

# BHLH32 modulates several biochemical and morphological processes that respond to P<sub>i</sub> starvation in *Arabidopsis*

Zhi-Hui CHEN<sup>1</sup>, Gillian A. NIMMO, Gareth I. JENKINS and Hugh G. NIMMO<sup>2</sup>

Plant Science Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, U.K.

P<sub>i</sub> (inorganic phosphate) limitation severely impairs plant growth and reduces crop yield. Hence plants have evolved several biochemical and morphological responses to P<sub>i</sub> starvation that both enhance uptake and conserve use. The mechanisms involved in P<sub>i</sub> sensing and signal transduction are not completely understood. In the present study we report that a previously uncharacterized transcription factor, BHLH32, acts as a negative regulator of a range of P<sub>i</sub> starvation-induced processes in *Arabidopsis*. In *bhlh32* mutant plants in P<sub>i</sub>-sufficient conditions, expression of several P<sub>i</sub> starvation-induced genes, formation of anthocyanins, total P<sub>i</sub> content and root hair formation were all significantly increased compared with the wild-type. Among the genes negatively regulated by BHLH32 are those encoding PPCK (phosphoenolpyruvate carboxylase kinase), which is involved in modifying metabolism so that P<sub>i</sub> is spared. The present study has shown that

PPCK genes are rapidly induced by P<sub>i</sub> starvation leading to increased phosphorylation of phosphoenolpyruvate carboxylase. Furthermore, several *Arabidopsis* proteins that regulate epidermal cell differentiation [TTG1 (TRANSPARENT TESTA GLABRA1), GL3 (GLABRA3) and EGL3 (ENHANCER OF GL3)] positively regulate PPCK gene expression in response to P<sub>i</sub> starvation. BHLH32 can physically interact with TTG1 and GL3. We propose that BHLH32 interferes with the function of TTG1-containing complexes and thereby affects several biochemical and morphological processes that respond to P<sub>i</sub> availability.

**Key words:** *Arabidopsis*, basic helix–loop–helix (bHLH) protein, gene expression, inorganic phosphate (P<sub>i</sub>) starvation, phosphoenolpyruvate carboxylase kinase (PPCK), root hair formation.

## INTRODUCTION

Phosphorus is an essential macronutrient for the growth, development and reproduction of plants. It is absorbed by plants almost entirely in the form of P<sub>i</sub> (inorganic phosphate), whose availability frequently limits crop yield [1]. Although P<sub>i</sub> is relatively abundant in many soils, much of it is unavailable for uptake because it forms insoluble complexes with cations. Plants respond to P<sub>i</sub> limitation in several ways [1–4]. They enhance P<sub>i</sub> uptake by modifying root architecture and growth, including an increase in the number and length of root hairs, expression of P<sub>i</sub> transporters, induction of endogenous and secreted phosphatases and RNases, and exudation of organic acids. They also limit the metabolic requirements for P<sub>i</sub> by engaging alternative respiratory pathways and bypassing P<sub>i</sub>-requiring steps, and by increasing the accumulation of anthocyanins. To achieve these responses, the expression of many genes are altered in P<sub>i</sub> starvation. Microarray experiments have identified genes showing different response kinetics to P<sub>i</sub> starvation [5–7]. For example, ‘early’ responses (observed after 4 h of P<sub>i</sub> starvation) can be distinguished from ‘late’ responses (observed after 100 h) [5]. Many of the ‘early’ responsive genes are also sensitive to other types of stress [5].

Over the last five years, some of the molecular mechanisms through which cellular P<sub>i</sub> status is sensed in plants have been revealed, but the picture is far from complete. The *Arabidopsis* gene *PHR1* (PHOSPHATE STARVATION RESPONSE 1) encodes a single MYB transcription factor required for the induction of a subset of the genes whose expression is activated in P<sub>i</sub> starvation [8]. Genes that are controlled by PHR1 include the P<sub>i</sub> transporter *AtPT1* (*ARATH*, *Pht1;1*), the acid phosphatase

*AtACP5* and the ribonuclease *AtRNS1*. PHR1 is thought to be a positive regulator of some, but not all, P<sub>i</sub> starvation-induced gene expression. Mutations in *Arabidopsis PSRI* (PHOSPHATE STARVATION RESPONSE 1) reduce the activities of P<sub>i</sub> starvation-inducible isoforms of ribonuclease and acid phosphatase, whereas *PDR2* (PHOSPHATE DEFICIENCY RESPONSE 2) is thought to link P<sub>i</sub>-sensing to root development [9,10], but neither gene has been characterized. Recently several *lpi* (low P<sub>i</sub> insensitive) mutants have been reported [11]. In a similar manner to *PHR1*, the *LPI* genes affect only a subset of P<sub>i</sub> starvation responses; for *LPI* genes, these include root development and expression of some P<sub>i</sub> starvation-induced genes, but neither anthocyanin accumulation nor root hair formation. In P<sub>i</sub> starvation some of the effects of *PHR1* are mediated by expression of the microRNA miR399. This suppresses expression of UBC24 (an E2 ubiquitin-conjugating enzyme), the product of the *PHO2* gene, and leads to over-accumulation of P<sub>i</sub> [12–15]. The targets of the ubiquitin-conjugating enzyme, and the functional effects of their ubiquitination, are not known, but the miR399/*PHO2* pathway is thought to affect the allocation of P<sub>i</sub> within the plant [14].

The *phr1* mutation does not affect the increased number and length of root hairs found in P<sub>i</sub> starvation [8]. Recently it was shown that the *Arabidopsis* SUMO E3 ligase *AtSIZ1* negatively regulates some P<sub>i</sub> starvation responses such as increased root hair number and length that do not involve PHR1 [16]. Furthermore, mutations in the inositol polyphosphate kinase gene *AtIPK1* also impinge on P<sub>i</sub> status signalling to root hair development: mutants have an elevated P<sub>i</sub> content but fail to repress root hair growth fully in P<sub>i</sub> sufficiency [17]. As well as its role as a negative

Abbreviations used: AtPT1, phosphate transporter 1; bHLH, basic helix–loop–helix; CPC, CAPRICE; Ct, threshold cycle value; EGL3, ENHANCER OF GLABRA3; GFP, green fluorescent protein; GL3, GLABRA3; GST, glutathione S-transferase; LPI, low P<sub>i</sub> insensitive; MS, Murashige–Skoog; PEPC, phosphoenolpyruvate carboxylase; PEPC, PEPC kinase; PHR1, PHOSPHATE STARVATION RESPONSE 1; P<sub>i</sub>, inorganic phosphate; RT, reverse transcriptase; SQD1, UDP-sulfoquinovose synthase 1; TTG1, TRANSPARENT TESTA GLABRA1; WER, WEREWOLF.

<sup>1</sup> Present address: Division of Cell and Developmental Biology, School of Life Sciences, University of Dundee, Dundee DD1 5EH, U.K.

<sup>2</sup> To whom correspondence should be addressed (email h.g.nimmo@bio.gla.ac.uk).

regulator, AtSIZ1 also positively regulates some  $P_i$  starvation responses in which *PHR1* is involved, and can SUMOylate PHR1 *in vitro* [16]. There is also evidence for negative regulation in the induction of the  $P_i$  transporter gene *AtPT2* (*ARATH*, *Pht1;4*) in  $P_i$  starvation [18]. Thus the available data suggest that plants respond to  $P_i$  starvation through multiple different sensing and signalling mechanisms and that several components remain to be identified.

