Plant Physiol. (1995) 107: 1075–1082

Cytokinin Action Is Coupled to Ethylene in Its Effects on the Inhibition of Root and Hypocotyl Elongation in *Arabidopsis thaliana* Seedlings[1]

Andrew J. Cary, Wennuan Liu, and Stephen H. Howell*

Boyce Thompson Institute, Cornell University, Ithaca, New York 14853

Cytokinins have profound effects on seedling development in *Arabidopsis thaliana*. Benzyladenine (BA) inhibits root elongation in light- or dark-grown seedlings, and in dark-grown seedlings BA inhibits hypocotyl elongation and exaggerates the curvature of apical hooks. The latter are characteristic ethylene responses and, therefore, the possible involvement of ethylene in BA responses was examined in seedlings. It was found that the inhibitory effects of BA on root and hypocotyl elongation were partially blocked by the action of ethylene inhibitors or ethylene-resistant mutations (*ein1–1* and *ein2–1*). Ethylene production was stimulated by submicromolar concentrations of BA and could account, in part, for the inhibition of root and hypocotyl elongation. It was demonstrated further that BA did not affect the sensitivity of seedlings to ethylene. Thus, the effect of cytokinin on root and hypocotyl elongation in *Arabidopsis* appears to be mediated largely by the production of ethylene. The coupling between cytokinin and ethylene responses is further supported by the discovery that the cytokinin-resistant mutant *ckr1* is resistant to ethylene and is allelic to the ethylene-resistant mutant *ein2*.

The responses of seedlings to developmental and environmental cues have been useful in studying signal transduction pathways in plants. *Arabidopsis thaliana* seedlings respond to exogenously added or endogenous cytokinins, and in light-grown seedlings the application of exogenous cytokinins inhibits primary root growth. On the basis of the root inhibition response, Su and Howell (1992) selected *ckr1* mutants in *Arabidopsis* that were resistant to cytokinin. *Arabidopsis* mutants that overproduce endogenous cytokinins, called *amp1* mutants, have also been reported (Chaudhury et al., 1993). The *amp1* mutants have multiple cotyledons and produce more leaves.

Cytokinin produces certain effects in dark-grown *Arabidopsis* seedlings that are similar to those caused by ethylene (Lieberman, 1979) and light (Chory et al., 1994). Ethylene produces a characteristic "triple response" in seedlings that includes inhibition of hypocotyl growth and expansion of the hypocotyl base (Crocker et al., 1913). In addition, ethylene produces an exaggeration in the curvature of the apical hook and inhibits primary root elongation (Guzmán and Ecker, 1990). Some of these features have been utilized in the isolation of mutants with altered responses to ethylene (Rothenberg and Ecker, 1993). Ethylene-resistant mutants (*ein* or *etr* mutants) were identified by selecting seedlings that fail to express the triple response in the presence of ethylene. Other mutants were identified that produced a triple-response phenotype constitutively, i.e. when no ethylene was applied. These mutants have been grouped into two classes, ethylene overproducers (*eto*) and constitutive triple-response mutants (*ctr*), distinguished on the basis of whether the phenotype is suppressed by inhibitors of ethylene biosynthesis or action (Guzmán and Ecker, 1990). Through epistasis analysis of the mutants, ethylene-response genes have been ordered on an unbranched pathway (Kieber and Ecker, 1993; Kieber et al., 1993). Interestingly, *ein* or *etr* mutants appear to have very little effect on the normal growth and development of light-grown *Arabidopsis* seedlings in the absence of supplied ethylene.

We have examined the relationship between cytokinin and ethylene responses in *Arabidopsis* seedlings and have found that ethylene largely mediates a number of responses to exogenous cytokinin, such as the inhibition of root and hypocotyl elongation. Cytokinin stimulates ethylene production, which, in turn, inhibits root and dark-grown hypocotyl elongation. Because of that, the effects of cytokinin on root and hypocotyl elongation can be blocked, in part, by the action of ethylene inhibitors or by ethylene mutants. These results constitute genetic proof of the idea put forth by Lieberman (1979) that cytokinin is coupled to ethylene action in seedlings.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All plants, including mutant lines, were of the *Arabidopsis thaliana* Columbia (Col-0) ecotype. *ckr1* mutants were described by Su and Howell (1992). The *ckr1*-109 line used in this study was recovered from two back-crosses of the mutant to *wt* plants. Ethylene-resistant mutants (*ein1–1* and *ein2–1*) were obtained from the Arabidopsis Biological Resource Center at The Ohio State University. Prior to sowing, seeds were surface sterilized for 7 min in 70% ethanol

[1] Support was provided by the U.S. Department of Agriculture National Research Initiative Competitive Grants Program (91–37301–6420).

* Corresponding author; e-mail shh5@cornell.edu; fax 1–607–254–1242.

Abbreviations: AVG, aminoethoxyvinylglycine; CAPS, cleaved amplified polymorphic sequences; RFLP, restriction fragment length polymorphism; *wt*, wild type.

and for 15 min in 30% (v/v) bleach and 0.01% Triton X-100 with periodic agitation, and then rinsed three times with sterile, deionized water. Seedlings were grown in sterile conditions on mineral nutrient medium (Lincoln et al., 1990), with cytokinin (BA), ACC, AVG, or $AgNO_3$ added to the medium after autoclaving. After sowing, seeds were stratified in the dark for 3 d at 4°C and then exposed to white light (30 μE m^{-2} s^{-1}) for 2 h. Although *wt* seeds of Columbia ecotype do not require light for germination, seeds were exposed to light to ensure germination of mutants and to increase synchrony of germination. All seeds, except those used to determine ethylene evolution, were sown on media in 100 × 20 mm Petri dishes at a density of about 100 seeds per plate. Four days after germination, morphological measurements were made on at least 20 seedlings for each treatment, except where noted.

