Different Pathways Are Involved in Phosphate and Iron Stress-Induced Alterations of Root Epidermal Cell Development[1]

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Low bioavailability of phosphorus (P) and iron (Fe) induces morphogenetic changes in roots that lead to a higher surface-to-volume ratio. In Arabidopsis, an enlargement in the absorptive surface area is achieved by an increase in the length and frequency of hairs in roots of Fe- and P-deficient plants. The extra root hairs are often located in positions that are occupied with non-hair cells under normal conditions, i.e. over a tangential wall of underlying cortical cells. An involvement of auxin and ethylene in root epidermis cell development of Fe- and P-deficient plants was inferred from phenotypical analysis of hormone-related Arabidopsis mutants and from the application of substances that interfere with either synthesis, transport, or perception of the hormones. Application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid or the auxin analog 2,4-D caused a marked increase in root hair density in plants of all growth types and confers a phenotype characteristic of ethylene-overproducing mutants. Hormone insensitivity and application of hormone antagonists inhibited the initiation of extranumerary root hairs induced by Fe deficiency, but did not counteract the formation of extra hairs in response to P deprivation. A model is presented summarizing putative pathways for alterations in root epidermal cell patterning induced by environmental stress.

P and Fe are essential mineral elements for virtually all organisms. Although the total amount of both nutrients in the soil is often well beyond a level limiting growth, plants may suffer from P and Fe deficiency due to the presence of these minerals in forms that are not readily available for uptake. To maintain an adequate supply, plants have evolved multifaceted adaptive mechanisms that help to enhance the mobilization of poorly soluble P and Fe and facilitate the uptake of these nutrients. Production of root exudates, acidification of the rhizosphere, and enhanced expression of specific transport proteins are components of such strategies that have been reported for higher plants (Schachtman et al., 1998; Mori, 1999; Raghothama, 1999; Schmidt, 1999). A further set of adaptive processes is concerned with alterations in root architecture and morphology, resulting in a higher ratio of surface area to volume. In P-starved plants, the exploration of topsoil resources is enhanced by alterations in gravitropism of basal roots and by a shift in biomass allocation from basal to adventitious roots (Bonser et al., 1996). In some taxa, mainly in members of the Proteaceae, clusters of rootlets ("proteoid roots") are formed that are highly efficient in extruding organic acids, acid phosphatases, and other compounds that facilitate the mobilization of nutrients from soils (Watt and Evans, 1999). Root proliferation and root elongation as well as the formation of mycorrhiza are further hallmarks of plant responses to low P availability (Smith and Read, 1997). In roots grown under conditions of Fe limitation, changes in root morphology comprise a decrease in lateral root length and a reduction in inter-lateral distance. An increase in the number of laterals and an enhancement of root diameter, caused by an enlargement of cortical cells, are additional changes found in various species under Fe shortage (Schmidt, 1999). The production of extranumerary root hairs is probably the most common morphological response to P and Fe deficiency. Root hairs are tubular-shaped outgrowths of epidermal cells that play an important role in the acquisition of water and nutrients, especially phosphate.

The mechanisms underlying cell fate specification have been studied pharmacologically and by analysis of mutants harboring defects that cause alterations in root hair patterning (e.g. Masucci and Schiefelbein, 1996; Woeste and Kieber, 2000). Root hair morphogenesis is controlled by a set of genes that negatively regulate hair fate (Benfey, 1999). In addition, the plant hormones ethylene and auxin promote hair differentiation by acting downstream of the cell specification genes and may also be involved in a number of other alterations in root morphology such as formation of laterals and adventitious roots (Muday et al., 1995; Smalle and Van der Straeten, 1997; Visser et al., 1997). Several loci

