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ETHYLENE AND PHOSPHORUS RESPONSES IN PLANTS

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Abstract

Plant roots react to low phosphorus availability in several ways that may permit them to acquire more phosphorus, including distributing more roots in upper, phosphorus -rich soil horizons, growing relatively more roots, i.e. higher root/shoot ratio, and producing longer and denser root hairs to increase the surface area. These alterations in growth may also influence tolerance to other stresses, such as water stress. The objectives of this study were to examine the involvement of ethylene in phosphorus regulation of root hair density and length and basal root angle. I also evaluated the effects of alumina-buffered phosphorus (Al-P), a novel phosphorus fertilizer that releases phosphorus at a much lower rate than conventional fertilizers, on growth and drought tolerance of woody plants and on seedling establishment of several tree species grown in containers with soilless media.

Low phosphorus induced longer and denser root hairs in roots of *Arabidopsis thaliana* ecotype Columbia. The increase in root hair density partially resulted from increased number of cortical cells compared with roots grown in high phosphorus, which had eight large cortical cells. The increase in cortical cell number resulted in an increased number of trichoblasts. Ethylene manipulation affected root hair length and density, but not cortical cell number or trichoblast number. For example, the ethylene precursor, 1-aminocyclopropane-1-carboxylate (ACC) added to high phosphorus medium could mimic the low phosphorus effect on the root hair growth, while ethylene synthesis and action inhibitors including aminoethoxyvinyl glycine (AVG), MCP (1-methylcyclopropene), or silver thiosulfate (STS) reduced root hair growth under low phosphorus conditions. Ethylene insensitive mutants, including *ein4*, *ein2-1*, *ein3-1*, *ein5-1*, *ein6*, *ein7*, and the mutant *eir1-1*, showed a reduced but still significant response to low phosphorus. However, results of root cross-sectioning showed that ethylene manipulation did not affect the number of cortical cells or trichoblasts in roots. Ethylene-insensitive mutants grown with low phosphorus produced more and smaller cortical cells,

similar to wild-type plants. The pattern of trichoblast length change in response to phosphorus availability for both of the ethylene-insensitive mutants tested (*ein2-1* and *ein4*) was similar to the wild type. These results suggest that low phosphorus and ethylene might operate via separate pathways to influence root hair density.

The genotype G19833 of common bean (*Phaseolus vulgaris* L.) had shallow basal roots that became shallower as phosphorus availability decreased, while DOR364 had deep basal roots that were affected by phosphorus availability to a lesser extent. These genotypes and recombinant inbred lines (RILs) produced from their F1 hybrid were used to study the role of ethylene in basal root gravitropic responses to low phosphorus. Basal roots grew shallower when ACC or gaseous ethylene was added to high phosphorus medium, especially the shallow genotypes. AVG and STS, on the other hand, prevented the change in basal root angle induced by low phosphorus. The extent of basal root growth angle change was proportional to the concentrations of ACC or AVG added to the media. A strong correlation was observed for the shallow genotype G198333 between basal root shallowness and the endogenous ethylene production rate. These results suggest that ethylene might mediate the response of roots to phosphorus availability and that genetic variation in ethylene responsiveness could be linked to basal root plasticity in response to phosphorus availability.

Al-P reduced phosphorus leaching while improving plant drought tolerance. When plants were fertilized with Al-P prepared at a phosphorus desorption rate of 74 μM, vegetative growth of rhododendron (*Rhododendron catawbiense* Michx.), forsythia (*Forsythia intermedia* Zab.), Ohio buckeye (*Aesculus glabra* Willd.), and bur oak (*Quercus macrocarpa* Michx.), measured as plant height, stem caliper, and/or biomass, was as fast as, or faster than that observed with Osmocote or monoammonium phosphate fertilizer. Imposition of summer drought during the first growth season slightly reduced growth of rhododendron, with a stronger effect in the second year, while forsythia was more affected in the first season. The drought effect on growth was mitigated by Al-P fertilization in forsythia but not in rhododendron. When Al-P was used, more rhododendron plants produced flower buds in the first year, and at the lower desorption

concentration, drought caused no reduction in the percent of plants producing flower buds.

These results are important in understanding the regulatory mechanism of the responses of roots to low phosphorus limitation, and should be useful for breeders to develop crops with improved nutrient (especially phosphorus) uptake characteristics. The results can also provide information for agronomists and horticulturists to develop better cultural practices to reduce phosphorus fertilization and leaching.

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Chapter 1

Introduction

As an essential macronutrient element for plants, phosphorus plays important roles in many biochemical processes and plants need a large quantity of phosphorus to survive and thrive. Therefore, providing plants with adequate phosphorus is critical to natural and agricultural systems. However, phosphorus in the soil can react with aluminum, iron, manganese, and calcium to form insoluble precipitates which are not available to plants. Therefore, phosphorus is a primary limitation over most terrestrial areas in the world, especially in vast regions in tropical and subtropical areas, because phosphorus availability in soils in these areas is usually too low to provide adequate phosphorus for plant growth and development.

Application of phosphorus fertilizers is one solution to low phosphorus stress. This solution, however, has several drawbacks. Firstly, crop production systems suffering most from low phosphorus availability in soils are in tropical and subtropical areas in developing countries in Asia, Africa, and South America where farmers cannot afford to apply phosphorus fertilizers. Secondly, phosphorus ores are a mined, non- renewable resource and subject to depletion. Thirdly, phosphorus fertilization can cause pollution. Excessive phosphorus run-off from the soil surface can enter lakes or streams, over-stimulating plant and algae growth in lakes and streams, reducing water quality, and harming aquatic wild life.

An alternative and a better solution is to use crop cultivars that can acquire phosphorus more efficiently from the soil. Phosphorus efficient crops would require much less phosphorus fertilizer for adequate growth and development, so this solution can minimize the above mentioned problems. Phosphorus efficiency is a relatively new objective for plant breeders, who have traditionally bred crops for high fertility production systems. Therefore basic physiological studies have to be done before breeders can effectively integrate phosphorus efficiency into their breeding programs.

The first step is to identify plant traits that are important for efficient uptake of phosphorus from the soil; then we need to identify sources of genetic variation for those traits. The result can be used by breeders in conventional or molecular breeding programs.

A third solution is the development of more phosphorus efficient cultural practices for crop production. This also requires the understanding of mechanism how plants react to low phosphorus stress: the physiological, biochemical, and molecular characteristics of these traits.

Root responses that influence the spatial distribution of roots in soil determine plant ability to acquire relatively immobile resources such as phosphorus. It is important, therefore, to study how plants react to phosphorus deficiency. The study of roots is relevant to the understanding of the adaptation of plants to adverse environments because roots are not just passive victims of phosphorus deficiency, but active explorers for available phosphorus in soils.

It has been observed that plants use one or several of the following strategies to acquire more phosphorus under low phosphorus conditions: changing the root architecture so that more roots are distributed where more phosphorus is available, usually the top soil; increasing absorbing surface area mainly by developing more and longer root hairs and by increasing association with mycorrhizal fungi; and exuding acid phosphatase to potentially liberate phosphorus from the organic pool. However, we have very little knowledge so far about the physiological regulation of these responses to low phosphorus.

The goals of this study are to provide information for breeders to develop crops with improved nutrient uptake characteristics and provide information for agriculturists to develop better cultural practices. We focused on the regulatory mechanism of root hair growth and basal root gravitropism in response to low phosphorus availability, especially the possible involvement of ethylene, by manipulating ethylene and phosphorus

availability in the growth media. For root hair growth, we compared effects of low phosphorus and ethylene on root hair density, root hair length, trichoblast file number, and trichoblast cell length. For basal root gravitropism, we examined whether ethylene could mimic low phosphorus in inducing shallower basal roots. We also observed the responses of containerized woody ornamental plants to different levels of phosphorus in root and shoot growth, reproduction, and drought resistance; the result should be useful to nurseries to reduce phosphorus fertilization and leaching.

Chapter 2

Literature Review

Phosphorus deficiency alters plant growth and development. Changes in roots caused by phosphorus deficiency include altered root architecture such as reduced gravitropism and over-growth of root hairs. Interestingly, ethylene has effects similar to phosphorus deficiency on roots. These similarities suggest a possible regulatory role for ethylene in plant response to low phosphorus stress. In this review, I will focus on the following topics: 1. Ethylene biosynthesis and signal transduction pathways, and the relationship between nutrient stress and ethylene; 2. Root hairs, including root hair development, genetic control, and epidermal cell patterning; 3. Root gravitropism; and 4. Interaction of drought and phosphorus.

1. Ethylene Biosynthesis and Signal Transduction Pathways

Ethylene Biosynthesis Pathway

Ethylene is a gaseous growth regulator involved in an array of cellular, developmental and stress-related processes in plants. Ethylene production in plants is induced by physiological, pathological, and environmental events such as germination, ripening, pollination, water stress, wounding, and pathogen attack (Beyer *et al.*, 1984). Ethylene has important roles in floral induction, sex determination, flooding-induced shoot elongation, senescence, and leaf abscission.

Ethylene biosynthesis starts from the amino acid methionine (Met), which is converted to S-adenosyl-L-Methionine (SAM). The direct precursor of ethylene, 1-aminocyclopropane-1-carboxylate (ACC), is produced from SAM by the action of ACC synthase (ACS). The terminal step of ethylene biosynthesis is under the control of ACC oxidase (ACO) (Adams & Yang, 1979) (Fig. 2.1).

ACS is the key enzyme catalyzing the limiting step of ethylene biosynthesis (Kende, 1993). External stimuli, such as mechanical impedance (Botella *et al.*, 1995; He *et al.*, 1996), water stress (Tudela & Primo-Millo, 1992; Zarembinski & Theologis, 1997), hypoxia (He *et al.*, 1996), pathogen attack (Knoester *et al.*, 1995), ozone (Schlagnhaufer *et al.*, 1997; Tuomainen *et al.*, 1997), wounding, and auxin (Botella *et al.*, 1992; Imaseki, 1989), can enhance ACS gene expression and enzyme activity, and endogenous ethylene regulates ACS activity in a feedback loop (Kende, 1993). ACS is encoded by a multigene family, comprising at least 5 genes in *Arabidopsis* (Liang *et al.*, 1992), and at least 7 genes in tomato (Lincoln *et al.*, 1993; Oetiker *et al.*, 1997; Olson *et al.*, 1995). In most cases, stress-enhanced ACS activity is regulated at the transcriptional level. In *Arabidopsis*, ACS1 is expressed in young tissues and the expression is strongly correlated with lateral root formation (Rodrigues-Pousada *et al.*, 1993). ACS4 is found to be a primary IAA-responsive gene (Abel *et al.*, 1995). In tomato, LE-ACS4 responds to ripening and wounding (Lincoln *et al.*, 1993). In potato (*Solanum tuberosum* L.) leaves, ST-ACS5 and ACS4 are sequentially expressed in response to biotic and abiotic stresses (Schlagnhaufer *et al.*, 1997). In rice (*Oryza sativa* L. cv. Habiganj Aman II), partial submergence in water induces expression of OS-ACS1 in all zones of the elongating internode, but suppresses expression of OS-ACS2, and OS-ACS3 and OS-ACS5 are not expressed in all zones (Zarembinski & Theologis, 1997).

In vegetative tissues of pea (Peck & Kende, 1995) and mung bean (Kim *et al.*, 1997), ACO reacts to stress by increasing ACO gene expression and ACO activity. Since this response is blocked by inhibitors of ethylene action, it is believed to be positively feedback-regulated by ethylene. The expression of ACO gene(s) is therefore a good molecular marker to determine ethylene production and responsiveness by plant tissues (Peck *et al.*, 1998). In tomato, ACO is encoded by 3 genes (Barry *et al.*, 1996).

Several mutants that overproduce ethylene (*ethylene over production (eto)*) were identified in *Arabidopsis* by the phenotype of displaying characteristics of ethylene exposure even in the absence of the gas (Kieber *et al*., 1993). Higher levels of ACC synthase were found in *eto1*, *eto2*, and *eto3* than in wild type (Woeste *et al*., 1999). ETO2

encodes the ACS-5 protein and the *eto2-1* mutant protein lacks a region of the protein that negatively regulates the activity of the enzyme, resulting in constitutive activity of ACC synthase and thus ethylene overproduction (Vogel *et al*., 1998).

Ethylene Signal Transduction Pathway

The ethylene signal transduction pathway, in which *Arabidopsis* plants perceive and respond to ethylene, starts with five genes encoding ethylene receptors with redundant functions: ETR1, ETR2, EIN4, ERS1, and ERS2 (Chang and Shockey, 1999; Urao *et al*., 2000). The membrane-localized ethylene binding sites require a copper cofactor and the delivery of copper depends upon the copper transporter RAN1 (Urao *et al*., 2000). These genes are redundant because if one is missing another one of the receptors can act in its place (Hua and Meyerowitz, 1998). The five receptors fall into two subfamilies based on their gene and protein structure: ETR1/ERS1 and EIN4/ETR2/ERS2 (Chang and Shockey, 1999). The ethylene receptors are catalytic receptor kinases similar to bacterial two-component system, composed of a sensory histidine kinase and a response regulator. In bacteria, the sensory kinase senses a signal and interacts with the response regulator, which in turn transduces the signal to its downstream pathway. In wild type *Arabidopsis*, ETR1 is in an active state in the absence of ethylene and activates CTR1, a kinase similar to a Raf family of protein kinases, that negatively regulates the downstream components EIN2 and EIN3, leading to the repression of ethylene responses (Urao *et al*., 2000). Binding of ethylene to ETR1 stops activation of CTR1, thus lifting the repression and allowing ethylene responses (Fig. 2.2).

Nutrient Stress and Ethylene

Nutrient stress, either deficiency or toxicity, can change ethylene biosynthesis and/or responsiveness (Lynch & Brown, 1997). Tomato leaves provided with NH_4-N produced more ethylene than those provided with NO_3-N, and a large amount of ethylene was produced by leaves under NH_4-N toxicity combined with deficiency of K, Ca, or Mg (Feng & Barker, 1993; Corey & Barker, 1989; Baker & Corey, 1988). The responses of cucumber roots to iron deficiency were inhibited by ethylene synthesis or action inhibitors but enhanced by ACC, implying that ethylene is involved in the process

(Romera, 1994). In Arabidopsis, iron deficiency induced overproduction of root hairs. Ethylene insensitive mutants or ethylene biosynthesis and action inhibitors can block the response of plants to iron deficiency (Schmidt & Schikora, 2001). Nutrient deficiency may affect tissue sensitivity (or responsiveness) without changing ethylene production (He *et al.*, 1992; Baker & Corey, 1988).

Plant responses to phosphorus concentrations are mixed in terms of ethylene production. Ethylene production by tomato/avocado fruit slices, carrot root, pea seedlings, and tomato segments is strongly reduced if the intercellular concentration of phosphate is increased (Chalutz *et al.*, 1980). Phosphorus deficiency did not change ethylene production by tomato leaves (Feng & Baker, 1993), but reduced ethylene production by corn adventitious roots (Drew *et al.*, 1989). Borch *et al.* (1999) found that phosphorus deficient common bean roots produced twice as much ethylene per dry weight than roots with adequate phosphorus. Plants may increase sensitivity, or increase ethylene production, or both, under phosphorus deficiency.

2. Root Hairs

Root Hair Growth and Development

The root tip consists of three distinct developmental zones. The meristematic zone contains the meristematic initials and dividing cells of the root. Proximal to this zone is the elongation zone. Next is the differentiation zone in which elongated cells mature into fully differentiated cells. Root hairs, arising from root epidermal cells known as trichoblasts, emerge just behind the elongation zone (Ridge, 1996) (Fig. 2.3).

Under natural conditions, diameters of root hairs range from 5 μm in *Fraxinus lanceolata* to 17 μm in *Glycine max* (Jungk, 2001). The hair length also varies among species, and can be as long as 4 mm (Meisner and Karnock, 1991). Jungk (2001) found that in 7 species observed, root hair number ranges from only 1 per mm root length in onion to 71 per mm root length in spinach. The functional longevity of root hairs of

barley, determined by neutral red staining (McElgunn and Harrison, 1969), increases from 40 hrs at 26 ºC to 55 hrs as temperature decreases to 15 ºC. In *Arabidopsis*, hairs grow slowly at first and then more quickly after they reach a length of 20-40 μm (Dolan *et al*., 1994). The root hair has a maximal growth rate of approximately 0.12 mm/hour, and phosphorus deficiency in the media can increase the growth rate (Bates and Lynch, 1996). The root hair stops growing after 5.5 hours. Again, low phosphorus can prolong the growth period (Bates and Lynch, 1996).

Root hair development occurs in 5 distinct stages: epidermal cell specification, hair initiation, bulge formation, tip growth, and maturation (Grierson *et al*., 2001; Schiefelbein, 2000). Genetic analysis using *Arabidopsis* mutants has resulted in the discovery of over 40 genes regulating root hair development (Fig. 2.4). At the stage of cell specification, the fate of each immature epidermal cell, to become a trichoblast or an atrichoblast, is decided by a simple either-or switch. Genes involved in cell specification include TTG, R, WER, and CPC (Fig. 2.4). Root hair initiation is the first visible sign of root hair morphogenesis after an immature epidermal cell has adopted the root hair cell fate. In *Arabidopsis* the location of hair outgrowth is predictable. Under natural conditions, it occurs at the basal end of the cell, implying participation of cell polarity during the process (Masucci and Schiefelbein, 1994). Bulge formation is characterized as the swelling at the site of hair outgrowth (Grierson *et al*., 2001), resulting from localized loosening of the epidermal cell wall. After swelling, the endoplasmic reticulum and Golgi bodies begin to produce vesicles full of new cell wall materials and the transition from bulge formation to tip growth occurs (Grierson *et al*., 2001). Tip growth is the stage when the major portion of the root hair is formed by a specialized type of cell expansion employed to generate tubular-shaped cells.

Root Epidermal Cell Patterning

Root epidermal cells make up the outmost layer of most vascular plant roots, and the tip-growing extensions of a root epidermal cell form a root hair. In addition to epidermal cells, root hairs may also originate from the cortex in a few species (Pinkerton, 1936). Two types of root epidermal cells can be recognized: those that develop a root hair

(trichoblasts, or potential hair cells), and those that remain hairless (atrichoblasts, or non-hair cells). In angiosperms, trichoblasts and atrichoblasts are arranged in three types of patterns (Dolan, 1996; Dolan & Costa, 2001; Pemberton *et al.*, 2001): alternate patterning, random patterning, and striped patterning. The alternate patterning is characterized as asymmetric cell divisions of an epidermal cell in the meristematic zone, giving rise to a large cell which remains hairless (atrichoblast) and a smaller cell that develop into a root hair (trichoblast). In random patterning, epidermal cells are morphologically undistinguishable from each other, and root hairs can develop from any epidermal cell. Therefore all epidermal cells are trichoblasts, although not all cells will produce a root hair. Plants with striped patterning develop root hairs in cell files separated by one to three files of non-hair cells.

The distribution of these root epidermal cell patterns in vascular plant taxa has been investigated (Dolan, 1996 & 2001; Pemberton *et al.*, 2001). Alternate patterning is found in some families of monocotyledons (Dolan, 2001) but not in any of 77 eudicotyledonous species from 43 families surveyed by Pemberton *et al.* (2001). Random patterning appears to be the most widespread among dicotyledons as well as many monocotyledonous taxa. Striped patterning was first described for species in the family Brassicaceae by Cormack (1935). Pemberton *et al.* (2001) found this type of patterning occurring more widely in eudicotyledons than had been previously thought. In addition to Brassicaceae, two other families in the Brassicales – Limnanthaceae and Resedaceae – have striped patterning. This type of patterning also occurs in six families of the Caryophyllales: Amaranthaceae, Basellaceae, Caryophyllaceae, Plumbaginaceae, Polygonaceae, and Portulaceae. One species (*Nemophila maculata*) in Boraginaceae also has striped patterning (Pemberton *et al.*, 2001).

Epidermal Cell Patterning in *Arabidopsis*

The *Arabidopsis* primary root is made up of single cell-thick, concentric layers of epidermis, cortex, endodermis, and pericycle tissues surrounding the central stele, with a constant number of eight cells for the cortical and endodermal layers (Dolan *et al.*, 1993). The root epidermal cells, 16 or more in number, are arranged as the striped form of

patterning (Dolan *et al*., 1993, 1994; Pemberton *et al*., 2001). Trichoblasts form from epidermal cells overlying the junction of two cortical cells, while the epidermal cells overlying periclinal cortical cell walls remain hairless. The morphological difference between hair and non-hair epidermal cells is evident early in their development and, at maturity, hair cells are shorter than non-hair cells and have denser cytoplasm (Dolan *et al*., 1993, 1994). Epidermal cell length at a distance of 600 μm from the central cells of the quiescent center is around 200 μm for atrichoblasts; while for trichoblasts, the length ranges from below 150 μm (Dolan *et al*., 1994), to over 170 μm (Ma *et al*., 2001a).

Genetic Control of Root Hair Development

In *Arabidopsis*, at least 40 genes affecting root hair development have been reported (Grierson *et al*., 2001). These genes, according to their mutant phenotypes, are positioned along the genetic pathway corresponding to the hair developmental stages, i.e., epidermal cell specification, site selection and hair initiation, bulge formation, tip growth, and growth duration (Fig. 2.4).

Epidermal cell specification is controlled by TTG (Galway *et al*., 1994), R (Galway *et al*., 1994), WER (Lee and Schiefelbein, 1999), and CPC (Wada *et al*., 1997). TTG either promotes non-hair cell specification, or suppresses hair cell specification. The R gene encodes a bHLH transcription factor that also promotes non-hair cell specification and acts downstream from TTG. The GL2 gene also positively influences non-hair cell specification and is influenced by TTG and R genes. The WER gene works closely with a bHLH protein. In contrast to the effects of above genes, CPC promotes hair cell specification. Schiefelbein (2000) proposed a model for epidermal cell specification: WER interacts with bHLH and the TTG protein and the interaction generates a transcription complex to activate downstream gene (GL2) expression, leading to the non-hair cell specification pathway; the interaction of CPC with bHLH and TTG protein, on the other hand, leads to hair cell specification (Fig. 2.5).

The selection of an initiation site within the hair cell depends on the gene RHD6 (Masucci and Schiefelbein, 1994) and is regulated by AXR2, a gene for auxin action, and

CTR1, a gene in ethylene signal transduction pathway (Masucci and Schiefelbein, 1996). Mutations in the RHD1 (Schiefelbein and Somerville, 1990) and TIP1 (Ryan *et al*., 1998) genes result in hairs with larger swellings at their bases than those of wild type hairs. RHD1 and TIP1 are believed to restrict the degree of cell wall loosing during hair initiation.

Once a swelling has formed, the hair begins tip growth, which is controlled by COW1, RHD2, RHD3, RHD4, TIP1, SHV1, SHV2, SCN1, and KJK (Grierson *et al*., 2001). Mutations of these genes can result in tip growth cessation, branched hairs, or wavy hairs. Fig. 2.4 lists some key genes for hair development from epidermal cell specification to hair growth duration. It is interesting to note that genes in ethylene pathways are important for hair development, as one gene (CTR1) controls hair initiation and three genes (EIN2, ETR1, and ETO1) control the hair elongation along with other genes.

Root Hair Development in Response to Nutrient Deficiency

Root hair production is affected by a number of environmental factors, including the supply of mineral nutrients (Michael, 2001), and is stimulated particularly by the deficiency of nitrogen and P, as well as by iron and manganese (Foehse & Jungk, 1983; Bates & Lynch, 1996; Gahoonia & Nielsen, 1997 & 1998; Ma *et al*., 2001a; Schmidt & Schikora, 2001). Dense and long root hairs occur only when the concentration of nitrate is low; root hair formation is largely suppressed by nitrate if its concentrations are above 100 μM for tomato and rape, and 1000 μM for spinach (Fohse and Jungk, 1983). Comparable results are obtained for phosphorus with these species as well. The same influence of phosphorus is obtained in sugar beet (Hoffman and Jungk, 1995), barley (Gahoonia *et al*., 1999), and *Arabidopsis* (Bates, 1998; Bates and Lynch, 2000a and 2000b; Ma *et al*., 2001a). In *Arabidopsis*, more and longer root hairs are induced by low phosphorus availability. Root hair density linearly decreases as the logarithm of phosphorus concentration increases, and root hair formation is almost completely suppressed at 2000 μM or higher concentrations of phosphorus (Ma *et al*., 2001a). Root hair development not only responds to phosphorus availability in the surrounding

environment, but also to the internal phosphorus concentrations. According to Fohse and Jungk (1983), root hair length in tomato, spinach and rape has a negative linear relation with phosphorus concentration in the shoot tissue.

Contradictory results have been reported on whether root hair formation responds to phosphorus in the immediate environment or to overall phosphorus status of the plant. When spinach roots are grown in a split-root system, root hair length is proportional to the percentage of roots exposed to either low (2 µM) or high (1000 µM) phosphorus (Fohse and Jungk, 1983). The hair length of the roots in the low phosphorus solution increases markedly with the decrease in the percentage of roots in the high phosphorus solution. On the other hand, roots grown in high phosphorus also produce sizable hairs, provided less than 75% of them are exposed to the high phosphorus solution. This result supports the view that it is the phosphorus concentration in the plant tissue that regulates root hair formation, not the phosphorus availability at the root surface (Fohse and Jungk, 1983). However, a transfer study and a well study in *Arabidopsis* indicate that the response of root hairs to low phosphorus is a local event (Bates and Lynch, 1996). When roots growing in low or high phosphorus medium are transferred to a contrasting phosphorus medium or are allowed to grow through a tunnel into a well which contains a contrasting phosphorus medium, length of new root hairs corresponds to the external phosphorus concentration (Bates and Lynch, 1996). Since comparative studies of these species have not been reported, it is not known whether different species respond to phosphorus differently in root hair growth.

Ma *et al*. (2001a) reported that the increase in root hair density under phosphorus starvation was caused by increased trichoblast number, and also by the increased percentage of trichoblasts that produced root hairs (90% for low phosphorus vs. 24% for high phosphorus). Schmidt and Schikora (2001) found that phosphorus starvation induced more root hairs, 6% of which were found in ectopic positions. Although low phosphorus stress increased the number of trichoblasts, the cell length of trichoblasts was not affected (Ma *et al*., 2001a).

Root Hairs and Phosphorus Uptake

The important roles of root hairs include anchoring the plant in the soil, providing sites associated with nodule formation in legumes, and most importantly, increasing the root surface area and thereby the uptake of water and nutrients, especially of the less mobile nutrients such as phosphorus (Esau, 1969; Foehse and Jungk, 1983; Gilroy and Jones, 2000; Itoh and Barber, 1983; Jungk, 2001). Since the concentration of mineral nutrients in the soil solution is often insufficient for an adequate rate of uptake by roots, the flux to absorbing surfaces is small, and roots must have large surface areas and be distributed throughout the soil in order to allow adequate quantities of the soil nutrients to reach the root surface by diffusion.

The transfer of nutrients from soil into plants is the result of interactions between nutrient uptake by roots and rate of transport of nutrients from soil to the root surface. Transport of phosphorus from soil to the root is governed by diffusion, a process usually much slower than the root can take up phosphorus. As a result, a concentration gradient from the soil towards the root surface is created. The driving force for net diffusion is a concentration gradient. Mathematical models predict that root hairs can create a steeper gradient than the bare root cylinder, thus speeding up the rate of diffusion from soil (Jungk, 2001). The calculations are confirmed by biological experiments showing that phosphorus uptake per unit root length increases as the root hair length and density increase (Bates and Lynch, 2000a and 2000b). Without root hairs, as in Arabidopsis hairless mutants, plants accumulate less biomass and phosphorus, and produce fewer seeds when planted with wildtype plants under low phosphorus availability (Bates and Lynch, 2001). Gahoonia *et al*. (1999) measured root hair length and phosphorus uptake of several barley varieties under field conditions. They found a positive correlation between the total length of root hairs (length * number) per unit root length and phosphorus uptake, and the contribution of root hairs to the phosphorus supply can be up to 50%. Computer simulation also indicates that longer and/or denser root hairs have higher phosphorus acquisition efficiency (PAE) than shorter and/or fewer root hairs (Ma *et al*., 2001b).

The beneficial role of root hairs in phosphorus acquisition is also confirmed by genetic analysis of *Arabidopsis*. Narang and Altmann (2001) crossed two morphologically and physiologically divergent *Arabidopsis* accessions, *C24* and *Col-O* to study the inheritance of PAE-related morphological and physiological traits in *Arabidopsis*. *C24* has longer and denser root hairs, and higher I_{max} (the maximum influx), and therefore higher PAE at low phosphorus availability. I_{max}, describing the transporter's kinetic characteristics, is a key parameter of the PAE (Gardiner and Christensen, 1997; Liu *et al*., 1995). The other genotype, *Col-O*, has longer roots and higher PAE when phosphorus availability is high. The resulting hybrid showed superior acquisition of phosphate comparing with either parent at low phosphorus availability. The data suggest that the superiority of the F1 hybrid is due to the accumulation of favorable dominant genes at numerous loci. The hybrid inherited the long root hair length trait from *C24* and the long root length trait of *Col-O*.

Root Hair Development and Ethylene

Immature epidermal cells located over the junction between underlying cortical cells develop into trichoblasts, whereas atrichoblasts are located directly over a single cortical cell (Dolan *et al.*, 1994). This cell position-dependent differentiation is possibly affected by ethylene transported outside from the inner root through the apoplast (Michael, 2001). There is evidence showing that ethylene is required for proper root hair development (Dolan, 1996 & 2001). For example, *Arabidopsis eto* mutants overproduce ethylene and also produce longer root hairs (Cao *et al*., 1999; Pitts *et al*., 1998). The ethylene precursor ACC (1-aminocyclopropane-1-carboxylate) added to the growth medium increases root hair formation and elongation (Bates and Lynch, 1996; Ma *et al*., 2001a). The role of ethylene is confirmed in studies where ethylene biosynthesis is blocked by AVG (aminoethoxyvinylglycine), or ethylene action is blocked by Ag^+ (Masucci and Schiefelbein, 1994; Tanimoto *et al*., 1995). Genes in the ethylene signal transduction pathway are also required for root hair development. In *etr1*, an ethylene receptor mutant in *Arabidopsis*, root hairs are much shorter than those of the wild type, and *ein2* mutants also have short root hairs (Pitts *et al*., 1998). In *ctr1*, a constitutive ethylene response

mutant in *Arabidopsis,* on the other hand, root hairs are longer than wild type (Dolan *et al.*, 1994).