Ethylene Application and Measurement

For treatment with exogenous ethylene, seedlings in Petri dishes were placed in 2.5-L flow-through aeration chambers. Air was scrubbed of ethylene by passage through Purafil (Purafil Inc., Atlanta, GA) and pumped to a four-outlet manifold (Lee and LaRue, 1992). Ethylene was then added through micro valves (Nupro Co., Willoughby, OH) to three outlets to give concentrations ranging from 0.05 to 1.0 μL L^{-1}. Seedlings received air at a flow rate of 0.6 L min^{-1} through the sample chambers, and ethylene levels were monitored daily. Ethylene was measured using a Varian (Sunnyvale, CA) model 3700 gas chromatograph fitted with a Porapak Q column and a flame ionization detector.

Ethylene Evolution

To measure ethylene production, 500 *Arabidopsis* seeds were sown on the surface of agar in a 5.9-mL (1 dram) shell vial. Each vial contained 1.8 mL of agar medium that was allowed to solidify at about a 45° slant to increase the surface area. At that density the seeds spread into a monolayer on the surface of the medium. The seeds were germinated in the dark as described above. Three days after germination the vials were sealed with a septum, and ethylene was collected for the next 24 h. A 1-mL air sample was removed from each vial for measurement. Ethylene values represent the average of three separate samples and experiments were repeated at least three times.

CKR1 Gene Mapping

DNA samples were extracted as described by Bernatzky and Tanksley (1986) from 90 F_2 individuals or F_3 families of BA-resistant lines from Su and Howell (1992). The chromosomal location of the *CKR1* gene was determined by measuring the recombination frequency between the mutation and CAPS that mark different *Arabidopsis* chromosomes (Konieczny and Ausubel, 1993). The *CKR1* gene was located at the top of chromosome 5, and the map position was further refined by scoring the recombination frequency relative to nearby markers on chromosome 5, RFLP marker AR119 (Rose et al., 1992), and a CAPS marker generated from the published sequence of CTR1 (Kieber et al., 1993). The CTR1 CAPS marker was amplified with oligonucleotide primers, 5′-CGATCCTGCATCAGGTATTCC-3′ and 5′-CTTACTTAGACGCGAAGGC-3′, and cleaved with *BclI*.

RESULTS

Cytokinin and the Triple Response

Arabidopsis seedlings are very sensitive to applied cytokinin. Previously, it was shown that root elongation in light-grown seedlings was inhibited by submicromolar to micromolar levels of BA (Su and Howell, 1992). However, when seedlings were grown in the dark, BA produced other effects more characteristic of the triple response produced by ethylene. In the dark, BA inhibited both the growth of the roots, as it did in the light, and the growth of hypocotyls (Fig. 1A). In addition, BA accentuated the curvature of apical hooks (Fig. 1C) in a manner similar to that seen when seedlings were grown in the presence of exogenous ethylene (Fig. 1D).

Ethylene-Resistant Mutants Are Resistant to Cytokinin

Because BA-treated seedlings show triple-response characteristics, we tested the idea that certain BA effects are mediated by ethylene. As a first test we examined the effects of BA on the growth of two well-characterized

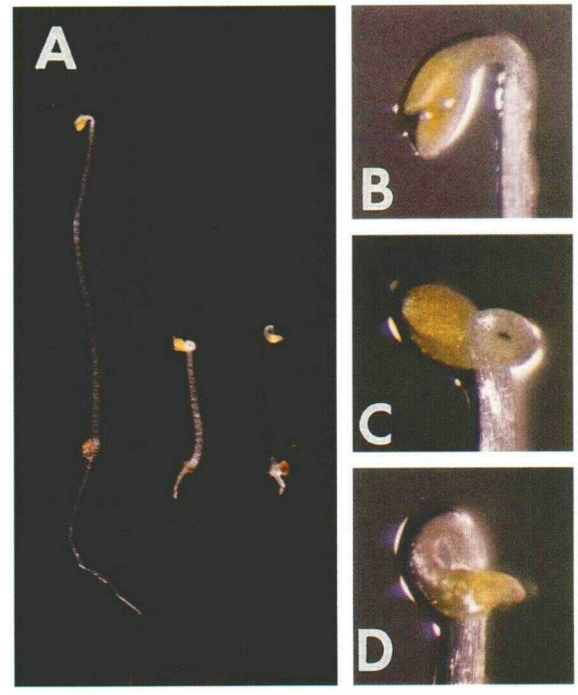

Figure 1. Effect of cytokinin and ethylene on the growth of dark-grown seedlings in *Arabidopsis*. *wt* seedlings (A) were grown for 4 d in the absence of hormone (left), on 5 μM BA (center), or in 0.7 μL L^{-1} ethylene in air (right). Enlargements show detail on cotyledons and apical hooks in the seedlings grown in the absence of hormone (B) and in the presence of BA (C) or ethylene (D).

ethylene-resistant mutants, *ein1–1* and *ein2–1*. Root growth (expressed as relative growth = [length of the root in the presence of BA/length in the absence] × 100) was inhibited in *wt* seedlings by concentrations of BA as low as 10 nM (Fig. 2A). Root growth in both of the ethylene-resistant mutants, *ein1–1* and *ein2–1*, was more resistant to BA than it was in *wt*. As a control, the growth of a standard allele of the cytokinin-resistant mutant, *ckr1–109*, was also examined and showed marked resistance, as previously reported (Su and Howell, 1992). In fact, the degree of BA resistance in the *ein* mutants was indistinguishable from that in *ckr1-109*.

