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# The responses of wild-type and ABA mutant *Arabidopsis thaliana* plants to phosphorus starvation

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## ABSTRACT

**The growth patterns of plants subjected to phosphorus starvation resemble those caused by treatment with ABA, suggesting that ABA could mediate the response of the plant to phosphorus starvation. We examined the role of ABA in phosphorus stress by comparing growth and biochemical responses of *Arabidopsis thaliana* ABA mutants *aba-1* and *abi2-1* to those of wild-type plants. We first characterized acid phosphatase production of wild-type *Arabidopsis* in response to phosphorus starvation. We found that several acid phosphatase isozymes are present in roots and shoots, but only a subset of these isozymes are induced by phosphorus stress, and they are induced in both organs. Production of acid phosphatase in response to phosphorus stress was not affected by the *aba-1* or *abi2-1* mutations. Low phosphorus also resulted in decreased growth of both wild-type and ABA mutant plants, and the root-to-shoot ratio was increased in both wild type and mutants. Anthocyanins accumulated in response to phosphorus stress in both wild-type and mutant plants, but the increase was reduced in the *aba-1* mutant. Thus, two different ABA mutants responded normally in most respects to phosphorus stress. Our data do not support a major role for ABA in coordinating the phosphorus-stress response.**

*Key-words*: *Arabidopsis thaliana;* Brassicaceae; *aba-1*, *abi2-1*; abscisic acid; acid phosphatase; anthocyanin; phosphorus starvation.

## INTRODUCTION

Phosphorus availability in native soils is seldom adequate for optimal plant growth. In large part this is because phosphorus is commonly bound to many soil constituents that make it unavailable or only sparingly available to plants, including Fe and Al oxides, and recalcitrant organic matter (Sample, Roper & Racz 1980). Up to 50% of total soil phosphorus can be complexed with organic matter (Ozanne 1980). In agricultural systems, this problem has been addressed through the application of concentrated phosphorus fertilizers. This is only a partial solution, however, since applied fertilizer is only marginally effective in some soils due to chemical immobilization (Sanchez & Uehara 1980). In addition, agricultural systems in many parts of the world have limited access to fertilizers, sources of high-grade phosphorus ore for fertilizer processing are limited and non-renewable (Cathcart 1980), and fertilizers are costly (an estimated 10 billion dollars is spent on phosphorus fertilizers by US farmers each year; Netzer 1987). Finally, intensive fertilization is a primary source of runoff pollution which threatens surface water resources in the US and other developed nations (Natural Research Council 1989; Francis, Flora, & King 1990). Understanding how plants respond to phosphorus-deficient conditions may result in development of strategies for optimizing phosphorus use.

Growth inhibition is a predictable effect of phosphorus deficiency, but careful observation of growth in a number of different species subjected to phosphorus starvation revealed that growth inhibition is selective, affecting shoots much more than roots. For example, Loneragan & Asher (1967) examined eight annual pasture species in low-phosphorus solution culture, and found that the growth of shoots was reduced more than the growth of roots. Similarly, under phosphorus-deficient conditions, tomato seedlings showed a decrease in dry weight accumulation but an increase in the root-to-shoot ratio (Goldstein, Baertlein, & McDaniel 1988a). A detailed study of the growth of bean plants, which analysed component production rates of shoots and roots separately over time, confirmed that root growth is less sensitive than shoot growth to phosphorus deficiency (Lynch, Lauchli, & Epstein 1991).

An increase in root-to-shoot ratios is also found in maize plants subjected to water stress (Sharp & Davies 1979; Saab *et al.* 1990), and in plants sprayed with abscisic acid (ABA; Watts *et al.* 1981). In water-stressed maize plants, an increase in endogenous ABA content is associated with the promotion of root elongation and the inhibition of shoot elongation (Saab *et al.* 1990). Treating plants with ABA also increases the root-to-shoot ratio by limiting shoot growth but not root growth (Watts *et al.* 1981). The similarity in growth patterns of plants stressed by phosphorus deficiency and by drought suggested that ABA may

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play a role in altering growth of phosphorus-stressed plants, as it appears to do in water-stressed plants.

The role of ABA in phosphorus stress was addressed by Radin (1984). He did not observe differences in ABA concentration in phosphorus-stressed cotton plants except when water stress was also applied. However, he measured an increase in stomatal responsiveness to applied ABA in phosphorus-stressed plants, and he concluded that sensitivity to ABA was altered by phosphorus stress. We decided to explore further the role of ABA in the plant response to phosphorus stress by utilizing *Arabidopsis* mutants that are deficient in ABA biosynthesis or sensitivity.

