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Factors regulating ethylene biosynthesis in etiolated *Arabidopsis thaliana* seedlings

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We examined the effect on etiolated *Arabidopsis thaliana* seedlings of a wide variety of factors that are known to induce ethylene in other plant tissues. Auxin, cytokinin, brassinosteroid and cupric ion were found to highly elevate ethylene production in these seedlings, but several other signaling compounds, as well as wounding and mechanical stimulation, had little or no effect. A mutant that disrupts the *ACS5* gene (*cin5*) was partially defective in the induction of ethylene in the presence of brassinosteroids, suggesting a role for this isoform in mediating this response. Cytokinin displayed a synergistic interaction with both brassinosteroid and auxin, while the other interactions tested were essentially additive. Auxin and cytokinin have been shown to act synergistically to elevate ethylene biosynthesis in many other plant tissues. We show that the synergism between cytokinin and auxin in *Arabidopsis* is due to an enhancement of the effects of auxin, but not by increased elevation of *ACS4* mRNA levels. These results suggest that cytokinin acts post-transcriptionally to increase *ACS4* function, which, coupled with the observation that auxin elevates *ACS4* mRNA levels, accounts for the synergistic interaction.

Introduction

The simple gas ethylene has been recognized as a plant hormone since the turn of the century. It has been shown to influence a diverse array of plant growth and developmental processes, including germination, leaf and flower senescence and abscission, fruit ripening and the response to a wide variety of stresses (Mattoo and Suttle 1991, Abeles et al. 1992). The ethylene biosynthetic pathway has been elucidated in a series of elegant studies by a number of laboratories (reviewed in: Yang and Hoffman 1984, Kende 1993) and the genes encoding the biosynthetic enzymes have been isolated. The first committed and generally the rate-limiting step in ethylene biosynthesis is the conversion of *S*-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase. In the species that have been previously examined, ACC synthase is encoded by a small gene family comprised of three to six members. Distinct subsets of ACC synthase genes are expressed in response to various developmental, environmental and hormonal factors (e.g., Olson et al. 1991, 1995, Rottmann et al. 1991, Botella et al. 1993, 1995, Schlagnhaufer et al. 1995).

Almost all plant tissues have the capacity to make ethylene, but ethylene production increases dramatically during a number of developmental events such as germination, leaf and flower senescence and abscission and fruit ripening (Yang and Hoffman 1984, Mattoo and Suttle 1991, Abeles et al. 1992). A diverse group of inducers increase ethylene biosynthesis, and a major effect of most of these factors is to increase the steady-state level of ACC synthase mRNA, though in some cases significant post-translational control has been demonstrated (Nakajima et al. 1990b, Spanu et al. 1994, Oetiker et al. 1997, Vogel et al. 1998b). These inducers include a wide variety of stresses including wounding, flooding, temperature shifts, physical loads and heavy metals such as lithium and copper, as well as light and various other plant signaling molecules including auxin, cytokinins, jasmonic acid, brassinosteroids and ethylene itself (reviewed in: Yang and Hoffman 1984, Mattoo and Suttle 1991). The expression of *ACS2* has been examined using a fusion of the presumed regulatory sequences to a GUS reporter gene (Rodrigues-Pousada et al. 1993). *ACS2*

Abbreviations – ACC: 1-aminocyclopropane-1-carboxylic acid; BA: benzyladenine; 2,4-D: 2,4-dichlorophenoxyacetic acid.
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expression is higher in young, developing leaves and flowers as compared to more mature tissues from these organs, and is also correlated with the initial stages of lateral root formation.

