

# Chromosaponin I Specifically Interacts with AUX1 Protein in Regulating the Gravitropic Response of Arabidopsis Roots

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We have found that chromosaponin I (CSI), a  $\gamma$ -pyronyl-triterpenoid saponin isolated from pea (*Pisum sativum* L. cv Alaska), specifically interacts with AUX1 protein in regulating the gravitropic response of Arabidopsis roots. Application of 60  $\mu\text{M}$  CSI disrupts the vertically oriented elongation of wild-type roots grown on agar plates but orients the elongation of agravitropic mutant *aux1-7* roots toward the gravity. The CSI-induced restoration of gravitropic response in *aux1-7* roots was not observed in other agravitropic mutants, *axr2* and *eir1-1*. Because the *aux1-7* mutant is reduced in sensitivity to auxin and ethylene, we examined the effects of CSI on another auxin-resistant mutant, *axr1-3*, and ethylene-insensitive mutant *ein2-1*. In *aux1-7* roots, CSI stimulated the uptake of [ $^3\text{H}$ ]indole-3-acetic acid (IAA) and induced gravitropic bending. In contrast, in wild-type, *axr1-3*, and *ein2-1* roots, CSI slowed down the rates of gravitropic bending and inhibited IAA uptake. In the null allele of *aux1*, *aux1-22*, the agravitropic nature of the roots and IAA uptake were not affected by CSI. This close correlation between auxin uptake and gravitropic bending suggests that CSI may regulate gravitropic response by inhibiting or stimulating the uptake of endogenous auxin in root cells. CSI exhibits selective influence toward IAA versus 1-naphthaleneacetic acid as to auxin-induced inhibition in root growth and auxin uptake. The selective action of CSI toward IAA along with the complete insensitivity of the null mutant *aux1-22* toward CSI strongly suggest that CSI specifically interacts with AUX1 protein.

Every aspect of growth and development of a plant is being influenced by plant hormones (Davies, 1995). Among the hormones, indole-3-acetic acid (IAA), the major form of auxin in higher plants, is considered unique because of its prominent feature of polar transport (for review, see Goldsmith, 1977). Physiological studies revealed that auxin plays an important regulatory role in gravitropism (Kaufman and Song, 1987). The recent development in molecular genetic studies of Arabidopsis mutants further supports the idea. Nine loci involved in auxin response have been mutationally defined in Arabidopsis: *aux1* (Maher and Martindale, 1980), *dwf* (Mirza and Maher, 1987), *axr1* (Estelle and Somerville, 1987), *axr2* (Wilson et al., 1990), *axr3* (Leyser et al., 1996), *axr4* (Hobbie and Estelle, 1995), *axr6* (Hobbie et al., 2000), *tir1* (Ruegger et al., 1998), and *ask1* (Gray et al., 1999). Almost all of these auxin-resistant mutants have defects in root gravitropism, confirming the importance of auxin in this process (Hobbie and Estelle, 1995). It has long been postulated that the presence of influx and efflux carriers on the plasma membrane facilitates the transport of auxin (Rubery and Shelldrake, 1974). The *EIR1/AGR1/AtPIN2* gene has recently been cloned (Chen et al., 1998; Luschnig et al., 1998; Müller

et al., 1998; Utsuno et al., 1998), and its product shown to act as an efflux carrier in Arabidopsis roots. Bennett et al. (1996) has cloned the *AUX1* gene of Arabidopsis and shown that *AUX1* encodes an amino acid permease-like protein. Since IAA is structurally similar to Trp and the *AUX1* product is a permease, they suggested *AUX1* as a putative uptake carrier of auxin in roots. The longer latent period for inhibition of root growth by auxin in *aux1-7* roots than in other auxin-resistant mutant roots suggested that *aux1* is defective in auxin uptake (Evans et al., 1994). Agravitropic nature of *aux1* and *eir1/agr1/Atpin2* roots implies that both influx and efflux of auxin are required for gravitropic response of roots.

Chromosaponin I (CSI), a  $\gamma$ -pyronyl-triterpenoid saponin isolated from pea (*Pisum sativum* L. cv Alaska) (Tsurumi et al., 1991, 1992) and other leguminous plants (Kudou et al., 1992, 1993; Massiot et al., 1992), has been shown to influence the growth of roots in several plants (Tsurumi and Wada, 1995). CSI increases the mechanical extensibility of root cell walls, increases the cell length, and reduces the root diameter (Tsurumi et al., 1996; Tsurumi and Ishizawa, 1997) while stimulating the root growth. We reported recently that Arabidopsis roots are the most sensitive organ with regard to CSI action among those organs that we have tested. CSI stimulates both cell division and cell elongation in the wild-type

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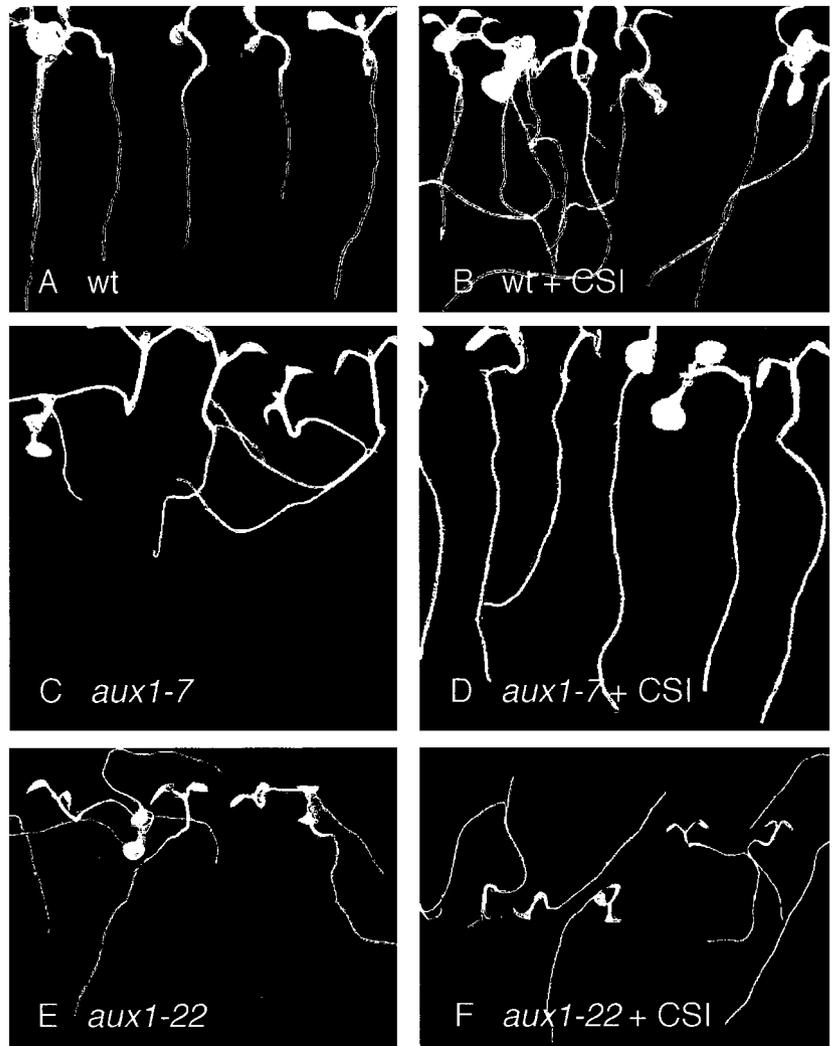
roots (Rahman et al., 2000). By using several ethylene mutants we also suggested that CSI inhibits ethylene signaling at or downstream of CTR1. In these experiments Arabidopsis roots were grown horizontally on wetted filter paper and the wild-type roots exhibited a wavy growth pattern in the absence of CSI, while CSI-treated wild-type roots grew straight (Tsurumi et al., 2000). The CSI-induced straight growth of roots is similar to the root phenotype of agravitropic mutants, including *aux1-7* when grown horizontally. However, to our surprise CSI-treatment of the *aux1-7* roots resulted in a restoration of gravity response to the levels in wild-type roots. The complete opposite effects of CSI on the growth patterns of wild-type and *aux1-7* roots prompted us to investigate the CSI action on root gravitropism in detail. In the present paper we show that CSI can regulate the gravitropic response of roots in Arabidopsis seedlings and also suggest the possible interaction of CSI with AUX1 protein in this process.