*Arabidopsis* mutants that are deficient in ABA biosynthesis or that are insensitive to applied ABA have been isolated (Koornneef *et al.* 1982; Koornneef, Reuling & Karssen 1984). The ABA-deficient mutant *aba* appears to block the epoxidation of zeaxanthin, resulting in lower levels of ABA (Reid & Howell 1995). The leaves of *aba-1* mutants that were water stressed had 5% of the wild-type levels of ABA (Rock, Heath & Zeevaart 1992). Interestingly, there was no increase in ABA levels in the mutant in response to water stress, unlike the wild type which experienced a 5·7-fold increase in ABA levels (Rock *et al.* 1992). The *aba* phenotype includes reduced seed dormancy, smaller plant size, and an increased rate of water loss. ABA-insensitive mutants (*abi*) have been isolated at five different loci (Koornneef *et al.* 1984; Finkelstein 1994a). None of these mutants is insensitive for all ABA-inducible responses. The *abi1* and *abi2* mutants affect water relations and seed dormancy, while the major effects of *abi3*, *abi4*, *abi5* are on seed development (Finkelstein & Somerville 1990; Finkelstein 1994a).

Our analysis of *Arabidopsis* ABA mutants included characterization of both growth and biochemical responses to phosphorus deficiency. The biochemical responses we focused on included anthocyanin accumulation and acid phosphatase secretion. Anthocyanin accumulation is a symptom of phosphorus deficiency in many species (Bergmann 1992; Halsted & Lynch 1996). However, even among nutrient stresses, its accumulation is not completely specific to phosphorus stress, since in some species, including *Arabidopsis*, anthocyanins accumulate in response to nitrogen stress as well (Bergmann 1992; Bariola *et al.* 1994). Anthocyanins also accumulate in response to a large number of other environmental stresses including drought stress (Do & Cormier 1990), UV light, high light intensity, microbial challenge, heavy metal ions, and ozone pollution (Heller & Forkmann 1988). It is possible that induction of anthocyanin accumulation is an indirect and non-specific side-effect of these stress responses.

Acid phosphatase secretion in response to low phosphorus has been studied in a number of species, including tomato (Goldstein *et al.* 1988a), rice, wheat, lupin, soybean and several other crops (Tadano *et al.* 1993). Secreted acid phosphatase is thought to liberate phosphorus from organic sources in the soil, making it available to the plant (Duff, Sarath & Plaxton 1994). An increase in acid phosphatase activity in response to low phosphorus has also been reported for *Brassica nigra* suspension cultures (Lefebvre *et al.* 1990), and tomato suspension cultures (Goldstein *et al.* 1988a). In addition, acid phosphatases are induced in shoots in response to phosphorus deficiency (McLachlan *et al.* 1987). In leaves, acid phosphatases accumulate in vacuoles and are assumed to function to scavenge phosphorus (Duff *et al.* 1994).

In this paper, we report our characterization of the response of wild-type and ABA mutant *Arabidopsis* plants to phosphorus starvation. We characterized the induction of acid phosphatase by phosphorus starvation in *Arabidopsis*, and found that only a subset of acid phosphatase isozymes are affected by phosphorus starvation. We compared growth, phosphorus accumulation, anthocyanin accumulation, and acid phosphatase activity of wild type and the ABA mutants *aba-1* and *abi2-1*. The ABA mutants responded normally in most respects to phosphorus stress, indicating that ABA is probably not the major signal that coordinates the phosphorus-stress response.

## MATERIALS AND METHODS

### Plant material and growth conditions

The initial characterization of acid phosphatase activity was done using the *Arabidopsis thaliana* ecotype Columbia. Seeds for the ABA-deficient mutant *aba-1* and the ABA-insensitive mutant *abi2-1* were obtained from the *Arabidopsis* Biological Resource Centre at Ohio State University. The phenotypes were verified by germination on ABA and by wilting assays (Finkelstein 1994b). For comparison with ABA mutants, wild-type *Arabidopsis* plants of the background ecotype Landsberg erecta were used.

