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RESEARCH PAPER

Ethylene and phosphorus availability have interacting yet distinct effects on root hair development

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Abstract

The hypothesis that ethylene participates in the regulation of root hair development by phosphorus availability in *Arabidopsis thaliana* was tested by chemically manipulating ethylene synthesis and response and with ethylene-insensitive mutants. Low phosphorus-induced root hair development could be mimicked by adding the ethylene precursor, 1-aminocyclopropane-1-carboxylate (ACC), to high phosphorus media, and inhibited by adding ethylene inhibitors to low phosphorus media. Ethylene-insensitive mutants showed a reduced response to low phosphorus, indicating ethylene involvement in root hair responses to phosphorus deficiency. To dissect the nature of this involvement, the morphological and anatomical changes associated with increased root hair density were investigated. Growth in low phosphorus resulted in smaller, more numerous cortical cells, resulting in a larger number of root hair-bearing epidermal cell files. Cortical cell number was not affected by ethylene inhibitors, ACC, or mutations reducing ethylene sensitivity in roots grown with low phosphorus, indicating that ethylene does not participate in this response. The exception was the *eir1* mutation, which strongly reduced this change in radial anatomy, supporting a role for polar auxin transport in this process. Trichoblast cell length was reduced by low phosphorus availability in all genotypes, but even more so for *ein2-1* and *ein4*. The proportion of epidermal cells forming hairs and root hair length were reduced in ethylene-insensitive mutants, especially in the presence of low phosphorus. These results demonstrate multiple effects of low phosphorus from the earliest stages of root hair development, and cross-talk between ethylene and phosphorus in the control of a subset of the low phosphorus effects, concentrating on those later in development.

Key words: *Arabidopsis thaliana*, ethylene, ethylene-insensitive mutants, phosphorus, root hair density, root hair length, trichoblast.

Introduction

Root hairs are subcellular protrusions of epidermal cells that are important for the acquisition of immobile nutrients such as phosphorus (Clarkson, 1985; Jungk, 2001). Root hair production is stimulated by the deficiency of several nutrients, including iron, zinc, manganese, and phosphorus, but, at least in *Arabidopsis*, phosphorus has a greater effect than other nutrients on root hair density and length (Bates and Lynch, 1996; Foehse and Jungk, 1983; Gahoonia and Nielsen, 1997; Ma *et al*., 2001*a*; Schmidt and Schikora, 2001). The effects of low phosphorus availability on root hair density, length, and distribution act synergistically to increase the effective volume of soil from which the plant can acquire phosphorus, and therefore its growth and competitiveness when phosphorus availability is limiting (Bates and Lynch, 2000*b*, 2001; Ma *et al*., 2001*b*).

In *Arabidopsis*, root hairs are formed from specific epidermal cells located over the intercellular space between underlying cortical cells, i.e. cells in the H position, while cells located directly over a single cortical cell (in the N position) do not form hairs (Dolan *et al*., 1994). Not all epidermal cells in the H position actually form hairs, i.e. become trichoblasts, and ectopic hairs may occur on cells in the N position with ethylene and other treatments (Dolan *et al*., 1994; Ma *et al*., 2001*a*; Schmidt and Schikora, 2001; Tanimoto *et al*., 1995).

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Trichoblast files are typically separated by one to three files of atrichoblasts (non-hair-bearing cells) (Dolan and Costa, 2001; Pemberton *et al*., 2001). Most reports indicate that there are eight cortical cells in cross-section and correspondingly eight trichoblast files (Dolan and Costa, 2001). Ma *et al*. (2001*a*) found that *Arabidopsis* grown under phosphorus deficiency had 12 cortical cell files, and therefore 12 trichoblast files. This increase in the number of potential hair-bearing cells, along with an increase in the proportion of H cells forming root hairs, accounted for most of the increased root hair density observed under phosphorus deficiency (Ma *et al*., 2001*a*).

It has been proposed that the position-dependent differentiation of trichoblasts may be regulated by ethylene transported from the inner root through the apoplast (Michael, 2001; Tanimoto *et al*., 1995). This was based on the enhancement of root hair production by ethylene, ACC, and the *ctr1* (constitutive ethylene response) mutation, and the inhibition of root hair production by the ethylene inhibitors aminoethoxyvinylglycine (AVG) and Ag^+ (Dolan *et al*., 1994; Kieber *et al*., 1993; Masucci and Schiefelbein, 1996; Michael, 2001; Tanimoto *et al*., 1995). The fact that *ctr1* and ACC could cause the development of ectopic hairs supported the idea that ethylene was responsible for the differentiation of epidermal cells to form hairs. However, later work showed that neither 1-methylcyclopropene (1-MCP), an ethylene response inhibitor, nor mutants with impaired ethylene response showed a reduction in root hair density unless root hair formation was stimulated by hormonal or environmental stimuli (Cho and Cosgrove, 2002).

The similarities in root hair response to low phosphorus and ethylene suggest a role for ethylene in mediating this nutrient deficiency response. Nutrient stress, either deficiency or toxicity, can change ethylene biosynthesis and/or responsiveness (Lynch and Brown, 1997). Borch *et al*. (1999) found that phosphorus-deficient bean roots produced twice as much ethylene per unit dry weight as roots supplied with adequate phosphorus. It is therefore reasonable to suggest that ethylene might mediate root hair production in response to phosphorus deficiency. However, Schmidt and Schikora (Schmidt and Schikora, 2001) found that phosphorus deficiency increased root hair density of *Arabidopsis* even in the presence of the ethylene antagonists AVG, amino-oxyacetic acid (AOA), Co^{2+}, and STS. Based on this result, the authors proposed a hypothetical model in which phosphorus deficiency may not affect ethylene synthesis and signal transduction pathways, but interacts directly with ethylene-responsive genes associated with root hair formation (Schmidt, 2001; Schmidt and Schikora, 2001).

In this paper, the hypothesis was tested that ethylene and phosphorus interact to control root hair density and length. The root hair responses to phosphorus deficiency and ethylene manipulation were compared and the ability of ethylene-insensitive mutants to respond to phosphorus was investigated. Since root hair formation involves many developmental stages (Parker *et al*., 2000; Schiefelbein, 2000) and low phosphorus availability affects several of these (Ma *et al*., 2001*a*), the effects of phosphorus and ethylene manipulation on specific aspects of root anatomy known to contribute to increased root hair density were examined.