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involved in the ethylene signal transduction pathway have been identified through their mutant phenotypes (for recent reviews, see Kieber, 1997; Johnson and Ecker, 1998; Theologis, 1998; Chang and Shockey, 1999). As shown by epistasis analysis, a family of ethylene receptors with partially redundant functions act through CTR1 (*constitutive triple response*), which represents a central component in the ethylene signaling pathway situated downstream of thereceptor (Kieber et al., 1993). *CTR1* encodes a Raf-like protein kinase, suggesting the involvement of a mitogen-activated protein kinase cascade in the signal transduction pathway. Inactivation of CTR1 by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) causes non-hair cells to differentiate into hairs (Kieber et al., 1993; Tanimoto et al., 1995). A fine regulation of the diversity of plant responses to ethylene may be mediated by a hierarchy of transcription factors for stress-responsive genes, acting downstream of the components of the ethylene signaling pathway (Solano et al., 1998; Fujimoto et al., 2000). These factors include ethylene-responsive factors (ERFs), a group of novel DNA-binding proteins that activate genes containing ethylene-responsive elements by interacting directly with a cis-regulatory sequence referred to as the GCC box (Ohme-Takagi and Shinshi, 1995).

The pathways involved in the translation of environmental signals into changes in epidermal cell fate are not understood. Ethylene levels are increased in response to P and Fe deficiency (Borch et al., 1999; Romera et al., 1999), and treatment with auxin and ethylene mimics the effects of P and Fe stress on root morphology, suggesting that some of the adaptive reactions are induced by ethylene and/or auxin-ethylene interactions. *ctr1* mutants and transgenic plants overexpressing *ERF1* display continuous activation of the pathway and resemble wild-type seedlings grown in ethylene. In the present study, we investigated the effects of P and Fe deficiency on root hair formation in various hormone-related Arabidopsis mutants. It is shown that the initiation of root hairs in response to P and Fe deficiency is differentially affected by defects in ethylene signaling and by antagonists of auxin and ethylene.

RESULTS

P and Fe Stress-Induced Formation of Root Hairs Is Differentially Affected in Hormone-Related Arabidopsis Mutants

To investigate the role of hormones in P and Fe deficiency-induced alterations in epidermal patterning, various hormone-related mutants in Arabidopsis and the *Col-0* wild-type were grown either under control conditions or in the absence of P or Fe. The most obvious effects of Fe and P starvation on the morphology of wild-type roots were increases in the length and frequency of root hairs, leading to a manifold enlargement of the root surface area (Fig. 1, B and C). Formation of extranumerary root hairs in response to P deficiency was observed 2 d after transferring the plants into P-free medium; development of extra root hairs in Fe-deficient plants was evident 3 d after the onset of treatment. In roots of Arabidopsis only certain cells within the epidermis, i.e. those lying over anticlinal cortical cell walls (trichoblasts), are capable of producing root hairs, whereas other cells (atrichoblasts) remain hairless. Upon growth in P- or Fe-free medium, this array of root hair and non-root hair cells is altered by the formation of ectopic hairs in positions that are occupied by non-hair cells under ordinary conditions (e.g. overlying periclinal cortical cell walls). Such ectopic root hairs were formed in roots of both P- and Fe-deficient plants. When compared with Fe-free grown plants, the density of root hairs was markedly higher in response to P deficiency, partly due to the formation of ectopic hairs (Table I; Fig. 1).

axr1 and *axr2* are mutants that are resistant to ethylene and auxin and that display a reduced number of root hairs (Estelle and Somerville, 1987; Wilson et al., 1990). Under the present conditions, roots of *axr2* were almost completely devoid of hairs when the seedlings were grown under ordinary conditions (Table I; Fig. 1G). No changes in root hair formation were observed after transferring the seedlings to Fe-free nutrient solution (Fig. 1H). In contrast, growing the plants under conditions of P deficiency resulted in the development of a pattern typical of P-deficient wild-type roots (Fig. 1I). A similar behavior has been described previously for *axr2* (Bates and Lynch, 1996) and was observed with the auxin-insensitive mutant *aux1* in the present study (Table I). Hair density of P-starved *aux1* roots was somewhat lower relative to the wild type and root hairs were formed mainly in normal position. In contrast with *axr2*, *axr1* and *aux1* developed some hairs under control conditions, although the frequency was clearly reduced when compared with the wild type (Table I; Fig. 1, D–F).

