Hormones Act Downstream of TTG and GL2 to Promote Root Hair Outgrowth during Epidermis Development in the Arabidopsis Root

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The Arabidopsis root produces a position-dependent pattern of hair-bearing and hairless cell types during epidermis development. Five loci (TRANSPARENT TESTA GLABRA [TTG], GLABRA2 [GL2], ROOT HAIR DEFECTIVE6 [RHD6], CONSTITUTIVE TRIPLE RESPONSE1 [CTR1], and AUXIN RESISTANT2 [AXR2]) and the plant hormones ethylene and auxin have been reported to affect the production of root hair and hairless cells in the Arabidopsis root. In this study, genetic, molecular, and physiological tests were employed to define the roles of these loci and hormones. Epistasis tests and reporter gene studies indicated that the hairless cell-promoting genes TTG and GL2 are likely to act early to negatively regulate the ethylene and auxin pathways. Studies of the developmental timing of the hormone effects indicated that ethylene and auxin pathways promote root hair outgrowth after cell-type differentiation has been initiated. The genetic analysis of ethylene- and auxin-related mutations showed that root hair formation is influenced by a network of hormone pathways, including a partially redundant ethylene signaling pathway. A model is proposed in which the patterning of root epidermal cells in Arabidopsis is regulated by the cell position-dependent action of the TTG/GL2 pathway, and the ethylene and auxin hormone pathways act to promote root hair outgrowth at a relatively late stage of differentiation.

INTRODUCTION

The acquisition of cell-type characteristics in plants is a complex, progressive process that involves positional cues, environmental signals, and internal genetic programs. The plant growth regulators, also known as plant hormones, are known to influence many physiological and developmental processes, including some aspects of cell differentiation (Davies, 1987). An important and unresolved issue concerns the precise role of plant hormones in cell differentiation. In many instances, it is not clear whether the hormones primarily influence differentiation by acting as early regulators of cell fate or by acting during relatively late stages, such as cell expansion or cell morphogenesis.

The root epidermis is well suited for studies of cell differentiation in higher plants. In many species, the root epidermis consists of two cell types, root hair-bearing cells and hairless cells, which simplifies the study of cell-type specification and differentiation in this tissue (Cormack, 1947, 1949; Bunning, 1951; Cutter, 1978). Furthermore, the developing epidermal cells, like many other cells of the root, are organized into columns (or files) along the length of the root, with the newly formed cells located near the apex (in the meristematic region) and the more developmentally advanced cells located farther from the tip. In numerous cases, epidermal cell differentiation has been shown to be influenced by plant hormones. For example, ethylene enhances root hair production in pea, faba bean, and lupine, and it induces root hair formation in tulip, a species that normally lacks root hairs (Abeles et al., 1992).

The small number and simple organization of cells in the epidermis of the Arabidopsis root make it an attractive tissue for the study of pattern formation and cell differentiation in plants (Dolan et al., 1993, 1994; Galway et al., 1994; Scheres et al., 1994). Within the Arabidopsis root epidermis, cells adopt distinct fates in a position-dependent manner. Epidermal cells located outside an anticlinal (radial) wall separating two cortical cells differentiate into root hair cells, whereas epidermal cells that lie outside periclinal cortical cell walls differentiate into mature hairless cells. The precursors to the two mature cell types can be distinguished from one another throughout their differentiation (Dolan et al., 1994; Galway et al., 1994), as outlined in Figure 1. The earliest distinctions are apparent when the cells are still located within the meristematic region of the root; here the two differentiating cell types exhibit differences in cytoplasmic density, with differentiating root hair cells possessing relatively dense, intensely staining cytoplasm. During later stages of epidermis development, the two differentiating cell types differ in their rate of vacuolation, in the extent of elongation, and ultimately in the presence or absence of root hairs. Thus, the cells of the root epidermis begin to acquire cell-type-specific characteristics in a position-dependent manner.
Differential Cell Vacuolation
Differential Cytoplasmic Staining

Figure 1. Drawing of the Epidermal Cells of a Wild-Type Arabidopsis Root.

Longitudinal files of epidermal cells originating at the meristem initials are shown. The developmental regions of the root and the location of differences between differentiating root hair and nonhair cells are indicated. The earliest cytological difference between the two differentiating epidermal cell types is apparent in cells from the late meristematic region (~150 to 300 μm from the central cells of the apical meristem), where differential cytoplasmic staining and differential vacuole formation are evident in transverse sections. Note that root hairs emerge from epidermal cells at a relatively late stage of development. The single curved line at the root apex indicates the outline of the root cap. Bar = 100 μm.

manner at an early stage of epidermis development, and the emergence of root hairs may be considered a late event in cell differentiation.

The genetic control of cell-type differentiation in the Arabidopsis root epidermis has begun to be explored. Five loci are reported to influence the production of root hair and/or hairless cells in the Arabidopsis root: TRANSPARENT TESTA GLABRA (TTG; Galway et al., 1994), GLABRA2 (GL2; Masucci et al., 1996), CONSTITUTIVE TRIPLE RESPONSE1 (CTR1; Dolan et al., 1994), ROOT HAIR DEFECTIVE6 (RHD6; Masucci and Schiefelbein, 1994), and AUXIN RESISTANT2 (AXR2; Wilson et al., 1990). In addition to their effects on root epidermis development, each of these loci, with the exception of RHD6, influences development in other tissues or organs of the Arabidopsis plant.

The TTG and GL2 genes appear to encode negative regulators of the root hair cell differentiation pathway. Recessive mutations in the TTG gene cause nearly all root epidermal cells to exhibit the characteristics of root hair cells throughout their development (Galway et al., 1994). Because the ttg defects can be suppressed by constitutive expression of the maize R cDNA in Arabidopsis, the TTG gene has been suggested to encode or activate an R homolog (a myc-like transcriptional activator) (Lloyd et al., 1992; Galway et al., 1994). Like the ttg mutations, recessive mutations in the GL2 gene cause root hairs to form on essentially every root epidermal cell (Masucci et al., 1996). However, the differentiating gl2 epidermal cells retain the characteristic cellular differences that distinguish developing root hair and hairless cells in the wild type (e.g., differences in cell vacuolation and cytoplasmic density). The GL2 gene encodes a homeodomain protein and is expressed preferentially in the differentiating hairless epidermal cells within the meristematic and elongation regions of the root (Rerie et al., 1994; Masucci et al., 1996). Thus, TTG and GL2 may be considered negative regulators of root hair cell differentiation, with TTG inhibiting all aspects of root hair cell differentiation and GL2 affecting a subset of these processes (Galway et al., 1994; Masucci et al., 1996).