## RESULTS

### CSI Disrupts the Vertically Oriented Growth of Wild-Type Roots But Orients the Growth of *aux1-7* Roots toward the Gravity

Wild-type and *aux1-7* seedlings were grown in the presence or absence of 60  $\mu\text{M}$  CSI on vertically oriented agar plates under continuous irradiation for 4 d at 23°C. Figure 1 represents typical results showing the direction of root growth. The roots of wild-type seedlings grew vertically in the absence of CSI (Fig. 1A), but treatment with CSI changed the orientation of roots away from the vertical direction and some roots grew horizontally (Fig. 1B). The growth of the agravitropic mutant *aux1-7* roots was not toward the gravity rather random (Fig. 1C) as reported by Maher and Martindale (1980). To our surprise, CSI-treated *aux1-7* roots grew toward the gravity as if they were the wild type (Fig. 1D).

**Figure 1.** CSI disrupts the vertically oriented growth of wild-type roots (A and B) but orients the growth of *aux1-7* roots toward the gravity (C and D) in the light, whereas in *aux1-22* roots CSI fails to induce any change (E and F). Arabidopsis seedlings were grown on vertical agar plates in the presence (B, D, and F) or absence (A, C, and E) of 60  $\mu\text{M}$  CSI under continuous irradiation for 4 d. Bar = 1 mm.



In the experiments of Figure 1, *Arabidopsis* seedlings were irradiated from the upper light source. Because the roots of wild-type and *aux1-7* seedlings show negative phototropism (Okada and Shimura, 1992), the results shown in Figure 1 is the summation of gravitropic and phototropic responses. To rule out the involvement of negative phototropism we performed the growth experiments in the dark condition, and the results are shown in Figure 2. CSI-treated wild-type roots grew in random directions (Fig. 2B), whereas CSI-treated *aux1-7* roots grew with the gravity vector (Fig. 2D). These results clearly indicated that CSI disrupted the gravitropic response in wild-type roots and induced it in *aux1-7* roots.

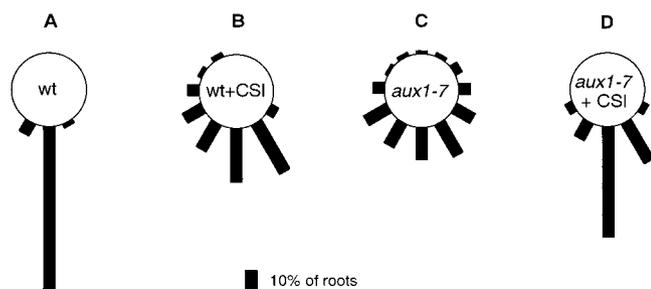
We also examined effects of CSI on the null allele of *aux1*, *aux1-22* seedlings (Fig. 1, E and F). In contrast to *aux1-7* roots (Fig. 1D), *aux1-22* roots retained their agravitropic nature even in the presence of 60  $\mu\text{M}$  CSI both in the light (Fig. 1F) and dark conditions (data not shown).

#### Effects of CSI on the Growth and Gravitropic Response of Wild-Type and *aux1-7* Roots

Figure 3A shows the dose-response of the growth of roots in wild-type and *aux1-7* seedlings against various concentrations of CSI. CSI did not show any significant effects on the growth of roots at concentrations less than 150  $\mu\text{M}$ .

To see the effects of CSI on gravitropic response we used the root tip re-orientation assay method (Lincoln et al., 1990). In this assay *Arabidopsis* seedlings were rotated by 90°, and the bending of roots was measured. After 6 h of gravistimulation the wild-type roots achieved an angle approximately 75°, whereas CSI-treated wild-type roots showed less response to gravistimulation over the concentrations of 60 to 300  $\mu\text{M}$  (Fig. 3B).

Because the roots of *aux1-7* seedlings grew in random directions in the absence of CSI, it was difficult to provide the same gravistimulus to each root by



**Figure 2.** Histogram showing the CSI-induced disruption of gravitropic response in wild-type roots and the CSI-induced gravitropic response in *aux1-7* roots in the dark. *Arabidopsis* seedlings were grown on vertical agar plates in the presence (B and D) or absence (A and C) of 60  $\mu\text{M}$  CSI in the dark for 4 d. Each root was assigned to one of 12 30° sectors. A, Wild-type control ( $n = 55$ ); B, wild type + 60  $\mu\text{M}$  CSI ( $n = 55$ ); C, *aux1-7* control ( $n = 102$ ); D, *aux1-7* + 60  $\mu\text{M}$  CSI ( $n = 59$ ).

rotating the plate. So we selected the roots growing relatively toward the gravity vector (less than 30° away from the gravity vector), and performed the root tip orientation assay. In contrast, in the presence of CSI at concentrations greater than 20  $\mu\text{M}$ , *aux1-7* roots grew vertically (Fig. 1D) so that it was easy to measure the curvature of roots in the root tip re-orientation assay. The CSI-induced curvature of *aux1-7* roots reached approximately 50° after 6 h of gravistimulation over the concentrations of 60 to 300  $\mu\text{M}$ . In the re-orientation assay, 60  $\mu\text{M}$  of CSI was enough not only to reduce the gravitropic bending of wild-type roots but also to induce it in *aux1-7* roots (Fig. 3B). Furthermore, this concentration of CSI had no inhibitory effect on the growth rate (Fig. 3A). Hence we used 60  $\mu\text{M}$  CSI for following experiments.