Excessive ethylene can cause the formation of ectopic root hairs in *eto* mutants (Cao *et al.*, 1999) or in wild type plants grown with ACC (Tanimoto *et al.*, 1995). In *ctr1*, root hair overproduction results from ectopic root hair production (Dolan *et al.*, 1994). In addition, roots treated with ethylene form hairs at the extreme basal ends of the trichoblast cells. In the wild type, the hair initiation site is 5-10 μm away from the basal end (Masucci and Schiefelbein, 1994). The *rhd6* mutant displays defects including a reduction in the number of root hairs and variable positions of hair initiation along the trichoblast. This phenotype is rescued if ACC is included in the growth medium (Masucci and Schiefelbein, 1994). It is likely the positioning of the hair initiation is controlled by ethylene.

3. Root Gravitropism

A root is a heterogeneous organ and can be divided into several parts according to the tissue origin and position. For example, the taproot, elongated from the radicle, grows straight downward into the soil. In the apical region of the root tip, active cell division occurs; some distance away root hairs emerge. Lateral roots emerge from more differentiated regions of the taproot, growing at an angle from the taproot. Basal roots, a type of primary root, develop from the basal part of the taproot in many species (Fig. 2.6). Adventitious roots originate from organs other than roots. For many plant species such as tomatoes (*Lycopersicon esculentum*) and beans (*Phaseolus* spp.) and mung beans (*Vigna radiata*), adventitious root emergence occurs from the basal part of the hypocotyl or lower stem. All types of roots can produce root hairs. Different root types may react to environmental stimuli differently.

Root gravitropism is the growth process by which roots grow with a particular orientation with respect to the direction of gravity. Root gravitropism has an important impact on agriculture because it determines the spatial distribution of the root system in

the soil. Since resources in the soil are not evenly distributed, root growth in reacting to gravity determines the plant's ability to acquire the heterogeneously located, limited resources.

Gravitropic Set-Point Angle

Firn and Digby (1997) introduced the concept of the "gravitropic set-point angle" (GSA) to describe root gravitropism. Taproots usually grow vertically down, a GSA of 0°. Other roots, such as basal and lateral roots, take an orientation greater than 0°. Root GSA is controlled developmentally and by environmental factors, and even by gravity itself (Firn and Digby, 1997). GSA is a unifying concept which does not treat the mechanism of an organ (root) growing diagravitropically (GSA = 90°) as being any different from an organ operating orthogravitropically (GSA = 0° or 180°). In the traditional view, however, they are indeed considered as two different responses (Leopold and Wettlaufer, 1988).

Root gravitropism consists of gravity perception, signal transduction, and the growth response (Salisbury, 1993; Chen *et al*., 1999). Gravity perception is hypothesized to occur in the root-cap region in columella cells during gravistimulation, and a signal originating from the cap is transmitted to the elongation zone, across which the response (differential growth) occurs (Chen *et al*., 1999) (Fig. 2.7).

Starch-Statolith Hypothesis

The principal model for the perception stage of gravitropism is the starch-statolith hypothesis. According to the hypothesis, perception occurs via dense organelles interacting with cytoplasmic structures (Sack, 1997). These dense organelles, or statoliths, are believed to be amyloplasts. Each amyloplast contains two or more starch grains and can settle to the physical bottom of the cell (statocyte, a cell with statoliths) in response to gravity and thus provide the basic perception mechanism (Fig. 2.7). Root tips are covered and protected by the root cap. The root cap cells are derived from the root cap meristem that pushes cells forward into the cap region. These cells are arranged in columns and are therefore known as columella cells, which are rich in amyloplasts. This

hypothesis is supported by the facts that firstly, the root cap is necessary for root gravitropism, since Theophil Ciesielski reported in 1871 that decapped roots lost the ability to respond to gravistimulation (Salisbury and Ross, 1992), and roots with larger caps are more graviresponsive than roots with smaller caps (Moore, 1985). Secondly, in contrast to wild type *Arabidopsis* seedlings, roots of starch-deficient mutants are not as oriented along the gravity vector, and they respond more slowly to gravistimulation (Kiss *et al*., 1996). Thirdly, hypergravity could restore the sensitivity of starch-deficient mutants of *Arabidopsis*, and the restoration of gravitropic sensitivity was correlated with the sedimentation of plastids toward the distal cell wall. Even in wild type plants, hypergravity can cause greater sedimentation of plastids and improved gravitropic response capability (Fitzelle and Kiss, 2001). Fourthly, there is a close temporal correlation between the rate of amyloplast settling and the minimum time to elicit a gravitropic response (Iversen and Larsen, 1973). Fifthly, amyloplasts in columella cells of root caps in the microgravity of outer space are distributed randomly (Moore and Evans, 1986). Lastly, removal of the innermost columella cells by laser ablation caused the strongest inhibitory effect on root curvature, while ablation of the peripheral cap cells and tip cells did not alter root curvature. Between the two inner columella stories, the central cells of story 2 contributed the most to root gravitropism; these cells also exhibited the largest amyloplast sedimentation velocities (Blancaflor *et al*., 1998).

Cholodny-Went Theory

Cholodny-Went theory (Salisbury and Ross, 1992) is widely regarded as the leading model for gravitropic curvature. According to this model, the gravitropic curvature is a consequence of differential cell elongation on opposite sides of the organ (root or stem) and mediated by a lateral auxin gradient (Chen *et al*., 1999). Auxin transport has been reported to occur in two distinct polarities, acropetally and basipetally, in two different root tissues. The preferential direction of movement is acropetal (apex seeking, from the base to the cap) in the stele. The polar transport of auxin through epidermal cells involves auxin-influx and -efflux carriers. A basipetal movement is localized in the epidermal and outer cortical cells. Horizontally placed roots have more IAA moving basipetally in the lower cortical cells than the upper cortex. Consequently, more auxin

accumulates at the bottom than at the top of the root. The resulting auxin gradient is transmitted from the root cap into the elongation zone, where it promotes a differential rate of cellular elongation on opposite flanks, which is responsible for the curvature (Pilet, 1996).

It is basipetal transport, not acropetal transport, of auxin that controls root gravitropism (Rashotte *et al.*, 2000). Inhibition of basipetal IAA transport in roots by local application of the auxin transport inhibitor naphthylphthalamic acid (NPA) blocked the gravity response, while inhibition of acropetal IAA transport by application of NPA at the root-shoot junction only partially reduced the gravity response, and only at high NPA concentrations. Excised root tips, which do not receive auxin from the shoot (i.e. acropetal transport), exhibited a normal response to gravity. In addition, the *Arabidopsis eir1* mutant, which has agravitropic roots, exhibited reduced basipetal IAA transport but wild-type levels of acropetal IAA transport.

The initial development of auxin asymmetry is also localized in the root cap and calcium plays an important role in the asymmetric distribution of auxin (see review by Merkys and Darginaviciene, 1997). Manipulation of the calcium gradient in the root cap can change the gravitropic response (Poovaiah and Reddy, 1996). When roots respond to a change in the gravity vector, the amyloplasts shift their position. During this shift there is an interaction with the endoplasmic reticulum, leading to the release of free calcium ions from the endoplasmic store. The local transient increase in cytoplasmic free Ca^{2+} levels would modify the activities of certain enzymes and receptor proteins, including the IAA-receptor systems. Consequentially, the lateral gradient of IAA concentration is formed in the horizontally oriented plant organ (Merkys and Darginaviciene, 1997).

The Role of Ethylene

Ethylene might play a non-primary, yet still significant, role in modulating the gravitropic responses (Madlung *et al.*, 1999). The tomato mutant *dgt* (*diageotropica*), an auxin resistant mutant with reduced responsiveness to ethylene, has an altered gravitropic phenotype (Kelly & Bradford, 1986). Small amounts of ethylene have been reported to

restore a normal gravitropic response in *dgt* (Madlung *et al*., 1999; Zobal, 1974); slightly higher concentrations of ethylene suppress gravitropic responses (Madlung *et al*., 1999). The apical hook is caused by asymmetric cell elongation, and in peas the apical hook is mediated by ethylene (Peck *et al*., 1998), and auxin plays a non-primary role in the hook formation (Du and Kende, 2001). Since ethylene production by gravistimulated plants increased hours after the gravitropic response had already been initiated (Clifford *et al*., 1983; Koffman *et al*., 1985), ethylene may modulate rather than initiate the gravitropic response (Madlung *et al*., 1999).

Basal Root Gravitropism

Because of its origin, the basal root distributes root length near the soil surface where phosphorus has the greatest availability (Pothuluri *et al*., 1986). Since the distribution of phosphorus in soil horizons decreases from the soil surface to the deep soil and phosphorus mobility is dominated by diffusion, which is a slow process (Jungk, 2001), shallow basal roots should be beneficial for phosphorus uptake. Some genotypes of common bean can change their root architecture, especially basal root gravitropism, in order to acquire more phosphorus under low phosphorus availability (Bonser *et al*., 1996; Liao *et al*., 2001). The basal root gravitropic response to low phosphorus is genetically controlled (Lynch & Beebe, 1995; Liao *et al.*, 1999; Yan *et al.*, 1998; Bonser *et al.*, 1996). Computer simulation (Ge *et al.*, 2000; Lynch *et al.*, 1997) and growth experiments show that phosphorus uptake efficiency is positively correlated with the shallowness of the basal root of common beans (Bonser *et al*., 1996; Ge *et al.*, 2000; Liao *et al*., 1998 & 2001).

4. Phosphorus and Drought

Drought is a primary limitation for plant growth in many parts of the world. Water stress has often been directly or indirectly related to plant mineral nutrition, such as phosphorus. Drought has a critical effect on the availability of phosphorus to plants because it affects both the concentration of phosphorus in the soil solution and phosphorus transport to the roots. There are two major forms of ion movement from the

soil to the root surface: mass flow and diffusion. Mass flow is a process where ions in solution move through soil toward the root down the water potential gradient caused by transpiration. By diffusion, on the other hand, ions move down concentration gradients created by the uptake of ions at the root surface. Diffusion accounts for over 99% of phosphate uptake (Barber, 1974). Because the rate of diffusion to the root surface is usually the rate-limiting step in phosphorus uptake, drought might inhibit phosphorus uptake by reducing phosphorus diffusion to roots (Ackerson, 1985). Diffusion coefficient is proportional to soil water content, and therefore drier soil has a lower diffusion coefficient (Barber, 1974). Water stress can reduce the effectiveness of phosphorus fertilizers by between 20 to 60% (Bolland, 1994).

Phosphorus can affect plant tolerance to drought stress. Nelsen and Safir (1982) indicate that mycorrhizal fungus increases onion plant drought tolerance by improving phosphorus nutrition of plants. However, too much phosphorus may decrease drought tolerance. Results from red clover (*Trifolium pratense* L.) indicate that plants with the highest phosphorus treatment maintain high stomatal conductance during drought, while all lower phosphorus treatments have much reduced conductance (Fitter, 1988). A lower conductance should be beneficial for plants to prevent further water loss during drought, and a higher conductance would result in a lower drought tolerance when leaf water potential is decreasing. Saneoka *et al*. (1990) also show that the stomatal resistance, which is the reverse of conductance, increases more at the lower than at the higher levels of phosphorus nutrition.

Al-P, a novel phosphorus fertilizer, can provide much lower but more stable amount of phosphorus to plants throughout the growing seasons than the conventional phosphorus fertilizers do. With Al-P, marigold (*Tagetes patula*) and impatiens (*Impatiens wallerana*) are more drought resistant (Borch *et al*., 1998). In addition to higher stomatal resistance, another possible mechanism by which low phosphorus nutrition improves drought resistance is through the growth of root. Roots of impatiens grown with low phosphorus are evenly distributed through the medium (Borch *et al*., 1998). In bean (*Phaseolus* spp.) and sorghum (*Sorghum bicolor*), drought resistant cultivars generally

have better root growth (longer root length) than drought sensitive cultivars (Al-Karaki *et al*., 1995).

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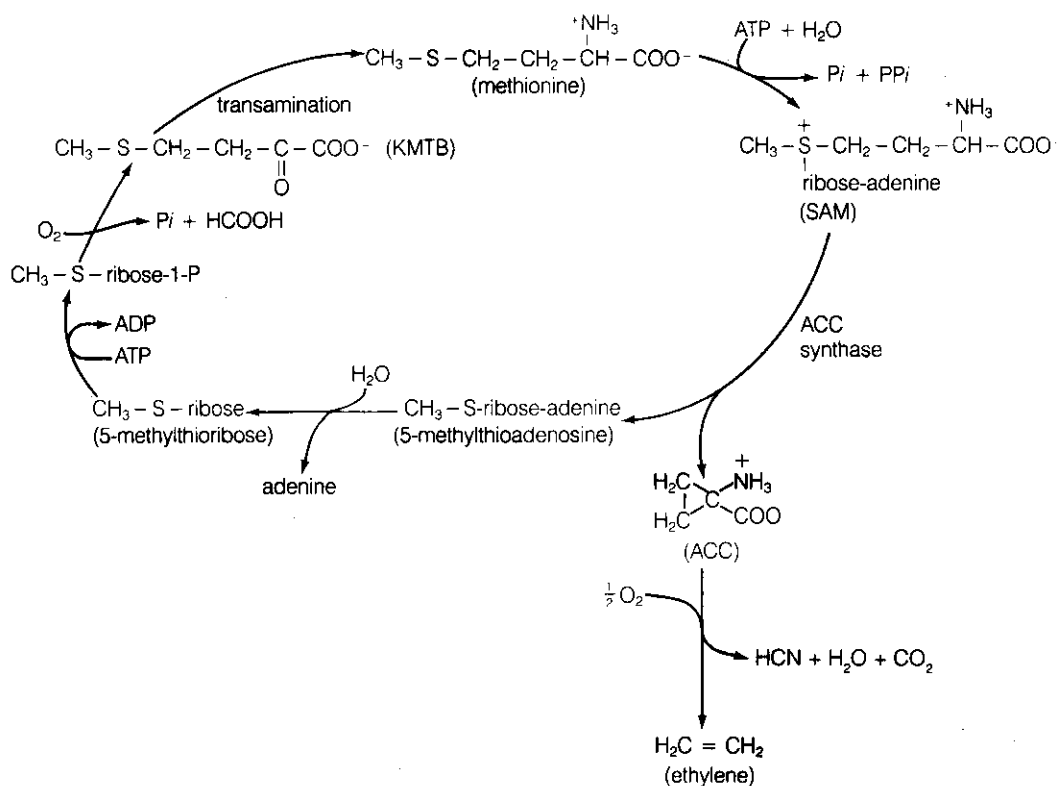
Fig. 2.1. The ethylene biosynthesis pathway starts from the conversion of the amino methionine to SAM, which is catalyzed by ACC synthase to form ACC, the direct precursor of ethylene. Ethylene production from ACC is under control of ACC oxidase. In many cases, ACC synthase is the key enzyme in the pathway.
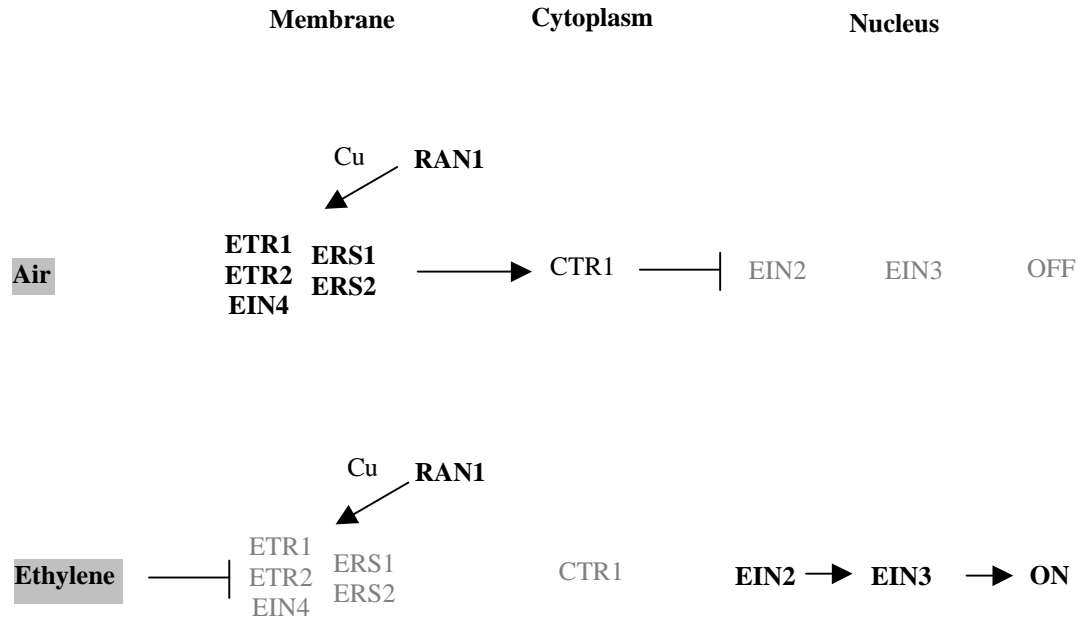
Fig. 2.2. A model of ethylene transduction pathway. The ethylene receptors (ETR1, ETR2, EIN4, ERS1, and ERS2) and CTR1 negatively regulates ethylene signal transduction. In the absence of ethylene (i.e., in the air), receptors are in an active state, and they activate CTR1. The activated CTR1 represses the ethylene responses by inactivating the downstream components EIN2 and EIN3. The binding of ethylene to its receptors inactivates the repressive receptor – CTR1 complex, thus permits the signal to transduce to target tissues. An Arabidopsis mutant, responsive-to-antagonist 1(ran1), shows ethylene-treated phenotypes in response to receptor antagonists. Adapted from Fig. 1 in Chang and Shokey (1999) and Fig. 3 in Urao et al. (2000).
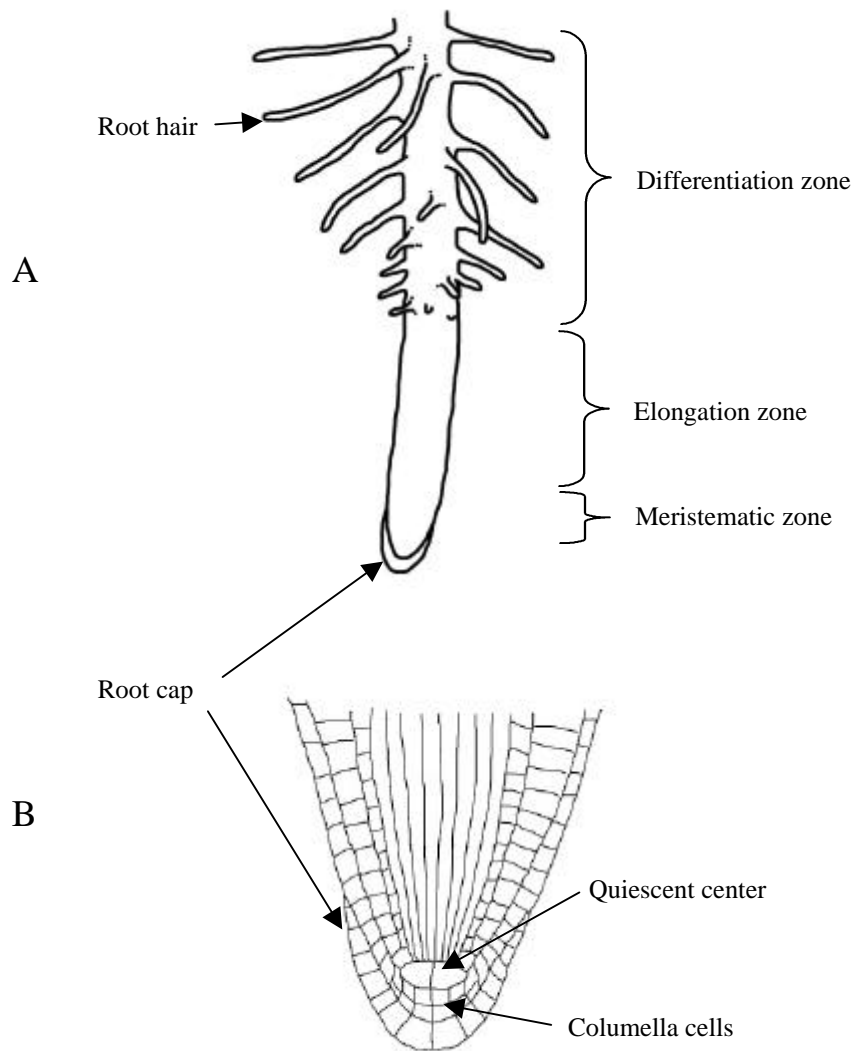
Fig. 2.3. Simplified diagram of *Arabidopsis* root. A. Root tip showing root cap, meristematic zone, elongation zone, and zone of differentiation with root hairs. B. enlarged meristematic zone and root cap in A. Image B is redrawn from figure 1c of Benfey and Schiefelbein (1994).
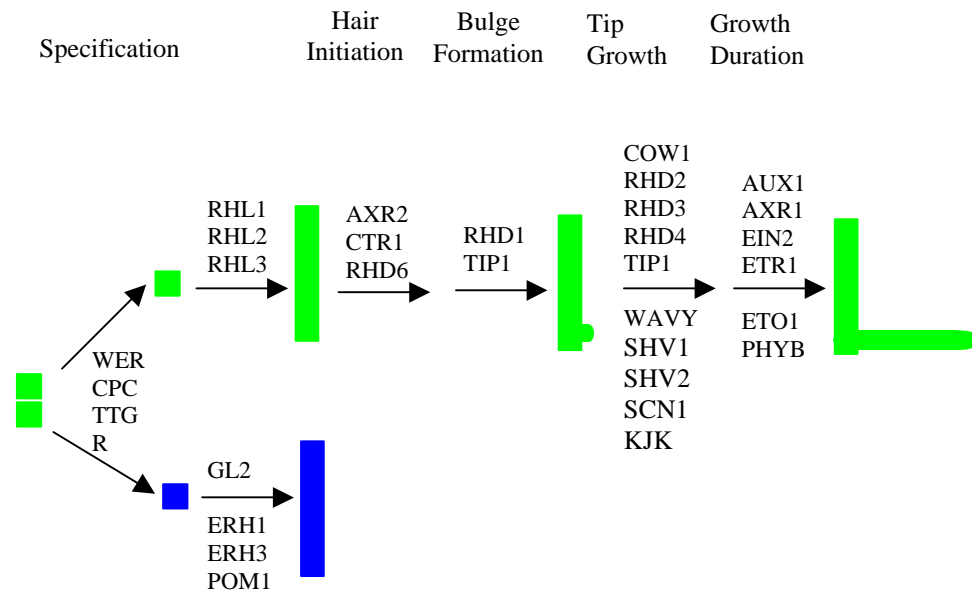
Fig. 2.4. Root hair developmental stages and their corresponding genetic pathway in Arabidopsis. The box represents an epidermal cell, and the basal end of these cells is at the bottom of each image. The default cell fate is a root hair cell (green). After cell specification, some epidermal cells are turned into non-hair cells (blue). Genes controlling each step are labeled. Adapted from Schiefelbein (2000).
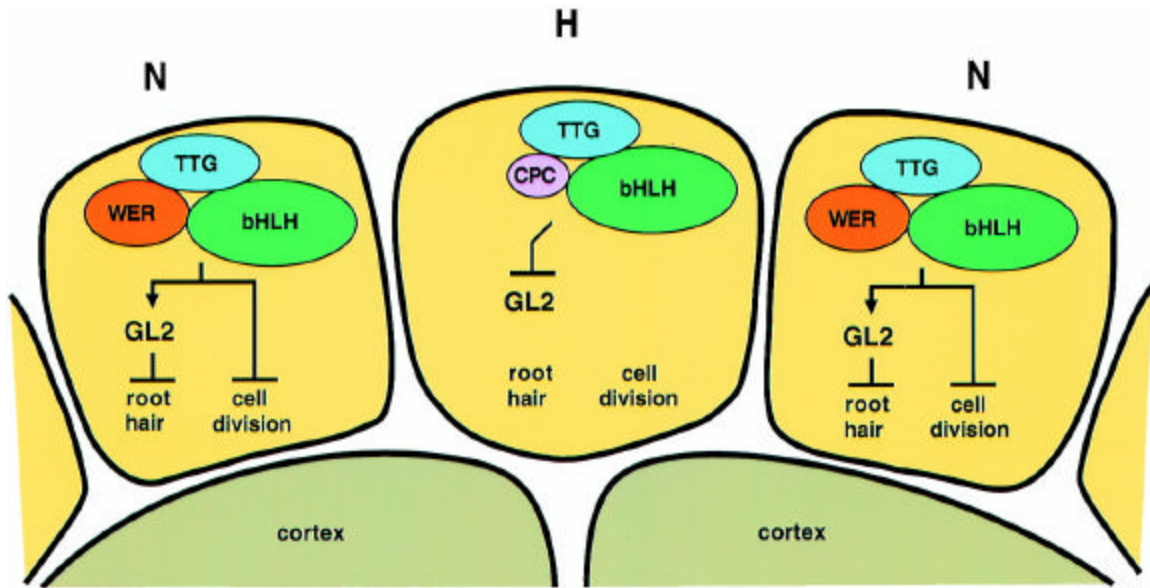
Fig. 2.5. Model for cell specification of Arabidopsis root epidermal cells. The default cell fate is the hair cell (H). With the interaction of proposed cell fate regulators shown in the model, the epidermal cells are destined to be hair cells or non-hair cells (N). Arrows indicate positive control, and blunted lines indicate negative control. Copied from figure 1 in Schiefelbein (2000).
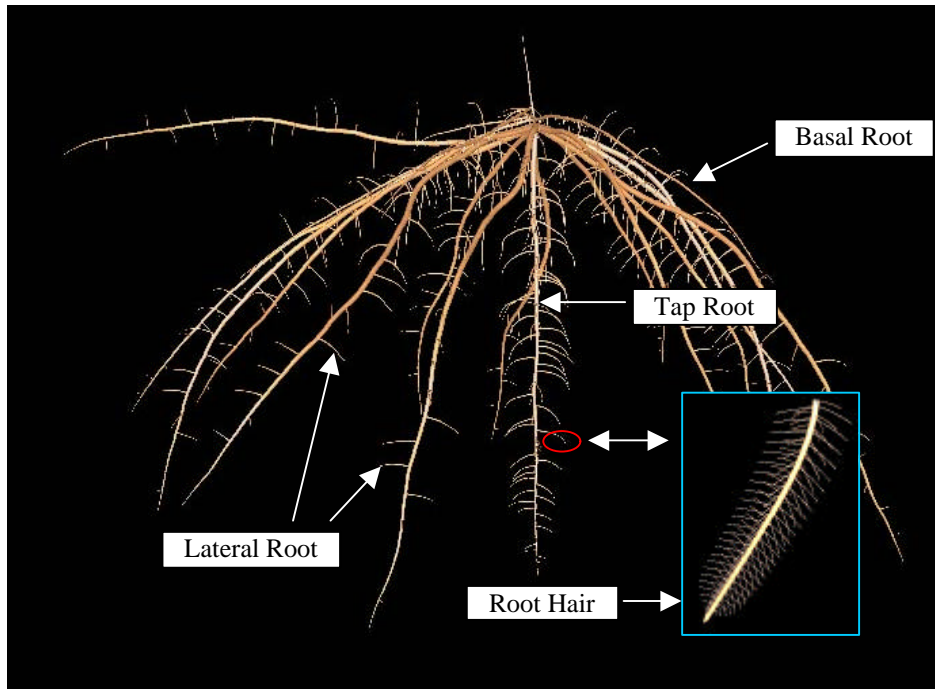
Fig. 2.6. A typical bean root system. Image generated from *SimRoot*, a computer program for root simulation (Lynch et al., 1997).
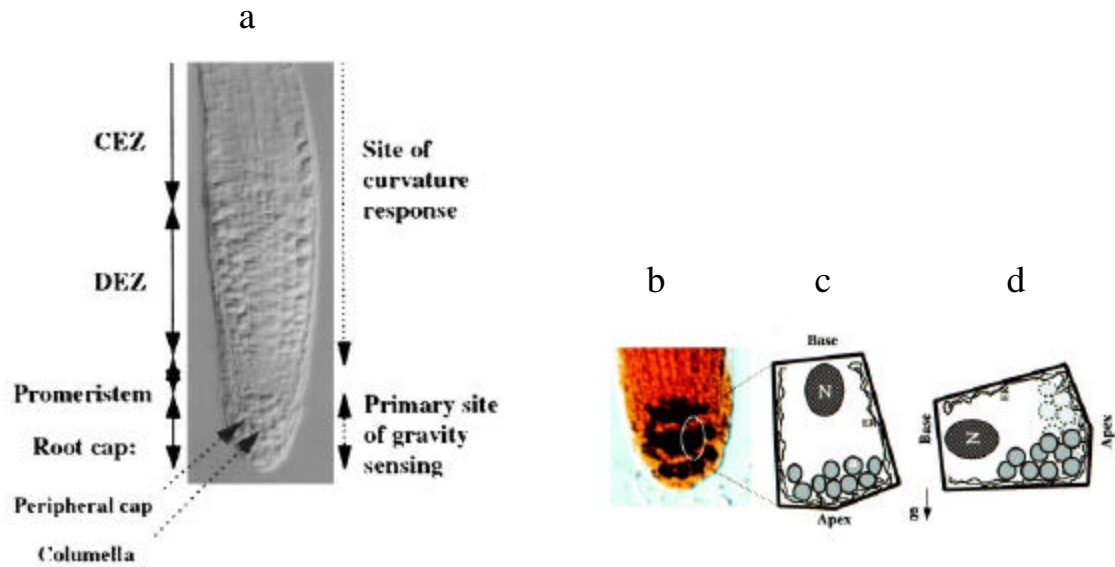
Fig. 2.7. Gravity perception and response by the root. a. The root tip of a 5-d-old Arabidopsis seedling. It contains a root cap made of columella and peripheral cells, a promeristem, a DEZ in which cells undergo division, and a CEZ where cells elongate. Gravity perception occurs in the columella cells (statocytes) of the root cap, whereas the curvature response occurs in the DEZ and CEZ. b. A vertically growing root-cap where columella cells are filled with amyloplasts (dark brown). c. The columella cell contains a nucleus (N) at the top, a peripheral ER, and amyloplasts at the bottom. d, Upon gravistimulation, amyloplasts sediment from their original position to the new physical bottom of the cell. The gravity vector is shown by arrow (g). DEZ: cell division and expansion zone. CEZ: cell elongation zone. Copied from Figures 2 & 3 in Chen et al. (1999).