The increased formation of root hairs in  $P_i$  starvation is particularly striking because it can increase the total root surface area by an order of magnitude, and is more sensitive to  $P_i$  limitation than to deficiencies in most other nutrients [19]. Root epidermal cells differentiate in a position-dependent manner, with hair cells overlying two cortical cell files and non-hair cells overlying a single cortical file [20]. Much information is now available about genes involved in these differentiation events. *GL3* (*GLABRA3*) and *EGL3* (*ENHANCER OF GL3*) encode bHLH (basic helix–loop–helix) proteins that together specify non-hair cell fate. *GL3* and *EGL3* act redundantly in a complex with the WD40 protein TTG1 (*TRANSPARENT TESTA GLABRA1*) and the R2R3 MYB protein WER (*WEREWOLF*). This complex functions by inducing expression of the homeodomain protein *GL2* (*GLABRA2*) in non-hair cells. Hair cell fate is specified by the single MYB proteins *CPC* (*CAPRICE*) and *TRY* (*TRIPTYCHON*), negative regulators of *GL2* [21,22]. Surprisingly, expression of *GL3* and *EGL3* occurs primarily in developing hair cells. The current model [23] involves movement of *GL3* and *EGL3* from hair to non-hair cells, where the TTG1–*GL3*–*EGL3*–*WER* complex induces expression of *GL2* and *CPC*, and reciprocal movement of *CPC* to hair cells where it reduces expression of *GL2* but increases expression of *GL3* and *EGL3*. Hence the specification of non-hair versus hair cell fate depends on the cellular distribution of TTG1, *GL3*, *EGL3* and *CPC*. Any factor that perturbs this balance could affect root hair formation either positively or negatively.  $P_i$  starvation leads to ectopic development of hairs in the non-hair position [24], though, in addition, a change in anatomy leads to an increase in hair cell files [25]. However, the mechanisms by which  $P_i$  starvation is first sensed and then subverts the normal differentiation programme are not known.

One of the metabolic responses to  $P_i$  starvation is thought to be the bypass of the pyruvate kinase step in glycolysis via induction of PEPC (phosphoenolpyruvate carboxylase) [1,3,4,26]. PEPC is itself regulated by phosphorylation, which activates the enzyme by reducing its sensitivity to feedback inhibition by malate [27,28]. Control of PEPC is mainly exerted at the level of expression of *PPCK* (PEPC kinase) genes [29,30]. In the present study we show that expression of the *PPCK1* and *PPCK2* genes in *Arabidopsis* is rapidly induced in  $P_i$  starvation, consistent with a role for activation of flux through PEPC as an early part of the  $P_i$  starvation response. This has led us to identify several genes that affect  $P_i$  starvation responses, some of which have previously been implicated in controlling epidermal cell differentiation and gene expression. One newly identified component, *BHLH32*, functions as a negative regulator of a range of biochemical and morphological processes that are induced in  $P_i$  starvation.

## EXPERIMENTAL

### Plant growth conditions

*Arabidopsis* suspension culture cells were derived from the Landsberg *erecta* ecotype [31]. Cells were grown photomixotrophically in 500 ml flasks containing 200 ml of culture medium [1 × MS (Murashige–Skoog) salts with minimal organics, 0.5 mg/

l  $\alpha$ -naphthaleneacetic acid, 0.05 mg/l kinetin (all obtained from Sigma) and 3% (w/v) sucrose (pH 5.8)]. Suspension cultures were grown at 20 °C in a continuous low fluence rate of white light (20  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ) with constant shaking (110 rev./min) and were subcultured every week by a 1:10 dilution [32]. For studies of the effects of  $P_i$ , 7-day-old cells were collected and then cultured in MS micronutrient medium (6.2 mg/l  $\text{H}_2\text{BO}_3$ , 0.025 mg/l  $\text{CoCl}_2$ , 0.025 mg/l  $\text{CuSO}_4$ , 27.8 mg/l  $\text{FeSO}_4$ , 16.9 mg/l  $\text{MgSO}_4$ , 0.25 mg/l  $\text{NaMoO}_3$ , 0.83 mg/l KI and 8.6 mg/l  $\text{ZnSO}_4$ ; all from Sigma) containing 50 mM sucrose, 5 mM  $\text{KNO}_3$  and  $\text{KH}_2\text{PO}_4$  as indicated. Cells were then collected, frozen in liquid nitrogen and stored at –80 °C.

For seedling culture, the *phr1* and *bhlh32* (SALK\_013517) lines were in the Columbia background, while *gl3*, *egl3* and *ttg1* were in the Landsberg *erecta* background. The Salk\_013517 T-DNA insert line (obtained from the Nottingham *Arabidopsis* Stock Centre) was self-fertilized to obtain a homozygous line as judged by PCR. Seeds were sterilized and then germinated in a 250 ml flask of 100 ml 1/5 × MS macronutrients with 1.25 mM  $\text{KH}_2\text{PO}_4$ , 1 × MS micronutrient medium containing 50 mM sucrose and 2.5 mM Mes buffered to pH 6.0 by addition of Bis/Tris propane (Sigma). Seedlings were grown at 22 °C in a continuous low fluence rate of white light (20  $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ) with constant shaking (110 rev./min). Seven days after germination seedlings were transferred to 1/5 × MS without  $\text{KH}_2\text{PO}_4$ , 1 × MS micronutrient medium containing 50 mM sucrose and 2.5 mM Mes (pH 6.0) for 12 h or as indicated. The whole seedlings were harvested for gene expression analysis.

For analysis of root hair development, seeds were germinated on 1 × MS complete medium, 3% sucrose, 2.5 mM Mes (pH 5.7), 0.8% agar for 5 days, then transferred to 1/20 × MS macronutrients, 1 × MS micronutrients, 3% sucrose, 2.5 mM Mes (pH 5.7), 1.2% agar with a total  $P_i$  concentration of either 1.25 mM or 0.06 mM for 3 days. Roots were photographed on agar.

### RNA extraction, RT (reverse transcriptase)-PCR and real-time quantitative PCR

Total RNA was extracted using RNeasy plant mini kits (Qiagen) and then treated with DNase (DNA-free; Ambion) to remove contaminating genomic DNA. Total RNA (2.5  $\mu\text{g}$ ) was used to synthesize first strand cDNA and subsequently PCR was performed using specific primers for *PPCK1*, *PPCK2* and *ACTIN2* as described previously [32]: primers for *AtPT1* (At5g43350), forward 5'-acagatatcgagcttgagga-3', reverse 5'-ttggcgaagaagaagtaag-3'; primers for *AtPT2* (At2g38940), forward 5'-cgatcattccacttctct-3', reverse 5'-acagcttttgctcatgtcc-3'; primers for *SQD1* (UDP-sulfoquinovose synthase; At4g33030), forward 5'-ctactgctctcactgtcc-3', reverse 5'-ttggagtccactactacc-3'; primers for *BHLH32* (At3g25710), forward 5'-gtctctctctctctccatgg-3', reverse 5'-cttgacttaattaactataaacatcg-3'. All PCR was performed using the same programme: 94 °C for 2 min, 25 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; and a final extension of 72 °C for 5 min.

Real-time quantitative PCR was performed in 25  $\mu\text{l}$  reaction mixtures containing 5  $\mu\text{l}$  of 10-fold diluted first-strand cDNA, 0.3  $\mu\text{M}$  of each primer and 1 × QuantiTect SYBR Green PCR Master Mix (Qiagen). The following primers were used: *PPCK1* forward (5'-gaagattctcaaccgaacaagcatt-3'), *PPCK1* reverse (5'-ttggtagctggcgaatcta-3'); *PPCK2* forward (5'-tgtatcatcggaagcgaaggatt-3'), *PPCK2* reverse (5'-ttgctgaacacacaactactgttg-3'). The relative expression level was calculated using the comparative *Ct* (threshold cycle value) method. The expression level of *UBQ10* (At4g05320) was measured using the primers described in [14] and was taken as the housekeeping control. Fold changes

( $2^{-\Delta\Delta Ct}$ ) were expressed relative to wild-type seedlings without  $P_i$  starvation (zero time).

