# Isolation and Characterization of Arabidopsis Mutants Defective in the Induction of Ethylene Biosynthesis by Cytokinin

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ABSTRACT

Cytokinins elevate ethylene biosynthesis in etiolated Arabidopsis seedlings via a post-transcriptional modification of one isoform of the key biosynthetic enzyme ACC synthase. In order to begin to dissect the signaling events leading from cytokinin perception to this modification, we have isolated a series of mutants that lack the ethylene-mediated triple response in the presence of cytokinin due to their failure to increase ethylene biosynthesis. Analysis of genetic complementation and mapping revealed that these Cin mutants (cytokinin-insensitive) represent four distinct complementation groups, one of which, *cin4*, is allelic to the constitutive photomorphogenic mutant *fus9*/*cop10*. The Cin mutants have subtle effects on the morphology of adult plants. We further characterized the Cin mutants by analyzing ethylene biosynthesis in response to various other inducers and in adult tissues, as well as by assaying additional cytokinin responses. The *cin3* mutant did not disrupt ethylene biosynthesis under any other conditions, nor did it disrupt any other cytokinin responses. Only *cin2* disrupted ethylene biosynthesis in multiple circumstances. *cin1* and *cin2* made less anthocyanin in response to cytokinin. *cin1* also displayed reduced shoot initiation in tissue culture in response to cytokinin, suggesting that it affects a cytokinin signaling element.

PLANT development is modulated by interactions between hormones, an illustration of which is the induction of the biosynthesis of one hormone in response to another. Cytokinins, $N^6$-substituted adenine derivatives, are one of the many factors that modulate the biosynthesis of the gaseous hormone ethylene (Yang and Hoffman 1984; Mattoo and Suttle 1991; Abeles *et al.* 1992). We are characterizing the induction of ethylene biosynthesis by cytokinins in Arabidopsis in order to understand how these hormones interact to regulate plant development.

Cytokinins were first identified as factors that acted synergistically with auxin to promote cell division *in vitro* and acted antagonistically to auxin to promote shoot and root initiation from callus cultures (Miller *et al.* 1955, 1956; Skoog and Miller 1957). Cytokinins have been implicated in many aspects of plant growth and development, including cell division, shoot initiation and growth, leaf senescence, and photomorphogenesis (Binns 1994; Brzobohaty *et al.* 1994; Mok and Mok 1994). In contrast to the wealth of knowledge concerning the physiological effects of cytokinins, the molecular mechanisms underlying cytokinin perception and action remain largely unknown.

One powerful approach that has been employed in the analysis of other plant hormone signaling pathways has been the isolation of mutants defective in hormone responsiveness. However, identifying cytokinin-insensitive (Cin) mutants has been hampered by the lack of a suitable genetic screen. Part of the problem is that it is difficult to fully anticipate the phenotype of a Cin mutant. Screens for cytokinin-insensitivity using inhibition of root elongation, inhibition of germination, cotyledon expansion and growth in tissue culture have met with limited success. The recessive tobacco mutant *zea3* was selected for its ability to germinate on cytokinin levels which completely inhibit germination of wild-type plants (Faure *et al.* 1994). *zea3* does not seem to be a general cytokinin response mutant because it is only resistant to cytokinin during germination. *zea3* also affects cotyledon development and carbon-nitrogen metabolism. Recent work suggests that *ZEA3* may be a negative regulator of cytokinin responsiveness (Martin *et al.* 1997). In Arabidopsis, the *cyr1* (cytokinin response) and *ckr1* (cytokinin resistant) mutants were identified by the ability to elongate their roots on inhibitory concentrations of cytokinin (Su and Howell 1992; Deikman and Ulrich 1995). *ckr1* has been found to be allelic to the ethylene-insensitive mutation *ein2*, and was identified because it was resistant to the ethylene produced in response to exogenous cytokinin (Cary *et al.* 1995). The *cyr1* phenotype (pale green leaves, abbreviated shoot development and incomplete cotyledon and leaf expansion) is consistent with the predicted phenotype of a cytokinin-insensitive plant (Deikman and Ulrich 1995). *cyr1* plants are less responsive to exogenous cytokinin in root inhibition

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assays, anthocyanin accumulation assays and *in vitro* shoot initiation assays. *cyr1* plants are also affected in abscisic acid responsiveness in root inhibition assays. One other potential cytokinin response mutant from Arabidopsis is *stp1* (Baskin *et al.* 1995). The *stp1* mutant displayed decreased sensitivity to cytokinin in root inhibition assays, but had normal sensitivity to cytokinin in callus and shoot initiation assays. *stp1* had wild-type sensitivity to auxin, ethylene, gibberellin and abscisic acid in root inhibition assays.

Mutants that overproduce cytokinins have been identified. In the moss *Physcomitrella patens*, the *OVEA*, *OVEB* and *OVEC* mutants were identified because they overproduce gametophores due to an elevation of cytokinin levels up to 100-fold (Ashton *et al.* 1979; Wang *et al.* 1981; Featherstone *et al.* 1990). In Arabidopsis, the *amp1* mutant (altered meristem program) produces sixfold more cytokinin than wild-type plants (Chaudhury *et al.* 1993; Chinatkins *et al.* 1996). Several *amp1* phenotypes, such as a lack of apical dominance, delayed senescence and increased shoot regeneration in tissue culture, are consistent with the predicted phenotype of a cytokinin over-producing mutant. However, other *amp1* phenotypes, such as polycotyly, precocious flowering and abnormal phyllotaxy, have not been observed in plants treated with exogenous cytokinin or in transgenic plants containing the cytokinin biosynthetic gene *ipt*. The pleiotropic phenotype of *amp1* makes it difficult to conclude that increased cytokinin is the primary defect.

A gene implicated in cytokinin signaling was recently identified in Arabidopsis by selecting for T-DNA::CaMv 35S promotor insertions that conferred cytokinin-independent shoot initiation in tissue culture (Kakimoto 1996). The *CKI1* gene identified in this screen is similar to bacterial two-component histidine kinases, ubiquitous signaling elements in prokaryotic cells (Hoch and Silhavy 1995). CKI1 may be a cytokinin receptor, much like the ethylene receptor ETR1, which is also highly homologous to two-component histidine kinases (Chang *et al.* 1993).