Plants were grown on media containing half-strength nutrient salts (Jonson *et al.* 1957), 100 mg $dm^{-3}$ myo-inositol, 1 mg $dm^{-3}$ thiamine-HCl, 0·5 mg $dm^{-3}$ pyridoxine-HCl, 0·5 mg $dm^{-3}$ nicotinic acid, 1% sucrose and 0·5 g $dm^{-3}$ MES, pH 5·7, prepared as described by Bates & Lynch (1996). In the low-phosphorus medium, $NH_4NO_3$ was substituted for $NH_4H_2PO_4$. We found that commercially available solidifying agents such as Phytagar (Gibco BRL) and Phytagel (Sigma) contributed levels of phosphorus to the medium varying from 10 to 85 mmol $m^{-3}$. Therefore, to create low-phosphorus conditions, media were solidified with phytagel treated to remove phosphorus as described by Doner & Douds (1995), except we used a mixed bed resin (TMD-8, Sigma) for 4 h at 60 °C. The amount of phosphorus in the final medium was determined by the method of Murphy & Riley (1962), and was either 6 mmol $m^{-3}$ phosphorus (low phosphorus) or 1 mol $m^{-3}$ phosphorus (high phosphorus). To determine whether acid phosphatase induction was a general nutrient-stress response, plants were also grown on medium lacking potassium. For the low-potassium medium $NH_4NO_3$ was substituted for $KNO_3$, and in both high- and low-potassium media $NH_4Cl$ was substituted for KCl.

Twelve sterilized seeds were placed in a row on plates containing 30 $cm^3$ of media, and the plates were orientated at a 35° angle and placed at 4 °C for 2 d. Plants were grown at 22 °C with a 16 h day length and staged after 5

by eliminating seedlings that did not have both open cotyledons and the root touching the lower Petri plate.

### Anthocyanin determination

Anthocyanins were extracted with propanol-HCl and quantified by measuring absorbance at 535 nm (Schmidt & Mohr 1981).

### Phosphorus determination

Phosphorus concentration was determined in both the shoots and roots after dry ashing by using the procedure described by Murphy & Riley (1962).

### Acid phosphatase assays

For acid phosphatase assays, plants were grown as described above. Plants were removed from the medium 7, 11, 15 and 19 d after germination, and the shoots were discarded. Since much of the acid phosphatase activity was expected to be on the outside of the root, or in the surrounding medium, the roots were not rinsed. Any large pieces of media, however, were discarded. Roots were weighed, ground and centrifuged, and the supernatant was assayed for acid phosphatase activity using *p*-nitrophenyl phosphate as described by Basha (1984). Data are reported as the absorbance of *p*-nitrophenol at 405 nm $g^{-1}$ fresh weight.

### Acid phosphatase isozyme analysis

Plants were grown as described above on media supplemented with 3 mol $m^{-3}$ K and 1 mol $m^{-3}$ P or on media deficient in either phosphorus or potassium. Roots and shoots were harvested 3, 7, 11 and 15 d after germination. Proteins for isozyme analysis were isolated from both roots and shoots as described by Aarts *et al.* (1991) and run on non-denaturing 10% polyacrylamide gels at 4 °C. The gels were soaked in buffer (50 mol $m^{-3}$ sodium acetate, pH 5·5; 10 mol $m^{-3}$ $MgCl_2$) for two 15 min incubations and then stained for acid phosphatase activity in buffer containing 1 mg $cm^{-3}$ Fast Black K Salt and 0·03% $\beta$-naphthyl acid phosphate (Aarts *et al.* 1991). A denaturing polyacrylamide gel was loaded with duplicate samples and stained for protein using silver nitrate (Morrissey 1981) to check for equal loading.

## RESULTS

In initial experiments, we confirmed that wild-type *Arabidopsis* plants respond to phosphorus starvation conditions similarly to previously studied crop species. Although there was a general reduction in the dry weight of the plants, a greater percentage of the dry weight was in the roots (Table 1). For example, by 11 d after germination 49·4% of the weight of the phosphorus-starved plants was due to roots compared to 22·2% for plants grown under phosphorus-sufficient conditions. Total phosphorus accumulation was greatly reduced by growth on low-phosphorus medium (Table 1). Roots of phosphorus-starved plants generally accumulated more phosphorus $g^{-1}$ dry weight than did shoots. (The large increase in phosphorus in shoots of plants grown on low-phosphorus medium seen at day 15 was not reproducible.) Finally, within 7 d after germination there was already a 30-fold increase in anthocyanin pigments $g^{-1}$ fresh weight (Table 1).