Materials and methods

Seeds of *Arabidopsis thaliana* L. (Heynh) Columbia and isogenic ethylene-insensitive mutants *ein2-1*, *ein3-1*, *ein4*, *ein5-1*, *ein7*, and *eir1-1*, as well as *ein6*, which is in the Landsberg erecta background, were obtained from the Arabidopsis Biological Resource Center, of the Ohio State University. Seeds propagated in the greenhouse were used in all experiments reported here.

Growth media composition and preparation, seed germination, seedling culture, and root hair observation were carried out according to Ma *et al*. (2001*a*), except for the use of a dark period, in a 16/8 h light/dark alternation. A completely randomized design was used with at least six replicates per treatment. $NH_4H_2PO_4$ was added to give targeted phosphorus concentrations of 1, 10, 100 or 1000 μM, and $(NH_4)_2SO_4$ was used to balance the N among phosphorus treatments. Treatments with 1 or 1000 μM phosphate are referred to as low phosphorus or high phosphorus, respectively. To manipulate ethylene production and sensitivity, fresh media containing the ethylene precursor ACC, the ethylene production inhibitor AVG, or the ethylene action inhibitor STS (silver thiosulphate), were added to the existing media after seedlings had grown 12 d, as described by Bates and Lynch (1996). For treatment with the gaseous ethylene action inhibitor 1-MCP (EthylBloc®: 0.43% 1-methylcyclopropene, Floralife Inc. 751 Thunderbolt Drive, Walterboro, SC), open Petri dishes with 12-d-old plants were placed in an 8.0 l, air-tight container, which also contained a 5 ml vial containing 2 mg EthylBloc l^{-1} of chamber volume. The container was surface-disinfected with 70% alcohol before use. The chamber was sealed, and 0.04 ml distilled water was added to the vial through a septum to release 1-MCP gas. The treatment lasted 2 d until root hair measurement. At 14 d of growth (2 d after adding ethylene inhibitors or ACC, or after the initiation of 1-MCP treatment), six plants of each treatment were evaluated under a stereomicroscope for root hair length and density. The experiment was repeated four times.

For the root anatomical observations, roots of at least five plants were sampled from plants of the same age as used for root hair measurement, and root segments (2–3 mm long) were taken from the same region for root hair observation. Root fixation, embedding, cross-sectioning, and staining were done according to Ma *et al*. (2001*a*).

To measure trichoblast cell length, Columbia, *ein2-1* and *ein4* were used, each of which was transformed with β-glucuronidase (GUS) reporter gene under the control of the promoter of AtEXP7, an α-type cell wall expansin specifically expressed in hair-bearing epidermal cells (Cho and Cosgrove, 2002). For AtEXP7::GUS-transformed materials, treatments included low phosphorus, low phosphorus plus 3 μM AVG, high phosphorus, and high phosphorus plus 1 μM ACC. Living roots of transformed plants were stained at 37 °C for 1 h with 1.0 mM X-glucuronide in the buffer (0.1 M NaH_2PO_4, 10 mM EDTA, 0.5 mM K-ferricyanide, 0.1% Triton X-100, pH adjusted to 7.0 with 1 N NaOH), washed three times with the same buffer, then stained with 0.05% aqueous solution of Neutral red for 5 min at room temperature. For non-transformed plants, living

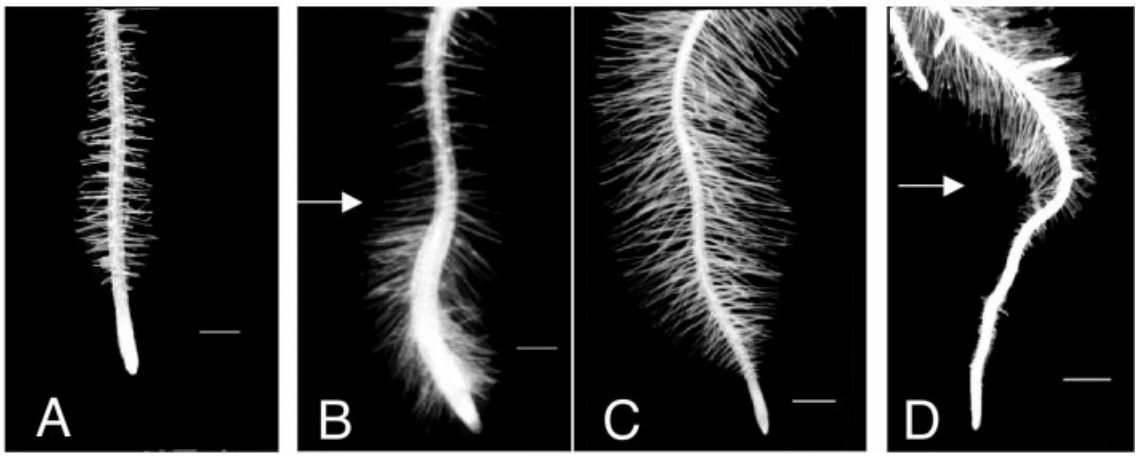

Fig. 1. Root hair growth of Columbia plants grown with high or low phosphorus, with the addition of ACC or AVG after 12 d growth. Photos were taken after 14 d. (A) High P (1000 μM); (B) high P then 1 μM ACC; (C) low P (1 μM); (D) low P then 2 μM AVG. Bar=0.5 mm.

Table 1. *Effect of ethylene manipulation on root hair density and length of* Arabidopsis *plants*

Plants were grown in high P (1000 μM) or low P (1 μM). Contrast (*t*-test) of ACC or inhibitor treatments with same P control showed that all treatments significantly affected root hair density and length (P <0.01). Values shown are means ±SE (n=6).

Treatment	Root hair density (hairs mm^{-1})	Root hair length (mm)
Low P	130.8±15	1.30±0.03
High P	38.0±2.8	0.34±0.01
High P+ACC (1 μM)	144.8±5.7	0.78±0.10
Low P+STS (5 μM)	43.5±7.4	0.20±0.04
Low P+1-MCP (200 nl l^{-1})	21.5±8,8	0.16±0.04
Low P+AVG (2 μM)	22.0±5.7	0.10±0.04

roots were stained with 0.05% Toluidine Blue O in 0.1 M Na_2HPO_4/NaH_2PO_4 buffer (pH 6.8) for 5 min at room temperature. Stained root images were recorded with a digital camera connected with a microscope, and the cell length was then measured with Photoshop (Adobe Photoshop® 6.0, Adobe System Incorporated). Trichoblast cell lengths were measured on six cells from each of six plants.