### Antibody production and Western blot analysis

The soybean *GmPPCK2* gene [33] was cloned into a modified version of pPICZ $\alpha$  (Invitrogen) which places FLAG and His<sub>10</sub> tags at the N-terminus [34] and was expressed in *Pichia pastoris* SMD116H cells (Invitrogen). The protein was purified from the extracellular fluid using chromatography on Phenyl Sepharose [35] and Chelating Sepharose Fast Flow (Amersham Biosciences), and antisera were raised in rabbits. PPCK was detected after SDS/PAGE as described in [36].

### Analytical methods

For measurements of anthocyanin and  $P_i$  content, seedlings were grown on agar plates with  $1/2 \times$  MS complete medium for 5 days and then transferred to plates plus (0.625 mM) or minus (0.04 mM)  $P_i$  for 7 days. Whole seedlings were extracted. The contents of anthocyanins and  $P_i$  were measured as described in [37] and [17] respectively. Crude extracts of seedlings or suspension cultured cells were prepared and used for determination of the L-malate sensitivity of PEPC as described previously [30]. Nuclei were isolated from 7-day-old seedlings using the CelLytic™ PN (plant nuclei) isolation kit (Sigma) according to the manufacturer's instructions. The GFP (green fluorescent protein)–BHLH32 fusion was detected using anti-GFP as the primary antibody as described in [36].

### Complementation of *bhlh32*

A full-length cDNA encoding BHLH32 was amplified by PCR (forward primer 5'-caccatgtacgcaatgaagaagaag-3', reverse primer 5'-tcccattttggatccctaattaactaaccc-3') and cloned into the Gateway entry vector pENTR™/SD/D-TOPO (Invitrogen). The fragment was transferred into the binary vector pGWB6 (Invitrogen) to yield the cauliflower mosaic virus 35S promoter:GFP–BHLH32 fusion using the Gateway LR recombination reaction. The construct was stably transformed into *bhlh32* mutant plants and homozygous lines were selected.

### Clone construction, transformation and yeast-two-hybrid analysis

Full-length cDNAs encoding PHR1, BHLH32, GL3, EGL3 and TTG1 were amplified by PCR. Primers for PHR1 were: forward 5'-ctaggaattcatggaggctcgtcc-3', reverse 5'-tccgaggatcccagcaagacca-3'; primers for BHLH32, forward 5'-acgaattcatgacgcaatgaagaagaag-3', reverse 5'-tcccattttggatccctaattaactaaccc-3'; primers for GL3, forward 5'-gggagctcgaattcgcattgctaccggacaaacaga-3', reverse 5'-ggtctagaggatcctcaacagatccatgcaacc-3'; primers for EGL3, forward 5'-atgggatgaagaattcggcaaccggag-3', reverse 5'-ctaaacctggatccttaacatcatcgc-3'; and primers for TTG1, forward 5'-cacacgaattcatggataattcagctcc-3', reverse 5'-aggcctagcctcaggaaattatgtagc-3'. Products were cloned into pTOPO-TA (Invitrogen). The full-length cDNAs for PHR1, BHLH32, GL3 and EGL3 were then released from their respective pTOPO clones as EcoRI/BamHI fragments, except for TTG1 which was released as an EcoRI/PstI fragment, and the fragments were ligated into the corresponding sites of the vectors pGBKT7 or pGADT7 (BD Biosciences). Pairs of different combinations of these pGBKT7 and pGADT7 vectors were co-transformed into yeast strain AH109 competent cells (BD Biosciences) and plated onto synthetic dropout medium lacking leucine, tryptophan, and histidine and supplemented with X- $\alpha$ -gal to investigate the interaction of these hybrid proteins.

$\alpha$ -Galactopyranosidase activity was assayed with *p*-nitrophenyl  $\alpha$ -galactopyranoside (Sigma) according to a protocol provided with the Matchmaker system (BD Biosciences). One unit of  $\alpha$ -galactopyranosidase was defined as the amount of enzyme that hydrolyses 1  $\mu$ mol PNPG (*p*-nitrophenyl  $\beta$ -D-glucopyranoside) to *p*-nitrophenol and D-galactose in 1 min at 30 °C in acetate buffer (pH 4.5).

### In vitro pulldown assay

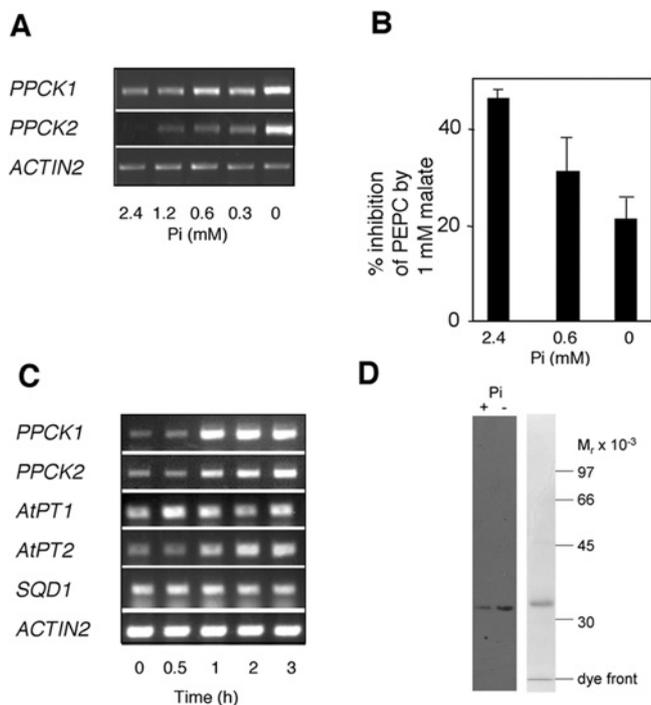
BHLH32, GL3 and TTG1 were recombined in-frame to pGBKT7 as described above. They were used as templates in a coupled transcription–translation system (TNT®T7; Promega) to synthesize protein to be used as prey. BHLH32 was also recombined in-frame to pET-41a(+) (Novagen) to produce GST (glutathione S-transferase)-tagged protein as a bait. Subsequently the pulldown assay was performed according to the manufacturer's protocol for the MagneGST™ Pull-Down System (Promega).

## RESULTS AND DISCUSSION

### PEPCK gene expression responds rapidly to $P_i$ starvation

In experiments on *PPCK* expression in an *Arabidopsis* cell culture [31] we noted that expression increased towards the end of each 7-day growth period and decreased following transfer to fresh medium. Systematic investigation of this phenomenon showed that depletion of  $P_i$  in the medium led to induction of *PPCK* expression. The effect of exposing *Arabidopsis* cells to reduced  $P_i$  concentrations for 3 h is illustrated by semi-quantitative RT-PCR in Figure 1(A). Expression of *PPCK2* changes dramatically over the range 0–2.4 mM  $P_i$ , whereas there is a lesser change of expression of *PPCK1*. To test whether the  $P_i$  starvation-induced expression of *PPCK* genes affected the phosphorylation state of PEPC, we measured the sensitivity of PEPC to inhibition by malate (a well-established indicator of the phosphorylation state) [29]. The increase in *PPCK* expression in response to  $P_i$  starvation was accompanied by a decrease in the malate sensitivity of PEPC (Figure 1B). These data indicate that  $P_i$  starvation increases the phosphorylation state of PEPC, thus demonstrating the functional effect of increased *PPCK* expression.

