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pho3: a phosphorus-deficient mutant of *Arabidopsis thaliana* (L.) Heynh.

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Abstract. A novel P-deficient mutant of *Arabidopsis thaliana*, *pho3*, was isolated by screening for root acid phosphatase (APase) activity in plants grown under low-P conditions. *pho3* had 30% less APase activity in roots than the wild type and, in contrast to wild-type plants, root APase activity did not increase in response to growth in low P. However, shoot APase activity was higher in *pho3* than in the wild-type plants. In addition, the *pho3* mutant had a P-deficient phenotype, even when grown in P-sufficient conditions. The total P content of 11-d-old *pho3* plants, grown in agar media with a plentiful supply of P, was about 25% lower than the wild-type level in the shoot, and about 65% lower in the roots. In the rosette leaves of mature soil-grown *pho3* plants the total P content was again reduced, to about 50% of wild-type levels. *pho3* exhibited a number of characteristics normally associated with low-P stress, including severely reduced growth, increased anthocyanin content (at least 100-fold greater than the wild type in soil-grown plants) and starch accumulation. The results suggest that the mutant is unable to respond to low internal P levels, and may lack a transporter or a signalling component involved in regulating P nutrition.

Key words: Acid phosphatase – *Arabidopsis* (*pho3* mutant) – Mutant (*Arabidopsis*, phosphorus) Phosphate metabolism – Phosphorus nutrition

Introduction

Phosphorus is an essential macronutrient for plant growth and is also a key regulator of many biochemical processes (Bieleski 1973; Marschner 1995). Plants obtain P as the inorganic phosphate ion (Pi), which is taken up from soil by the roots; however, the availability of this ion is often limiting, even in soils with a high total P content (Marschner 1995; Schachtman et al. 1998). Plants respond to low-P stress with a rapid increase in the rate of Pi uptake, and a corresponding increase in the expression of a number of Pi transporters has recently been reported (Leggewie et al. 1997; Smith et al. 1997; Daram et al. 1998; Liu et al. 1998; Muchhal and Raghothama 1999). The levels of other enzymes that may assist in the mobilisation of P from organic compounds, including RNases and acid phosphatases, also increase in response to low-P stress (Tadano et al. 1993; Bariola et al. 1994; Duff et al. 1994; Dodds et al. 1996). These results indicate that changes in gene transcription may underlie some of the low-P stress responses observed in plants, as in other organisms including bacteria and yeast. Additionally, some plant species have the ability to secrete protons or organic acids from their roots to solubilise P-containing compounds in the soil (Lipton et al. 1987). Low-P stress also leads to more general responses by the plant, which coordinate growth and metabolism with nutrient availability (Bieleski 1973; Marschner 1995). Within the plant, cytosolic Pi levels are maintained within narrow limits by regulating Pi uptake, remobilising reserves and by transport between organs, cells and organelles (Lauer et al. 1989; Mimura et al. 1996). It is clear from these observations that plant P nutrition is highly regulated, and responsive to the external supply of P. Although the mechanisms that regulate these fluxes of P have not yet been elucidated, significant progress is being made through the application of molecular approaches (Lynch and Deikman 1998).

Two *A. thaliana* mutants with aberrant P metabolism, *pho1* and *pho2*, have been isolated by measuring leaf P content directly (Poirier et al. 1991; Delhaize and Randall 1995). *pho1* has normal levels of P in roots but the shoots are severely P deficient. Studies of Pi uptake in *pho1* have indicated that the defect in this mutant lies in the loading of Pi into the xylem (Poirier

Abbreviations: APase = acid phosphatase; BCIP = 5-bromo-4-chloro-3-indolyl-phosphate; βNAP = β-naphthyl acid phosphate; *pho3* = phosphorus-deficient mutant; P = phosphorus; Pi = inorganic phosphate

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et al. 1991; Delhaize and Randall 1995). In contrast, *pho2* accumulates abnormally high levels of P in leaf tissue, indicating that transport of Pi from the shoot to the root is impaired (Delhaize and Randall 1995; Dong et al. 1998). Although the genes involved have not been identified, it is clear that *pho1* and *pho2* will be valuable tools to begin to explore the pathways and mechanisms that allow plants to regulate their P nutrition.