Chapter 3

Ethylene does not directly mediate the regulation of root hair growth by phosphorus availability

Abstract

Root hair growth of *Arabidopsis thaliana* ecotype 'Columbia' was enhanced by low phosphorus availability. Both length and density of root hairs increased as phosphorus availability decreased from 1000 μM to 1 μM. Low phosphorus induced root hair growth could be mimicked by adding the ethylene precursor, 1-aminocyclopropane-1-carboxylate (ACC), to high phosphorus media, and inhibited by adding ethylene inhibitors aminoethoxyvinyl glycine (AVG), MCP (active ingredient: 0.43% 1-methylcyclopropene), or silver thiosulfate (STS) to low phosphorus media. Ethylene insensitive mutants, including *ein4*, *ein2-1*, *ein3-1*, *ein5-1*, *ein6*, *ein7*, and the mutant *eir1-1*, showed a reduced but still significant response to low phosphorus. Growth in low phosphorus resulted in more, smaller cortical cells compared with high phosphorus grown plants. The number of trichoblasts increased under low phosphorus as a result of the increase in the number of cortical cells. ACC did not change the number of cortical cells or trichoblasts in roots grown with high phosphorus; neither did AVG treatments to plants grown with low phosphorus. Epidermal cells in the atrichoblast position produced root hairs when plants were grown with ACC, but not when they were grown with low phosphorus. The length of trichoblasts with low phosphorus treatment was only 50 – 60% of that for high phosphorus. ACC reduced trichoblast length under high phosphorus and AVG increased the trichoblast length under low phosphorus. Ethylene-insensitive mutants grown with low phosphorus produced more and smaller cortical cells, similar to wild-type plants. The pattern of trichoblast length change in response to phosphorus availability for two observed mutants (*ein2-1* and *ein4*) was similar to the wild type. These results suggest that low phosphorus and ethylene influence root hair growth through different mechanisms.

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 affected by a number of environmental factors, including nutrient supply (Michael, 2001) and is stimulated by the deficiency of nutrients, such as iron, zinc, manganese, and phosphorus (Foehse and Jungk, 1983; Bates and Lynch, 1996; Gahoonia and Nielsen, 1997, 1998; Ma et al., 2001a; Schmidt and Schikora, 2001). In *Arabidopsis*, more and longer root hairs are induced by low phosphorus availability (Bates and Lynch, 1996; Ma et al., 2001a). This change has adaptive value, since phosphorus uptake per unit root length increases as the root hair length and density increase (Bates, 1998; Bates and Lynch, 2000a, 2000b, 2001; Ma et al., 2001a). Gahoonia et al. (2001) found that barley (*Hordeum vulgare* L.) cultivars with longer root hairs absorbed more phosphorus and produced more shoot biomass than shorter root hair cultivars under low phosphorus conditions. Hairless mutants of *Arabidopsis* accumulate less biomass and phosphorus, and produce fewer seeds when planted with wildtype plants under low phosphorus availability (Bates and Lynch, 2001), indicating that plants with root hair overproduction under low phosphorus have more chances to survive and produce progeny. Geometric modelling confirms that longer and denser root hairs induced by low phosphorus conditions are beneficial adaptations for phosphorus acquisition (Ma et al., 2001b).

Root hairs are formed from specific epidermal cells called trichoblasts. Epidermal cell fate is determined by cell position (Dolan et al., 1994). Immature epidermal cells, located over the intercellular space between underlying cortical cells, develop into trichoblasts, whereas atrichoblasts are located directly over a single cortical cell. This cell position-dependent differentiation may be regulated by ethylene transported from the inner root through the apoplast. Ethylene is positively involved in the determination of root epidermal cell fate and is required for regulation of hair development (Dolan, 1996, 2001). Tanimoto et al. (1995) reported that atrichoblasts formed ectopic root hairs when the ethylene precursor ACC (1-aminocyclopropane-1-carboxylate) was added, while the

ethylene synthesis inhibitor AVG (aminoethoxyvinyl glycine), and the ethylene action inhibitor Ag$^+$, reduced the proportion of trichoblasts forming hairs. In *ctr1*, a constitutive ethylene response mutant in *Arabidopsis,* root hair overproduction results from ectopic root hair production (Dolan et al., 1994). The *rhd6* mutant displays three defects including a reduction in the number of root hairs. This phenotype is rescued if ACC is included in the growth medium (Masucci and Schiefelbein, 1994). These results support the hypothesis that ethylene positively regulates root hair development.

In *Arabidopsis*, files of trichoblasts are separated by one to three files of atrichoblasts (Dolan and Costa, 2001; Pemberton et al., 2001). Most reports indicate that there are 8 cortical cells in cross section and correspondingly 8 trichoblast files (Dolan and Costa, 2001). (Ma et al., 2001a) found that *Arabidopsis* grown under phosphorus deficiency had 12 cortical cells, and therefore 12 trichoblast files. However, not all trichoblasts will actually form hairs, and ectopic hairs may occur on atrichoblasts under unusual conditions (Dolan et al., 1994; Tanimoto et al., 1995; Ma et al., 2001a; Schmidt and Schikora, 2001). Ma et al. (2001a) reported that the increase in root hair density under phosphorus starvation resulted from an increased number of trichoblast files and from an increase in the percentage of trichoblasts which formed root hairs (90% for low phosphorus vs. 24% for high phosphorus). Although low phosphorus stress increased the number of trichoblasts, the cell length of trichoblasts was not affected (Ma et al., 2001a). Schmidt and Schikora (2001) also found that phosphorus starvation induced more root hairs, and reported that 6% of these were from ectopic positions, i.e. on atrichoblasts.

In addition to root hair density, root hair length is also very plastic in response to phosphorus availability, with longer hairs under low phosphorus (Bates and Lynch, 1996; Yan and Lynch, 1998). In *Arabidopsis*, root hair length decreased from over 1.0 mm at 1 µM phosphorus to 0.3 mm at 1000 µM phosphorus, and hair elongation is completely suppressed at 3000 µM phosphorus (Bates and Lynch, 1996). Ethylene is important in root hair elongation. Plants grown in the presence of ethylene synthesis inhibitor aminoethoxyvinyl glycine (AVG) have shorter root hairs than wild type (Tanimoto et al., 1995). *Arabidopsis eto* mutants overproduce ethylene and exhibit longer hairs (Kieber et

al., 1993). For example, *eto1-1* hairs are 50% longer than wild type. Genes that are in the ethylene signal transduction pathway, such as EIN2 and ETR1, are required for root hair elongation (Schiefelbein, 2000).

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 common bean roots produced twice as much ethylene per unit dry weight as roots with adequate phosphorus. These results suggest that there might exist a close relationship between root hair production in response to phosphorus availability and ethylene. However, Schmidt and Schikora (2001) found that the formation of dense root hairs in *Arabidopsis* wild type 'Columbia' grown in phosphorus deficient conditions was not inhibited by ethylene antagonists AVG, 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 response genes (Schmidt, 2001; Schmidt and Schikora, 2001).

If ethylene does mediate the low phosphorus response, then we would expect that genotypes with impaired ethylene response should have a reduced ability to respond to low phosphorus. Several mutations in the ethylene signal transduction pathway have been isolated which vary in the extent the inhibition of ethylene induction of the triple response (Roman et al., 1995). In the *ein* series, *ein2-1* and *ein4* are the strongest mutants; *ein6* is an intermediate mutant; and *ein3-1*, *ein3-2*, *ein5-1*, *ein5-2*, and *ein7* are the weakest, based on the extent of the triple response at the presence of ethylene. *eir1-1* is an insensitive mutant whose root has a reduced sensitivity to ethylene although the primary lesion is on an auxin efflux carrier (Roman et al., 1995; Luschnig et al., 1998; Luschnig and Fink, 1999; Sieberer et al., 2000). The sensivity of *eir1-1* to ethylene is comparable to the weakest mutants in the *ein* series (Roman et al., 1995). In the signal transduction pathway, EIN4 is in a gene family encoding ethylene receptor homodimers,

and EIN2 is responsible for signal propagation from CTR1 to EIN3 and to other genes in the nucleus (Chang and Shockey, 1999).

Since root hair formation from trichoblasts involves several developmental stages (Parker et al., 2000; Schiefelbein, 2000) and low phosphorus conditions increase root hair density partly through increasing the number of trichoblasts (Ma et al., 2001a), investigation of root anatomy is needed to clarify whether ethylene is involved in root hair production under phosphorus deficiency. In this paper, we tested the hypothesis that ethylene mediates the increase in root hair density and length which occurs in response to low phosphorus availability by manipulating ethylene and by using ethylene insensitive mutants. We did so by examining the effects of phosphorus and ethylene manipulation on specific aspects of root anatomy known to contribute to increased root hair density.

Materials and Methods

Original seeds of *Arabidopsis thaliana* L. (Heynh) 'Columbia' ecotype and isogenic ethylene insensitive mutants *ein*2-1, *ein*3-1, *ein*4, *ein*5-1, *ein*7, and *eir*1-1, as well as *ein*6, which is in the Landsberg erecta background, were obtained from *Arabidopsis* Biological Resource Center, the Ohio State University. Seeds were propagated in our greenhouse and 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. (2001a), 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 6 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 are referred to as low phosphorus or high phosphorus, respectively. To manipulate ethylene production and sensitivity, fresh media containing the ethylene precursor ACC (0.05, 0.5, 1.0, 2.0, 4.0, 6.0, or 10.0 µM), the ethylene production inhibitor AVG (1.0, 2.0, or 5.0 µM), or the ethylene action inhibitor STS (silver thiosulfate) (1.0, 5.0, or 10.0 µM), were

added to the existing media after seedlings had grown 12 days, as described by Bates and Lynch (1996). For treatment with the gaseous ethylene action inhibitor MCP (active ingredient: 0.43% 1-methylcyclopropene. EthylBloc®, Floralife®, Inc. 751 Thunderbolt Drive, Walterboro, SC 29488), open Petri dishes with 12-day old plants were placed in an 8-liter, airtight container, which also contained a 5-ml vial containing 1.0, 2.0, 3.0 or 4.0 mg MCP per liter of chamber volume. The container was surface-disinfected with 70% alcohol before use. The chamber was sealed, and 0.02 ml distilled water per mg MCP was added to the vial through a septum to release MCP gas. The treatment lasted 2 d until root hair measurement. At 14 days of growth (2 days after adding ethylene inhibitors or ACC, or after the initiation of MCP treatment), 6 plants of each treatment were evaluated under a stereomicroscope for root hair length and density. The experiment was repeated 4 times.

For the root anatomical experiments, roots of at least 5 plants were sampled from the same age of plants 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. (2001a).

To measure trichoblast cell length, we used the wild type 'Columbia' and two mutants *ein2-1* and *ein4*, and their genetic transformants with *b*-glucuronidase (GUS) reporter gene under the control of the promoter of Exp7, a α-type cell wall expansin specifically expressed in hair-bearing epidermal cells. For 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 GUS-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, 0.5 mM k-ferrocyanide, 0.1% triton X-100, pH adjusted to 7.0 with 1 M NaOH), washed 3 times with the same buffer, then stained with 0.05% aqueous solution of Neutral red for 5 min at room temperature. For non-tranformed plants, living roots were stained with 0.05% Toluidine Blue O in 0.1 M Na_2HPO_4/NaH_2PO_4 buffer (pH6.8) for 5 min at room temperature. The image of the stained root was 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 length of six cells of each of 6 plants were measured.

Root growth rate under low or high phosphorus was recorded daily by marking, with colored markers, the root tip position on the bottom of the Petri dish corresponding to one day's growth of the root, starting from day 9 of culture. Daily root growth was estimated by scanning the Petri dish into a digital image and analyzed with WinRHIZO program (Regent Instruments Inc., 4040 rue Blain, Quebec, Qc. G2B 5C3 Canada, Version 3.10b). Each treatment was repeated 6 times.

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

We tested phosphorus dosage response of the wild type 'Columbia' and two ethylene insensitive mutants, *eir1-1* and *ein7*. The root hair density of all genotypes was statistically the same with 1000 µM phosphorus, but 'Columbia' increased its hair density more sharply than the mutants as phosphorus concentration decreased logarithmically in the range of 1000 to 1 µM in the media (Fig. 3.1). Similarly, the mutants were less sensitive than the wild type 'Columbia' to the effects of reduced phosphorus availability on root hair length (Fig. 3.1, 3.2 & 3.3). As a result, both mutants had shorter hairs than "Columbia" when plants were grown in 1 µM phosphorus, even though at phosphorus 1000 µM, *ein7* had the longest hairs among the three genotypes. The slopes of the regression lines for root hair density and length were much smaller for the mutants than for 'Columbia' (Fig. 3.1). The two mutants had similar responses to phosphorus for density but not for length.

Other ethylene mutants, including *ein2-1*, *ein3-1*, *ein4*, *ein5-1*, and *ein6*, were evaluated for their root hair density and length under low (1 µM) or high phosphorus

(1000 µM) (Fig. 3.3 & 3.4). For all genotypes, root hair density and length in low phosphorus media were significantly greater than in high phosphorus media. However, the difference or ratio of means between low and high phosphorus treatments was the greatest for the wild type 'Columbia', and all the mutants were less responsive to phosphorus availability in the media (Table 3.1, Fig. 3.2 & 3). For example, the root hair density and length of 'Columbia' increased 3.44- and 3.86-fold, respectively, as phosphorus concentration decreased from 1000 µM to 1 µM, while for the mutants, the increase was below 3.0-fold except the density of *ein2-1*, which was 3.32-fold. The lowest ratio was 1.65 (*ein6*, density), less than half of the ratio for 'Columbia' (Table 3.1).

To further evaluate the effect of ethylene on root hair growth in response to phosphorus availability, we manipulated ethylene production and action using the ethylene production inhibitor AVG, the ethylene action inhibitors STS or MCP, or the ethylene precursor ACC. The inhibitors reduced root hair density and length of 'Columbia' plants grown in low phosphorus medium (Fig. 3.2-d & 3.5), while ACC increased root hair density and length of plants grown in high phosphorus (Figs. 2-b & 6). The low phosphorus effect could be completely mimicked by 1 µM ACC for density but not for length (Table 3.2). Increasing ACC up to 10 µM still failed to mimic the low phosphorus effect for length (Table 3.1, Fig. 3.6). Root hair density and length were lower in low phosphorus plus ethylene inhibitors than in high phosphorus, except low phosphorus plus STS (5 µM), which gave statistically the same hair density as high phosphorus treatment (Table 3.2).

The mutant *eir1-1* also responded to ACC similarly as 'Columbia' for length, but to a much less extent than 'Columbia' for density. Fig. 3.6 shows the hair density and length of 'Columbia' and *eir1-1* in media with 0, 2, 4, 6, or 10 µM ACC. This result indicated that this mutant retained some responsiveness to ethylene.

Effects of low phosphorus and ethylene on root anatomy

When 'Columbia' plants were grown with high phosphorus, we always observed the typical eight files of cortical cells, and root hairs formed only on cells in the trichoblast position (Fig. 3.7a). Low phosphorus stress resulted in production of more and smaller cortical cells (Table 3.3; Fig. 3.7c). As a result, more epidermal cells were located over the junction of two cortical cells, i.e. in the trichoblast position. Sometimes this resulted in no atrichoblast cell files between trichoblast files, so that two adjacent epidermal cells could be hair cells, as shown in Fig. 3.7c.

ACC added to the high phosphorus media failed to induce a change in cortical cell organization like that found with the low phosphorus treatment (Table 3.3), but promoted more hair formation from both trichoblasts and atrichoblasts (Fig. 3.7b). AVG added to the low phosphorus media did not prevent the change the cortical cell organization (Table 3.3). Cortical cell number was not statistically different in low phosphorus plants with and without AVG (Fig. 3.7c & d).

We also examined the anatomy of roots of ethylene insensitive mutants *ein2-1*, *ein4*, and *eir1-1* grown with low or high phosphorus treatment. The mutants *ein2-1* and *ein4* grown in low phosphorus had an anatomy similar to 'Columbia', since they produced more cortical cells than plants grown in high phosphorus (Table 3.3). We occasionally observed ectopic hairs on the roots of *ein4* grown in low phosphorus media (Fig. 3.8d). However, the difference in cortical cell number between low and high phosphorus for *eir1-1* was less than that of 'Columbia' (Table 3.3; Fig. 3.8e & f).

To exclude the possibility that the difference in cortical cell size and number was caused by different root developmental stages in low and high phosphorus, we sectioned roots of both phosphorus treatments from the tip to the first lateral root, and found that the cortical cell number was constant along the observed differentiation zone.

The root of *Arabidopsis* wildtype 'Columbia' and ethylene insensitive mutants *ein2-1* and *ein4* grown with high phosphorus was made up of single cell-thick, concentric layers

of epidermis, cortex, endodermis, and pericycle tissues surrounding the central stele (Fig. 3.7a &3.8a, c and e). Low phosphorus treatments increased the number of cortical cell layers (Fig. 3.7c & 3.8b,d and f).

Shorter trichoblasts under low phosphorus

Arabidopsis genotypes 'Columbia', *ein2*, and *ein4*, transformed with the GUS gene attached to an expansin gene promoter, were used for trichoblast identification and cell length measurements. The specific expression of the GUS gene in trichoblast cells allowed easy measurement of these traits. For all three genotypes, there were more trichoblast files under low phosphorus than under high phosphorus (Fig. 3.9a & b, and Fig. 3.10). From observations under microscope, we estimated 8 trichoblast files for high phosphorus and 16 or more files for low phosphorus for all genotypes used. Trichoblast cells were much shorter under low phosphorus than under high phosphorus for all genotypes (Fig. 3.9 & 3.10; Table 3.4). However, *ein2* seemed to be more responsive to phosphorus availability in terms of trichoblast length, since the length under high phosphorus was 2.8 fold higher than under low phosphorus (Table 3.4). The genotypes 'Columbia' and *ein4* had similar trichoblast lengths with high phosphorus or low phosphorus treatments (Table 3.4). 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 3.4; Fig. 3.9). AVG and ACC were not specific to trichoblasts; they also had effects on overall growth of the root, with root growth promoted by AVG and inhibited by ACC.

To examine whether the GUS insert affected trichoblast length, we also examined the same genotypes without GUS transformation. The non-transformed 'Columbia' and *ein4* had more extreme responses to phosphorus availability than their GUS-transformed counterparts, but the overall pattern was similar, i.e., low phosphorus reduced trichoblast elongation and *ein2* was again the most responsive genotype for trichoblast length (Table 3.5, Fig. 3.11).

Roots grown with low phosphorus were of larger diameter than those grown with high P; ACC also increased root diameter but AVG decreased it. Low phosphorus also increased root diameter of *ein2* and *ein4*, but the absolute and proportional increase in root diameter of *ein2* was larger than the other two genotypes, and this genotype had the thinnest roots under both phosphorus treatments (Table 3.6).

Percentage of trichoblasts forming hairs (PTH)

Phosphorus availability did not change PTH for the wild type 'Columbia', since low and high phosphorus treatments did not differ in their PTH, which were 90.2 and 89.9%, respectively (Table 3.7). ACC increased PTH to almost 100%, while AVG reduced PTH by half. For ethylene mutants *ein2-1* and *ein4*, low phosphorus treatment had lower PTH than high phosphorus treatment (Table 3.7).

Daily root growth rate

We measured the root growth rate in the last 5 days before harvest, when the region of the root used in our experiments was developing. Since phosphorus availability in the medium is finite and not replenished in our experiments, we wanted to check whether phosphorus depletion was affecting growth during the course of these experiments, especially under low phosphorus. Phosphorus availability had a profound effect on root elongation, the extent of which varied with genotype (Fig. 3.12). The difference in growth rate between low and high phosphorus was the largest for *ein2* (roots grew about 5 times faster with high phosphorus than with low phosphorus); while the growth rate for 'Columbia' and *ein4* was similar at each phosphorus level (about 0.8 cm/day for high phosphorus, and about 0.4 cm/day for low phosphorus). The percentage of low phosphorus/high phosphorus for root growth rate of 'Columbia' for each day is 45.1, 42.5, 40.3, 40.6, and 40.6%, respectively, and the average is 41.8%. The root growth rate for all three genotypes with either low or high phosphorus was constant (Fig. 3.12), implying constant cell elongation and differentiation. Therefore root hair density differences were not due to reduction in overall root growth rate.

Discussion

Ethylene and phosphorus deficiency might operate through different mechanisms

Our results show that ethylene and low phosphorus have similar impacts on root hair development in *Arabidopsis*. First, ethylene (ACC) and low phosphorus significantly increase hair density and length (Fig. 3.1, 3.2, 3.3 & 3.6), and significantly reduce the trichoblast length (Tables 4 & 5; Fig. 3.9 & 3.11); second, inhibiting ethylene synthesis and action prevents the low phosphorus response (Tables 4 & 5; Fig. 3.2, 3.5, and 3.9); and third, inhibiting ethylene action via ethylene insensitive mutants reduces the low phosphorus response (Tables 4 & 5; Fig. 3.4, 3.6, and 3.10). Bates and Lynch (1996) and Ma et al. (2001a) also found that ACC treatments increased root hair length and density in *Arabidopsis*. It is interesting that the hair density from low phosphorus or ACC treatment in this experiment (Table 3.2) is at about the same level as *eto3* (an ethylene overproducing mutant) reported by Schmidt and Schikora (2001). These results are consistent with the hypothesis that ethylene mediates the responses of root hair growth to low phosphorus.

However, 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 triple response (Roman et al., 1995). For example, *ein7* was the least responsive mutant to reduced phosphorus levels (Table 3.1) but was one of the weaker mutants in terms of the lesion in the ability of ethylene to initiate the triple response; and *ein2-1* and *ein4* were strong for all aspects of triple responses (Roman et al., 1995), but not for the phosphorus effect (Table 3.1). Not only were differences in phosphorus responsiveness among mutants not correlated with lesions in the triple response, but the phosphorus responses of the two root hair traits, root hair density and root hair length, were not consistent (Fig. 3.3; Table 3.1). 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.

Schmidt and Schikora (2001) used two *Arabidopsis* ethylene insensitive mutants, *etr1-3* and *ein2-1* in their root hair density study. ETR1 codes for an ethylene receptor protein (Chang and Shockey, 1999). They found that these two mutants exhibited a slightly reduced number of hairs when cultivated with phosphorus-free medium, in comparison with 'Columbia'. Based on the fact that these ethylene response mutations do not affect the root hair density induced by phosphorus deficiency, they proposed that a phosphorus-deficiency stress signal may interact directly with components of an ethylene-independent pathway, which is not dependent on genes involved in ethylene signaling (Schmidt and Schikora, 2001). In our experiments *ein2-1* had only a weak effect on low phosphorus enhancement of root hair density too, and we did not use *etr*. However, the other mutants had a stronger effect (Fig. 3.4; Table 3.1).

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 trichoblasts elongated into hairs (PTH). As we did not find ectopic hairs induced by low phosphorus treatment, the increase of root hair density for low phosphorus should be from these components changed by low phosphorus.

Normally, trichoblast file number is determined by the underlying cortical cell organization (Dolan et al., 1993; Dolan et al., 1994). Our results show that low phosphorus and ethylene have distinct effects on cortical cell organization. Low phosphorus has impacts on tissue organization that ethylene cannot mimic, i.e., smaller and more cortical cells. Ethylene (ACC) induces swelling of cortical cells but has no effect on cortical cell number, and AVG and ethylene insensitive mutants have no effect on cortical cell number. Under normal conditions, trichoblast cell files form at the juncture of two underlying cortical cell files (Benfey and Schiefelbein, 1994; Dolan et al., 1994; Dolan, 1996). Low phosphorus increases trichoblast cell file number partially by increasing the number of cortical cells (Fig. 3.7 & 3.8; Table 3.3) (Ma et al., 2001a). Since low phosphorus increases cortical cell numbers but ethylene does not, low

phosphorus and ethylene increase hair density through at least one distinct mechanism. Specifically, ethylene increases hair density through promoting hair initiation from trichoblasts and, occasionally, inducing root hair formation from atrichoblasts (Fig. 3.7b & 3.9).

Root hair development can be divided into cell specification, hair initiation, bulge formation, tip growth, and growth duration (Parker et al., 2000; Schiefelbein, 2000; Foreman and Dolan, 2001). In *Arabidopsis*, at least 40 genes that affect root hair development, including genes in the ethylene biosynthesis pathway and signal transduction pathway (ETO1, ETR1, CTR1 and EIN2) affecting initiation and tip growth, have been reported (Grierson et al., 2001). Some of the genes affecting root hair development that are not in ethylene pathways are known to be related to, or affected by ethylene, such as AUR2 and RHD6. It is possible that some genes important for root hair differentiation are affected by phosphorus but not by ethylene. For example, GLABRA2 (GL2) is a positive regulator of trichoblast/non-hair fate (Masucci and Schiefelbein, 1996), but the pattern of GL2 expression is not altered by *ctr1* mutant or ethylene, indicating that ethylene acts either after or independently of GL2. It is important to note that the genes listed by Grierson et al. (2001), as responsible for hair development including hair cell specification, hair initiation, bulge formation, tip growth, and growth duration, are not known to have functions in cortical cell division. Our results and those of Ma et al. (2001a) imply that there are genes controlling cortical cell division, and that phosphorus deficiency, but not ethylene, triggers their expression.

Trichoblast cell length is an important factor contributing to root hair density, since shorter trichoblast cells would increase the number of potential hairs per unit length of root. Our results show that ethylene might be a factor in reducing trichoblast length under low phosphorus. Except for a recent report by Ma et al. (2001a), trichoblast length and cortical cell number are two aspects that are ignored in root hair studies, especially in gene identification and isolation. We do not know yet what genes control trichoblast elongation and cortical cell division.

Table 3.7 summarizes the effects of low phosphorus and ethylene on root hair density. The observed and calculated percentage of trichoblasts forming hairs (PTH) have a similar pattern with phosphorus availability and ethylene treatments, though generally observed PTH is a little higher than calculated PTH. Low phosphorus affects hair density through trichoblast file number and trichoblast length, while the observed and calculated PTH remains about the same (Table 3.7). Ethylene, on the other hand, acts through trichoblast length and percentage of trichoblasts forming hairs, but trichoblast file number is the same. For example, 'Columbia' plants grown with high phosphorus plus ACC have shorter trichoblasts and much higher PTH in high phosphorus, and plants grown with low phosphorus plus AVG have longer trichoblasts and much lower PTH than low phosphorus alone. Mutants *ein2-1* and *ein4* have lower PTH under low phosphorus than high phosphorus. These results show that ethylene has a positive effect on PTH, and that ethylene and phosphorus have distinct mechanisms.

Root hair length

Root hair elongation (length) is promoted by both low phosphorus and ethylene (Fig. 3.3, 3.4, & 3.5). Although the low phosphorus effect is significantly reduced in mutants *ein2-1*, *ein3-1*, *ein4*, *ein5*, *ein7*, and *eir1-1*, the reduction is not as strong as that produced by ethylene biosynthesis or action inhibitors (Fig. 3.4 & 3.5). This result indicates that these mutants retain residual responsiveness to ethylene, an idea borne out by the fact that root hair density still increases in response to ACC (Fig. 3.6). From this result, it seems that ethylene mediates low phosphorus effects on hair elongation, or low phosphorus affects hair elongation through genes in ethylene pathways. But hair elongation is controlled by many genes, only some of which are in ethylene pathways (Parker et al., 2000; Schiefelbein, 2000; Grierson et al., 2001). Therefore, it is possible that ethylene mutants affect hair elongation through genes in ethylene pathways and low phosphorus through other genes.

Extent of root hair density increase under low phosphorus is affected by cultural conditions

Although the hair density of 'Columbia' plants grown with high or low phosphorus from our results and others' (Bates and Lynch, 1996; Ma et al., 2001a; Schmidt and Schikora, 2001) show similar pattern, i.e., hair density increases as phosphorus availability decreases, there is a great variation in data of hair density of the same genotype (*A. thaliana* ecotype Columbia) among different authors. For example, the density (number of hairs per mm) for low phosphorus/high phosphorus is 39.0/27.8 from Bates and Lynch (1996), 61.0/13.0 from Ma et al. (2001a), and 55.0/30.0 from Schmidt and Schikora (2001), and ours is 130.8/38.0. We noticed that root hair development is very sensitive to cultural conditions, which may explain the density differences among experiments. Schmidt and Schikora (2001) used a different basal medium from that used by Bates and Lynch (1996) and Ma et al. (2001a), and all of these experiments used continuous light. In this experiment, we grew plants in the same basal medium as Bates and Lynch (1996) and Ma et al. (2001a) used, but with 16-8 hrs alternating light-dark. But as Table 3.8 shows, continuous and alternating light had only a slight effect on root hair density of plants grown with low phosphorus, and trichoblast length and root growth rate were not statistically different with either photoperiod regime.