The CTR1, AXR2, and RHD6 genes are each associated with ethylene and/or auxin effects on root epidermis development. The CTR1 gene encodes a Raf-like protein kinase proposed to negatively regulate the ethylene signal transduction pathway (Kieber et al., 1993), and recessive ctr1 mutations cause root hairs to form on epidermal cells that are normally hairless (Dolan et al., 1994). The dominant mutations of the axr2 gene confer insensitivity to high concentrations of auxin, ethylene, and abscisic acid, and they reduce root hair formation (Wilson et al., 1990). The recessive rhd6 mutations cause a dramatic reduction in root hair production, possibly due to a defect in the initiation of root hair outgrowth, and the mutant phenotype can be suppressed by the inclusion of an auxin (indole-3-acetic acid; IAA) or the ethylene precursor l-amino-cyclopropane-1-carboxylic acid (ACC) in the growth media (Masucci and Schiefelbein, 1994).

In addition to the ctr1, axr2, and rhd6 mutant phenotypes, a role for ethylene in root hair formation has been indicated by the effects of ethylene inhibitors and an ethylene precursor on wild-type Arabidopsis seedlings. Plants grown in the presence of either aminooxyacetic acid (AVG; an ethylene synthesis inhibitor) or Ag+ (an inhibitor of ethylene action) display a reduction in the frequency of root hairs (Masucci and Schiefelbein, 1994; Tanimoto et al., 1995), whereas roots grown
on high concentrations of ACC possess ectopic root hairs (Tanimoto et al., 1995). Together, these observations imply that ethylene and auxin may act as positive regulators of root hair cell differentiation in Arabidopsis. However, a precise role for these genes and hormones has not been established, in part because an analysis of the developing root epidermis in these mutants and treated plants has not been conducted.

Based on the preceding observations, at least two pathways are likely to exist to influence root epidermis cell–type differentiation in Arabidopsis. One pathway involves the T7G and GL2 gene products (putative negative regulators of root hair cell differentiation), and another pathway involves ethylene and/or auxin and their associated signal transduction cascades (putative positive regulators of root hair cell differentiation). Thus, two simple models could explain the position-dependent pattern of root epidermal cell types. In one model, the T7G/GL2 pathway would negatively regulate the ethylene/auxin pathway in differentiating hairless cells. Alternatively, the ethylene/auxin pathway could negatively regulate the T7G/GL2 pathway in differentiating root hair cells. In this study, a variety of experimental approaches was employed to distinguish between these alternatives and to define the genetic and hormonal interactions that control epidermal cell differentiation in the Arabidopsis root. Our results show that a network of ethylene and auxin pathways acts to promote root hair emergence at a relatively late stage in root epidermal cell differentiation and that the hormones are not likely to act at an early stage to initiate cell-type differentiation. We propose a model in which the T7G/GL2 pathway controls the patterning of epidermal cell types by negatively regulating the root hair–promoting activities of the ethylene/auxin pathway in a cell position–dependent manner.

RESULTS

Root Epidermis Cell Types in Mutants and Hormone-Treated Plants

To provide a framework for the analysis of genes and hormones affecting the root epidermis, the proportion of root hair and hairless epidermal cell types was first determined in wild-type, mutant, and hormone-treated seedling roots. This assay provides a convenient measure of the effect of a mutation or factor on cell-type differentiation, because the formation of a root hair represents the defining difference between the two epidermal cell types. In this assay, any epidermal cell with a hairlike outgrowth, regardless of how small, was classified as a root hair cell. In addition to analyzing the proportion of epidermal cell types, the location of the cell types relative to the underlying cortical cells was assessed by examining transverse sections of seedling roots.

In wild-type roots (ecotypes Columbia and Landsberg erecta), ~40% of the epidermal cells bear root hairs and 60% of the cells are hairless, as shown in Table 1. The root hair cells are almost exclusively located in the crevice between adjacent cortical cells (outside an anticlinal cortical cell wall). Root hair–bearing cells located in the alternative position (outside a periclinal cortical cell wall) are referred to as ectopic root hair cells.

The ttg, gl2, and ctrl mutants exhibit an increase in the proportion of root hair cells and a corresponding decrease in the proportion of hairless cells, relative to the wild type. In the ttg and gl2 mutants, almost all epidermal cells produce root hairs, regardless of their position (Table 1). The ctrl mutation causes a modest increase in the proportion of root hair cells (62%), with a corresponding frequency of ectopic root hair cells (32% ectopic hair cells; Table 1). Unlike the ttg and gl2 mutations, ctrl has a significant negative effect on epidermal cell length, indicating that ctrl has multiple effects on epidermis development (Table 1). The rh6 and axr2 mutants display an abnormally low frequency of root hair cells. Only 4% of the rh6 epidermal cells and 27% of the axr2 epidermal cells formed root hairs (Table 1). In each mutant, the root hair cells are located in the appropriate position (Table 1). The results of these tests with ttg, gl2, ctrl, rh6, and axr2 are consistent with previously published data (Wilson et al., 1990; Dolan et al., 1994; Galway et al., 1994; Masucci and Schiefelbein, 1994; Masucci et al., 1996).

To examine the effect of plant hormones on root epidermis differentiation, seedlings were grown on unsupplemented media (Figure 2A), transferred to media supplemented with a hormone precursor or hormone inhibitor, and then analyzed for their epidermal cell types. As shown in Table 2 and Figure 2B, media containing the ethylene precursor ACC caused wild-

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Percentage of Root Hair Cells</th>
<th>Percentage of Ectopic Root Hair Cells</th>
<th>Epidermal Cell Length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Columbia&lt;sup&gt;c&lt;/sup&gt;</td>
<td>42 ± 2 (5)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1 (111)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>198 ± 43</td>
</tr>
<tr>
<td>Landsberg erecta&lt;sup&gt;c&lt;/sup&gt;</td>
<td>39 ± 5 (3)</td>
<td>3 (65)</td>
<td>155 ± 37</td>
</tr>
<tr>
<td>ttg</td>
<td>94 ± 1 (2)</td>
<td>51 (77)</td>
<td>155 ± 50</td>
</tr>
<tr>
<td>gl2</td>
<td>97 ± 3 (2)</td>
<td>53 (78)</td>
<td>186 ± 50</td>
</tr>
<tr>
<td>ctrl</td>
<td>62 ± 2 (2)</td>
<td>32 (88)</td>
<td>43 ± 6</td>
</tr>
<tr>
<td>rh6</td>
<td>4 ± 2 (2)</td>
<td>3 (36)</td>
<td>192 ± 68</td>
</tr>
<tr>
<td>axr2</td>
<td>27 ± 4 (2)</td>
<td>6 (37)</td>
<td>236 ± 68</td>
</tr>
<tr>
<td>axr2 ttg</td>
<td>25 ± 14 (3)</td>
<td>40 (105)</td>
<td>217 ± 50</td>
</tr>
<tr>
<td>axr2 gl2</td>
<td>28 ± 6 (2)</td>
<td>25 (100)</td>
<td>229 ± 62</td>
</tr>
<tr>
<td>rh6 ttg</td>
<td>23 ± 13 (4)</td>
<td>13 (131)</td>
<td>248 ± 43</td>
</tr>
<tr>
<td>rh6 gl2</td>
<td>17 ± 8 (3)</td>
<td>4 (108)</td>
<td>186 ± 43</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean ± standard deviation.