**Table 1.** Growth responses of wild-type *Arabidopsis* on high phosphorus (1 mol $m^{-3}$) or low phosphorus (6 mmol $m^{-3}$) media. Values are reported as mean (standard error); $n = 6$ to 10

| | | Days after germination | | | |
|---|---|---|---|---|---|
| | | 7 | 11 | 15 | 19 |
| Dry weight (mg) | High P | 0·55 (0·04) | 1·34 (0·08) | 3·19 (0·59) | 4·58 (0·40) |
| | Low P | 0·11 (0·01) | 0·23 (0·02) | 0·34 (0·02) | 0·39 (0·03) |
| % weight due to roots | High P | 19·6 (1·4) | 22·2 (1·4) | 23·1 (1·4) | 24·7 (0·7) |
| | Low P | 33·3 (4·0) | 49·4 (2·8) | 50·0 (2·2) | 54·6 (1·9) |
| P content (µmol) | High P | 0·232 (0·021) | 0·837 (0·071) | 1·157 (0·207) | 1·094 (0·131) |
| | Low P | 0·037 (0·008) | 0·022 (0·002) | 0·064 (0·013) | 0·015 (0·001) |
| Shoot P as % dry weight | High P | 1·28 (0·15) | 1·97 (0·12) | 1·13 (0·10) | 0·70 (0·06) |
| | Low P | 0·65 (0·15) | 0·16 (0·02) | 0·78 (0·23) | 0·09 (0·01) |
| Root P as % dry weight | High P | 1·59 (0·18) | 1·74 (0·09) | 1·31 (0·08) | 0·81 (0·04) |
| | Low P | 1·91 (0·45) | 0·49 (0·08) | 0·41 (0·03) | 0·14 (0·01) |
| Anthocyanins[1] | High P | 2·0 (0·3) | 0·7 (0·1) | 0·8 (0·2) | 1·3 (0·3) |
| | Low P | 59·7 (10·0) | 67·2 (7·9) | 65·9 (6·3) | 59·2 (6·4) |

[1]Absorbance at 535 nm $g^{-1}$ fresh weight.

### Induction of acid phosphatase

We found an approximately 2-fold increase in acid phosphatase activity in roots under low-phosphorus conditions after 7, 11, 15 and 19 d of growth (Fig. 1). Under both low- and high-phosphorus conditions, acid phosphatase activity peaked at 11 d and declined by 19 d. To examine acid phosphatase isozymes, proteins were isolated from shoots and roots after growth on medium containing high phosphorus and potassium or on media deficient in either phosphorus or potassium for 3, 7, 11 or 15 d after germination. A comparison with a potassium-deficient treatment was carried out to test the specificity of any response to phosphorus deficiency. Proteins were separated on non-denaturing polyacrylamide gels and stained for acid phosphatase activity. Silver staining of denaturing gels loaded with duplicate samples indicated that loading of each lane on the gel was equal (data not shown). At least four isozymes were evident in the shoots (Fig. 2A) and at least five isozymes were visible in the roots (Fig. 2B). One of these isozymes was induced by low phosphorus but not by low potassium in both the shoots and the roots by 3 d after germination (Fig. 2, arrow). In older plants, the activity of all of the slowly migrating bands appeared to be increased (Fig. 2). However, the activity of the fastest isozymes was not influenced by phosphorus starvation.

### Responses of ABA mutants to low phosphorus

To examine whether ABA is involved in plant response to phosphorus starvation, we examined the induction of acid phosphatase isozymes in the *Arabidopsis* ABA-deficient mutant *aba-1* and an ABA-insensitive mutant, *abi2-1*. An examination of the acid phosphatase isozymes on a non-denaturing polyacrylamide gel indicated that both ABA mutants had the same number of isozymes as wild type, and that the level of increase in activity of the inducible isozymes in response to low phosphorus was comparable to wild type (Fig. 3).

We also measured several other parameters of plant response to phosphorus deficiency in the ABA mutants. The *aba-1* mutant had no significant difference in phosphorus concentration in the shoots and roots after 15 d compared to wild type (Figs 4A & B). For *abi2-1*, however, there was significantly less phosphorus in the shoots and roots under high-phosphorus conditions (Figs 4A & B, Student's *t*-test, $P < 0{\cdot}01$). Overall growth of mutant and wild-type plants was similar (data not shown), and for both mutants there was an increase in the root-to-shoot ratio under phosphorus-deficient conditions similar to that seen with wild-type plants (Fig. 4C). Anthocyanins accumulated in both *aba-1* and *abi2-1* on low-phosphorus medium (Fig. 4D). However, in *aba-1* the accumulation of anthocyanins in response to low phosphorus was significantly decreased in comparison to wild type (Fig. 4D; $P < 0{\cdot}01$). For both *aba-1* and *abi2-1*, total soluble acid phosphatase activity increased at least two-fold in response to low phosphorus, as it does in wild-type plants (Fig. 4E).