Schmidt and Schikora (2001) reported that ectopic hairs were induced by phosphorus deficiency in the wild type 'Columbia' and in ethylene mutants. They define "ectopic hairs" as those occurring "in positions that are occupied by non-hair cells under ordinary conditions". The meaning of non-hair cells in their paper is equivalent to atrichoblasts, which according to Dolan et al. (1994), are cell files directly over cortical cells. As we have shown, low phosphorus induced more and smaller cortical cells, which, in turn, support more trichoblasts in the ordinary position, over the junction of cortical cells. It is therefore possible that many hairs, which appeared to be from "ectopic positions" reported by Schmidt and Schikora (2001), might actually have elongated from epidermal cells over the junction of cortical cells (or normal positions). Even if the hairs designated as ectopic by Schmidt and Schikora (2001) actually did form from epidermal cells that would have been atrichoblasts, the number must be very low, since we observed none,

and they observed a maximum of 6%. Ectopic hair production would therefore not contribute significantly to increased root hair density under phosphorus deficiency.

Ma et al. (2001a) reported that the length of trichoblasts with low phosphorus was only slightly shorter than that from high phosphorus, and the increase in root hair density was due to increased number of trichoblasts per cross section and increased rate of trichoblasts elongated to hairs (Ma et al., 2001a). According to their result (Ma et al., 2001a), the percent of trichoblasts forming hairs was the most important factor explaining the increased root hair density, followed by the increased number of trichoblast cell files (Table 3.9). In our experiment, the percentage of trichoblasts producing hairs is only slightly higher for low phosphorus than for high phosphorus, and the increased proportion of trichoblasts forming hairs can explain less than 2% of the increase in root hair density under low phosphorus (Table 3.9). The trichoblast length and the number of trichoblasts per cross section, on the other hand, account for most of the hair density increase. We found that low phosphorus treated roots grew at a substantially slower rate (Fig. 3.12), a result consistent with Ma *et al*. (unpublished data). Since low phosphorus did not reduce the trichoblast length in Ma et al. (2001a), the longitudinal root cell division rate must have been slower under low phosphorus than under high phosphorus while trichoblast elongation remained the same in Ma et al. (2001a). In our experiment, however, the trichoblasts were shortened by low phosphorus (Tables 4 & 5). Data in Table 3.5 and Fig. 3.12 were from the same set of experiment, making trichoblast elongation and root growth comparable. For 'Columbia', the trichoblast length under low phosphorus is 43.1% of that under high phosphorus (Table 3.5), the same reduction as for root growth rate under low phosphorus (41.8%). Therefore, the slower root growth under low phosphorus must have been caused by a slower trichoblast elongation while the longitudinal cell division rate of trichoblasts might have remained the same under both phosphorus treatments.

Our model

Since cortical cell number and trichoblast length are important in root hair density, we need to consider cortical cell division and trichoblast elongation when assessing the effects of phosphorus and ethylene. Cortical cell division should precede cell specification since cell specification is position-dependent, and trichoblast elongation is parallel in time to stages from cell specification until bulge formation when the trichoblast reaches its maximal length (Grierson et al., 2001). We can now summarize the similarities and differences of low phosphorus and ethylene in different stages of root hair development. 1. Low phosphorus affects trichoblast patterning by inducing division of cortical cells, an early stage in root hair development in which ethylene is not involved. 2. Low phosphorus reduces cortical cell size while ethylene enlarges the cortical cells. 3. Both low phosphorus and ethylene shorten the length of trichoblast cells. 4. Both increase the root hair length. 5. Ethylene increases percentage of trichoblasts forming hairs (or, hair initiation) where low phosphorus is not a factor. Based on the above comparison, we propose that low phosphorus and ethylene work independently in early stages of hair development, and converge at later stages (initiation to tip growth) (Fig. 3.13).

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Table 3.1. Ratio of root hair density and length in low P over in high P in *Arabidopsis thaliana* ecotype 'Columbia' and ethylene insensitive mutants

Genotype	Ratio (low P/high P)	
	Density	Length
Columbia	3.44	3.86
ein2-1	3.32	2.61
ein3-1	2.73	2.65
ein4	2.16	2.93
ein5-1	2.55	2.99
ein6	1.65	2.83
ein7	1.94	1.78
eir1-1	1.79	2.87

Table 3.2. Student t-test of root hair density and length of *Arabidopsis thaliana* ecotype 'Columbia' grown in high P (1000 μM) plus ACC 1 μM (high P + ACC 1) against low P (1 μM), Low P plus ethylene inhibitors (STS at 5 μM, MCP at 2 μM, or AVG at 2 μM) against high P, to examine whether ethylene mimics low P effect. ** indicates significant at α = 0.01 level.

Treatment	Density (hairs/mm)	Length (mm)
low P	130.8	1.302
high P + ACC 1	144.75	0.7825**
high P	38.0	0.337
low P + STS 5	43.5	0.205**
low P + MCP 2	21.5**	0.1575**
low P + AVG 2	22.0**	0.1**

Table 3.3. Root cortical cell number of *Arabidopsis thaliana* ecotype 'Columbia' and ethylene insensitive mutants *ein2-1*, *ein4*, and *eir1-1*. Cell number is mean ± standard error, from at least 5 plants.

Genotype	Treatment	Cortical Cell Number
Columbia	High P	8.0 ± 0.0
	Low P	16.4 ± 0.92
	High P + ACC	8.7 ± 0.90
	Low P + AVG	15.5 ± 0.58
ein2-1	High P	8.0 ± 0.0
	Low P	17.7 ± 1.26
ein4	High P	8.0 ± 0.0
	Low P	15.0 ± 0.0
eir1-1	High P	9.0 ± 0.0
	Low P	10.2 ± 0.84

Table 3.4. Trichoblast cell length of GUS-transformed *Arabidopsis*. Cell length = mean of 6 plants ± standard error. For each plant, length of 6 cells was measured.

genotype	treatment	Cell Length (μm)
Columbia	low P	103.0 ± 7.58
Columbia	low P + 3 μm AVG	148.9 ± 14.32
Columbia	high P	165.5 ± 3.03
Columbia	high P + 1 μm ACC	59.1 ± 2.11
ein2	low P	77.5 ± 4.85
ein2	high P	220.0 ± 32.96
ein4	low P	94.2 ± 4.47
ein4	high P	162.5 ± 11.21

Table 3.5. Trichoblast cell length of *Arabidopsis* (not GUS-transformed). Cell length = mean of 6 plants ± standard error. For each plant, length of 6 cells was measured.

genotype	P level	Cell Length (μm)
Columbia	low P	79.6 ± 5.76
Columbia	high P	184.7 ± 15.22
ein2	low P	71.3 ± 7.14
ein2	high P	212.6 ± 5.07
ein4	low P	77.8 ± 5.46
ein4	high P	174.8 ± 8.46

Table 3.6. Effects of P availability and ethylene on *Arabidopsis* root diameter. Root diameter = mean of 6 plants ± standard error.

Genotype	Treatment	Root Diameter (μm)
Columbia	low P	187.4 ± 4.05
Columbia	low P + 3 μm AVG	172.1 ± 5.39
Columbia	high P	179.4 ± 3.35
Columbia	high P + 1 μm ACC	221.9 ± 7.00
ein2	low P	176.3 ± 4.34
ein2	high P	141.5 ± 7.41
ein4	low P	193.4 ± 5.11
ein4	high P	183.7 ± 8.97

Table 3.7. Effects of low P and ethylene on different components constituting root hair density. Materials used are transgenic with GUS.

	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 (mm) (L)	165.5	59.1	103	148.9	212.6	71.3	174.8	77.8
Number of trichoblast/1000 μm long of root (U = 1000/L)	6.04	16.92	9.71	6.72	4.70	14.03	5.72	12.85
Number of trichoblast/cross section (N)	8	8.7	16.4	15.5	8	17.7	8	15
Theoretic hair density (number of hairs/mm root) (T = U * N)	48.30	147.21	159.20	104.10	37.63	248.25	45.77	192.80
Observed hair density (number of hairs/mm root) (O)	38	144.8	130.8	22	24.8	82.4	37.8	72
% trichoblasts forming hairs (PTH)								
Calculated. PTH = (O/T) * 100%	78.6	98.3	82.1	21.1	65.9	33.2	82.6	37.3
Observed.	90.2	99.2	89.9	47.4	71.7	51.2	69.0	54.5

Table 3.8. Effects of photoperiod on trichoblast length, root hair density, and root growth rate. Values represent means (SE). Six plants for trichoblast length and hair density, and 12 plants for root growth rate.

Treatment	Trichoblast length (μm)	Hair density (per mm root)	Root growth rate (cm/day)				
			day 10	day 11	day 12	day 13	day 14
high P, 16 h light	178.9 (8.11)	38.3 (1.98)	0.91 (0.0495)	0.87 (0.0264)	0.90 (0.0445)	0.96 (0.0453)	0.90 (0.0147)
high P, 24 h light	173.9 (9.63)	40.8 (1.72)	0.89 (0.0253)	0.80 (0.0245)	0.82 (0.0239)	0.80 (0.0475)	0.77 (0.0496)
low P, 16 h light	92.4 (6.57)	114.2 (4.46)	0.39 (0.0438)	0.34 (0.0455)	0.30 (0.0502)	0.32 (0.0302)	0.31 (0.0226)
low P, 24 h light	102.0 (7.63)	94.0 (2.11)	0.43 (0.0175)	0.38 (0.0254)	0.31 (0.0296)	0.34 (0.0303)	0.33 (0.0180)

Table 3.9. Comparison of hair density between this paper and Ma et al. (2001).

Components of root hair density	This paper		Ma et $al.$ (2001)	
	High P	Low P	High P	Low P
Length of trichoblasts (μm) (L)	165.5	103.0	181.6	171.3
Number of trichoblast/1000 μm long of root (U = 1000/L)	6.04	9.71	5.51	5.84
Number of trichoblast/cross section (N)	8.0	16.4	8.3	12.0
Theoretic hair density (number of hairs/mm root) (T = U * N)	48.3	159.2	46.0	70.0
Observed hair density (number of hairs/mm root) (O)	38.0	130.8	11.0	63.0
% trichoblasts forming hairs (PTH) (R = (O/T) * 100%)	78.60%	82.10%	24%	90%
The increase of hair density under low P*:				
1. due to increased % trichoblasts forming hairs (F = ((R_{lowP} − R_{highP}) * T_{highP})/(O_{lowP} - O_{highP})) * 100%)		1.8%		58.4%
2. due to trichoblast changes (C = ((T_{lowP} − T_{highP}) * R_{highP})/(O_{lowP} - O_{highP})) * 100%)		93.9%		11.1%
2.1. due to trichoblast file number (DN = ((R_{lowP}/(O_{lowp} - O_{highP})) * U_{highP} * (N_{lowP} - N_{highP})) * 100%)		43.0%		9.4%
2.2. due to trichoblast length (DL =((R_{lowP}/(O_{lowp} - O_{highP})) * N_{highP}*(U_{lowP} - U_{highP}) * 100%)		24.8%		1.3%
2.3. due to trichoblast length * file number (D(N*L) = ((R_{lowP}/(O_{lowp} - O_{highP})) * (N_{lowP} - N_{highP}) *(U_{lowP} - U_{highP}) * 100% = C - DN - DL)		26.2%		0.7%
3. Due to both % trichoblasts forming hairs and trichoblast changes (B = ((R_{lowP} − R_{highP}) * (T_{lowP} − T_{highP}))/(O_{lowP} - O_{highP})) * 100% = 100% - F - C)		4.3%		30.5%

* Hair density increase under low P = O_{lowP}−O_{highP}

O_{lowP}−O_{highP} = T_{lowP}*R_{lowP} - T_{highP} * R_{highP}

= (T_{highP} + (T_{lowP} - T_{highP})) * (R_{highP} + (R_{lowP} - R_{highP})) - T_{highP} * R_{highP}

= T_{highP}* (R_{lowP} - R_{highP}) + R_{highP} * (T_{lowP} - T_{highP}) + (T_{lowP} - T_{highP}) * (R_{lowP} -R_{highP})

T_{highP} * (R_{lowP} -R_{highP}): due to % trichoblasts forming hair

R_{highP} * (T_{lowP} -T_{highP}): due to trichoblast file number and length both of which determine theoretical trichoblast cell number

(T_{lowP} - T_{highP}) * (R_{lowP} -R_{highP}): due to both % trichoblasts forming hair and trichoblast changes

Similarly, we can split R_{highP} * (T_{lowP} -T_{highP}) (due to trichoblast change) into three parts, as shown in the table: due to trichoblast file number, due to trichoblast length, and due to their interaction.
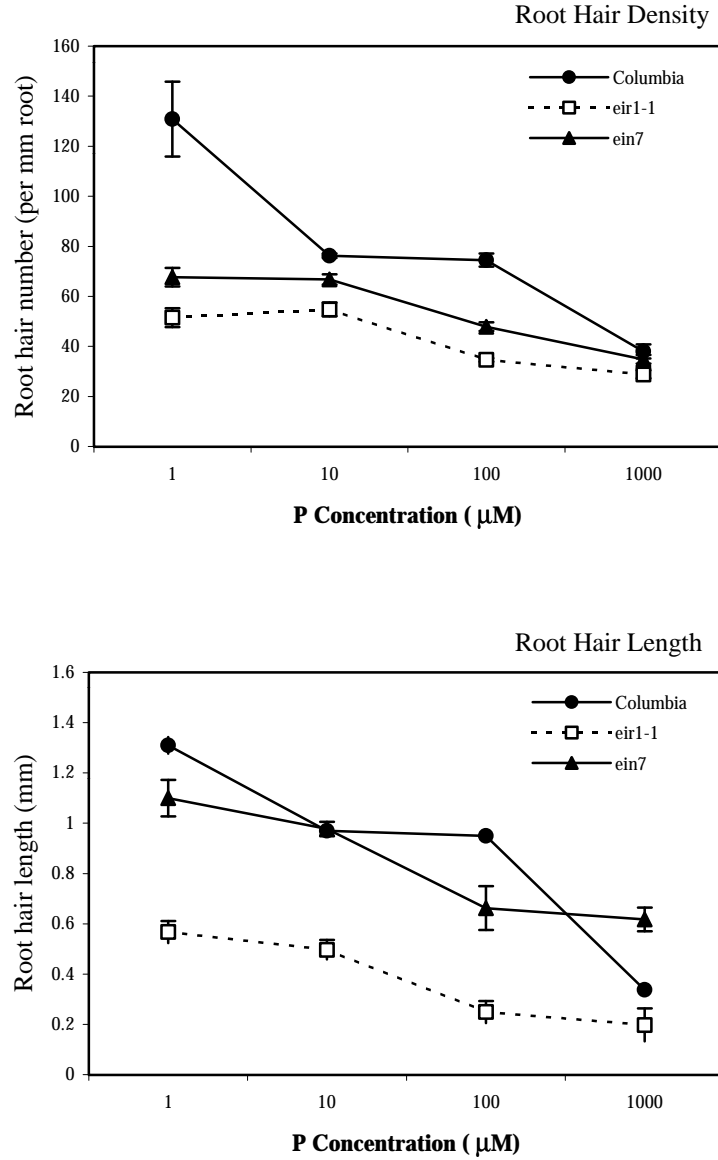
Fig. 3.1. Root hair density and length of Arabidopsis thaliana ecotype 'Columbia' and its ethylene insensitive mutants, eir1-1 and ein7, in response to P dosage. Regression analysis of root hair density and length (Y_{den} and Y_{len}) against logarithm of P concentrations (X) is the following (as $Y_{den} = a + bX$ or $Y_{len} = a + bX$): (1) Columbia $Y_{den} = 123 - 28.6* X$; $Y_{len} = 1.41 - 0.317*X$; (2) eir1-1 $Y_{den} = 54.9 - 8.78* X$; $Y_{len} = 0.573 - 0.135*X$; (3) ein7 $Y_{den} = 74.0 - 12.6* X$; $Y_{len} = 1.14 - 0.191*X$. For all regression equations, $a_{prob} < 0.001$, $b_{prob} < 0.001$.
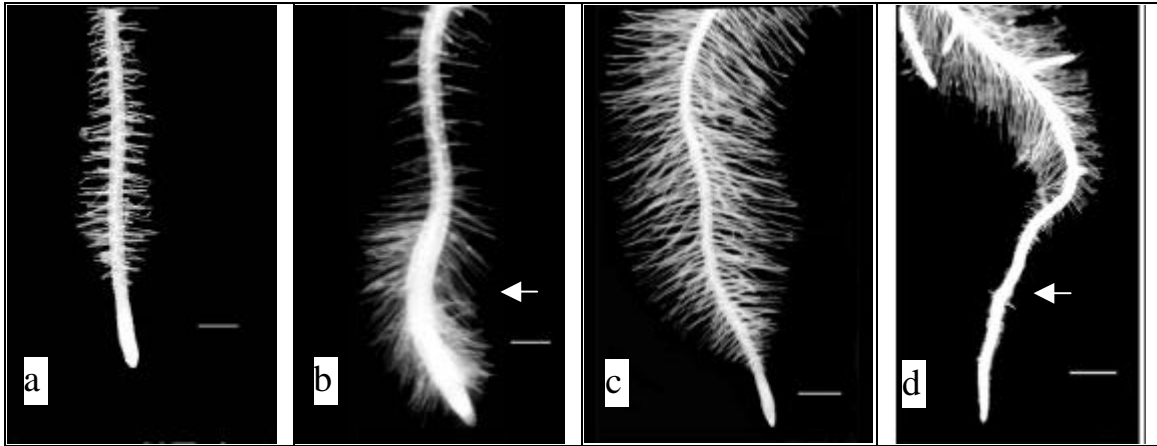
Fig. 3.2. Root hair growth of Columbia. a. high P (1000 µM); b. high P for 12 d then transferred to high P plus ACC 1 µM for 2 d. More and longer hairs are formed with ACC (arrow); c. low P (1 µM); d. low P for 12 d then transferred to low P plus AVG 2 µM for 2 d. Hair production is inhibited by AVG (arrow). Bar = 0.5 mm.
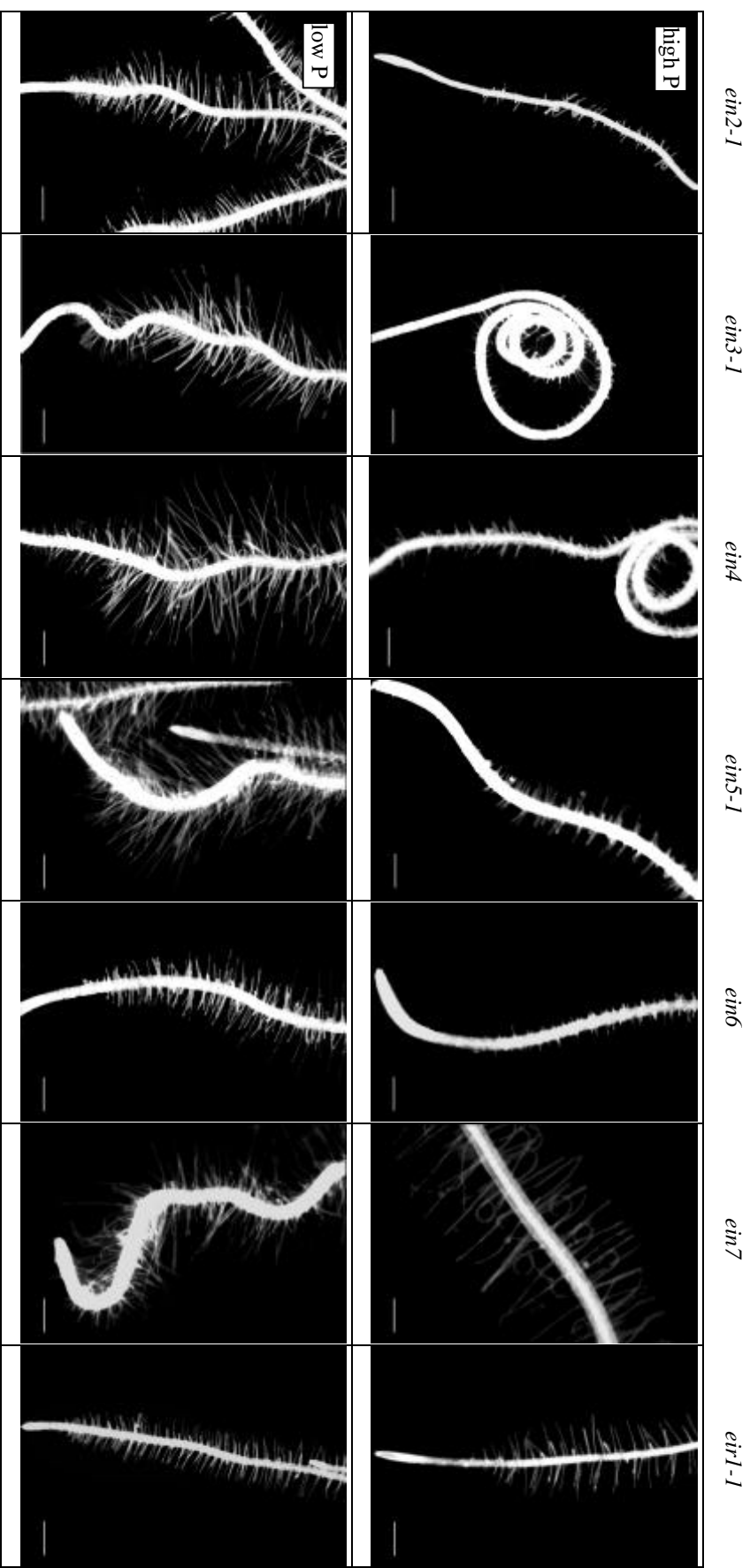
Fig. 3.3. Root hair growth of Arabidopsis mutants. First row: high P (1000 μM); second row: low P (1 μM). Bar = 0.5 mm.

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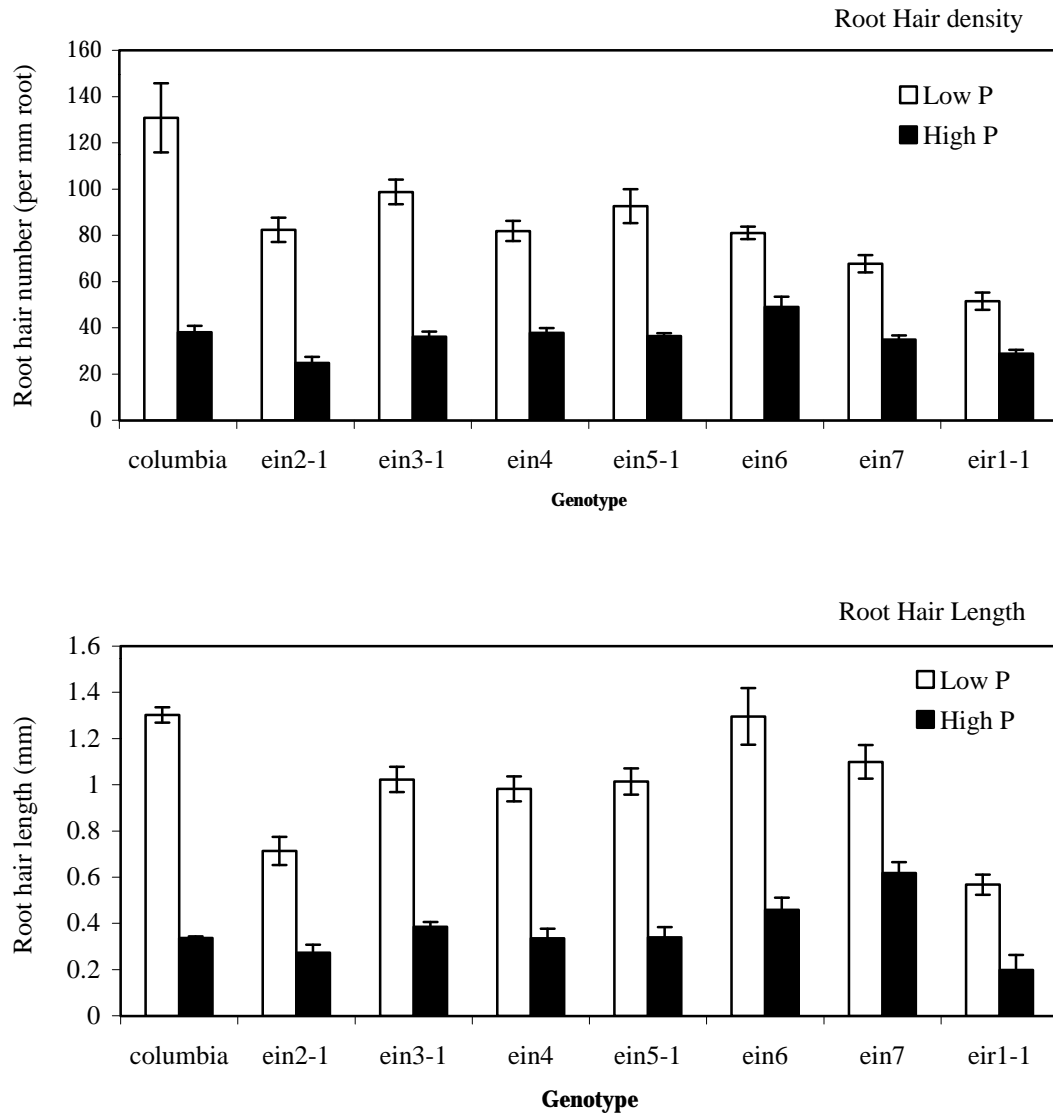
Fig. 3.4. Root hair density and length of Arabidopsis thaliana ecotype 'Columbia' and its ethylene insensitive mutants grown in media with low P (1 μM) and high P (1000 μ N Contrast (t-test) between low P and high P treatments for root hair density and length indicates significant at 1% level for all genotypes.