<sup>b</sup>Percentage of the root hair–bearing cells that are located over a periclinal cortical cell wall (ectopic position).

<sup>c</sup>Wild type.

<sup>d</sup>Numbers within parentheses indicate the number of trials (125 cells examined per trial).

<sup>e</sup>Numbers within parentheses indicate the total number of root hair–bearing cells scored for their location.
agarose-solidified media without supplementation.

(A) Wild-type seedlings (Columbia ecotype) grown for 4 days on media supplemented with 50 μM ACC, an ethylene precursor. Note that root length is dramatically reduced due to a reduction in cell length (Table 2).

(B) Wild-type seedlings (Columbia ecotype) grown for 4 days on media supplemented with 25 nM AVG, an ethylene biosynthesis inhibitor. Magnifications are the same for (A) to (C).

Figure 2. Ethylene Alters Root Epidermis Development in Arabidopsis.

(A) Wild-type seedlings (Columbia ecotype) grown for 4 days on agarose-solidified media without supplementation.

(B) Wild-type seedlings (Columbia ecotype) grown for 4 days on media supplemented with 50 μM ACC, an ethylene precursor. Note that root length is dramatically reduced due to a reduction in cell length (Table 2).

(C) Wild-type seedlings (Columbia ecotype) grown for 4 days on media supplemented with 25 μM AVG, an ethylene biosynthesis inhibitor. Magnifications are the same for (A) to (C).

Several lines of evidence indicate that the ACC treatments affect root epidermis development through an ethylene pathway. First, as previously reported (Masucci and Schiefelbein, 1994), the AVG-induced defects in root epidermis cell differentiation can be reversed by ACC. This reversal was confirmed in this study, when wild-type seedlings treated with 5 μM AVG and 5 μM ACC were found to produce 32% root hair cells. Second, the ACC-treated seedlings have shorter and radially expanded root epidermal cells, similar to those of ethylene-treated seedlings (Table 2; data not shown). Furthermore, the ctr1 mutant produces ectopic root hair cells, like the ACC-treated seedlings (discussed above). Together, the effect of ethylene precursors and inhibitors indicates that ethylene promotes root hair formation, as previously discussed (Masucci and Schiefelbein, 1994; Tanimoto et al., 1995).

Because IAA, like ACC, is able to suppress the rhd6 mutant phenotype (Masucci and Schiefelbein, 1994) and because the axr2 mutation is resistant to auxin as well as ethylene (Wilson et al., 1990), wild-type seedlings were grown in the presence of high IAA concentrations to determine the effect of auxin on root hair formation. Although the IAA treatments had significant effects on cell elongation, no change in root hair production or ectopic hair cell formation was observed (Table 2).

Interactions between the TTG/GL2 and the RHD6/Ethylene/Auxin Pathways

Double Mutant Tests

The opposing effects of the ttg and gl2 mutations (excess root hair cell formation) and the rhd6 and axr2 mutations (reduced root hair cell formation) were exploited in double mutant analyses to test for epistatic interactions. As shown in Table 1, the phenotypes of the axr2 ttg and axr2 gl2 double mutants resemble the phenotype of the axr2 mutant with respect to the frequencies of the epidermal cell types. However, the axr2 ttg and axr2 gl2 double mutants possess a large proportion of root hair cells in ectopic positions (40 and 25%, respectively; Table 1). Thus, a functional AXR2 gene product is required for induction of a high frequency of root hair cells by the ttg and gl2 mutations. These results would be expected if the TTG and GL2 genes normally act to negatively regulate the AXR2 gene pathway in the developing hairless epidermal cells.

In the rhd6 ttg and rhd6 gl2 double mutants, the proportion of root hair cells (23 and 17%, respectively) is considerably less than that observed in wild-type plants but greater than that observed in rhd6 mutants (Table 1). The rhd6-containing double mutants also produced some ectopic root hair cells (Table 1). This observation indicates that a functional RHD6 gene

Table 2. Root Epidermis Phenotypes in Wild-Type and Hormone-Treated Seedlings

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of Root Hair Cells†</th>
<th>Percentage of Ectopic Root Hair Cells‡</th>
<th>Epidermal Cell Length (μm)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Columbia</td>
<td>42 ± 2 (5)</td>
<td>1 (111)</td>
<td>98 ± 43</td>
</tr>
<tr>
<td>5 μM ACC</td>
<td>48 ± 7 (5)</td>
<td>9 (71)</td>
<td>62 ± 37</td>
</tr>
<tr>
<td>50 μM ACC</td>
<td>53 ± 5 (4)</td>
<td>22 (161)</td>
<td>56 ± 19</td>
</tr>
<tr>
<td>1 μM AVG</td>
<td>42 ± 4 (3)</td>
<td>3 (64)</td>
<td>236 ± 56</td>
</tr>
<tr>
<td>5 μM AVG</td>
<td>11 ± 7 (6)</td>
<td>0 (55)</td>
<td>124 ± 25</td>
</tr>
<tr>
<td>25 μM AVG</td>
<td>1 ± 0 (2)</td>
<td>ND†</td>
<td>149 ± 37</td>
</tr>
<tr>
<td>30 nM IAA</td>
<td>45 ± 2 (2)</td>
<td>1 (82)</td>
<td>56 ± 19</td>
</tr>
<tr>
<td>300 nM IAA</td>
<td>46 ± 2 (2)</td>
<td>3 (113)</td>
<td>25 ± 6</td>
</tr>
</tbody>
</table>

† Mean ± standard deviation.
‡ Percentage of the root hair–bearing cells that are located over a periclinical cortical cell wall (ectopic position).
§ Wild type.
Numbers within parentheses indicate the number of trials (125 cells examined per trial).
Numbers within parentheses indicate the total number of root hair–bearing cells scored for their location.
ND, not determined.
product is necessary for the ttg and gl2 effects, and it is consistent with the possibility that the RHD6 pathway is negatively regulated by, or acts separately from, the TTG/GL2 pathway during epidermis development.

Hormone Treatments

Both ACC and IAA are able to suppress the rhd6 mutant defects (Masucci and Schiefelbein, 1994; Table 3). To determine whether these hormone treatments could suppress the rhd6-dependent defects in the rhd6 ttg and rhd6 gl2 double mutants, these seedlings were grown on media supplemented with either 5 μM ACC or 30 nM IAA (concentrations that rescue the rhd6 mutant phenotype). As shown in Table 3, the rhd6 ttg and rhd6 gl2 double mutants treated in this manner possess ttg- and gl2-like phenotypes, respectively. Because these treatments are able to bypass the rhd6 defect in the ttg and gl2 backgrounds, the ACC and IAA must not act through a pathway that requires the TTG or GL2 gene products. This concept implies that these hormones act downstream of the TTG/GL2 pathway to influence root hair formation.