The rapid induction of *PPCK* gene expression in response to  $P_i$  starvation can also be detected using seedlings in liquid culture. Increased expression of *PPCK* genes was detectable within 1 h of the transfer of seedlings to  $P_i$ -deficient medium (Figure 1C), and an increase in the abundance of *PPCK* protein was evident by 3 h after transfer as judged by Western blot analysis (Figure 1D; the purified tagged soybean *PPCK*, against which the antiserum used in the present study was raised, is shown in the right-hand panel). The phosphate transporter gene *AtPT2* was also rapidly induced, whereas *AtPT1* was not affected in the time scale used. *SQD1*, which is involved in sulfolipid synthesis [38], also showed no increase in expression within 3 h of  $P_i$  starvation. This is in agreement with data from real-time quantitative RT-PCR experiments using hydroponically grown *Arabidopsis*, in which expression of *SQD1* was not induced after 4 h of  $P_i$  starvation [5]. In that study [5] it was reported that many of the genes that respond rapidly to  $P_i$  starvation are also induced by other stresses, and it was argued that rapid responses to  $P_i$  starvation are actually stress responses. However *PPCK* expression in *Arabidopsis* is not increased by such stresses as drought, high light or cold ([32]; Z. H. Chen and H. G. Nimmo, unpublished work). Moreover, as is the case for several genes induced by  $P_i$  starvation [39,40], repression of *PPCK* expression by  $P_i$  was replicated by the

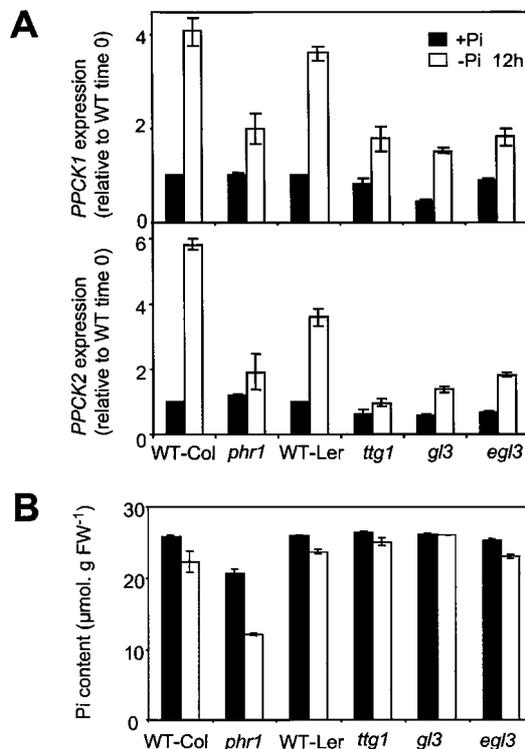


**Figure 1**  $P_i$  starvation induces *PPCK* gene expression and increases the phosphorylation state of PEPC

(A) and (B) 7-day-old cells were transferred to medium containing the indicated concentration of  $KH_2PO_4$  for 3 h. (A) Cells were collected, RNA was isolated and gene expression was analysed by RT-PCR. (B) Cells were treated as in (A). The malate sensitivity of PEPC in cell extracts is expressed as the percentage inhibition given by 1 mM L-malate. Error bars are S.D. for  $n=5$  measurements in one representative experiment. Relative to the value at 2.4 mM  $P_i$ , malate sensitivity is significantly reduced at 0.6 mM  $P_i$  and at zero  $P_i$  ( $P < 0.01$  and  $P < 0.001$  respectively, by Student's *t* test). (C) 7-day-old seedlings were transferred to MS micronutrient medium containing 50 mM sucrose and 5 mM  $KNO_3$  without  $P_i$  for the times indicated. Seedlings were collected, RNA was isolated and gene expression was analysed by RT-PCR. (D) Left-hand panel, seedlings were harvested after growth for 7 days with 1.25 mM  $P_i$  or after a further 3 h without  $P_i$  and PPCK protein was detected by Western blot analysis following SDS/PAGE of extracts (15  $\mu$ g protein); right-hand panel, purified tagged soybean PPCK resolved on SDS/PAGE and stained with Coomassie blue. The  $M_r$  of this protein is slightly bigger than those of the two *Arabidopsis* PPCKs.

non-metabolizable analogue phosphite (results not shown), indicating that  $P_i$  status affects *PPCK* expression rapidly through a signalling system that detects  $P_i$  and phosphite rather than through direct effects on metabolism or a stress response. Thus the induction of *PPCK* expression and the resultant phosphorylation of PEPC seem to be two of the fastest known responses specifically to  $P_i$  starvation.

These data thus define a new, rapid response to  $P_i$  starvation in *Arabidopsis*, namely the induction of *PPCK* gene expression. The consequence is increased phosphorylation of PEPC, one of the key enzymes involved in central metabolism in plants. These findings significantly extend the microarray experiments that showed induction of the *PPCK* genes in  $P_i$  starvation [7] by emphasizing the speed and the functional consequence of the response. There have been several reports that genes encoding plant PEPCs are induced in  $P_i$  starvation [7,26,41–43], including the three *Arabidopsis* *ppc* genes that encode the phosphorylatable, 'higher plant-type' PEPCs [44]. The proposed function of the induction of PEPC in  $P_i$  starvation is to provide a bypass of the pyruvate kinase step in glycolysis, reduce the requirement for adenine nucleotides and thus 'spare'  $P_i$  for use in essential processes. Several other metabolic adaptations to  $P_i$  starvation



**Figure 2** Gene expression in mutants under  $P_i$  starvation

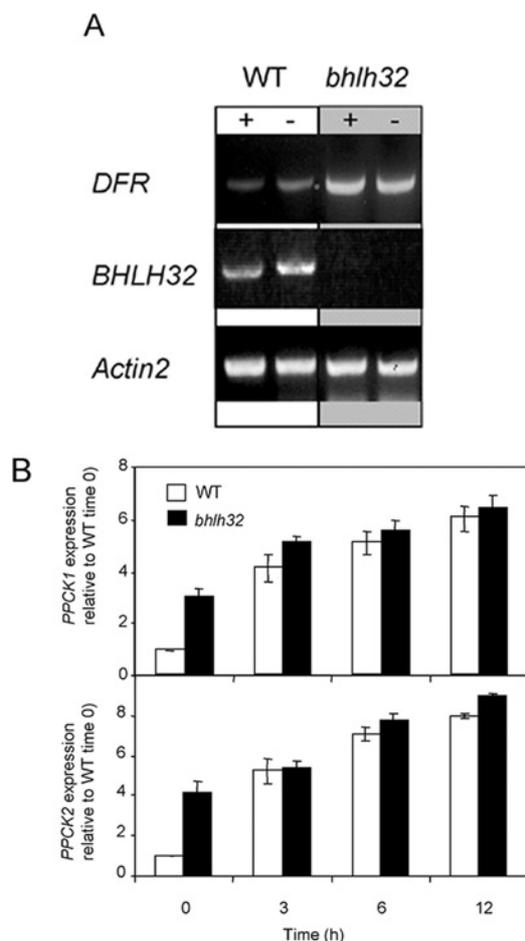
Seedlings (7 days) were transferred to  $1/5 \times$  MS without  $KH_2PO_4$ ,  $1 \times$  MS micronutrient medium containing 50 mM sucrose and 2.5 mM Mes (pH 6.0) for 12 h prior to harvesting. (A) Quantitative real-time PCR analysis of *PPCK* transcript abundance relative to that of *UBQ10*, values are means  $\pm$  S.D. ( $n = 4$ ). (B) Total  $P_i$  contents, means  $\pm$  S.D. ( $n = 4$ ). WT, wild-type.

are thought to provide the same general function of sparing  $P_i$  [1,3,4,26]. Hence it seems probable that the induction of *PPCK* expression and the resulting phosphorylation of PEPC serve to provide a rapid metabolic response to  $P_i$  starvation.