Root Hair density
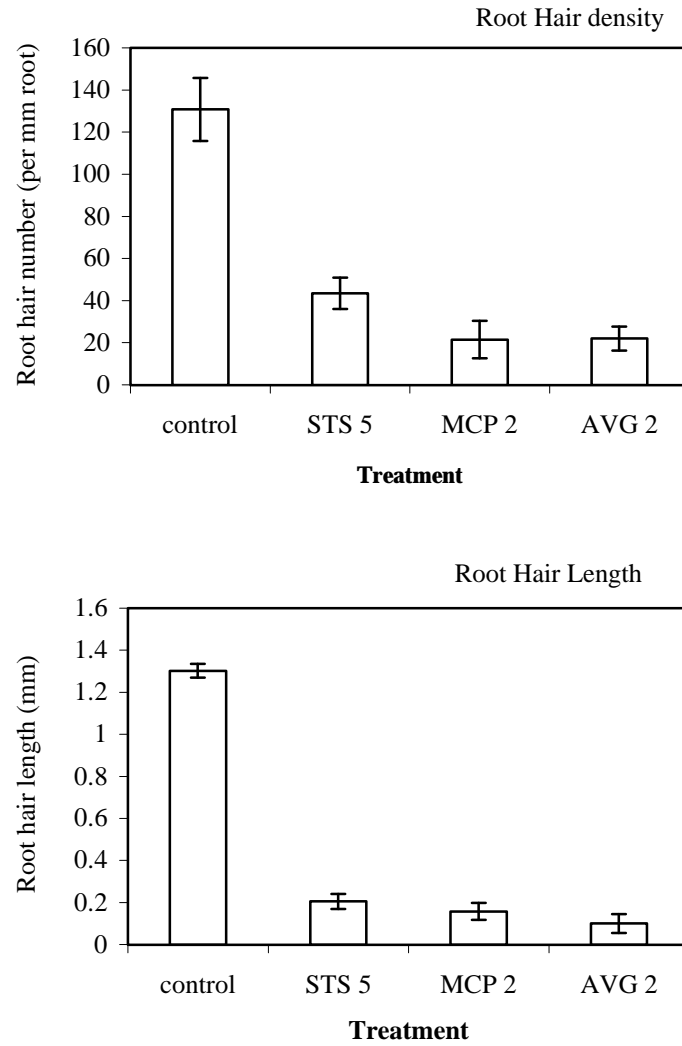
Root Hair Length

Fig. 3.5. Root hair density and length of Arabidopsis thaliana ecotype 'Columbia' grown in low P (1 μM) media without inhibitors (control), or with ethylene production inhibitor AVG (2 μM), ethylene action inhibitor STS (5 μM), or MCP (2 μM). Contrast (t-test) of inhibitor treatments against control showed that all inhibitors significantly reduced root hair density and length (p <0.01).
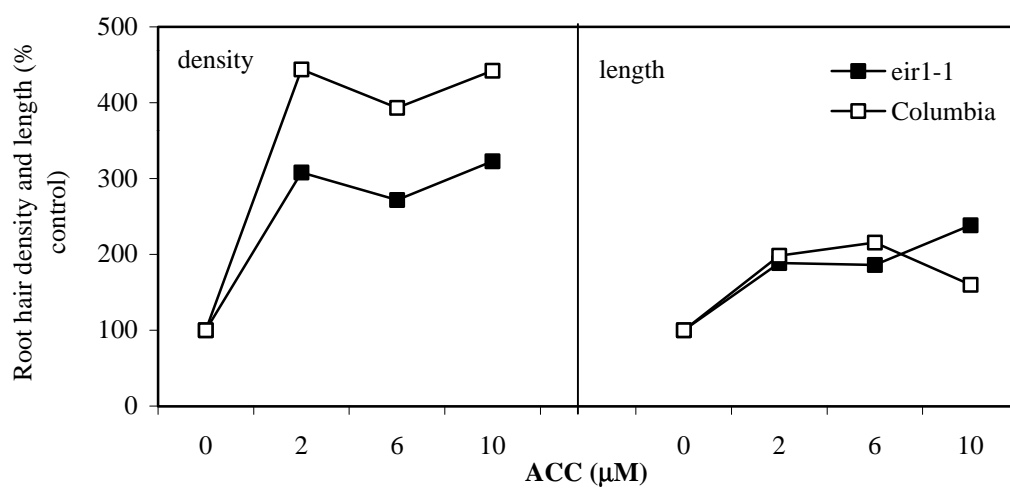
Fig. 3.6. Effect of ACC on root hair density and length in Arabidopsis plants grown in high P.
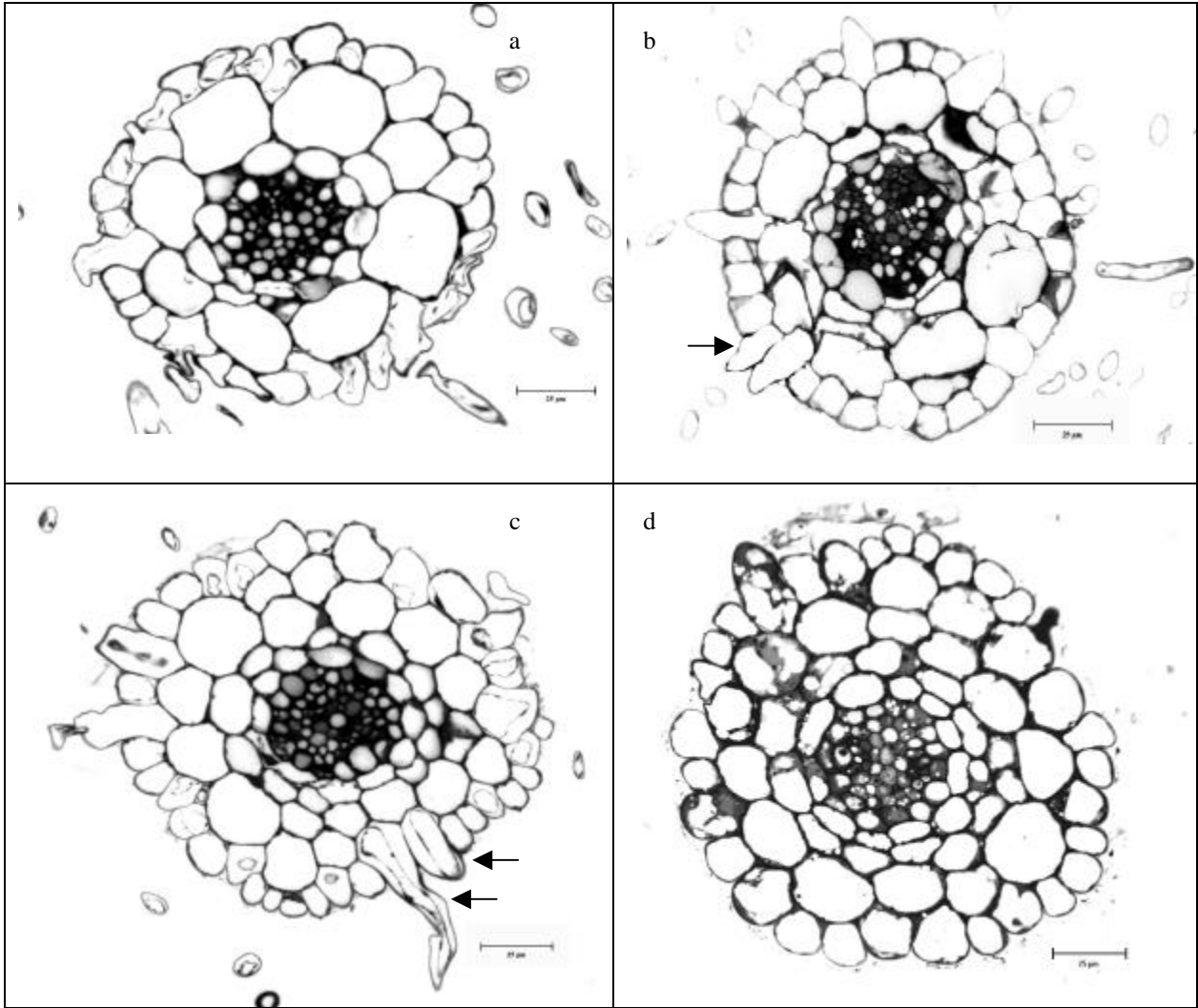
Fig. 3.7. Root cross-sectioning *of Arabidopsis thaliana* ecotype 'Columbia'. a. high P (1000 µM); b. high P plus ACC (1 µM), arrow indicates a hair in an ectopic location; c. low P (1 µM), arrows indicate two adjacent hairs; d. low P plus AVG (2 µM). Bar = 25 µ m
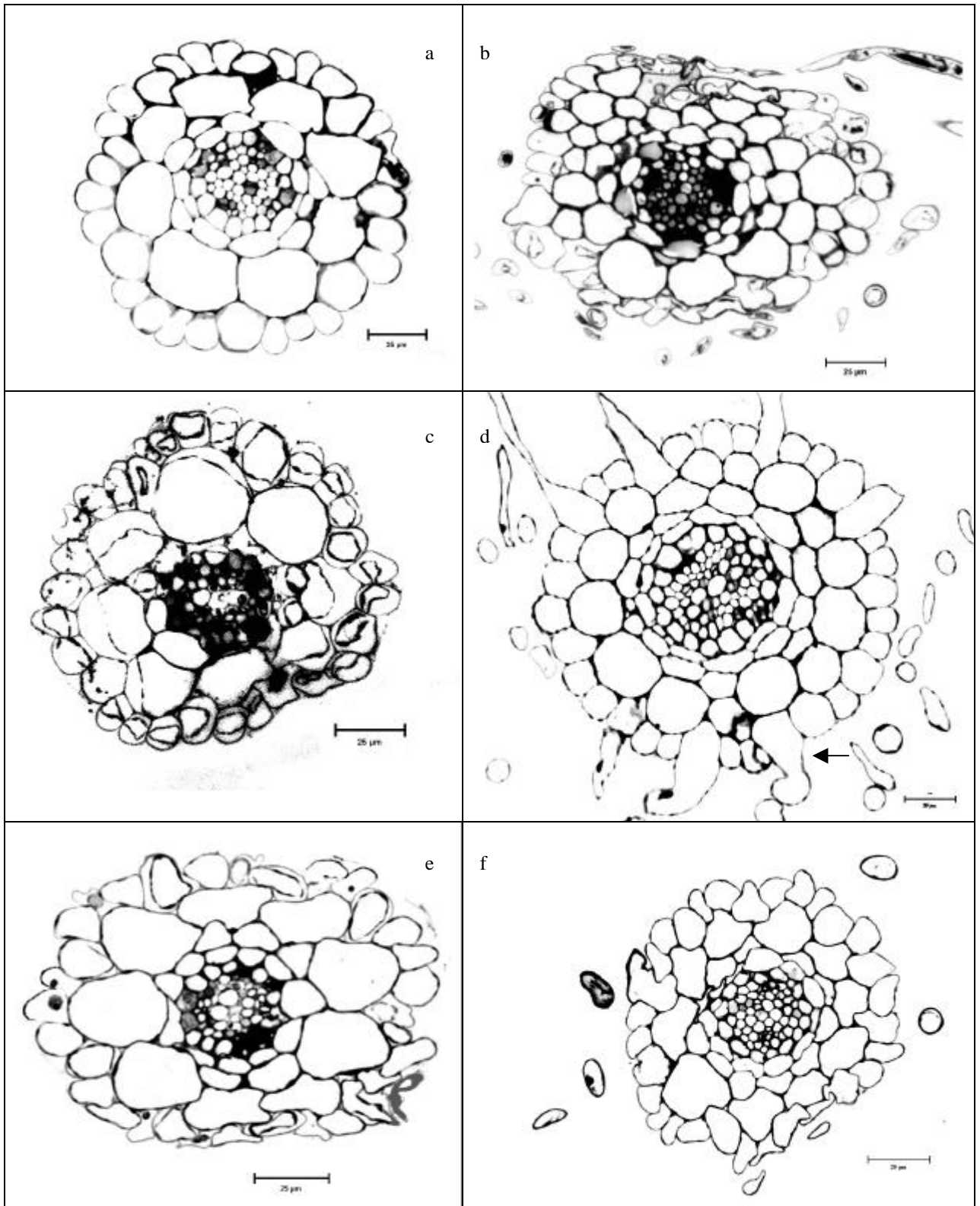
Fig. 3.8. Root cross-section of *Arabidopsis thaliana* ethylene insensitive mutants. a. *ein2-1*, high P (1000 μM); b. *ein2-1*, low P (1 μM); c. *ein4*, high P; d. *ein4*, low P. Arrow indicates a hair in an ectopic location; e. *eir1-1*, high P; f. *eir1-1*, low P. Bar = 25 μ m.
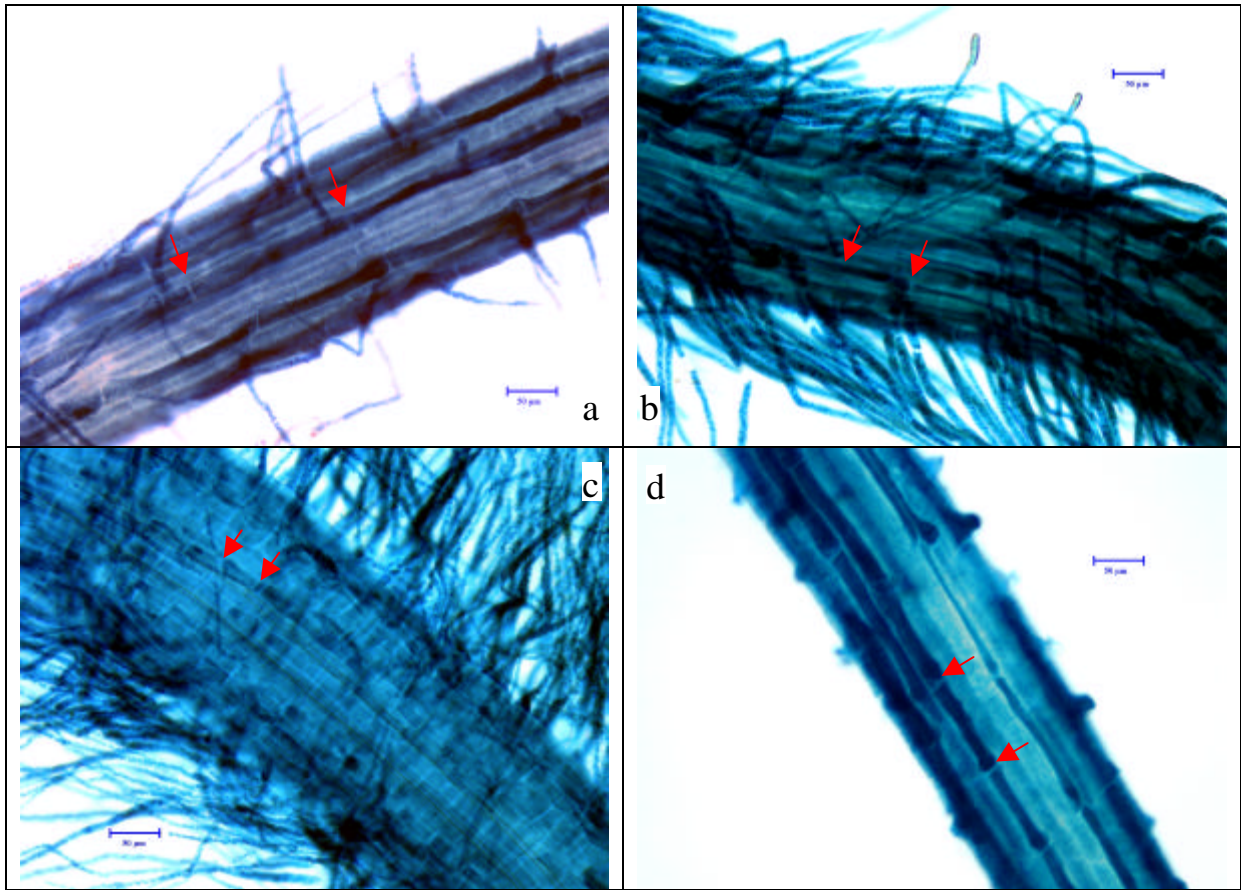
Fig. 3.9. Effects of P availability and ethylene on root hair cell length of 'Columbia' transformed with GUS on a trichoblast-specific promoter. (a) high P; (b) low P; (c) high P + 1 μM ACC; (d) low P + 3 μM AVG. Arrows indicate cell walls at each end of a trichoblast cell. Bar = 50 μm.

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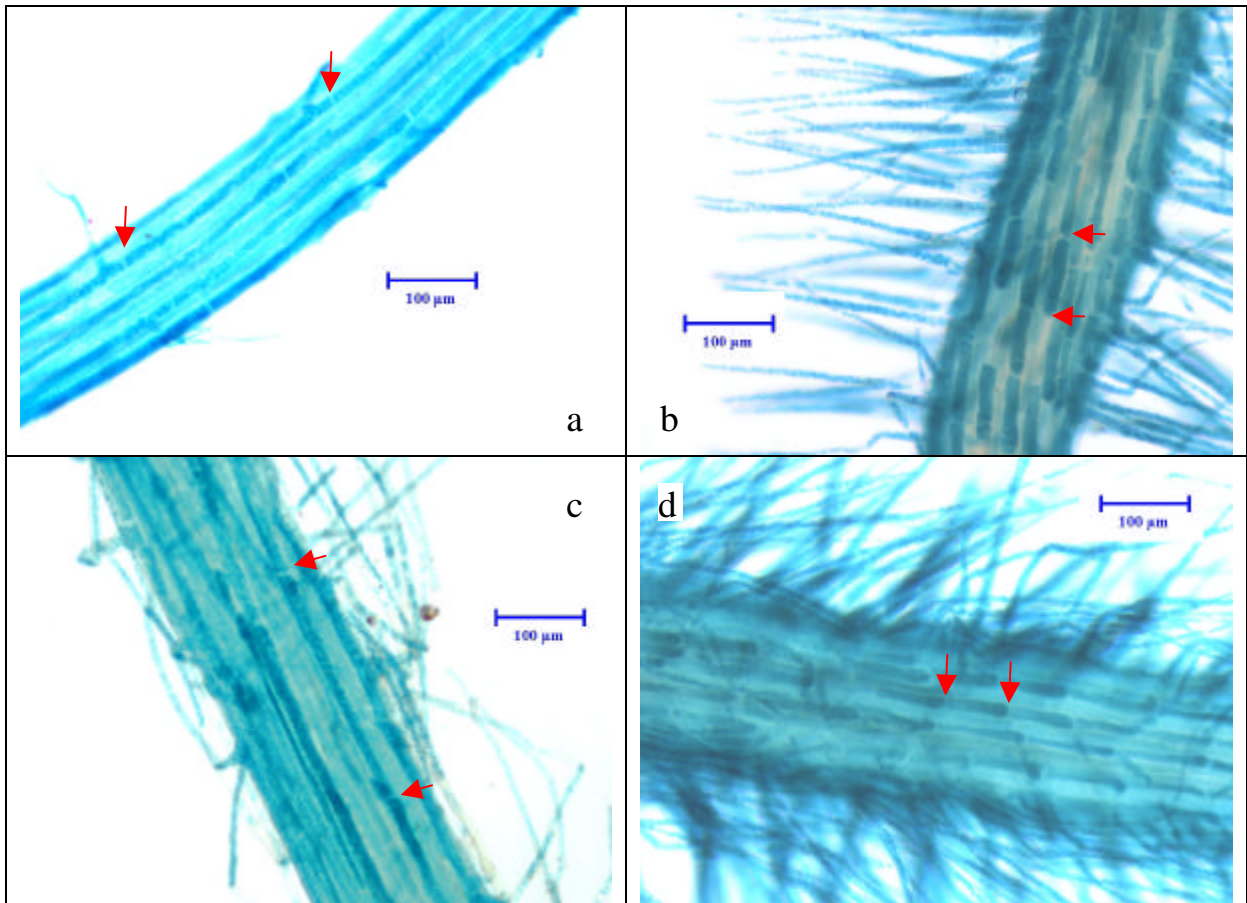
Fig. 3.10. Effects of P availability on root hair cell length of ethylene insensitive mutants *ein2* and *ein4* transformed with GUS on a trichoblast-specific promoter. (a) *ein2-1* high P; (b) *ein2-1* low P; (c) *ein4* high P; d: *ein4* low P. Arrows indicate cell walls at each end of a trichoblast cell. Bar = 100 μm.
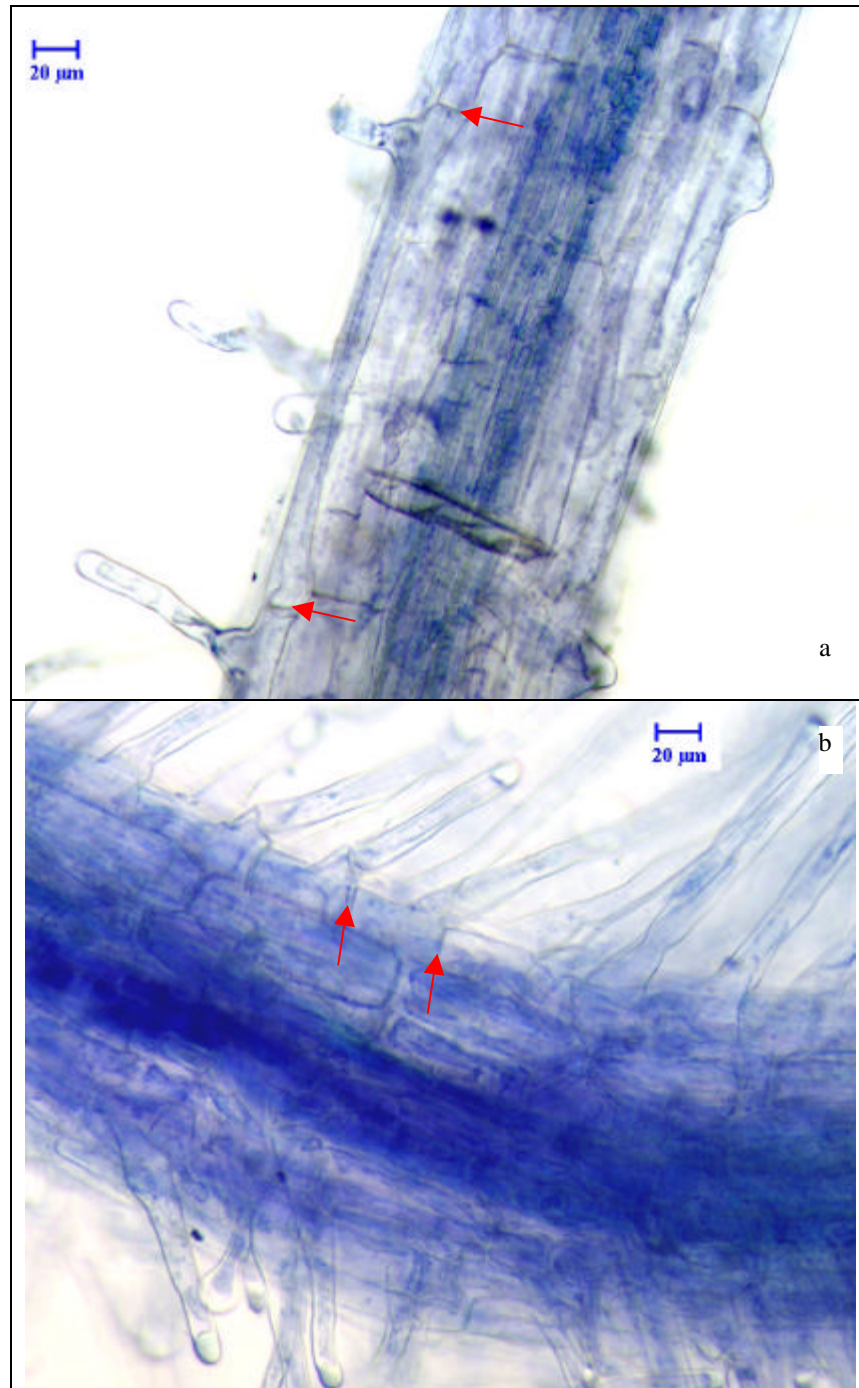
Fig. 3.11. Root hair cell length of *Arabidopsis* wild type 'Columbia'. Roots were stained with Toluidine Blue O. a: high P; b: low P. Arrows indicate cell walls at each end of a trichoblast cell. Bar = 20 µm.
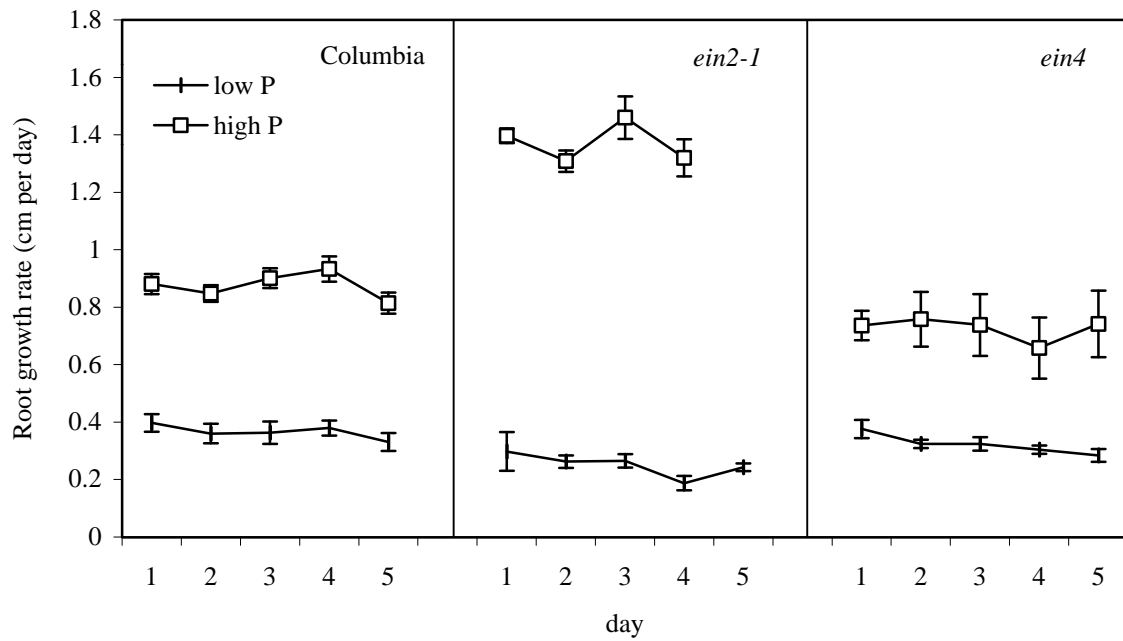
Fig. 3.12. Root growth rate of *Arabidopsis* 'Columbia' and ethylene insensitive mutants *ein2-1* and *ein4* in the last 5 days (starting from day 9) before harvest (at day 14 of culture) in low or high P medium.
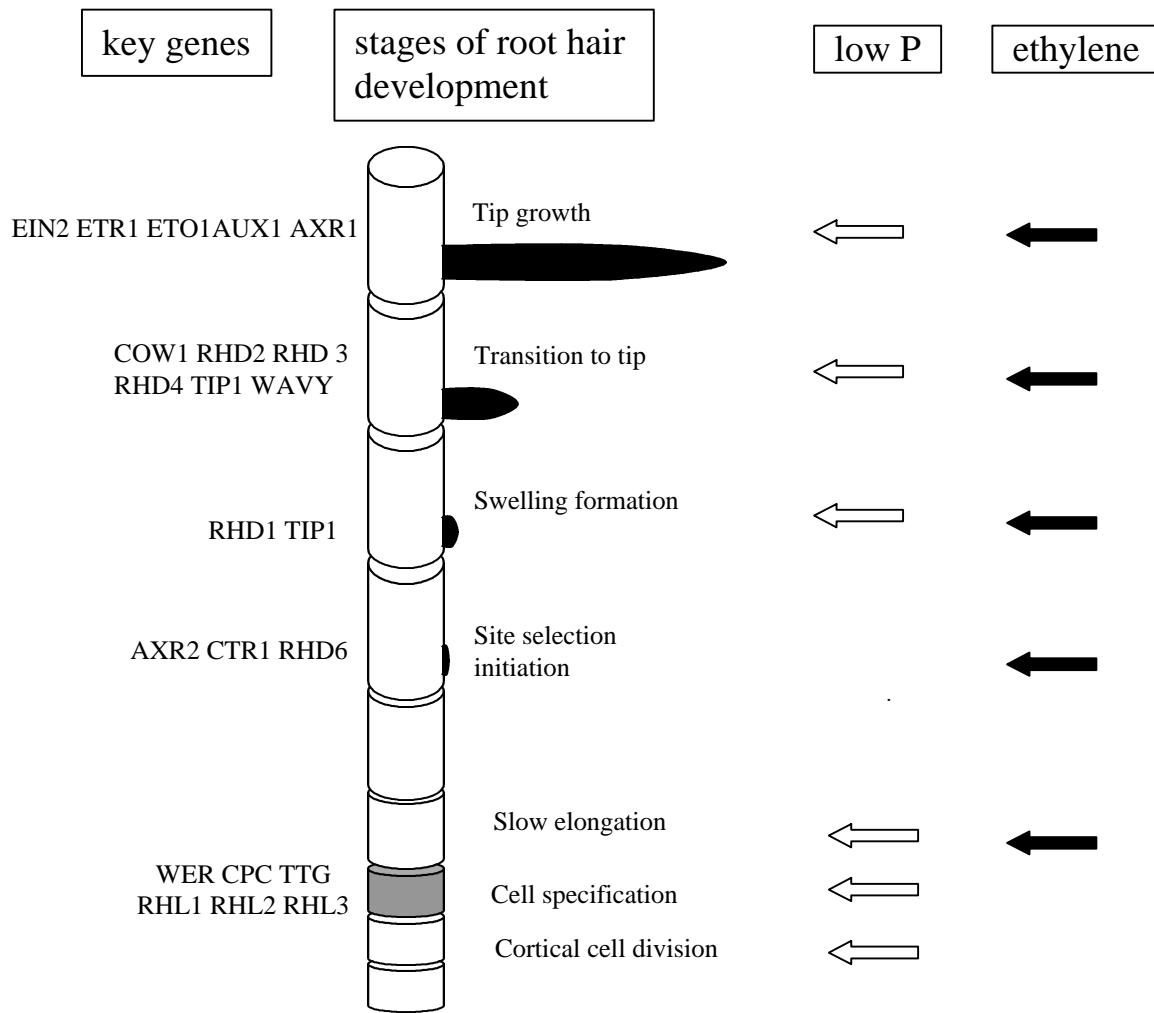
Fig. 3.13. Diagram of root hair developmental stages, corresponding key genes and possible stages affected by low P and ethylene. Low P affects cortical cell division (and, therefore indirectly, cell specification), trichoblast elongation, and hair elongation. Ethylene affects trichoblast elongation, hair initiation, and hair elongation. Ethylene enhances hair elongation through ethylene genes; low P may or may not through ethylene genes. Adapted from Figures 1A and 2 in Grierson *et al.* (2001) and Figure 2 from Schiefelbein (2000).

Chapter 4

Ethylene mediates basal root gravitropism response to phosphorus deficiency

Abstract

Plant roots react to low phosphorus availability in several ways that may permit them to acquire more phosphorus, including distributing more roots in upper, phosphorus -rich soil horizons. G19833, a phosphorus efficient common bean (*Phaseolus vulgaris* L.) genotype, has shallow basal roots that are shallower with phosphorus deficiency. DOR364, a phosphorus inefficient genotype, has deep basal roots which are affected to a less extent by phosphorus limitation. These contrasting genotypes and recombinant inbred lines (RILs) derived from an F1 hybrid of these genotypes were used to investigate ethylene involvement in basal root gravitropic responses to phosphorus deficiency. Aminoethoxyvinyl glycine (AVG), an inhibitor of ethylene synthesis, and silver thiosulfate (STS), an inhibitor of ethylene response, prevented the change in basal root angle induced by low phosphorus. 1-aminocyclopropane-1-carboxylic acid (ACC) or gaseous ethylene treatment, on the other hand, increased shallowness of basal roots even with high phosphorus availability, and the extent of basal root growth angle change was proportional to the concentrations of ACC or AVG added to the media. Shallow genotypes were more responsive to ethylene manipulation than deep genotypes. A strong correlation was found for the shallow genotype G198333 between its basal root shallowness and the endogenous ethylene production rate. These results suggest that ethylene mediates the response of roots to phosphorus availability and that genotype affects ethylene responsiveness.

Key words: *Phaseolus vulgaris*, low phosphorus, basal root shallowness, ethylene

Introduction

Root architecture, or the spatial configuration of the root system in the soil, determines the ability of the root system to explore the spatial domains in the soil and the ability to respond dynamically to localized soil resources. Phosphorus, a relatively immobile nutrient which is essential for plant growth, is heterogeneously distributed in most soils, with greatest availability in surface horizons and decreasing availability with depth (Pothuluri et al. 1986). Root architecture is particularly important for phosphorus acquisition in the soil because phosphorus mobility is dominated by diffusion, which is a slow process (Jungk 1996). Plants change their root architecture in order to acquire more phosphorus, especially when phosphorus availability is low (Bonser et al. 1996; Borch et al. 1999; Lynch and Brown 2001).

Common bean (*Phaseolus vulgaris* L.) is an important crop cultivated in many parts of the world. Its root system includes one tap root which emerges directly from the seed, varied number of basal roots which originate from the base of the tap root, and lateral roots from the tap, basal, or other lateral roots. Because of their origin, basal roots are important for exploring the upper layers of soil where the phosphorus availability is usually the highest. Studies show that basal roots of bean grow shallower with decreased phosphorus availability in the medium (Bonser et al. 1996). This response to low phosphorus is genetically controlled. Comparative analysis of common bean genotypes indicates that the basal root gravitropism regulated by phosphorus availability is genotype dependent and closely correlated with phosphorus efficiency. The phosphorus efficient genotypes respond to low phosphorus by increasing the shallowness of basal root growth, while inefficient genotypes do not (Bonser et al. 1996). This basal root architectural response is specifically induced by phosphorus deficiency and cannot be induced by other nutrient deficiencies. Computer modeling and plant growth studies indicate that shallow basal root angle is a desirable trait in agriculture. SimRoot, a computer simulation program developed by Lynch et al. (1997) , shows that phosphorus uptake efficiency is positively correlated with the shallowness of the basal root of common beans (Ge et al. 2000). Bonser et al. (1996) reported a significant correlation between yield of various

common bean genotypes in the field and their basal root growth angle, measured from a 2-D pouch experiment system. A smaller growth angle, meaning a shallower basal root, was associated with higher yield. Using sand cultures with stratified phosphorus levels to mimic field phosphorus distributions, Liao et al. (1998) and Liao et al. (2001) showed that plants with increased root length in upper soil layers had higher phosphorus content. In other words, shallower roots confer phosphorus efficiency.