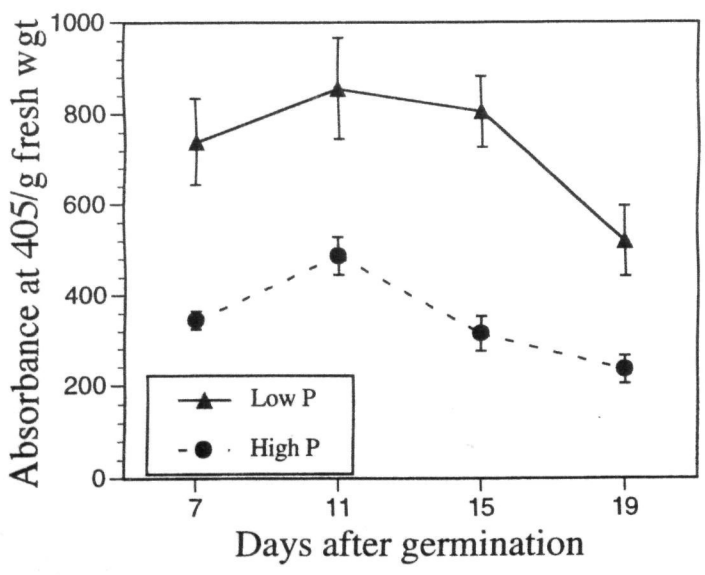


**Figure 1.** Root acid phosphatase activity on low- and high-phosphorus media. Root extracts were assayed for acid phosphatase activity using *p*-nitrophenyl phosphate. Activity is reported as absorbance of *p*-nitrophenol at 405 nm $g^{-1}$ fresh weight. The error bars represent the standard error of the means. $n = 9$ or 10.

## DISCUSSION

The production of acid phosphatase in response to low-phosphorus conditions has been observed in many species (Tadano *et al.* 1993; Duff *et al.* 1994). We have shown that in *Arabidopsis* acid phosphatase activity is increased in shoots and roots in response to phosphorus starvation (Fig. 2). It is not increased in response to potassium deficiency, which indicates that it is not a generalized response to nutrient deficiency. Thus, this biochemical response is a good marker for response to phosphorus deficiency in *Arabidopsis*, as it is in other species (McLachlan *et al.* 1987).

We observed four acid phosphatase isozymes in shoots and five isozymes in roots. Interestingly, it appears that the same isozymes that are induced in the roots are also induced in the shoots. Multiple acid phosphatase isozymes have been reported in a variety of plant species (McLachlan *et al.* 1987; Goldstein *et al.* 1988b; Panara, Pasqualini, & Antonielli 1990; Guthrie, McLachlan & DeMarco 1991). In wheat leaves, there are at least five acid phosphatase isozymes (McLachlan *et al.* 1987), and the activity of two of these is detectable only under phosphorus-deficient conditions (Guthrie *et al.* 1991). In tomato suspension cultures, there are at least two isozymes that are secreted in response to starvation levels of phosphorus (Goldstein *et al.* 1988b).

While we observed an approximately 2-fold increase in acid phosphatase activity in response to phosphorus starvation when activity was determined by solution assay (Fig. 1), the increase in activity appears much greater on native polyacrylamide gels (Fig. 2). This difference could be due to variation in the ability of different isozymes to hydrolyse particular substrates, or to the presence of inhibitors of acid phosphatase activity in the extracts from phosphorus-deficient plants. These putative inhibitors would be separated from the acid phosphatase isozymes upon gel electrophoresis.

