Auxin and Ethylene Response Interactions during Arabidopsis Root Hair Development Dissected by Auxin Influx Modulators

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The plant hormones auxin and ethylene have been shown to play important roles during root hair development. However, cross talk between auxin and ethylene makes it difficult to understand the independent role of either hormone. To dissect their respective roles, we examined the effects of two compounds, chromosaponin I (CSI) and 1-naphthoxyacetic acid (1-NOA), on the root hair developmental process in wild-type Arabidopsis, ethylene-insensitive mutant ein2-1, and auxin influx mutants aux1-7, aux1-22, and double mutant aux1-7 ein2. β-Glucuronidase (GUS) expression analysis in the BA-GUS transgenic line, consisting of auxin-responsive domains of 5S-IAA4/5 promoter and GUS reporter, revealed that 1-NOA and CSI act as auxin uptake inhibitors in Arabidopsis roots. The frequency of root hairs in ein2-1 roots was greatly reduced in the presence of CSI or 1-NOA, suggesting that endogenous auxin plays a critical role for the root hair initiation in the absence of an ethylene response. All of these mutants showed a reduced in root hair length, however, the root hair length could be restored with a variable concentration of 1-naphthaleneacetic acid (NAA). NAA (10 nm) restored the root hair length of aux1 mutants to wild-type level, whereas 100 nm NAA was needed for ein2-1 and aux1-7 ein2 mutants. Our results suggest that insensitivity in ethylene response affects the auxin-driven root hair elongation. CSI exhibited a similar effect to 1-NOA, reducing root hair growth and the number of root hair-bearing cells in wild-type and ein2-1 roots, while stimulating these traits in aux1-7 and aux1-7 ein2 roots, confirming that CSI is a unique modulator of AUX1.

Root hairs are tip-growing, tubular-shaped outgrowths that help to anchor roots, interact with soil microorganisms, and assist in the uptake of water and nutrients (Cutter, 1978). The relatively simple and invariant cellular organization of the primary roots of Arabidopsis and the ease of isolation and characterization of mutants make it a very attractive material for studying the root hair developmental process. The first committed step for root hair development is epidermal cell specification. In many species, including Arabidopsis, the root epidermis consists of two epidermal cell types, root hair-forming trichoblast cells and hairless atrichoblast cells (Cormack, 1947, 1949; Bunning, 1951; Cutter, 1978). Within the Arabidopsis root epidermis, cells adopt distinct fates in a position-dependent manner. Epidermal cells that overlay the junction between two cortical cell files adopt a root hair cell fate, whereas the epidermal cells that contact only one cortical cell file become hairless cells (Dolan et al., 1994; Galway et al., 1994; Berger et al., 1998).

Once the immature epidermal cell adopts a root hair cell fate, it goes through characteristic changes in its shape and size (Schiefelbein, 2000). Genetic analysis revealed that the root hair initiation mutations axr2 (Wilson et al., 1990), axr3 (Leyser et al., 1996), and ctrl (Kieber et al., 1993) exhibit changes in their response to two important plant hormones, auxin and ethylene. The root hair initiation defect of the rhd6 mutant can be suppressed by application of 1-aminocyclopropane-1-carboxylic acid (ACC; an ethylene precursor) or indole-3-acetic acid (IAA; endogenous form of auxin; Masucci and Schiefelbein, 1994), further confirming the roles of these two hormones in this process. After initiation, the root hair starts to grow through the process of tip growth. Mutants with altered responses to ethylene and auxin also show defects in root hair length (Reed et al., 1993; Okada and Shimura, 1994; Pitts et al., 1998), suggesting that these two hormones play important roles in controlling the root hair growth. Physiological experimental data with auxin, auxin transport inhibitors, and ACC further support this idea (Masucci and Schiefelbein, 1994; Okada and Shimura, 1994; Pitts et al., 1998). Collectively, these results clearly suggest that after cell specification, auxin and...
ethylen play indispensable roles regulating root hair morphogenesis.