Since hypocotyl elongation was also inhibited by cytokinin, the BA sensitivity of hypocotyl elongation was compared in the seedlings of *wt*, *ckr1*, and *ein* mutants. Hypocotyl elongation in *wt* seedlings was as sensitive to BA as was root elongation (Fig. 2B). In the ethylene-resistant mutants, *ein1–1* and *ein2–1*, hypocotyl elongation was as resistant to BA, if not more so, than root elongation. The *ckr1–109* seedlings were also resistant to BA in a manner indistinguishable from the *ein* mutants.

Ethylene Inhibitors Partially Relieve Cytokinin Inhibition

As a second test of the involvement of ethylene in the action of cytokinin, we determined if the effects of cytokinin on hypocotyl and root elongation could be blocked by ethylene inhibitors (as in Corriveau and Krul, 1986). Silver ions (from 20 μM $AgNO_3$), an ethylene-response inhibitor (Beyer, 1979), were able to overcome to a large extent the

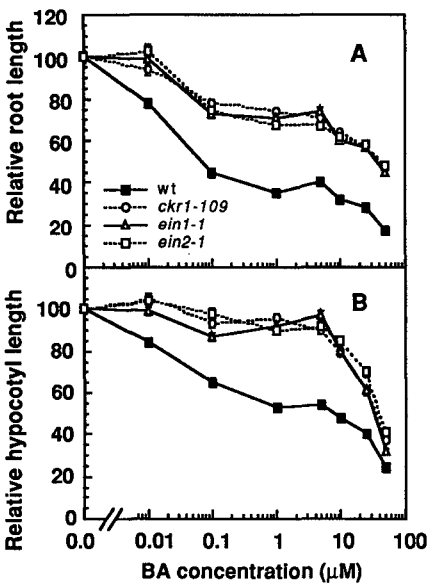

Figure 2. Dose response of *wt* and various *Arabidopsis* mutants (*ckr1–109*, *ein1–1*, and *ein2–1*) to cytokinin. Seedlings were germinated in the dark on medium containing varying concentrations of BA. Root and hypocotyl length were measured 4 d after germination. Root (A) and hypocotyl (B) lengths are expressed as relative lengths (compared to seedlings grown in the absence of BA). Each point represents the measurement of at least 20 seedlings, and the error bars represent the SE.

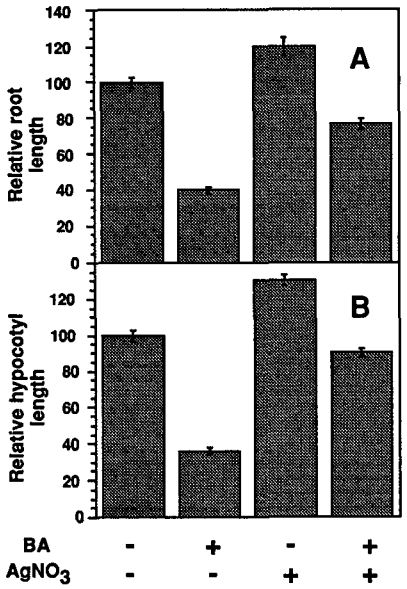

Figure 3. The effects of Ag^+, an ethylene-response inhibitor, on the BA-induced inhibition of root and hypocotyl elongation. *wt* seedlings were germinated in the dark on medium containing 5 μM BA and/or 20 μM $AgNO_3$, as indicated. Root (A) and hypocotyl (B) lengths were measured and are expressed as in Figure 2.

inhibition of root and hypocotyl elongation brought on by 5 μM BA in *wt* seedlings (Fig. 3). Five micromolar BA limited elongation of roots and hypocotyls to about 40% of untreated controls. In the absence of BA, 20 μM $AgNO_3$ stimulated both root and hypocotyl elongation somewhat, probably by counteracting the growth-inhibitory effects of endogenous ethylene production. When added with 5 μM BA, 20 μM $AgNO_3$ was able to overcome much but not all of the inhibition caused by cytokinin. Lower or higher concentrations of $AgNO_3$ were unable to relieve the BA inhibition further (not shown). Thus, an inhibitor of ethylene response blocked the action of cytokinin and was able to restore, in part, normal root and hypocotyl growth.

A similar approach was used to determine whether cytokinin action was blocked by an inhibitor of ethylene biosynthesis, AVG, an inhibitor of ACC synthase (Yang and Hoffman, 1984). AVG (at 2 μM) also restored, albeit not completely, normal root and hypocotyl growth in the presence of 5 μM BA (Fig. 4), similar to the effects of Ag^+. As with Ag^+, lower or higher concentrations of AVG were unable to relieve the BA inhibition further (not shown). Because Ag^+ and AVG blocked BA action, it was concluded that cytokinin acts "upstream" from the ethylene-response pathway and that ethylene mediates a major portion of the action of BA on the inhibition of root and hypocotyl elongation.

