.29 nL/g
ein2-1	
Etiolated seedlings	20.69 ± 2.09 nL
Leaves	136.59 ± 26.89 nL/g

Ethylene accumulation was measured as described in Methods.

Table 3. Effects of Ethylene Biosynthesis and Action Inhibitors in Etiolated Wild-Type and *eto1-1* Seedlings

	No Inhibitor	AVG	AIB	*trans*-Cyclooctene	$AgNO_3$
Triple response[a]					
Wild type	–	–	–	–	–
eto1-1	+	–	–	–	–
Ethylene production (nL)					
Wild type	6.74 ± 0.10	0.41 ± 0.60	1.28 ± 1.13	10.11 ± 2.81	47.65 ± 7.13
eto1-1	276.62 ± 53.70	1.90 ± 0.29	2.61 ± 1.41	445.87 ± 23.40	769.31 ± 161.10

Ethylene production was measured as described in Methods.
[a] (–) represents the wild-type morphology as shown in Figure 2B. (+) represents the constitutive triple response morphology as shown in Figure 2C.

also caused an increase in ethylene production in both wild-type and *eto1-1* seedlings (Table 3). It is likely that the block in ethylene action affects the autoregulation (autoinhibition) of ethylene biosynthesis and, thus, results in increased ethylene production (Yang and Hoffman, 1984). As a consequence of the inhibition of ethylene biosynthesis and action, etiolated wild-type seedlings grown in media containing AVG, AIB, *trans*-cyclooctene, or Ag^+ showed more elongation of the hypocotyl than untreated seedlings (data not shown). Thus, it is likely that ethylene plays a role in the regulation of cell elongation during seedling development.

Because ethylene production in *eto1-1* seedlings was found to proceed via the previously established biosynthetic pathway (Figure 1), we reasoned that the *eto1-1* mutation may be affecting the conversion of SAM to ACC, the rate-limiting step. Attempts to quantitate in vitro the formation of ACC in etiolated *Arabidopsis* seedlings have thus far been unsuccessful (P. Guzmán, unpublished results). Therefore, we decided to determine whether the conversion of ACC to ethylene was affected by the *eto1-1* mutation. The accumulation of ethylene by seedlings grown in media containing different concentrations of ACC was compared; the formation of endogenous ACC in these experiments was inhibited with AVG. As shown in Figure 3, *eto1-1* and wild-type seedlings produced similar amounts of ethylene over a broad range of ACC concentrations. Thus, the alteration in the *eto1-1* mutant occurs before the step of the conversion of ACC to ethylene.

Hookless Mutants

A role for ethylene has been demonstrated in the regulation of shape in the apical hook region (reviewed by Abeles, 1973). An exaggeration of the curvature in the apical region of the hypocotyl was observed after ethylene treatment of etiolated *Arabidopsis* seedlings (Figure 2A). We reasoned that mutants that did not display the apical hook might be altered either in the production or in the perception of ethylene. Considering this, a screen was performed in the absence of exogenous ethylene for seedlings that were hookless (see Methods). Two mutants were identified that showed straight hypocotyls and normal elongation of the hypocotyl and root (Figure 2D). Genetic analysis performed with these mutants is summarized in Table 1. The two mutations segregated as single recessive traits (Table 1B, lines 1 and 2). We designated these previous unidentified mutations *hls* for *h*ook*l*e*s*s. Complementation analysis of the Hls plants indicated that the two mutations were not allelic (Table 1B, line 3). We chose to analyze the *hls1-1* mutant in more depth.

The absence of apical curvature in the *hls1-1* mutant

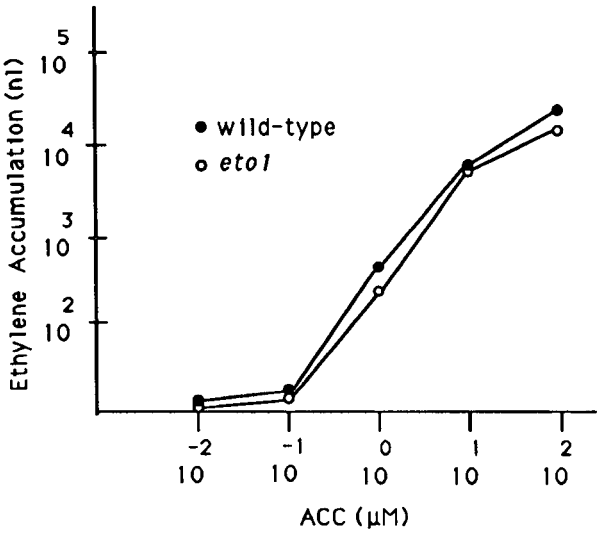

Figure 3. Assay for EFE, Conversion of Exogenous ACC by Etiolated *eto1* and Wild-Type Seedlings.