Growth of etiolated Arabidopsis seedlings in the presence of exogenous cytokinin causes an elevation of ethylene biosynthesis that has recently been shown to be due to induction of a single ACC synthase isoform (Vogel *et al.* 1998). ACC synthase converts S-adenosyl methionine into 1-aminocyclopropane-1-carboxylic acid (ACC) and is the first committed step in ethylene biosynthesis (Yang and Hoffman 1984; Kende 1989, 1993). The Arabidopsis ACC synthase gene family consists of at least five members called *ACS1–ACS5* (Liang *et al.* 1992; Van Der Straeten *et al.* 1992; Liang *et al.* 1995). Recently, we have found that loss-of-function *acs5* mutations disrupt the induction of ethylene at low cytokinin concentrations ($<10$ μmol) but not at higher concentrations (Vogel *et al.* 1998), indicating that this ACS isoform is responsible for the increase in ethylene biosynthesis observed in response to low levels of cytokinin. Furthermore, northern analysis indicated that alterations in the steady-state level of wild-type *ACS5* mRNA did not significantly contribute to this increased ACS5 function. This, coupled with the finding that the dominant ethylene overproducing mutant *eto2* is the result of a perturbation of the 11 carboxy-terminal amino acids of ACS5 (Vogel *et al.* 1998), suggests that cytokinin acts by a post-transcriptional modification of ACS5.

The increased ethylene biosynthesis induced by cytokinin results in seedlings that display what is known as the triple response (Figure 1). In Arabidopsis, the triple response consists of an inhibition of hypocotyl and root elongation, radial expansion of the hypocotyl and exaggeration of curvature of the apical hook. This simple response has been invaluable in elucidating the signaling mechanism of ethylene (Bleecker *et al.* 1988; Guzman and Ecker 1990; Kieber *et al.* 1993; Roman *et al.* 1995; Kieber 1997). In this study, we utilized this response to isolate mutants that are disrupted in the elevation of ethylene biosynthesis in response to cytokinin. The genetics and hormone responsiveness of these Cin mutants is presented. These mutants identify elements necessary for cytokinin signaling to ACS5 and represent the first step in the elucidation of this signaling pathway.

## MATERIALS AND METHODS

**Plant lines and growth conditions:** *Arabidopsis thaliana* (L) Heynh. ecotypes Columbia (Col), Wassilewskija (WS) and Landsberg Erecta (Ler) were used in this study as indicated. *fus9-1* seed was obtained from the Arabidopsis Biological Resource Center at Ohio State University. For experiments involving plants grown under sterile conditions, seeds were surface-sterilized and plated on murashige and skoog salts (MS) medium (Gibco, Grand Island, NY) solidified with 0.8% agar (MS agar) as described (Vogel *et al.* 1998). Gamborg's B5 medium (Gibco) was used for plates that were incubated in the light. Adult plants were grown in Metro mix 250 (Grace) under continuous illumination at 23° and were fertilized weekly with Peter's Professional Fertilizer (20-20-20). Seeds were mutagenized with methane-sulfonic acid ethyl ester as described (Vogel *et al.* 1998). Two different ecotypes (WS and Col) of Arabidopsis were used in the genetic screens to increase the chance of finding mutants that might be masked by modifiers in one ecotype.

**Ethylene measurement:** Seedlings (about 15 per vial) were sown on 3 ml MS agar in 22-ml gas chromatography vials and incubated at 4° for four days to ensure uniform germination. They were then incubated in the dark at 23° for 48 hr. Appropriate supplements, as indicated, were added in a volume of 200 μl (controls were 200 μl water) just before capping. For the cytokinin dose response, various concentrations of kinetin were included directly in the medium. The vials were flushed with hydrocarbon-free air and then capped for the indicated times. The accumulated ethylene was measured using a gas chromatograph (Perkin Elmer, Norwalk, CT) fitted with a PoraPLOT U column, a cryofocusing attachment and a flame ionization detector. A sample of headspace from each vial was loaded onto the column at −50° via an autosampler, and the column was warmed to 30°. Quantification and analysis of the ethylene peaks was by Turbochrome 4 software (Perkin Elmer)

based on comparison to a 1 μl/liter ethylene standard. 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.

To measure ethylene from adult plants, tissues were detached, weighed and then placed in 22-ml vials containing 3 ml of MS agar. The vials were flushed with hydrocarbon-free air, sealed and incubated in the light for the indicated times. The ethylene was then measured as above. To determine the effect of wounding on ethylene biosynthesis, fully expanded leaves were sliced with a razor blade at 1 mm intervals (tissues were not sliced all the way through), placed in vials containing 3 ml MS agar and capped. Accumulated ethylene was measured 24–28 hr later. The amount of ethylene produced by light-grown seedlings was determined by placing capped vials in a lighted growth chamber and measuring the ethylene that accumulated during the first 72 hr of growth.

**Isolation of mutants:** Mutagenized seeds were plated on MS agar containing 0.5 μmol of the synthetic cytokinin $N^6$-benzyladenine (BA). After incubation for three days at 23° in the dark, tall seedlings were selected. Self-set seeds from putative mutants were retested on MS agar containing 0.5 μmol BA. Seeds from plant lines which re-tested for a reduced triple response on BA were plated on MS agar containing 10 μmol 1-aminocyclopropane-1-carboxylic acid (ACC) to determine if they were ethylene-insensitive. ACC is the immediate precursor of ethylene and is converted to ethylene by constitutively-expressed ACC oxidase. Lines that were wild type for the triple response in the presence of ACC were further analyzed by measuring the ethylene produced in response to cytokinin.

**Genetic mapping:** Mutants were crossed with different ecotypes to create $F_2$ mapping populations. $F_2$ seeds were plated on MS agar supplemented with 0.5 μmol BA and mutant seedlings for *cin1*, *cin2* and *cin4*, and wild-type seedlings for the *cin3* cross, were transferred to MS agar, without BA, and allowed to grow for one week. DNA was extracted using a minipreparation method previously described (Edwards *et al.* 1991). Simple sequence length polymorphism markers polymorphic between ecotypes were amplified using PCR, separated by gel electrophoresis in 4% agarose and visualized by ethidium bromide staining essentially as described (Bell and Ecker 1994).

**Cytokinin sensitivity assays:** To assay shoot initiation, root explants (2 cm) were taken from two-week-old light-grown sterile plants and placed on callus-inducing medium (0.25 μmol 2,4-dichlorophenoxyacetic acid and 2 μmol kinetin) for one week and then transferred to shoot inducing medium (0.75 μmol indole acetic acid and varying concentrations of 2-isopentyladenine). Clusters of shoots were counted after 22 days on shoot inducing medium. At least 40 explants were analyzed for each mutant at each dose. Gamborg's medium (Gibco) solidified with 0.8 % agar (Gamborg's agar) was used as the base media for all stages.