To test further the relationship between ethylene and the TTG/GL2 pathway, the ttg and gl2 mutant seedlings were grown on media containing the ethylene inhibitor AVG. If ethylene is necessary for later stages of root hair differentiation rather than for early regulation of the TTG/GL2 pathway, then AVG is expected to block root hair formation in the ttg and gl2 mutants. When treated with 5 μM AVG, ~30% of the epidermal cells in the ttg and gl2 mutant roots formed root hairs (rather than nearly 100% in the untreated mutant seedlings; Table 1; data not shown). This effect is comparable to the reduction in root hair formation in wild-type seedlings treated with 5 μM AVG (26%; Table 2), and it provides additional support for the notion that ethylene acts downstream of the TTG and GL2 gene pathway.

**Table 3. Effect of Ethylene and Auxin Mutations and Treatments on Root Epidermal Cell Differentiation**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. Supplement</th>
<th>5 μM ACC</th>
<th>30 nM IAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Columbia</td>
<td>42 ± 2 (5)</td>
<td>48 ± 7 (4)</td>
<td>45 ± 2 (2)</td>
</tr>
<tr>
<td>rhd6</td>
<td>4 ± 2 (2)</td>
<td>33 ± 4 (2)</td>
<td>36 ± 3 (2)</td>
</tr>
<tr>
<td>rhd6 ttg</td>
<td>23 ± 13 (4)</td>
<td>74 ± 5 (2)</td>
<td>97 ± 1 (2)</td>
</tr>
<tr>
<td>rhd6 gl2</td>
<td>17 ± 8 (3)</td>
<td>89 (1)</td>
<td>94 (1)</td>
</tr>
<tr>
<td>rhd6 ctrl</td>
<td>43 ± 1 (2)</td>
<td>ND†</td>
<td>ND</td>
</tr>
<tr>
<td>rhd6 etol</td>
<td>27 ± 4 (2)</td>
<td>31 ± 1 (2)</td>
<td>34 ± 6 (3)</td>
</tr>
<tr>
<td>rhd6 axr2</td>
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<td>0 ± 0 (2)</td>
<td>0 ± 0 (2)</td>
</tr>
<tr>
<td>axr1</td>
<td>48 ± 4 (3)</td>
<td>50 ± 0 (2)</td>
<td>45 ± 4 (2)</td>
</tr>
<tr>
<td>rhd6 axr1</td>
<td>9 ± 3 (3)</td>
<td>27 ± 0 (2)</td>
<td>16 ± 8 (2)</td>
</tr>
<tr>
<td>eto1</td>
<td>43 ± 2 (2)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>rhd6 eto1</td>
<td>38 ± 9 (3)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ein2</td>
<td>41 ± 4 (3)</td>
<td>43 ± 0 (2)</td>
<td>45 ± 1 (2)</td>
</tr>
<tr>
<td>rhd6 ein2</td>
<td>35 ± 9 (3)</td>
<td>10 ± 8 (3)</td>
<td>38 ± 0 (2)</td>
</tr>
<tr>
<td>etr1</td>
<td>43 ± 1 (3)</td>
<td>35 (1)</td>
<td>42 (1)</td>
</tr>
<tr>
<td>aux1</td>
<td>38 ± 0 (3)</td>
<td>38 ± 2 (2)</td>
<td>46 ± 3 (2)</td>
</tr>
<tr>
<td>aux1 ein2</td>
<td>41 ± 3 (3)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>aux1 etr1</td>
<td>24 ± 3 (2)</td>
<td>15 ± 4 (2)</td>
<td>40 ± 0 (2)</td>
</tr>
</tbody>
</table>

† Percentage of the root epidermal cells that possess root hairs, given as the mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>5 μM ACC</th>
<th>30 nM IAA</th>
</tr>
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<tbody>
<tr>
<td>mutant background</td>
<td>5 μM ACC</td>
<td>30 nM IAA</td>
</tr>
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<td>Columbia</td>
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<tr>
<td>rhd6</td>
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<td>36 ± 3 (2)</td>
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<td>rhd6 ttg</td>
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</tr>
<tr>
<td>rhd6 gl2</td>
<td>89 (1)</td>
<td>94 (1)</td>
</tr>
<tr>
<td>rhd6 ctrl</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>rhd6 etol</td>
<td>31 ± 1 (2)</td>
<td>34 ± 6 (3)</td>
</tr>
<tr>
<td>rhd6 axr2</td>
<td>0 ± 0 (2)</td>
<td>0 ± 0 (2)</td>
</tr>
<tr>
<td>axr1</td>
<td>50 ± 0 (2)</td>
<td>45 ± 4 (2)</td>
</tr>
<tr>
<td>rhd6 axr1</td>
<td>27 ± 0 (2)</td>
<td>16 ± 8 (2)</td>
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<tr>
<td>eto1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>rhd6 eto1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ein2</td>
<td>43 ± 0 (2)</td>
<td>45 ± 1 (2)</td>
</tr>
<tr>
<td>rhd6 ein2</td>
<td>10 ± 8 (3)</td>
<td>38 ± 0 (2)</td>
</tr>
<tr>
<td>etr1</td>
<td>35 (1)</td>
<td>42 (1)</td>
</tr>
<tr>
<td>aux1</td>
<td>38 ± 2 (2)</td>
<td>46 ± 3 (2)</td>
</tr>
<tr>
<td>aux1 etr1</td>
<td>ND</td>
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<tr>
<td>aux1 etr1 eto1</td>
<td>15 ± 4 (2)</td>
<td>40 ± 0 (2)</td>
</tr>
</tbody>
</table>

† Percentage of the root epidermal cells that possess root hairs, given as the mean ± standard deviation.

† Numbers within parentheses indicate the number of trials (125 cells examined per trial).

† ND, not determined.

In another assay for interactions between the TTG/GL2 pathway and the hormone-related genes, the effect of the ctr1, rhd6, and axr2 mutations on the expression of a GL2-promoter--β-glucuronidase (GUS) reporter gene fusion was determined. In wild-type plants harboring the GL2-GUS construct, GUS activity is preferentially detected in developing hairless epidermal cells within the meristematic and elongation zones of the Arabidopsis root, which reflects the pattern of GL2 transcript accumulation (Masucci et al., 1996). Thus, the GL2–GUS reporter fusion represents an excellent marker for studying genes that may regulate GL2 gene activity directly or indirectly. For example, if the AXR2, RHD6, or CTR1 genes normally exert their effects on epidermal cell differentiation by regulating the TTG/GL2 pathway, the pattern of GUS expression would be expected to be altered in plants harboring mutations in those genes. However, when the GL2–GUS construct was introduced into the axr2, rhd6, and ctr1 mutant backgrounds and GUS activity was assessed, no deviation in GUS expression was observed, as shown in Figures 3A to 3D. Furthermore, an examination of transverse sections of these roots showed that the GUS-expressing cells are located over the periclinal cortical cell walls (i.e., the position of the differentiating hairless epidermal cells in wild-type plants; data not shown). This indicates that the AXR2, RHD6, and CTR1 genes are not likely to regulate the GL2 gene.