Root growth rate of untransformed plants grown with low or high phosphorus was recorded daily by marking the root tip position on the bottom of the Petri dish, starting from day 9 of culture. Daily root growth was estimated by scanning the Petri dish and analysing the digital image with WinRHIZO (Regent Instruments Inc., 4040 Rue Blain, Quebec, G2B 5C3 Canada, Version 3.10b). Each treatment was repeated six times. Preliminary experiments showed a steady growth rate from day 9 to day 13; values taken from day 9 to day 10 are presented.

ANOVA of root hair data was conducted using statistical software MiniTab (Minitab Inc.).

Results

Root hair growth in response to low phosphorus availability and ethylene

Ethylene production and action were manipulated using the ethylene production inhibitor AVG, the ethylene action inhibitors STS or 1-MCP, or the ethylene precursor ACC. The inhibitors reduced root hair density and length of Columbia plants grown in low phosphorus, while ACC increased root hair density and length of plants grown in high phosphorus (Table 1). Ethylene inhibitors reduced root hair density and length as much or more than high phosphorus availability. Root hair growth could be manipulated in the growing plant by adding ACC or AVG to the medium with similar results (Fig. 1).

Root hair density and length of the ethylene response mutants were evaluated under low (1 μM) or high phosphorus (1000 μM) (Fig. 2). For all genotypes, root hair density and length in low phosphorus media were significantly greater than in high phosphorus media. In high phosphorus, ethylene-insensitive genotypes had root hair lengths and densities varying from greater than to less than those of Columbia. All mutant genotypes had shorter and less dense root hairs than Columbia with low phosphorus, except *ein6*, which had root hair length equal to Columbia. The responsiveness to low phosphorus was defined as the ratio of means between low and high phosphorus treatments, which was the greatest for Columbia (Fig. 2). All the mutants were less responsive to phosphorus availability than Columbia.

Columbia and two ethylene-insensitive mutants, *eir1-1* and *ein7*, displayed increasing root hair density and length as phosphorus availability declined (Fig. 3). The root hair density of these genotypes was the same with 1000 μM phosphorus, but Columbia increased its hair density more sharply than the mutants as phosphorus concentration decreased to 1 μM (Fig. 3A). Similarly, the mutants were less sensitive than Columbia to the effects of reduced phosphorus availability on root hair length (Fig. 3B). As a result, both mutants had shorter hairs than Columbia when plants were grown in 1 μM phosphorus, even though at 1000 μM phosphorus, *ein7* had the longest hairs of the three genotypes. The two mutants had similar responses to phosphorus for density but not for length.

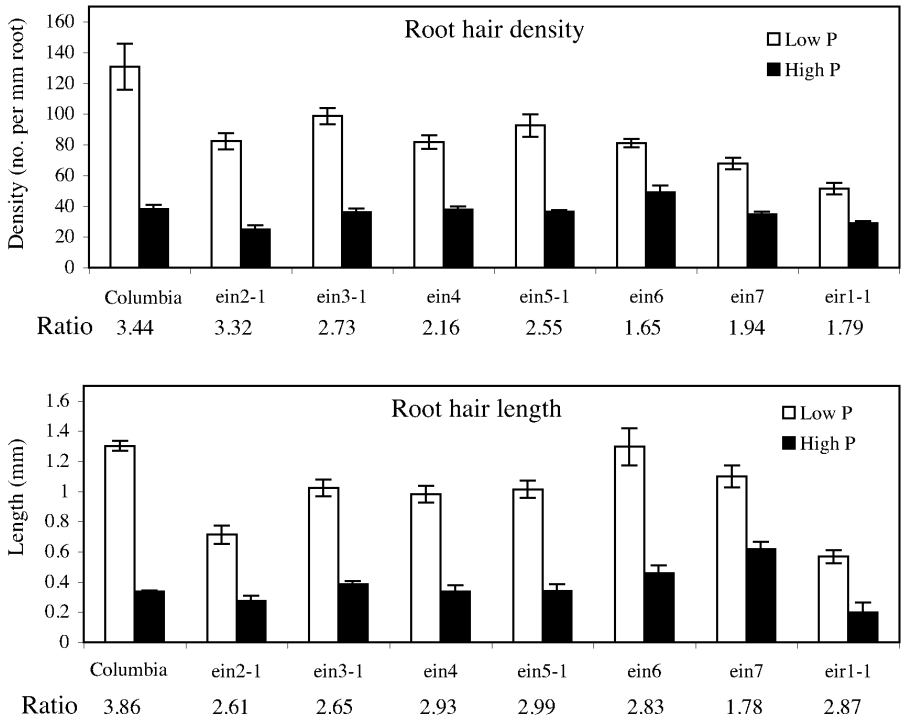

Fig. 2. Root hair density and length of Columbia and ethylene-insensitive mutants grown with low P (1 μM) or high P (1000 μM). Values shown are means of six observations ±SE. Contrast (*t*-test) between low P and high P treatments for root hair density and length indicates significant differences at 1% level for all genotypes. Numbers below genotype names are low P/high P ratios.

Low phosphorus and ethylene have different effects on radial anatomy

When Columbia plants were grown with high phosphorus, the typical eight files of cortical cells were always observed, and root hairs formed only on cells in the H position (Table 2; Fig. 4A). Low phosphorus stress resulted in the production of more and smaller cortical cells (Table 2; Fig. 4C). As a result, more epidermal cells were located in the H position, as previously found by Ma *et al*. (2001*a*). Sometimes this resulted in no atrichoblast cell files between trichoblast files, so that two adjacent epidermal cells were hair-bearing cells (Fig. 4C). Root diameters were slightly (5%) larger in low phosphorus plants (Table 3), as previously reported (Ma *et al*., 2001*a*).

ACC added to high phosphorus media failed to induce a change in cortical cell number like that found with the low phosphorus treatment (Table 2), but promoted hair formation on both H and N cells (Fig. 4B). AVG added to low phosphorus media did not significantly affect cortical cell number (Table 2; Fig. 4D).

The ethylene-insensitive mutants *ein2-1* and *ein4* grown in low phosphorus had an anatomy similar to Columbia, since they produced more cortical cell files, and therefore more trichoblast cell files, than plants grown in high phosphorus (Table 2; Fig. 5A–D). Ectopic hairs were occasionally observed on the roots of *ein4* grown in low phosphorus medium (Fig. 5D). The *eir1-1* mutant showed very little increase in cortical cell number under phosphorus deficiency (Table 2; Fig. 5E, F).