To determine the effect of cytokinin on anthocyanin accumulation, seeds were plated on Gamborg's agar supplemented with increasing concentrations of BA. Anthocyanin was measured after 10 days of growth. Anthocyanin was extracted and quantified from individual seedlings or entire plates of seedlings (about 20 seedlings) as previously described (Deikman and Hammer 1995).

The effect of cytokinin on de-etiolation was determined by plating seeds on MS agar supplemented with 0, 5 or 50 μmol BA. Plates were incubated in the dark at 23° for two weeks.

To determine the effect of cytokinin on senescence, leaves were cut from two-week-old plants grown on Gamborg's agar and placed inside a petri plate on sterile filter paper that was soaked with the appropriate cytokinin solution. To prevent the filter paper from drying out, the petri plates were kept in high humidity conditions. When the controls were visibly yellow, the leaves were weighed and the chlorophyll was extracted by placing a leaf in a 1.5-ml centrifuge tube with 1 ml methanol. After overnight incubation at room temperature, the concentration of chlorophyll was determined spectroscopically (Porra *et al.* 1989).

**Northern blot analysis:** Seedlings were grown (about 2000 per 100-mm plate) on MS agar for three days as described above except sterile filter paper was placed on top of the agar. 10 ml of liquid MS or liquid MS + 5 μmol BA was added and the seedlings harvested after 15 minutes. Total RNA was prepared by extraction with phenol/chloroform and 15 μg analyzed by northern blotting as described (Ausubel *et al.* 1994). Equal loading of RNA was confirmed by ethidium bromide staining of an agarose gel of the samples and by probing the blots with an rDNA probe (not shown). Signals were visualized and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Abscisic acid dose response:** Seven-day-old light-grown seedlings were transferred to MS agar containing abscisic acid (ABA). Seedlings were laid on the surface of the agar and the location of the root tip marked on the back of the plate. Plates were placed vertically in the light so that the roots would grow along the surface of the agar. After three days, root growth was measured.

## RESULTS

**Isolation of mutants:** Low doses of cytokinin (0.5–10 μmol) elevate ethylene biosynthesis in etiolated Arabidopsis seedlings to a level that is sufficient to induce a triple response (Figures 1 and 2). The triple response of etiolated dicotyledonous seedlings to ethylene was first described in peas by Neljubov (1901) and, in Arabidopsis, consists of a shortening and radial swelling of the hypocotyl, inhibition of root elongation and exaggeration of the curvature of the apical hook (Figure 1). Approximately $10^6$ $M_2$ Arabidopsis seedlings (from approximately 50,000 $M_1$ plants in 50 independent lots) were screened for those lacking a triple response in the presence of 0.5 μmol of the cytokinin benzyladenine (BA). The lowest concentration of BA to give a moderately-strong triple response was chosen for screening in order to increase the chance of recovering weak mutants. This screen has several advantages over previous genetic screens used to identify cytokinin-insensitive mutants: it is very easy to identify the mutant plants (tall seedlings protruding above a lawn of short seedlings); it is fast (>100,000 seedlings can be easily screened in a day); the response can be easily quantified by measuring the ethylene produced in response to cytokinin; and the triple response morphology is very distinctive and specific for ethylene. The ability to screen large numbers of mutagenized seedlings increases the likelihood that we can overcome the potential problem of lethality resulting from cytokinin-insensitivity by allowing the identification of rare, weak alleles. Three classes of mutants were found using this screen: ethylene-insensitive mutants, mutants in the ethylene biosynthetic gene *ACS5* and mutants upstream of *ACS5*. The ethylene-insensitive

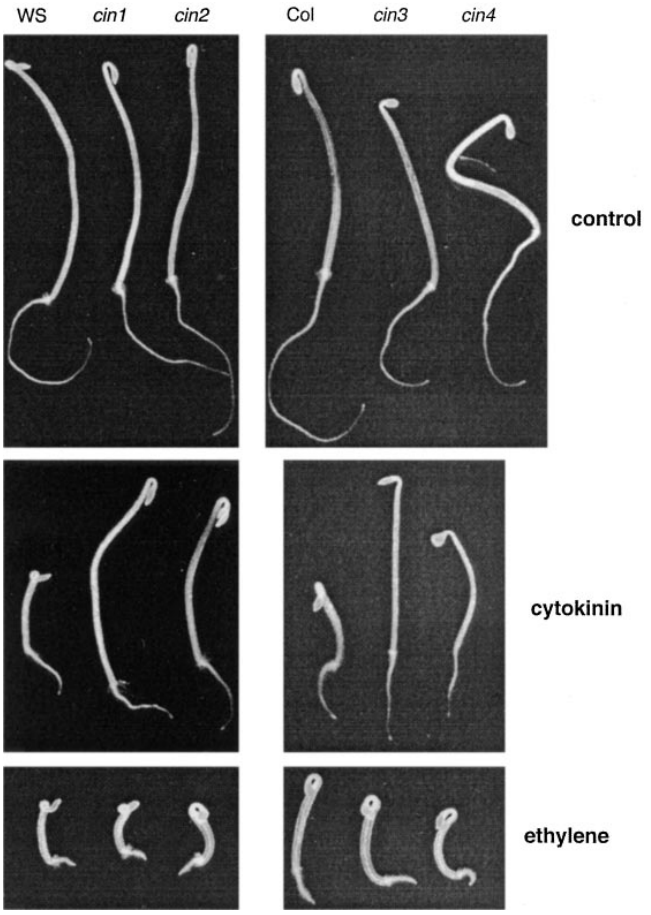


Figure 1.—Phenotype of wild-type and mutant etiolated seedlings. Seedlings were grown for three days in the dark at 23° under the following conditions: no hormones (control); 0.5 μmol BA (cytokinin); and 10 μl/liter ethylene. Representative seedlings were picked and photographed. Genotypes are noted at the top. WS and Col are the wild-type controls.

mutants were identified by their failure to adopt a triple response morphology in the presence of ethylene. We identified 79 such ethylene-insensitive mutants in this screen (at least 38 of which were independent). The *acs5 (cin5)* mutations have been described, and reveal that cytokinin acts via a post-transcriptional modification of the ACC synthase 5 isoform (Vogel *et al.* 1998). We identified four mutants that failed to display a triple response in the presence of 0.5 μmol BA, which displayed wild-type sensitivity to ethylene and which were not allelic to *acs5*. These mutants display a normal triple response when grown in 10 μl/liter ethylene (Figure 1), and were comparable to wild type in a dose response analysis using the ethylene precursor ACC (Figure 3). The hypocotyl length of the *cin4* mutant was shorter at all levels of ACC, but the slope of the dose response curve is essentially the same as wild-type seedlings.