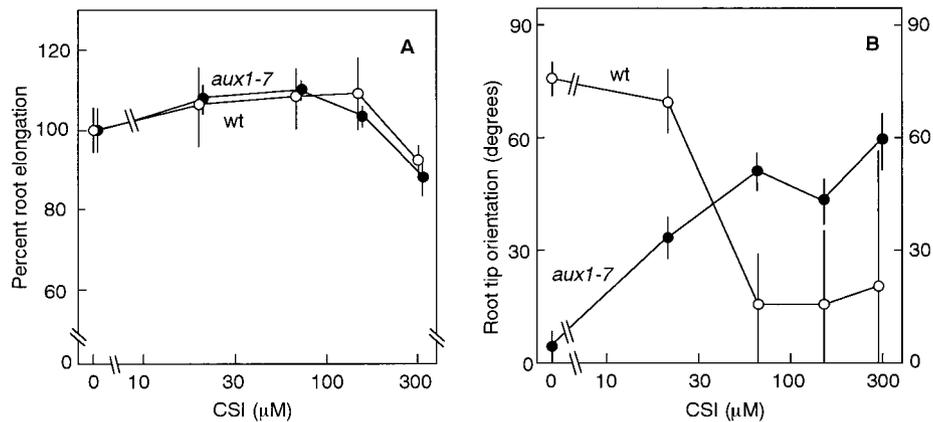
#### Time Course of CSI-Induced Inhibition and Induction of Gravitropic Bending in Roots

To analyze the CSI action on gravitropic response, we measured time courses of bending of wild-type and *aux1-7* roots after re-orientation. CSI-treated wild-type roots showed slower and less response to gravistimulus than control roots (Fig. 4A). The angle of curvature in CSI-treated roots did not reach 90° even 24 h after re-orientation and the roots grew in an oblique direction to the gravity. In striking contrast, CSI-treated *aux1-7* roots bent toward the gravity as if they were the wild-type roots (Fig. 4B), although the angle of bending of *aux1-7* roots was slightly less compared with normal gravitropic bending of wild-type roots (Fig. 4A).

#### Specific Interaction of CSI with *aux1-7* Roots to Induce Gravitropic Response

To demonstrate the specificity of CSI action on *aux1-7* mutant, we tested the effects of CSI on other agravitropic mutants *eir1-1* (Luschnig et al., 1998) and *axr2* (Wilson et al., 1990). The *eir1* gene is related to auxin efflux carrier of root cells (Luschnig et al., 1998) and the *axr2* gene encodes a member of the Aux/IAA protein family (Nagpal et al., 2000). CSI did not change the agravitropic nature of these mutants (data not shown). Since CSI is an amphipathic compound, we also tested other detergents including Triton X-100, Tween 20, SDS, nonanoyl-*N*-methylglucamide, and CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid), but none of them restored gravitropic response in *aux1-7* roots (data not shown). The restoration of gravitropic response in *aux1-7* roots is specific to CSI but not general to detergents.

The *aux1-7* mutant is resistant to both auxin and ethylene (Pickett et al., 1990). We examined other auxin-resistant mutants, *axr1-3* (Lincoln et al., 1990) and *axr4-2* (Hobbie and Estelle, 1995) and also ethylene-insensitive mutant *ein2-1* (Guzmán and Ecker, 1990). The *axr1-3* gene is related with ubiq-

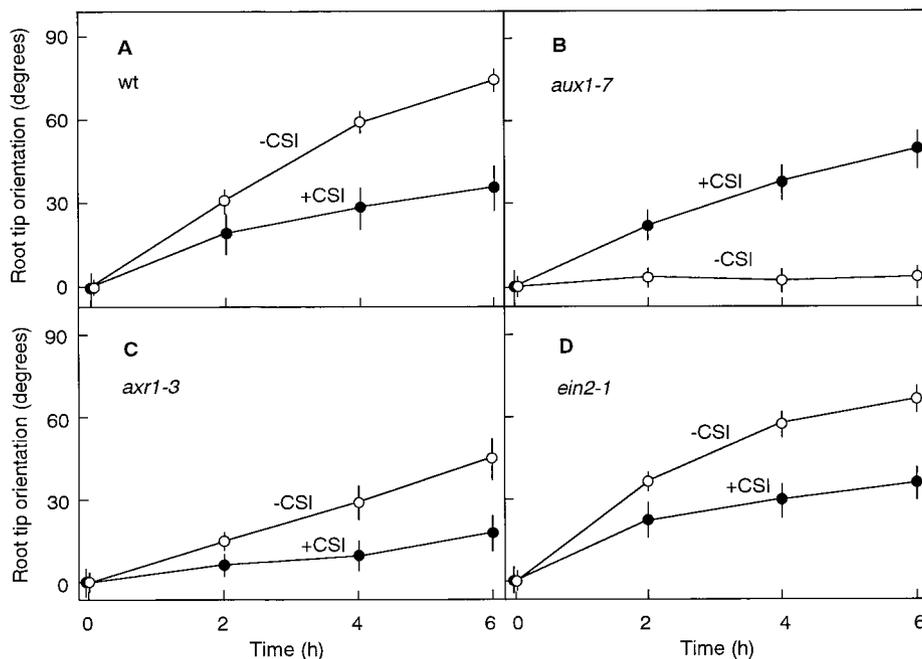


**Figure 3.** Effect of CSI on the growth (A) and gravitropic bending (B) of roots in wild-type (wt,  $\circ$ ) and *aux1-7* ( $\bullet$ ) seedlings. Arabidopsis seedlings were grown on vertical agar plates with various concentrations of CSI (0, 20, 60, 150, and 300  $\mu\text{M}$ ) in the light. Data are the averages from 10 to 15 seedlings ( $\pm$ SE). A, Elongation of roots was measured on d 3. Mean values for 100% root elongation were  $7.80 \pm 0.44$  mm (wt) and  $8.40 \pm 0.45$  mm (*aux1-7*). B, For the root tip orientation assay, on d 2 a gravity stimulus was given by rotating the plates  $90^\circ$ . Angles of curvature were measured 6 h after the rotation. In the case of control *aux1-7* roots, the roots growing relatively toward the gravity vector (less than  $30^\circ$  away from the gravity vector) were selected and root tip orientation assay was performed.

uitin pathway of auxin response (Leyser et al., 1993) and *axr4-2* gene is not yet characterized. The gravitropic bending of *axr1-3* roots was less and slower than that of wild-type roots as reported previously by Lincoln et al. (1990). The angle of curvature of *axr1-3* roots after 6 h of gravistimulation was approximately  $45^\circ$ , and treatment with CSI further

reduced the angle of bending (Fig. 4C). Another auxin-resistant mutant *axr4-2* roots showed less gravitropic response in the similar fashion to *axr1-3* roots and treatment with CSI further reduced the bending (data not shown).