The effect of hormone precursors or inhibitors on GL2 gene activity was also assessed by subjecting wild-type plants harboring the GL2–GUS construct to 50 μM ACC or 5 μM AVG. As shown in Figures 3E and 3F, a normal pattern of GUS activity was detected in the hormone-treated roots, despite the fact that these hormone treatments significantly altered the proportion of root hair and hairless cells (Table 2). The AVG-treated roots did exhibit a reduction in the length of the staining zone, which may be due to the rapid cellular differentiation induced by AVG (see above). In transverse sections of these roots, the GUS-expressing cells were located over periclinal cortical cell walls, like the GUS-expressing cells in the wild-type background (data not shown). These results show that the changes in epidermal cell–type differentiation induced by ACC and AVG are not due to direct or indirect effects on GL2 gene activity. Thus, these ethylene-related treatments affect a pathway that is likely to act downstream of the GL2 gene pathway in the development of the root epidermis.
Timing of Gene and Hormone Action during Epidermis Differentiation

Cellular Characteristics in the Developing Epidermis

The effects of the rhd6, axr2, and ctr1 mutations on cell-type differentiation in the root epidermis could be exerted at several possible stages. To determine whether the earliest aspects of cell differentiation are altered by these mutations, transverse sections were obtained from the root apex of wild-type and mutant Arabidopsis seedlings. As shown in Figure 4A, two sets of differentiating epidermal cells can be distinguished in wild-type sections taken ~150 to 300 μm above the central cells of the root meristem (in a region of the root where a single lateral root cap layer is present). The epidermal cells located over anticlinal cortical cell walls (cells destined to bear root hairs) exhibit more intensely staining cytoplasm and a reduced degree of vacuole formation than their neighboring cells located over periclinal cortical cell walls (cells destined to remain hairless). In previous studies, these cellular differences were found to be present in the developing epidermis of gl2 mutants (Masucci et al., 1996) but absent from the ttg mutants (Galway et al., 1994).

If AXR2, RHD6, and CTR1 act at a late stage of epidermis development, then the cellular differences present in the early epidermis of the wild type should exist in the axr2, rhd6, and ctr1 mutants. As shown in Figures 4B to 4D, each of the axr2, rhd6, and ctr1 roots exhibits differential cytoplasmic staining and differential vacuole formation that is dependent on the position of the epidermal cells. The degree of differential staining is reduced in the rhd6 and axr2 mutants, and vacuole formation is reduced in the ctr1 mutant (Figure 4). Nonetheless, these results show that the axr2, rhd6, and ctr1 mutations do not abolish the early developmental events associated with the position-dependent differentiation of the epidermal cells.

The cellular characteristics of the developing epidermis were also examined in roots treated with ACC or AVG to determine whether the cell-type alterations induced by these treatments may be due to early changes in cell differentiation. Roots treated with 50 μM ACC exhibited the position-dependent differences in cytoplasmic staining and vacuole formation present in wild-type roots (Figure 4E). In roots treated with 5 μM AVG, epidermis differentiation appeared to advance more rapidly than it did in wild-type roots, and differential cytoplasmic staining and cell vacuolation were observed in sections taken closer to the root apex (at an earlier stage in development) than in the wild type (data not shown). Sections taken at the usual later stage (in the region of the root in which a single lateral root cap layer is present) exhibited a diminished degree of differential cytoplasmic staining and differential vacuolation in the epidermal cells (Figure 4F). Because position-dependent differences in epidermal cell characteristics were observed in these ACC- and AVG-treated roots, ethylene is not likely to regulate early stages of epidermis development.

Figure 3. The Spatial Expression Pattern of the GL2–GUS Reporter Gene Fusion Construct in the Arabidopsis Root Is Not Affected by Hormone Mutations or by Ethylene Treatments.

Five-day-old seedlings harboring the GL2 promoter–GUS reporter gene were assayed for GUS activity. (A) Wild-type root (Columbia ecotype). (B) axr2 mutant. (C) rhd6 mutant. (D) ctrl mutant. (E) Wild-type seedling grown on media supplemented with 50 μM ACC. (F) Wild-type seedling grown on media supplemented with 5 μM AVG. Note the presence of root hair cells closer to the apex in the ctr1 and ACC-treated roots in (D) and (E). Differences in staining intensity reflect differences in incubation times. Magnifications are the same for (A) to (F). Bar in (A) = 100 μm.

Bead-Labeling Study

Although the results from the experiments above indicated that ethylene acts relatively late in epidermis development, the actual developmental period in which ethylene alters epidermal cell–type differentiation was not clear. Because the seeding
root is a continuously growing organ with constant cell proliferation and cell differentiation, we were able to define the latest point in epidermis development at which ACC can induce ectopic root hairs. When wild-type seedlings are transferred from unsupplemented media to media supplemented with ACC, there is a period of time before the first ectopic hair forms. This lag period indicates that some of the differentiating epidermal cells are unable to switch their developmental program in response to the treatment, and the period provides a means to identify the oldest (most developmentally advanced) epidermal cell capable of being affected by the treatment.

In this experiment, polylysine-coated beads were bound to wild-type seedlings to mark the developing root epidermal cells. The youngest root hair–bearing cell (the epidermal cell with the shortest root hair) was used as a convenient reference point for developing cells within a particular file. The marked seedlings were transferred to media supplemented with 50 μM ACC and were allowed to grow for various lengths of time. The root epidermis was then examined to identify ectopic root hairs and to determine the number of cells present between the youngest root hair–bearing cell (at the time of transfer) and the ectopic root hairs (after growth on ACC). This number, which varied from seven to 21 (mean of 14), provides an estimate of the number of developing cells located below the position of the youngest root hair–bearing cell within a file that fail to respond to the ACC. Because the ACC concentration used (50 μM) causes only 22% ectopic root hair cells (Table 2), a better estimate of the actual number of cells unresponsive to ACC is probably at the lower end of this range (seven to 10 cells per file). Thus, ACC is able to alter the differentiation of epidermal cells that have not progressed beyond a point located approximately seven to 10 cells below the position of the youngest root hair–bearing cell. The location of this point is within the elongation zone of the root (see Figure 1), indicating that ethylene can induce ectopic hairs on differentiating epidermal cells that have progressed to the relatively late stage of cell elongation.