Although both AVG and Ag^+ blocked the effect of BA, they did not do so completely (Figs. 3 and 4). Because of this, it was asked whether the inhibitors blocked the ethylene response incompletely or whether BA inhibition involved more than one mechanism. To answer this question we tested the ability of Ag^+ to block the effects of applied ethylene. In contrast to its effect on BA inhibition, Ag^+ (at

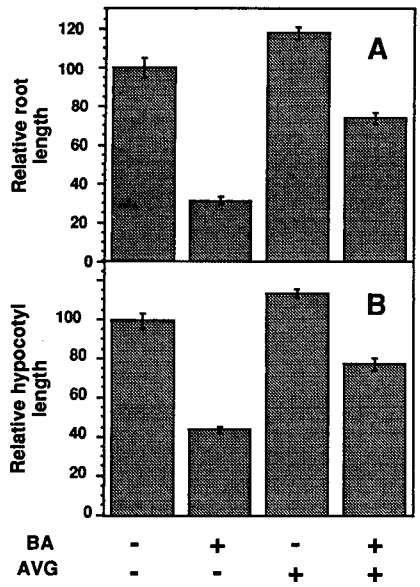

Figure 4. The effects of the ethylene biosynthesis inhibitor AVG on the BA-induced inhibition of root and hypocotyl elongation. *wt* seedlings were germinated in the dark on medium containing 5 μM BA and/or 2 μM AVG, as indicated. Root (A) and hypocotyl (B) lengths were measured and are expressed as in Figure 2.

the concentration described above) was able to relieve completely ethylene-induced inhibition of root and hypocotyl elongation (data not shown).

ckr1 Is Resistant to Ethylene and Allelic to *ein2*

Since the action of cytokinin can be blocked by ethylene inhibitors, it was important to determine whether the *ckr1* mutants were also resistant to ethylene. It was found that, indeed, root and hypocotyl elongation in *ckr1–109* was as resistant to the growth-inhibitory effects of ethylene as it was in the *ein* mutants (Fig. 5, A and B). Both root and hypocotyl elongation were inhibited above 0.1 μL L^{-1} ethylene (in air) in *wt* seedlings, whereas no statistically significant effects were observed in *ein1–1*, *ein2–1*, or *ckr1–109* up to about 1 μL L^{-1} ethylene.

Because *ckr1–109* is resistant to ethylene, the relationship of the genes responsible for *ckr1* and known ethylene mutants was examined. In an earlier report, *ckr1* was mapped to chromosome 5 in the *Arabidopsis* genome. Further mapping analysis confirmed that *ckr1* was indeed located on chromosome 5 but not near RFLP marker 211, as previously reported. Using CAPS markers, we found that *ckr1* was located near the top of chromosome 5, in the vicinity of *CTR1* (Fig. 6). A CAPS marker was developed for *CTR1* based on the published sequence, but it was found that *ckr1* did not co-segregate consistently with *CTR1*, but was located about 2 centimorgans away, close to the ethylene-resistance gene *EIN2*. *EIN2* was originally reported on chromosome 4 (Kieber et al., 1993); however, the map position of *EIN2* has been corrected to chromosome 5 (*Arabidopsis* data base). To determine whether *ckr1* is allelic to *ein2*, *ckr1–109* and *ckr1–50* were crossed with *ein2–1*. The F_1 progeny were cytokinin resistant (non-complementing phenotype), indicating that the *ckr1–109*, *ckr1–50*, and *ein2–1* are allelic.

Cytokinin Stimulates Ethylene Production

The above data show that ethylene appears to mediate the cytokinin inhibition response; in part, however, they do not address the question whether cytokinin stimulates the production of ethylene. Therefore, ethylene production in response to varying concentrations of BA was measured over a 24-h period between 3 and 4 d after germination. (This time was chosen because it represents a period in seedling development when the difference in root and hypocotyl growth between untreated and BA-treated seedlings was most apparent.) The amount of ethylene produced in response to different doses of BA was compared to the inhibition of root and hypocotyl elongation. Ethylene production was detected at low concentrations of BA but was most significant above 0.1 μM BA (Fig. 7). There was a close correlation between the increase in ethylene production and the inhibition of root and hypocotyl elongation. (The dose responses are somewhat different from those shown in Fig. 1. To collect detectable quantities of ethylene, seeds had to be sown more densely. The seedlings may be more sensitive to BA when sown at higher density because of the accumulation of ethylene and CO_2 and the depletion of O_2.)

To determine whether the amount of ethylene produced in response to cytokinin could account for the inhibition in root and hypocotyl elongation, an approach was taken similar to that described by Bertell and Eliasson (1992). The amount of ethylene generated in response to cytokinin that produces a given growth inhibition effect was compared to

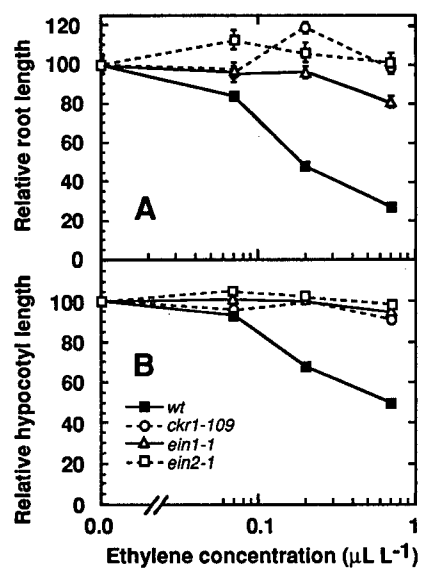

Figure 5. Dose response of *wt* and various *Arabidopsis* mutants to ethylene. Seedlings were germinated in the dark and exposed to varying concentrations of ethylene. Root and hypocotyl length were measured 4 d after germination. Root (A) and hypocotyl (B) lengths are expressed as described in Figure 2.