The assay and the conditions to measure ethylene accumulation are described in Methods.

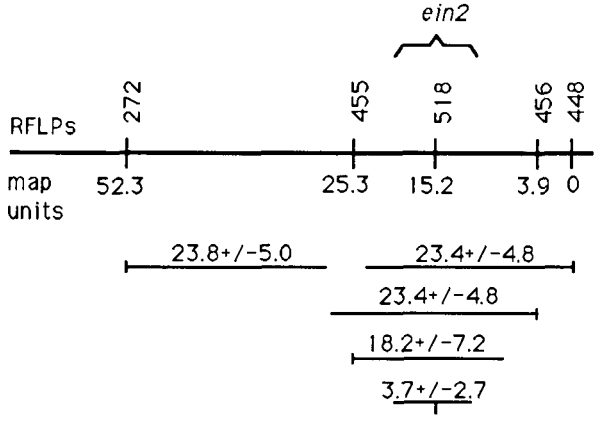

Figure 4. RFLP Mapping of *ein2-1* to Chromosome 4.

The heavy line represents chromosome 4. RFLP positions and map units have been published (Chang et al., 1988). Values in the lower part of the figure indicate the percent of recombination between RFLPs and *ein2-1*. The inferred location of *ein2* is noted by the horizontal bracket.

could be related to defects in either the production or perception of ethylene. Analysis of ethylene production from etiolated seedlings and adult tissue revealed that *hls1-1* plants produced reduced amounts of the hormone (Table 2). The difference between etiolated wild-type and etiolated *hls1-1* seedlings was not significant. It is unlikely that this decrease could account for the profound morphological change in the hook region of *hls1-1* seedlings. Accordingly, wild-type and *eto1-1* seedlings still showed significant apical curvature even in the presence of high concentrations of inhibitors of ethylene biosynthesis or action (Table 3). After incubating *hls1-1* seedlings in the dark for 3 days at 23°C with 10 μL/L ethylene, they exhibited the same extent of root and hypocotyl inhibition as the wild type. However, like the hypocotyl in untreated *hls1-1* seedlings, no evidence of apical curvature could be detected (data not shown). These results demonstrate that both the hypocotyl and root of *hls1-1* seedlings can respond normally to ethylene.

Ethylene-Insensitive Mutants

A third screen was initiated to identify *Arabidopsis* mutants that are insensitive to high concentrations of ethylene. This strategy has already been useful in the isolation of one *Arabidopsis* mutant that was reported to be affected in several responses to ethylene (Bleecker et al., 1988). To determine whether more than one locus was involved in insensitivity to ethylene, we isolated 25 independent ethylene-insensitive mutants. Six mutants, which showed at least threefold difference in the length of the hypocotyl compared with ethylene-treated wild-type hypocotyl, were further characterized (see Methods). In these mutants, the apical hook was either present, absent, or showed some curvature in the apical region. The appearance of the apical curvature was dependent on the duration of the incubation. After more than 3 days of incubation in the dark with 10 μL/L ethylene, the apical curvature was absent (data not shown). We named this phenotype Ein for ethylene *in*sensitive (Figure 2E). A summary of the Mendelian analysis is shown in Table 1. This analysis indicated that insensitivity to ethylene was inherited as either a dominant (Table 1C, lines 1 and 2) or a recessive (Table 1C, lines 3 and 4) trait depending on the mutation studied. To determine whether more than one locus was involved in this phenotype, complementation analysis was performed with five recessive mutants. The results of these studies indicated that all five recessive mutations were allelic (Table 1C, line 5). To determine whether the recessive and dominant *ein* mutations were allelic, the Ein phenotype was tested for linkage to nine visible markers (see Methods). The dominant *ein* mutation was mapped close to the mutation *ap-1* locus (7.0 ± 4.0% recombination) on chromosome 1. We named this mutation *ein1-1*. None of the nine markers showed linkage to the recessive *ein* mutation (data not shown). Thus, restriction fragment length polymorphism (RFLP) analysis was performed to map this mutation. Randomly selected RFLP probes were initially used to assess linkage (Chang et al., 1988). After testing probes from three different chromosomes, linkage was detected to one RFLP from chromosome 4. This observation was confirmed further using additional RFLP probes from the same chromosome. We named this locus *ein2*. The map position of the *ein2* locus is shown in Figure 4.