We recently reported that chromosaponin I (CSI), a γ-pyronyl-triterpenoid saponin isolated from pea (Pisum sativum) and other leguminous plants (Tsurumi et al., 1991, 1992; Kudou et al., 1992, 1993; Massiot et al., 1992), specifically interacts with auxin influx carrier AUX1 (Bennett et al., 1996) and changes the response of Arabidopsis roots toward auxin and ethylene by controlling auxin uptake (Rahman et al., 2001a). Application of 60 μM CSI inhibited the auxin uptake in the roots of Arabidopsis expressing the wild-type AUX1 protein and slowed down the gravitropic response of roots. In the auxin influx mutant aux1-7, CSI conversely stimulated the uptake of auxin and partially restored the gravitropic response (Rahman et al., 2001a). We also observed that the CSI-induced change in auxin influx consequently affected the ethylene response of roots. CSI made the wild-type roots resistant to ethylene while it restored ethylene response in the ethylene-resistant mutant aux1-7 roots (Rahman et al., 2001a). In a later study, we showed that application of low concentrations of 1-naphthaleneacetic acid (NAA) restored the ethylene response in aux1-7, suggesting that the intracellular level of auxin plays an important role in regulating the ethylene response in Arabidopsis root growth (Rahman et al., 2001b).

Imhoff et al. (2000) characterized a large group of aryloxyalkykarboxylic acids as potent inhibitors of auxin influx in suspension-cultured tobacco (Nicotiana tabacum) cells. Parry et al. (2001) recently investigated the effect of the aryloxyalkykarboxylic acids including 1-naphthoxyacetic acid (1-NOA) on intact Arabidopsis seedlings. The authors concluded that 1-NOA was a useful auxin influx inhibitor because 1-NOA phenocopied the agravitropic aux1 root phenotype in wild type and did not show any effect on auxin efflux. Interestingly, application of 30 μM 1-NOA to wild-type roots mimicked the effect of 60 μM CSI in a root growth assay and in disrupting the root gravitropism. Although auxin and ethylene play indispensable roles during root hair development, cross talk between the two hormones (Rahman et al., 2001b) makes it difficult to resolve their independent roles. In the present paper we clarify the role of auxin by modulating its concentration in roots using the novel compounds CSI and 1-NOA.

**RESULTS**

**Effects of CSI and 1-NOA on the Root Hair Developmental Process in Wild-Type Arabidopsis Seedlings**

We reported earlier that application of 60 μM CSI disrupted the gravitropic response and auxin uptake in wild-type Arabidopsis roots (Rahman et al., 2001a). In the present study, we used the same concentration of CSI to see its effect on root hair development.

**1-NOA and CSI Specifically Inhibit the IAA-Induced β-Glucuronidase (GUS) Expression in BA-GUS Reporter Line**

The Arabidopsis BA-GUS transgenic line (BA3) encodes the auxin-responsive A and B domains of the PS-IAA4/5 promoter fused to a GUS reporter gene (Ballas et al., 1995; Oono et al., 1998). Specific GUS expression in the root elongation zone can be increased by modulating its concentration in roots using the novel compounds CSI and 1-NOA.

**Figure 1.** Effect of auxin, CSI, and 1-NOA on root hair length (A) and root length (B). Wild-type Arabidopsis seedlings were grown on vertical agar plates under continuous light for 3 d. Vertical bars indicate se.
duced by exogenous application of auxin in this reporter line (Oono et al., 1998). By using two different auxins, IAA, which requires an uptake carrier to enter the cell, and NAA, which enters the cell mainly by diffusion (Delbarre et al., 1996; Yamamoto and Yamamoto, 1998; Marchant et al., 1999; Rahman et al., 2001a), we investigated the effect of 1-NOA and CSI on auxin influx machinery.

Figure 2 represents the typical effect of 1-NOA and CSI on the IAA- and NAA-induced GUS expression in the root elongation zone of BA-GUS seedlings. 1-NOA (30 μM) and 60 μM CSI completely blocked 0.1 μM IAA-induced GUS expression in these seedlings (Fig. 2, second panel), but they failed to show any effect on 1 μM NAA-induced GUS expression (Fig. 2, third panel). 1-NOA or CSI alone did not show any effect (Fig. 2, first panel). We used a 10-fold higher concentration of NAA because of the lack of response of BA-GUS transgenic line to 0.1 μM NAA. Because of a 10-fold difference in auxin concentration, one may argue that 1-NOA or CSI could not inhibit the NAA-induced GUS expression simply by the presence of a high concentration of auxin. To address this question, we investigated the effects of 1-NOA and CSI on 1 μM IAA-induced GUS expres-