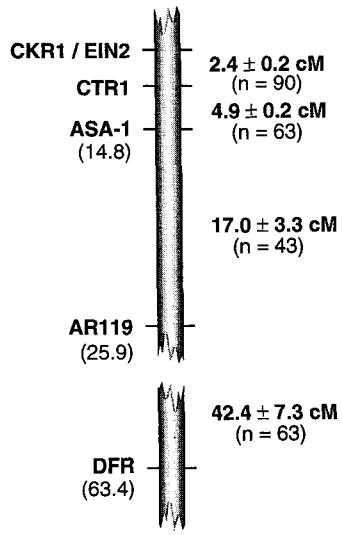

Figure 6. Location of *CKR1* on chromosome 5 of the *Arabidopsis* genome. The map position of *CKR1* was determined in relationship to standard CAPS markers *ASA-1* and *DFR*, to a CAPS marker generated from the sequence of *CTR1*, and to an RFLP marker, *AR119*. The markers and their map positions, in parentheses, are shown on the left. The distance in centimorgans (±SE) between *CKR1* and the other markers is shown on the right. The number (*n*) of F_2 progeny used in the mapping analysis is indicated in parentheses below the map distances.

the amount of ethylene that gives the same inhibitory effect in response to ACC, a precursor of ethylene (Fig. 8). If all the growth inhibition from cytokinin resulted from the production of ethylene, then the amount of ethylene generated in response to cytokinin should be the same as the ethylene levels generated in response to concentrations of ACC that inhibited hypocotyl elongation to a similar extent. We found that the amount of ethylene produced by BA application corresponding to a 50% reduction in growth of the hypocotyl (22 μL h^{-1} seedling^{-1} 10^{-8}) was less than that produced by ACC application at a similar level of inhibition (48 μL h^{-1} seedling^{-1} 10^{-8}). The differ-

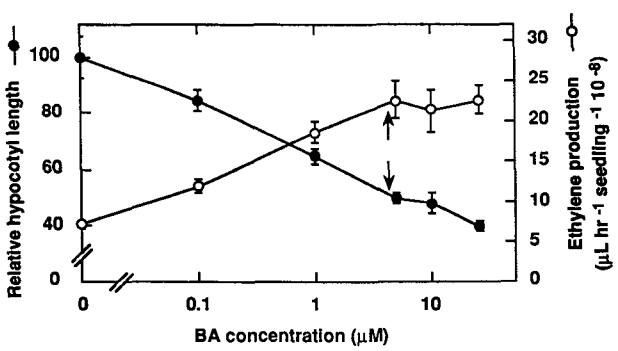

Figure 7. Ethylene production in dark-grown, *wt Arabidopsis* seedlings in response to various concentrations of BA. Ethylene was collected over a 24-h period between 3 and 4 d after germination. Hypocotyl lengths were measured and are expressed as in Figure 2. Arrows indicate the rate of ethylene production corresponding to 50% inhibition of hypocotyl elongation.

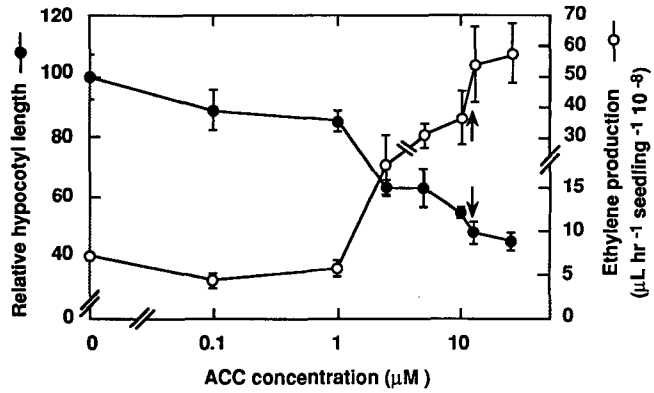

Figure 8. Ethylene production in dark-grown, *wt Arabidopsis* seedlings in response to various concentrations of ACC. Ethylene was collected over a 24-h period between 3 and 4 d after germination. Hypocotyl lengths were measured and are expressed as in Figure 2. Arrows indicate the rate of ethylene production corresponding to 50% inhibition of hypocotyl elongation.

ences in the amount of ethylene evolved (in the linear phases of ethylene evolution) were generally greater for ACC treatment than for BA, but the differences were more pronounced at higher levels of inhibition. Roots showed a similar relationship between the levels of ethylene produced in response to BA and ACC at comparable levels of growth inhibition (data not shown).