Growth Features of Triple Response Mutants

Preliminary observations were made on the effects of the *eto1-1*, *hls1-1*, *ein1-1*, and *ein2-1* mutations on the growth of plants to maturity. Plants homozygous for these mutations grew to maturity without displaying any dramatic morphological changes; all were fertile and set seed normally. However, several differences in growth can be readily identified; some of these characteristics are summarized in Table 4. When transplanted to soil, *eto1-1* plants grew more slowly and showed a reduction in the size of the rosette; however, they bolted after the same period of time as wild-type plants. Several other characteristics of *eto1-1* plants were consistently observed but not quantitated: the rosette leaves were darker green and the main inflorescence and lateral branches showed purple pigmentation when they were 1 cm to 2 cm in length. In contrast, *hls1-1* plants bolted and senesced very early when compared with wild type, and the rosette leaves were small

Table 4. Growth Features of Triple Response Mutants[a]

	Strains				
	Wild Type	*eto1-1*	*hls1-1*	*ein1-1*	*ein2-1*
Rosette size (diameter in cm)					
8 days	0.66 ± 0.10	0.46 ± 0.10	0.90 ± 0.16	0.58 ± 0.17	0.52 ± 0.07
24 days	7.80 ± 0.50	5.80 ± 0.50	3.18 ± 0.54	8.90 ± 1.24	9.10 ± 0.96
Bolting (50%)[b]					
(days after planting)	21–23	22–24	12–14	24–26	24–26

[a] Seeds were planted in soil, cold treated at 4°C for 4 days, and grown as described in Methods.
[b] A plant was considered bolted when the stem showed 1 cm to 2 cm in length.

and narrow. The rosette of *ein1-1* and *ein2-1* plants was larger compared with the wild-type rosette, and a delay in bolting was observed. These observations indicated that the ethylene-related mutations identified at the seedling stage exerted significant effects during adult stages of growth.

DISCUSSION

In this report, we described the isolation and physiological and genetic characterization of *A. thaliana* mutants that show alterations in the triple response. We define the triple response in *Arabidopsis* in a way similar to that described for pea (Knight and Crocker, 1913). In response to exogenously applied ethylene, etiolated *Arabidopsis* seedlings show (1) inhibition of hypocotyl and root elongation, (2) swelling of the hypocotyl, and (3) exaggerated tightening of the apical hook (Figure 2A). Three types of triple response mutants were isolated: *eto*, *hls*, and *ein*. Our studies on these mutants suggested that ethylene production can be controlled by various mechanisms. Each of the triple response mutations had a different effect on the shape of the seedling and a different level of ethylene production during seedling or adult growth (Table 2). The occurrence of these mutant phenotypes has provided important information about the regulation of ethylene production and the role of ethylene during plant growth.

The Constitutive Triple Response Is Caused by Ethylene Overproduction

Plants emerging from the soil or experimentally incubated in the dark are highly elongated, show an apical hook, and have unexpanded leaves. This morphology is believed to allow the seedling to reach the surface of the soil rapidly without damage to the apical meristem and leaves. As the seedling is irradiated with light, symmetrical growth in the apical region results in the opening of the apical hook and in the expansion of the leaves. Leaves become photosynthetically competent, and a new developmental program begins (Darwin, 1896; Cosgrove, 1986). Changes in the environment during germination in the dark can elicit ethylene production, and subsequent morphological changes are readily observed (Goeschl et al., 1966).

Analysis of the *eto1-1* mutant revealed that etiolated seedlings can produce high levels of ethylene and that this results in a morphological response (Figure 2C; Table 3). The recessive nature of the *eto1-1* mutation (Table 1A) suggested that a mechanism of negative control is affected in the mutant. Our results indicated that wild-type and *eto1-1* seedlings converted ACC to ethylene equally well (Figure 3). These data suggested that in the *eto1-1* mutant the alteration that affects the biosynthesis of ethylene is likely to occur at a step before the conversion of ACC to ethylene. For instance, the conversion of SAM to ACC (Figure 1) could be deregulated in this mutant. Recently, a gene encoding ACC synthase was cloned (Sato and Theologis, 1989). ACC synthase is the enzyme that catalyzes the conversion of SAM to ACC. It has been observed that the steady-state level of ACC synthase mRNA is markedly increased by treating plant cells with cyclohexamide (W. Rottmann and A. Theologis, personal communication). Thus, there is some molecular evidence of negative regulation in this step. The formation of ACC from SAM is the key step in ethylene biosynthesis and it is known to be regulated by a variety of stimuli (Yang and Hoffman, 1984). Thus, the *ETO1* gene product could play a role in the regulation of ACC synthase gene expression.