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<tr>
<th>Treatment</th>
<th>Percentage of Root Hair Cells (%)</th>
<th>Epidermal Cell Length (μm)</th>
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<tr>
<td>Untreated wild type</td>
<td>39.3 ± 1.5</td>
<td>147.37 ± 3.97</td>
</tr>
<tr>
<td>Wild type + 60 μM CSI</td>
<td>30.7 ± 1.7</td>
<td>167.53 ± 3.79</td>
</tr>
<tr>
<td>Wild type + 30 μM 1-NOA</td>
<td>33.7 ± 2.1</td>
<td>156.43 ± 1.98</td>
</tr>
<tr>
<td>Wild type + 10 nM NAA</td>
<td>54.3 ± 1.4</td>
<td>163.06 ± 4.07</td>
</tr>
<tr>
<td>Wild type + 10 μM IAA</td>
<td>54.0 ± 1.5</td>
<td>161.96 ± 4.30</td>
</tr>
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</table>
We found a requirement to increase the 1-NOA or CSI concentration to completely block the 1 μM IAA-induced GUS expression. A 5- to 10-fold increase in concentrations of these compounds (200–300 μM) could completely inhibit the 1 μM IAA-induced GUS expression (Fig. 2, fourth panel), whereas these concentrations did not inhibit the GUS expression induced by 1 μM NAA (Fig. 2, bottom panel). The inability of these compounds to inhibit NAA-induced GUS expression provides functional evidence that 1-NOA and CSI interfere with the auxin influx machinery of Arabidopsis roots by acting as potent auxin influx inhibitors.

**Inhibition of Auxin Influx Blocks the Root Hair Developmental Process in Ethylene-Insensitive Mutant ein2-1**

The plant hormones auxin and ethylene have been proposed to act as important regulators of root hair development (Masucci and Schiefelbein, 1996; Pitts et al., 1998), but the cross talk between these hormones (Rahman et al., 2001b) makes it difficult to separate their roles during this process. To dissect the roles of two hormones, we examined the effects of CSI and 1-NOA on the ethylene-insensitive mutant ein2-1 (Guzmán and Ecker, 1990). Untreated ein2-1 roots grew longer compared with wild-type roots (Figs. 1A and 4A), but the length of root hairs in this mutant was extremely short (Figs. 3, a and e, 1B, and 4B), as observed previously by Pitts et al. (1998). ein2-1 roots grown in the presence of CSI had fewer root hair-bearing cells and shorter root hairs compared with control (Figs. 3f and 4B). Only approximately 20% of epidermal cells formed root hairs in CSI-treated ein2-1 roots, compared with approximately 40% of untreated ein2-1 roots (Table II). 1-NOA treatment also showed similar reductions in the number of root hair-forming cells and in the length of root hairs in this mutant root (Figs. 3g and 4B; Table II). In contrast, the growth of roots and the length of mature epidermal cells were not affected by either compound (Fig. 4A; Table II). Because CSI or 1-NOA acts to block auxin influx in roots (Fig. 2), the effect of these compounds in blocking root hair initiation and elongation in ein2-1 suggests that endogenous auxin plays a critical role in both processes. Although application of 10 nM NAA to ein2-1 roots could not restore the length of root hairs to a wild-type level, a 10-fold increase in exogenous NAA (100 nM) restored root hair length to the wild-type level (Figs. 3, a and h, 1B, and 4B). The latter concentration of NAA also increased the percentage of root hair-bearing cells to approximately 50% (Table II). However, we observed...
approximately 40% of both wild-type and (Table III). This value is less compared with approximately 30% of the epidermal cells formed root hairs (Figs. 3, a, e, and i, 1B, 4B, and 5B). These results suggest that the normal level of endogenous auxin or the normal response to ethylene is required for both root hair initiation and root hair elongation. Application of 60 \( \mu M \) CSI dramatically changed the aux1-7 root hair phenotype (Figs. 3, i and j). CSI stimulated root hair length (Fig. 5B) and also increased the percentage of root hair-bearing cells to approximately 50% (Table III) without altering root length and epidermal cell length (Fig. 5A; Table III). Application of 10 \( \mu M \) NAA, which has been suggested to enter into the cell mainly by diffusion (Delbarre et al., 1996), mimicked CSI treatment to rescue aux1-7 root hair development (Fig. 5B). The percentage of root hair-bearing cells in NAA-treated aux1-7 roots also increased from approximately 30% of control to approximately 50%, which is similar to that of CSI-treated roots (Table III). Application of IAA, suggested to be taken up by the uptake carrier AUX1 (Delbarre et al., 1996; Yamamoto and Yamamoto, 1998; Marchant et al., 1999; Rahman et

CSI Phenocopies the Wild-Type Root Hair Phenotype in the Auxin-Influx Mutant aux1-7

CSI has been described to exhibit opposite effects in aux1-7 mutant and wild-type roots. CSI inhibits gravitropic response, auxin influx, and ethylene-mediated growth response in wild-type roots (Rahman et al., 2001a) but stimulates all of them in aux1-7 roots (Rahman et al., 2001a). The unique effects of CSI on aux1-7 roots prompted us to investigate its effect on root hair development in this mutant. The aux1-7 mutant has a defect in auxin influx and is also resistant to ethylene (Pickett et al., 1990; Rahman et al., 2001a, 2001b). In the aux1-7 mutant root, approximately 30% of the epidermal cells formed root hairs (Table III). This value is less compared with approximately 40% of both wild-type and ein2-1 roots (Tables I and II). The root hair length of aux1-7 is also considerably shorter than that of wild type but slightly longer than ein2-1 (Figs. 3, a, e, and i, 1B, 4B, and 5B).