**Cytokinin dose response:** The four Cin mutants were backcrossed to wild-type plants of their respective ecotype two times before further physiological analysis. To verify that the mutants were defective in the induction of ethylene by cytokinin, rather than ethylene perception, and to quantify the strength of the mutants, the amount of ethylene produced by three-day-old etiolated seedlings in response to various concentrations of the cytokinin kinetin was measured (Figure 2). The Cin mutants produced much less ethylene than wild type at kinetin concentrations less then 10 μmol, but near wild-type levels in response to 50 μmol kinetin. This is consistent with previous work that suggested that there are independent low and high dose cytokinin response pathways in etiolated Arabidopsis seedlings (Vogel *et al.* 1998), and suggests that these Cin mutants are primarily affected in the low-dose response pathway.

**Genetic analysis:** Backcrosses of the mutants to wild type revealed that *cin1*, *cin2* and *cin4* are recessive: the

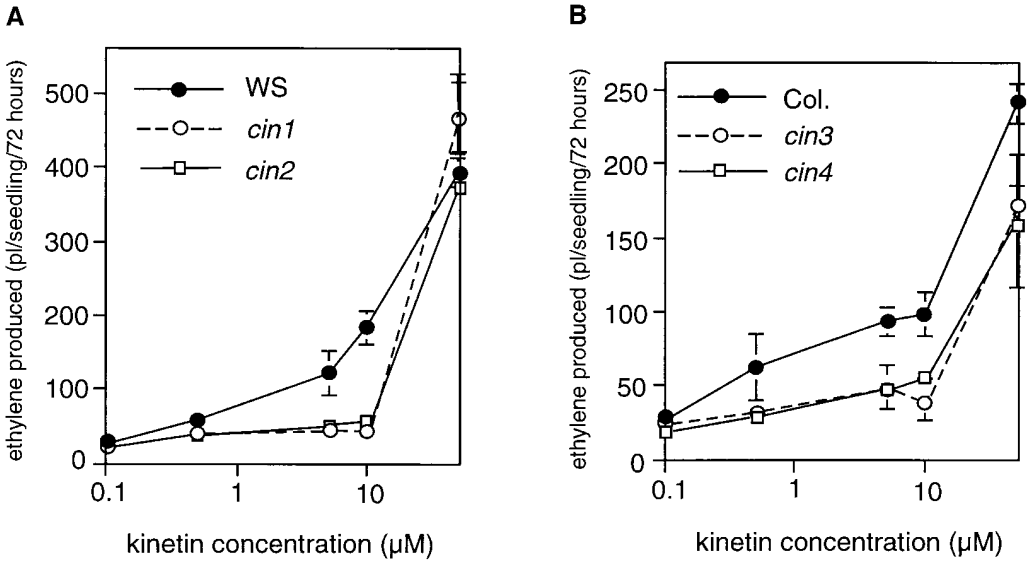


Figure 2.—Kinetin dose response. Seeds were sown on MS agar containing the indicated concentration of kinetin. Accumulated ethylene was measured after 72 hr of growth at 23° in the dark. Error bars are ± SD based on three replicates. (A) WS ecotype mutants. (B) Col ecotype mutants.

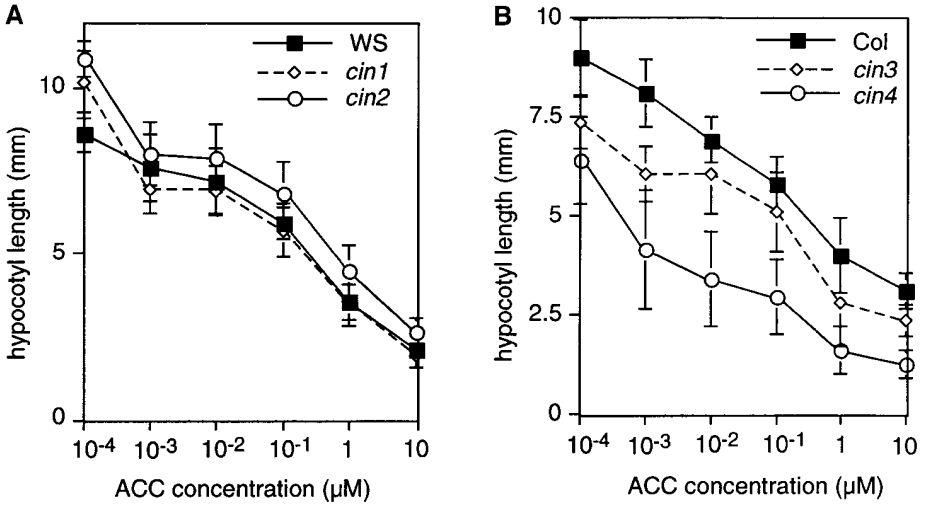


Figure 3.—Response of the mutants to ACC. Seeds were plated on MS agar supplemented with the indicated concentration of ACC and hypocotyl lengths were measured three days after germination at 23° in the dark. Points are the mean ± SD of 10 observations. (A) WS ecotype mutants. (B) Col ecotype mutants.

$F_1$ of a backcross has a wild-type phenotype and the $F_2$ segregates in a ratio consistent with 3:1, wild type:mutant (Table 1). The *cin3* mutation is dominant, but an $F_2$ backcross population does not fit the expected 1:3, wild type:mutant ratio. The under-representation of mutant seedlings is most likely due to incomplete penetrance of the *cin3* heterozygotes: while homozygous *cin3* mutant seedlings are uniformly tall on BA, the heterozygotes display a range of hypocotyl heights in the presence of BA, some comparable to that of wild-type seedlings (not shown). The *cin1* and *cin2* mutations complement each other, indicating that they disrupt two distinct genes. *cin1*, *cin2* and *cin4* all complement *acs5*, indicating that they are novel loci. Genetic mapping suggests that *cin4* as well as *cin3* represent independent loci (see below).

