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A Single Genetic Locus, *Ckr1*, Defines *Arabidopsis* Mutants in which Root Growth Is Resistant to Low Concentrations of Cytokinin[1]

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ABSTRACT

***Arabidopsis* mutants resistant to cytokinin (benzyladenine [BA]) have been isolated with the intent to find plants defective in cytokinin perception or response. At low concentrations, BA produces a "cytokinin root syndrome" in which primary root elongation is inhibited, but root hair elongation is stimulated. Five independent mutants that did not express this syndrome in the presence of BA were selected. All five mutants were recessive, and crosses between them indicated that they were in the same complementation group. The genetic locus represented by these mutations has been designated *ckr1* and mapped to chromosome 5.**

The analysis of mutants with altered responses to hormones is an important approach to understanding hormone-signaling mechanisms in plants. Because high levels of hormones usually inhibit rather than promote plant growth, mutant plants resistant to high concentrations of exogenously supplied hormones have been selected in a variety of plant systems. Mutant plants with altered responses to the plant hormones auxin, gibberellin, ABA, and ethylene have been described (see recent reviews in refs. 9, 13, and 15).

Much progress has been made in isolating and characterizing mutants with altered responses to auxin. Estelle and Somerville (6) isolated a number of auxin-resistant mutants in *Arabidopsis* in which root growth was resistant to high levels of auxin. Mutants in one group were recessive at a locus called *axr1* (11). Although one *axr1* mutant line was less severe than the others, all affected leaf and flower morphology. Mutant plants were shorter than nonmutants and displayed reduced apical dominance. Another mutant line carried a dominant mutation at a locus called *axr2*. These mutants were dwarfed, had abnormal gravitropic responses, and were resistant to growth-inhibiting concentrations of ethylene and ABA. Bitoun *et al.* (1) isolated *Nicotiana plumbaginifolia* mutants by selection for auxin resistance at the seedling stage. The authors obtained recessive mutants that defined three different complementation groups, *iba1*, *iba2*, and *iba3*. The *iba1/iba1* plants also displayed cross-resistance to ABA and to an inhibitor of GA_3 biosynthesis, paclobutrazol. The authors suggested that the cross-resistance was a result of the interaction of the hormones in promoting normal growth. Recently, new *N. plumbaginifolia* mutants have been isolated that are not resistant but are more sensitive to auxin (5).

Less progress has been made in isolating mutant plants with altered responses to cytokinin. There is only one report of a higher plant mutant selected on the basis of its resistance to cytokinin. The defect in the mutant does not appear to be specific to cytokinin. Instead, the mutant seems to be less growth inhibited in response to general stresses produced by high cytokinin concentrations (2).

In this report, we describe the isolation and characterization of cytokinin-resistant mutants in *Arabidopsis*. To avoid mutants with defects not specifically related to cytokinin responses, we selected mutants at relatively low concentrations of cytokinin. Five independent mutants were identified and found to be mutations in the same complementation group, defining a locus called *ckr1* in *Arabidopsis*.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All mutant lines described in this report were derived from the *Arabidopsis thaliana* Columbia ecotype (Col-0). For growing sterile plants, seeds were sterilized and grown on 0.8% agar medium as described by Estelle and Somerville (6) in an incubator at 21°C with continuous illumination at 30 to 80 $\mu E/m^2/s$. Selective medium contained 2.5 μM of N^6-BA added after autoclaving. Nonsterile plants were grown in soil at 22 to 24°C with continuous illumination at 75 to 100 $\mu E/m^2/s$.

Cytokinin-resistant mutants were selected from EMS[2]-mutagenized M_2 seedling populations. *Arabidopsis* seeds were subjected to EMS mutagenesis according to the method of Lincoln *et al.* (11). To select for mutants, M_2 seeds were sterilized and sown from a suspension in 0.1% agar into slits cut in sterile agar medium in Petri plates. Usually, three slits were cut in a plate, and about 100 seeds were sown per slit. Seeds were introduced into the slit to force root growth through the agar and not on the surface. Plates were sealed

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[2] Abbreviations: EMS, ethyl methane sulfonate; RFLP, restriction fragment length polymorphism; SSC, standard sodium citrate.

with surgical tape and placed on edge in a vertical position in the incubator.