Table II. Effect of auxin, CSI, and 1-NOA on root hair formation in ethylene-insensitive mutant ein2-1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of Root Hair Cells</th>
<th>Epidermal Cell Length</th>
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</thead>
<tbody>
<tr>
<td>Untreated ein2-1</td>
<td>39.6 ± 1.7</td>
<td>173.24 ± 3.67</td>
</tr>
<tr>
<td>ein2-1 + 60 ( \mu M ) CSI</td>
<td>17.9 ± 1.6</td>
<td>173.31 ± 2.79</td>
</tr>
<tr>
<td>ein2-1 + 30 ( \mu M ) 1-NOA</td>
<td>20.0 ± 2.2</td>
<td>172.46 ± 1.95</td>
</tr>
<tr>
<td>ein2-1 + 10 ( \mu M ) NAA</td>
<td>43.3 ± 1.9</td>
<td>169.87 ± 2.63</td>
</tr>
<tr>
<td>ein2-1 + 100 ( \mu M ) NAA</td>
<td>47.0 ± 1.2</td>
<td>123.52 ± 3.52</td>
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Table III. Effect of auxin, CSI, and 1-NOA on root hair formation in auxin influx mutants aux1-7 and aux1-22

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of Root Hair Cells</th>
<th>Epidermal Cell Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated aux1-7</td>
<td>32.5 ± 2.3</td>
<td>179.54 ± 2.87</td>
</tr>
<tr>
<td>aux1-7 + 60 ( \mu M ) CSI</td>
<td>51.4 ± 2.8</td>
<td>175.30 ± 2.94</td>
</tr>
<tr>
<td>aux1-7 + 30 ( \mu M ) 1-NOA</td>
<td>31.0 ± 2.8</td>
<td>179.46 ± 2.68</td>
</tr>
<tr>
<td>aux1-7 + 10 ( \mu M ) NAA</td>
<td>51.0 ± 1.9</td>
<td>189.68 ± 5.06</td>
</tr>
<tr>
<td>aux1-7 + 10 ( \mu M ) IAA</td>
<td>29.0 ± 2.3</td>
<td>185.79 ± 5.70</td>
</tr>
<tr>
<td>Untreated aux1-22</td>
<td>32.2 ± 2.8</td>
<td>180.56 ± 3.50</td>
</tr>
<tr>
<td>aux1-22 + 60 ( \mu M ) CSI</td>
<td>29.9 ± 1.7</td>
<td>175.81 ± 3.20</td>
</tr>
<tr>
<td>aux1-22 + 30 ( \mu M ) 1-NOA</td>
<td>33.7 ± 2.5</td>
<td>178.48 ± 4.24</td>
</tr>
<tr>
<td>aux1-22 + 10 ( \mu M ) NAA</td>
<td>44.0 ± 2.2</td>
<td>185.68 ± 3.85</td>
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Figure 4. Effect of auxin, CSI, and 1-NOA on root length (A) and root hair length (B). ein2-1 seedlings were grown on vertical agar plates under continuous light for 3 d. Vertical bars indicate se.

Figure 5. Effect of auxin, CSI, and 1-NOA on root length (A) and root hair length (B). aux1-7 and aux1-22 seedlings were grown on vertical agar plates under continuous light for 3 d. Vertical bars indicate se.
al., 2001a), did not show any effect on root hair developmental process of aux1-7, confirming the idea that the auxin influx carrier protein is mutated in aux1-7 (Bennett et al., 1996). These results collectively suggest that CSI phenocopied the wild-type root hair phenotype by increasing the intracellular level of auxin in aux1-7 roots. In contrast, 1-NOA failed to induce any change in aux1-7 root hair phenotype (Figs. 3k and 5B; Table III).