#### Identification of transcription factors required for the $P_i$ starvation-induced expression of *PPCK* genes

The use of seedlings in liquid culture allowed us to test the involvement of several gene products in the response to  $P_i$  starvation using a number of well-defined mutants. Figure 2(A) shows results from quantitative RT-PCR experiments. In the *phr1* mutant the induction of *PPCK1* and *PPCK2* expression in  $P_i$  starvation is markedly reduced but not eliminated. Another well-known effect of  $P_i$  starvation is to increase anthocyanin synthesis, and many regulatory genes for this pathway have been identified. Several of these regulators are additionally involved in controlling epidermal cell fate [45–47]. Figure 2(A) shows that the effect of  $P_i$  starvation on *PPCK* expression is compromised in *ttg1*, *gl3* and *egl3* mutant seedlings. However it is unaffected in *hy5* (*elongated hypocotyl5*) mutant seedlings (results not shown). Figure 2(B) shows that the total  $P_i$  content of wild-type, *ttg1*, *gl3* and *egl3* seedlings is barely affected by 12 h in  $P_i$ -deficient medium. *PPCK* expression is clearly sensitive to a small pool of  $P_i$  rather than to total  $P_i$  content. The effects of mutations in *TTG1*, *GL3* and *EGL3* on induction of *PPCK* expression by  $P_i$  starvation are not simply due to a change in total  $P_i$  content.

*TTG1* is a pleiotropic gene encoding a WD40 repeat protein involved in root hair initiation, trichome development, production of seed coat mucilage and anthocyanin synthesis [45]. *GL3* and *EGL3* are bHLH transcription factors that can interact



**Figure 3** Increased expression of *PPCK* and *DFR* genes in *bhlh32*

(A) Seedlings were harvested 3 h after transfer to medium with (+) or without (–)  $P_i$ . Expression of *BHLH32* and *DFR* was assessed by RT-PCR. The T-DNA insertion in *bhlh32* (Salk\_013517) is immediately after the end of the first exon of At3g25710 (results not shown). (B) Seedlings were transferred to medium without  $P_i$  for the indicated times. *PPCK* expression was assessed by quantitative real-time PCR as described for Figure 2. WT, wild-type.

with TTG1 in heterotrimeric TTG1–BHLH–MYB complexes to control various processes in the epidermis [47,48]. Since bHLH proteins are well-known for their ability to form heterodimers, we sought to identify other bHLHs with a role in  $P_i$  starvation. Microarray experiments had indicated that expression of *BHLH32* is induced after 48 h of  $P_i$  starvation [6]. We therefore obtained a T-DNA insertion *bhlh32* mutant from the Salk collection and isolated a homozygous line completely devoid of *BHLH32* transcripts (Figure 3A). Figure 3(B) shows the response of *bhlh32* to  $P_i$  starvation compared with the wild-type as time-courses of *PPCK* expression measured using quantitative RT-PCR following removal of  $P_i$  from the medium. Notably, the expression of *PPCK2* in  $P_i$ -sufficient medium (zero time in Figure 3B) is some 4-fold higher in *bhlh32* than in the wild-type, whereas that of *PPCK1* is some 3-fold higher. In line with this, the malate sensitivity of PEPC is significantly lower in  $P_i$ -sufficient *bhlh32* seedlings than in the wild-type (results not shown). However, the inductive effect of  $P_i$  starvation on *PPCK* expression is seen in both the wild-type and *bhlh32*. Thus *BHLH32* acts as a negative regulator of *PPCK* expression in  $P_i$  sufficiency, but does not directly affect induction in  $P_i$  starvation.

TTG1 acts in the root, seed and shoot epidermis in conjunction with specific transcription factors to control either cell fate

or biosynthetic processes [45,46,48].  $P_i$  starvation affects both root biochemistry and root development. For example, it causes increases in secretion of organic acids, root/shoot ratio, root hair proliferation and length and lateral root number. It is therefore not surprising that TTG1 is involved in at least some of these responses, and our results show that a subset of bHLH proteins act with TTG1 in specific responses to  $P_i$  starvation. The morphological and biochemical responses of roots to  $P_i$  starvation may be linked by the involvement of these proteins.

### BHLH32 affects root hair development, anthocyanin formation and $P_i$ content

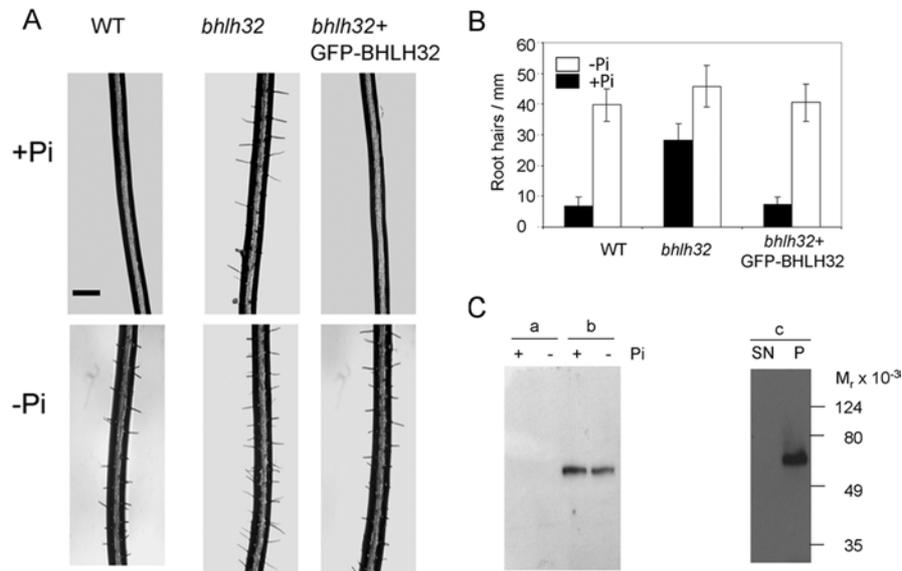
Because of the well-established connection between  $P_i$  nutrition and root hair development [2], we studied root hair production in *bhlh32*. Figures 4(A) and 4(B) clearly show that, in contrast to the wild-type, root hair formation in *bhlh32* is not suppressed by growth in the presence of high levels of  $P_i$ . This phenotype is reversed by expression of a GFP–BHLH32 fusion protein from the cauliflower mosaic virus 35S promoter. The location of the fusion protein was examined first by centrifugation of seedling extracts and subsequently using isolated nuclei. Figure 4(C) shows that the fusion protein was predominantly nuclear, and was not extracted by high salt, suggesting that it was tightly bound to DNA. The *bhlh32* mutant had no obvious growth phenotype apart from this effect on root hairs.

When grown in  $P_i$ -sufficient conditions, *bhlh32* contained significantly more total  $P_i$  and more anthocyanin than the wild-type (Figure 5), whereas *phr1*, in agreement with previous work [8], showed the opposite effect. We also monitored the expression of *DFR*, which encodes dihydroflavonol reductase, the key step in the formation of anthocyanins. Figure 3(A) shows that *DFR* expression in  $P_i$ -sufficient conditions was substantially increased in the *bhlh32* mutant. Thus *BHLH32* acts as a negative regulator of three processes, *PPCK* expression, root hair formation and anthocyanin production, all of which have in common the property that they respond to  $P_i$  starvation. Furthermore, complexes containing TTG1 were required for correct functioning of all of these processes.