In *Arabidopsis*, six ACC synthase genes have been identified (*ACS1-6*), at least four of which are expressed and encode active ACC synthase enzymes (Liang et al. 1992, Van der Straeten et al. 1992, Woeste et al. in press). Wounding, auxin, cycloheximide, LiCl and anaerobiosis differentially elevate the steady-state level of these *Arabidopsis* genes (Liang et al. 1992, 1996). Auxin specifically induces transcription of *ACS4*, and several auxin-response elements (AuxREs) have been identified upstream of the *ACS4* coding region (Abel et al. 1995). The steady-state level of *ACS5* mRNA is elevated by treatment with lithium ion (Liang et al. 1996). *ACS5* is also involved in the response of etiolated seedlings to low doses of cytokinin by a post-transcriptional mechanism (Vogel et al. 1998b). The *ACS3* gene is most likely a pseudogene and *ACS1* encodes a non-functional ACC synthase (Liang et al. 1995). The steady-state level of mRNA in all four active *ACS* genes is increased by treatment with the protein synthesis inhibitor cycloheximide, suggesting that they are under the control of a short-lived negative regulator (Liang et al. 1992, Woeste et al. in press).

The final step of ethylene biosynthesis, the conversion of ACC to ethylene, is catalyzed by the enzyme ACC oxidase, which may also play an important role in regulating ethylene biosynthesis, especially during conditions of high ethylene production (Nadeau et al. 1993, Kim and Yang 1994, Tang et al. 1994, Barry et al. 1996, Lasserre et al. 1996, Mekhedov and Kende 1996). ACC oxidases, like ACC synthases, are generally encoded by a gene family whose members are differentially regulated in response to environmental and developmental cues. In *Arabidopsis*, at least one member of the *ACO* gene family is induced by ethylene itself (Gomez-Lim et al. 1993).

We have chosen 3-day-old etiolated *Arabidopsis* seedlings as a model system to begin to decipher the circuitry regulating ethylene biosynthesis. As a first step toward this goal, we examined a number of factors that have been demonstrated to influence ethylene biosynthesis in other plant species. Here we present the characterization of the effect of these factors on ethylene production in etiolated *Arabidopsis* seedlings. This work, together with a variety of isolated mutants that affect ethylene biosynthesis in etiolated *Arabidopsis* seedlings (Guzman and Ecker 1990, Kieber et al. 1993, Vogel et al. 1998a), provides a foundation to understand the various pathways that regulate ethylene biosynthesis, and how they are integrated in this model system.

Materials and methods

Plant lines and growth conditions

The Wassilewskija (WS) ecotype of *Arabidopsis* and the *cin5-1* allele were used in this study. Seeds were surface sterilized as described in Vogel et al. (1998b), resuspended in a suitable volume of top agar (0.8%, w/v, low-melt agarose) and spread onto MS agar (MS salts, [Gibco, Grand Island, NY, USA], 2%, w/v, sucrose and 0.8%, w/v, agar at pH 5.7). Seeds were cold-treated for 4 days (4°C), exposed to light for 2 h and then moved to a dark incubator at 23°C. Adult plants were grown in Metro mix 250 (Grace, Boca Raton, FL, USA) under continuous illumination at 23°C.

Seedlings treatments and ethylene measurement

Hormones and other potential inducers were added in a volume of 200 µl to 2-day-old etiolated seedlings that were grown on 3 ml MS agar in 22-ml GC vials (23°C). An equal volume of solvent was added to the control treatments. These vials were then flushed with hydrocarbon-free air, capped for 24 h and the accumulated ethylene measured as described in Vogel et al. (1998b). In one case (the kinetin dose response), kinetin was dissolved in the medium onto which the seeds were sown and the vials capped when they were moved to 23°C. The accumulated ethylene was measured 72 h later. Note that the concentrations of cytokinin in this experiment differ from those in which the cytokinin was added as a supplement, as in the latter case the hormone diffuses into the 3 ml of MS agar medium. To measure the effect of wounding on ethylene biosynthesis, 6-day-old etiolated seedlings were sliced with a razor blade at 1 mm intervals (tissues were not sliced all the way through), placed in vials containing MS agar and capped for 24 h. For thigmostimulation, seedlings were germinated on sterile filter paper disks placed on top of 3 ml MS agar (Table 1), so that they did not get pushed into the agar, or they were grown in sand saturated with liquid MS (not shown). A sterile foam impediment was placed at various distances above the medium in the GC vials such that the seedlings would first make contact with the block at approximately 60 h or just after germination; the accumulated ethylene was measured at 84 h. In all cases, ethylene production was normalized to the number of seedlings and the time between capping and sampling. All observations are from at least three replicates and each experiment was repeated at least once with comparable results.