Similar bead-labeling and transfer experiments were performed with the rhd6 mutant. These experiments were designed to take advantage of the ability of ACC and IAA to induce normal root hair formation on the rhd6 mutant root. Bead-marked wild-type and rhd6 seedlings were simultaneously transferred to media supplemented with either 5 μM ACC or 30 nM IAA and allowed to grow for various lengths of time. The number of new root hair–bearing cells that formed (per file) in the wild type was compared with the number of root hair–bearing cells that formed (per file) in the treated rhd6 mutant. The difference between these numbers represents the number of rhd6 epidermal cells (within a file) that were unable to respond to the hormone and produce a root hair. The average number of cells incapable of responding to ACC was 7.2 ± 2.7 and to IAA was 8.0 ± 2.8. Therefore, the oldest rhd6 epidermal cell capable of altering its developmental program in response to ACC or IAA is approximately six to nine cells below the youngest root hair cell, which corresponds to cells located within the early portion of the elongation region of the root (see Figure 1). These results are consistent with the data on wild-type roots presented above and indicate that plant hormone treatments can induce root hair formation on cells at a relatively late stage of differentiation.
Genetic Analysis of the Hormone Pathways Affecting Root Hair Formation

Additional Hormone-Related Mutations

The results above indicate that the ethylene and auxin pathways are likely to be involved in promoting root hair formation in Arabidopsis. To understand these genetic pathway(s) in greater detail, we analyzed the root epidermis in seedlings harboring mutations in additional loci associated with hormone biosynthesis or response. Seedlings possessing mutations in the following loci were tested for their root hair production: axr1 (recessive, auxin insensitive [Estelle and Somerville, 1987; Lincoln et al., 1990], and slightly ethylene insensitive [Timpte et al., 1995], and gene product is related to ubiquitin-conjugating enzymes [Leyser et al., 1993]), eto1 (ethylene overproducer [Guzman and Ecker, 1990]), etr1 (dominant, ethylene insensitive [Bleecker et al., 1988], and encodes putative ethylene receptor with similarity to two-component regulators [Chang et al., 1993; Schaller and Bleecker, 1995]), ein2 (recessive, ethylene insensitive, and slight ethylene overproducer [Guzman and Ecker, 1990]), and aux1 (auxin and ethylene insensitive in a root-specific manner [Pickett et al., 1990]). In each of these five mutants, the root epidermis was determined to possess a normal proportion of root hair and hairless cells (Table 3).

The lack of an observable effect of these mutations on the formation of root epidermis cell types may be due to redundancy in the hormone pathways involved in root hair formation. To test this possibility, two double mutants were constructed that included the aux1 mutation, because the aux1 defect appears to be root specific (Pickett et al., 1990). The aux1 ein2 double mutant produces a normal proportion of root hair cells (Table 3), indicating that these genes either do not act in redundant pathways or that they do not participate in root hair formation. In contrast, the aux1 etr1 double mutant produces a significantly lower frequency of root hair cells than does the wild type or either single mutant alone (Table 3). Thus, AUX1 and ETR1 may normally act in separate, redundant pathways to promote root hair formation.

Double Mutants with rhd6

To examine further the role of the hormone-related genes in root hair formation, the effect of ACC and IAA treatments was assessed in seedlings bearing the hormone mutations. Because the effect of ACC and IAA on root hair production is most clearly observed in the rhd6 mutant, the hormone-related mutations were introduced into the rhd6 mutant background, and these homozygous double mutants were used to determine the extent to which ACC or IAA could induce root hairs.

The axr2 mutants, which produce a reduced frequency of root hair cells relative to wild type (Table 1), are not significantly affected by ACC or IAA treatments (Table 3). The rhd6 axr2 double mutant completely lacks root hair cells, and root hairs could not be induced in the double mutant by ACC or IAA treatments (Table 3). Therefore, a functional AXR2 gene is required for normal root hair formation, and it is required for ACC or IAA to induce root hairs.

The rhd6 axr1 double mutant exhibits a phenotype similar to the rhd6 single mutant (Table 3). However, when the rhd6 axr1 mutant was treated with ACC or IAA, complete suppression of the mutant phenotype did not occur. Instead, ACC treatment led to only 27% root hair cells, and IAA treatment generated only 16% root hair cells (Table 3). This result indicates that ACC and IAA require a functional AXR1 gene to fully promote root hair initiation and that an AXR1-independent pathway may be mediating an ethylene and auxin response.

The rhd6 eto1 double mutant has an essentially normal proportion of epidermal cell types on unsupplemented media (Table 3). Thus, the eto1 mutation is able to suppress the hairless phenotype of the rhd6 mutant, perhaps due to the excess ethylene that is produced by eto1 plants.

Like the eto1 mutation, ein2 is able to suppress the rhd6 defect in rhd6 ein2 double mutants (Table 3), perhaps because of the enhanced ethylene production in ein2 (Guzman and Ecker, 1990). The rhd6 ein2 double mutant is unusual because root hair formation appears to be delayed within the cell files relative to the wild type (data not shown), and treatment with ACC actually reduces root hair formation (Table 3).

The rhd6 ctr1 double mutant is most similar to the ctr1 single mutant phenotype (Table 3). In rhd6 ctr1, ~11% of the root hair cells are in ectopic locations and the epidermal cell length is reduced (51 ± 10 μm), similar to the ctr1 single mutant (Table 1). Thus, root hair formation induced by mutations in the negative regulator CTR1 does not require a functional RHD6 product, which implies that CTR1 acts at a step downstream of RHD6.

Effect of Auxin

Auxin is known to enhance ethylene production in roots (Abeles et al., 1992). To examine the possibility that the auxin effects on root hair formation are actually due to ethylene, we determined the effect of IAA on AVG-treated seedlings. Whereas growth of wild-type seedlings on 5 μM AVG causes only 11% root hair cell production (Table 2), the addition of 30 nM IAA to the AVG media results in normal root hair production (45%). Therefore, the AVG-induced defects in root hair cell differentiation can be suppressed by IAA. Another indication of the separate nature of the ethylene and auxin pathways was obtained by measuring the effect of ACC and IAA on the aux1 etr1 double mutant. The reduced-hair phenotype of aux1 etr1 seedlings is not suppressed by ACC, but it is suppressed by IAA (Table 3). These results show that auxin and ethylene pathways are separable and that IAA is not likely to affect root hair formation merely by enhancing ethylene production.
DISCUSSION

Control of Cell-Type Patterning during Root Epidermis Development

In the Arabidopsis root, a position-dependent pattern of root hair cells and hairless cells is generated during epidermis development. This study analyzed the genetic control of epidermal cell-type patterning by defining the relationship between genes acting in the hairless-cell differentiation pathway(s) (TTG and GL2) and the genes and hormones that participate in the root hair cell differentiation pathway(s) (AXR2, CTR1, RHD6, ethylene, and auxin). It was important to determine whether the cell-type pattern is due to the position-dependent expression of the developmental pathways or due to the ethylene/auxin pathway negatively regulating the TTG/GL2 pathway in the differentiating hairless cells (located over the periclinal cortical cell walls) or due to the ethylene/auxin pathway negatively regulating the TTG/GL2 pathway in the differentiating root hair cells (located over the anticlinal cortical cell walls). Several lines of evidence indicate that the former is most likely correct.

First, epistasis tests with double mutants containing either ttg or gl2 and rhd6 or axr2 showed that RHD6 and AXR2 are likely to be negatively regulated by, or acting in a separate pathway from, TTG and GL2. Second, the ethylene biosynthesis inhibitor AVG blocked root hair formation in ttg and gl2 mutants, and IAA and ACC suppressed the hairless phenotype of the rhd6 ttg and rhd6 gl2 double mutants, showing that the ethylene/auxin pathways do not influence root epidermis development by acting through the TTG/GL2 pathway. Finally, neither the axr2, rhd6, and ctrl mutations nor the treatments with ACC and AVG affected the position-dependent GL2 gene activity, as assessed by using a GL2 promoter–GUS reporter gene fusion. Together, these results indicate that the TTG/GL2 pathway acts upstream of, or independently from, the ethylene/auxin pathway to influence root epidermal cell identity. Thus, we conclude that the normal pattern of cell types in the root epidermis is not instigated by position-dependent hormone signaling but rather by the position-dependent expression of the TTG/GL2 pathway, which acts to repress the ethylene/auxin pathway in developing hairless cells.