With respect to the ethylene mutants, analogous root hair patterns were observed in the ethylene-insensitive *ein2* and the ethylene-resistant *etr1* mutant (Table I). Both mutants display similar responses as the auxin mutants, e.g. production of hairs only under −P conditions but a reduced number in Fe-free medium and under control conditions. Under P-deficient conditions root hair elongation was restricted, being more severe in *etr1*. Compared with the wild type, *etr1* and *ein2* exhibited a slightly decreased number of hairs when cultivated in P-free medium. In both mutants, P starvation caused the formation of root hairs in ectopic positions, although in a low frequency. The ethylene overproducer *eto3* displayed formation of extra root hairs irrespective of

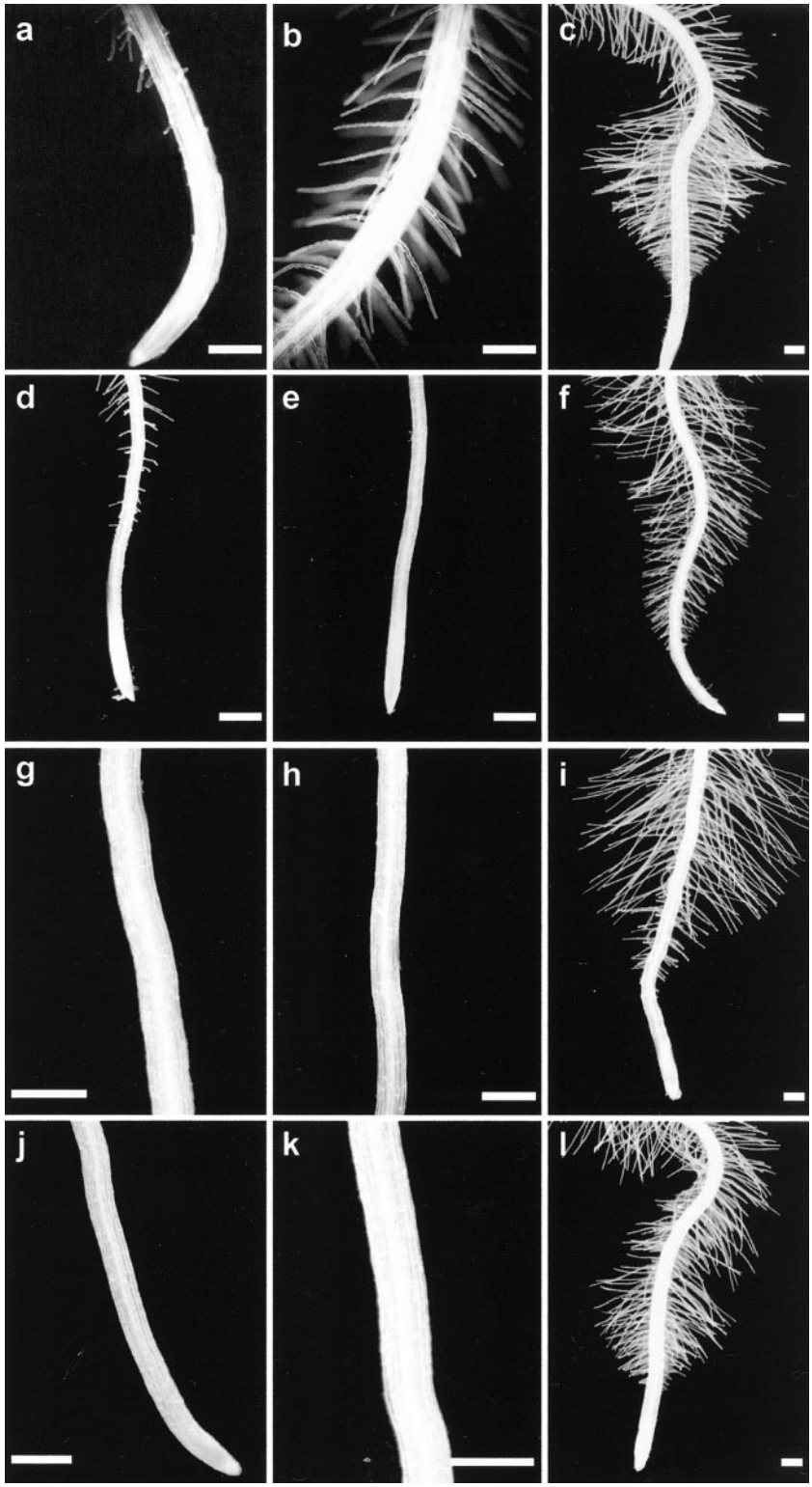

Figure 1. (Continued on facing page.)