Table 1. Ethylene production by etiolated seedlings in response to wounding and thigmostimulation. Seedlings were grown and treated as described in Materials and methods. For thigmostimulation, the vials were capped upon transfer to 23°C and grown for 3 days. The seedlings reached 5 mm at approximately 60 h and never reached the foam in the control group. For wounding, 5-day-old etiolated seedlings were wounded and then the vials capped and incubated for an additional 24 h at 23°C. Values are the mean ± the SD from three replicates. *P* values were calculated using a Student's t-test.

Treatment	Ethylene produced
	$pl \cdot seedlings^{-1} \cdot 3\ day^{-1}$
Control	28 ± 0.6
Foam at 1 mm	34 ± 5
Foam at 5 mm	34 ± 3
	$pl \cdot seedlings^{-1} \cdot 24\ h^{-1}$
Unwounded	22 ± 4
Wounded	45 ± 3
Statistical differences	
Thigmostimulation	$P > 0.05$
Wounding	$P \leq 0.01$

Table 2. Effect of cytokinin and auxin on ethylene biosynthesis in wild-type and *cin5* mutant etiolated seedlings. 200 µl of solution containg the indicated coincentration of BA, kinetin and/or 2,4-D was added to 2-day-old etiolated seedlings. The tubes were capped and the accumulated ethylene measured after 24 additional h of growth in the dark. Values represent the mean ± the SD of three replicates.

[2,4-D] (µ*M*)	Line	Ethylene ($pl \cdot seedling^{-1} \cdot h^{-1}$)					
		Kinetin			BA		
		0 µ*M*	5 µ*M*	50 µ*M*	0 µ*M*	5 µ*M*	50 µ*M*
0	WS	0.19 ± 0.05	0.6 ± 0.1	1.3 ± 0.1	0.28 ± 0.06	1.5 ± 0.2	1.8 ± 0.7
0	*cin5*	0.14 ± 0.01	0.4 ± 0.1	0.34 ± 0.05	0.22 ± 0.08	0.6 ± 0.1	0.7 ± 0.2
5	WS	0.9 ± 0.2	1.5 ± 0.1	3.9 ± 0.4	1.1 ± 0.2	2.9 ± 0.3	3.4 ± 0.5
5	*cin5*	1.0 ± 0.2	1.5 ± 0.1	2.2 ± 0.6	2.1 ± 0.6	3.4 ± 1.1	3.7 ± 1.1
50	WS	2.9 ± 0.3	5.2 ± 0.4	9.1 ± 1.7	2.0 ± 0.2	6.4 ± 1.9	6.1 ± 0.8
50	*cin5*	3.6 ± 0.4	5.8 ± 1.4	9.1 ± 1.0	3.2 ± 0.2	7.4 ± 1.6	9.1 ± 1.2

Northern analysis

Seedlings (about 5000 per plate) were grown on sterile filter paper placed on top of a 150 mm petri plate containing MS agar. Hormones were added after 48 h of growth in a total volume of 15 ml, and the seedlings harvested 12 h later. Total RNA was prepared as previously described (Ausubel et al. 1994) and polyA$^+$RNA isolated using Oligotex-dT columns as described by the manufacturer (Qiagen, Chatsworth, CA, USA). Five µg of polyA$^+$mRNA was separated on an agarose gel, blotted to a Nylon membrane and hybridized to radiolabeled probes (as described in Ausubel et al. 1994). The *ACS4* and *β-tubulin* fragments were obtained by amplifying the respective genes from *Arabidopsis* genomic DNA using the polymerase chain reaction with gene-specific oligonucleotide primers derived from the published sequences. The signals were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) and normalized to the level of the *β-tubulin* control.