The change of basal root growth angle of common beans in response to phosphorus availability is a gravitropic response. In the literature, gravitropic responses are usually observed in orthogravitropic organs in response to gravistimulation, i.e. rotation of the root system to change the growth angle with respect to gravity. However, few roots are actually orthogravitropic, but rather grow at some other angle with respect to the gravity vector. According to Firn & Digby (1997), every organ has a "gravitropic set point angle" (GSA), which changes over time and is controlled by developmental and environmental factors. When a basal root grows more shallowly in response to low phosphorus, this represents a gravitropic response of the basal root because it is a specific orientation of growth in response to gravity.

The understanding of low phosphorus regulation of basal root angle has important theoretical and practical implications. Root gravitropism has been a classical research topic since the time of Charles Darwin, but a clear, detailed understanding of the process has not been produced (Firn and Digby 1997). Understanding how phosphorus affects the root gravitropic response should help the understanding of gravitropism as a whole and should help breeders to produce phosphorus-efficient crop varieties, which adapt well in low phosphorus soil and need less phosphorus fertilization.

Ethylene, a natural plant growth regulator, is likely to be important for basal root gravitropic responses to low phosphorus availability (Lynch and Brown 2001). First, there are reports indicating that ethylene plays a role in modulating gravitropic responses (Madlung et al. 1999; Peck et al. 1998; Zobel 1974). The tomato mutant *dgt* (*diageotropica*) has an altered gravitropic phenotype (Kelly and Bradford 1986), and

small amounts of ethylene have been reported to restore a normal gravitropic response in *dgt* (Zobel 1974). The apical hook is caused by asymmetric cell elongation, which is mediated by ethylene (Peck et al. 1998). Second, there exists a close relation between ethylene and roots responding to low phosphorus (Lynch and Brown 2001). Borch et al. (1999) reported that common bean roots under phosphorus stress produced twice as much ethylene as unstressed roots. We also observed that low phosphorus availability increased ethylene production by roots of 2 cultivars of tomato plants (Lynch and Brown 2001). Phosphorus starvation can induce aerenchyma formation in corn roots and the process is mediated by ethylene (Eshel et al. 1995; He et al. 1992). We hypothesize that ethylene might be important in basal root gravitropism in response to low phosphorus. Specifically, phosphorus deficiency is a stress to plants. In responding to this stress, roots might increase ethylene production and/or increase tissue sensitivity to ethylene, and this might make basal roots grow shallower under low phosphorus.

To study this problem, using common bean as the material has advantages over other species: the basal root responses to low phosphorus availability have been well characterized in pouch and sand cultures and in the field (Bonser et al. 1996; Liao et al. 2001). Genotypes contrasting in basal root plasticity, and recombinant inbred lines resulting from F1 hybrids of contrasting parents showing a whole spectrum of basal root responses to low phosphorus are available for clarifying the role of ethylene in regulating the basal root gravitropism and its genetic control. Data obtained from this experiment should be useful in further genetic mapping of this trait.

In this study, we used the pouch culture system to examine whether ethylene is involved in regulating the basal root gravitropic response to low phosphorus, through manipulating ethylene.

Materials and Methods

Seeds of all genotypes used in this experiment were obtained from the global Phaseolus germplasm collection at CIAT headquarters in Cali, Colombia. Common bean (*Phaseolus vulgaris* L.) genotypes G19833 and DOR364 are contrasting genotypes, with

different genetic origins (Singh et al. 1991). G19833 is better adapted to phosphorus limited conditions than DOR364 (Beebe et al. 1997; Lynch and Beebe 1995); it also has shallower root system compared to DOR364 (Bonser et al. 1996; Liao and Yan 1999) (Fig. 4.1). These two genotypes were crossed and the progenies advanced by single seed descent to the F5 generation, then advanced by mass selection within each family to F12 recombinant inbred lines (RILs). Two parental genotypes and six RILs were selected for this experiment. The selected RILs genotypes have deep (RILs 32 and 38), intermediate (RILs 32 and 38), or shallow (RILs 7 and 33) basal roots, according to a preliminary screening result under low phosphorus availability (Liao et al., unpublished data).

The seeds of parental and RIL genotypes were surface sterilized with 10% bleach (contains 6% sodium hypochlorite) and germinated at 25 C in the dark in rolled No.76 brown germination paper (size 25.5 $*$ 37.5 cm, Packaging Convertors, 1501 Swasey, Hudson, WI 54016, USA) moistened with either low or high phosphorus nutrient solution, which was composed of (in µM) 3000 KNO_3, 2000 $Ca(NO_3)_2$, 250 $MgSO_4$, 25 KCl, 12.5 H_3BO_3, 1 $MnSO_4$, 1 $ZnSO_4$, 0.25 $CuSO_4$, 0.25 $(NH_4)_6Mo_7O_{24}$, 25 Fe-Na-EDTA. For high phosphorus solution, 1000 µM $NH_4H_2PO_4$ was added; for low phosphorus, 500 µM $(NH_4)_2SO_4$ was added. Germinated seeds with 2-3 cm long radicals were transferred to growth pouches containing the same low or high phosphorus solutions. The growth pouch culture system was as described by Liao et al. (2001), and as shown in Fig. 4.2. A complete randomized block design with 4-6 replicates was used in this experiment. After transferring to the pouches, plants were kept in the dark for 2 days, and then grown in light for 12 h each day at 400 µmol m^{-2} s^{-1} of photosynthetically active radiation. Plants were harvested 6 d after transplanting. Roots were scanned into a computer and the digital images were analyzed with Photoshop (Adobe Photoshop® 6.0, Adobe System Incorporated) by measuring basal root growth angles, which is expressed as degrees from horizontal (Liao et al. 2001) (Fig. 4.1). When basal root angles are reported for roots arising from different positions, roots were numbered from the most apical position as indicated in figure 4.1. Radical diameter was measured under a dissecting microscope. Statistical analyses of data were performed with MiniTab (MINITAB® for Windows, Minitab Release 13.1, Minitab Inc.).

For ethylene measurement, harvested basal roots were cut into 1 cm segments and about 0.1 g root samples (or about 10 pieces) were put into 4-ml glass vials capped with septa. Ethylene was sampled with a 1-cc syringe from the headspace of the vial 2 hr later and ethylene was measured by gas-chromatography (HP6890 gas chromatograph equipped with a flame ionization detector and an activated alumina column, Hewlett-Packard Company, 2850 Centerville Road, Wilmington, DE 19808-1610, USA).

To manipulate ethylene level in plants, 1-aminocyclopropane-1-carboxylic acid (ACC) at 5, 10, or 15 µM, or aminoethoxyvinyl glycine (AVG) at 1.0, 3.0, or 5.0 µM, or silver thiosulfate (STS) 10 µM was added to the nutrient solution. For exogenous ethylene treatment, germinated seeds were transferred to the growth pouch and were immediately exposed to 1.0 µL/L ethylene for 24 hrs at room temperature in an airtight container. ACC and AVG treatments were applied to all genotypes, but only the parents and RILs 7 and 32 were used for STS and ethylene treatments.

To determine the optimal concentrations of the ethylene inhibitors AVG and STS and the ethylene precursor ACC, two parental genotypes were tested. The criterion for the optimal concentration is the concentration that causes significant changes in the basal root angle without producing visible abnormalities in growth. For example, AVG at 1, 3, and 5 µM reduced the basal root shallowness of G19833 grown in low phosphorus by 0.0, 33.3, and 33.9%. Both 3 and 5 µM AVG gave the same effect and no physiological disorder of plant growth was observed. Therefore, 5 µM AVG was used throughout the rest of this experiment. Since ACC at 20 µM or higher concentration greatly reduced tap root growth, 10 µM ACC was used. Similarly, 10 µM STS was chosen for this experiment.

Results

The two-dimensional pouch experimental system is fast, sensitive laboratory system for the evaluation of basal root growth angles in common bean in response to phosphorus

availability (Bonser et al. 1996; Liao et al. 2001). The results from the pouch system are comparable to those from solid media culture in term of basal root shallowness (Liao et al. 2001).

Basal root gravitropic response to low phosphorus

Basal roots of shallow genotypes, including the parental genotype G19833 and shallow RILs, grew even shallower under low phosphorus (Fig. 4.3). For deep genotypes, including the parental genotype DOR364, basal roots were much less sensitive to phosphorus availability (Fig. 4.3). On average, shallow genotypes decreased their basal root growth angle by 41% when they were grown with low phosphorus. For deep genotypes, however, the reduction was only 2.2%. RIL10, classified as an intermediate genotype in basal root shallowness in pre-screening by Liao et al. (unpublished data), was actually shallow under either high and low phosphorus conditions (Fig. 4.3). RIL66, the other intermediate genotype used in this experiment, was more responsive to phosphorus availability than any other genotype (basal root growth angle decreased 65% from high phosphorus to low phosphorus) (Fig. 4. 3).

The basal root gravitropic response depended not only on genotype, but also on the position from which the basal root arose. The basal root arising in the most apical position (closest to the surface of the soil) was the most responsive. For example, all 4 observed pairs of basal roots of G19833 grew more shallowly under low phosphorus, but the extent of responsiveness decreased in roots arising from more basal positions. For the deep genotype DOR364, however, there were no significant differences in root angle with phosphorus availability regardless of their positions (Fig. 4.4).

Effects of ACC and gaseous ethylene

ACC and gaseous ethylene applied to plants grown with high phosphorus solutions induced shallower basal roots (Fig. 4.5 & 4.6). Increasing ACC concentration from 0 to 10 μM has an increasing effect on basal root gravitropic responses (Fig. 4.5). Further increasing ACC concentration to 15 μM did not increase the response, indicating that the

response to ACC is saturated at 10 µM. Application of 1 µL/l ethylene reduced basal root angle, but not as much as 10 µM ACC (Fig. 4.6). We did not test gaseous ethylene dosage on basal root gravitropic response, so the concentration of 1 µL/L used in the experiment may not be optimal. Neither ACC nor gaseous ethylene affected basal root angle of plants grown with low phosphorus (not shown).

Ethylene production in response to added ACC was measured as a rough assay of *in vivo* ACC oxidase activity in basal root tissues and the ability of roots to respond to ACC by increasing ethylene production. ACC increased ethylene production for some genotypes, but not for others (Fig. 4.7). The ability of ACC to increase ethylene production did not correlate with ACC effects on basal root growth angle. For example, the deep genotype RIL32 did not grow shallower with ACC, even though ethylene production was higher with ACC, while the shallow genotypes G19833 and RIL33 did not produce more ethylene with ACC treatment, even though ACC reduced their basal root growth angle (compare Fig. 4.6 & 4.7).

Effects of ethylene inhibitors

Both the ethylene synthesis inhibitor AVG and the action inhibitor STS could reverse the low phosphorus effect on basal root gravitropism, i.e., basal roots of plants grown with low phosphorus plus AVG or STS grew deeper (Fig. 4.8). In fact, AVG and STS induce basal roots as deep as, or even deeper than basal roots grown with high phosphorus, except for DOR364, where STS does not affect basal root angle. AVG and STS had no significant effect under high phosphorus (data not shown). Fig. 4.9 shows that the effect of AVG on basal root shallowness is a result of interaction between AVG and genotype. The shallow genotype G19833 grew increasingly deeper as higher concentrations of AVG were added to low phosphorus medium. DOR364 was already deep and did not respond to AVG. AVG and STS did not significantly affect basal root growth angle in plants grown with high phosphorus, with the exception that STS made G19833 deeper (Fig. 4.8).

Correlation between basal root ethylene production and shallowness

Statistical analysis shows that, for shallow genotypes (G19833, RILs 7 and 33), there was a significant negative correlation between basal root ethylene production and basal root angle when treatments with high phosphorus, low phosphorus, ACC and AVG were included in the analysis (Fig. 4.10). For deep genotypes (DOR364, RILs 32 and 38), the correlation was not statistically significant (Fig. 4.10). When the correlation was done for just one shallow genotype treated with various concentrations of AVG to reduce ethylene production, there was a significant correlation between ethylene production and basal root growth angle (Fig. 4.11).

The relationship between radical morphology and basal root angle

We examined the hypothesis that basal root angle is determined by the morphology of the surface from which the root emerges. If we assume that the basal roots arise perpendicular to the radical, then their initial angle would be determined by the slope of the radical surface. The upper part of the radical was somewhat conical for both genotypes, with a larger diameter at the upper end than at the lower end (Table 4.1). When the expansion of the upper radical was compared over the 3-day period during which basal roots form, differences in expansion for low vs. high phosphorus availability were found in the shallow genotype G19833 but not in the deep genotype DOR364. (Fig. 4.12, Table 4.1). We observed that, for G19833 under low phosphorus, the lower end of the region expanded faster than the upper end (Table 4.1). The situation is the opposite with high phosphorus treatment because the upper end grew faster than the lower end. The deep genotype DOR364 did not show any difference of growth in the upper radical between low and high phosphorus treatments (Table 4.1).

Discussion

Our results support the hypothesis that the change in basal root growth angle with low phosphorus availability is mediated by ethylene, at least in some genotypes. Specifically, low phosphorus induces shallower basal roots through increasing ethylene synthesis or by increasing tissue sensitivity to ethylene, which, in turn, changes the root gravitropic

responses. If ethylene regulates basal root gravitropic responses, then the following should be true: (1) ethylene manipulation should affect basal root shallowness, i.e., increasing ethylene synthesis should have the opposite effect as decreasing it or blocking ethylene action; (2) there should be a dosage response to ethylene manipulation: the basal root growth angle should respond to exogenous ethylene or ethylene inhibitors in proportion to the applied concentration; and (3) the endogenous ethylene level or response to ethylene should be a good indicator of basal root shallowness. As we have shown, basal roots grow shallower under high phosphorus with applied ethylene or ACC (Fig. 4.5 & 4.6), and conversely, ethylene synthesis or action inhibitors AVG and STS prevent basal roots from growing shallow under low phosphorus (Fig. 4.8). The extent of basal root growth angle change is proportional to the concentrations of ACC or AVG added to the media (Fig. 4.5, 4.9), and ethylene production by basal roots is strongly correlated with the basal root growth angle (Fig. 4.10-4.11). These results suggest that ethylene positively regulates basal root shallowness. However, ethylene production by roots of most genotypes examined was statistically the same for both low and high phosphorus (Fig. 4.7). This implies that ethylene biosynthesis is not the only factor determining the basal root growth angle in response to phosphorus availability.

The tissue sensitivity to ethylene could be just as important in the phosphorus response as the absolute amount of ethylene produced by the basal root. Basal roots grown with low phosphorus were more sensitive to AVG and STS than under high phosphorus, and the shallow genotypes were affected to a greater extent by ethylene inhibitors under low phosphorus than the deep genotypes (Fig. 4.8 & 4.9), implying that phosphorus availability and genotype together determine the basal root sensitivity to ethylene. This result also suggests that ethylene sensitivity is more important for plants grown with low phosphorus than high phosphorus, and ethylene biosynthesis is more important for plants grown with high phosphorus than low phosphorus, in terms of basal root growth angle change. It is likely that low phosphorus decreases the threshold of ethylene required by the basal root to change the angle, so the basal root grows shallower even the ethylene synthesis remains about the same as with high phosphorus (Fig. 4.10). Exogenous ethylene treatment fails to reduce basal root angle for plants grown with low

phosphorus, possibly because the response has already been saturated. Likewise, ethylene inhibitors fail to affect basal root angle for roots grown with high phosphorus because the ethylene levels are already below the response threshold. Some basal roots respond to ACC by changing the growth angle even though there is no measurable increase in ethylene production (Fig. 4.6-4.7).

For the shallow genotype G19833, the growth angles of the basal roots in response to phosphorus availability depended on their position (Fig. 4.4), with the roots closest to the surface being the most sensitive to phosphorus availability, and the further away from the surface, the less sensitive the root. However, the roots nearest to the surface (or shallower roots) were less sensitive to ACC dosage than the roots that were farther from the surface (deeper roots) (Fig. 4.5). The possible reason for this is that phosphorus availability affects ethylene sensitivity the most for the root, and least for the root farthest from the surface. So the root closest to the surface is the most sensitive to ethylene under low phosphorus, and least sensitive under high phosphorus. It is possible that the basal root plasticity, or the extent that the root can change its growth angle in response to environmental stimuli, is pre-determined genetically and developmentally, and phosphorus availability and ethylene are among those stimuli that can cause the basal roots to realize their potential plasticity. The differences among roots in response to phosphorus availability related to their position on the radical implies that, within a root system, some basal roots are more responsive to factors affecting angle than others.

The asymmetric expansion of the upper- and lower end of the basal region of the radical, from which basal roots originate, might play a role affecting basal root growth angle, by a geometric effect. This might be partially the reason the basal root shallowness does not change much for DOR364 under high and low phosphorus (Fig. 4.3). However, since basal roots do not grow perfectly straight (Fig. 4.1), the uneven growth of the base region of the radical is not likely to have a big influence on the basal root growth angle. Results from computer simulation confirms that the influence of the uneven growth of the base is limited (not shown). Instead the differential growth may be another thing that varies with genotype and phosphorus treatment and may be a result of some of the same

factors that also affect the basal root angle. Since the basal root continuously adjusts its growth angle (or GSA) during its development (Fig. 4.1), the initial GSA will not have a long effect on its shallowness.

Our result that basal root growth angle could be altered by phosphorus and genotype, supports the hypothesis that this process be regulated by ethylene. The alteration of basal root growth angle illustrates the concept of GSA, which is controlled genetically and developmentally (Firn and Digby 1997). The implication of this work to agriculture include accessing and evaluating root traits important for phosphorus acquisition, which should be an objective of crop breeding programs for enhancing phosphorus acquisition efficiency.

Spatially relocating the root closest to the possible phosphorus sources in the soil is an economic and efficient strategy for plants to minimize the cost associated with such relocation and maximize the gain. This ability is a desirable agricultural trait for improving phosphorus acquisition efficiency in phosphorus deficient regions.

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Table 4.1. The relative growth of the upper and lower ends of the region of the radical where basal roots emerge. Values inside parentheses are SE of mean. N = 20.

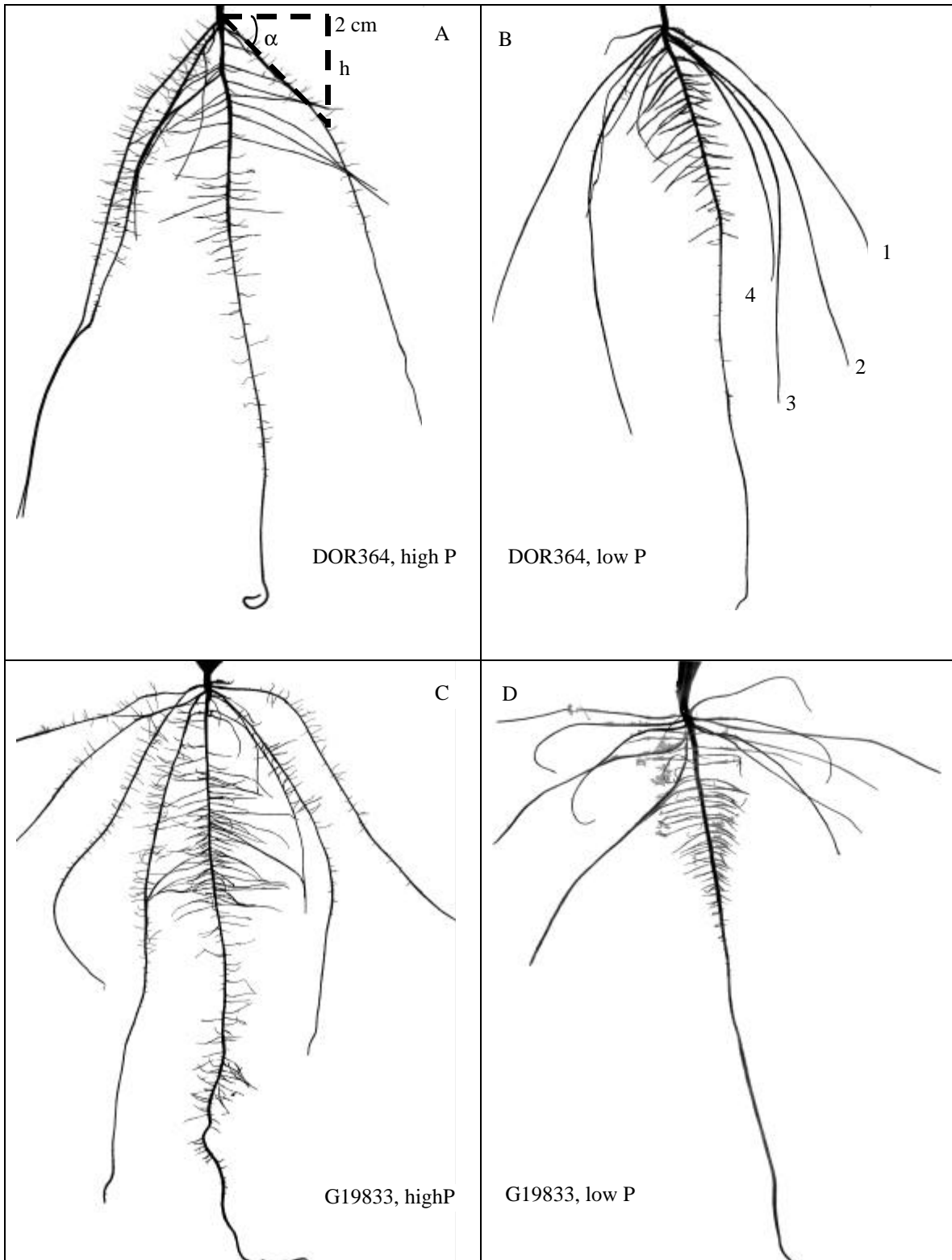| Days after germination	Diameters (mm)					
	Day 2		Day 5		Growth increment	
	Lower end	Upper end	Lower end	Upper end	Lower end	Upper end
DOR364, low P	2.3 (0.050)	2.9 (0.038)	2.7 (0.049)	3.2 (0.07)	0.4	0.3
DOR364, high P	2.1 (0.049)	2.7 (0.057)	2.5 (0.044)	2.9 (0.068)	0.4	0.2
G19833, low P	2.3 (0.041)	3.2 (0.063)	2.9 (0.051)	3.5 (0.060)	0.6	0.3
G19833, high P	2.6 (0.058)	3.2 (0.052)	2.7 (0.066)	3.7 (0.069)	0.1	0.5

Fig. 4.1. Scanned 2-D images of roots of two parental genotypes used in this experiment. DOR364 is a deep genotype with a larger basal root growth angle than genotype G19833. As shown in image A, the growth angle 'α' is estimated by measuring 'h' 2 cm away from the origin of the basal root, and α = atan(h/2) * 180/PI. Image B shows numbered basal roots according to their positions.
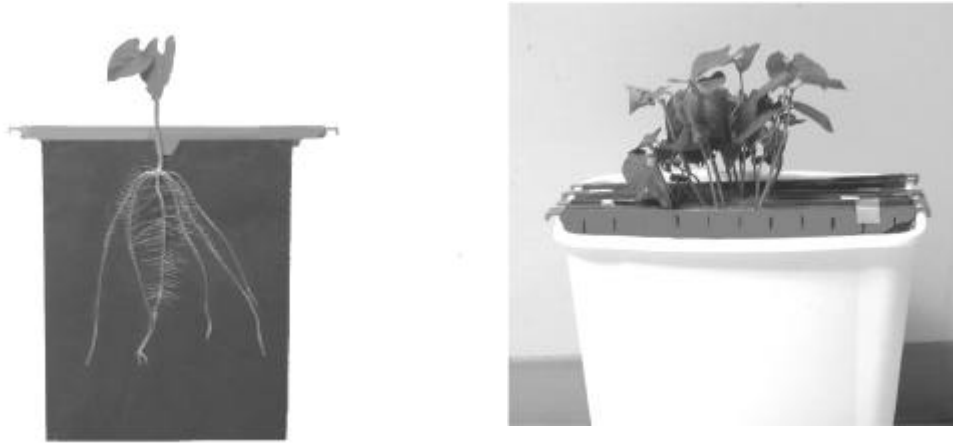
Fig. 4.2. A common bean plant cultured in plastic pouch (left), which was hung in the trash can (right) containing nutrient solution with high P or no P. The phosphorus-free blue germination paper was placed inside a polyethylene bag, which was punctured evenly with small holes (r = 5 mm) to improve aeration. A germinated seed was placed 2 - 3 cm below the top of the germination paper.
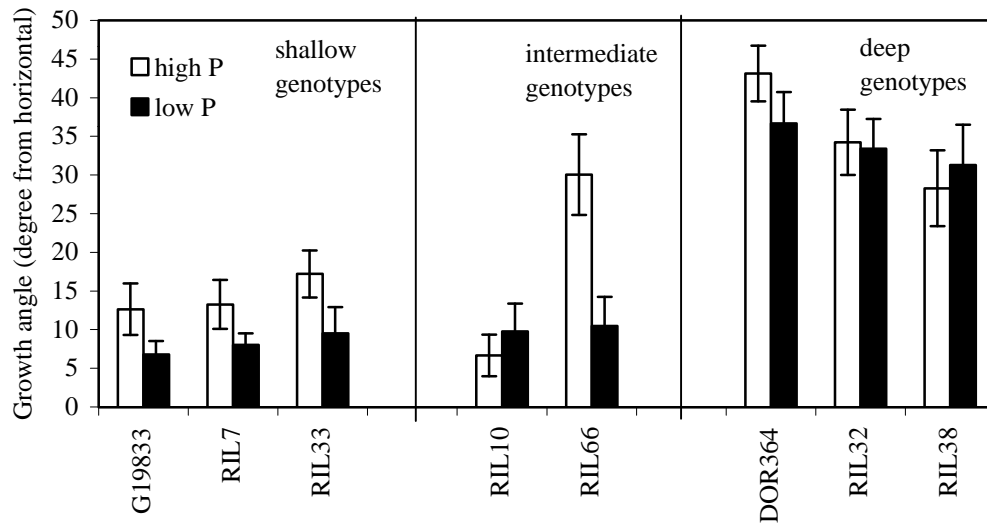
Fig. 4. 3. Response of basal roots to P availability. Genotypes were grouped into shallow, intermediate, and deep classes based on previous work. All shallow genotypes became shallower under low P (unpaired t-test of basal root angle between low and high P is significant for all shallow genotypes at $\alpha = 0.05$ level). The deep genotypes remained deep under low P. RIL 10 had shallow basal roots under both high and low P in this experiment. RIL 66 was significantly shallower under low P compared to high P ($\alpha = 0.05$). Bar: standard error of mean.
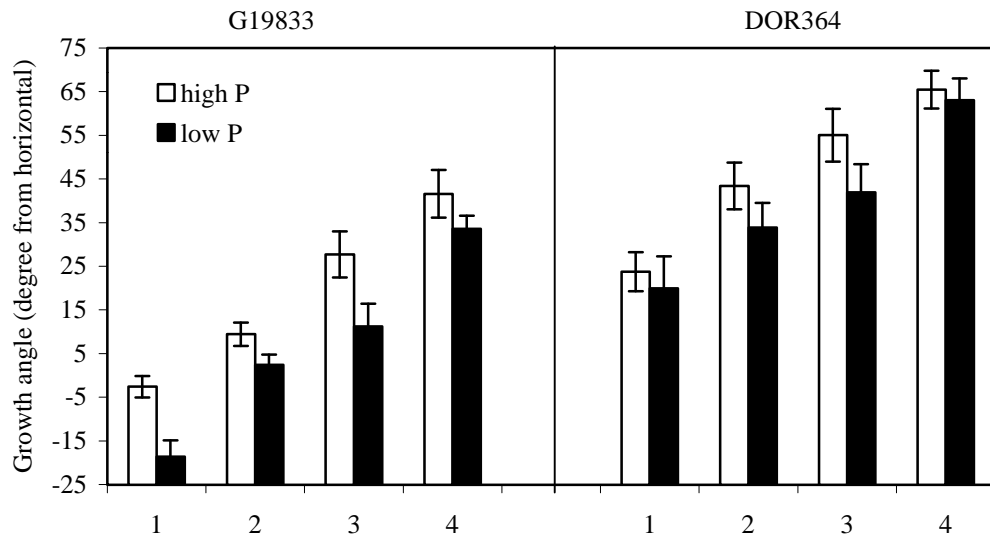
Fig. 4.4. Basal root angle depends on genotype, P availability, and position of roots. The first basal root is the closest to the surface, so it is the shallowest root; the fourth is farthest from the surface, so it is deep. The shallow genotype G19833 responds to low P by growing shallower for basal roots 1, 2, and 3. All 4 observed basal roots of DOR364 are not sensitive to P availability in graviresponse. Bar: standard error of mean.
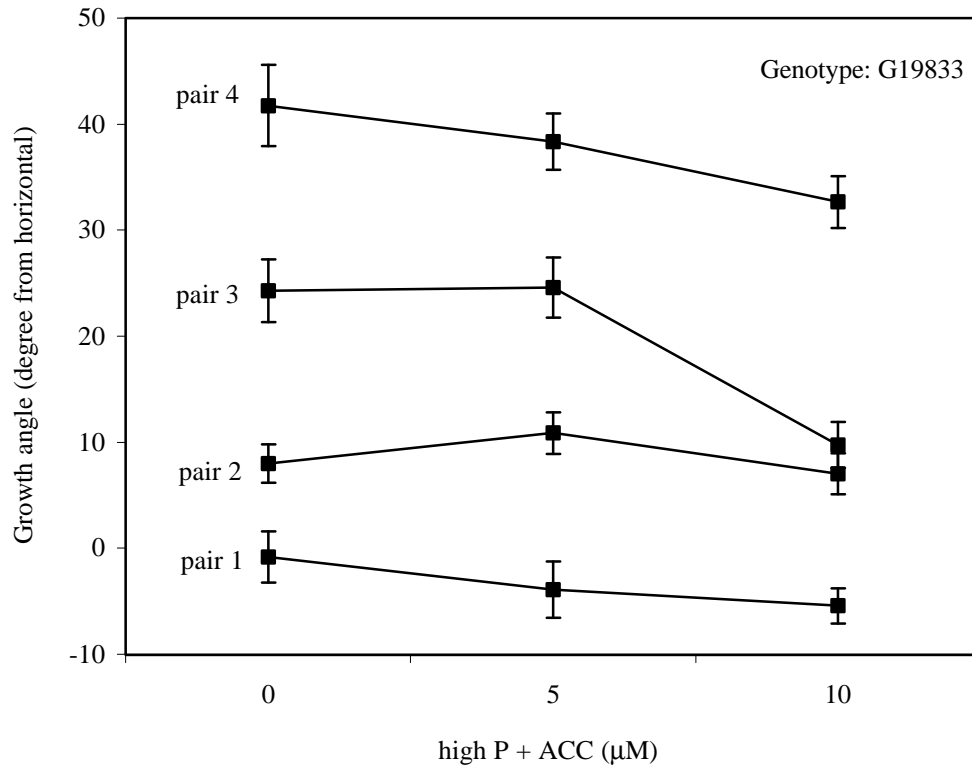
Fig. 4.5. Effects of ACC dosage on the growth angle of basal roots grown with high P. Pair 1 refers to the basal roots closest to the surface, and pair 4 is the farthest from the surface. Bar: standard error of mean.