As an alternative to the direct measurements of P content used to isolate the *A. thaliana pho1* and *pho2* mutants, induction of root acid phosphatase (APase) activity in response to low-P stress has been proposed as an approach to isolate plants with mutations in P nutrition (Goldstein et al. 1989). This secreted APase activity can be detected visually in the roots of *A. thaliana* plants grown in vitro on low-P medium, using a chromogenic substrate, 5-bromo-4-chloro-3-indolylphosphate (BCIP). Recently, an *A. thaliana* mutant (*pup1*) lacking one inducible APase isoform was isolated using this approach (Trull and Deikman 1998). In this paper we describe the isolation and preliminary characterisation of *pho3*, a mutant with a markedly reduced P content, identified using a modified version of this screening method based on inducible root APase activity.

Materials and methods

Mutant screening and plant growth

Mutant screening was carried out using T-DNA-tagged *Arabidopsis thaliana* (L.) Heynh. (ecotype Wassilewskija) (Feldmann et al. 1989). Seeds were surface-sterilised and cold-treated (at least 2 d at 4 °C) prior to sowing on a low-Pi agar medium, containing half-strength MS salts (Murashige and Skoog 1962) lacking Pi, MS vitamins, 1.0 mg l^{-1} glycine, 0.5 g l^{-1} Mes, 3% sucrose, 0.9% agar. The Pi content of this low-P medium was 11.31 ± 0.82 μM. Seeds were plated in rows and plates were held in a vertical position in a growth cabinet (16 h photoperiod, 24 °C). The roots of 7- to 8-d-old plants were overlaid with 0.6% agar that contained 0.01% (w/v) BCIP and 1-3 d later the plants were scored for APase staining intensity in the roots. Putative mutants with white or pale-blue roots were transferred to fresh medium, supplemented with 0.63 mM PO_4^{3-}, and grown for a further 7–10 d before transfer into soil. Plants were allowed to self-pollinate and their progeny (100–200 seeds per individual plant) re-screened to confirm the mutant phenotype. Selected mutants were backcrossed to the wild-type parent prior to analysis.

For studies on mature tissue, plants were germinated and grown in agar medium containing Pi for 7 d before transfer to compost (Fisons Levington, F2) with a 16-h daylength at 24 °C.

Acid phosphatase analysis

Proteins were isolated according to Aarts et al. (1991) and separated by 10% SDS-PAGE using a discontinuous buffer system (Laemmli 1970). After electrophoresis, gels were equilibrated for 2 × 15 min in 50 mM sodium acetate (pH 4.9) 10 mM $MgCl_2$. Acid phosphatase activity was made visible in the gel by staining with 0.5 mg ml^{-1} Fast Black K and 0.3 mg ml^{-1} β-naphthyl acid phosphate (βNAP) (added immediately before use) in 50 mM sodium acetate (pH 4.9) 10 mM $MgCl_2$ at 37 °C in the dark for 3–4 h. An alternative staining protocol using the substrate BCIP (0.04% BCIP in 50 mM sodium acetate (pH 4.9) 10 mM $MgCl_2$ overnight) was also used.

Acid phosphatase activity was measured in vitro using BCIP as a substrate. Protein extracts (8–10 μl) were incubated in 800 μl of 4 mM BCIP in 50 mM sodium acetate (pH 4.9) 10 mM $MgCl_2$ for 25 min at 37 °C and the reactions terminated by the addition of 1 ml of 1 N HCl. Samples were allowed to precipitate for 25 min, then centrifuged for 5 min at 10,000*g* and the precipitate dissolved in 1 ml of dimethyl sulfoxide (DMSO). Acid phosphatase activity was measured as the change in absorbance at 635 nm.