The ethylene-insensitive mutant *ein2-1* roots showed a similar pattern of gravitropic curvature



**Figure 4.** Time course of CSI-induced inhibition and induction of gravitropic bending of roots in Arabidopsis seedlings; wild-type (A), *aux1-7* (B), *axr1-3* (C), and *ein2-1* (D). Seedlings were grown on vertical agar plates with ( $\bullet$ ) or without ( $\circ$ ) 60  $\mu\text{M}$  CSI in the light for 2 d. On d 2, a gravity stimulus was given by rotating the plates  $90^\circ$ . Angles of curvature were measured in regular time interval (0, 2, 4, and 6 h) after the rotation. In case of control *aux1-7* roots, the roots growing relatively toward the gravity vector (less than  $30^\circ$  away from the gravity vector) were selected and root tip orientation assay was performed. Data are the averages from 10 to 15 seedlings ( $\pm$ SE).

like the wild type (Fig. 4, A and D). The CSI-treated *ein2-1* roots showed a slower response to gravistimulus as CSI-treated wild-type roots did. The similarity in gravitropic bending between *ein2-1* and wild-type roots suggests that ethylene does not play a major role in root gravitropism.

In all *Arabidopsis* seedlings we examined, CSI reduced the gravitropic curvature of roots except agravitropic mutants. CSI specifically induced gravitropic response of roots only in *aux1-7* mutant but not in other agravitropic mutants including the null allele of *aux1*, *aux1-22*. Since the *aux1* gene has been shown to be related to auxin uptake carrier (Bennett et al., 1996; Marchant et al., 1999), these results raise the possibility that CSI may play some function in regulating auxin uptake.

#### CSI Inhibits the Uptake of [<sup>3</sup>H]IAA in Wild-Type Roots But Stimulates It in *aux1-7* Roots

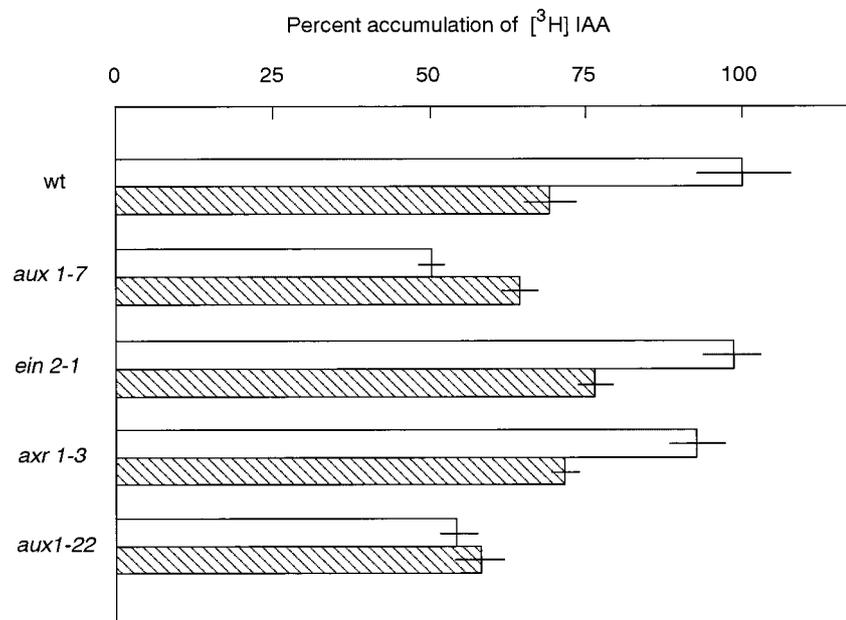
We examined the uptake of labeled IAA using apical 3-mm root tips. The root tips were incubated in the buffer supplemented with 30 nM [<sup>3</sup>H]IAA. Since the accumulation of radioactivity in root tips increased linearly up to 2 h and then saturated, the accumulation for the first 1 h was measured. The uptake of [<sup>3</sup>H]IAA in *aux1-7* roots was almost one-half of that in wild-type roots (Fig. 5). The less accumulation of labeled IAA in *aux1-7* mutant agrees well with the results reported by Marchant et al. (1999), although they measured the uptake of 2,4-dichlorophenoxyacetic acid (2,4-D) using a different method from ours. Since there was a less accumulation of radioactivity in *aux1-7* mutant roots compared with wild-type roots, we assume that carrier mediated auxin influx plays the major role for the accumula-

tion of radioactive compound in root tips in these experiments.