the treatment (Fig. 1, P–R). The frequency of root hairs was more than 2-fold higher than that of the wild type under control and −Fe conditions. Growing the plants under P deficiency caused a further increase in hair number, reaching a 3-fold higher density when compared with −P wild-type plants (Table I).

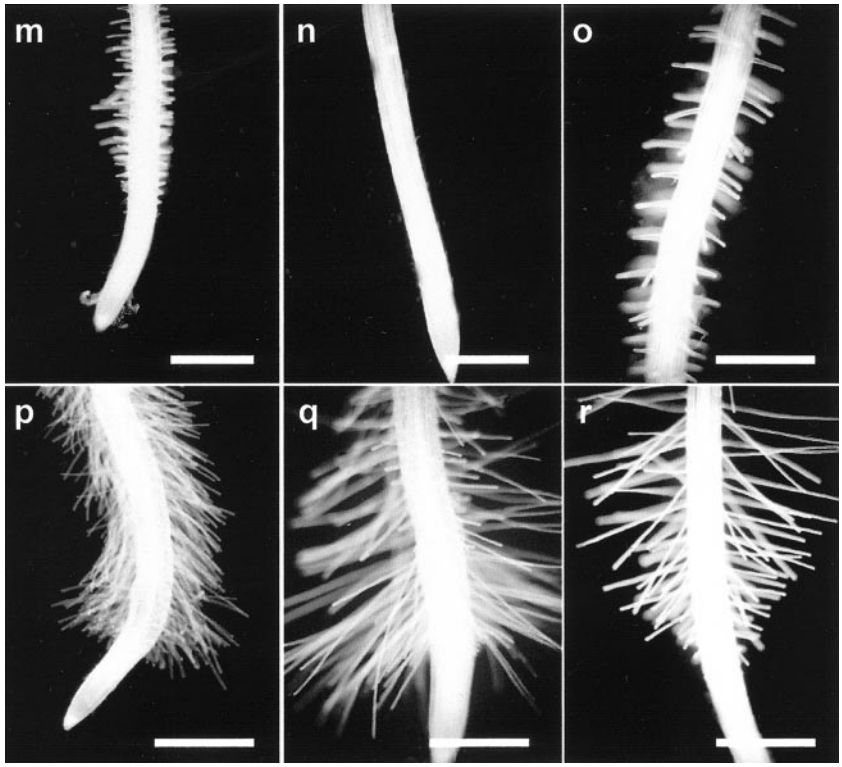

Figure 1. (Continued from facing page.)
Root tips of Arabidopsis wild type and of various hormone-related mutant plants grown under control conditions or in the absence of P or Fe. A, *Col-0* control; B, *Col-0* −Fe; C, *Col-0* −P; D, *axr1* control; E, *axr1* −Fe; F, *axr1* −P; G, *axr2* control; H, *axr2* −Fe; I, *axr2* −P; J, *ein2* control; K, *ein2* −Fe; L, *ein2* −P; M, *etr1* control; N, *etr1* −Fe; O, *etr1* −P; P, *eto3* control; Q, *eto3* −Fe; R, *eto3* −P. *aux1* exhibited a phenotype similar to that of *axr2* under all growth conditions and is not shown in the figure. Bar = 0.25 mm.