Phosphate analysis

Total P in plant samples was determined using the method described by Poirier et al. (1991).

Pigment analysis

For chlorophyll analysis, samples were ground in a chilled mortar in 80% acetone. After centrifugation the supernatants were measured at 663 nm and 646 nm and total chlorophyll content was calculated as 17.3 A_{464} + 7.18 A_{663} (Harborne 1984). Anthocyanins were extracted with propanol:HCl:H_2O (81:1:18, by vol.) for 5 min at room temperature and 3 min at 100 °C. Samples were then left for 24 h at 4–10 °C in the dark and absorption measured at 530 nm and 652 nm. Anthocyanin content was calculated as A_{530}–2.2A_{652} (Lange et al. 1971).

Starch staining

Leaves were immersed in ethanol (96%) for 6 h to remove pigments and stained with 1% iodine for 30 min to make the starch granules visible.

Results

Isolation of low-APase mutants

The screening approach developed to identify mutants with aberrant P metabolism was based on induction of secreted root APase activity by low-P stress, first reported by Goldstein et al. (1989). In a modification to this method, the colourless substrate BCIP was applied in an agar overlay to the roots of plants germinated and grown in medium containing no added supply of Pi. This adaptation avoided the toxicity evident when seedlings were germinated in medium containing BCIP (Goldstein et al. 1989). In low-P stress conditions the roots of wild-type plants stained blue, as the induced root APase cleaved BCIP to release the coloured product. Potential mutants were identified as having white or pale-blue roots under these conditions. Out of approximately 79,000 T-DNA mutagenised seedlings screened, three mutants were isolated with reduced APase staining (pale-blue roots) that persisted through three generations. One of these mutants, *pho3*, was chosen for further analysis. When *pho3* was backcrossed to the wild-type parent the F1 progeny resembled the wild type, indicating that the *pho3* mutation was recessive. However, the ratio of kanamycin-resistant:kanamycin-sensitive F2 progeny was very close to 15:1, suggesting that *pho3* originally contained

two independently assorting T-DNA copies ($\chi^2 = 0.3$, $P > 0.05$). Subsequently a number of F3 families segregating the mutant phenotype and kanamycin resistance 3:1 have been identified, suggesting linkage between the mutation and the T-DNA.

Acid phosphatase activity in pho3

The reduced histochemical staining observed in the roots of *pho3* during screening by agar overlay was confirmed by using BCIP as a substrate for an in-gel activity assay. A comparison of the intensity of staining in protein extracts from roots of plants grown in agar medium with no additional Pi showed that less APase activity was present in roots of *pho3* than wild-type roots (Fig. 1A, panel a). Staining of extracts from shoots with BCIP revealed that one band (the lower band observed on the gel) was reduced or absent in *pho3*, while the other main band (the upper band) was increased compared to wild type. Similar results were obtained when βNAP, a more frequently used APase substrate, was used for activity staining of extracts, although more bands were detected due to the broader substrate specificity of some of the APase isozymes. In general, for agar-grown plants the intensity of staining in extracts from the *pho3* mutant was lower than for the wild-type in roots, but higher than for the wild-type in shoots (Fig. 1A, panel b).

Total APase activity was measured spectrophotometrically in protein extracts from wild-type and *pho3* plants (Fig. 1B). In wild-type plants, APase activity increased in response to growth in medium containing no added Pi in both shoots (by 150%) and roots (by 60%). In *pho3*, shoot APase activity was 2-fold higher than in wild-type plants and also increased in response to growth in low Pi. However, in contrast to the shoots, the roots of *pho3* contained 30% less APase activity than those of the wild type and this level actually dropped in response to growth in low Pi (Fig. 1B). This resulted in a 5-fold difference in root APase activity between the wild type and mutant plants when grown in medium containing no added supply of Pi.