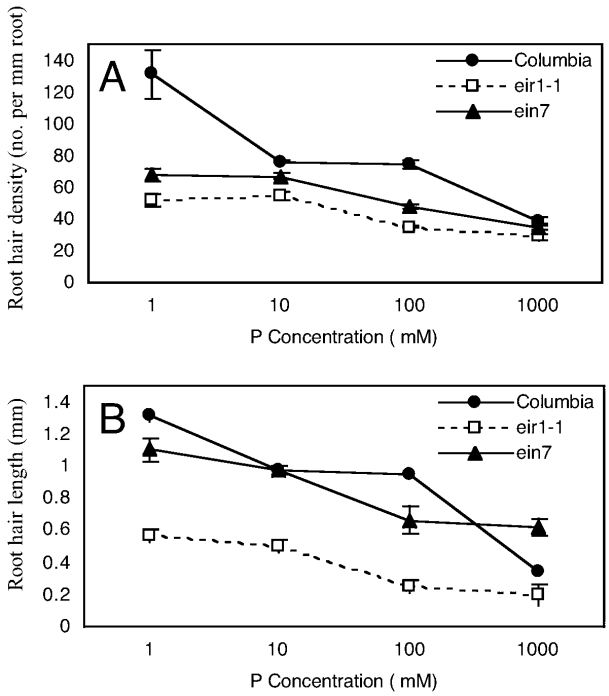

Fig. 3. Root hair density (A) and length (B) of Columbia and the ethylene-insensitive mutants *eir1-1* and *ein7* in response to phosphorus. Values shown are means of six observations ±SE.

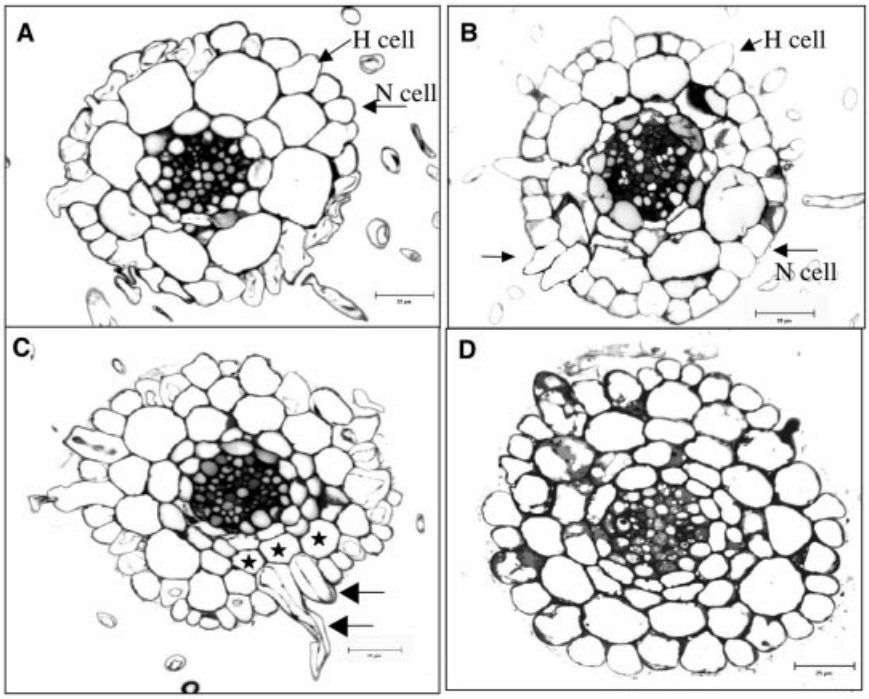

Fig. 4. Root cross-sections of Columbia showing cortical cell numbers and trichoblast cell files. (A) High P (1000 μM); (B) high P plus 1 μM ACC, arrow indicates an ectopic hair; (C) low P (1 μM), arrows indicate two adjacent hairs and stars indicate three cortical cells below these two hair cells; (D) low P plus 2 μM AVG. Bar=25 μm. H (hair) cells are shown in the position over the junction of two cortical cells, while N (non-hair) cells are shown over just one cortical cell.

Table 2. *Root cortical cell number of Columbia and ethylene-insensitive mutants*

Values shown are means ±SE (*n*=5)

Genotype	Treatment	Cortical cell number	
		High P	Low P
Columbia	Control	8.0±0	16.4±0.9
	ACC (1 μM)	8.7±0.9	–
	AVG (3 μM)	–	15.5±0.6
ein2-1	Control	8.0±0	17.7±1.3
ein4	Control	8.0±0	15.0±0
eir1-1	Control	9.0±0	10.2±0.8

To exclude the possibility that the difference in cortical cell size and number was caused by differences in root development associated with low phosphorus stress, roots of both phosphorus treatments were sectioned from the middle of the hair-free zone near the root tip to the first lateral root, and found that the cortical cell number was constant throughout the root hair differentiation zone (data not shown).

Phosphorus and ethylene affect trichoblast cell length

Arabidopsis genotypes Columbia, *ein2*, and *ein4* transformed with the GUS gene attached to an expansin gene (AtEXP7) promoter, were used for trichoblast identification and cell length measurements. This promoter was previously demonstrated to be specific for cells forming root hairs (Cho and Cosgrove, 2002) and allows easy visualization of the cross walls. Trichoblast cells were much shorter under low phosphorus than under high phosphorus for all genotypes (Table 3). ACC added to high phosphorus medium reduced trichoblast length of Columbia roots by 65%, while AVG added to low phosphorus medium increased trichoblast length by 45% (Table 3). ACC also significantly increased root diameter (Table 3), consistent with the known effects of ethylene. Columbia and *ein4* had similar trichoblast lengths and root diameters at both phosphorus levels (Table 3). However, *ein2* seemed to be more responsive than Columbia to phosphorus availability, since trichoblast length increased with high phosphorus and decreased with low phosphorus (Table 3). Root diameter was reduced by *ein2* at both phosphorus levels, but more so at low phosphorus (Table 3).

To examine whether the AtEXP7::GUS insert affected trichoblast length, the same genotypes were examined without transformation (data not shown). Although the absolute values varied slightly from the previous experiment, the patterns of response were identical, i.e. low phosphorus reduced trichoblast length to a similar extent in Columbia and *ein4*, but *ein2* had a more exaggerated response to phosphorus.