DNA Manipulation and the RFLP Mapping

The chromosomal location of the *Ckr1* gene was determined by measuring the recombination frequency between the mutant gene and RFLP markers mapped by Chang *et al.* (3). A homozygous *ckr1-7* plant (Columbia ecotype) was crossed to a wild-type plant of the Landsberg ecotype (La-0), and the resulting F_1 plants were selfed to generate F_2 plants segregating for both the *ckr1* mutation and RFLPs between the Columbia and Landsberg ecotypes. Seeds were collected from F_2 plants and tested for cytokinin resistance. About 100 seedlings resistant to cytokinin were used to establish F_3 lines for DNA blot analysis.

Total DNA was isolated from leaves by the method of Cone (4). Approximately 5 to 10 μg of DNA were digested overnight with a fivefold excess of a restriction enzyme and subjected to gel electrophoresis through a 0.8% agarose gel at 650 V × h. DNA was blotted and cross-linked to Hybond-N filters (Amersham) following the manufacturer's instructions. ^{32}P-labeled DNA probes were generated using the random priming method (7). Filters were prehybridized for 6 to 10 h and hybridized (10^6–10^7 cpm/mL) for 18 to 24 h at 65°C in a solution containing 5× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate), 0.6% SDS, 50 μM sodium phosphate (pH 7.5), 2.5 μM EDTA, 0.1% Ficoll, 0.1% PVP, 0.1% BSA, and 5% dextran sulfate. Filters were washed at 65°C in 2× SSC containing 0.1% SDS for 30 min, 1× SSC for 30 min and 0.5× SSC for 30 min. Filters were exposed to x-ray film for 1 to 10 d at −80°C with an intensifying screen or analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Inhibition of Root Growth

To select mutants with altered responses to cytokinin, we characterized the growth of *Arabidopsis* seedlings at various concentrations of exogenously supplied cytokinin. We found that *Arabidopsis* seedlings were sensitive to cytokinins over a wide range of concentrations. At high concentration (100 μM BA), seedling growth was inhibited, the emerging leaves were thickened and cupped, and no growth of the primary root was observed (data not shown). However, because high BA concentrations might produce nonspecific stress responses, an effort was made to select for specific mutants resistant to much lower concentrations of BA.

We found that growth of the primary root and root hairs was very sensitive to BA. Inhibition of primary root growth was observed with concentrations of BA as low as 5 nM, and root growth was generally inhibited in proportion to the log of the BA concentration up to 50 μM (Fig. 1). It was interesting to find that, in as much as primary root growth was inhibited, root hair elongation was stimulated. A dose-response curve showed steady root hair elongation up to 10 μM BA. Hence, *Arabidopsis* seedlings grown in 2.5 μM BA have a characteristic "cytokinin root syndrome" with shorter, fuzzier roots than those grown in the absence of BA (Fig. 2). (Note: the effects of BA on root hair elongation were more pronounced when the roots were grown in agar rather than on the surface of agar. The seedlings depicted in Fig. 2 were grown on the surface of agar for photographic purposes.)

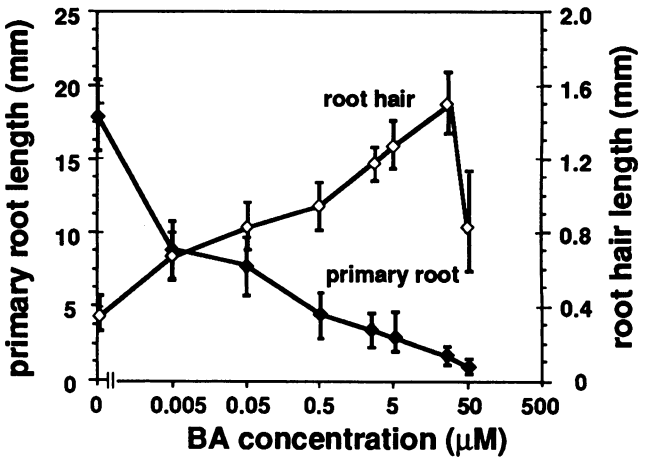

Figure 1. Dose-response curve demonstrating the effects of cytokinin (BA) on primary root growth and root hair elongation in wild-type *Arabidopsis* plants. Primary root length and root hair length were measured after 7 d of growth. Each point represents the mean of a minimum of 20 measurements, and the bar represents the SD from the mean.