The position of each mutant on the Arabidopsis genetic map was determined using SSLP markers (Table 2). Each mutant mapped to a different chromosome. None of the recessive mutants map close to the position of the previously-identified *acs5* mutant, consistent with genetic complementation tests (Table 1). The *cin3* mutation mapped to the same chromosome arm as the cytokinin-insensitive mutant *cyr1*, but 20 m. u. distal to it. *cin4* mapped very close to *fus9-1* (Castle and Meinke 1994; Wei *et al.* 1994) and when *cin4* mutants are grown in the dark, a portion of seedlings display a de-etiolated phenotype (see below). Thus, we crossed *fus9 -1* heterozygotes (heterozygotes were used because *fus9 -1* is lethal) with *cin4* homozygotes to determine if they were allelic. The $F_1$ segregated in a ratio of 32:29, wild type:

**TABLE 1**

**Genetic analysis of cytokinin-insensitive mutants**

| Cross[a] | Type | Total | Seedling phenotype: Tall | Seedling phenotype: Triple response | $\chi^2$ |
|---|---|---|---|---|---|
| *CIN1/CIN1* (WS) × *cin1/cin1* | $F_1$ | 24 | 0 | 24 | |
| | $F_2$ | 279 | 63 | 216 | 0.87[b]; $P > 0.05$ |
| *CIN2/CIN2* (WS) × *cin2/cin2* | $F_1$ | 33 | 0 | 33 | |
| | $F_2$ | 613 | 144 | 469 | 0.74[b]; $P > 0.05$ |
| *cin2/cin2* × *cin1/cin1* | $F_1$ | 18 | 0 | 18 | |
| | $F_2$ | 108 | 42 | 66 | 1.0[c]; $P > 0.05$ |
| *cin1/cin1* × *acs5/acs5* | $F_1$ | 31 | 0 | 31 | |
| *cin2/cin2* × *acs5/acs5* | $F_1$ | 11 | 0 | 11 | |
| *CIN3/CIN3* (Col) × *cin3/cin3* | $F_1$ | 18 | 18 | 0 | |
| | $F_2$ | 430 | 244 | 186 | 76.4[d]; $P < 0.05$ |
| *CIN4/CIN4* (Col) × *cin4/cin4* | $F_1$ | 27 | 0 | 27 | |
| | $F_2$ | 262 | 59 | 203 | 0.86[b]; $P > 0.05$ |
| *cin4/cin4* × *acs5/acs5* | $F_1$ | 48 | 0 | 48 | |

[a] Ecotype shown in parenthesis.
[b] Chi-squared calculated for an expected 3:1, wild type:mutant ratio.
[c] Chi-squared calculated for an expected 9:7, wild type:mutant ratio.
[d] Chi-squared calculated for an expected 1:3, wild type:mutant ratio.

**TABLE 2**

**Map positions of cytokinin-insensitive mutants**

| Mutation | Marker | Chromosome | Chromosomes scored (*n*) | Recombination[a] (%) |
|---|---|---|---|---|
| *cin1* | nga1107 | 4 | 88 | 16 ± 5 |
| *cin2* | nga111 | 1 | 86 | 19 ± 4 |
| *cin2* | nga128 | 1 | 88 | 42 ± 5 |
| *cin3* | AthS0191 | 5 | 68 | 38 ± 6 |
| *cin3* | nga129 | 5 | 44 | 23 ± 6 |
| *cin4* | nga162 | 3 | 98 | 5 ± 2 |
| *cin4* | nga172 | 3 | 92 | 11 ± 3 |

[a] Recombination frequency was corrected using Haldane's function. A 95% confidence interval based on a normal distribution is given.

weakly de-etiolated (Figure 4), consistent with the 1:1, wild type:mutant ratio that is expected if these mutations fail to complement. Thus, *cin4* is allelic to *fus9-1* and we have re-named it *fus9-2*. *fus9-1* etiolated seedlings show little hypocotyl elongation, have opened cotyledons and accumulate high levels of anthocyanin, and light-grown plants are severely stunted and do not produce seed. (Castle and Meinke 1994; Wei *et al.* 1994). The phenotype of *cin4* is consistent with it being a weak *cop10*/*fus9* allele: only 10% of dark-grown seedlings are de-etiolated; anthocyanin production is only slightly elevated; and the hypocotyls of etiolated seedlings are only slightly shorter than wild type (see below). In addition to these phenotypes, etiolated *cin4* seedlings have enlarged cotyledons and their hypocotyls twist or spiral, sometimes plunging the cotyledons into the media. The *cin4* rosettes are much smaller than wild type, their leaves are narrower and twisted, and the inflorescences are shorter (not shown). These phenotypes are consistent with a weak *cop10*/*fus9* allele. The *cin4* adult phenotype cosegregates with cytokinin-insensitivity: 164 out of 164 $F_2$ plants that displayed the *cin4* seedling phenotype also had the *cin4* adult phenotype.

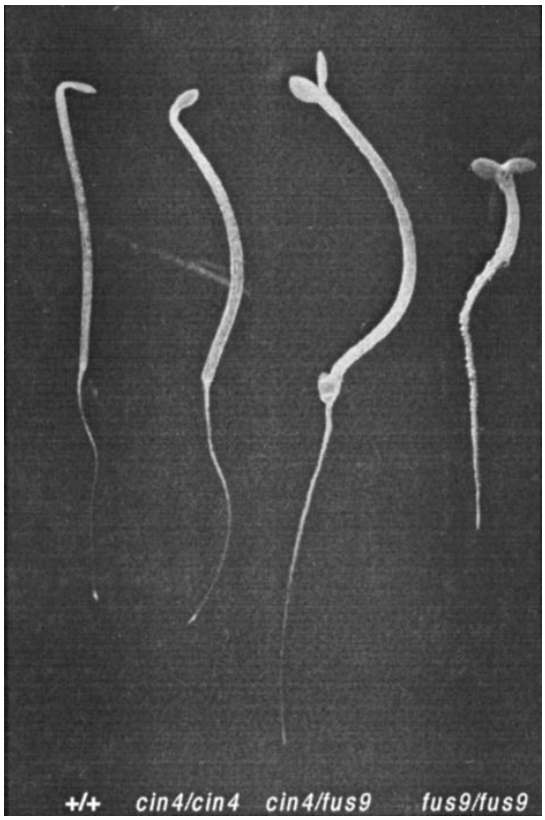


Figure 4.—Phenotype associated with *fus9*/*cop10* alleles. Seedlings were grown for three days at 23° in the dark. Representative seedlings were picked and photographed. The genotype of each seedling is noted below.