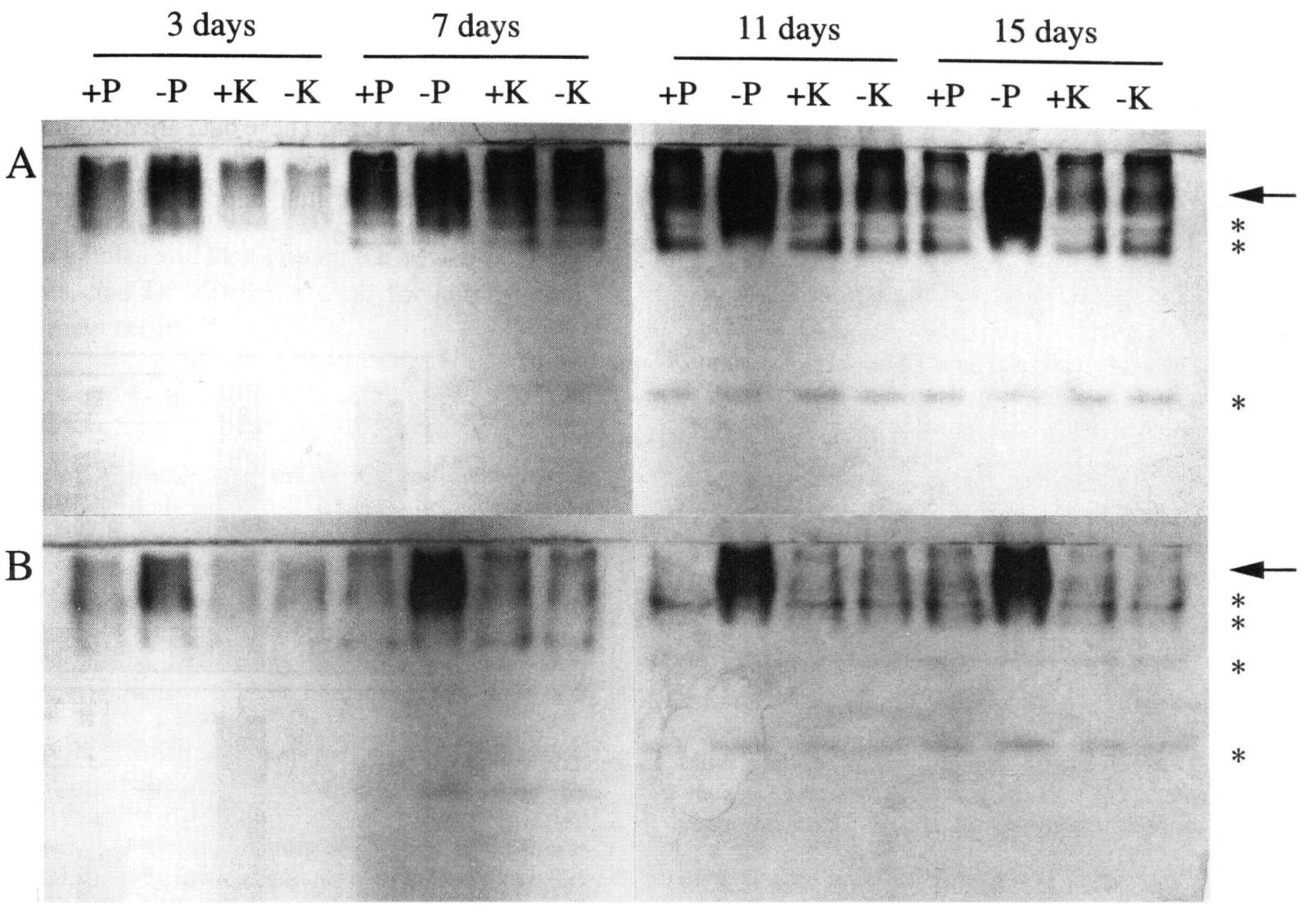


**Figure 2.** Analysis of acid phosphatase isozymes of *Arabidopsis* plants 3, 7, 11 or 15 d after germination on media supplemented with 1 mol $m^{-3}$ P and 3 mol $m^{-3}$ K or on media deficient in either phosphorus or potassium. Shoot (A) and root (B) protein extracts were run on non-denaturing polyacrylamide gels and stained for acid phosphatase activity. In the 7 d shoot samples, the –P lane is underloaded compared to the +P, +K, and –K lanes. Arrows indicate the isozyme that increases in activity the most rapidly after growth on low-phosphorus medium. Asterisks denote other acid phosphatase activities.

To investigate the role of ABA in the response of *Arabidopsis* to low phosphorus, we examined the induction of acid phosphatase in *Arabidopsis* ABA mutants *aba-1* and *abi2-1* subjected to phosphorus stress. We found that both the total amount of acid phosphatase in the roots (Fig. 4E) and the amount of the inducible isozyme in roots and shoots (Fig. 3) were indistinguishable from wild type, suggesting that ABA is not required for acid phosphatase production in response to phosphorus stress.