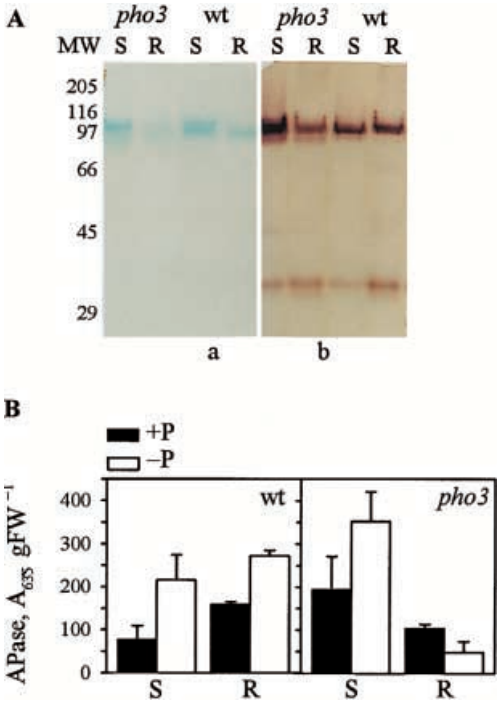

Fig. 1A,B. Analysis of acid phosphatase activity in roots and shoots of *pho3*. **A** Proteins were isolated from shoots (*S*) and roots (*R*) of 18-d-old wild-type (*wt*) and *pho3* plants grown in agar containing no added phosphate, separated by SDS-PAGE and stained for APase activity using either BCIP (*a*) or βNAP (*b*) as a substrate. **B** Acid phosphatase activity was assayed spectrophotometrically in shoot (*S*) and root (*R*) extracts from 11-d-old plants grown in agar containing no added phosphate (*−P*) or supplemented with 0.63 mM phosphate (*+P*). Values represent the mean of 3–4 independent experiments ± SD

Phosphate content

The P status of mutant and wild-type plants was also investigated. Analysis of the total P content of 11-d-old plants grown in agar medium with a plentiful supply of Pi showed that *pho3* contained lower levels of P than wild-type *A. thaliana*. Under these growth conditions the total P content of the shoots of *pho3* was about 75% of the wild-type level, and in the roots was even lower, at only 35% of wild-type levels (Fig. 2A). In both wild-type and mutant plants the P content of the shoots was higher than that of the roots; however, in the mutant this difference was greater, suggesting that the shoot P content of the mutant was maintained at the expense of the roots. When plants were grown on medium containing no added Pi the levels of P in both the wild type and *pho3* were severely reduced (Fig. 2A). To determine the P content of *pho3* during growth and maturation, levels of P were measured in the rosette leaves of soil-grown plants (Fig. 2B). During the early period in soil there was no apparent difference between wild-type and mutant plants, but at 20 d a small decrease in the P content in the leaves of *pho3* plants was seen. From 25 d onwards the P content of *pho3* rosette leaves was

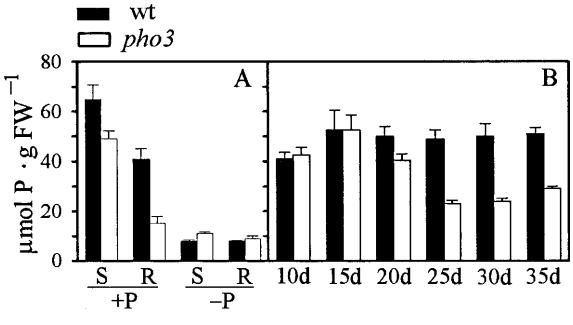

Fig. 2A,B. Phosphate content of *pho3*. Total P was measured in shoots (*S*) and roots (*R*) of 11-d-old seedlings grown in agar supplemented with 0.63 mM phosphate (*+P*) or containing no added phosphate (*−P*) (**A**), and rosette leaves of plants transferred from +P medium to soil at 7 d (**B**). Values represent the mean of 10–12 measurements ± SD

severely reduced, at only 50% of wild-type levels (Fig. 2B).

pho3 *has a phosphorus-deficient phenotype*

Plants experiencing low-P stress exhibit a number of characteristic changes to their growth and metabolism and it was of interest to observe these parameters in *pho3*. The growth of *pho3* was severely reduced when compared to the wild type. In Pi-supplemented agar medium the fresh weight of both shoots and roots of *pho3* was reduced by at least 30%. The roots were most affected, resulting in a small increase in the shoot:root ratio of *pho3* (Table 1). Growth in medium containing no added Pi led to a severe reduction in growth and a markedly lower shoot:root ratio of both mutant and wild-type plants, although the shoot:root ratio of *pho3* remained higher than that of the wild-type plants (Table 1).