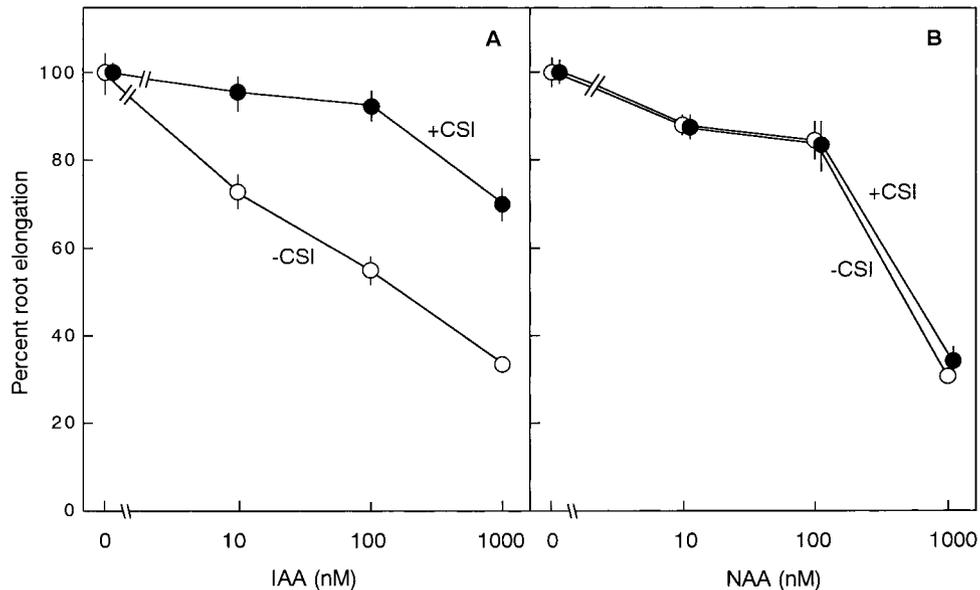
Wild-type and *aux1-7* roots exhibited opposite responses with respect to auxin uptake following CSI treatment. In CSI-treated wild-type roots the accumulation of [<sup>3</sup>H]IAA was reduced to approximately 70% compared with untreated roots ( $0.005 > P > 0.002$ ) (Fig. 5). This result is consistent with the CSI-induced reduction in gravitropic bending of wild-type roots (Fig. 4A). On the other hand, in CSI-treated *aux1-7* roots the accumulation of labeled compound was greater than that of untreated roots ( $0.002 > P > 0.001$ ). This result is in accordance with the CSI-induced gravitropic bending in this mutant root (Fig. 4B).

The ethylene-insensitive mutant *ein2-1* and the auxin-resistant mutant *axr1-3* showed a similar level of [<sup>3</sup>H]IAA uptake in roots like wild-type roots (Fig. 5). CSI treatment of these roots resulted in a reduction in [<sup>3</sup>H]IAA uptake ( $0.002 > P > 0.001$ ) as observed in wild-type roots. These results are comparable with CSI-induced reduction in gravitropic bending of these mutant roots (Fig. 4, C and D).

In all *Arabidopsis* genotypes we examined except *aux1* mutants, CSI reduced both [<sup>3</sup>H]IAA uptake and gravitropic bending. In contrast, CSI stimulated [<sup>3</sup>H]IAA uptake and induced gravitropic response in *aux1-7* roots. This close correlation between the effects of CSI on auxin uptake and gravitropic response suggests that CSI may regulate gravitropic response by altering the uptake of endogenous auxin in root cells. This idea is further supported by the uptake experiments in the roots of the null allele of *aux1*, *aux1-22*. CSI did not change [<sup>3</sup>H]IAA uptake in this mutant roots, whereas auxin accumulation in control



**Figure 5.** Effect of CSI on the uptake of [<sup>3</sup>H]IAA in the root tips of wild-type, *aux1-7*, *ein2-1*, *axr1-3*, and *aux1-22*. *Arabidopsis* seedlings were grown on vertical agar plate with (shaded bar) or without (white bar) 60  $\mu$ M CSI for 4 d in the light. Ten root tips of 3 mm in length were incubated with 30 nM [<sup>3</sup>H]IAA for 1 h. After the incubation, root tips were washed and the radioactivity was counted. Data are the averages of 12 experiments. 100% means the accumulation of labeled IAA in wild-type roots.



**Figure 6.** Selective influence of CSI on IAA versus NAA in auxin-induced inhibition of root growth of wild-type seedlings. Arabidopsis seedlings were grown on vertical agar plates with (●) or without (○) 60  $\mu\text{M}$  CSI in the presence of various concentrations of IAA (A) or NAA (B) for 3 d. Data are the averages from 10 to 15 seedlings ( $\pm\text{SE}$ ). Mean values for 100% root elongation in (A) were  $7.80 \pm 0.38$  mm (control) and  $8.90 \pm 0.23$  mm (CSI), in (B)  $7.21 \pm 0.24$  mm (control) and  $9.00 \pm 0.23$  mm (CSI), respectively.

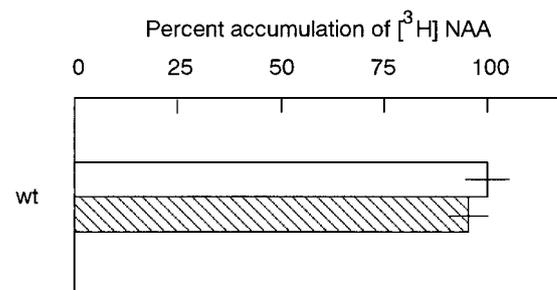
*aux1-22* roots was about a one-half of that in wild-type roots and similar to *aux1-7* roots (Fig. 5).

#### Selective Influence of CSI toward IAA versus 1-Naphthaleneacetic Acid as to Both Auxin-Induced Inhibition in Root Growth and Auxin Uptake

Delbarre et al. (1996) reported that an influx carrier facilitates the uptake of IAA but not 1-naphthaleneacetic acid (NAA), which enters the cells through diffusion. Yamamoto and Yamamoto (1998) and Marchant et al. (1999) showed that this concept is applicable to Arabidopsis roots using *aux1* mutants. To clarify our idea that CSI may interfere with the auxin influx carrier in wild-type roots, Arabidopsis seedlings were grown in the presence of IAA or NAA supplemented with or without CSI. Exogenous IAA and NAA, depending on their concentrations (Fig. 6, A and B) inhibited the growth of wild-type roots. Treatment with 60  $\mu\text{M}$  CSI counteracted the inhibitory effect of IAA in root growth over a broad range of concentrations, whereas CSI did not reduce the inhibitory effect of NAA. These differential actions of CSI to counteract the inhibitory effects of auxin in root growth are consistent with our idea. We also investigated the effect of CSI on the uptake of labeled NAA in root tips that were incubated with 30 nM [ $^3\text{H}$ ]NAA for 1 h. CSI did not inhibit the uptake of NAA in contrast to IAA (Figs. 5 and 7). This selective action of CSI toward IAA versus NAA strongly supports the idea that CSI reduces the activity of auxin influx carrier protein AUX1 in wild-type roots.