Results

Effect of various compounds on ethylene biosynthesis in etiolated *Arabidopsis* seedlings

We examined the effect of a large number of factors, all of which have been demonstrated to influence ethylene biosynthesis in other plant tissues, on the level of ethylene production from etiolated *Arabidopsis* seedlings. A number of these compounds had little detectable effect on the basal level of ethylene produced from etiolated seedlings, including ABA, GA, salicylic acid and jasmonic acid (not shown). Surprisingly, in contrast to other plants, mechanical impedance had no significant effect on ethylene biosynthesis in etiolated *Arabidopsis* seedlings (Table 1). Wounding also had only a minor effect (about 2-fold), much less than in adult *Arabidopsis* leaves (Vogel et al. 1998a). Of the compounds that we tested, the strongest inducers were the cytokinins BA and kinetin, the auxin 2,4-D, the brassinosteroid 24-epibrasinolide and $CuSO_4$. We performed a dose response for these compounds by either growing seedlings in their presence (kinetin) or by adding as a 200 µl supplement (onto 3 ml MS agar; see Materials and methods) after 48 h (Fig. 1). Growth in the presence of varying concentrations of kinetin revealed a biphasic dose response, as had been previously noted (Vogel et al. 1998a,b). 2,4-D and $CuSO_4$ displayed monophasic responses, with a half maximal induction at approximately 50 µ*M* and 1 m*M*, respectively. 24-epibrassinolide displayed a peak induction at 0.5 µ*M*, with a decline at higher concentrations.

We examined the induction of ethylene biosynthesis in response to brassinosteroids in *cin5* mutant seedlings in order to assess the role of the ACS5 isoform. *cin5* is a loss-of-function mutation in the *ACS5* member of the ACC synthase gene family, which has been shown to disrupt ethylene biosynthesis in response to low doses of cytokinin (Vogel et al. 1998b). This mutation had no effect on the induction of ethylene by 2,4-D (Vogel et al. 1998b), consistent with previous reports that auxin acts by increasing the steady-state level of the *ACS4* gene specifically (Abel et al. 1995). However, the induction of ethylene by 24-epibrassinolide was significantly reduced in *cin5* seedlings (7.5 ± 2.9 $pl \cdot seedling^{-1} \cdot h^{-1}$ for wild-type verses 3.7 ± 2.9 $pl \cdot seedling^{-1} \cdot h^{-1}$ for *cin5* mutant, 3-day-old etiolated seedlings treated with 5 µ*M* 24-epibrassinolide), suggesting a role for this isoform, though this does not preclude the involvement of other *ACS* genes as well.

Interactions of inducers

To begin to understand how these factors interact to regulate ethylene biosynthesis, we examined the effect of simultaneous addition of two inducers. As was previously observed in several plant tissues (Burg and Burg 1968, Imaseki et al. 1975, Kondo et al. 1975, Lau and Yang, 1976, Lau et al. 1977, Yip and Yang 1986), cytokinin (both BA and kinetin) and 2,4-D were synergistic in their effect on ethylene biosynthesis in etiolated *Arabidopsis* seedlings (Table 2). That is, the level of ethylene produced in response to the two compounds was greater than the sum of the two individual effects. In fact, the level of ethylene produced was close to that expected from the multiplication of each individual fold-induction. 24-epibrassinolide displayed a similar synergistic interaction with BA (Table 3). In contrast, $CuSO_4$ showed an approximately additive interaction with both BA and 2,4-D, as did 2,4-D and 24-epibrassinolide (Table 4).

The synergism of cytokinin and auxin has been hypothesized to be due to an enhancement of auxin action (Lau and Yang 1973). To test this model, we examined a mutant defective in cytokinin-induced ethylene biosynthesis, *cin5*.