Hormone Antagonists Inhibit the Formation of Root Hairs Induced by Fe Shortage, But Not Those Induced in Response to P Deficiency

To determine whether the mutant phenotypes can be copied in the wild type, we applied hormones or inhibitors of either auxin transport (naphthylphthalamic acid [NPA] and 2,3,5-triiodobenzoic acid [TIBA]), ethylene synthesis (aminoethoxyvinylglycine [AVG], aminooxyacetic acid [AOA], and Co^{2+}), or ethylene action (silver thiosulfate [STS]) to wild-type seedlings. The results are shown in Table II. 2,4-D and ACC markedly enhanced the number of root hairs and caused the formation of ectopic hairs. Application of 2,4-D caused no alterations in roots grown in the absence of Fe but enhanced root hair frequency in −P plants. In −Fe and −P plants, the presence of ACC led to a root hair frequency typical of *eto3* seedlings grown under similar con-

Table I. *Effect of Fe and P deficiency on root hair formation in Arabidopsis wild-type and mutant plants*
Values represent no. of root hairs (means ± SE) of root hairs per millimeter. Twenty roots were scored for each genotype/treatment.

Gene	Mutant Phenotype	Control		−Fe		−P		Reference
		Root hair density	Ectopic hairs	Root hair density	Ectopic hairs	Root hair density	Ectopic hairs	
			%		%		%	
Col-0	Columbia	30 ± 1	0	45 ± 2	4	55 ± 2	6	
axr1-3	Auxin resistant, display resistance to various hormones	7 ± 1	0	6 ± 2	0	56 ± 2	8	Estelle and Somerville (1987)
axr2	Auxin resistant, displays resistance to various hormones	<1	0	0	0	50 ± 3	9	Wilson et al. (1990)
aux1-7	Auxin insensitive	11 ± 2	0	4 ± 1	0	35 ± 2	2	Pickett et al. (1990)
ein2-1	Ethylene insensitive	10 ± 2	0	6 ± 2	0	42 ± 1	2	Kieber et al. (1993)
etr1-3	Ethylene resistant	13 ± 2	0	7 ± 3	0	41 ± 2	2	Bleecker et al. (1988)
eto3	Ethylene overproducer	129 ± 8	>10	124 ± 10	>10	148 ± 10	>10	Guzmán and Ecker (1990)

Table II. *Effects of ACC, 2,4-D, and various inhibitors on hair formation in Arabidopsis wild-type roots grown under P and Fe deficiency*

Values represent no. of root hairs (means ± SE) of root hairs per millimeter. Twenty roots were scored for each treatment.

Treatment	Control	−Fe	−P
None	30 ± 1	45 ± 2	55 ± 2
2,4-D	95 ± 5	94 ± 1	126 ± 10
ACC	80[a] ± 8	130 ± 11	148 ± 11
NPA	0	1 ± 1	90 ± 12
TIBA	7[a] ± 2	0	78 ± 7
AVG	1[b] ± 1	5[a,b] ± 2	49[b] ± 4
AOA	23 ± 4	7 ± 3	57 ± 5
Co^{2+}	21[a] ± 4	2[a] ± 1	44[a] ± 3
STS	3 ± 2	0	71 ± 8

[a] Inhibited root hair elongation. [b] Leaves of this growth type showed chlorosis symptoms.

ditions. With respect to the inhibitors, root hair density was reduced under control and −Fe conditions and unaffected or increased in P-deficient plants. The auxin antagonists TIBA and NPA caused an almost complete absence of root hairs in control and −Fe plants, whereas the density of root hairs was considerably increased in P-deficient plants (Table II). A similar pattern was observed after application of STS. Application of Co^{2+} ions additionally caused an inhibition of root hair elongation (Table II). Restricted elongation of hairs was also noted in ACC- and TIBA-treated roots grown in normal medium and in Fe-deficient plants after application of AVG. The formation of dense root hairs in −P plants was not markedly affected by ethylene antagonists. Because plants treated with AVG showed leaf chlorosis under all conditions, the effect of AVG may not be specific to the inhibition of ethylene synthesis.