Sensitivity to Ethylene Is Unaffected by Cytokinin

We have shown above that BA stimulates ethylene production, which can, in part, account for the inhibitory effect of cytokinin on seedling growth. It is possible that BA might also enhance the sensitivity of seedlings to ethylene. To examine this possibility, dose responses to ethylene were determined in seedlings grown in the presence of various concentrations of cytokinin. Because it has been shown that BA stimulates the production of ethylene, the production of endogenous ethylene was controlled by 5 μM AVG. Under these conditions a family of dose-response curves was generated, each at a different concentration of BA (Fig. 9). It can be seen that the ethylene dose responses at different concentrations of BA are similar, indicating that under these conditions cytokinin did not significantly alter the seedling response to ethylene.

DISCUSSION

The role of cytokinin in seedling developmental processes is not well established. Many of the morphogenic changes produced by cytokinin in *Arabidopsis* seedlings are similar to those that result from the action of ethylene (Guzmán and Ecker, 1990). In our experiments we have seen diagnostic signs of an ethylene response after treatment of seedlings with BA. In addition, we have shown that cytokinin generates ethylene in seedlings and that the production of ethylene accounts in large part for action of BA on root and hypocotyl elongation. However, as has been pointed out by others, cytokinin also causes morphogenic changes in seedlings that are similar to those pro-

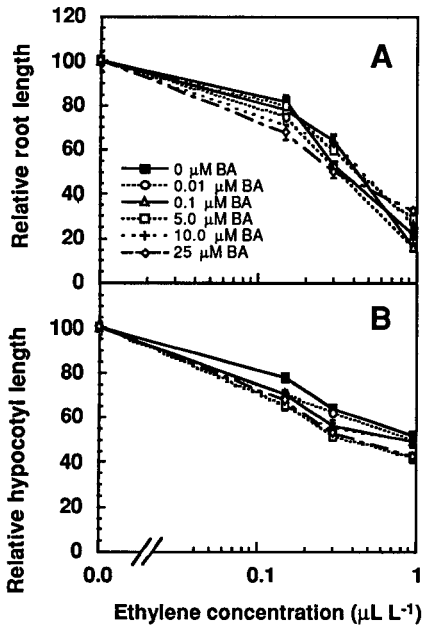

Figure 9. Dose response to ethylene of dark-grown, *wt Arabidopsis* seedlings grown in various concentrations of BA in the presence of 2 μM AVG. Ethylene dose responses were generated as described in Figure 2. Root (A) and hypocotyl (B) lengths were measured and are expressed as in Figure 2.

duced by light (Chory et al., 1994). In particular, the de-etiolated phenotype exhibited by *det* mutants is reminiscent of cytokinin action. Because of that it has even been suggested that cytokinin may be an intermediate in the light-response pathways. However, our results show that some of the prominent cytokinin responses, such as inhibition of hypocotyl elongation, are largely mediated by ethylene. Obviously, light, cytokinin, and ethylene responses are not serially coupled to effectuate the action of light. If they were, then light-induced de-etiolation effects could be blocked by ethylene inhibitors or ethylene-resistant mutants. In addition, as has been pointed out by workers in the ethylene field, the effects of light and cytokinin/ethylene on hypocotyl elongation are different (Eisinger, 1983). Ethylene inhibits hypocotyl elongation by redirecting longitudinal growth in a radial direction.

The coupling between cytokinin and ethylene was not obvious in establishing conditions used to screen for cytokinin-resistant mutants (Su and Howell, 1992). First, the levels of BA used in the screens were low (micromolar to submicromolar) to avoid the general stress responses usually associated with ethylene action. Second, the mutants were selected in the light (Su and Howell, 1992), and light masks most ethylene growth responses except for its effects on root growth. Third, even in the dark, BA-treated seedlings did not always display diagnostic ethylene responses. A possible reason is that the morphology of BA-treated seedlings appears to be a composite of cytokinin acting on its own and cytokinin activating other responses. For example, the tight coiling of the apical hook, one of the more obvious diagnostic features of an ethylene response in *Arabidopsis* seedlings (Guzmán and Ecker, 1990), is BA dose dependent, and at high BA concentrations coiling appears to be counteracted by other effects of cytokinin on the apical hook (C. Smart, unpublished observations).

We have shown in this study that the effects of exogenous cytokinin on root and hypocotyl elongation can be largely, although not entirely, accounted for by the production of ethylene. The argument is based on several experiments, including an approach originally described by Bertell and Eliasson (1992) in which one compares the amount of ethylene evolved in response to two different agents (BA and ACC) that give the same level of biological effect. In addition, we have found that the effects of cytokinin on root and hypocotyl elongation can be reversed, although not entirely, by ethylene inhibitors. The same concentrations of these inhibitors can entirely reverse the action of exogenous ethylene. Therefore, it appears that BA has a residual effect on root and hypocotyl elongation that is not mediated by ethylene. Whether that effect is mediated directly by cytokinin or by yet another growth regulator is an unanswered question.

Coupling Cytokinin Action to Ethylene

Ethylene is coupled to the action of other plant hormones. For example, auxin inhibition of apical hook opening in bean seedlings is largely mediated by ethylene (Kang et al., 1967). Auxin stimulates ethylene biosynthesis (Jones and Kende, 1979) and the expression of the ACC synthase gene encoding the controlling enzyme in ethylene biosynthesis (Sato and Theologis, 1989). To distinguish between auxin and ethylene action, a common approach has been to use ethylene inhibitors. Romano et al. (1993) employed the action of ACC deaminase to uncouple auxin effects from ethylene production. The coupling between auxin and ethylene action may affect the phenotypes of auxin mutants. Two *Arabidopsis* mutants selected for resistance to auxin at the seedling stage, the dominant mutant *AXR2* and the recessive mutant *aux1*, are both resistant to ethylene (Wilson et al., 1990).