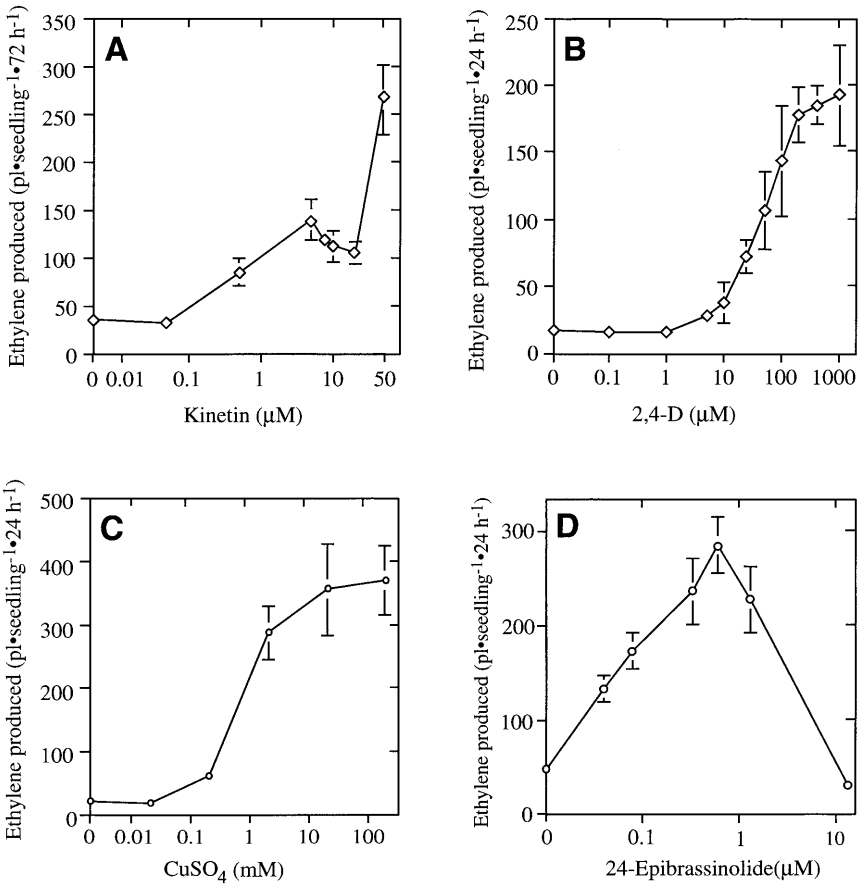

Fig. 1. Dose response of various ethylene inducers. (A) Response to the cytokinin kinetin. Seedlings were grown on MS agar containing the indicated level of kinetin for 3 days in the dark at 23°C and the accumulated ethylene measured as described in the Materials and methods section. For (B), (C) and (D), 48-h-old etiolated seedlings were treated with the indicated concentration of 2,4-D (B), $CuSO_4$ (C) or 24-epibrasinolide (D) for 24 h and the accumulated ethylene measured.

Cytokinin has been demonstrated to elevate ethylene biosynthesis by increasing mainly ACS5 function via a post-transcriptional mechanism (Vogel et al. 1998b) and auxin has been shown to act primarily through increased transcription of the *ACS4* gene (Abel et al. 1995). Thus, if cytokinin acts to enhance the effect of auxin, the *cin5* mutation should not disrupt the synergism between the two. Conversely, if auxin increases the potency of cytokinin, the synergism should be greatly diminished in the *cin5* mutant. As shown in Table 2, the *cin5* mutation did not affect the synergism of 2,4-D with either of the cytokinins BA or kinetin, although it significantly diminished the response to cytokinin in the absence of auxin. This is consistent with the notion that cytokinins potentiate the ability of auxin to elevate ethylene biosynthesis.