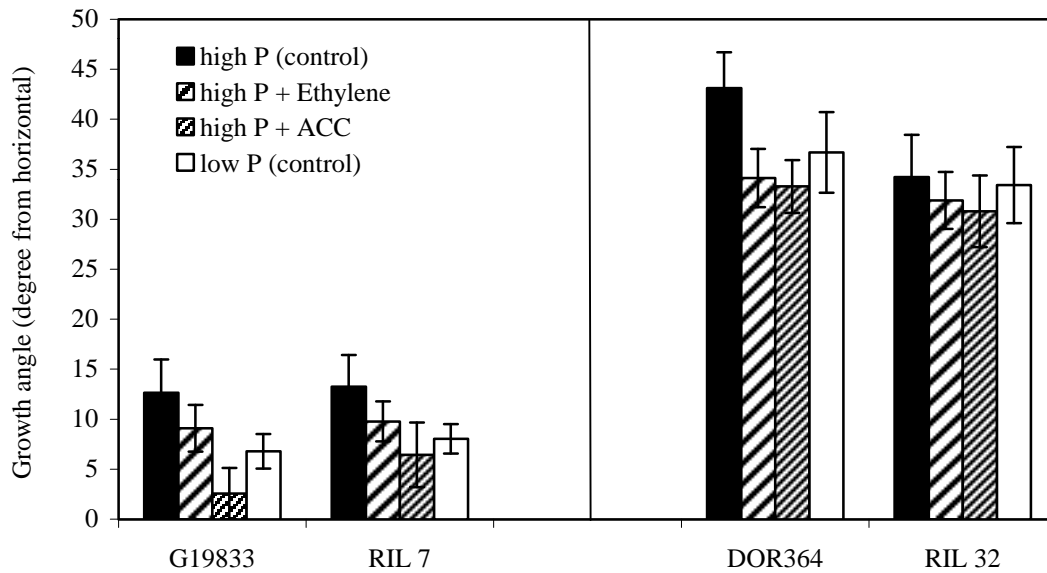Fig. 4.6. Effect of gaseous ethylene (1 μL/L) and ACC (10 μM) on basal root shallowness. Both made basal roots shallower with high P. Significant differences (α = 0.05) of growth angle existed in high P vs. high P + ACC for G19833, and high P vs. high P + ACC and high P + Ethylene for DOR364. They have no effect under low P (not shown). Bar: standard error of mean.
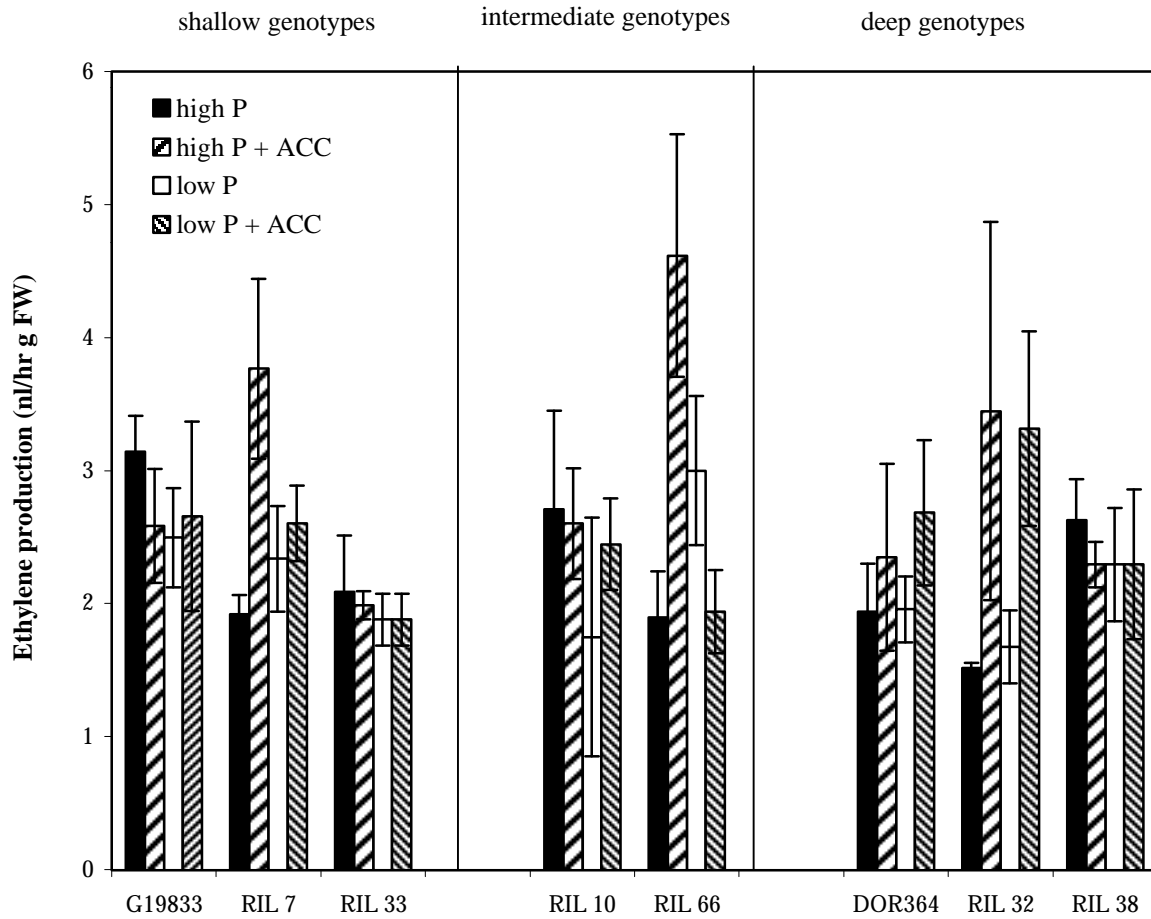
Fig. 4.7. Effect of 5 μM ACC on ethylene production by basal roots. ACC significantly increased ethylene production for RILs 7, 66 and 32 under high P, and RIL32 under low P (α = 0.05), but did not affect other genotypes. Bar: standard error of mean.

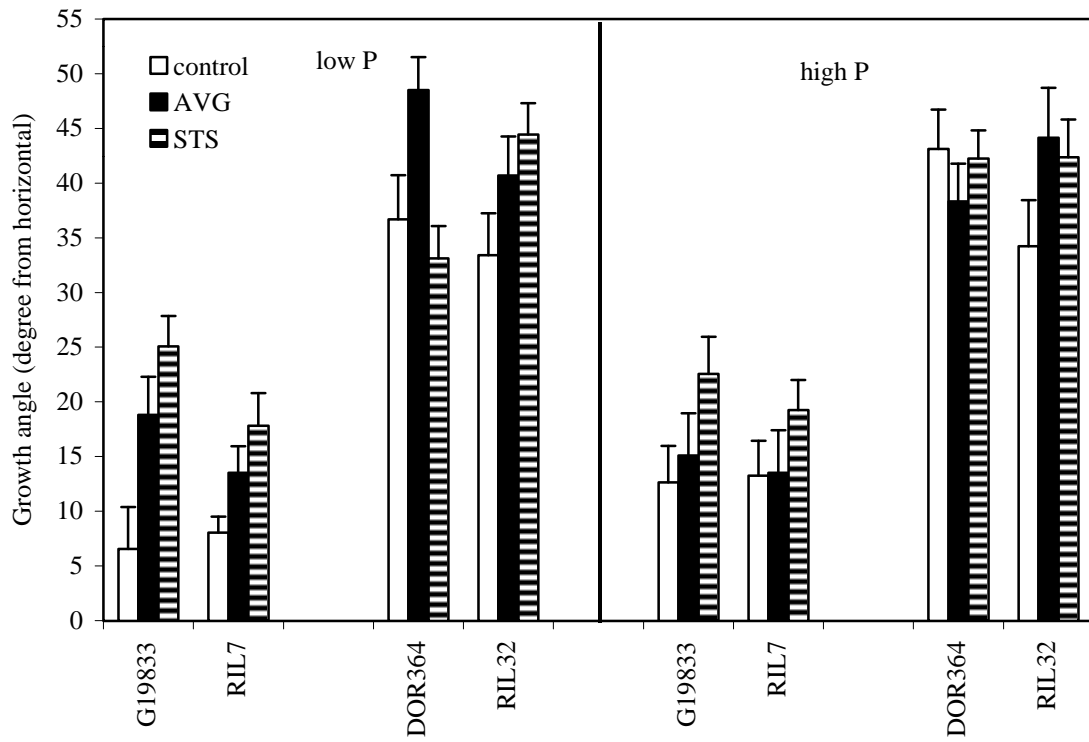Fig. 4.8. Effects of AVG (5 μM) and STS (10 μM) on basal root shallowness. AVG and STS had a strong effect under low P. Growth angle increased by 77.4% for AVG and 106.9% for STS. They had no significant effect under high P (data not shown). Bar: standard error of mean.
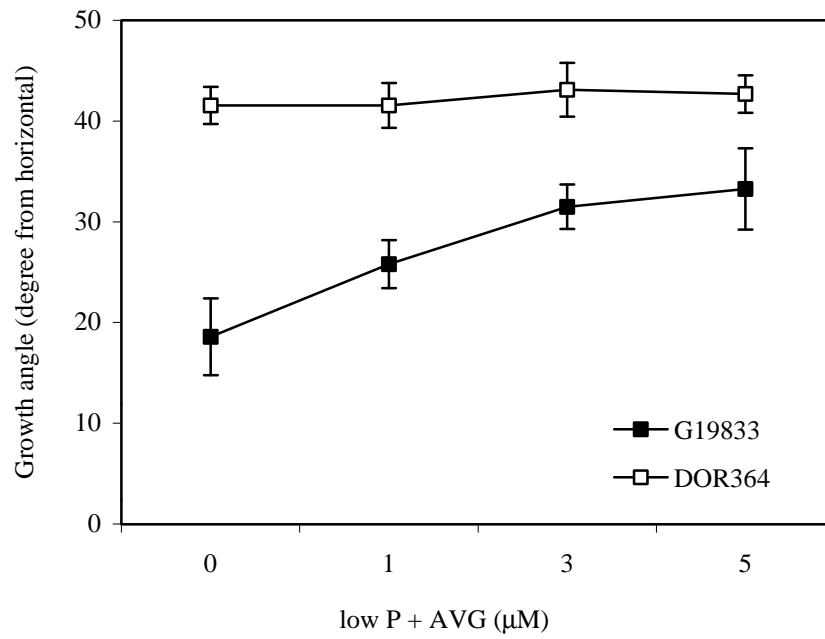
Fig. 4.9. Effects of AVG dosage on shallowness. Plants were grown in low P medium without AVG (control), or with 1, 3 or 5 μM AVG. Bar: standard error of mean.
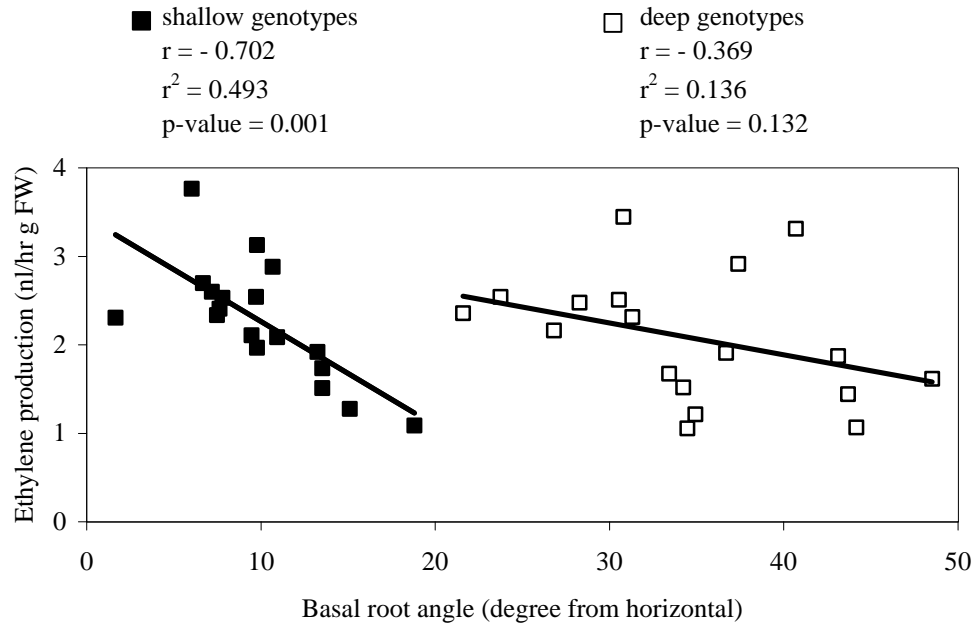
Fig. 4.10. Correlation between basal root angle and ethylene production by shallow genotypes (G19833, RILs 7 and 33) or deep genotypes (DOR364, RILs 32 and 38) for all the data, including ACC and AVG treatments. A high correlation was found for shallow genotypes, but the correlation for deep genotypes was not significant.

Fig. 4.11. High correlation between ethylene production and basal root angle of G19833. Correlation coefficient r = -0.624, P-value = 0.03

Fig. 4.12. Diameters of the region where basal roots emerge were measured at d1, which is the lower end of the region, and d2 (the upper end). Bar = 1.0 mm.

Chapter 5

Improvement in Quality and Drought Tolerance of Woody Ornamental Plants Grown in Buffered Phosphorus Fertilizer

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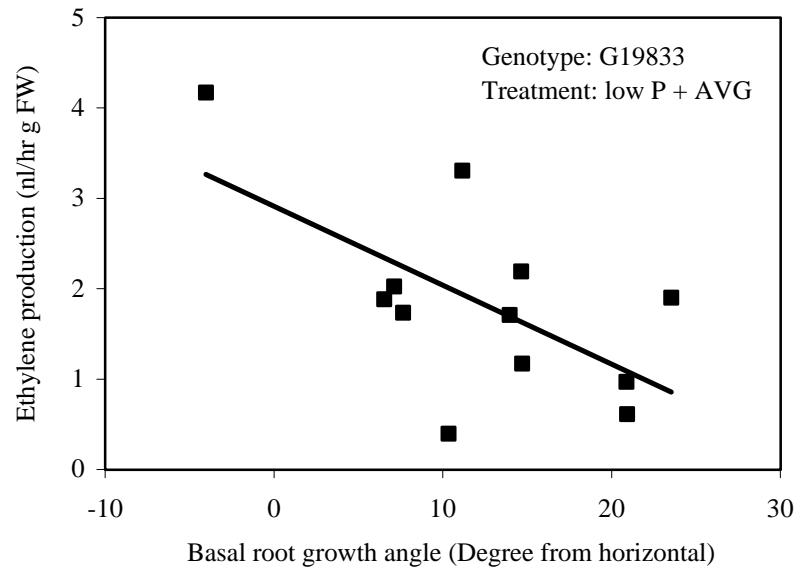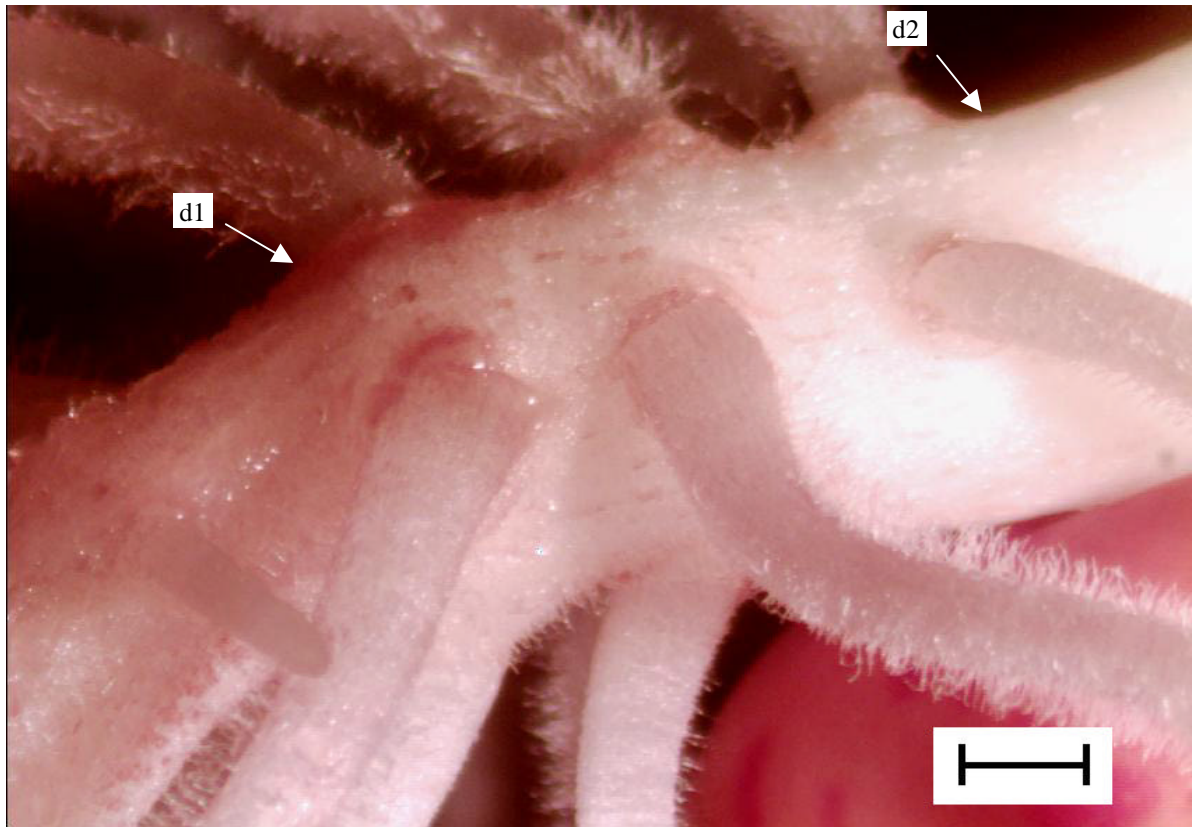
Abstract

The effects of alumina-buffered phosphorus (Al-P) were evaluated on growth and drought tolerance of woody plants and on seedling establishment of several tree species grown in containers with soilless media. Al-P reduced phosphorus leaching while improving plant drought tolerance. When plants were fertilized with Al-P prepared at a phosphorus desorption rate of 74 μM, vegetative growth of Rhododendron (*Rhododendron catawbiense* Michx. cv. 'English Roseum'), Forsythia (*Forsythia intermedia* Zab. cv. 'Spring Glory'), Ohio buckeye (*Aesculus glabra* Willd.), and Bur oak (*Quercus macrocarpa* Michx.), measured as plant height, stem caliper, and/or biomass, was as fast as, or faster than that observed with Osmocote or monoammonium phosphate fertilizer. Imposition of summer drought during the first growth season slightly reduced growth of Rhododendron, with a stronger effect in the second year, while Forsythia was more affected in the first season. The drought effect on growth was mitigated by Al-P fertilization in Forsythia but not in Rhododendron. When Al-P was used, more Rhododendron plants produced flower buds in the first year, and at the lower desorbing concentration, drought caused no reduction in percent of plants producing flower buds. Al-P reduced branching and flowering in the second year, possibly as a result of damage from the recharging treatment.

Index words: leaching, *Forsythia*, *Rhododendron*, *Ohio Buckeye*, *Bur Oak*

Species used in this study: Rhododendron (*Rhododendron catawbiense* Michx.), Forsythia (*Forsythia intermedia* Zab.), Ohio buckeye (*Aesculus glabra* Willd.), Bur oak (*Quercus macrocarpa* Michx.)

Solid-phase buffered phosphorus (Al-P), a novel phosphorus fertilizer that dynamically provides phosphorus to plant roots based on actual plant phosphorus requirements, has been evaluated in soilless culture of flowers including marigold and impatiens (1, 2, 7), and shrubs including Rhododendron and Forsythia (3). With Al-P, phosphorus leaching is dramatically reduced compared with conventional fertilization methods (1, 3, 7). Lin *et al*. (7) found that marigolds grown with Al-P showed superior growth and produced higher biomass compared to marigolds grown using commercial fertilizers. Borch *et al*. (1) reported that marigold and impatiens plants grown with the Al-P fertilizer were more resistant to drought than conventionally grown plants, and floral wilting was reduced for both species grown with Al-P. In addition, marigold plants grown with Al-P produced more flowers, and their roots were more evenly distributed through the medium. Al-P improved plant growth of two woody species, Forsythia and Rhododendron during a single season compared with soluble or slow-release phosphorus fertilizers (3). For Forsythia, the highest shoot dry mass was obtained from mixing Al-P with the growth medium at 0.5% w/v; while for Rhododendron, 1.0% Al-P treatment gave the largest plants. In this paper, we describe the growth responses to Al-P of Rhododendron and Forsythia plants over two growing seasons and after the imposition of drought stress. We hypothesized that woody plants, like bedding plants, may be more tolerant of drought stress when grown with Al-P.

We have also tested the effects of Al-P fertilization on the growth of young tree seedlings. Culture of such seedlings may provide the industry with an improved method to produce container-grown shade trees. However, one difficulty of doing so is that seedlings of some tree species establish a long taproot. When this root is cut during transplanting, the tree may take several years to establish an adequate root system. We had found that low phosphorus availability stimulated the root growth of common bean, marigolds, and other species (1, 6, 8). Therefore, low but consistent phosphorus supplied by Al-P might be beneficial to establishment of tree seedlings by improving root proliferation.

Materials and Methods

One-year old rooted cuttings of Rhododendron (*Rhododendron catawbiense* Michx. cv. 'English Roseum') and Forsythia (*Forsythia intermedia* Zab. cv. 'Spring Glory') were purchased from Appalachian Nurseries, Inc., Waynesboro, PA, in spring, 1999. Ohio buckeye (*Aesculus glabra* Willd.) and Bur oak (*Quercus macrocarpa* Michx.) seeds were collected in the Fall of 1998 from trees growing in State College, PA, and stratified in moist peat moss at 3 C for three months. The seeds were planted on February 25, 1999 in D-40 DeePots (DeePots[TM], Stuewe & Sons, Inc., 2290 SE Kiger Island Drive, Corvallis, Oregon 97333-9425, USA) in Sunshine No. 4 medium (Canadian Sphagnum peat moss, perlite, gypsum, and dolomitic lime, J. R. Johnson Supply, Inc., 2582 Long Lake Road, St Paul, MN 55113, USA). Fertilizer (21-7-7, with micronutrients) solution was applied twice per week to supply 2 mM (60 ppm) N for the first month and 3 mM (90 ppm) N for the second month. Germination and cultivation were carried out in a Pennsylvania State University greenhouse located in University Park, PA (longitude: W77.8, latitude: N40.8). Day-night average temperature was maintained in the range of 28-18 C.

On May 13, 1999, seedlings were transplanted to #2 nursery pots. The inside of the pots was painted with Spinout (Griffin Corp., Valdosta, Georgia, active ingredient 7.1% copper hydroxide in latex paint) prior to planting. The growth medium used was Fafard Mix No. 52 (Fafard Peat Moss Co., Ltd., 422 Chemin Pallot, Inkerman, NB, Canada E8P 1B5). It consists of processed pine bark (60%), Canadian sphagnum peat, and vermiculite. Conventional phosphorus or Al-P fertilizers were mixed with the medium to obtain comparable availability of all nutrients except phosphorus (Table 5.1). Throughout this paper, the treatment with Al-P desorbing at 74 μM phosphorus will be referred to as Al-P/74, and that with Al-P desorbing at 127 μM phosphorus will be referred to as Al-P/127. Al-P was prepared according to Lynch *et al*. (9).

Plants were grown at Penn State's Russell E. Larson Research Facility in Rock Springs, PA, under daily irrigation during the growth season. Plants were covered between Dec. 9, 1999 and Feb. 20, 2000 with plastic ThermoBlanket (Cady Bag Co., Inc.

PO Box 68, Pearson, GA 31642, USA) to protect the roots. In June 2000, Al-P was recharged by adding a 5 mM KH_2PO_4 solution (pH 5.2) at 1 L/pot for Al-P/127, and 2.5 mM for Al-P/74. Pots were leached thoroughly after 2 days. All other fertilizers were re-applied to the top of the medium, at the same rate as in the first year. There was no irrigation during recharging. The phosphorus content of the irrigation water was 0.4 µM in the greenhouse and 0.07 µM at Rock Springs.

Leachate samples were collected from saucers beneath the pots 15 min after irrigation with 500 ml water in addition to the daily irrigation. Phosphorus concentration in leachate was measured according to Murphy & Riley (11).

Drought treatment of Rhododendron and Forsythia started on August 9, 1999, and lasted for 2 weeks. Plants were placed in a shed covered with transparent plastic film (roof only). During the study, stomatal conductance of leaves was monitored with a steady state porometer (Model LI-1600, LI-COR, Inc., Lincoln, NE) around 10 AM every day until values became too low to measure. The date of visible wilting was recorded. Table 5.2 lists daily high and low temperatures, relative humidity (RH, %), wind speed, and precipitation during the treatment.

Plant height and stem caliper of all species, flower bud differentiation of Rhododendron and Forsythia, and shoot and root weight of Forsythia were measured at the end of November in 1999 and 2000. Plant height was measured from the surface of the medium to the top of the plant. For stem caliper measurement, stems were marked 2-3 cm above the medium with a permanent mark, and the same place was measured with a caliper after 2 years. Forsythia shoots were pruned to a height of 15 cm, and FW and DW of pruned branches were recorded. For leaf analysis, fully expanded leaves were collected, dried in an oven at 60C, and dried samples were analyzed at Penn State's Agricultural Analytical Services Laboratory.

For Buckeye and Bur oak, a single factor (fertilizer treatment) randomized block design was used. For Rhododendron and Forsythia, a second factor, drought, was

combined with the factor of phosphorus fertilizer in a two-factor randomized block design. Each treatment was replicated 3 times with 4 plants per replication serving as subsamples, for a total of 12 plants per treatment. ANOVA of the data was done with the statistical software package MiniTab® (Minitab Inc., 3081 Enterprise Drive, State College, PA 16801-3008, USA).

Results and Discussion

*Al-P reduced phosphorus leaching***.** The phosphorus levels in leachate from Al-P with both rates of phosphorus desorption were stable and low in comparison with Osmocote or MAP in both 1999 and 2000 (Fig. 5.1). Phosphorus released from Osmocote treated pots increased during the season, reaching a peak in mid-summer and then decreasing as temperature dropped (Fig. 5.1). The phosphorus level in leachate from pots with MAP was 1791 ± 209 µM at first measurement, a level about one order of magnitude higher than that from other treatments, and then decreased sharply in the second and third samplings (Fig. 5.1). As shown previously (3), plants grown with Al-P maintained a relatively low and steady rate of phosphorus leaching within the range of 1.3 to 79.0 µM phosphorus (Fig. 5.1).

At the beginning of the second season, additional MAP and Osmocote was added to the surface of the medium. Phosphorus leaching during the second season was considerably less than in the first season, when fertilizers were incorporated, but continued to be higher from Osmocote or MAP treated plants than from Al-P treated plants (Fig. 5.1). For example, the leachate sample of Osmocote treatment for Forsythia on September 9, 2000, contained 72.7 µM phosphorus, much higher than Al-P /74 (3.9 µM) and Al-P/127 (3.4 µM) treatments. We estimate by integrating the phosphorus leaching curve that Al-P reduced leaching by 86-88% compared with the control (Osmocote) for Rhododendron and Forsythia in 1999, and by 56-59% for Rhododendron and 72-78% for Forsythia in 2000.

Since the P in the Al-P fertilizer would eventually become depleted, we recharged the Al-P *in situ* early in the 2000 season by adding 1 L of 5 mM KH_2PO_4 solution to each pot, leaving it for 2 days, then leaching. After the *in situ* phosphorus recharging of alumina, both Rhododendron and Forsythia showed leaf chlorosis, with more severe symptoms in Forsythia. Leaf analysis indicated that Al-P grown plants of both species contained much higher K (0.96% vs. 0.66% for Forsythia; 0.92% vs. 0.71% for Rhododendron) and Mn ($\mu g/g$: 772 vs. 608 for Forsythia; 1272 vs. 416 for Rhododendron) than controls. The recharging solution contained a high concentration of K^+, which may have temporarily altered rhizosphere pH or microbial population following the period of recharging, leading to greater Mn uptake. The chlorosis disappeared in two weeks. Reapplication of Osmocote and MAP did not cause any visible phytotoxic effects. Further study is needed to optimize phosphorus recharging, including the use of lower concentrations of KH_2PO_4, and/or a shorter duration of recharging.