### Physical interactions between transcription factors involved in $P_i$ starvation

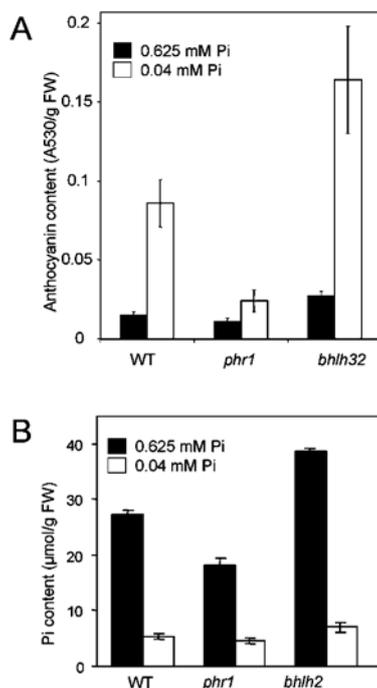
Short-term  $P_i$  starvation of seedlings (1–2 h) did not affect the transcript abundance of *PHR1*, *TTG1*, *GL3*, *EGL3* and *BHLH32* (results not shown). We therefore reasoned that early responses to  $P_i$  starvation may affect the location or activity of complexes involving some or all of these factors rather than the absolute abundance of the proteins, although induction of *BHLH32* [6] may be involved in longer-term effects. As a first step towards identifying such complexes we have used the yeast-two-hybrid system. Figures 6(A) and 6(B) show that *BHLH32* interacted physically with itself, with TTG1 and with GL3. However it did not appear to interact with either PHR1 or EGL3. Our data confirm the already reported interactions of TTG1 with GL3 and with EGL3 and the formation of GL3–EGL3 heterodimers [47,48]. PHR1 and GL3 both result in auto-activation when fused to the DNA-binding domain (results not shown) so we were unable to test whether they interacted. However, PHR1 did not seem to interact with either TTG1 or EGL3 (Figures 6A and 6B).

To confirm these interactions using another method, we expressed a GST–BHLH32 fusion protein in *Escherichia coli* and immobilized it onto glutathione-agarose beads. We then used the beads to probe  $^{35}S$ -labelled proteins expressed by *in vitro* transcription/translation. Figure 6(C) shows that labelled *BHLH32*,



**Figure 4** Expression of GFP-BHLH32 corrects the root hair phenotype of *bhlh32*

Seedlings were grown as described in the Experimental section. (A) Representative images of Col0, *bhlh32* and *bhlh32* transformed with the GFP-BHLH32 fusion. The scale bar represents 100  $\mu\text{m}$ . (B) Root hairs were counted from 0.75 mm to 1.75 mm from the root tip, values are means  $\pm$  S.D. ( $n = 5$ ). The *bhlh32* line contains significantly more root hairs in the presence of  $\text{P}_i$  than the wild-type or *bhlh32* expressing GFP-BHLH32 ( $P < 0.001$ , Student's *t* test). (C) Western blot analysis of the location of GFP-BHLH32. Left-hand panel, seedlings of *bhlh32* transformed with the GFP-BHLH32 fusion were grown as described in the Experimental section and harvested before (+ $\text{P}_i$ ) or after ( $-\text{P}_i$ ) 3 h of  $\text{P}_i$  starvation. Extracts were centrifuged at 12000 *g* and equal amounts (15  $\mu\text{g}$  protein) were analysed by SDS/PAGE and Western blot analysis with an anti-GFP antibody. Lanes a, supernatants; lanes b, pellets. Right-hand panel, nuclei from seedlings in  $\text{P}_i$ -sufficient medium were prepared and subjected to high salt extraction (see the Experimental section); SN and P represent the soluble and particulate material from this extraction respectively.



**Figure 5** *bhlh32* contains increased anthocyanin and  $\text{P}_i$

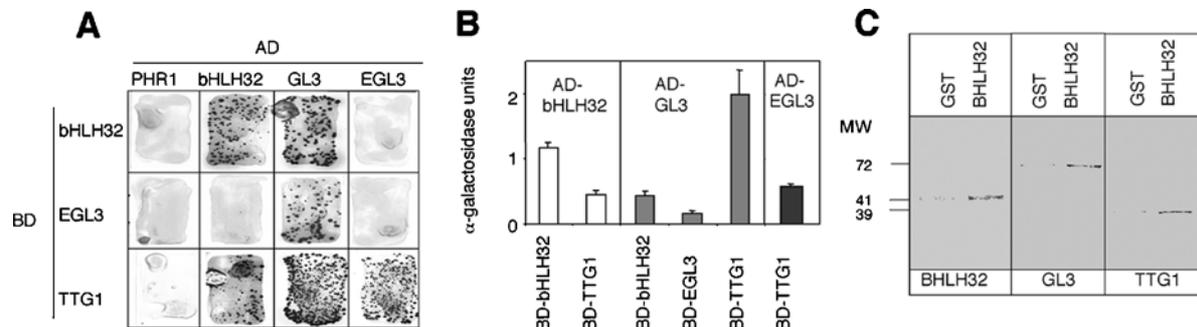
Seedlings were grown as described in the Experimental section. (A) Anthocyanin content and (B)  $\text{P}_i$  content, values are means  $\pm$  S.D. ( $n = 4$ ). Relative to the wild-type, *bhlh32* contains significantly more anthocyanin at both 0.625 mM  $\text{P}_i$  and 0.04 mM  $\text{P}_i$ ,  $P < 0.001$  and  $P < 0.01$  respectively, and significantly more  $\text{P}_i$  at both concentrations,  $P < 0.001$  and  $P < 0.05$  respectively.

GL3 and TTG1 were all captured by immobilized GST-BHLH32 but not by immobilized GST, thus confirming the results of the yeast-two-hybrid experiments.

### The involvement of transcription factors in $\text{P}_i$ starvation

The only transcription factor previously implicated in  $\text{P}_i$  starvation in *Arabidopsis* is the single MYB protein PHR1 [8]. This is involved in the induction of *PPCK* genes in  $\text{P}_i$  starvation but, in addition, it is clear that TTG1, GL3 and EGL3 are also required for full induction (Figure 2). In other systems, the role of TTG1 seems to be in a complex with bHLH and R2R3 MYB proteins [47]. In these complexes the bHLH interacts with both TTG1 and the MYB, which do not interact with each other. PHR1 does not contain the bHLH-interaction domain and we have no evidence to show that it interacts with any of the other factors that we have implicated in  $\text{P}_i$  starvation responses. We therefore suggest that the involvement of PHR1 in  $\text{P}_i$  starvation is quite separate to that of a TTG1-bHLH-MYB complex. This is reminiscent of seed mucilage production which is regulated by both TTG1-dependent and TTG1-independent pathways [49].

The three bHLH proteins previously implicated as positive factors in TTG1-dependent pathways, GL3, EGL3 and TT8 (TRANSPARENT TESTA8), show significant amino acid sequence similarity. As judged by yeast-two-hybrid analysis of truncated fragments, they all possess an N-terminal MYB-interacting domain, a central TTG1-interacting domain and a C-terminal bHLH domain [47,48]. BHLH32 is a much smaller protein, apparently lacking a MYB-interacting domain, though it can clearly bind to TTG1 and to some bHLHs, including itself (Figure 6). The function of *BHLH32* in *Arabidopsis* has not previously been reported. Our data show that it negatively regulates root hair formation, anthocyanin accumulation and *PPCK* expression, all of which are induced by  $\text{P}_i$  starvation (Figures 3–5). In addition  $\text{P}_i$  uptake and storage are de-regulated in the *bhlh32* mutant. However *BHLH32* is not apparently required itself for the induction of root hair production, anthocyanin formation and *PPCK* expression (Figures 3–5).