#### CSI Also Induces Opposite Effects on Sensing Ethylene in Wild-Type and *aux1-7* Roots

The *aux1-7* mutant is less sensitive to both IAA and ethylene inhibition of root growth (Pickett et al., 1990). Since CSI stimulated the [ $^3\text{H}$ ] IAA uptake and restored the gravitropic response in *aux1-7* roots, we became interested to know the CSI effect on ethylene sensitivity of this mutant. Hence, we examined the effects of CSI on ethylene-induced inhibition in root growth. To our surprise, the effects of CSI were again opposite between wild-type and *aux1-7* roots. In wild-type roots the ethylene-induced inhibition of root growth was less in the presence of CSI than in



**Figure 7.** Effect of CSI on the uptake of [ $^3\text{H}$ ]NAA in the root tips of wild-type seedlings. Arabidopsis seedlings were grown on vertical agar plates with (shaded bar) or without (white bar) 60  $\mu\text{M}$  CSI for 4 d in the light. Ten root tips of 3 mm in length were incubated with 30 nM [ $^3\text{H}$ ]NAA for 1 h. After the incubation, root tips were washed and the radioactivity was counted. Data are the averages of 12 experiments. 100% means the accumulation of labeled NAA in wild-type roots.

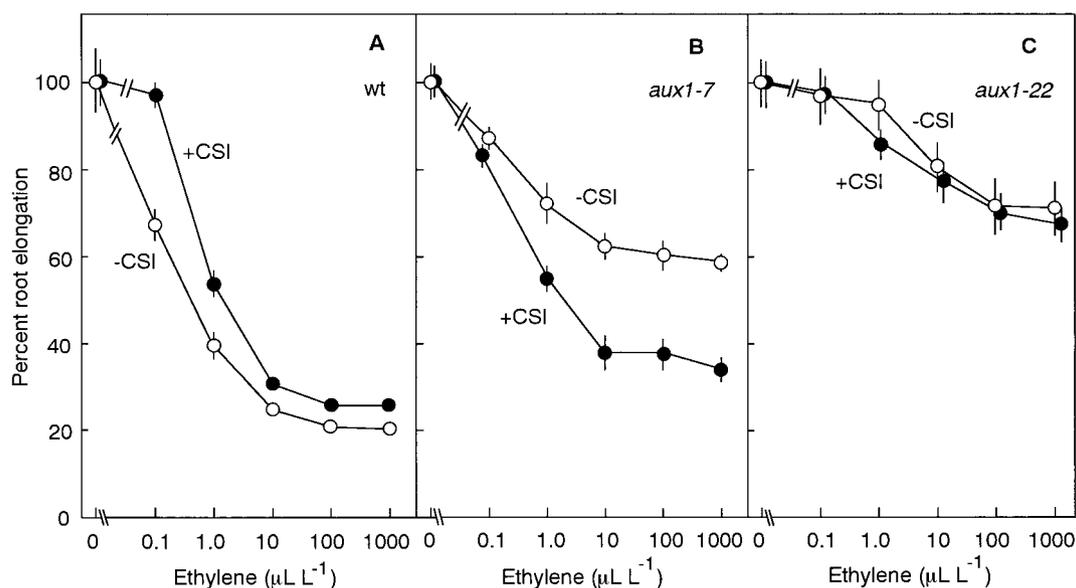
the absence ( $0.001 > P$  at  $0.1 \mu\text{L L}^{-1}$  ethylene and  $0.01 > P > 0.005$  in the range of  $1\text{--}1,000 \mu\text{L L}^{-1}$  ethylene) (Fig. 8A), whereas application of CSI to *aux1-7* roots provided a greater ethylene inhibition than control, that is, CSI made the *aux1-7* roots more responsive toward exogenous ethylene (Fig. 8B). The restoration of ethylene response in *aux1-7* roots along with induction of gravitropic response and an increase in [ $^3\text{H}$ ]IAA uptake by CSI suggest that CSI may specifically interact with AUX1 protein. This idea is consistent with the fact that CSI was unable to induce any change in the ethylene sensitivity of the null mutant *aux1-22* roots (Fig. 8C).

## DISCUSSION

*AUX1* gene encodes an amino acid permease-like protein and has been suggested to be an uptake carrier of auxin in roots (Bennett et al., 1996). In fact, the uptake of [ $^3\text{H}$ ]IAA in *aux1-7* and *aux1-22* roots was almost one-half of that in wild-type roots (Fig. 5). The less accumulation of labeled IAA in *aux1* roots is in close agreement with the results reported by Marchant et al. (1999) who measured the uptake of 2,4-D in *aux1-100* roots. The agravitropic nature of *aux1* mutant roots indicates that AUX1 protein is required for gravitropic response of roots. Delbarre et al. (1996) observed that an influx carrier facilitates the uptake of IAA but not NAA, which enters the cells through diffusion. Yamamoto and Yamamoto (1998) and Marchant et al. (1999) reported that NAA but not IAA was able to fully restore gravitropic bending of roots in *aux1* seedlings. These results

further supported the idea that AUX1 protein is the auxin influx carrier and is involved in gravitropic response of roots.

We have obtained several lines of experimental evidences indicating that CSI specifically interacts with AUX1 protein to regulate the gravitropic response of Arabidopsis roots. First of all is the close correlation between the effects of CSI on auxin uptake and on gravitropic response. CSI inhibited the uptake of [ $^3\text{H}$ ]IAA in wild-type, *axr1-3*, and *ein2-1* roots and slowed down the gravitropic bending of these roots (Figs. 4 and 5). In striking contrast, in *aux1-7* roots, CSI stimulated the uptake of [ $^3\text{H}$ ]IAA and restored gravitropic response in this agravitropic mutant root. These results suggest that CSI may regulate gravitropic response by inhibiting or stimulating the uptake of endogenous auxin in root cells. Second, CSI selectively influenced the uptake of auxin, which requires carrier-mediated influx. CSI counteracted IAA-induced inhibition in the growth of wild-type roots, whereas it was unable to counteract NAA-induced inhibition (Fig. 6). Furthermore, CSI inhibited the uptake of IAA in wild-type roots but it did not show any effects on NAA uptake (Figs. 5 and 7). The selective influence of CSI toward IAA is a good evidence for the interaction of CSI with the auxin influx carrier protein. Third, CSI regulates ethylene response (Fig. 8) as well as auxin uptake. The *aux1* roots not only have a defect in auxin uptake but are also less sensitive to ethylene's inhibition of root growth (Pickett et al., 1990). Application of CSI to wild-type seedlings reduced auxin uptake in roots and made the roots more resistant to ethylene (Fig.



**Figure 8.** CSI induces opposite effects on sensing ethylene in wild-type (A) and *aux1-7* roots (B) but does not induce any change in *aux1-22* roots (C). Arabidopsis seedlings were grown on vertical agar plates with (●) or without (○)  $60 \mu\text{M}$  CSI in the presence of various concentrations of ethylene for 3 d in the light. Data are the averages from 10 to 15 seedlings ( $\pm\text{SE}$ ). Mean values for 100% root elongation in (A) were  $7.47 \pm 0.43$  mm (control) and  $8.00 \pm 0.40$  mm (CSI), in (B)  $9.50 \pm 0.32$  mm (control) and  $10.00 \pm 0.36$  mm (CSI), and in (C)  $9.01 \pm 0.47$  mm (control) and  $9.13 \pm 0.47$  mm (CSI), respectively.