When sown directly in soil, *pho3* seed germinated, but only around 15% of plants became established and grew to maturity. In contrast, when seeds were germinated and grown in sucrose-supplemented agar medium before transfer to soil, all the seedlings were capable of continued growth. The growth of plants transferred from agar to soil at 7 d was measured, and revealed that the fresh weight of the aerial parts of soil-grown *pho3* plants was severely reduced when compared to wild-type plants (Fig. 3A). A visual examination of the *pho3* phenotype clearly showed that the size of the plant was much reduced, with smaller leaves and a thin, usually single, stem (Fig. 3B). Although the mutant was fertile it was delayed in flowering by 2–3 weeks and had reduced fertility when compared to the wild type (producing less than 300 seeds per plant).

The leaves of soil-grown *pho3* plants showed a distinctive pattern of pigment accumulation (Fig. 3C). Purple pigmentation appeared in a developmental manner as leaves matured, visible first in the leaf tips and then spreading from the edge of the leaf towards the centre. *pho3* accumulated a 3-fold higher level of anthocyanins than wild-type plants when grown on agar medium containing Pi (Fig. 4A). However, when grown on medium with no added Pi, the anthocyanin content of *pho3* did not increase to the same extent as that of the wild-type plants (Fig. 4A). In keeping with the striking visual appearance of *pho3,* the levels of anthocyanins in leaves of 2-week-old soil-grown *pho3* plants were found to be more than 100 times higher than wild-type levels (data not shown).

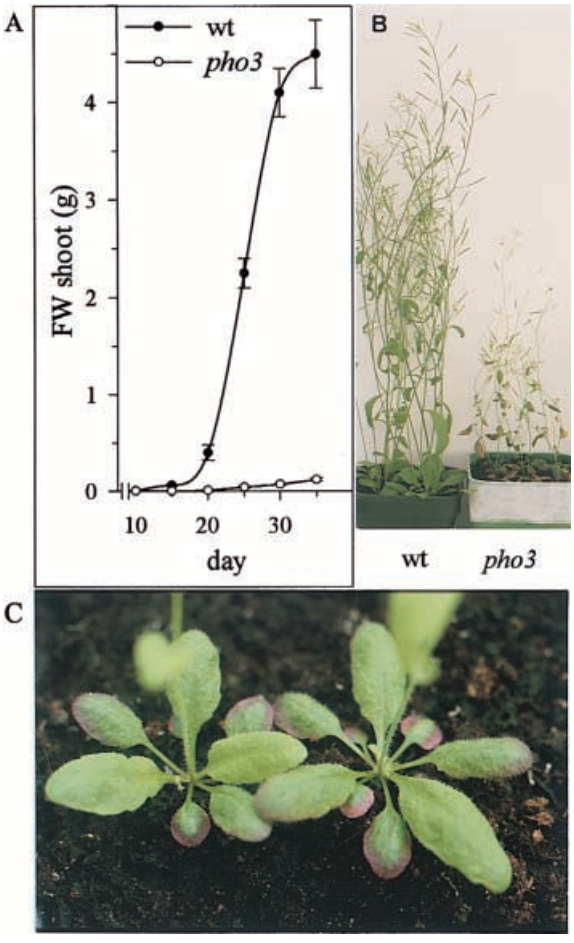

Fig. 3A–C. Growth and appearance of *pho3*. **A, B** The fresh weight of aerial parts of soil grown, wild type and *pho3* plants (**A**) and appearance at maturity (**B**). **C** Characteristic pigmentation of rosette leaves of 3-week-old *pho3* plants

The chlorophyll content of *pho3* was 50% lower than that of the wild-type plant when grown in medium containing additional Pi, and showed no further reduction in response to low-P stress (Fig. 4B). This was in contrast to wild-type plants, which responded to low-P stress with a 50% reduction in their chlorophyll content, to approximately the same level found in the mutant.