At Least Two Loci Are Involved in Insensitivity to Ethylene

Little is known about the molecular basis of ethylene perception; the genes coding for receptor(s) or involved in the signal transduction pathway have not been identified. Recently, an *Arabidopsis* mutant that alters various responses to ethylene has been isolated. This mutation maps to chromosome 1 near the *ap-1* locus (Bleecker et al., 1988). Our genetic analysis indicated that at least two loci

confer insensitivity to ethylene. Given the chromosomal location of the *ein1-1* mutation and of the previously identified mutation, it is possible that these two mutants are allelic. The RFLP data have allowed the assignment of a new ethylene-insensitive locus, *ein2*, to chromosome 4. Because more than one gene is involved in this phenotype, it is likely that a more complex pathway for ethylene perception and signal transduction may exist than previously assumed (Bleecker et al., 1988). Mutations in *ein1* and *ein2* loci may alter different steps in this pathway (Figure 1).

Regulation of ethylene biosynthesis by ethylene is known to occur in plants; both autocatalysis and autoinhibition of ethylene production have been observed (Riov and Yang, 1982; Yang and Hoffman, 1984). *ein1-1* and *ein2-1* mutants displayed a phenotype opposite to that of *eto1-1*: they were highly elongated and the apical hook readily opened (Figure 2E). Interestingly, ethylene production was also increased significantly both in the dominant *ein1-1* mutant and recessive *ein2-1* mutant (Table 2). These differences in ethylene production may indicate an alteration in the autoinhibition control of ethylene biosynthesis as a consequence of the defect in ethylene perception. Similar conclusions were inferred from the previously isolated ethylene-resistant *Arabidopsis* mutant (Bleecker et al., 1988).

Hookless Mutants Uncouple Components of the Triple Response

The occurrence of Ein and Hls phenotypes suggested that in the seedling the apical hook and the elongating hypocotyl and root have independent responses to ethylene. This assumption was supported by the fact that wild-type and *eto1-1* seedlings displayed an apical hook in the presence of ethylene biosynthesis and action inhibitors (Figure 3). The Hls phenotype indicated that the inhibition of hypocotyl elongation is separate from the hook response. Several factors are thought to participate in the formation and opening of the apical hook; the shape of the hook is probably the result of differential cell elongation (Kang, et al., 1967; Abeles, 1973). In fact, more than one locus was involved in the Hls phenotype (Table 1B; P. Guzmán unpublished results). The absence of an apical hook in ethylene-treated etiolated *hls1-1* seedlings was perhaps an indication of absence of diageotropism, which is one feature of the triple response in pea. In pea, exogenously applied ethylene causes the stem to grow horizontally, independent of the orientation in which the growing etiolated pea seedlings are placed (Neljubow, 1901). In *Arabidopsis*, diageotropism may contribute to the exaggerated tightening of the apical hook (Figure 2A).

One can speculate that an antagonist of ethylene action is produced in the outer region of the apical hook. The existence of inhibitors of ethylene action has been inferred from the observation that some fruits do not ripen while attached to the tree. When the fruit is detached, ethylene production increases after a few days, and ripening is initiated (Burg and Burg, 1964; Tingwa and Young, 1975; Brady, 1987). In the *hls1-1* mutant, a putative antagonist would not be present; therefore, cells in the apex of the hypocotyl would respond to ethylene equally well and the apical curvature would not be formed. This event may result in an increase in ethylene perception. Given the autoregulation of ethylene biosynthesis, a reduction in the levels of ethylene production would be expected. Contrary to the effect observed in the *ein1-1* and *ein2-1* mutants, which are affected in ethylene responses and show an increase in ethylene production, the *hls1-1* mutant showed a reduction in ethylene production (Table 2). Thus, the *HLS1* gene product perhaps antagonizes or modulates ethylene action (Figure 1).