A null allele of aux1, aux1-22 (Marchant and Bennett, 1998), exhibited a similar root hair phenotype to aux1-7, i.e. the root hair length of aux1-22 was reduced (Fig. 5B) and the percentage of root hair-bearing cells was approximately 30% (Table III). However, application of CSI failed to induce any change in the root hair phenotype in this mutant (Fig. 5B; Table III), yet NAA completely restored the root hair phenotype to wild type (Fig. 5B; Table III). These results confirm that CSI specifically interacts with AUX1 protein in regulating the auxin uptake in Arabidopsis roots and can partially compensate for the absence of an ethylene response.

### DISCUSSION

The plant hormones auxin and ethylene have been suggested to act after root hair cell specification in Arabidopsis (Masucci and Schiefelbein, 1996; for review, see Schiefelbein, 2000). The cross talk between auxin and ethylene in Arabidopsis roots (Rahman et al., 2001b) makes it difficult to understand the independent role of either hormone in root hair developmental process, as illustrated by the cross resistance of the auxin-resistant mutants (e.g. axr1, axr2, axr3, and aux1) toward ethylene. Several studies have used the ethylene biosynthetic inhibitor AVG to elucidate the role of ethylene during this developmental process (Tanimoto et al., 1995; Masucci and Schiefelbein, 1996), but no such work is available to date to determine the role of auxin. In the present paper, we dissected the role of auxin as well as ethylene during root hair development using two interesting compounds, CSI and 1-NOA.

### 1-NOA and CSI Are Potent Auxin Influx Inhibitors

The exogenous requirement of auxin to induce GUS expression in the root elongation zone of BA-GUS transgenic line makes it an excellent reporter to investigate the interaction of 1-NOA and CSI with the auxin influx components in Arabidopsis. Although application of 0.1 μM IAA induced the GUS expression in the BA-GUS transgenic line, 0.1 μM NAA failed to do so (data not shown). We also found a difference in the response of the transgenic line toward another auxin, 2,4-dichlorophenoxyacetic acid (2,4-D). Like NAA, at least 1 μM 2,4-D was required to induce GUS expression in the BA-GUS line (Y. Oono and A. Rahman, unpublished data). We found that 30 μM 1-NOA or 60 μM CSI completely blocked 0.1 μM IAA-induced GUS expression (Fig. 2, second panel) and that 300 μM 1-NOA or CSI was required to block 1 μM IAA-induced GUS expression (Fig. 2, fourth panel). On the other hand, these concentrations of 1-NOA and CSI were unable to block the 1 μM NAA-induced GUS expression in this line (Fig. 2, third and bottom panels). Because IAA enters the cell through an uptake carrier while NAA enters by diffusion (Delbarre et al., 1996), these results indicate that 1-NOA and CSI interfere with the auxin influx.

### Table IV. Effect of auxin, CSI, and 1-NOA on root hair formation in aux1-7 ein2 double mutant

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of Root Hair Cells</th>
<th>Epidermal Cell Length</th>
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<tbody>
<tr>
<td>Untreated</td>
<td>13.9 ± 1.9</td>
<td>187.81 ± 2.41</td>
</tr>
<tr>
<td>aux1-7 ein2</td>
<td>27.0 ± 2.0</td>
<td>185.39 ± 1.72</td>
</tr>
<tr>
<td>aux1-7 ein2 + 60 μM CSI</td>
<td>16.1 ± 2.1</td>
<td>182.28 ± 1.98</td>
</tr>
<tr>
<td>aux1-7 ein2 + 30 μM 1-NOA</td>
<td>52.1 ± 2.7</td>
<td>138.50 ± 3.37</td>
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</table>

Data are means ± se.
component of Arabidopsis roots. These results are also in agreement with our previous finding that CSI specifically inhibited \[^3H\]IAA uptake in Arabidopsis roots yet failed to block \[^3H\]NAA uptake (Rahman et al., 2001a).

**Auxin Plays a Compensating Role in Root Hair Developmental Process in Arabidopsis Roots in the Absence of Ethylene**

Several lines of evidence support the argument that auxin can control root hair development in the absence of an ethylene response. The first line of evidence is the root hair phenotype of ethylene-insensitive mutant ein2-1. Ethylene signaling is disrupted in ein2-1 mutant because of a mutation in the bifunctional transducer protein EIN2 (Alonso et al., 1999), which mediates an essential step in the signal propagation between CTR1 and EIN3/EIL (Roman et al., 1995; Chao et al., 1997). Even in the absence of an ethylene response, approximately 40% of ein2-1 epidermal cells form root hairs (Table II). The frequency of root hairs in ein2-1 roots is similar to that of wild-type roots (Table I). Masucci and Schiefelbein (1996) also previously reported that root hair number is not altered in ein2-1 or in another ethylene-insensitive mutant eir1-1. These results suggest that for the root hair initiation process, the absence of ethylene response can be compensated by another factor. Because auxin and ethylene have been proposed to act during root hair development (Masucci and Schiefelbein, 1996; Pitts et al., 1998), auxin represents a likely candidate as the compensating factor.