To determine if cytokinin acts by enhancing the sensitivity or persistence of the auxin signal, or alternatively if it acts through a distinct mechanism, we determined if auxin and cytokinin together superinduce the steady-state level of *ACS4* mRNA. Northern blot analysis (Fig. 2) revealed that *ACS4* mRNA levels were not higher in seedlings treated with 2,4-D and BA simultaneously as compared to only 2,4-D. Furthermore, cytokinin does not affect the steady-state level of *ACS5* under these conditions (Fig. 2). This suggests that cytokinin does not simply increase the perception of auxin, but rather may affect an interdependent process, such as the post-transcriptional regulation of *ACS4*, in a manner similar to the effect of cytokinin on ACS5.

Discussion

As a complement to genetic studies that have identified genes involved in regulating ethylene biosynthesis in etiolated *Arabidopsis* seedlings (Guzman and Ecker 1990, Kieber et al. 1993, Vogel et al. 1998a,b), we determined the level of ethylene produced by such seedlings in response to a variety of factors in order to identify the various regulatory inputs. We found that application of the plant hormones cytokinin, auxin and brassinosteroid had large effects on ethylene production by etiolated seedlings, as did cupric ion. These compounds have been shown to also affect the level of ethylene biosynthesis in other plant species and tissues (Yang and Hoffman 1984, Arteca 1990, Abeles et al.

Table 3. Interactions with brassinosteroid, cytokinin and auxin on ethylene biosynthesis in etiolated seedlings. 200 μl of solution containing the indicated concentration of 24-epibrassinolide (Brass.) and BA or 2,4-D was added to 2-day-old etiolated seedlings. The tubes were capped and the accumulated ethylene measured after 24 additional h of growth in the dark. Values represent the mean ± the SD of three replicates.

Compound	Ethylene ($pl \cdot seedling^{-1} \cdot h^{-1}$)	
	0 μ*M* Brass.	5 μ*M* Brass.
Control	0.3 ± 0.03	7.5 ± 2.9
BA, 10 μ*M*	1.5 ± 0.2	17.9 ± 6.7
BA, 75 μ*M*	1.8 ± 0.7	23.4 ± 12.8
2,4-D, 10 μ*M*	1.1 ± 0.2	12.8 ± 2.2
2,4-D, 50 μ*M*	2.0 ± 0.2	9.9 ± 1.7

Table 4. Interactions of various inducers of ethylene biosynthesis. 200 µl of solution containing the indicated concentration of BA, 2,4-D and/or $CuSO_4$ was added to 2-day-old etiolated seedlings. The tubes were capped and the accumulated ethylene measured after 24 additional h of growth in the dark. Values represent the mean ± the SD of the three replicates.

Compound	Ethylene ($pl \cdot seedling^{-1} \cdot h^{-1}$)		
	0 m*M* $CuSO_4$	2 m*M* $CuSO_4$	20 m*M* $CuSO_4$
BA, 0 µ*M*	0.35 ± 0.02	4.8 ± 0.9	3.9 ± 1.1
BA, 5 µ*M*	1.2 ± 0.3	4.8 ± 0.5	4.7 ± 0.9
BA, 50 µ*M*	1.5 ± 0.3	5.4 ± 0.4	6.1 ± 2.9
2,4-D, 0 µ*M*	0.32 ± 0.03	4.3 ± 0.4	4.7 ± 1.0
2,4-D, 5 µ*M*	0.84 ± 0.27	4.5 ± 0.8	5.8 ± 2.0
2,4-D, 50 µ*M*	4.2 ± 0.6	6.4 ± 1.0	6.6 ± 0.7

1992). In particular, auxin has been extensively studied and in many cases has been linked to an increase in the steady-state level of a subset of *ACS* genes. The effect of these hormones on ethylene biosynthesis in etiolated *Arabidopsis* seedlings indicates that there is cross-talk between ethylene and these hormonal signals, and in some cases this can be linked to physiological responses. For example, ethylene, auxin and perhaps cytokinin interact to direct the differential cell expansion leading to the curvature of the apical hook (e.g. see: Schwark and Schierle 1992, Lehman et al. 1996). Brassinosteroids, like ethylene and auxin, are involved in the regulation of cell expansion and also affect the de-etiolation response (Sasse 1997, Yokota 1997) which includes opening of the apical hook (Chory et al. 1994, Li et al. 1996), a process that most likely involves changes in the level of ethylene biosynthesis (Goeschl et al. 1967).