Like auxins, cytokinins also stimulate ethylene production (Mattoo and White, 1991), especially in stressed tissue (Wright, 1980; McKeon et al., 1982; Kao and Yang, 1983; Khan et al., 1988). Cytokinins are more effective in stimulating ethylene production in conjunction with auxin, particularly in young tissue (Fuchs and Lieberman, 1968; Aharoni et al., 1979). Lau and Yang (1974) reported that the stimulation of ethylene production by cytokinin in conjunction with auxin was different from the stimulation by cytokinin plus Ca^{2+}. In particular, the time course for the production of ethylene differed under the two conditions. Like auxin, BA has been shown to stimulate ACC synthase activity in mung bean hypocotyls (Yoshii and Imaseki, 1981).

Some of the characteristics of cytokinin overproduction in transgenic plants expressing the bacterial isopentenyl transferase gene may be, in fact, ethylene responses. For example, cytokinin overproduction inhibits root formation and reduces stem and leaf area (Memelink et al., 1987; Medford et al., 1989). In addition, cytokinin overproduction stimulates the expression of genes normally expressed

in response to stress (Memelink et al., 1987; Harding and Smigocki, 1994). These responses might be ethylene responses elicited indirectly by the action of cytokinin. Some of the properties of *amp1*, the *Arabidopsis* mutant with altered levels of cytokinin, might also be a consequence of ethylene production. For example, the *amp1* mutant has a de-etiolated phenotype with a shortened hypocotyl (Chaudhury et al., 1993). It needs to be examined if the hypocotyl phenotype in the mutant is largely an ethylene response.

ckr Mutants

Consistent with the observations in this study that the action of cytokinin is coupled to the ethylene responses in *Arabidopsis* seedlings, we have found that mutations in the *CKR1* gene are allelic to mutations in *EIN2*, a gene that encodes a component of the ethylene-response pathway. The ethylene-response pathway has been defined by mutational analysis and largely represents a linear cascade of protein phosphorylation steps. *EIN2* occupies a position downstream from *CTR1* (Kieber and Ecker, 1993). Mutations in *EIN2* are epistatic to those in *CTR1* in that the *ctr1* phenotype is not expressed in the double *ctr1 ein2* mutants (Ecker, 1994). Although *ckr1* is allelic to *ein2*, that fact may not have any special significance with respect to how the pathways are coupled. We have shown that the coupling occurs largely through ethylene production and not through ethylene responses. Therefore, the cytokinin signal is probably not fed into the ethylene pathway at the site where *EIN2* acts. The fact that the five cytokinin-resistant mutants isolated so far have turned out to be *ein2* alleles, as opposed to other ethylene-pathway mutations, may only reflect the fact that *ein2* mutations have a severe phenotype and are more frequently picked up even in a search for ethylene-resistant mutants (J.R. Ecker, personal communication). However, it would be interesting to determine if the alleles obtained through cytokinin screening are a distinct class compared to those obtained by screening directly with ethylene.

Cytokinin has other effects on seedling development that do not appear to be mediated by ethylene, such as cotyledon opening and expansion (A.J. Cary and C. Smart, unpublished observations). Those effects can be easily observed in *ein* mutant seedlings treated with BA. Additional cytokinin-response mutants could be obtained by screening for abnormal cotyledon opening and expansion responses to cytokinin in mutant seedling populations. In addition, it is possible that other cytokinin mutants can be obtained by the same procedures that were used to screen for the *ckr1* mutants. Cytokinin-response mutants that are defective in steps upstream from the ethylene pathway would be expected to have the same root and hypocotyl phenotype as *ckr1*, except that these mutants would be ethylene sensitive.

ACKNOWLEDGMENTS

Special thanks to Joe Ecker for sharing prepublished information, Peter Davies and Thomas LaRue for advice and use of equipment for measuring ethylene, Alan Rose for the pAR119 marker clone, and Christina Smart and Thomas Silva for helpful discussions.

Received October 18, 1994; accepted December 19, 1994.
Copyright Clearance Center: 0032–0889/95/107/1075/08.

LITERATURE CITED

Aharoni N, Anderson JD, Lieberman M (1979) Production and action of ethylene in senescing leaf discs. Effect of indoleacetic acid, kinetin, silver ion, and carbon dioxide. Plant Physiol **64:** 805–809

Bernatzky R, Tanksley SD (1986) Towards a saturated linkage map in tomato based on isozymes and cDNA clones. Genetics **112:** 887–898

Bertell G, Eliasson L (1992) Cytokinin effects on root growth and possible interactions with ethylene and indole-3-acetic acid. Physiol Plant **84:** 255–261

Beyer EM (1979) Effect of silver, carbon dioxide, and oxygen on ethylene action and metabolism. Plant Physiol **63:** 169–173

Chaudhury AM, Letham S, Craig S, Dennis ES (1993) *Amp1*—a mutant with high cytokinin levels and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis and precocious flowering. Plant J **4:** 907–916

Chory J, Reinecke D, Sim S, Washburn T, Brenner M (1994) A role for cytokinins in de-etiolation in *Arabidopsis*. *Det* mutants have an altered response to cytokinins. Plant Physiol **104:** 339–347