The second line of evidence is the effect of the CSI and 1-NOA on ein2-1 mutant. Both CSI and 1-NOA can act as inhibitors of auxin uptake in Arabidopsis roots (Fig. 2; Parry et al., 2001; Rahman et al., 2001a). We used these compounds to reduce the intracellular level of auxin in ein2-1 and investigated their effects on root hair initiation. As expected, we observed a significant reduction in the number of root hair-forming cells in ein2-1 roots grown in the presence of CSI or 1-NOA (Fig. 3, f and g). The frequency of root hairs was reduced to approximately 20% from approximately 40% of untreated control (Table II). These results suggest that the normal root hair initiation in the ethylene-insensitive mutant ein2-1 is attributable to auxin.

Finally, the root hair phenotype of the double mutant aux1-7 ein2 further supports the idea. If auxin plays a complementary role in the ein2-1 mutant, one can expect that in the aux1-7 ein2 double mutant, the percentage of root hair-bearing cell would be reduced compared with the ein2-1 single mutant. We observed a reduced frequency (approximately 14%) of root hair initiation in the double mutant compared with approximately 40% in ein2-1 (Fig. 3, e and m; Tables II and IV). To rule out the possibility of over-looking minute bulging, we counted the root hairs at 100× magnification and obtained identical results. This reduction in the root hair frequency in aux1-7 ein2 also confirms the function of CSI and 1-NOA as auxin influx inhibitors, because we obtained a similar reduction in the root hair frequency in CSI- or 1-NOA-treated ein2-1 roots (Tables II and IV). All of these results suggest that endogenous auxin plays a critical role for root hair initiation in the absence of an ethylene response.

The auxin influx mutant aux1, which is also ethylene resistant (Pickett et al., 1990), showed a reduced number of root hair-bearing cells compared with wild type and ein2-1 (Tables I–III). We reported previously that a reduction in the intracellular level of auxin decreased the ethylene-mediated growth response in wild-type Arabidopsis roots (Rahman et al., 2001a) and that the application of a minute concentration of NAA (10 nm) restored the ethylene response in the ethylene-resistant mutants aux1-7 and eir1-1 (Rahman et al., 2001b). In the present study, we found that 10 nm NAA restored aux1 root hair initiation (Table III). Therefore, we argue that the reduction in the frequency of the root hair-bearing cells in aux1 mutants is attributable to a reduced level of endogenous auxin and the resulting alteration in ethylene response. This argument is further supported by the observation that CSI or 1-NOA application to wild-type roots mimicked the aux1 root hair phenotype, i.e. a reduction in the frequency of root hair-forming cells (Table I). These results collectively indicate that the reduction in the root hair frequency in both aux1 mutants and CSI/1-NOA-treated wild-type seedlings is attributable to the low level of endogenous auxin and the reduced response to ethylene, which is regulated by the intracellular level of auxin.

In contrast to root hair initiation, the regulation of root hair elongation is more complex. Although ein2-1 mutant shows a normal percentage of root hair-bearing cells (Table II), the root hair length is extremely short (Figs. 3e and 4B). These results apparently could lead to a conclusion that for the root hair elongation process, auxin may not work as a compensating factor. We also found that a low level of exogenous auxin (10 nm) did not restore ein2-1 root hair length to the wild-type level (Fig. 4B), whereas, a 10-fold increase in the concentration of exogenous auxin completely recovered root hair length (Figs. 3h and 4B). Because ethylene signaling is absent in the ein2-1 mutant, the recovery of the root hair length by exogenous auxin suggests that auxin can also facilitate root hair elongation. The root hair length of the aux1-7 ein2 double mutant is significantly shorter than that of ein2-1 (Figs. 3, e and m, and 6B). We also observed the similar decrease in the root hair length in CSI- or 1-NOA-treated ein2-1 mutant (Fig. 4B). These results collectively indicate that endogenous auxin plays a significant role in root hair outgrowth.
of the ein2-1 mutant and partially compensates for the loss of ethylene response.