8A). On the contrary, CSI treatment of *aux1-7* seedlings increased auxin uptake and made the roots more responsive to ethylene (Fig. 8B). Effects of CSI on wild-type roots partly mimic the phenotype of *aux1-7* mutant with regard to auxin uptake and ethylene sensing. Furthermore, CSI treatment of *aux1-7* mutant partly reversed both of these phenotypes to those of wild type. The ability of auxin uptake is likely to be related to sensing ethylene. The last and most convincing argument is that CSI showed no activity on the agravitropic nature of the AUX1 null allele *aux1-22* roots (Fig. 1, E and F). Moreover, the uptake of [<sup>3</sup>H]IAA and ethylene response of the null mutant roots were not influenced by CSI (Figs. 5 and 8C). All of these results are consistent with the concept that the AUX1 protein is the auxin uptake carrier and CSI specifically interacts with this protein.

The *aux1-7* mutant is a missense mutant where Gly-459 is changed to Asp-459 close to the carboxyl terminal end (Bennett et al., 1996). The quantification of the mutant *aux1* mRNA showed that all the missense alleles including *aux1-7* had similar levels of *aux1* mRNA to the wild type (Marchant and Bennett, 1998), and it has been shown by using anti-sera on western blots that *aux1-7* still makes the full length protein, yet it acts as a complete loss of function mutant (Dr. Bennett, personal communication). Although the reason is not clear why CSI restored the activity of the mutant protein to facilitate the uptake of auxin, it might be possible that CSI could modify the conformation of the mutant protein from inactive state to slightly active one. This idea is consistent with the inability of CSI to affect growth or auxin transport in the *aux1-22* mutant roots, which is a null allele of AUX1.

We reported earlier that CSI stimulates the elongation of Arabidopsis roots grown horizontally on wetted filter paper (Rahman et al., 2000). The CSI-induced stimulation in growth involves the increase in both cell division and cell elongation. We proposed that CSI stimulates cell elongation by interfering with ethylene signaling. In the present paper, Arabidopsis seedlings were grown on vertically oriented agar plates and CSI did not show any significant stimulatory effects on the growth of wild-type roots (Fig. 3A). This difference in CSI action is due to the difference in involvement of ethylene to regulate root elongation. On filter paper the length of wild-type roots was much shorter compared with the ethylene insensitive mutant *ein2-1* roots (Rahman et al., 2000), whereas length of roots of both genotypes is not significantly different from each other when grown vertically (data not shown). These results indicate that endogenous ethylene plays the major role to inhibit the elongation of wild-type roots grown horizontally, whereas it does not in the vertical condition, hence CSI does not stimulate root elongation in the present study. Although CSI did not influence root elongation in the absence of ethylene-induced

inhibition, CSI counteracted the inhibitory effect of ethylene in wild-type roots when applied exogenously (Fig. 8A), indicating that CSI has the ability to counteract ethylene action even in the vertical condition. Although ethylene is involved in the CSI action to stimulate root growth, it is not likely involved in the CSI-induced alteration of gravitropic response in roots, because CSI slowed down the gravitropic bending of roots in the ethylene-insensitive mutant *ein2-1* seedlings as observed in wild type (Fig. 4, A and D).

The interaction between ethylene and auxin is so far the best-characterized example of hormone interaction in plant. It has been shown that auxin treatment can increase the level of 1-aminocyclopropane-1-carboxylic acid, the immediate precursor of ethylene biosynthesis (Jones and Kende, 1979); ethylene inhibits auxin efflux in pea stem (Suttle, 1988) and the auxin-resistant Arabidopsis mutants *axr1*, *axr2*, *axr3*, *aux1*, and *dwf* show cross resistance to ethylene. Although *aux1-7* roots exhibit resistance to a broad range of ethylene concentrations, the resistance to ethylene was reduced in the presence of CSI; CSI treatment made the roots more responsive to ethylene (Fig. 8B). On the contrary, application of CSI to wild-type seedlings made the root more resistant to exogenous ethylene especially at a low concentration of ethylene (Fig. 8A). These results suggest that the ethylene response is related to the amount of auxin uptake in roots. In fact, recently we observed that the ethylene response in *aux1-7* roots was also restored in the presence of 10 nM NAA (Rahman et al., 2001). The simplest explanation for the restoration of ethylene response in *aux1-7* roots is that a certain level of auxin in root cells is required for ethylene response. This idea implies that the reduction of intracellular level of auxin may be, at least in part, the cause of the resistance to ethylene of *aux1-7* roots. Our experimental results are consistent with the above idea. CSI could reduce the intracellular level of auxin in root cells by inhibiting auxin uptake, which results in some resistance to exogenous ethylene (Fig. 8A). On the other hand, in *aux1-7* roots CSI could increase the intracellular auxin level by stimulating auxin uptake, resulting in restoration of ethylene response (Fig. 8B), whereas in the null mutant *aux1-22* CSI fails to induce any change (Fig. 8C).

The Cholodny-Went hypothesis holds that gravitropic curvature of a growing plant organ depends on regulated transport of auxin (Estelle, 1996). In the root, the presence of two auxin transport polarities has been proposed; basipetal transport from the root tip to the elongation zone through the epidermis or outer cortex and acropetal transport in the central stele of the elongation zone (Ohwaki and Tsurumi, 1976; Tsurumi and Ohwaki, 1978). Recent molecular genetic studies demonstrated that AUX1 and EIR1/AGR1/AtPIN2 proteins may be the influx and efflux carriers for auxin transport in roots, respectively

(Bennett et al., 1996; Chen et al., 1998; Luschnig et al., 1998; Müller et al., 1998; Utsuno et al., 1998). Furthermore, the cellular immunolocalization of these proteins clearly visualized the basipetal transport in the epidermis of Arabidopsis roots (Müller et al., 1998; Marchant et al., 1999). Mutations in AUX1 and EIR1/AGR1/AtPIN2 caused agravitropic root phenotypes indicating that the basipetal transport is of primary importance to root gravitropism (Marchant et al., 1999). Rashotte et al. (2000) recently provided evidence showing that the basipetal transport of auxin is required for gravitropic response of Arabidopsis roots. The most reasonable model for the agravitropic nature of *aux1-7* roots may be as follows; the mutation in AUX1 protein reduces auxin uptake and thereby reduces endogenous auxin level in root cells. As a result, the amount of auxin transported from the root tip toward the elongation zone may be reduced so that roots fail to bend toward the gravity. CSI-induced reduction in gravitropic response fits this model. CSI interacts with AUX1 protein and inhibits the uptake of endogenous auxin resulting in less flow of the basipetal auxin transport. The gravitropic response of roots is consequently, reduced. In case of *aux1-7* roots, CSI interacts with the mutant protein and partially restores the uptake of endogenous auxin, which results in an increased flow of the basipetal auxin transport so that the gravitropic response is partly restored in the *aux1-7* roots.