Screening for Mutants

Because the root growth syndrome was easy to detect in seedlings grown on vertical plates, we screened EMS-mutagenized populations of *Arabidopsis* M_2 seedlings for resistance to 2.5 μM BA. Groups of 3.5×10^4 M_2 seedlings derived from seven pools of M_1 plants (5000 plants/pool) were screened. We found that resistance appeared in about one of 10^4 seedlings. Nine putative mutants, designated *ckr* mutants, were recovered from 1.4×10^5 seedlings. Five independent *ckr* mutants were studied further, *ckr1*-7, -8, -12, -50, and -109. Three of the five mutants were from different pools, and two mutants were from the same pool. The two mutants from the same pool were judged to be independent because they were phenotypically different. However, the trait distinguishing the two mutants was found to be unrelated and unlinked to cytokinin resistance and was eliminated by backcrossing to the parental line.

The mutants were selfed, and M_3 seedlings were retested for BA resistance. The response to BA in M_3 seedlings is described for two of the mutants, *ckr1*-50 and -109, but the findings are applicable to all of the other mutants. Roots on wild-type seedlings showed the characteristic "cytokinin syndrome," short primary roots and long root hairs, when grown in the presence of 2.5 μM BA, whereas *ckr1*-109 M_3 seedlings did not (Fig. 2). In the absence of exogenous cytokinin, the primary roots on *ckr1*-109 and the other *ckr* mutants were somewhat longer, and the root hairs were shorter than wild type. Root growth in the mutants, as represented by *ckr1*-50, was more resistant to BA than the wild type over a wide range of BA concentrations (Fig. 3, upper panel). For example,

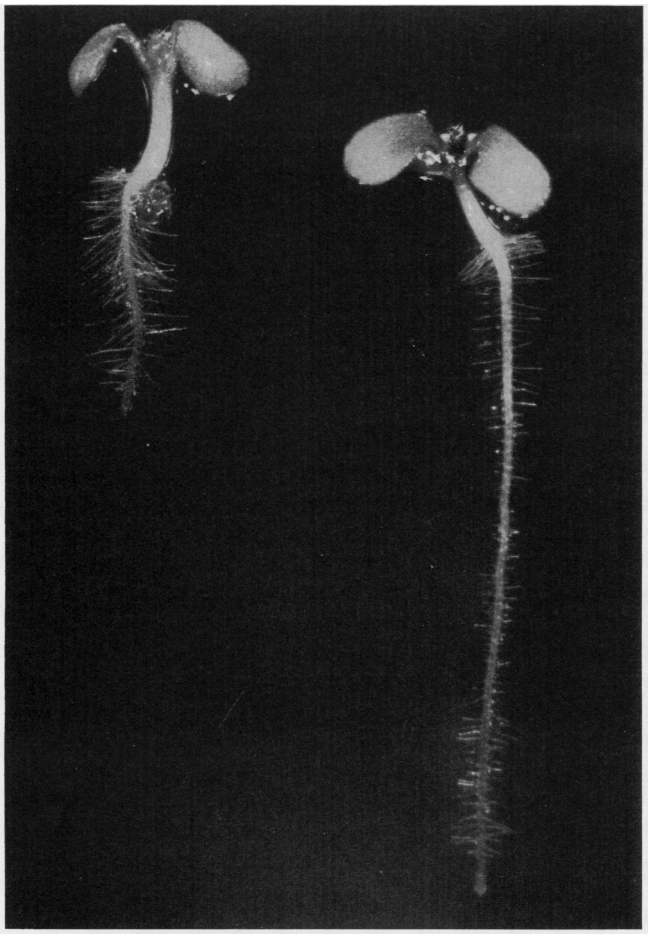

Figure 2. Root growth of wild-type (left) and *ckr1*-109 (right) *Arabidopsis* mutants in the presence of cytokinin (BA). Roots were grown on the surface on agar medium containing 2.5 μM BA. Petri plates containing the medium were incubated in a vertical position and photographed after 8 d of growth.