In the auxin influx mutant aux1, the root hair length was found to be significantly shorter than that of wild type (Figs. 3, a and i, 1B, and 5B) but slightly longer than ein2-1 (Figs. 3, e and i, 4B, and 5B). Application of a very low concentration (10 nm) of NAA could restore the root hair length of aux1 mutant to the wild-type level in contrast to ein2-1, which required 100 nm of NAA (Figs. 4B and 5B), suggesting that the loss of ethylene signaling makes the root less sensitive to auxin. It is also interesting to note that application of 10 nm NAA stimulated the percentage of root hair-bearing cells in both the wild-type and aux1 mutants to approximately 50%, whereas ein2-1 and aux1-7 ein2 mutants required 100 nm NAA to increase the root hair-bearing cells to that level. All of these results, along with the requirement of the higher concentration of NAA for recovering root hair growth in ein2-1 and aux1-7 ein2 mutants, suggest that insensitivity in ethylene response affects auxin-driven root hair elongation and initiation processes. These results confirm that the loss of ethylene sensitivity makes the root resistant to auxin to some extent. This idea is consistent with our observation that root elongation of ein2-1 is resistant to auxin (data not shown). It has also been cited earlier as an unpublished observation of the author that both ein2 and etr1 mutants show low levels of auxin resistance (Hobbie, 1998). Later, Hobbie et al. (2000) identified ein2 alleles in the screen of 2,4-D-resistant plants. Zolman et al. (2000) found ein2-1 to be resistant to indole-butyric acid.

The root hair developmental process seems to be divided into two steps. In the first step, endogenous auxin plays a compensating role in the absence of an ethylene response as observed in ein2-1 roots, and in the second step, endogenous auxin acts together with ethylene for root hair outgrowth. A higher level of auxin is required for facilitating the latter step in the absence of ethylene signaling.

CSI: a Novel Auxin Influx Modulator in Arabidopsis Roots

CSI exhibited a unique mode of action in controlling root hair developmental process in Arabidopsis roots. Although CSI behaved like 1-NOA to inhibit both root hair initiation and root hair growth in wild-type and ein2-1 roots (Figs. 1B and 4B; Tables I and II), in aux1-7 and aux1-7 ein2 roots, CSI showed completely opposite effects increasing the root hair length and the number of root hair-bearing cells (Figs. 3, j and n, 5B, and 6B; Tables III and IV), whereas 1-NOA did not show any effect on these mutant roots. These results are consistent with our previous findings that CSI partially restored the auxin influx in aux1-7 roots and restored both the gravitropic response and ethylene-induced inhibition of root growth in this mutant root (Rahman et al., 2001a). CSI was much less effective in recovering the frequency of root hair-bearing cells and root hair outgrowth in aux1-7 ein2 double mutant compared with those of aux1-7 single mutant (Figs. 3, j and n, 5B, and 6B; Tables III and IV). These results suggest that CSI-induced recovery in the root hair development of the aux1-7 mutant requires an ethylene response, highlighting the interaction between auxin and ethylene during root hair development in Arabidopsis.

We propose that CSI-induced change in the root hair phenotype of aux1-7 is mediated by restoration of auxin uptake, which consequently accelerates the response to endogenous ethylene and phenocopies the wild-type root hair phenotype. On the other hand, because of the absence of ethylene signaling in aux1-7 ein2 double mutant, CSI only partially restored the root hair phenotype (Fig. 6B; Table IV). We also observed a difference in NAA concentrations required to recover the wild-type root hair phenotype in aux1-7 and aux1-7 ein2 mutants (Table IV). For instance, 10 nm NAA increased the percentage of root hair-bearing cells to approximately 50% in aux1-7, whereas 100 nm NAA was required for the aux1-7 ein2 double mutant. A similar difference in the requirement of auxin concentration was observed for root hair growth (Figs. 5B and 6B) in these mutants. These results suggest that in the presence of ethylene signaling, a low level of auxin is enough to restore a wild-type root hair phenotype, whereas in the absence of an ethylene response, an increased level of auxin is required. The differential effect of CSI on aux1-7 and aux1-7 ein2 mutants along with the requirement of different concentrations of NAA to in-

![Figure 6. Effect of Auxin Influx Modulators on Root Hair Development](https://www.plantphysiol.org)

**Figure 6.** Effect of auxin, CSI, and 1-NOA on root length (A) and root hair length (B). aux1-7 ein2 seedlings were grown on vertical agar plates under continuous light for 3 d. Vertical bars indicate SE.
duce the similar changes in root hair phenotype clearly suggest that in the presence of ethylene signaling, auxin acts together with endogenous ethylene. This idea is consistent with our previous finding that auxin is a positive regulator for ethylene-mediated response in the growth of Arabidopsis roots (Rahman et al., 2001b).