DISCUSSION

Auxin and ethylene have been implicated in developmental adaptations of roots grown under P or Fe deficiency (Landsberg, 1981, 1996; Romera et al., 1994; Bates and Lynch, 1996; Schmidt and Bartels, 1996; Schmidt et al., 2000). This assumption was based on the effects of exogenously applied hormones that mimic the respective phenotypes, and on the fact that deficiencies in P and Fe supply increase the level of ethylene within the plant (Borch et al., 1999; Romera et al., 1999). This does not inevitably imply, however, that either hormone is involved in the signal transduction pathway. The analysis of root hair patterning in hormone-related Arabidopsis mutants in the present study showed a complete inhibition of root hair outgrowth in Fe-deficient mutant plants, but not in those grown under P deficiency.

Differences between Fe- and P-deficient plants in root hair patterning were evident both in auxin and ethylene-related mutants. *AXR2* corresponds with one of the auxin-inducible *Aux/IAA* genes, *IAA7* (Nagpal et al., 2000), and may play a key role in root hair morphogenesis because a functional *AXR2* product is required for normal root hair development and is also necessary for ACC and auxin to induce root hairs (Masucci and Schiefelbein, 1996). AUX1 and AXR1 are proposed to define a separate pathway for ethylene action (see below). ETR1 and EIN2 have important functions in the ethylene signaling pathway (Johnson and Ecker, 1998; Chang and Shockey, 1999). ETR1 is an ethylene receptor and mutants bearing a defect in this gene are defective in negative feedback of ethylene biosynthesis. EIN2 acts downstream of ETR1 and is thought to be involved in the signal transduction from CTR1 to downstream components of the pathway.

The results obtained with the hormone-related mutants indicate that ethylene and auxin are essential for the development of extra root hairs in response to Fe deficiency, but are apparently not required for root hairs induced by P deficiency stress. This assumption is supported by the results of the inhibitor studies. Although all inhibitors under study inhibited root hair initiation and, in the case of Co^{2+}, elongation of the hairs in Fe-deficient roots, only a marginal decrease in root hair density was observed in −P plants.

The results of the present study enable us to consider a model for root hair formation induced by P and Fe deficiency. According to the model outlined in Figure 2, Fe deficiency can activate two different pathways, probably via the production of putative stress signals. Both pathways ultimately lead to the formation of extra root hairs located in positions normally occupied by non-hair cells. One of the pathways is dependent on ethylene signaling and requires functional *EIN2* and *ETR1* genes. Based on analysis of auxin-related mutant phenotypes, an ethylene signaling-independent pathway involved in root hair cell differentiation involving AUX1 and/or AXR1 has been proposed by Masucci and Schiefelbein (1996). Although the data from the present study do not allow a possible interplay between these pathways to be deduced, ethylene may also act through the products of these genes in the Fe stress-induced formation of root hairs because defects in their products caused an inhibition of this process. Both ethylene pathways are suggested to converge at, or upstream of, AXR2, which thus represent a key component of the hormone response pathway.

The lack of influence of mutations in genes described above on epidermal cell differentiation induced by P deficiency suggests that a P deficiency-specific stress signal may interact directly with components of an ethylene-independent pathway. This pathway is not dependent on genes involved in ethylene or auxin signaling and may directly activate primary ethylene response genes. AtERF proteins are likely candidates for factors that are responsive to