The Etiolated *Arabidopsis* Seedling: A Model System To Study Ethylene-Related Processes in Plants

Ethylene-induced gene expression has been investigated in systems in which a rise of ethylene occurs during natural processes such as senescence of flower petals (Lawton et al., 1989), ripening of climacteric fruits (Lincoln et al., 1987; Davies and Grierson, 1989), or during pathogen attack (cf. Ecker and Davis, 1987; Broglie et al., 1989). The constitutive production of ethylene in *Arabidopsis* *eto1-1* plants makes this mutant a natural high-level ethylene-producing system. The possibility of using hormone biosynthesis and action inhibitors (Figure 3) makes this a unique system to analyze the role of ethylene in vivo. Multiple mechanisms for ethylene-induced gene expression have been found; specific genes display unique dose-response curves in response to exogenous ethylene (Lincoln and Fischer, 1988). The effects of endogenous and exogenous hormone levels in hormone-inducible processes can be compared in *eto1*, *hls1*, *ein1*, and *ein2* mutants. In addition, the constitutive ethylene response of etiolated *eto1-1* seedlings can be used as a rapid and efficient assay to identify specific inhibitors of ethylene action and ethylene biosynthesis. We have found that *trans*-cyclooctene, a recently reported ethylene antagonist (Sisler et al., 1990), effectively inhibited ethylene action in etiolated *eto1-1* seedlings (Table 3). This in vivo effect was likely due to the more specific action of this cyclic olefin compared with the previously reported olefins. Thus, binding of ethylene and ethylene antagonists to the ethylene receptor(s) can be investigated in wild-type and mutant seedlings.

Plant responses to hormones are often unique depending on the system, the stage of development, or the specific tissue involved. Components and regulatory mechanisms of hormone production and mode of action are very likely to be shared by the different systems. As a

strategy to understand the general regulatory principles that control hormone biosynthesis and signal transduction pathways at the molecular level, it may be necessary to approach such complex phenomena in the same plant system. *Arabidopsis* has received much attention as a system to study biological phenomena using a molecular genetic approach (Estelle and Somerville, 1986; Meyerowitz, 1989). The easily scored triple response phenotype of an ethylene-treated *Arabidopsis* seedling provides a powerful screen to identify ethylene-related mutants. Alterations in these mutants are also observed during adult growth, indicating that the effect of the mutations is not seedling specific (Table 4). Currently, tools for gene isolation in *Arabidopsis* are being developed (Feldmann and Marks, 1987; Van Sluys et al., 1987; Chang et al., 1988; Koncz et al., 1989; Nam et al., 1989), and efficient transformation procedures have been achieved (Valvekens et al., 1988; Damm and Willmitzer, 1989). Molecular cloning of genes, such as *ETO1*, *HLS1*, *EIN1*, and *EIN2*, that have been identified by mutational analysis may be possible by chromosome walking from a closely linked RFLP (Chang et al., 1988; Nam et al., 1989). This process may be facilitated by chromosome-walking strategies that utilize yeast artificial chromosome libraries (Burke et al., 1987; Guzmán and Ecker, 1988).

METHODS

Arabidopsis Strains and Growth Conditions

Arabidopsis thaliana ecotype Columbia was the parental strain for the isolation of triple response mutants. Ecotypes Lansberg and Niederzenz were used for mapping purposes. (Seeds were obtained from Dr. Nigel Crawford, University of California, San Diego.) Etiolated *Arabidopsis* seedlings were grown as follows: seeds were surface sterilized for 8 min in 5% sodium hypochlorite (Clorox) and plated in Petri plates containing growth medium consisting of Murashige and Skoog salts (MS, GIBCO) (pH 5.7) supplemented with 10 g/L sucrose, 1 mg/L thiamine HCl, 0.5 mg/L pyridoxine, 0.5 mg/L nicotinic acid, 100 mg/L inositol, and 0.8% agar (bacto-agar, Difco). Seeds were planted with top agarose consisting of 0.6% low-melting-point agarose in MS salts. After cold treatment at 4°C for 4 days, the plates were incubated in the dark at 23°C for 66 hr to 72 hr. Plants were grown to maturity in a growth chamber at 22°C to 25°C under continuous illumination with fluorescent and incandescent light. Seeds were planted in Metro-Mix 200 (Grace), and modified Hoagland's nutrient media (Feldmann and Marks, 1987) was subirrigated every 3 days.