**Figure 6** Physical interactions between components involved in the  $P_i$  starvation response

(A) and (B) show interactions analysed in the yeast-two-hybrid system. (A) The indicated combinations of BD (DNA-binding domain) and AD (activation domain) vectors were co-transformed into yeast strain AH109 and plated as described in the Experimental section. Positive interactions are shown by the growth of blue colonies. (B) The strength of positive interactions was assessed by  $\alpha$ -galactopyranosidase activity assays. Values are means  $\pm$  S.D. from four individual yeast colonies. (C) *In vitro* pull-down assay. BHLH32 was expressed as a GST-fusion protein in *E. coli* and immobilized onto glutathione-agarose beads and then incubated with  $^{35}S$ -labelled proteins expressed using the *in vitro* transcription-translation system. As a negative control, beads were loaded with GST. Bound proteins were resolved by SDS/PAGE and visualized using a Fuji phosphorimager.

How could BHLH32 affect such disparate processes, and could any of its effects be secondary? It is noteworthy that the *bhlh32* mutant contains elevated levels of  $P_i$ . This could simply reflect increased root hair formation, although disruption of  $P_i$  homeostasis may also be involved. However, while the increased  $P_i$  in *bhlh32* would be expected to reduce the expression of *PPCK* and *DFR*, and the accumulation of anthocyanins, the opposite is observed. Hence BHLH32 must affect these processes quite independently of  $P_i$  content.

TTG1-bHLH-MYB complexes are involved in the induction of both *DFR* and *PPCK* genes in  $P_i$  starvation, though in the latter case PHR1 is also involved. However the underlying mechanisms are not understood. Our data demonstrate that BHLH32 can interact physically with TTG1 and with GL3. We therefore suggest that BHLH32 can interfere with TTG1-containing complexes such that its loss in the *bhlh32* mutant partly mimics the effects of  $P_i$  starvation and thereby increases the expression of *DFR* and *PPCK* even in  $P_i$  sufficiency. This is compatible with the nuclear localization of the GFP-BHLH32 fusion protein (Figure 4). The events underlying the effects of  $P_i$  starvation on root hair formation are not understood, and this system is more complex because it involves intercellular movement of proteins [23]. Any factor that affects the cellular distribution of TTG1, GL3, EGL3 and CPC could affect root hair formation either positively or negatively. Hence it is possible that in root epidermal cells BHLH32 can interact with TTG1 and bHLHs and affect root hair formation in such a way that it is increased in the *bhlh32* mutant.

*BHLH32* thus seems to act as a negative regulator of several genes in  $P_i$ -sufficient conditions, in line with the concept proposed in [18]. This role of *BHLH32* is quite different from that of *PHR1*, which is viewed as a positive regulator of  $P_i$  starvation responses [8]. The combination of negative regulation in the absence of the stimulus (i.e.  $P_i$  sufficiency) and positive regulation in response to the stimulus ( $P_i$  starvation) resembles the mechanism of plant responses to other environmental stimuli, including light, where positively acting photoreceptor signalling pathways overcome negative regulation of photomorphogenesis in darkness by components such as DET1 (DE-ETIOLATED 1) and COP1 (CONSTITUTIVE PHOTOMORPHOGENIC locus 1) [50].

Cellular and Molecular Biology, University of Texas at Austin, Austin, Texas, U.S.A.) for the gift of *phr1* and *egl3* seed respectively. We thank Janet Laird for excellent technical assistance.

## REFERENCES

- Raghothama, K. G. (1999) Phosphate acquisition. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 665–693
- Bates, T. R. and Lynch, J. P. (1996) Stimulation of root hair elongation in *Arabidopsis thaliana* by low phosphorus availability. *Plant Cell Environ.* **19**, 529–538
- Vance, C. P., Uhde-Stone, C. and Allan, D. L. (2003) Phosphorus acquisition and use: critical adaptations by plants for securing a non-renewable resource. *New Phytol.* **157**, 423–447
- Ticconi, C. A. and Abel, S. (2004) Short on phosphate: plant surveillance and countermeasures. *Trends Plant Sci.* **9**, 548–555
- Hammond, J. P., Bennett, M. J., Bowen, H. C., Broadley, M. R., Eastwood, D. C., May, S. T., Rahn, C., Swarup, R., Woolaway, K. E. and White, P. J. (2003) Changes in gene expression in *Arabidopsis* shoots during phosphate starvation and the potential for developing smart plants. *Plant Physiol.* **132**, 578–596
- Wu, P., Ma, L., Hu, X., Wang, M., Wu, Y., Liu, F. and Deng, X. W. (2003) Phosphate starvation triggers distinct alterations of genome expression in *Arabidopsis* roots and leaves. *Plant Physiol.* **132**, 1260–1271
- Misson, J., Raghothama, K. G., Jain, A., Jouhet, J., Block, M. S., Bligny, R., Ortet, P., Creff, A., Somerville, S., Rolland, N. et al. (2005) A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate starvation. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 11934–11939
- Rubio, V., Linhares, F., Solano, R., Martín, A. C., Iglesias, J., Leyva, A. and Paz-Ares, J. (2001) A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. *Genes Dev.* **15**, 2122–2133
- Chen, D. L., Delatorre, C. A., Bakker, A. and Abel, S. (2000) Conditional identification of phosphate-starvation-response mutants in *Arabidopsis thaliana*. *Planta* **211**, 13–22
- Ticconi, C. A., Delatorre, C. A., Lahner, B., Salt, D. E. and Abel, S. (2004) *Arabidopsis pdr2* reveals a phosphate-sensitive checkpoint in root development. *Plant J.* **37**, 801–814
- Sánchez-Calderón, L., López-Bucio, J., Chacón-López, A., Gutiérrez-Ortega, A., Hernández-Abreu, E. and Herrera-Estrella, L. (2006) Characterization of low phosphorus insensitive mutants reveals a crosstalk between low phosphorus-induced determinate root development and the activation of genes involved in the adaptation of *Arabidopsis* to phosphorus deficiency. *Plant Physiol.* **140**, 879–889
- Fujii, H., Chiou, T. Z., Lin, S.-I., Aung, K. and Zhu, J.-K. (2005) A miRNA involved in phosphate-starvation response in *Arabidopsis*. *Curr. Biol.* **15**, 2038–2043
- Aung, K., Lin, S.-I., Wu, C.-C., Huang, Y.-T., Su, C.-L. and Chiou, T.-Z. (2006) *pho2*, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. *Plant Physiol.* **141**, 1000–1011
- Bari, R., Pant, B. D., Stitt, M. and Scheible, W.-R. (2006) PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiol.* **141**, 988–999
- Chiou, T.-Z., Aung, K., Lin, S.-I., Wu, C.-C., Chiang, S.-F. and Su, C.-L. (2006) Regulation of phosphate homeostasis by microRNA in *Arabidopsis*. *Plant Cell* **18**, 412–421

This work was supported by the BBSRC (Biotechnology and Biological Sciences Research Council). We thank Dr Javier Paz-Ares (Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología, Madrid, Spain) and Dr Alan Lloyd (The Institute for