Higher levels of starch accumulation were observed in *pho3* plants compared to wild-type when grown with a plentiful supply of Pi, both in agar medium (Fig. 5) and in soil (data not shown). In contrast, wild-type plants accumulated starch only in response to low-P stress or at high sucrose concentrations (3% sucrose; data not shown).

Table 1. Shoot and root biomass of *pho3* and wild-type (wt) *A. thaliana* plants. Plants were grown in agar supplemented with 0.63 mM phosphate (+P) or containing no added phosphate (−P) and harvested after 11–15 d. Fresh weight (FW) in each experiment was determined for 170–250 plants and normalised per plant. Data represent mean values for three experiments ± SD

	Shoot (mg FW)		Root (mg FW)		Shoot:root ratio	
	wt	*pho3*	wt	*pho3*	wt	*pho3*
+P	8.51 ± 2.88	4.68 ± 0.87	1.51 ± 0.70	0.61 ± 0.13	5.6	7.7
−P	0.74 ± 0.10	0.81 ± 0.07	0.43 ± 0.09	0.21 ± 0.04	1.7	3.9

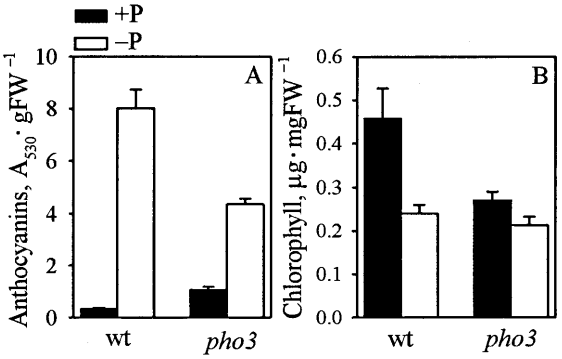

Fig. 4A,B. Pigment contents of 11-d-old wild-type and *pho3* *A. thaliana* plants. Seedlings were grown in agar supplemented with 0.63 mM phosphate or containing no added phosphate. Anthocyanin (**A**) and chlorophyll (**B**) levels were determined spectrophotometrically. Values represent the mean of 3–6 independent experiments ± SD

Discussion

The screening approach described in this paper was designed to identify mutants with defective responses to phosphate deprivation. This paper reports the isolation of a mutant in P nutrition, *pho3*, identified as having reduced levels of the secreted root APase normally induced by low-P stress. *pho3* was found to have a reduced P content compared to wild-type plants, even when grown in Pi-supplemented agar or soil. In keeping with these data, *pho3* exhibited many features characteristic of plants experiencing low-P stress, including an extremely slow growth rate, increased starch accumulation and high anthocyanin content (Bieleski 1973; Marschner 1995; Trull et al. 1997). The *Arabidopsis* mutant, *pup1*, also isolated by staining for root APase activity using BCIP, was shown to lack one inducible APase isoform (Trull and Deikman 1998). However, in contrast to *pho3*, the mutant