Our results are consistent with the view that all root epidermal cells initially have the capacity to differentiate into root hair cells (i.e., that the root hair cell differentiation pathway represents the default pathway), but some cells are induced to adopt an alternative fate and differentiate into mature hairless cells (discussed in Barlow, 1984; Galway et al., 1994; Masucci et al., 1996). To understand epidermal cell patterning further, an important future goal is to identify the spatial regulators of the TTG/GL2 pathway. One possibility is that TTG is involved in imparting the early positional information that specifies the hairless cell fate, because ttg mutations alter all aspects of epidermal cell differentiation (Galway et al., 1994). Also, although our results rule out a role for the ethylene/auxin pathway in regulating the TTG/GL2 pathway, a formal possibility remains that the ethylene/auxin pathway may act independently from the TTG/GL2 pathway to influence epidermal cell-type differentiation.

Ethylene and Auxin Promote Root Hair Outgrowth Late in Epidermal Cell Differentiation

The results from numerous pharmacological and genetic experiments indicate that ethylene and auxin influence root hair cell differentiation in Arabidopsis. These findings include the ability of AVG and the axr2 mutations to prevent root hair formation (Wilson et al., 1990; Masucci and Schiefelbein, 1994; Tanimoto et al., 1995; Table 1, this study), the ability of ACC and the ctrl mutation to induce some ectopic root hair cells (Dolan et al., 1994; Tanimoto et al., 1995; Table 1, this study), and the ability of ACC and IAA to suppress the hairless rhd6 phenotype (Masucci and Schiefelbein, 1994).

The results from this study suggest that ethylene and auxin affect root hair cell differentiation relatively late in epidermis development. First, the axr2, rhd6, and ctrl mutations and the ACC and AVG treatments fail to abolish the early position-dependent cellular differences that normally exist between developing root hair and hairless epidermal cells in the meristematic region of the root. Furthermore, these mutations and hormone treatments failed to alter the differences in GL2 gene activity (as assessed by the GL2–GUS reporter construct) that normally exist between developing root hair and hairless epidermal cells throughout the meristematic and elongation regions. These results show that although these hormone-related mutations and treatments alter the ability of epidermal cells to form root hair projections, they do not alter other position-dependent characteristics of the developing epidermal cells.

The developmental timing of the hormone effects also supports a late-acting role for ethylene and auxin in root hair outgrowth. ACC can induce ectopic root hairs in wild-type seedlings, and ACC and IAA can induce root hairs in the rhd6 mutant, on epidermal cells that have advanced into the cell elongation region of the root (Figure 1). Although these results do not rule out the possibility that ethylene and/or auxin also act at earlier stages of epidermis differentiation, they strongly implicate these hormones in the promotion of root hair outgrowth at a relatively late developmental stage.

The interpretation of the hormone effects as alterations in a late stage of cell differentiation is consistent with the extreme ethylene levels required to cause a moderate degree of ectopic hair formation. Both the ctrl mutation and the ACC concentration (50 μM) that induce 20 to 30% ectopic root hair cells caused a significant reduction in the length of epidermal cells (to ~25% of normal length; Tables 1 and 2). This implies that the ctrl mutation and the hormone treatments may induce ectopic root hairs not by switching cell fate but rather by overcoming the normal mechanisms controlling cell expansion or morphogenesis.
The precise mechanism by which ethylene and auxin promote root hair outgrowth in developing epidermal cells is not clear. However, a notable feature of differentiating epidermal cells at the time of root hair initiation is a change in the direction of cell expansion, from expansion predominantly in the longitudinal orientation to expansion in the radial orientation in a localized region of the cell (Corman, 1949, 1962). Both auxin and ethylene are known to influence cell expansion (Davies, 1987; Abeles et al., 1992). Auxin may act by inducing proton efflux leading to acidification of the cell wall and changes in wall plasticity (Evans, 1984; Cleland, 1988) and/or by altering the orientation of microtubules (Bergfeld et al., 1988; Nick et al., 1994; Shibaoka, 1994). Ethylene has been shown to induce radial cell expansion and reorientation of microtubules (Lang et al., 1982; Roberts et al., 1985; Shibaoka, 1994). Thus, it is possible that these hormones are involved in reorienting cell expansion, from the longitudinal to the radial direction, to promote root hair initiation.

A Model for Root Epidermal Cell Differentiation

The results of our study enable us to consider a model for the genetic control of epidermal cell differentiation in the Arabidopsis root. This proposed model is shown in Figure 5. Cell-type patterning is proposed to be achieved by the early action of the TTG and GL2 genes in developing epidermal cells located in particular positions (outside periclinal cortical cell walls) to inhibit the root hair differentiation pathway (as discussed earlier). As a result of the position-dependent action of the TTG/GL2 pathway, the genes that promote root hair formation (including RHD6 and the ethylene and auxin pathway genes) are proposed to act only in the developing epidermal cells located outside the anticlinal cortical cell walls.

The RHD6 gene appears to play a central role in root hair initiation. The rhd6 mutants exhibit a dramatic reduction in root hair formation, and they also exhibit defects in the selection of the root hair initiation site (Masucci and Schiefelbein, 1994). Because the rhd6 defect can be suppressed/bypassed by ACC and IAA treatments, the RHD6 gene is proposed to promote hair formation by acting through either the ethylene and auxin pathways or a separate pathway (Figure 5). The results from the cytoplasmic staining and double mutant tests leave open the possibility that RHD6 may act in a TTG/GL2-independent fashion to promote early aspects of root hair cell differentiation.

The AXR2 gene represents an important component of the hormone response pathway affecting root hair initiation, because axr2 mutations reduce root hair formation (Table 1; Wilson et al., 1990). In addition, axr2 mutants are unique because neither ACC nor IAA treatments induced root hairs in the axr2 single mutant or the axr2 rhd6 double mutants (Table 3). Thus, all pathways require a functional AXR2 product, and we propose that the auxin and ethylene hormone pathways converge at, or upstream of, the AXR2 product (Figure 5).

Redundancy in the ethylene response pathway for root hair formation is suggested by the genetic analysis of the aux1 and etr1 mutants. Neither mutation alone alters root hair formation; however, the aux1 etr1 double mutant exhibits a reduction in hair production (Table 3). This fact indicates that the AUX1 and ET1 gene products are each able to contribute to root hair formation and that each is sufficient to bypass defects in the other. Because ACC is unable to suppress the aux1 etr1 root hair phenotype, both of these gene products are probably acting in ethylene signal transduction. This idea is consistent with the ethylene-insensitive phenotype that each mutant exhibits for other ethylene responses (Bleecker et al., 1988; Pickett et al., 1990). Thus, we propose that ethylene acts through at least two pathways, one including AUX1 and the other ET1 (Figure 5). The ETR1 pathway is proposed to include the CTR1 and the EIN2 gene products, in a manner previously suggested for other ethylene response pathways (Ecker, 1995), although the importance of EIN2 in root hair formation is not clear (Figure 5).