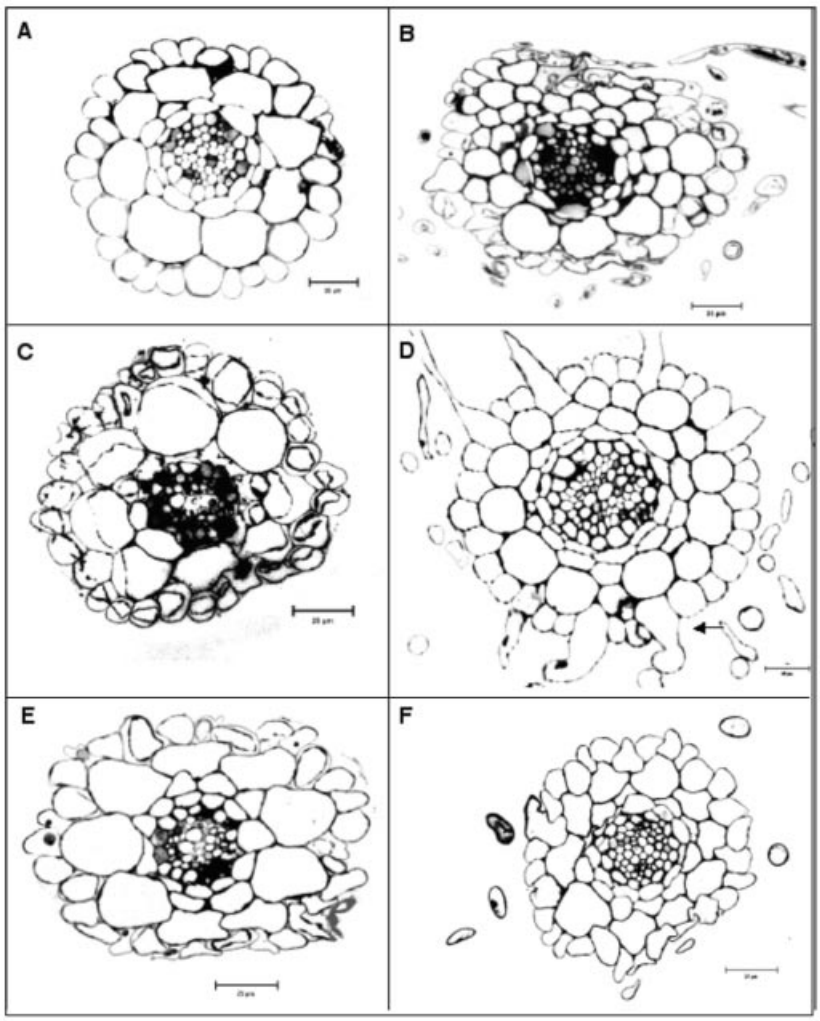

Fig. 5. Root cross-sections of ethylene-insensitive mutants. (A) *ein2-1*, high P (1000 μM); (B) *ein2-1*, low P (1 μM); (C) *ein4*, high P; (D) *ein4*, low P, arrow indicates an ectopic hair; (E) *eir1-1*, high P; (F) *eir1-1*, low P. Bar=25 μm.

Table 3. *Trichoblast cell length, root diameter, and root growth rate of* Arabidopsis *plants grown with high or low P*

Trichoblast cell lengths and root diameters were measured on six cells from each of six roots of transformed plants. Root growth rate was calculated from 9–10 d after planting on six non-transformed plants. Values shown are means ±SE.

Genotype	Treatment	Trichoblast cell length (μm)		Root diameter (μm)		Root growth rate (cm d^{-1})	
		High P	Low P	High P	Low P	High P	Low P
Columbia	Control	166±3	103±8	179±3	187±4	0.88±0.04	0.40±0.03
	ACC (1 μM)	59±2	–	222±7	–	0.88±0.04	0.40±0.03
	AVG (3 μM)	–	149±14	–	172±5	1.40±0.03	0.30±0.07
ein2	Control	220±33	78±5	142±7	176±4	1.40±0.03	0.30±0.07
ein4	Control	162±11	94±4	184±9	193±5	0.74±0.05	0.38±0.03

Percentage of H cells forming hairs

The percentage of H cells forming hairs was calculated from the other variables, trichoblast length, number of trichoblast cell files, and root hair density, and also measured directly. Both methods showed that most H cells formed hairs in Columbia, regardless of phosphorus availability (Table 4). ACC increased the percentage of H cells forming hairs to almost 100%, while AVG reduced it by half. The percentage of H cells forming hairs was reduced in *ein2-1* and *ein4*, especially with the low phosphorus treatment (Table 4).

Table 4. *Effects of low phosphorus and ethylene on components of* Arabidopsis *root hair density*

Transformed plants were used for observations of root hair density and percentage of H cells forming hairs.

Variable	Columbia				*ein2-1*		*ein4*	
	High P	High P+ACC	Low P	Low P+AVG	High P	Low P	High P	Low P
Length of trichoblasts (μm) (*L*)	166	59.1	103	149	213	71.3	175	77.8
Number of trichoblasts mm^{-1} root (U=1000/L)	6.04	16.9	9.71	6.72	4.70	14.0	5.72	12.8
Number of H cells per cross-section (*N*)	8	8.7	16.4	15.5	8	17.7	8	15
Theoretic hair density (no. of hairs mm^{-1} root) (T=U×N)	48.3	147	159	104	37.6	248	45.8	193
Observed hair density (no. of hairs mm^{-1} root) (*O*)	38	145	131	22	25	82	38	72
% H cells forming hairs (PH)								
Calculated PH=(O/T)×100%	79	98	82	21	66	33	83	37
Observed *PH*	90	99	90	47	72	51	69	55

Discussion

Gross effects of low phosphorus and ethylene are similar

Ethylene and low phosphorus have similar effects on root hair development in *Arabidopsis*. Ethylene (ACC) and low phosphorus significantly increase hair density and length (Fig. 1), and significantly reduce trichoblast cell length (Table 3). Inhibiting ethylene synthesis and action prevents the low phosphorus response (Table 1; Fig. 1), and inhibiting ethylene action via ethylene-insensitive mutants reduces the low phosphorus response (Figs 2, 3). These results are consistent with the hypothesis that ethylene mediates the responses of root hair growth to low phosphorus.

Ethylene-insensitive mutants reveal complex ethylene/phosphorus interaction

When the effects of low phosphorus on ethylene-insensitive mutants were investigated, it was found that all mutants responded to low phosphorus with increased root hair length and density, but that the ratio of values for low phosphorus/high phosphorus was reduced to an extent that varied among genotypes (Fig. 2). This is consistent with a partial involvement of ethylene in low phosphorus responses. There was no relationship between the root hair development of the mutants in response to low phosphorus and their responsiveness to ethylene as determined by the extent of inhibition of the triple response (Roman *et al*., 1995). For example, *ein7* was the mutant most responsive to reduced phosphorus levels (Fig. 2), but was one of the weaker mutants in terms of the lesion in the ability of ethylene to initiate the triple response, while *ein2-1* and *ein4* were strongly inhibited in the triple responses (Roman *et al*., 1995), but not in phosphorus responsiveness (Fig. 2). Not only were differences in phosphorus responsiveness among mutants not correlated with lesions in the triple response, but the extent of reduction in phosphorus responses for the two root hair traits, root hair density and root hair length, were not consistent among mutants (Fig. 2). The *ein2-1* mutant had the weakest inhibition of low phosphorus response for root hair density, but the second largest inhibition of low phosphorus response for root hair length. This lack of correlation among responses may indicate the extent to which ethylene is important for each response as well as the specific action of each mutant gene in signal transduction for ethylene responses related to seedling growth versus root hair development.