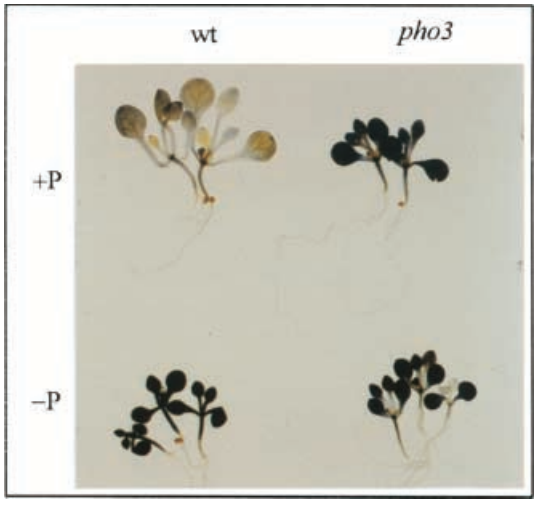

Fig. 5. Starch contents of 11-d-old wild type and *pho3* *A. thaliana* seedlings. Plants were grown in agar supplemented with 0.63 mM phosphate (*+P*) or containing no added phosphate (*−P*) and harvested in the middle of the photoperiod. Starch was made visible by iodine staining

was phenotypically wild type and was not P deficient, indicating that *pho3* and *pup1* are distinct mutants.

One possible explanation for the phenotype observed in *pho3* is that this mutant has a defect in Pi uptake by the roots. Normally, low internal levels of Pi stimulate an increase in the rate of Pi uptake (Glass 1983; Clarkson and Luttge 1991). However, this response appears to be absent in *pho3*, since the mutant remains P-deficient even when grown with an adequate supply of this nutrient. The increase in Pi uptake, in response to low-P stress, is thought to result from the activation, or increased expression, of a high-affinity Pi transporter in the roots. Recently a number of candidates for such high-affinity transporters have been identified on the basis of sequence analysis, complementation in yeast and expression studies (Muchhal et al. 1996; Leggewie et al. 1997; Smith et al. 1997; Daram et al. 1998). Even so, our understanding of the molecular biology of the transporters and other components involved in Pi uptake and distribution is still incomplete.

An alternative explanation for the *pho3* phenotype might be that this mutant is unable to regulate internal Pi levels, or respond to phosphate availability, as a result of a defect in P signalling. The response of *pho3* to low internal Pi levels differs from that of wild-type plants by a failure both to increase Pi uptake and to induce expression of the secreted root APase. Interestingly, yeast also expresses an inducible high-affinity Pi transporter and a secreted APase in response to low-P stress. A failure to increase expression of these two genes has been observed in several yeast mutants with defects in P signalling. The yeast cellular signalling system for perceiving low-P stress (the PHO system) has been well characterised (Lenburg and O'Shea 1996) and the yeast mutants with similarities to *pho3* have been shown to have defects in positive regulatory components of the low-P response, including both cytosolic signalling components and transcriptional regulators (Vogel et al. 1989; Schneider et al. 1994). These positive regulators were shown to have a role in activating transcription of the genes encoding the high-affinity H^+/Pi transporter and a secreted APase. If the basis of the *pho3* mutation was in a signalling component this might explain the observation that, in addition to the low content of *pho3*, this mutant also failed to induce the secreted root APase in response to low-P stress. Support for this suggestion might be provided by recent reports of *psr1*, a mutant of the unicellular green alga *Chlamydomonas reinhardtii* (Wykoff et al. 1999). This algal mutant is defective in a number of specific responses to P starvation, again including a failure to secrete acid phosphatase and to develop a high-affinity Pi transport system. Intriguingly, the *PSR1* gene encodes a nuclear protein with several features found in transcriptional regulators and has similarity to a number of higher-plant expressed sequence tags, although none to any yeast sequences.

Analysis of the total P content and distribution in *pho3* identifies it as a novel mutant in P metabolism and distinguishes it from *pho1* and *pho2*, two mutants in P nutrition described previously (Poirier et al. 1991; Delhaize and Randall 1995). The phenotype of *pho3*

has some similarities to *pho1,* since both have a low leaf P content and severely reduced growth. However, they differ substantially in their root P contents, which are reduced in *pho3,* but equivalent to, or higher than, wild type in *pho1*. In contrast to these two P-deficient mutants, *pho2* accumulates abnormally high P levels in leaf tissue. These comparisons indicate that the three, *pho* mutants have different defects in P uptake, distribution or signalling, and functional characterisation of their gene products may lead to the identification of novel components involved in P metabolism in plants.

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