Imhoff et al. (2000) carefully characterized recently a large group of aryl and aryloxyalkyl carboxylic acids as potent inhibitors of auxin influx and efflux carriers and showed the molecular requirement for auxin-influx inhibition, an aromatic moiety substituted by an acid side chain. The molecular structure of CSI does not seem to fit this requirement. They also described that many auxin-influx inhibitors inhibited auxin efflux. The possibility that CSI might also affect the auxin efflux carriers remained to be examined. Although a group of flavonoids including quercetin and apigenin has been shown as natural inhibitors of auxin efflux carrier (Jacobs and Rubery, 1988), to our knowledge the present study is the first report describing a natural compound as an inhibitor of auxin influx in root cells. Further works of CSI on auxin uptake will clarify the mechanism of action of CSI on AUX1 protein.

## MATERIALS AND METHODS

### Plant Materials and Growth Condition

All mutant lines were derived from Arabidopsis ecotype Columbia. Auxin-resistant mutants, *aux1-7* (Pickett et al., 1990), *axr1-3* (Lincoln et al., 1990), and *axr4-2* (Hobbie and Estelle, 1995), ethylene insensitive mutant *ein2-1* (Guzmán and Ecker, 1990), and agravitropic mutants *eir1-1* (Luschnig et al., 1998) and *axr2* (Wilson et al., 1990) were obtained from Arabidopsis Biological Resource Center (Columbus, OH). These mutants were propagated as described earlier

(Rahman et al., 2000). The AUX1 null allele *aux1-22* was a kind gift from Dr. Bennett.

Buffer solution was made of 5 mM KNO<sub>3</sub>, 2 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 2 mM MgSO<sub>4</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, and 20 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS), pH 6.6. The pH of the buffer was adjusted with KOH. Arabidopsis seeds were placed in a 2.6-cm Petri dish on filter paper (Advantec no. 2, Toyo Roshi Kaisha, Tokyo) wetted with 300 μL of the buffer. Two or 4 d after cold treatment at 4°C under nearly saturating humidity in the dark, seeds were irradiated to germinate for 1 or 2 d with white fluorescent lamps (FL 20SS-BRN/18, Toshiba, Tokyo) at an irradiance of approximately 17 μmol m<sup>-2</sup> s<sup>-1</sup>. For dark experiments, the irradiation time was 6 h for *aux1-7* and 1 d for wild type. The irradiated seeds were transferred on the surface of agar plate (2% w/v) containing the buffer solution described above in a rectangular plastic Petri dish (6 × 4 cm). Auxin and CSI were mixed with agar medium while the temperature of agar was 45°C to 50°C. Seedlings were grown on vertically oriented agar plate at 23°C under continuous irradiation or in dark condition. Pictures of seedlings were taken from the back of plates through the agar medium with a digital camera (RICOH DC-4T, Ricoh Company, Ltd., Japan).

### Chemicals

CSI was extracted from 7-d-old etiolated pea (*Pisum sativum* L. cv Alaska) seedlings with aqueous methanol and purified by HPLC as described previously (Tsurumi et al., 1992). The purified CSI was dried to white powder and kept under N<sub>2</sub> at -80°C. IAA, NAA, Triton X-100, and Tween 20 were purchased from Sigma (St. Louis). [5-<sup>3</sup>H]IAA (20 Ci mmol<sup>-1</sup>) and [4-<sup>3</sup>H]NAA (25 Ci mmol<sup>-1</sup>) were from American Radiolabeled Chemicals (St. Louis). MOPS, nonanoyl-*N*-methylglucamide,3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and Scintisol EX-H were from Dojindo Laboratories (Kumamoto, Japan). Other chemicals were from Wako Pure Chemical Industries (Osaka).

### Root Tip Re-Orientation Assay

Arabidopsis seedlings were grown with or without CSI under continuous irradiation at 23°C for 2 d. On d 2, a gravity stimulus was applied by rotating the agar plates 90°. To measure the curvature of roots, photographs of seedlings were taken by a digital camera and analyzed by an image analyzing software NIH image 1.62. In each case the mean (±SE) for 10 to 15 seedlings was calculated. Each assay was repeated at least three times. *P* values were evaluated using the Student's *t* test. Circular histograms in Figure 2 were drawn by MacDraw II 1.1.

### Root Growth Assay

Arabidopsis seedlings were grown with CSI, IAA, or NAA for 3 d under continuous irradiation at 23°C. For ethylene treatment, an agar plate containing germinated seeds was placed vertically in a sealed 140-mL plastic

cylinder. Ethylene was injected with a syringe into each cylinder through a small side hole to make various concentrations of ethylene (Tsurumi and Ishizawa, 1997). Length of roots was measured under a microscope. The mean ( $\pm$  SE) for 10 to 15 seedlings was calculated, and each assay was repeated at least three times.

### Auxin Uptake Assay

*Arabidopsis* seedlings were grown for 4 d under continuous irradiation. Root tips of 3 mm in length were excised from them and placed on a nylon mesh (1.5 cm<sup>2</sup>) with 250- $\mu$ m opening. The nylon mesh containing 10 root tips was transferred to a 2.6-cm Petri dish on a piece of filter paper of 1.2 cm<sup>2</sup> (Advantec no.2) wetted with 75  $\mu$ L of buffer solution supplemented with 30 nM [<sup>3</sup>H]IAA (22 KBq mL<sup>-1</sup>) or [<sup>3</sup>H]NAA (27.75 KBq mL<sup>-1</sup>) and incubated for 1 h under nearly saturating humidity. After incubation, the root tips were carefully transferred to a 3.5-cm Petri dish containing 3 mL of buffer without labeled compound and washed for 2 min with gentle shaking. The root tips were then soaked for overnight in 5-mL liquid scintillation fluid (Scintisol EX-H), and the radioactivity was measured with a scintillation counter (model LS6500, Beckman Instruments, Fullerton, CA). The assay was performed in quadruplicate and repeated at least three times.

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