**Other ethylene inducers:** To determine if the ethylene underproduction of the mutants was specific for cytokinin, the amount of ethylene produced by etiolated seedlings in response to other inducers of ethylene biosynthesis and by various adult tissues was measured. In addition to cytokinins, wild-type, etiolated Arabidopsis seedlings produce high levels of ethylene in response to cupric ion, auxin and brassinosteroids (K. Woeste, J. Vogel and J. Kieber, unpublished observations). Auxin has been found to increase the steady-state level of *ACS4* mRNA in Arabidopsis (Abel *et al.* 1995). The mechanism for induction of ethylene biosynthesis by cupric ion and brassinosteroid is unknown.

The ethylene produced by *cin1*, *cin3* and *cin4* in response to 2,4-D and $CuSO_4$ was not significantly different from wild type (Table 3). In addition, *cin1* and *cin3* flowers, siliques, leaves and wounded leaves all produced wild-type levels of ethylene. Therefore, it seems likely that *cin1* and *cin3* are not involved in the regulation of ethylene biosynthesis by the stimuli examined. *cin4* leaves produced slightly elevated levels of ethylene, but this may be due to an indirect effect of the mutation, such as an induction of stress responses. We can conclude from the amount of ethylene produced in response to auxin that *cin1*, *cin3* and *cin4* have wild-type auxin sensitivity, which was supported by analysis of root elongation in the presence of various doses of 2,4-D (not shown). The four Cin mutants also displayed wild-

**TABLE 3**

**Ethylene production in wild-type and mutant Arabidopsis**

| | WS | *cin1* | *cin2* | Col. | *cin3* | *cin4* |
|---|---|---|---|---|---|---|
| Treatment[a] | Ethylene produced (pl·seedling$^{-1}$·24 hr$^{-1}$)[b] | | | | | |
| Control | 21 ± 4 | 18 ± 3 | 12.5 ± 24 | 18 ± 4 | 15 ± 5 | 13 ± 3 |
| BA (10 μmol) | 151 ± 18 | 36 ± 2 | 52 ± 9 | 69 ± 11 | 28 ± 11 | 38 ± 4 |
| 2,4-D (50 μmol) | 179 ± 36 | 181 ± 44 | 116 ± 12 | 81 ± 13 | 95 ± 11 | 103 ± 15 |
| $CuSO_4$ (20 mM) | 256 ± 28 | 236 ± 32 | 110 ± 10 | 144 ± 14 | 146 ± 41 | 164 ± 36 |
| 24-epibrassinolide (600 nM) | 178 ± 34 | 128 ± 12 | 96 ± 19 | 141 ± 36 | 77 ± 12 | 221 ± 58 |
| Tissue | Ethylene produced (pl·mg$^{-1}$·hr$^{-1}$) | | | | | |
| Flowers | 36 ± 6 | 40 ± 2 | 11 ± 2 | 70 ± 28 | 80 ± 10 | 71 ± 20 |
| Siliques | 124 ± 5 | 124 ± 23 | 118 ± 14 | 202 ± 15 | 192 ± 18 | 136 ± 32 |
| Leaves | 0.9 ± 0.4 | 1.0 ± 0.2 | 1.0 ± 0.3 | 1.0 ± 0.3 | 1.3 ± 0.1 | 2.2 ± 0.7 |
| Wounded leaves | 8 ± 1 | 9 ± 2 | 9 ± 3 | 11 ± 2 | 10 ± 3 | 16 ± 6 |
| Light-grown seedlings | Ethylene produced (pl·seeding$^{-1}$·3 days$^{-1}$) | | | | | |
| | 102 ± 22 | 85 ± 9 | 87 ± 23 | 100 ± 9 | 94 ± 18 | 103 ± 21 |

[a] Refers to treatment of two-day-old etiolated seedlings. These concentrations of BA, 2,4-D, $CuSO_4$ and 24-epibrassinolide were chosen because they correspond to the peak of ethylene production observed in dose-reponse analysis in etiolated seedlings (K. Woeste, J. P. Vogel and J. J. Kieber, unpublished observations).

[b] Ethylene measurements are ± SD, based on 3 or 4 replicas.

type inhibition of root elongation in response to ABA (not shown) suggesting that they are not altered in ABA sensitivity. Etiolated *cin1*, *cin2* and *cin3* mutant seedlings make slightly less ethylene in response to the brassinosteroid 24-epibrassinosteroid than wild type. Brassinosteroids and cytokinins may interact in etiolated Arabidopsis seedlings: wild-type seedlings treated with cytokinins and mutants defective in brassinosteroid biosynthesis both adopt a de-etiolated morphology (Chory *et al.* 1994; Li *et al.* 1996). This interaction may explain the slight effect of *cin2* and *cin3* on ethylene biosynthesis in response to brassinosteroids. The *cin2* mutation affects ethylene biosynthesis under several additional conditions: *cin2* flowers produce 1/3 as much ethylene as wild-type flowers; etiolated *cin2* seedlings display a smaller induction of ethylene in response to auxin and $CuSO_4$. This suggests that CIN2 may be a general regulator of ethylene biosynthesis.

**Adult phenotypes:** *cin1* and *cin3* have very subtle adult phenotypes. *cin1* plants were yellow at the leaf edges under some growth conditions, and *cin3* plants were slightly larger than wild type. Siliques from *cin2* mutants were slightly shorter than wild type and were club shaped (not shown). In addition, *cin2* mutants contained slightly less total chlorophyll than wild-type leaves (not shown). As discussed above, *cin4* plants have a phenotype consistent with a weak *fus9/cop10* allele.

**Other cytokinin responses:** To determine if the mutants were affected in other cytokinin responses or only cytokinin-induced ethylene biosynthesis, the effect of cytokinin on shoot initiation, anthocyanin production, de-etiolation, senescence and gene expression was measured. To determine the level of shoot-initiation in response to cytokinin, root explants were placed on callus-inducing media for one week and then transferred to shoot-initiation media supplemented with various levels of the cytokinin 2iP. After one month, shoot clusters were counted. Clusters of shoots were counted rather than individual shoots because it was very difficult to identify individual shoots within a cluster. *cin1* showed significantly less shoot-initiation than wild type at all levels of 2iP tested, except for the highest (Table 4). In addition, *cin1* shoots were generally less developed than wild-type shoots (not shown). This indicates that *cin1* is resistant to the promotion of shoot-initiation by cytokinin. In contrast, the *cin2* mutant was indistinguishable from wild type in this assay. There was no shoot-initiation observed for the Columbia ecotype in these experiments, and thus we were unable to evaluate the response of the *cin3* or *cin4* mutants.