- 16 Miura, K., Rus, A., Sharkhuu, A., Yokoi, S., Karthikeyan, A. S., Raghothama, K. G., Baek, D., Koo, Y. D., Jin, J. B., Bressan, R. A., Yun, D.-J. and Hasegawa, P. M. (2005) The *Arabidopsis* SUMO E3 ligase SIZ1 controls phosphate deficiency responses. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 7760–7765
- 17 Stevenson-Paulik, J., Bastidas, R. J., Chiou, S.-T., Frye, R. A. and York, J. D. (2005) Generation of phytate-free seeds in *Arabidopsis* through disruption of inositol polyphosphate kinases. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 12612–12617
- 18 Mukatira, U. T., Liu, C., Varadarajan, D. K. and Raghothama, K. G. (2001) Negative regulation of phosphate starvation-induced genes. *Plant Physiol.* **127**, 1854–1862
- 19 Gilroy, S. and Jones, D. L. (2000) Through form to function: root hair development and nutrient uptake. *Trends Plant Sci.* **5**, 56–60
- 20 Schiefelbein, J. W. (2000) Constructing a plant cell. The genetic control of root hair development. *Plant Physiol.* **124**, 1525–1531
- 21 Schiefelbein, J. (2003) Cell-fate specification in the epidermis: a common patterning mechanism in the root and shoot. *Curr Opin. Plant Biol.* **6**, 74–78
- 22 Serna, L. (2004) A network of interacting factors triggering different cell fates. *Plant Cell* **16**, 2258–2263
- 23 Bernhardt, C., Zhao, M., Gonzalez, A., Lloyd, A. and Schiefelbein, J. (2005) The bHLH genes GL3 and EGL3 participate in an intercellular regulatory circuit that controls cell patterning in the *Arabidopsis* root epidermis. *Development* **132**, 291–298
- 24 Müller, M. and Schmidt, W. (2004) Environmentally induced plasticity of root hair development in *Arabidopsis*. *Plant Physiol.* **134**, 409–419
- 25 Ma, Z., Bielenberg, D. G., Brown, K. M. and Lynch, J. P. (2001) Regulation of root hair density by phosphorus availability in *Arabidopsis thaliana*. *Plant Cell Environ.* **24**, 459–467
- 26 Plaxton, W. C. and Carswell, M. C. (1999) Metabolic aspects of the phosphate starvation response in plants. In *Plant Responses to Environmental Stresses: From Phytohormones to Genome Reorganization* (Lerner, H. R., ed.), pp. 349–372. New York, Marcel Dekker
- 27 Vidal, J. and Chollet, R. (1997) Regulatory phosphorylation of C<sub>4</sub> PEP carboxylase. *Trends Plant Sci.* **2**, 230–237
- 28 Nimmo, H. G. (2000) The regulation of phosphoenolpyruvate carboxylase in CAM plants. *Trends Plant Sci.* **5**, 75–80
- 29 Nimmo, H. G. (2003) Control of the phosphorylation of phosphoenolpyruvate carboxylase in higher plants. *Arch. Biochem. Biophys.* **414**, 189–196
- 30 Hartwell, J., Gill, A., Nimmo, G. A., Wilkins, M. B., Jenkins, G. I. and Nimmo, H. G. (1999) Phosphoenolpyruvate carboxylase kinase is a novel protein kinase regulated at the level of expression. *Plant J.* **20**, 333–342
- 31 May, M. J. and Leaver, C. J. (1993) Oxidative stimulation of glutathione synthesis in *Arabidopsis thaliana* suspension cultures. *Plant Physiol.* **103**, 621–627
- 32 Fontaine, V., Hartwell, J., Jenkins, G. I. and Nimmo, H. G. (2002) *Arabidopsis thaliana* contains two phosphoenolpyruvate carboxylase kinase genes with different expression patterns. *Plant Cell Environ.* **25**, 115–122
- 33 Sullivan, S., Jenkins, G. I. and Nimmo, H. G. (2004) Roots, cycles and leaves. Expression of the phosphoenolpyruvate carboxylase kinase gene family in soybean. *Plant Physiol.* **135**, 2078–2087
- 34 Fraser, N. J. (2006) Expression and functional purification of a glycosylation deficient version of the human adenosine 2a receptor for structural studies. *Protein Expr. Purif.* **49**, 129–137
- 35 Nimmo, G. A., Wilkins, M. B. and Nimmo, H. G. (2001) Partial purification and characterization of a protein inhibitor of phosphoenolpyruvate carboxylase kinase. *Planta* **213**, 250–257
- 36 Chen, Z. H., Walker, R. P., Tecsli, L. I., Lea, P. J. and Leegood, R. C. (2004) Phosphoenolpyruvate carboxylase in cucumber plants is increased both by ammonium and by acidification, and is present in the phloem. *Planta* **219**, 48–58
- 37 Swain, T. and Hillis, H. E. (1959) Phenolic constituents of *Prunus domestica*. I. Quantitative analysis of phenolic constituents. *J. Sci. Food Agr.* **10**, 63–68
- 38 Essigman, B., Güler, S., Narang, R. A., Linke, D. and Benning, C. (1998) Phosphate availability affects the thylakoid lipid composition and the expression of *SQD1*, a gene required for sulfolipid biosynthesis in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1950–1955
- 39 Carswell, M. C., Grant, B. R. and Plaxton, W. C. (1997) Disruption of the phosphate-starvation response of oilseed rape suspension cells by the fungicide phosphonate. *Planta* **203**, 67–74
- 40 Ticconi, C. A., Delatorre, C. A. and Abel, S. (2001) Attenuation of phosphate starvation responses by phosphite in *Arabidopsis*. *Plant Physiol.* **127**, 963–972
- 41 Johnson, J. F., Allan, D. L. and Vance, C. P. (1994) Phosphorus stress-induced proteoid roots show altered metabolism in *Lupinus albus*. *Plant Physiol.* **104**, 657–665
- 42 Moraes, T. F. and Plaxton, W. C. (2000) Purification of phosphoenolpyruvate carboxylase from *Brassica napus* (rapeseed) suspension cell cultures. *Eur. J. Biochem.* **267**, 4465–4476
- 43 Toyota, K., Koizumi, N. and Sato, F. (2003) Transcriptional activation of phosphoenolpyruvate carboxylase by phosphorus deficiency in tobacco. *J. Exp. Bot.* **54**, 961–969
- 44 Sánchez, R. and Cejudo, F. J. (2003) Identification and expression analysis of a gene encoding a bacterial-type phosphoenolpyruvate carboxylase from *Arabidopsis* and rice. *Plant Physiol.* **132**, 949–957
- 45 Walker, A. R., Davison, P. A., Bolognesi-Winfield, A. C., James, C. A., Srinivasan, N., Blundell, T. M., Esch, J. J., Marks, M. D. and Gray, J. C. (1999) The *TRANSPARENT TESTA GLABRA1* locus, which regulates trichome differentiation and anthocyanin biosynthesis in *Arabidopsis*, encodes a WD40 repeat protein. *Plant Cell* **11**, 1337–1349
- 46 Nesi, N., Debeaujon, I., Jond, C., Pelletier, G., Caboche, M. and Lepiniec, L. (2000) The *TT8* gene encodes a basic helix-loop-helix domain protein required for expression of *DFR* and *BAN* genes in *Arabidopsis* siliques. *Plant Cell* **12**, 1863–1878
- 47 Zhang, F., Gonzalez, A., Zhao, M., Payne, C. T. and Lloyd, A. (2003) A network of redundant bHLH proteins functions in all TTG1-dependent pathways of *Arabidopsis*. *Development* **130**, 4859–4869
- 48 Payne, C. T., Zhang, F. and Lloyd, A. M. (2000) *GL3* encodes a bHLH protein that regulates trichome development in *Arabidopsis* through interaction with *GL1* and *TTG1*. *Genetics* **156**, 1349–1362
- 49 Western, T. L., Young, D. S., Dean, G. H., Tan, W. L., Samuels, L. and Haughn, G. W. (2004) *MUCILAGE-MODIFIED4* encodes a putative pectin biosynthetic enzyme developmentally regulated by *APETALA2*, *TRANSPARENT TESTA GLABRA1*, and *GLABRA2* in the *Arabidopsis* seed coat. *Plant Physiol.* **134**, 296–306
- 50 Chen, M., Chory, J. and Fankhauser, C. (2004) Light signal transduction in higher plants. *Annu. Rev. Genet.* **38**, 87–117

Received 18 January 2007/6 March 2007; accepted 21 March 2007

Published as BJ Immediate Publication 21 March 2007, doi:10.1042/BJ20070102