Drought stress response. Rhododendron plants grown with Al-P, especially at the low rate, wilted more slowly under drought stress than those grown with Osmocote. For example, of 12 plants treated with Al-P /74, Al-P /127 or Osmocote, 4, 7, and 9 plants, respectively, had wilted by day 8 of the drought treatment. The earlier stages of water stress were monitored by measuring stomatal conductance (Fig. 5.2). The higher stomatal conductance rate during the early part of the drought treatment for plants grown with Osmocote indicated that leaf stomata did not respond to drought as quickly as Al-P plants, which may have affected their ability to avoid water loss under stress. In plants that had been fertilized with Osmocote, young shoots had not stopped growth when drought stress started, while all shoots from Al-P treated plants had formed top buds. This difference in phenology may have affected the differences in time to wilting observed among the treatments.

Two-factor ANOVA (phosphorus level and drought) shows that for both 1999 and 2000, phosphorus treatments did not affect Rhododendron caliper or height (Table 5.3). This result suggests that Al-P provided adequate phosphorus for growth in terms of caliper and height. However, branching (assessed as total buds, since there were no blind

shoots) was significantly reduced in the Al-P treatments in the second year (Table 5.3, Fig. 5.3). Drought treatment imposed during 1999 had significant effects on height, stem caliper, and branching, especially at the end of the second season (Tables 5.3 and 5.4, Fig. 5.3 and 5.4). The reduction in branching of Al-P treated plants in the second year may have been caused by damage from the Al-P recharging treatment, described above. Another possibility could be that the Al-P fertilized plants did not receive adequate phosphorus during the second season. Except the first measurement, phosphorus concentration in the media with Al-P treatments was below 20 µM in 2000 (Fig. 5.1). However, leaf analysis showed no significant differences in tissue phosphorus concentrations among treatments. Phosphorus concentrations were always in the range 0.10-0.12%, and growth was not significantly reduced by Al-P, indicating that P supply was adequate.

Reproductive development was significantly affected by phosphorus treatment and drought (Tables 5.3 and 5.4). More plants grown with Al-P produced flower buds in the first year, and at 74 µM Al-P there was no effect of drought on flower bud formation. At the end of the 2000 season, all plants produced flower buds, but Al-P fertilized plants produced fewer branches and fewer flower buds than plants grown with Osmocote (Fig. 5.3). In addition, the proportion of shoots with flower buds rather than vegetative buds was 12-13% for Al-P plants and 24% for Osmocote-fertilized plants. The proportion of flower buds was unaffected by drought except in Al-P/127 plants, which had only 8% of buds as floral.

We observed better, more extensive root growth of Rhododendron from Al-P treatments (Fig. 5.5). Fine roots completely filled the medium throughout the pot for Al-P treatments, but barely extended into the lower half of the pot in the Osmocote treatment. The difference in root growth could have been responsible for the differences in drought resistance and flowering.

Forsythia plants did not have any noticeable difference in wilting during drought stress. The stomatal conductance of plants grown with Osmocote was lower than that of

Al-P plants at days 1 and 2 (Fig. 5.6). Since we did not measure initial stomatal conductance in well-irrigated plants, we do not know if this was a pre-existing difference or whether the Osmocote fertilized plants responded very quickly to the drought stress. The former possibility seems more likely because the plants were irrigated daily, so plants were not likely to be water stressed only 24 h after the last irrigation.

The fresh weight of pruned tissue, collected when Forsythia plants were pruned to 15 cm at the end of each growing season, was increased by Al-P fertilization during both years of production (Fig. 5.7). The lower rate of Al-P gave the greatest increase in both years (Fig. 5.7). The reductions caused by drought in fresh weight of pruned tissues in plants fertilized with Osmocote, Al-P /74 and Al-P /127 were 51%, 41%, and 25%, respectively. In the second year after drought, there was no residual effect of the drought treatment on growth of plants fertilized with Osmocote or Al-P /74. Plants fertilized with Al-P /127 and exposed to drought showed a 28.7% reduction in shoot fresh weight compared with irrigated controls, but still accumulated as much shoot weight as the Osmocote fertilized plants (Fig. 5.7). Forsythia plants fertilized with Al-P /74 had the highest shoot growth of all treatments. Leaves of plants from all treatments had tissue phosphorus concentrations between 0.10 and 0.12%.

In an experiment testing Al-P mixed with the medium at different rates, Brown *et al.* (3) reported that the highest biomass of Forsythia was from the treatment fertilized with 0.5% (w/v) Al-P (desorption rate at 200 μM), while for Rhododendron, 1% Al-P gave a higher biomass production. In this experiment, we used much lower desorption rates of Al-P (127 and 74 μM) at only one mixing rate (1% w/v). In this experiment, Rhododendron plants fertilized with Al-P at either desorption rate grew as fast as Osmocote treated plants. Al-P /74 gave a greater flower number after one season of growth (Table 5.4), which may be desirable for marketing. For Forsythia, there was no significant difference in flower production between Al-P and Osmocote plants (data not shown), but Al-P /74 plants had the highest growth rate (Fig. 5.7). The results from this paper and from Brown *et al.* (3) indicate that Forsythia growth is optimal at lower phosphorus availability.

Growth of tree seedlings fertilized with Al-P. The growth (stem caliper and plant height) of Buckeye and Bur oak was not significantly affected by phosphorus source, though buckeye grown with Al-P /74 produced a slightly taller plant (data not shown). During the experiment, buckeye grew slowly. For example, at the end of the 2 seasons of growth in this experiment, the average plant heights were below 20 cm. Bur oak plants were taller (the average plant height was in the range 55 – 75 cm), but plants usually did not branch. Al-P provided adequate phosphorus for growth of these species with reduced leaching but did not provide visible improvement in growth during the seedling stage.

Conclusions

1. Al-P substantially reduced phosphorus leaching from containerized woody plants compared with slow-release fertilizer (Osmocote) and conventional soluble fertilizer (MAP).

2. Al-P with a desorption rate of 74 µM provided adequate phosphorus for growth of woody plants.

3. Al-P promoted flower production in Rhododendron in first year.

4. Al-P increased growth rate of Forsythia and increased tolerance to drought.

Acknowledgements

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Table 5.1. Fertilizer Treatments for rhododendron, forsythia, burr oak, and buckeye

Sources of Nutrient	Fertilizer Treatment (g/L media)			
	MAP[1]	Osmocote	Al-P 74	Al-P 127
MAP 12-61-0	0.5	0	0	0
Osmocote 17-6-10 plus[2]	0	5	0	0
Osmocote 36-0-0[3]	2.5	0	2.5	2.5
Osmocote 0-0-44[3]	1.25	0	1.25	1.25
MicroMax	1	0	1	1
Pelletized lime	0.4	0	0.4	0.4
Al-P 74 μm[4]	0	0	10	0
Al-P127 μm[4]	0	0	0	10

(1). Treatment not included for rhododendron and forsythia.

(2). 12 month release formulation.

(3). 8-9 month release formulation.

(4). P desorption rate.

Table 5.2. Weather data during the drought treatment starting from August 9, 1999

Days after drought Treatment	Air temperature (^{o}C)			Average Dewpoint (^{o}C)	Average RH (%)	Wind speed (K meter/hr)	Precipitation. (mm)
	Average	Min.	Max.				
1	16.3	10.0	22.8	9.2	66.6	6.7	0
2	18.4	11.1	24.4	11.6	64.6	6.0	0
3	20.7	12.8	27.8	15.5	70.6	6.9	0
4	21.1	11.7	29.4	14.6	69.6	2.7	0
5	22.2	15.0	33.9	16.7	78.6	7.7	55.88
6	22.8	18.3	28.9	18.9	78.5	9.3	0
7	18.2	12.2	23.3	14.7	77.1	7.3	0
8	19.1	9.4	27.2	13.9	72.1	3.5	0
9	22.6	15.6	30.0	17.0	69.8	8.8	0.254
10	19.3	13.3	24.4	11.1	77.5	5.9	0.254
11	19.0	11.7	24.4	14.5	74.3	6.0	0
12	18.4	16.1	21.7	16.9	88.0	11.5	6.604
13	15.8	13.3	20.0	14.7	89.1	6.4	0.254

Table 5.3. P-values of two factor ANOVA (Rhododendron)

Factor	1999		2000			
	caliper	height	caliper	height	Number of Flower buds	Number of total buds
P	0.559	0.077	0.310	0.258	0.004	< 0.001
Drought	0.321	0.018	0.015	0.020	0.900	0.011
P*Drought	0.258	0.088	0.940	0.587	0.951	0.163

Table 5.4. Percentage of Rhododendron Plants Showing Flower Bud
Formation at the end of 1999 (in 2000 all plants produced flower buds)

P Source	Treatment	Plants with Flower Buds (%)
Osmocote	Drought	8.3
Osmocote	Control	16.7
Al-P 74	Drought	75.0
Al-P 74	Control	75.0
Al-P 127	Drought	16.7
Al-P 127	Control	41.7

1999   2000

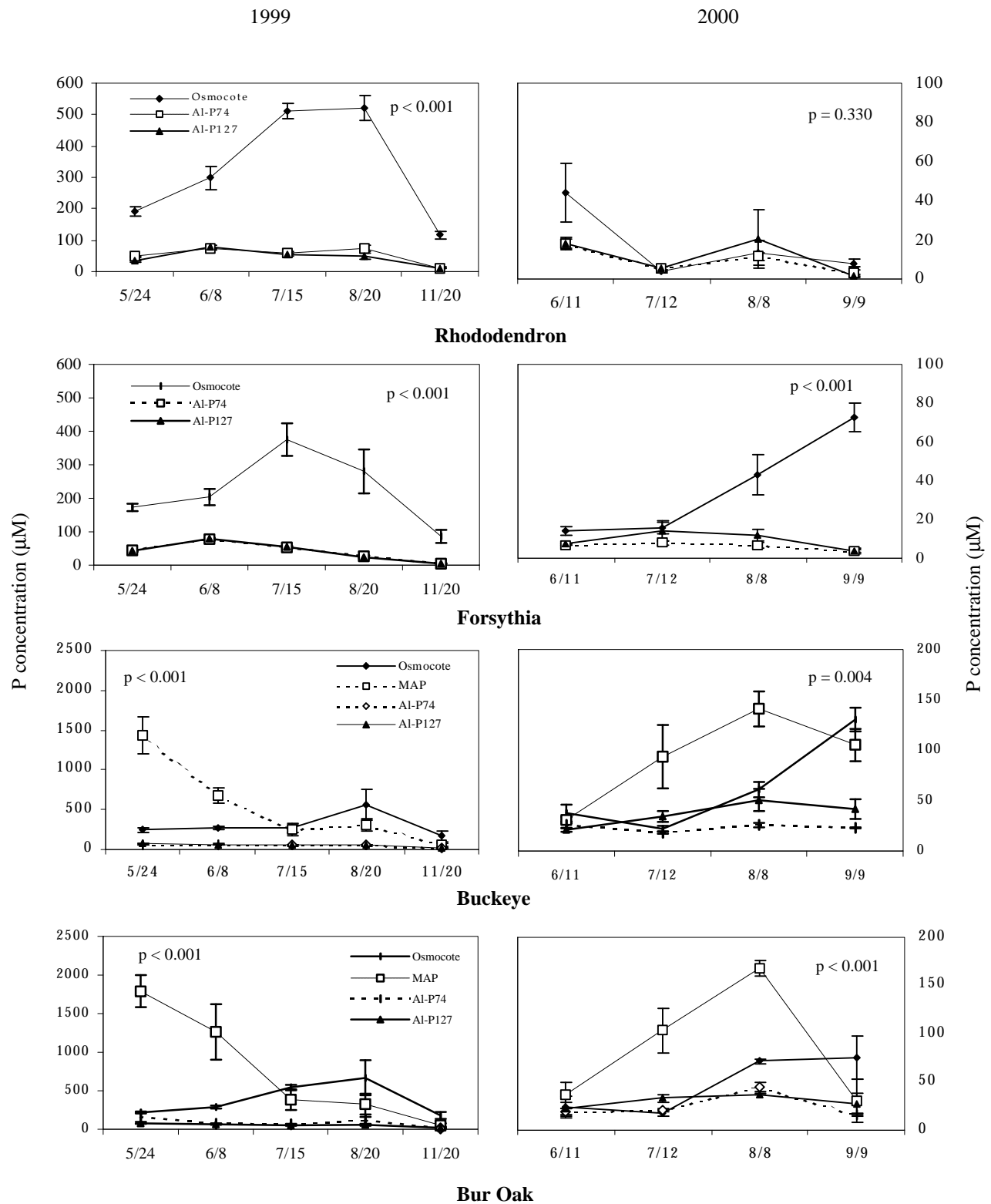**Rhododendron**

Forsythia

Buckeye

Bur Oak

Fig. 5.1. P concentration of leachate from Osmocote, MAP, and Al-P. Al-P/74 and Al-P/127: P desorption rate from Al-P is 74 or 127 µM. Bar represents standard error of mean. Figures in left column are for 1999 results, and right column for 2000 results. X coordinate: sampling date; Y coordinate: P concentration (µM) in leachate. The significance of treatment effects is shown as p-value from F-test. Note changes in scale of Y-axis.
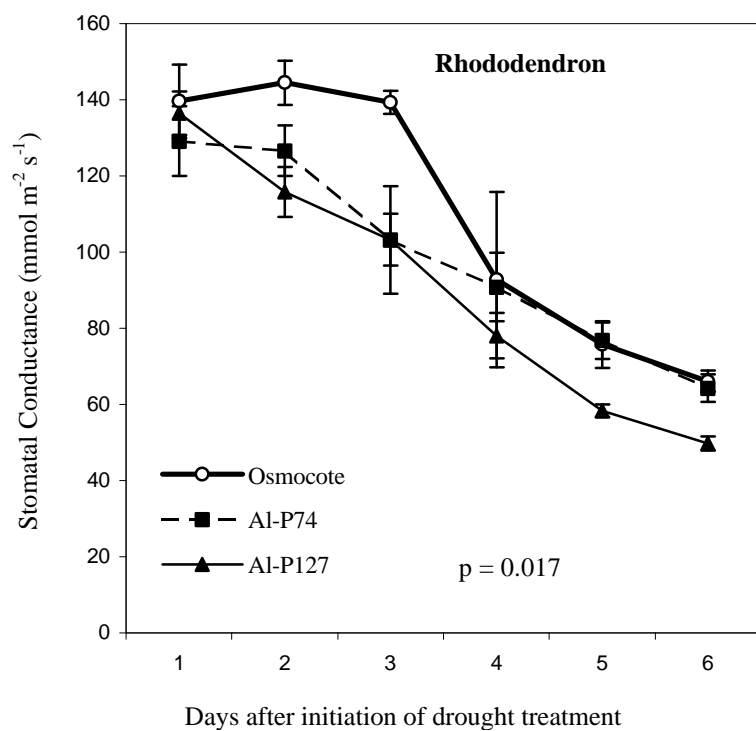
Fig. 5.2. Stomatal conductance of rhododendron leaves during drought stress. Bars represent standard error of mean. The significance of treatment effects is shown as p-value from F-test.
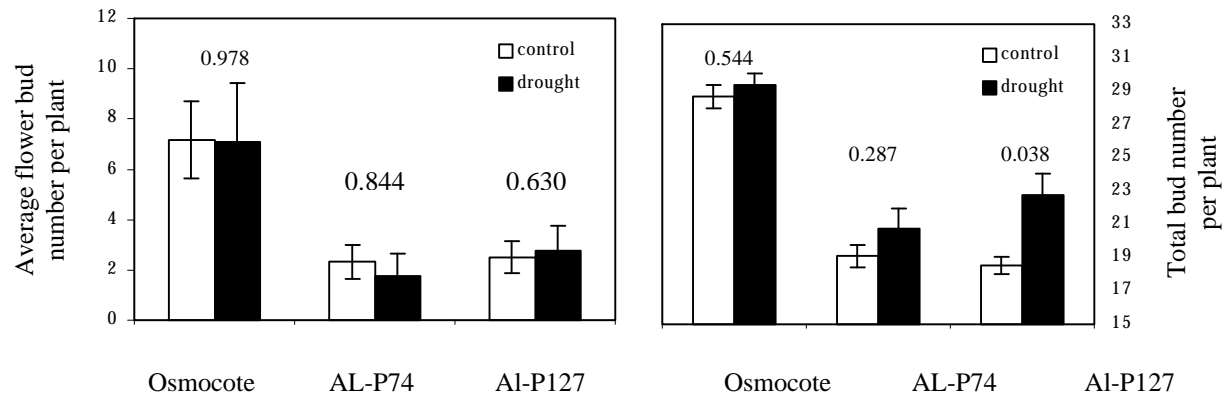
Fig. 5.3. Rhododendron flower bud number and total bud number at the end of 2000. These numbers are overall means of 12 plants for each treatment. Bar represents standard error of mean. P-values from t-test of treatments against irrigated controls are shown above the columns.
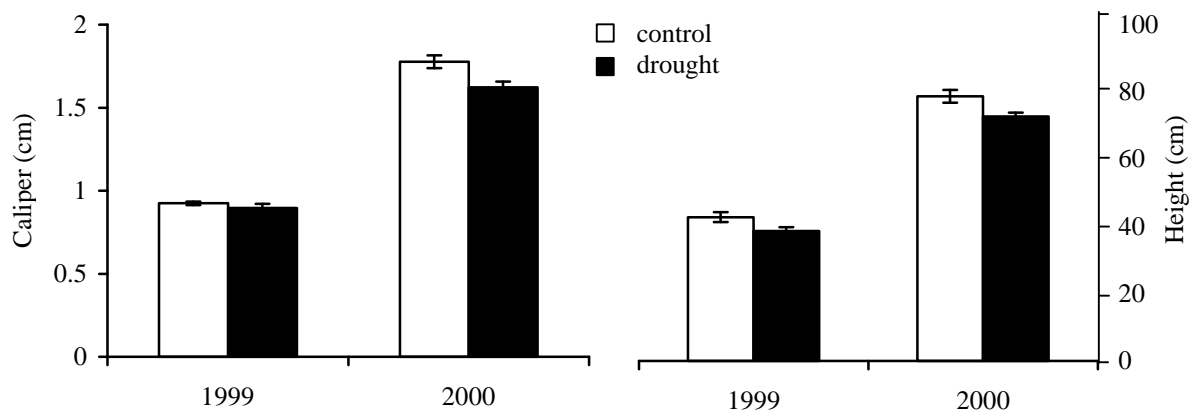
Fig. 5.4. Stem caliper and plant height of rhododendron at the end of 1999 and 2000.

Al-P/127　　　　　　Al-P/74　　　　　　Osmocote
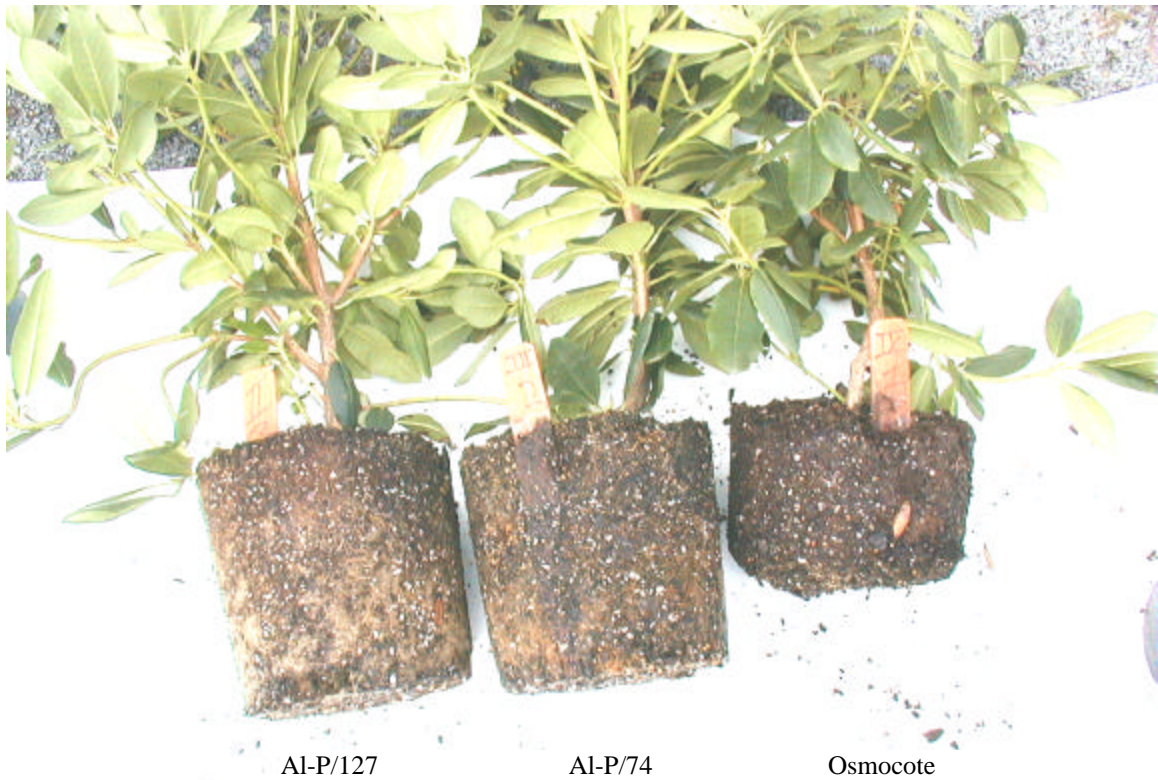
Fig. 5.5. Photo shows roots grown with P treatment. Treatment (left to right): Al-P/127, Al-P/74, and Osmocote. The root ball of Osmocote treatment is about half of that in two Al-P treatments. Photo was taken on Oct. 7, 2000.

Fig. 5.6. Stomatal conductance of forsythia leaves during drought stress. Bars represent standard error of mean. The significance of treatment effects is shown as p-value from F-test.

Fig. 5.7. Pruned shoot fresh weight of forsythia. Osmocote: 17-6-10 plus; AL-P 74: desorption rate of Al-P at 74 μM; AL-P 127: desorption rate of Al-P at 127 μM. Bars represent standard error of mean. P-values from t-test of treatm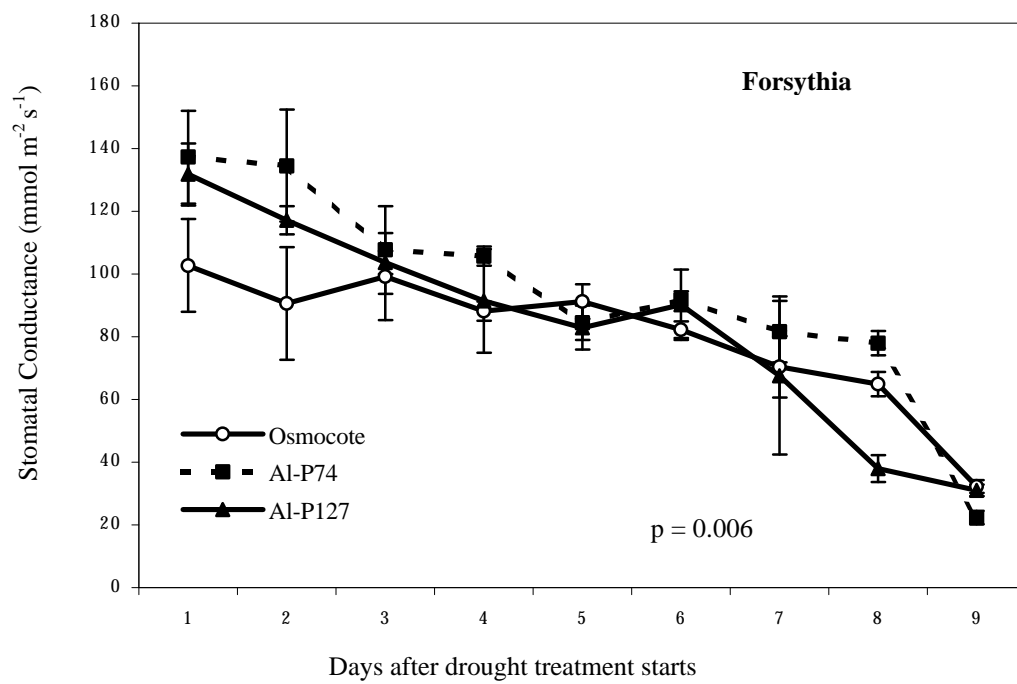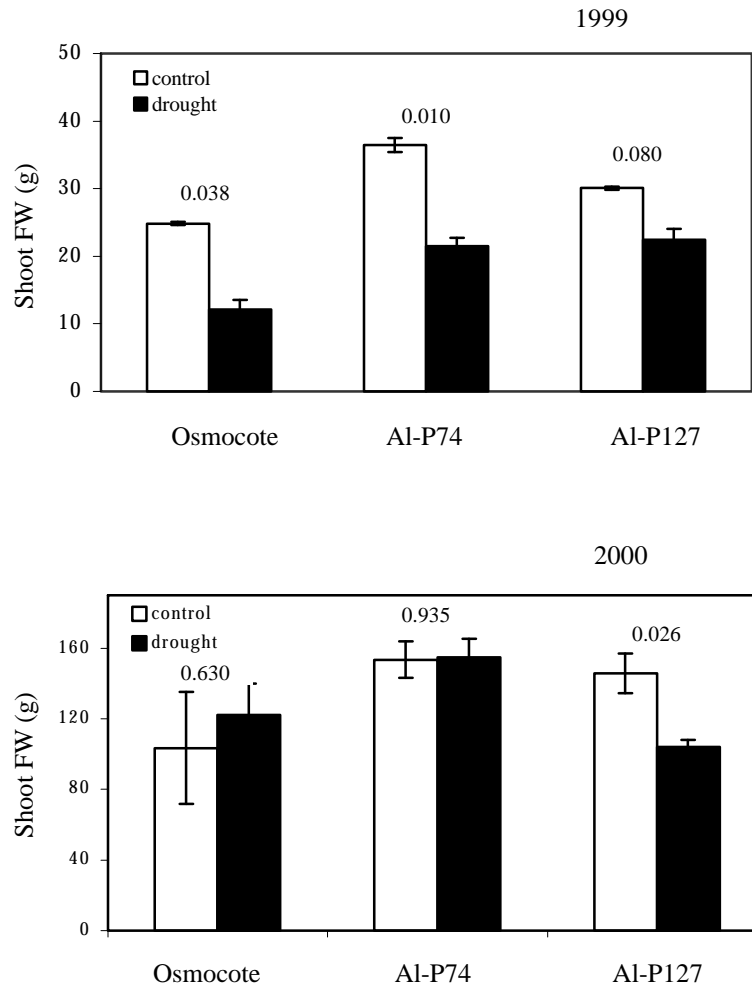ents against irrigated controls are shown above the columns. P-values of two-factor ANOVA for 1999 (2000) are 0.004 (0.076) for P; 0.001 (0.606) for drought; and 0.314 (0.216) for interaction.

Chapter 6

Directions for Future Research

This work suggests that the increase in root hair density caused by low phosphorus is not regulated by ethylene. The question remains unanswered: what regulates root hair density in response to low phosphorus? Potential candidates include auxin and cytokinin. Auxin and cytokinin are essential plant hormones which are known to control cell division (Mauseth, 1991). Our results indicate a close relationship between root hair density and cortical cell division, because *Arabidopsis* roots grown under low phosphorus have more cortical cells, which give rise to more trichoblasts. In other words, low phosphorus promoted cortical cell division.

In addition to higher hair density of roots grown with low phosphorus, root hairs are also longer in comparison with roots grown with high phosphorus. The regulation of root hair elongation under low phosphorus should be a topic in future research. Although root hair density is not likely regulated by ethylene, hair elongation might be controlled by ethylene, since our results show that ethylene insensitive mutants have reduced hair length, and root hairs are shorter with the presence of ethylene inhibitors in the growth media. Auxin might also be important in hair elongation, because root hair growth is also controlled by auxin genes (Fig. 2.4) (Bates and Lynch, 1996).

Future work for basal root shallowness may include whether ethylene plays a role in signal transduction in gravity perception or just plays a non-primary role in modulating the gravitropic responses (Madlung et al., 1999). Secondly, it would be interesting to know where the site is that the gravitropic response (curvature) occurs in basal roots in response to phosphorus availability. In addition to physiological aspects of basal root shallowness, we should also understand the regulatory mechanisms at cellular and molecular levels, and genetic control of the basal root shallowness in response to low phosphorus. In addition, focus should also be given to the balance of phosphorus availability in topsoil for shallower basal roots under low phosphorus stress and water

uptake, especially under drought conditions. In other words, we want to know whether plants adopt a compromise strategy when it is exposed to both phosphorus deficiency and drought stress. Since many genotypes/species produce adventitious roots which are closer to the soil surface than basal roots, it would be interesting to investigate the interaction of adventitious roots and basal roots in gravitropic-response to phosphorus availability. Particularly we want to know the interaction between basal roots and adventitious roots, and the gravitropic responses of adventitious roots under phosphorus deficiency. Since basal roots develop well before adventitious roots, adventitious roots would not be able to change the angle of basals. But with the presence of adventitious roots, do basal roots still grow shallower in low phosphorus? Do adventitious roots and basal roots compensate functionally or compete with each other for phosphorus uptake? Adventitious roots are mostly distributed in the soil surface. Do they change gravitropism in response to phosphorus availability, if so, to what extent?

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Selected publications
1. **Yuan Ji Zhang**, Xijin Mu, Qigui Cai, Yunluo Zhao, Xiaoping Wei, and Yingqian Qian. Plant regeneration from protoplast-culture of kiwifruit (*Actinidia*). *Plant Cell Reports*, 17:819-821(1998)
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