Some of the strongest inhibitions of the low phosphorus response were observed in mutants that have yet to be cloned and identified. The *ein6* and *ein7* mutants strongly reduced the low phosphorus/high phosphorus ratio for both root hair density and root hair length (Fig. 2). Compared with Columbia, the *ein6* mutant increased root hair density under high phosphorus and reduced it under low phosphorus (Fig. 2). EIN6 and EIN7 have been placed after CTR1 in ethylene signal transduction based on genetic analysis (Roman *et al*., 1995). The *ein6* mutant is the only ethylene-response mutant affected by the microtubule-destabilizing drug taxol (Johnson and Ecker, 1998) and the only ethylene-response mutant in which mechanical stimulation of TCH3 gene expression is inhibited (Wright *et al*., 2002). EIN6 may, therefore, be involved in responses to ethylene and other factors that affect the direction of growth, which could include phosphorus effects on trichoblast elongation.

There was also a strong reduction in the low phosphorus/high phosphorus ratio for root hair traits in *eir1-1* (Fig. 2), which was originally isolated as an ethylene-response mutant displaying interactions with the auxin-response pathway (Roman *et al*., 1995), and is now identified as carrying a mutated auxin-efflux carrier (Chen *et al*., 1998; Luschnig *et al*., 1998). This mutation reduces root hair

density and length at both phosphorus concentrations, but more so at low phosphorus (Fig. 2). In high phosphorus, *eir1-1* seedlings had longer roots than Columbia, indicating that increased root hair density could not have resulted from an inhibition of root elongation (Roman *et al*., 1995). Interestingly, *eir1-1* showed about 33% inhibition of response to 2 μM ACC for increased root hair density, but no inhibition of ACC enhancement of root hair length (data not shown), i.e. it retains ethylene sensitivity for that particular response. The fact that *eir1* had such a strong effect suggests that its effect on auxin transport is probably important for low phosphorus responses.

Schmidt and Schikora (2001) reported a lack of interaction between ethylene and phosphorus in controlling root hair density. They used two *Arabidopsis* ethylene-insensitive mutants, *etr1-3*, which has a mutated receptor protein (Schaller and Bleecker, 1995), and *ein2-1*, which has a mutation in the gene believed to transduce the signal across the nuclear membrane (Alonso *et al*., 1999). They found that these two mutants exhibited a slightly reduced number of hairs when transferred to phosphorus-free medium, compared with Columbia. Based on the fact that these ethylene-response mutations did not depress the enhancement of root hair density by phosphorus deficiency, and the lack of effect of the ethylene synthesis inhibitors AVG and AOA and the ethylene action inhibitor STS, they proposed that a phosphorus-deficiency stress signal was not dependent on genes involved in ethylene signalling (Schmidt and Schikora, 2001). In these experiments, *ein2-1* also had only a weak effect on low phosphorus enhancement of root hair density, and *etr1* was not used. However, the other mutants had a more pronounced effect (Fig. 2), and in this study, ethylene synthesis and action inhibitors also had a strong effect (Table 1; Fig. 1). To explain these contradictory observations, it is necessary to dissect the root hair density phenotype into its components.

Phenotypic components of root hair density

Three components contribute to root hair density (if ectopic hairs are not considered): trichoblast length, number of trichoblasts per cross-section (trichoblast file number), and percentage of H cells forming hairs. Ectopic hairs in Columbia plants grown with either phosphorus treatment were sought, but were not observed. Schmidt and Shikora (2001) found only a small number of ectopic hairs in *Arabidopsis* plants transferred from a very high phosphorus (2.5 mM) to a no-phosphorus medium; in that case the ectopic hairs could have resulted from the stress of transfer. Therefore, the increase in root hair density in plants grown with low phosphorus should be explained by alterations in trichoblast length, trichoblast file number, and/or percentage of H cells forming hairs.

Trichoblast cell length and root elongation reduced by low phosphorus and ethylene

Trichoblast cell length is an important factor contributing to root hair density, since shorter trichoblast cells would increase the number of H cells per unit length of root, and therefore the number of potential trichoblasts. Trichoblast cell length was reduced with low P, so that the number of trichoblasts per unit length of root is increased 1.6-fold (Table 3), accounting for about half the observed increase in root hair density. ACC reduced trichoblast cell length of low phosphorus plants, while AVG increased it in high phosphorus plants (Table 3). These results show that both phosphorus and ethylene influence trichoblast cell length, which would be expected to correlate with overall root elongation. Indeed, low phosphorus reduced root growth rate to 40–45% that of high phosphorus controls (Table 3). The *ein4* mutant had very little effect on trichoblast cell length or root growth rate (Table 3). The *ein2* mutant, on the other hand, had longer trichoblasts and a higher root growth rate when grown in high phosphorus, but shorter trichoblasts and slower growth under low phosphorus compared with Columbia (Table 3). This indicates that endogenous ethylene limits extension growth of roots under high phosphorus, but is below optimal for root extension under low phosphorus. Similar results were found for the main root growth of common bean roots treated with AVG (Borch *et al*., 1999) and for tap root growth of 1-MCP-treated *Arabidopsis* roots (Ma *et al*., 2003). In both mutants grown with low phosphorus, the root hair density was reduced, despite the increase in the number of trichoblasts per unit length, compared with Columbia (Fig. 2; Table 3). The effects on trichoblast cell length and root elongation must be counteracted by another variable to produce lower root hair densities. That variable is percent of H cells forming hairs (see below).