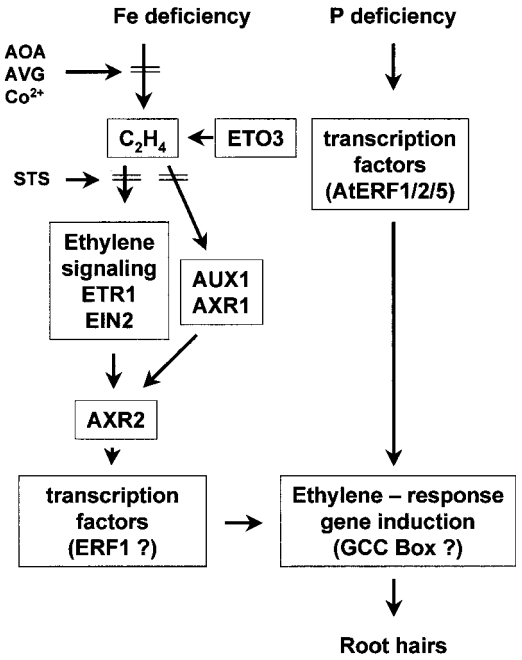

Figure 2. Model for changes in epidermal cell differentiation in response to P and Fe stress. Fe and P deficiency can cause the formation of extra root hairs. Under Fe-deficient conditions, root hair initiation is dependent on the ethylene signaling cascade. A possible auxin response pathway including AUX1 and/or AXR1 may represent a further pathway in which ethylene acts. Initiation of root hairs is inhibited in mutants defective in ethylene signaling and in those harboring defects in AUX1, AXR1, and AXR2, and by substances that interfere either with ethylene synthesis or perception. Root hair initiation induced by P deficiency is not affected in the respective mutants and application of hormone antagonists implying that these responses are controlled by an ethylene-independent pathway. This pathway may be defined by transcription factors that can induce expression of ethylene-response genes. Primary ethylene responsive genes like *ERF1* are assumed to be part of the ethylene-dependent pathway. Adapted from figures in Masucci and Schiefelbein (1996) and Fujimoto et al. (2000).

extracellular signals and a function of these proteins as stress signal-responsive factors was recently proposed by Fujimoto et al. (2000). Transcripts of AtERFs increased after exposure of Arabidopsis seedlings to ethylene in the wild type but not in the *ein2* mutant, whereas induction of AtERFs after abiotic stress was observed in both wild type and mutant. A further possibility is that P deficiency is translated by both pathways that are partially redundant. Whether or not ERFs are induced in response to Fe and P deficiency and whether other transcription factors are involved remains to be elucidated.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The genetic stocks were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). All mutants have been described elsewhere. Plants were grown in a growth chamber on an agar medium as described by Estelle and Somerville (1987). The seeds were surface sterilized by immersing them in 5% (v/v) NaOCl for 5 min and 96% (v/v) ethanol for 7 min, followed by four rinses in sterile water. The medium was composed of: KNO_3 (5 mM), $MgSO_4$ (2 mM), $Ca(NO_3)_2$ (2 mM), K_2PO_4 (2.5 mM), H_3BO_3 (70 μM), $MnCl_2$ (14 μM), $ZnSO_4$ (1 μM), $CuSO_4$ (0.5 μM), NaCl (10 μM), and Na_2MoO_4 (0.2 μM) and solidified with 0.5% (w/v) agar. Suc (43 mM) and 4.7 mM MES [2-(*N*-morpholino)ethanesulfonic acid] were included and the pH was adjusted to 6.0. Seeds were placed onto Petri dishes containing agar medium and kept for 3 d at 4°C in the dark, before the plates were transferred to a growth chamber and grown at 21°C in continuous light (150 μmol m^{-2} s^{-1}; TL lamps Philips, Eindhoven, The Netherlands). After 10 d, plants were grown for an additional 4 d either with 40 μM FeEDTA (+Fe plants), without P (−P plants) or without Fe and 100 μM 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate (FerroZine; −Fe plants). 2,4-D (0.1 μM) was added to the medium before autoclaving from a stock dissolved in 50% (w/v) ethanol. ACC (1 μM), NPA (10 μM), AVG (1 μM), AOA (10 μM), or Co^{2+} was added after autoclaving the medium.

Microscopy

Root hair patterns were analyzed by dark-field stereomicroscopy of fresh probes using a stereomicroscope (Stemi 2000-CS, Zeiss, Jena, Germany). Photographs were recorded on negative film (Superia 100, Fuji, Tokyo).

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