*Induction of anthocyanin:* The production of anthocyanin by Arabidopsis seedlings in response to cytokinin has been described (Deikman and Hammer 1995). Both the *cin1* and *cin2* mutants produced significantly less anthocyanin in response to several concentrations of BA (Figure 5). In addition, *cin1* and *cin2* produced less, but not significantly less, anthocyanin at the other BA concentrations tested. Ethylene does not seem to play a role in the accumulation of anthocyanin in response to cytokinin because *ein2*, a strong ethylene-insensitive mutant, showed wild-type accumulation of anthocyanin in response to cytokinin (not shown). *cin3* produced wild-type levels of anthocyanin and is therefore not resistant to cytokinin in this assay. The *cin4* mutant has slightly higher levels of anthocyanin in the controls and at all cytokinin concentrations. This is consistent with

**TABLE 4**

**Shoot initiation in Cin mutants**

| Genotype | Mean number of shoots per explant | | | |
|---|---|---|---|---|
| | 0.25 μmol 2iP | 0.5 μmol 2iP | 1.0 μmol 2iP | 5.0 μmol 2iP |
| Wild type (WS) | 1.4 ± 0.9 | 0.6 ± 0.3 | 0.9 ± 0.6 | 0.6 ± 0.5 |
| *cin1/cin1* | 0.7 ± 0.6* | 0.2 ± 0.2* | 0.3 ± 0.3* | 0.4 ± 0.4 |
| *cin2/cin2* | 1.9 ± 0.1 | 0.8 ± 0.5 | 0.8 ± 0.4 | 0.6 ± 0.2 |

*Significantly less ($P < 0.05$) than wild type as determined by ANOVA.

this being a weak allele of *fus9/cop10*, which contain high levels of anthocyanin.

*De-etiolation:* Arabidopsis plants grown on cytokinin in the dark have a de-etiolated, light-grown appearance: shortened hypocotyl, expanded cotyledons, expanded true leaves and partial conversion of etioplasts into chloroplasts (Chory *et al.* 1994). This striking effect on morphology was used as the basis for another cytokinin-sensitivity assay. It should be noted that the triple response observed in response to cytokinin occurs much sooner (three days for the triple response verses >10 days for de-etiolation) and at much lower concentrations of cytokinin than the de-etiolation response. Seeds were plated on medium supplemented with increasing concentrations of BA and incubated in the dark for two weeks. *cin1*, *cin2* and *cin3* appeared similar to wild type at all concentrations of cytokinin (not shown). Approximately ten percent (14 out of 134) of *cin4* plants grown on media without added BA displayed a de-etiolated morphology, and treatment with 5 μmol BA increased this proportion to 85% (66 out of 78). No wild-type plants grown on media without BA were de-etiolated, but in the presence of 5 μmol BA approximately ten percent (5 out of 47) displayed a de-etiolated phenotype. In the presence of 50 μmol BA, all *cin4* and wild-type seedlings were de-etiolated. These observations are consistent with *cin4* being a weak *fus9/cop10* allele.

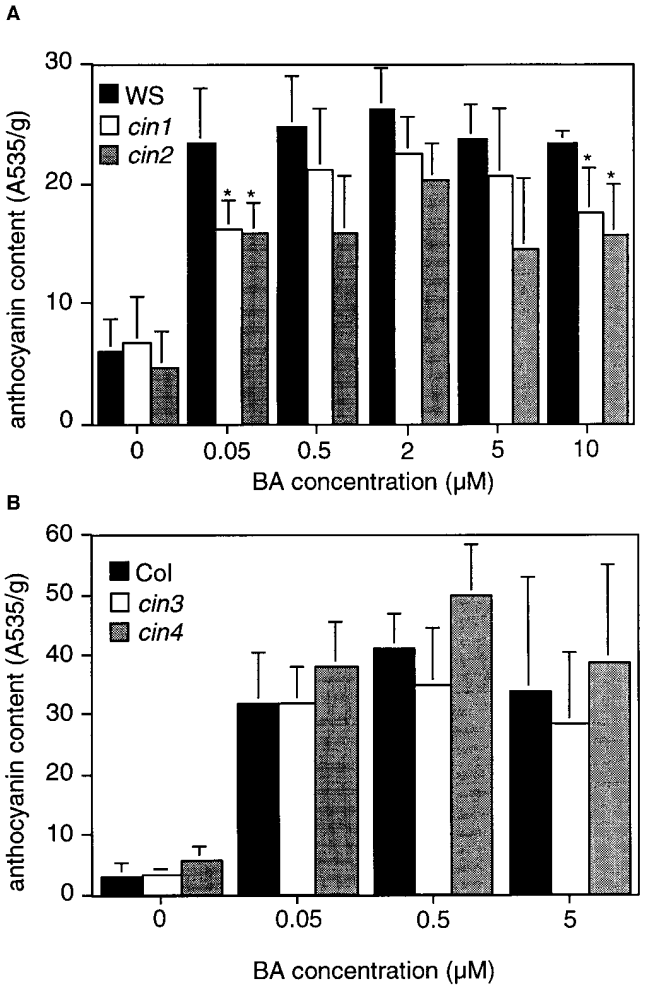


Figure 5.—Accumulation of anthocyanin in response to cytokinin. Anthocyanin was extracted from 10-day old plants grown in the light on media supplemented with the indicated level of BA. Anthocyanin was extracted from approximately 20 plants for each replicate. Error bars are ± SD based on four replicates. Bars marked with an * were significantly different from wild type, $P < 0.05$, based on ANOVA.

*Leaf senescence:* Exogenous application of cytokinin delays the senescence of detached leaves. Recently, an elegant study has clearly demonstrated the inhibition of senescence by endogenous cytokinin by transforming tobacco with the cytokinin biosynthetic gene *ipt*, from *Agrobacterium tumefaciens*, under the control of a senescence-specific promoter (Gan and Amasino 1995). The resulting transgenic plants showed greatly delayed senescence of detached leaves and intact plants. We assayed senescence in wild-type and mutant plants to determine if the Cin mutations affected this cytokinin response. The amount of chlorophyll present in leaves 10–14 days after detachment was used to infer the degree of senescence (see materials and methods). The senescence of all four Cin mutants was similar to wild type, indicating that they have wild-type cytokinin sensitivity in this assay (not shown).