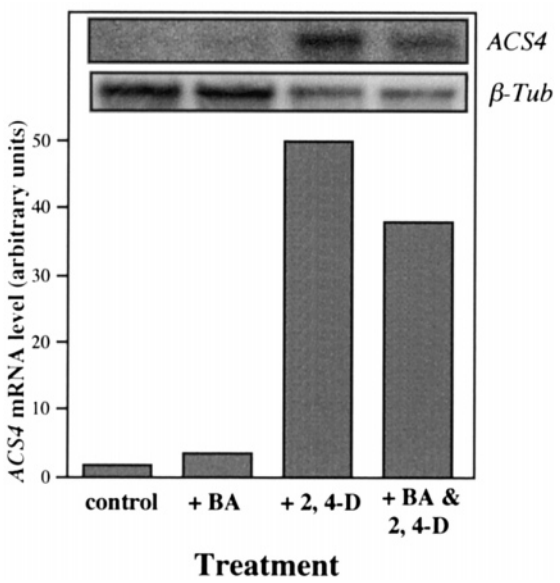

Fig. 2. Analysis of *ACS4* mRNA levels from etiolated seedlings treated with auxin and/or cytokinin. Etiolated seedlings, 48-h-old, were treated with no hormone (control), 50 µ*M* BA, 50 µ*M* 2,4-D or 50 µ*M* BA and 50 µ*M* 2,4-D, as indicated for 12 h. PolyA$^+$RNA was prepared and analyzed by northern blotting as described in Materials and methods. The level of *ACS4* signal was normalized to that of the *β-tubulin* loading control, and the level plotted using arbitrary units.

Cupric ion most likely acts by inducing oxidative stress, leading to an induction of ethylene (Mattoo et al. 1992, Lidon et al. 1995). Ozone, another inducer of oxidative stress, also induces ethylene in many plant tissues, and in at least one case has been linked to increased *ACS* gene expression (Schlagnhaufer et al. 1995). Treatment of *Arabidopsis* seedlings with aminoethoxyvinylglycine, a potent inhibitor of ACC synthase, eliminates the induction of ethylene by copper (not shown), indicating that copper-induced ethylene production is via the standard ACC pathway rather than by oxidation of lipids (Mattoo et al. 1985).

Surprisingly, wounding had only very minor effects on the level of ethylene biosynthesis by etiolated *Arabidopsis* seedlings. In other plant tissues, the level of ACC synthase mRNA has been shown to increase in response to wounding, suggesting that the regulation of wound-induced ethylene biosynthesis may lie at the level of transcription (Nakajima et al. 1990a, Olson et al. 1991, Lincoln et al. 1993). To verify this result, we tried wounding seedlings several different ways and never observed more than a 2-fold increase in ethylene production. Indeed, harsher treatments, such as crushing, often led to less ethylene production than unwounded controls, presumably due to extensive destruction of the tissue (not shown). Adult *Arabidopsis* leaves display a strong response to wounding (Vogel et al. 1998a). The different response of seedlings and adult plants to wounding may indicate that seedlings regulate ethylene biosynthesis differently than adults. This is supported by analysis of the Eto mutants. The *eto1*, *eto2* and *eto3* mutations result in a large increase in the amount of ethylene produced by etiolated seedlings, but have little or no effect on light-grown seedlings or any adult tissue (Guzman and Ecker 1990, Vogel et al. 1998b, Woeste et al. in press). Alternatively, the lack of a strong wounding response in etiolated *Arabidopsis* seedlings indicates that etiolated plants may regulate ethylene biosynthesis differently than light-grown plants.