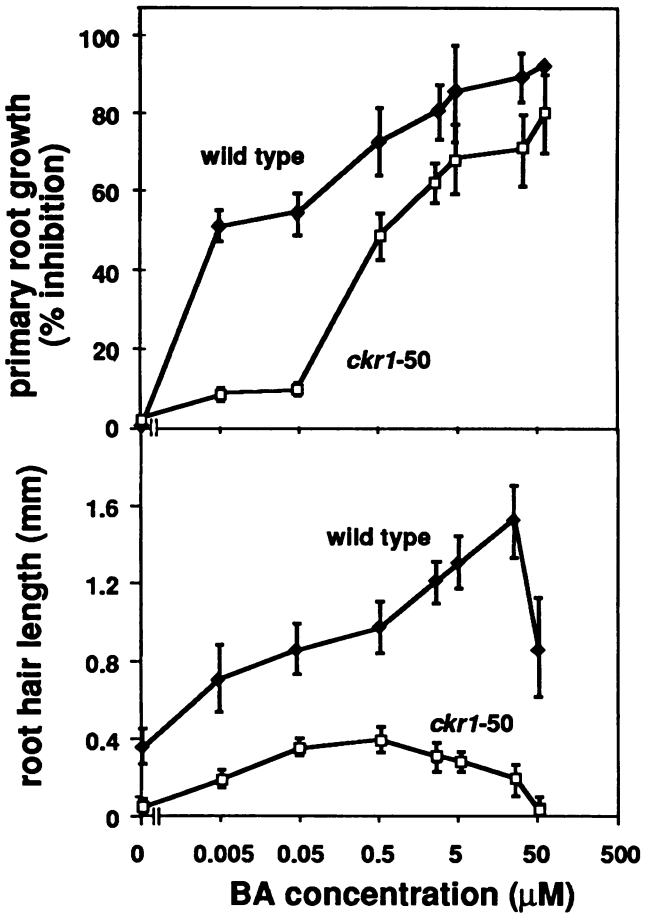

Figure 3. Dose-response curve showing the effects of cytokinin (BA) on root growth in wild-type and mutant plants. Primary root length and root hair length were measured after 7 d of growth. Each point represents the mean of a minimum of 20 measurements, and the bar represents the SD from the mean. Primary root length with no BA (0 μM) was 19.0 ± 2.4 mm for *ckr1*-50 and 17.9 ± 2.8 mm for wild type.

at 50 nM BA, primary root growth on *ckr1*-50 was inhibited <10%, whereas root growth on wild-type seedlings was inhibited by >50%. In the presence of exogenous cytokinin, root hairs on *ckr1*-50 were not stimulated to grow to the same length as roots hairs on wild-type plants at comparable concentrations of BA (Fig. 3, lower panel). In addition, root hairs of the *ckr1*-50 mutant were stimulated to grow to their maximum length (about the length of wild-type root hairs in the absence of BA) at 0.5 μM BA, whereas wild-type root hairs reached maximum length at about 10 μM BA.

Genetic Analysis

The genetic properties of the *ckr* mutants were examined by crossing the mutant lines with wild-type plants and determining the segregation of the resistance traits in the F_1 and F_2 progeny. In the F_1, all the progeny were nearly wild type (Table I), indicating that the resistance traits were recessive. However, on medium containing submicromolar concentrations of BA, the primary roots of the F_1 heterozygotes were somewhat longer than wild type, indicating that the wild-

Table I. *Genetic Analysis of Cytokinin-Resistant Mutants*

The analysis of F_1 and F_2 progeny from crosses between the homozygous *ckr1* mutants and wild-type (wt) *Arabidopsis* plants.