In the null allele of aux1, aux1-22 (Marchant and Bennett, 1998), we could not find any effect of CSI in changing the root hair phenotype, whereas NAA completely restored the root hair phenotype to wild type (Fig. 5B; Table III). These results are consistent with our previous hypothesis that CSI specifically interacts with AUX1 protein in regulating auxin influx and thereby affecting several root developmental processes including gravitropism and the ethylene-mediated growth response (Rahman et al., 2001a). In the present paper, we show that CSI influences both the root hair initiation and root hair elongation. All of these results confirm that CSI interacts via the AUX1 protein to regulate the intracellular level of auxin in Arabidopsis roots. In the two aux1 alleles, aux1-7 and aux1-22, 1-NOA did not show any effect on root hair elongation, root hair formation, and epidermal cell elongation, indicating that AUX1 function is required for 1-NOA action. This is the first strong evidence showing that 1-NOA action requires AUX1 function.

In summary, we conclude that endogenous auxin plays a complementary role for root hair development in the absence of an ethylene response in Arabidopsis. Auxin may act as a positive regulator for the endogenous ethylene-mediated root hair growth and root hair initiation. We have also demonstrated the physiological importance of auxin influx modulators in dissecting the roles of auxin and ethylene in root hair developmental process.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All mutant lines were derived from Arabidopsis (L.) Heynh. ecotype Columbia. Auxin-resistant mutant aux1-7 (Pickett et al., 1990), ethylene-insensitive mutant ein2-1 (Guzmán and Ecker, 1990), and double mutant aux1-7 ein2 were obtained from Arabidopsis Biological Resource Center (Ohio State University, Columbus). These mutants were propagated as described previously (Rahman et al., 2000). The AUX1 null allele aux1-22 was a kind gift from Dr. Bennett. BA-GUS transgenic Arabidopsis (BA3) line is described by Oono et al. (1998).

Buffer solution was made of 5 mM KNO$_3$, 2 mM Ca(NO$_3$)$_2$, 2 mM MgSO$_4$, 1 mM KH$_2$PO$_4$, and 5 mM 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, Ltd., Tokyo) wetted with 300 ml with KOH. Arabidopsis seeds were placed in a 2.6-cm Petri dish on filter paper (Advantec no. 2, Toyo Roshi Kaisha, Ltd., Tokyo) wetted with 300 ml with KOH. Arabidopsis seeds were placed in a 2.6-cm Petri dish on filter paper (Advantec no. 2, Toyo Roshi Kaisha, Ltd., Tokyo) wetted with 300 ml with KOH.

**Chemicals**

CSI was extracted from 7-d-old etiolated pea (Pisum sativum 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$_2$ at –80°C. IAA and NAA were purchased from Sigma-Aldrich (St. Louis). 1-NOA was from Aldrich Chemical Co. (Milwaukee) and toluidine blue N was from Schmidt GmbH Co. (Köngen/N, Germany). Other chemicals were from Wako Pure Chemical Industries, Ltd. (Osaka).

**Morphometric Analysis**

Seedlings were grown vertically as described above for 3 d. They were stained with a dilute toluidine blue (0.01%) solution and placed on a glass microscope slide under a coverslip. The number of root hairs in the 1-mm-region length at the midpoint of a root was counted under a light microscope (BX-50, Olympus, Tokyo) at 40× or 100× magnification depending on the sample type. From the midpoint of this 1-mm region, the length of 10 root hairs from each root was measured at 100× magnification. From the same zone the lengths of 10 mature epidermal cells per root were counted, and the total number of epidermal cells of this zone was calculated. Values from eight roots were used to determine the mean (± se) for percentage of root hair-bearing cells, root hair length, and epidermal cell length in each measurement. The measurement was repeated at least three to five times. P values were analyzed by Student’s t test. Root hairs that grew along the surface of the agar media were photographed at 50× magnification at the longitudinal midpoint of a root by using an Axioscop (Zeiss, Welwyn Garden City, UK)/MZFLIII (Leica, Wetzlar, Germany) imaging microscope equipped with an Olympus DP-50 digital camera.

**GUS Reporter Assay**

GUS assay was performed as described earlier (Oono et al., 1998). In brief, 4-d-old seedlings grown on agar plate as described above were treated with IAA/NAA supplemented with or without various concentrations of 1-NOA or CSI for 6 h in germination media. Seedlings were rinsed three times with staining buffer and incubated for 18 h in staining buffer containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-Glucuronide at 37°C in the dark. GUS expression in the root elongation zone was observed using a Leica MZFLIII dissecting microscope equipped with an Olympus DP-30 digital camera. Images were processed with Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA).

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