Corriveau JL, Krul WL (1986) Inhibition of benzyladenine-induced radial expansion in soybean hypocotyls by silver ion. J Plant Physiol **126:** 297–300

Crocker W, Knight LI, Rose RC (1913) A delicate seedling test. Science **37:** 380–381

Ecker JR (1994) The ethylene sensing apparatus in *Arabidopsis*. Paper presented at the Fourth International Congress on Plant Molecular Biology, Amsterdam, June 20–24

Eisinger W (1983) Regulation of pea internode expansion by ethylene. Annu Rev Plant Physiol **34:** 225–240

Fuchs Y, Lieberman M (1968) Effects of kinetin, IAA and gibberellin on ethylene production, and their interactions in growth of seedlings. Plant Physiol **43:** 2029–2036

Guzmán P, Ecker JR (1990) Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. Plant Cell **2:** 513–524

Harding SA, Smigocki AC (1994) Cytokinins modulate stress response genes in isopentenyl transferase-transformed *Nicotiana plumbaginifolia* plants. Physiol Plant **90:** 327–333

Jones JF, Kende H (1979) Auxin-induced ethylene biosynthesis in subapical stem sections of etiolated seedlings of *Pisum sativum* L. Planta **146:** 649–656

Kang BG, Yocum CS, Burg SP, Ray PM (1967) Ethylene and carbon dioxide: mediation of hypocotyl hook-opening response. Science **156:** 958–959

Kao CH, Yang SF (1983) Role of ethylene in senescence of detached rice leaves. Plant Physiol **73:** 881–885

Khan AA, Thakur R, Akbar M, HilleRisLambers D, Seshu DV (1988) Hormonal regulation of elongation in floating rice during submergence. Crop Sci **28:** 121–127

Kieber JJ, Ecker JR (1993) Ethylene gas: it's not just for ripening anymore. Trends Genet **9:** 356–361

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR (1993) CTR1, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the RAF family of protein kinases. Cell **72:** 1–20

Konieczny A, Ausubel FM (1993) A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specifc PCR-based markers. Plant J **4:** 403–410

Lau O-L, Yang SF (1974) Synergistic effect of calcium and kinetin on ethylene production by the mungbean hypocotyl. Planta **118:** 1–6

Lee KH, LaRue TA (1992) Exogenous ethylene inhibits nodulation of *Pisum sativum* L. cv Sparkle. Plant Physiol **100:** 1759–1763

Lieberman M (1979) Biosynthesis and action of ethylene. Annu Rev Plant Physiol **30:** 533–591

Lincoln C, Britton JH, Estelle M (1990) Growth and development of the *axr1* mutants of *Arabidopsis*. Plant Cell **2:** 1071–1080

Mattoo AK, White WB (1991) Regulation of ethylene biosynthesis. *In* AK Mattoo, JC Suttle, eds, The Plant Hormone Ethylene. CRC Press, Boca Raton, FL, pp 21–42

McKeon TA, Hoffman NE, Yang SF (1982) The effect of plant hormone pre-treatments on ethylene production and synthesis of 1-aminocyclopropane-1-carboxylic acid in water-stressed wheat leaves. Planta **155:** 437–443

Medford JI, Horgan R, El-Sawi Z, Klee HJ (1989) Alterations of endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene. Plant Cell **1:** 403–413

Memelink J, Hoge JHC, Schilperoort RA (1987) Cytokinin stress changes the developmental regulation of several defence-related genes in tobacco. EMBO J **6:** 3579–3583

Romano CP, Cooper ML, Klee HJ (1993) Uncoupling auxin and ethylene effects in transgenic tobacco and Arabidopsis plants. Plant Cell **5:** 181–189

Rose AB, Casselman AL, Last RL (1992) A phosphoribosylanthranilate transferase gene is defective in fluorescent *Arabidopsis thaliana* tryptophan mutants. Plant Physiol **100:** 582–592

Rothenberg M, Ecker JR (1993) Mutant analysis as an experimental approach towards understanding plant hormone action. Semin Dev Biol **4:** 3–13

Sato T, Theologis A (1989) Cloning the messenger encoding 1-aminocyclopropane-1-carboxylate synthase, the key enzyme for ethylene biosynthesis in plants. Proc Natl Acad Sci USA **86:** 6621–6625

Su W, Howell SH (1992) A single genetic locus, *ckr1*, defines *Arabidopsis* mutants in which root growth is resistant to low concentrations of cytokinin. Plant Physiol **99:** 1569–1574

Wilson AK, Pickett FB, Turner JC, Estelle M (1990) A dominant mutation in *Arabidopsis* confers resistance to auxin, ethylene and abscisic acid. Mol Gen Genet **222:** 377–383

Wright STC (1980) The effect of plant growth regulator treatments on the level of ethylene emanating from excised turgid and wilted leaves. Planta **148:** 381–388

Yang SF, Hoffman NE (1984) Ethylene biosynthesis and its regulation in higher plants. Annu Rev Plant Physiol **35:** 155–189

Yoshii H, Imaseki H (1981) Biosynthesis of auxin-induced ethylene. Effects of indole-3-acetic acid, benzyladenine and abscisic acid on endogenous levels of 1-aminocyclopropane-1-carboxylic acid (ACC) and ACC synthase. Plant Cell Physiol **22:** 369–379