Crosses[a]	Generation	No. of Plants			χ^{2b}
		Total	Wt	Mutant	
wt × *ckr1*-7	F_1	31	31	0	
	F_2	708	543	165	1.08[c]
wt × *ckr1*-8	F_1	25	25	0	
	F_2	526	389	137	0.3[c]
wt × *ckr1*-12	F_1	20	20	0	
	F_2	620	482	138	2.48[c]
wt × *ckr1*-50	F_1	13	130	0	
	F_2	292	221	71	0.072[c]
wt × *ckr1*-109	F_1	12	120	0	
	F_2	453	335	118	0.256[c]

[a] Female parent is listed first. [b] χ^2 calculated based on 3:1 ratio of wild type to mutant. [c] $P > 5\%$.

type allele was not completely dominant. Nonetheless, at the concentration of BA at which the progeny were tested, heterozygotes were difficult to distinguish from the homozygous wild type and, therefore, they were scored as wild type. In the F_2, the resistance traits segregated about 3:1, again confirming the near-recessive character of the resistance traits and demonstrating that the traits in all the mutant lines tested segregated as single Mendelian mutations.

To determine the number of complementation groups represented by the various mutant lines, the lines were crossed to each other. It was found that none of the mutants were able to complement each other, *i.e.* all of the F_1 progeny were cytokinin resistant (Table II). The failure to complement would be expected if the mutations were allelic. To determine whether that was the case, segregation of the cytokinin resistance in the F_2 generation was scored. No cytokinin-sensitive progeny were observed in the F_2 generation, even when more than 200 progeny were analyzed (Table II). Thus, the noncomplementing properties of these independently derived mutants appear to be due to the fact that the mutations are at the same genetic locus. This locus was designated the *ckr1* locus.

Mapping the *ckr1* Locus

The *ckr1* locus was mapped by crossing *ckr1-7* to the Landsberg ecotype and analyzing the DNA from F_3 lines for RFLPs. The DNA was analyzed with 20 different RFLP probes representing markers scattered throughout the genome (3). Cytokinin resistance and a marker at one end of chromosome number 5 (RFLP211) showed significant linkage (Table III).

Other Properties of the *ckr1* Mutants

The phenotypes of *ckr1* mutants were subtle when grown in soil (in the absence of cytokinin). The phenotypes were assessed for the progeny of mutants that had been subjected to two backcrosses with wild type. The cotyledon leaves on the *ckr1* mutant seedlings were usually larger and slightly yellower than the wild type. At the seedling stage, the rosette leaves of all the *ckr1* mutants were slightly cupped and yellower when grown under constant illumination but not when grown in a 12-h dark and 12-h light cycle. As mature plants the *ckr1* mutants had normal flowers, were fertile, and were similar in stature and branching pattern to wild-type plants (Fig. 4). Nonetheless, the roots in the mature mutant plants were longer than their wild-type counterparts. When germinated on the soil surface, the roots of seedlings (*ckr1-7* and *ckr1-12*) appeared to have a slightly altered gravitropic response. The roots tended to grow along the surface and penetrated the soil less easily.

The hormone resistance in *ckr1* mutants appeared to be specific to cytokinin. When different cytokinins, such as dihydrozeatin, kinetin, and zeatin, were tested, similar resistance responses of the *ckr1* mutants were observed (data not shown). Because several *Arabidopsis* mutants selected for resistance to auxin are also reported to be resistant to other hormones including cytokinin (10, 16), we determined whether the *ckr1* mutants were resistant to auxin (IAA). Our

Table III. *Cosegregation of ckr1 with RFLP Markers on Chromosome 5*

The analysis of F_3 lines from the cross between *ckr1-7* (Columbia) and wild-type (Landsberg) *Arabidopsis* plants.

Markers	Recombination Frequency[a]	No. Scored[b]
	%	
RFLP247	46.4 ± 6.6	28
RFLP558	42.4 ± 3.7	33
RFLP211	25.0 ± 4.6	44

[a] Recombination frequency (%) ± SD calculated according to method of Hinze *et al.* (8). [b] Number of F_3 lines scored.

Table II. *Complementation Analysis of ckr Mutants*

The analysis of F_1 progeny from crosses between homozygous *ckr1* mutants and F_2 progeny resulting from the selfing cross of F_2 plants.