Auxin, but not ethylene, required for increased trichoblast cell file number

Low phosphorus increases trichoblast cell file number partially by increasing the number of cortical cells (Ma *et al*., 2001*a*). These results show that low phosphorus and ethylene have distinct effects on cortical cell organization (Fig. 4; Table 2). Low phosphorus has impacts on tissue organization that ethylene cannot mimic, i.e. an increased number of files of smaller cortical cells. Ethylene (from ACC) induces swelling of cortical cells, but has no effect on cortical cell number, and likewise, AVG and ethylene-insensitive mutants have no effect on cortical cell number (Fig. 4; Table 2). The only exception was the *eir1-1* mutant, which showed only a slight increase in cortical cell number with low phosphorus (Table 2). Since the behaviour of *eir1-1* was inconsistent with AVG-treated Columbia or the other ethylene-insensitive mutants, its

failure to alter cortical cell organization in response to low phosphorus must result from the primary lesion in *eir1-1*, the impairment of auxin efflux (Luschnig *et al*., 1998), implicating auxin in the alterations of root anatomy resulting from phosphorus stress. Since low phosphorus increases cortical cell numbers but ethylene does not, low phosphorus increases root hair density through at least one mechanism that is completely independent of ethylene.

Ethylene is important for root hair formation on H cells

The final factor that could be responsible for increased root hair density is the percentage of H cells forming hairs. No significant effect of low phosphorus on this variable were observed, either when it was calculated from the other two variables or when it was observed directly (Table 4). However, ACC increased the percentage of H cells forming hairs to nearly 100%, while AVG and ethylene-response mutations reduced it. Although percentage of H cells forming hairs was not affected by phosphorus in Columbia, the *ein2* and *ein4* mutants had even greater reductions in the percentage of H cells forming hairs under low phosphorus than they did under high phosphorus. This confirms the importance of ethylene for root hair development (Masucci and Schiefelbein, 1994, 1996; Tanimoto *et al*., 1995) and also indicates that ethylene is more important for root hair development in plants grown with low phosphorus stress than in unstressed plants.

In another study, phosphorus had a significant effect on the percentage of H cells forming hairs (Ma *et al*., 2001*a*). High phosphorus treatment reduced the percentage of H cells forming hairs to only 24%, while in low phosphorus plants, 90% of H cells formed hairs, as in this paper. In that study, the percentage of H cells forming hairs was not measured directly, but calculated from the other components of root hair density (Ma *et al*., 2001*a*). In addition, the length of trichoblasts with low phosphorus was only slightly less than that from high phosphorus, so the increase in root hair density with low phosphorus was due to the increased number of trichoblast cell files and the increased percentage of H cells forming hairs (Ma *et al*., 2001*a*). In this study, reduced trichoblast length and the increased number of trichoblast cell files account for the root hair density increase. The reduction in trichoblast cell length was about the same as the reduction in overall root growth (Table 3). Each of these three components may contribute to changes in root hair density, depending on the experimental conditions and the extent of phosphorus deficiency. Only two of them, trichoblast cell length and percentage of H cells forming hairs, are influenced by ethylene.

Ethylene and phosphorus control root hair length

Root hair elongation (length) is promoted by low phosphorus or ACC and reduced by ethylene synthesis and action inhibitors (Fig. 1; Table 1). This is consistent with previous reports showing that low phosphorus increases both growth rate and duration of growth to produce longer root hairs (Bates and Lynch, 1996) and with the well-known effects of ethylene on root hair elongation (Abeles *et al*., 1992; Pitts *et al*., 1998; Schmidt and Schikora, 2001). The effects of ethylene response mutations on root hair length depend on phosphorus availability, indicating an interaction between ethylene and phosphorus (Figs 2, 3). All mutants except *ein6* had shorter root hairs than Columbia under low phosphorus, but the mutations had variable effects with high phosphorus. Likewise, Cho and Cosgrove (2002) found that root hair length was reduced in some, but not all, ethylene-insensitive genotypes at high phosphorus, while 1-MCP usually reduced root hair length. These results indicate that the role of ethylene in root hair elongation is not as simple as it may appear from work with ACC, AVG, and the first few mutants examined (Pitts *et al*., 1998). The fact that ethylene response mutations are more inhibitory at low phosphorus suggests that, as with root hair density, the action of ethylene appears to be more crucial when phosphorus is limiting.

Cross-talk between ethylene and phosphorus in root hair development

Phosphorus regulates root hair length and density by modifying some subset of the enormously complex set of processes regulating these traits, including root growth rate, trichoblast cell specification and growth, and all of the many events governing the development and elongation of the root hairs themselves (Grierson *et al*., 2001). Some of these processes may be responsive to environmental factors regulating root hair development, among which phosphorus is one of the most important (Bates and Lynch, 1996; Foehse and Jungk, 1983; Ma *et al*., 2001*a*). In its effect on cortical cell number, phosphorus affects the very earliest stages of root hair development by changing the patterns of meristematic cell division. Sections very close to the meristem show similar cortical cell numbers to those in the root hair zone shown in Fig. 4, indicating that these changes do, in fact, occur in or near the meristem and not later in root development (data not shown). This profound effect on radial organization does not involve ethylene action, but does require polar auxin transport, since this response was strongly suppressed in the *eir1-1* mutant (Table 2). The effect of phosphorus on radial organization affects cell specification (trichoblast versus atrichoblast) by influencing the number of epidermal cells in the H position.

Ethylene has effects on root hair development that are distinct from phosphorus effects. Ethylene, but not phosphorus, is important for the initiation of root hairs at the proper site on the trichoblast (Masucci and Schiefelbein, 1994). Abnormalities were not observed in root hair position in plants grown with a wide range of phosphorus concentrations, though alterations were clearly visible with

AVG treatment. Excess ethylene also causes root hair development from N cells, i.e. ectopic hairs (Dolan *et al*., 1994; Tanimoto *et al*., 1995), while phosphorus (at high or low availability) does not.

Low phosphorus has additional effects which result in an even greater increase in root hair density than would be achieved with the increase in trichoblast file number alone, including reduced root growth and trichoblast elongation and, under some conditions, an increase in the proportion of H cells forming hairs. Each of these responses appears to involve ethylene action. Once hairs form, low phosphorus increases their length by increasing both the growth rate and the duration of elongation (Bates and Lynch, 1996). Inhibition of ethylene under phosphorus deficiency results in reduced root growth, fewer H cells forming hairs, and reduced root hair length, all part of the programme to increase root hair density and length. These morphological changes are synergistic in their effects on phosphorus acquisition and increase performance and competitiveness when phosphorus is limiting (Bates and Lynch, 2000*a*, 2000b, 2001; Ma *et al*., 2001*b*). Ethylene is required for the plant to fully engage these adaptive responses to phosphorus deficiency.

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References

Abeles FB, Morgan PW, Saltveit ME. 1992. *Ethylene in plant biology*. San Diego: Academic Press Inc.

Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR. 1999. EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. *Science* **284,** 2148–2152.