*Regulation of a cytokinin-induced gene:* We have isolated a gene, *IBC6* (Induced By Cytokinin), that is rapidly (<10 min) and specifically induced by cytokinins in Arabidopsis (I. Brandstatter and J. Kieber, unpublished results). We examined the expression of *IBC6* in

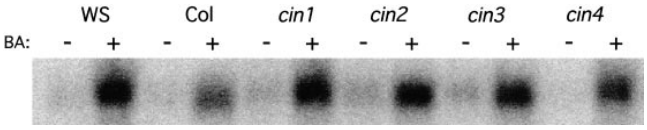


Figure 6.—Expression of a cytokinin-induced gene in the Cin mutants. Seedlings of the indicated genotype were either exposed to water (−) or to 5 μmol BA (+) for 15 min. Total RNA was extracted and 15 μg analyzed by northern blotting. Equal loading was confirmed by ethidium bromide staining (not shown). The blot was hybridized with an *IBC6* cDNA clone. Exposure to BA for 2 hr gave comparable results (not shown).

the Cin mutants in response to exogenous cytokinin by Northern blot analysis (Figure 6). In the four Cin mutants, as well as in the *cyr1* mutant (not shown), *IBC6* was induced to wild-type levels by BA, suggesting that these mutations do not disrupt the rapid expression of this gene.

## DISCUSSION

We have identified four mutants that fail to elevate ethylene biosynthesis in response to cytokinin. These mutants affect either cytokinin signaling elements, general ethylene regulatory elements or other regulatory pathways that feed into the signaling events between cytokinin perception and increased *ACS5* function. Based on our analysis of ethylene biosynthesis under various conditions in the Cin mutants and their responsiveness in other cytokinin assays, we can begin to distinguish among these possibilities for each mutant.

The *cin1* mutation disrupts ethylene biosynthesis specifically in response to cytokinins. *cin1* also affects the induction of shoots in culture and the production of anthocyanin in light grown seedlings in response to cytokinin. Thus, the *cin1* mutation affects three distinct cytokinin responses, which suggests that it disrupts a general cytokinin signaling element. However, the *cin1* mutation only slightly dampens these responses and has no significant effect on other cytokinin responses, including the induction of a cytokinin-regulated gene. In addition, *cin1* has only minor effects on the morphology of adult Arabidopsis plants, which is somewhat surprising for a cytokinin-insensitive mutation given the central role that cytokinins have been postulated to play in plant development. One model to account for these observations is that *CIN1* may be partially genetically redundant and/or is only active in a subset of tissues or cells. A second possibility is that there are multiple independent cytokinin signaling pathways in Arabidopsis that lead to different responses and *cin1* only disrupts a subset of these. A third possibility is that *CIN1* acts downstream of those signaling events leading to the regulation of leaf senescence and *IBC6* induction. Finally, it is possible that the *cin1* mutation is only a partial loss-of-function allele and that it lowers the threshold of cytokinin signaling below that required for some responses, such as the activation of ACS5, but not below that required for other cytokinin responses. This latter model is supported by the low frequency with which *cin1* was isolated (see also below).

The *cin2* mutation clearly affects multiple conditions of elevated ethylene production, suggesting that it may be a general regulator of ethylene biosynthesis. However, it also has a slight, but significant effect on cytokinin-induction of anthocyanin biosynthesis and also affects silique morphology as well as chlorophyll content of leaves. The pleiotropic nature of this mutation suggests that *CIN2* may participate in multiple regulatory pathways. Alternatively, it is possible that there are two closely-linked mutations in this line that are responsible for the diverse phenotypes. The isolation of additional *cin2* alleles of should address this point.

*cin3* is unaffected in any other cytokinin response examined and does not affect ethylene biosynthesis in response to any other inducer or in any other tissue. *CIN3* may not be a general cytokinin signaling element nor a general regulator of ethylene biosynthesis, but rather may act specifically in the signaling from cytokinin to ACS5.

The *cin4* mutation is clearly allelic to the constitutive photomorphogenic mutant *cop10/fus9*, which highlights the interaction between light and cytokinin in the regulation of ethylene biosynthesis. Cytokinins are known to regulate numerous light-regulated genes (Crowell and Amasino 1994), and growth of Arabidopsis seedlings in the dark for more than ten days in the presence of cytokinin results in a de-etiolated phenotype (Chory *et al.* 1994). Furthermore, the Arabidopsis de-etiolation mutants *det1* and *det2* display enhanced sensitivity to cytokinin in senescence and tissue culture assays (Chory *et al.* 1994). Light severely dampens the induction of ethylene biosynthesis by cytokinins (2–3-fold in the light *vs.* 8-fold in the dark), and in the light cytokinin does not act through the *ACS5* isoform as it does in the dark (Vogel *et al.* 1988). The genetic screen used in this study is for mutants with a longer hypocotyl than the wild type on cytokinin, and yet strong *fus9/cop10* alleles result in shortened hypocotyls because they mimic the effects of light (Castle and Meinke 1994; Wei *et al.* 1994). Indeed, in the absence of light, *fus9-2* (*cin4*) seedlings are slightly shorter (see Figure 3). This suggests that the *fus9-2* allele may partially uncouple the effects of light on cytokinin induction of ethylene from its effects on hypocotyl elongation.

The Cin mutants were identified at a low frequency (4 Cin mutants/$10^6$ $M_2$ seedlings *vs.* approximately 1 ethylene-insensitive mutant/$10^4$ $M_2$ seedlings) suggesting that they may not be simple loss-of-function alleles. One explanation for this low frequency is that strong alleles of these mutations could be lethal or infertile, which is certainly the case for strong alleles of *fus9/cop10*. Consistent with this, loss-of-function *acs5* alleles, which

have no effect on viability, were isolated at a five-fold higher frequency than *cin1-cin4* in this screen. This may explain why only subtle phenotypes are observed in the *cin1*, *cin2* and *cin3* alleles that we have obtained even though one might expect significant disruption of cytokinin signaling would lead to drastic morphological and/or developmental changes.

The mutants identified in this study represent the first step in elucidating the signaling events leading from cytokinin to increased ACS5 function. This screen is obviously far from saturated, as only single alleles have been found. Further characterization of these four Cin mutants, and ultimately of these genes, and the isolation of additional Cin loci should significantly enhance our understanding of this signaling pathway.

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