Crosses[a]	No. of F_1 Progeny			No. of F_2 Progeny		
	Total	Resistant	Sensitive	Total	Resistant	Sensitive
ckr1-7 × *ckr1-8*	3	3	0	73	73	0
ckr1-7 × *ckr1-12*	15	15	0			
ckr1-8 × *ckr1-7*	33	33	0			
ckr1-12 × *ckr1-7*	17	17	0	44	44	0
ckr1-12 × *ckr1-8*	11	11	0	190	190	0
ckr1-50 × *ckr1-7*	25	25	0	209	209	0
ckr1-50 × *ckr1-8*	14	14	0	83	83	0
ckr1-50 × *ckr1-12*	20	20	0	52	52	0
ckr1-50 × *ckr1-109*				61	61	0
ckr1-109 × *ckr1-7*	39	39	0			
ckr1-109 × *ckr1-8*	20	20	0	61	61	0
ckr1-109 × *ckr1-12*	5	5	0			
ckr1-109 × *ckr1-50*	13	13	0			

[a] Female parent is listed first.

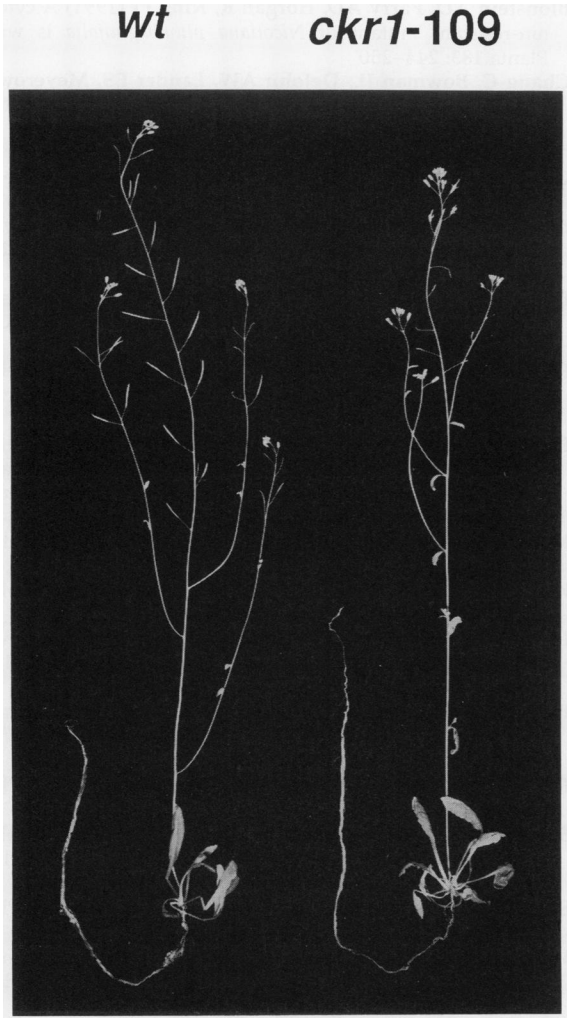

Figure 4. Comparison of mature wild-type (wt) and *ckr1*-109 plants grown in soil (in the absence of cytokinin).

results indicated that the *ckr* mutants, for which *ckr1*-50 was representative, were not resistant to auxin (Fig. 5).

DISCUSSION

Cytokinin-Resistant Mutants

To date, there is only one other report of a higher plant mutant selected on the basis of its resistance to cytokinin. The *ckr1* mutant in *N. plumbaginifolia* was selected at high concentrations of cytokinin (2). The primary lesion in this mutant is not known, but it appears that the defect may be unrelated to cytokinin metabolism or perception. Instead, the mutant seems less able to mount a response to general stresses such as high cytokinin concentrations. The mutant has a wilty phenotype, shows little ABA induction following stress, and apparently is blocked in the last step of ABA biosynthesis (12).

For these reasons, we sought another approach to isolate mutants with altered responses to cytokinin. We selected mutants resistant to much lower concentrations of cytokinins. We found that *Arabidopsis* roots were exquisitely sensitive to cytokinins. When BA was supplied in a range from 10^{-9} to 10^{-5} M, primary root growth was reduced, and root hair elongation was stimulated in proportion to the log of the BA concentration. The shorter, fuzzier root constituted a cytokinin root syndrome, and mutants were selected that did not show the root syndrome in the presence of BA.