The ability of IAA to suppress the aux1 etr1 phenotype indicates that IAA acts through an AUX1-independent pathway (Figure 5). The AXR1 gene is required for complete root hair formation in the rhd6 mutant in response to IAA and ACC (Table 3), and it has been shown recently to act in a separate pathway from AUX1 (Timpte et al., 1995). Thus, AXR1 is proposed to define an auxin response pathway for root hair formation, and it may represent a third pathway in which ethylene acts (Figure 5).

The results of this study show that ethylene and auxin act during the later stages of epidermal cell differentiation to promote root hair formation in Arabidopsis. These hormones have also been shown to affect root hair production in many other plant species (Corman, 1949, 1962; Barbieri and Galli, 1993).
Furthermore, these and other hormones affect root hair elongation. During the course of our experiments, we observed that 5 μM AVG caused a reduction in root hair length in wild-type plants and that root hair length was reduced in the axr2 mutant and the ein2 mutant (Wilson et al., 1990; J. Masucci and J. Schiefelbein, unpublished observations). In a related study, the hormone abscisic acid was reported to affect root hair length and to mimic the water-stress response in Arabidopsis root hairs (Schnall and Quatrano, 1992). Thus, an important role for plant hormones in epidermis differentiation may be to modulate the number and the length of root hairs, perhaps in response to various environmental conditions.

Our study provides a foundation for further investigations of the pathways that control epidermal cell differentiation. For example, many aspects of the proposed model for root epidermis development may be tested by using available gene probes, such as the AXR2, ETR1, and GL2 genes. It will also be of interest to determine the extent to which this model may apply to the development of the shoot epidermis, where TTG and GL2 are known to play important roles. The continued study of root epidermis development should provide new insights into the mechanisms by which hormones influence plant cell differentiation.

METHODS

Genetic Stocks and Growth Conditions

The following genetic stocks (with associated stock numbers) were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus): efrI (No. 237), eto1-1 (No. 3072), ein2-1 (No. 3071), aux1-7 (No. 3074), axr1-3 (No. 3075), axr2 (No. 3077), and g2-1 (No. 85). The rhd6-1 mutation was originally described by Masucci and Schiefelbein (1994). The cfrl-2 allele was obtained from J. Kieber (University of Illinois, Chicago) and was described previously by Kieber et al. (1993). The aux1-7 ein2 double mutant was obtained from L. Hobbie (Adelphi University, Garden City, NY).

Double mutants were constructed by crossing single mutant plants and examining F2 progeny for putative double mutant phenotypes at the seedling stage. The homozygous double mutant plants were confirmed by backcrossing to each of the single mutant lines. One double mutant line (rhd6 cfrl-2) was only tested by backcrossing to the rhd6 mutant because it displayed the cfrl-like short-root phenotype.

Unless otherwise noted, seedlings were grown on vertically oriented Petri dishes on agarose-solidified medium containing 1% sucrose, 0.6% agarose, and mineral nutrients (Estelle and Somerville, 1987) as previously described (Schiefelbein and Somerville, 1990). Media containing indole-3-acetic acid (IAA) (Sigma), 1-aminocyclopropane-1-carboxylic acid (ACC) (Sigma), or aminophloxyvinylglycine (AVG) (MAAG Agrochemicals, Ver0 Beach, FL) were prepared as previously described by Masucci and Schiefelbein (1994). When hormone-containing media were used, seedlings were initially germinated and grown for 3 days on unsupplemented media and then transferred to the hormone-supplemented media. As a control, a portion of the seedlings was transferred from unsupplemented media to fresh unsupplemented media.

Microscopy

To determine the number of root hair cells and hairless cells in the root epidermis, 5-day-old seedlings were mounted in artificial pond water (Schiefelbein et al., 1992) and viewed with differential interference contrast optics. In each trial, five epidermal cells were scored in each of five adjacent epidermal cell files from five roots for each line (total of 125 cells per line). Two to six trials were performed for each line. A cell was scored as a root hair cell if any protrusion was present, regardless of the root hair length. The length of root epidermal cells was determined by examining 50 to 75 mature cells from the root hair cell files of each line.

To determine the location of root hair–bearing cells, 5-day-old seedlings were submersed in molten 3% agarose. After solidification, transverse root sections were hand cut with a double-edged razor blade and stained with a solution of 0.002% toluidine blue dye in artificial pond water. The location of root hair cells relative to the underlying cortical cells was determined by viewing sections from at least five roots from each line.

The cytoplasmic density and vacuole formation in the developing epidermal cells were examined by using previously published protocols (Dolan et al., 1994; Masucci et al., 1996). Transverse sections (2 to 5 μm thick) were obtained from 5-day-old seedlings embedded in JB-4 resin (Polysciences Inc., Warrington, PA), stained with 0.05% toluidine blue O in 200 mM sodium acetate, pH 4.4, and viewed under a Leitz Laborlux S microscope (Wild Leitz Ltd., Heerbrugg, Switzerland) with differential interference contrast optics.

β-Glucuronidase Reporter Gene Assay

The GL2-β-glucuronidase (GUS) reporter gene construct contains a 4-kb DNA fragment from the 5’ upstream region of the GL2 gene fused to the GUS coding region (Jefferson et al., 1987), as previously described (Masucci et al., 1996). Five-day-old seedlings harboring the transgene were assayed for GUS activity, according to established methods (Gallagher, 1992), and photographed after incubation for 30 to 60 min. The GL2-GUS construct was introduced into the axr2, rhd6, and cfrl mutant backgrounds by crossing plants harboring the markers and analyzing F2 seedlings for homozygous mutants.

Latex Bead Labeling of Roots

Latex beads (6 μm in diameter; Polysciences Inc.) were coated with polylysine, according to the method of Staebell and Soll (1985). Four-day-old wild-type (ecotype Columbia) seedlings, grown on agarose-solidified medium, were submerged in a diluted suspension of the polylysine-coated beads and transferred to agarose-solidified media with or without hormone supplements. The location of the beads in relation to the youngest root hair–bearing cells was noted. Seedlings (n = 22) were allowed to grow for 12 to 27 hr on the new media, and the relative position of ectopic root hair cells was determined. Four-day-old rhd6 seedlings were treated and tested in the same manner as given above, except that wild-type roots were treated at the same time as the rhd6 roots. After the roots were incubated for 6 to 21 hr, the number of root hair cells formed per cell file in the rhd6 mutant was compared with the wild type. For the transfers to ACC-containing media, seedlings (n = 31) were incubated for 6, 8, 8.5, 20.5, and 21 hr. For the transfers to IAA-containing media, seedlings (n = 34) were incubated for 9, 10, 18.5, and 21 hr.
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