We also examined other aspects of plant response to phosphorus starvation in the ABA mutants, and found that the mutants exhibited responses similar to those of wild type. For example, the growth patterns of the mutants were comparable to those of wild type, and displayed the typical increase in the root-to-shoot ratio in response to low phosphorus (Fig. 4C). In addition, the mutants were able to accumulate the same amount of phosphorus in roots and shoots as wild type under phosphorus-limiting conditions (Figs 4A & B). Finally, both mutants accumulated large amounts of anthocyanins in response to low phosphorus (Fig. 4D). Although anthocyanin accumulation occurs in response to many stresses, and may be a secondary response, this result indicates that the ABA mutants sensed phosphorus deficiency and experienced stress.

Although the *aba-1* mutants did accumulate a significant amount of anthocyanin pigments in response to phosphorus stress, we consistently found that they did not accumulate as much anthocyanin as wild type in response to phosphorus stress (Fig. 4D). The same result was obtained with *aba-3*, a weaker allele of *aba-1* (Rock & Zeevaart 1991; data not shown). This result indicates that ABA is required for full activation of the anthocyanin biosynthetic pathway, and it would be interesting to see whether *aba-1* is capable of producing wild-type amounts of anthocyanins in response to other stimuli. This result further suggests that ABA does play some role in the plant response to phosphorus stress, and ABA may regulate aspects of the response to phosphorus that we did not measure. In addition, *abi2-1* accumulated wild-type amounts of anthocyanins, suggesting that anthocyanin accumulation in response to phosphorus stress is not regulated by the signal transduction pathway in which *abi2* participates. The fact that the effect of the *aba-1* mutation on anthocyanin biosynthesis was partial may be due to the leaky nature of the mutation, or perhaps anthocyanin accumulation in response to phosphorus stress is controlled independently by more than one signalling pathway.

Both the ABA biosynthetic mutant *aba-1* and the ABA perception mutant *abi2-1* had wild-type responses to phos-