Finding that all of the mutants selected by this very general screen represent mutations at a single genetic locus was very surprising. It might be expected that the pathways from hormone perception to root growth response involve many steps, and all of those steps might be subject to mutation. However, mutations at certain steps in a hormone signal transduction pathway might be lethal. Of course, we cannot discount the fact that the *ckr1* mutation might not have anything to do with the normal cytokinin-signaling pathway, *e.g.* the mutant might be involved in a generalized stress response to exogenous cytokinin. We tried to minimize that possibility by selecting for resistance to low concentrations of cytokinin. Although the *ckr1* mutants have an altered root morphology in the absence of exogenous cytokinin, we contend that the *ckr1* mutants are, indeed, cytokinin response mutants and not simply root morphology mutants. This is because these mutants show a differential sensitivity to cytokinin relative to wild type but not to other agents that affect root growth, such as auxin.

The phenotypes of the *ckr1* mutants are less severe than one might expect for plants with dysfunctional cytokinin response pathways. If the mutations are genuinely involved in cytokinin responses, then we conclude either that the *ckr1* mutants are leaky or that there are alternative cytokinin response pathways. On the other hand, it is possible that we have selected for mutants in which the defect in cytokinin perception or response is organ specific. The shoot is usually

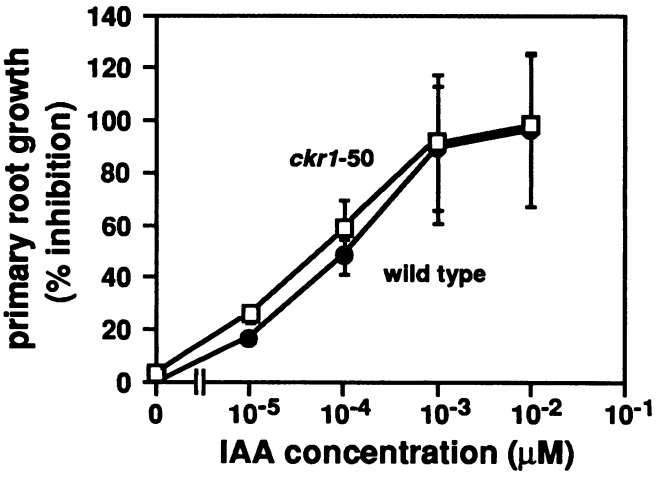

Figure 5. Dose-response curve for demonstrating the effects of auxin (IAA) on primary root growth in wild-type and *ckr1*-50 mutant seedlings. Primary root length was measured after 10 d of growth. Each point represents the mean of a minimum of 20 measurements, and the bar represents the SD from the mean. Primary root length with no BA (0 μM) was 33.6 ± 5.0 mm for *ckr1*-50 and 26.0 ± 4.2 mm for wild type.

considered the target for cytokinin action. In these mutants, the shoot is reasonably normal, although the young rosette leaves are more cup shaped and yellow.

In the absence of exogenous cytokinin, the primary roots of the *ckr1* mutants are longer, and the root hairs are shorter than wild type. We propose that endogenous cytokinins may counteract the growth of primary roots and stimulate the growth of root hairs in normal plants. Therefore, the longer roots and shorter root hairs in the mutant may be due to the fact that the mutant fails to perceive normal levels of endogenous cytokinins.

The *ckr1* mutants are not root hair mutants, such as those described by Schiefelbein and Somerville (14), because the root hair defect in the *ckr1* mutants can be rescued by the addition of cytokinin. We have shown that root growth in wild-type *Arabidopsis* seedlings is the most sensitive indicator of exogenous cytokinins. However, the organ-specific pattern of sensitivity may or may not relate to the fact that the perception and response to low levels of exogenous cytokinin is confined to roots. The levels and patterns of endogenous cytokinins could influence the apparent organ-specific response to exogenous hormone.

The *ckr1* mutation maps near the end of chromosome 5, which sets it apart from other described hormone response mutations. For example, the *AXR1* gene lies on chromosome 1 (11).

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