Bates TR, Lynch JP. 1996. Stimulation of root hair elongation in *Arabidopsis thaliana* by low phosphorus availability. *Plant, Cell and Environment* **19,** 529–538.

Bates T, Lynch J. 2000*a*. The efficiency of *Arabidopsis thaliana* (Brassicaceae) root hairs in phosphorus acquisition. *American Journal of Botany* **87,** 964–970.

Bates T, Lynch J. 2000*b*. Plant growth and phosphorus accumulation of wild type and two root hair mutants of *Arabidopsis thaliana* (Brassicaceae). *American Journal of Botany* **87,** 958–963.

Bates TR, Lynch JP. 2001. Root hairs confer a competitive advantage under low phosphorus availability. *Plant and Soil* **236,** 243–250.

Borch K, Bouma TJ, Lynch JP, Brown KM. 1999. Ethylene: a regulator of root architectural responses to soil phosphorus availability. *Plant, Cell and Environment* **22,** 425–431.

Chen R, Hilson P, Sedbrook J, Rosen E, Caspar T, Masson PH. 1998. The *Arabidopsis thaliana* AGRAVITROPIC 1 gene encodes a component of the polar-auxin-transport efflux carrier. *Proceedings of the National Academy of Sciences, USA* **95,** 15112–15117.

Cho H-T, Cosgrove D. 2002. Regulation of root hair initiation and expansin gene expression in *Arabidopsis*. *The Plant Cell* **14,** 3237–3253.

Clarkson D. 1985. Factors affecting mineral nutrient acquisition by plants. *Annual Review of Plant Physiology* **36,** 77–115.

Dolan L, Costa S. 2001. Evolution and genetics of root hair stripes in the root epidermis. *Journal of Experimental Botany* **52,** 413–417.

Dolan L, Duckett CM, Grierson C, Linstead P, Schneider K, Lawson E, Dean C, Poethig S, Roberts K. 1994. Clonal relationships and cell patterning in the root epidermis of *Arabidopsis*. *Development* **120,** 2465–2474.

Foehse D, Jungk A. 1983. Influence of phosphate and nitrate supply on root hair formation of rape, spinach and tomato plants. *Plant and Soil* **74,** 359–368.

Gahoonia TS, Nielsen NE. 1997. Variation in root hairs of barley cultivars doubled soil phosphorus uptake. *Euphytica* **98,** 177–182.

Grierson CS, Parker JS, Kemp AC. 2001. *Arabidopsis* genes with roles in root hair development. *Journal of Plant Nutrition and Soil Science* **164,** 131–140.

Johnson P, Ecker J. 1998. The ethylene gas signal transduction pathway: a molecular perspective. *Annual Review of Genetics* **32,** 227–254.

Jungk A. 2001. Root hairs and the acquisition of plant nutrients from soil. *Journal of Plant Nutrition and Soil Science* **164,** 121–129.

Kieber JJ, Rothenberg M, Roman G, Feldmann K, Ecker JR. 1993. CTR1, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the raf family of protein kinases. *Cell* **72,** 427–441.

Luschnig C, Gaxiola RA, Grisafi P, Fink GR. 1998. EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in *Arabidopsis thaliana*. *Genes and Development* **12,** 2175–2187.

Lynch J, Brown KM. 1997. Ethylene and plant responses to nutritional stress. *Physiologia Plantarum* **100,** 613–619.

Ma Z, Baskin TI, Brown KM, Lynch JP. 2003. Regulation of root elongation under phosphorus stress involves changes in ethylene responsiveness. *Plant Physiology* **131,** 1381–1390.

Ma Z, Bielenberg DG, Brown KM, Lynch JP. 2001*a*. Regulation of root hair density by phosphorus availability in *Arabidopsis thaliana*. *Plant, Cell and Environment* **24,** 459–467.

Ma Z, Walk TC, Marcus A, Lynch JP. 2001*b*. Morphological synergism in root hair length, density, initiation and geometry for phosphorus acquisition in *Arabidopsis thaliana*: a modeling approach. *Plant and Soil* **236,** 221–235.

Masucci JD, Schiefelbein JW. 1994. The *rhd6* mutation of *Arabidopsis thaliana* alters root-hair initiation through an auxin- and ethylene-associated process. *Plant Physiology* **106,** 1335–1346.

Masucci JD, Schiefelbein JW. 1996. Hormones act downstream of TTG and GL2 to promote root hair outgrowth during epidermis development in the *Arabidopsis* root. *The Plant Cell* **8,** 1505–1517.

Michael G. 2001. The control of root hair formation: suggested mechanisms. *Journal of Plant Nutrition and Soil Science* **164,** 111–119.

Parker JS, Cavell A, Dolan L, Roberts K, Grierson C. 2000. Genetic interactions during root hair morphogenesis in *Arabidopsis*. *The Plant Cell* **12,** 1961–1974.

Pemberton LMS, Tsai SL, Lovell PH, Harris PJ. 2001. Epidermal patterning in seedling roots of eudicotyledons. *Annals of Botany* **87,** 649–654.

Pitts RJ, Cernac A, Estelle M. 1998. Auxin and ethylene promote root hair elongation in *Arabidopsis*. *The Plant Journal* **16,** 553–560.

Roman G, Lubarsky B, Kieber JJ, Rothenberg M, Ecker JR. 1995. Genetic analysis of ethylene signal transduction in

Arabidopsis thaliana: five novel mutant loci integrated into a stress response pathway. *Genetics* **139,** 1393–1409.

Schaller GE, Bleecker AB. 1995. Ethylene-binding sites generated in yeast expressing the *Arabidopsis ETR1* gene. *Science* **270,** 1809–1811.

Schiefelbein JW. 2000. Constructing a plant cell. The genetic control of root hair development. *Plant Physiology* **124,** 1525–1531.

Schmidt W. 2001. From faith to fate: ethylene signaling in morphogenic responses to P and Fe deficiency. *Journal of Plant Nutrition and Soil Science* **164,** 147–154.

Schmidt W, Schikora A. 2001. Different pathways are involved in phosphate and iron stress-induced alterations of root epidermal cell development. *Plant Physiology* **125,** 2078–2084.

Tanimoto M, Roberts K, Dolan L. 1995. Ethylene is a positive regulator of root hair development in *Arabidopsis thaliana*. *The Plant Journal* **8,** 943–948.

Wright AJ, Knight H, Knight MR. 2002. Mechanically stimulated TCH3 gene expression in *Arabidopsis* involves protein phosphorylation and EIN6 downstream of calcium. *Plant Physiology* **128,** 1402–1409.