Mechanical stimulation strongly induces ethylene biosynthesis in etiolated pea seedlings, and this touch-induced ethylene is thought to be the relevant stimulus leading to the triple response, which then allows the seedlings to germinate around obstacles in the soil (Goeschl et al. 1966). Consistent with this, ethylene-insensitive mutants of *Arabidopsis* fail to germinate through compacted sand (Harpham et al. 1991). Thus, it is somewhat surprising that mechanical stimulation has little or no effect on the amount of ethylene produced by etiolated *Arabidopsis* seedlings. It is possible that etiolated *Arabidopsis* seedlings are very sensitive to even small changes in the concentration of endogenous ethylene. Consistent with this, increasing the basal level of ethylene only 2-fold by treatment with 0.5 µ*M* BA results in seedlings that adopt a moderately strong triple response phenotype (Vogel et al. 1998b). This high sensitivity to endogenous ethylene, perhaps coupled with the increased concentration of ethylene that would result from restricted airflow within the soil, may account for the link between the triple response and mechanical stimulation during germination. Alternatively, it is possible that that the touch stimulus was not presented in the correct manner in our experiments, or growth on MS media may preclude the thigmostimulation

of ethylene. It is also possible that additional factors in the soil play a role in elevating ethylene biosynthesis.

We examined the effect of the simultaneous addition of two compounds to begin to determine how the pathways that regulate ethylene biosynthesis interact. In a number of cases the level of ethylene produced by the simultaneous addition of two inducers was close to the summation of each individual effect (e.g. auxin and brassinosteroids; auxin and $CuSO_4$). This suggests that these factors may increase ethylene biosynthesis by parallel pathways, possibly through distinct ACS isoforms. Previous workers have found that auxin and brassinosteroid act synergistically to enhance ethylene biosynthesis in mung bean hypocotyls (Arteca et al. 1988). The difference between these results and those presented here may be explained by the different plant species used in each case. In some cases, the amount of ethylene produced by the simultaneous addition of two inducers was greater than what would be expected from an additive interaction. This synergism has been previously noted in other plant species treated with auxin and cytokinin. This suggests that these hormones are acting through an interdependent mechanism to elevate ethylene production. It has been proposed that cytokinin increases the half-life of auxin in plants (Lau and Yang 1973). This predicts that cytokinin should increase the effect of auxin on *ACS4* transcription. However, we found no superinduction of *ACS4* when cytokinin was present in addition to auxin, which suggests that this model is incorrect, at least in etiolated *Arabidopsis* seedlings. Because cytokinin has been shown to act post-transcriptionally, we propose a distinct model to account for the observed synergism: cytokinin acts post-transcriptionally to increase the function of the auxin-elevated *ACS4* mRNA. This predicts that the level of ethylene produced in the presence of these two inducers should be close to the multiplication of the fold-induction produced by each inducer, which is similar to what is observed. The nature of the modification effected by cytokinin is unknown, but it is thought to be targeted to the variable carboxy-terminus in ACS5 (Vogel et al. 1998b). One strong candidate is protein phosphorylation, which has been linked to the regulation of soybean ACC synthase in response to elicitor (Spanu et al. 1994). Brassinosteroid and cytokinin are also strongly synergistic, which may be explained by a similar model if brassinosteroid acts by elevating the transcription of an ACS gene(s) that is post-transcriptionally modified by cytokinin.

These studies have identified a number of regulatory inputs into the ethylene biosynthetic pathway in etiolated *Arabidopsis* seedlings. The action of these compounds in *Arabidopsis* mutants affected in the regulation of ethylene biosynthesis should be informative with respect to how these signaling pathways interact, and should begin to provide further glimpses into the molecular circuitry regulating ethylene biosynthesis.

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