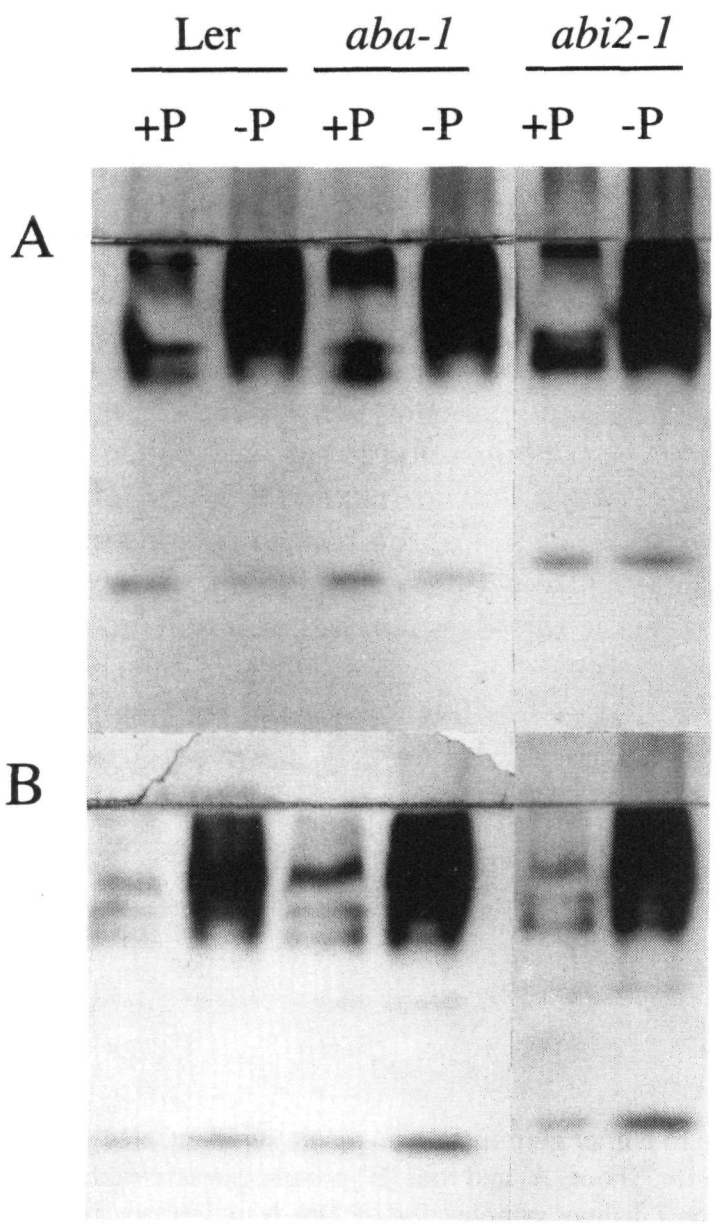


**Figure 3.** Acid phosphatase isozyme analysis of wild-type ecotype Landsberg erecta (Ler), ABA mutants *aba-1* and *abi2-1*. Proteins were extracted from shoots and roots 15 d after germination on low- or high-phosphorus media. Extracts were run on non-denaturing polyacrylamide gels and stained for acid phosphatase activity. (A) Acid phosphatase isozymes in the shoots. (B) Acid phosphatase isozymes in the roots.

phorus starvation in terms of acid phosphatase production and alterations in growth. For these responses, there was no reduction in the magnitude of the response between mutants and wild type. These data are not consistent with a central role for ABA in coordinating the major growth and biochemical changes that happen in plants subjected to phosphorus starvation.

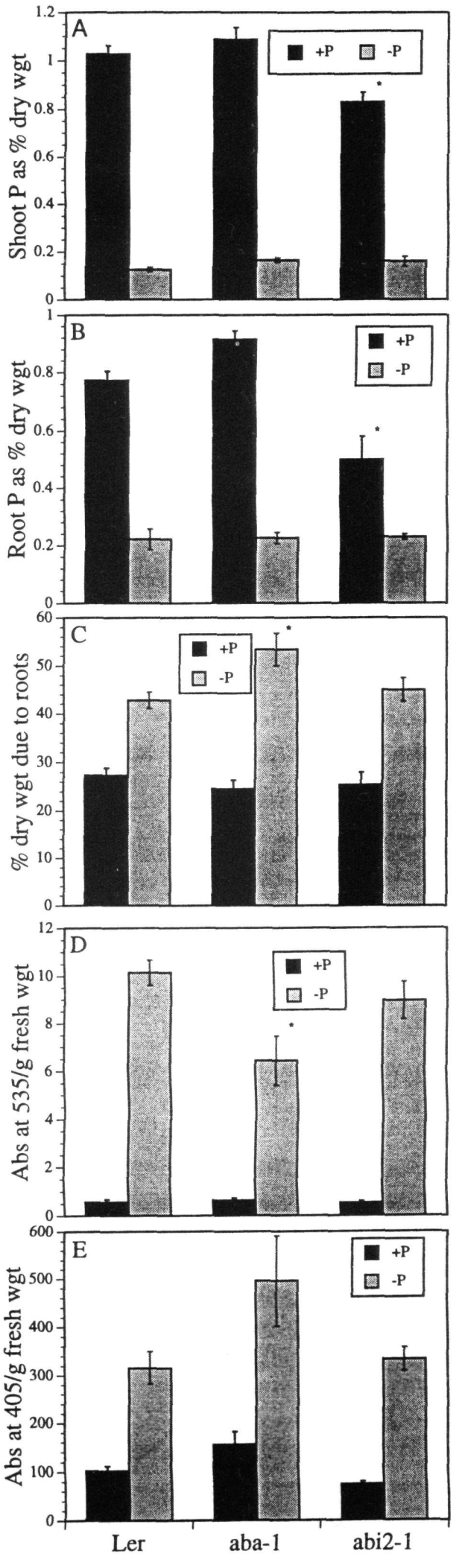


**Figure 4.** Comparison of ABA-deficient mutant *aba-1* and ABA-insensitive mutant *abi2-1* with wild-type ecotype Landsberg erecta (Ler) 15 d after germination on high- or low-phosphorus media. The low-phosphorus medium was 6 mmol $m^{-3}$ P and the high-phosphorus medium was 1 mol $m^{-3}$ P. Error bars represent the standard error of the means. * indicates significantly different from the wild type grown on the same phosphorus level as determined by the Student's *t*-test, $P < 0{\cdot}01$. $n = 4$–20. (A) Phosphorus concentration in the shoots reported as a percentage of shoot dry weight. (B) Phosphorus concentration in the roots reported as a percentage of root dry weight. (C) Percentage of total dry weight due to roots. (D) Anthocyanin accumulation reported as absorbance at 535 nm $g^{-1}$ fresh weight. (E) Root acid phosphatase activity. Root extracts were assayed for acid phosphatase activity using *p*-nitrophenyl phosphate. Activity is reported as absorbance of *p*-nitrophenol at 405 nm $g^{-1}$ fresh weight.

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