Induction and Isolation of Mutants

Stocks of mutagenized seeds were obtained by chemical mutagenesis with ethyl methanesulfonate (EMS). *A. thaliana* (Columbia) seeds were hydrated in distilled water overnight at 4°C and then treated with 0.4% EMS (Sigma) buffered in 100 mM sodium phosphate (pH 7.0) for 8 hr. Lots of 1500 to 2000 M1 seeds were planted separately to obtain independent populations of mutagenized M2 generation seeds. Approximately 15,000 to 20,000 M2 seeds were screened per lot. M2 seeds were surface sterilized and plated with low-melting-point agarose in Petri plates containing growth media. After 4 days of incubation at 4°C, seeds were placed at 23°C in the dark for 66 hr to 72 hr. To avoid the possible effects of ethylene accumulation in Eto and Hls screens, M2 seeds were planted at low density, about 1000 seeds per Petri plate (150 × 15 mm). The Ein screen was performed by incubating seeds in the dark in a flow-through chamber where 10 μL/L ethylene (Airco) was continuously delivered; M2 seeds were planted at a density of 10,000 per Petri plate (150 × 15 mm).

After selection, the putative mutants were transferred to the soil and allowed to self-pollinate, and the progeny (M3 seeds) were retested for the mutant phenotype. For Eto, 64 seedlings were identified from eight lots of M2 seeds; out of 26 plants that survived, two showed the phenotype. For Hls, 27 seedlings were isolated from six lots of M2 seed; of eight plants that survived, two showed the mutant phenotype. For Ein, 25 seedlings that showed the mutant phenotype survived and six were further analyzed.

Genetic Analysis

Crosses were performed as follows: two or three unopened flowers from the main inflorescence were emasculated with the help of a pair of fine forceps under a dissecting microscope, and several anthers from the male donor were used to pollinate the recipient stigmas. The rest of the flowers from the inflorescence were removed. To obtain the chromosomal location of the *ein* mutations, W100 (ecotype Lansberg) (Koornneef et al., 1987) was used as a mapping strain. W100 has two visible markers on each of the five chromosomes; our W100 stock does not carry the *ms-1* (male sterile) marker. Pollen from the W100 tester strain was crossed to *ein1-1* and *ein2-1* plants. The Ein F2 plants were chosen and segregation of visible markers was determined. An average population of 49 plants was examined for each cross.

To obtain the progeny for RFLP analysis, *ein2* mutants (ecotype Columbia) were crossed to wild-type plants of the ecotype Niederzenz. The F1 plants were allowed to self-fertilize, and DNA from single F2 plants homozygous recessive for the *ein2* mutation was prepared. An average population of 64 plants was considered for each analysis. Recombination frequencies were calculated using the RECF2 program developed by M. Koornneef (Agricultural University, Wageningen). Procedures for plant DNA isolation (Deblaere et al., 1987), probe preparation (Blattner et al., 1978; Feinberg and Vogelstein, 1983), and blot hybridization (Maniatis et al., 1989) have been described.

Determination of Ethylene Accumulation

Before physiological examination the mutants were back-crossed to the wild type at least once. More than 95% germination was obtained from wild-type *eto1*, *hls1*, *ein1*, and *ein2* strains when they were cold-treated for 4 days before germination. Surface-sterilized seeds (approximately 500) were germinated and grown for 66 hr to 72 hr in the dark at 23°C in 20-mL gas chromatograph vials containing 15 mL of growth medium. To measure the con-

version of exogenous ACC to ethylene, seedlings were grown in 1% low-melting-point agarose buffered with 3 mM Mes at pH 5.8. In this solid support no chemical formation of ethylene from ACC was detected at any of the concentrations of ACC employed. Ethylene accumulation from tissues of mature plants (100 mg) was measured after overnight incubation in 20-mL gas chromatograph vials. Leaves and inflorescence were taken from 24-day-old to 28-day-old plants, siliques from 32-day-old to 36-day-old plants. Each value represents the mean obtained from three to six samples. Accumulation of ethylene was determined by gas chromatography using a photo-ionization detector (HNU) and a Hewlett Packard HP5890A gas chromatograph equipped with an automated headspace sampler. A certified standard of 10 μL/L ethylene (Airco) was used to calculate ethylene concentrations. The concentration of the inhibitors of ethylene biosynthesis and ethylene action was determined empirically. AVG, α-aminoisobutyric acid, and $AgNO_3$ were supplemented to the media at 5 μM, 2 mM, and 0.1 mM, respectively. *trans*-Cyclooctene (17 μL/L) was injected to the vial after the cold treatment.

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