

Signaling of phosphorus deficiency-induced gene expression in white lupin requires sugar and phloem transport

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Received 29 June 2004; revised 28 September 2004; accepted 22 October 2004.

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Summary

Roots of phosphorus (P)-deficient white lupin exhibit striking changes in morphology and gene expression. In this report we provide further insight into genetic elements affecting transcription of P-deficiency-induced genes. Moreover, we also show that sugars and photosynthates are integrally related to P-deficiency-induced gene expression. White lupin phosphate transporter (*LaPT1*) and secreted acid phosphatase (*LaSAP1*) promoter-reporter genes when transformed into alfalfa, a heterologous legume, showed significant induction in roots specifically in response to P-deficiency. In addition, both promoters were active in nitrogen-fixing root nodules but not in ineffective nodules indicating a link between P-deficiency and factors related to nitrogen fixation/metabolism. As sugars play a role in signal transduction during nitrogen assimilation and are required for effective nitrogen fixation, we tested the relationship of sugars to P-deficiency-induced gene expression. Exogenous sucrose, glucose, and fructose stimulated *LaPT1* and *LaSAP1* transcript accumulation in dark-grown P-sufficient white lupin seedlings. Furthermore, in intact P-deficient white lupin plants, *LaPT1* and *LaSAP1* expression in cluster roots was strikingly reduced in dark-adapted plants with expression rapidly restored upon reexposure to light. Likewise, interruption of phloem supply to P-deficient roots resulted in a rapid decline in *LaPT1* and *LaSAP1* transcript accumulation. Similar results were also obtained with a third lupin P-deficiency-induced gene encoding a putative multidrug and toxin efflux protein (*LaMATE*). Taken together, our data show that the regulation of P-deficiency-induced genes is conserved across plant species and sugars/photosynthates are crucial for P-deficiency signal transduction.

Keywords: phosphorus deficiency, acid phosphatase, phosphate transporter, phloem transport.

Introduction

Plants are frequently phosphorus (P)-starved due to the extremely low soil bioavailability of this macronutrient (<1 μM). Phosphorus is the most limiting nutrient for plant growth and development on much of the world's arable land (Marschner, 1995). In response to P limitation, plants have evolved various biochemical, metabolic, and morphological adaptations to enhance P acquisition, including: increased synthesis and secretion of organic acids and acid phosphatases (APs); higher root/shoot ratio and lateral root growth; and increased root hair length and density for a larger root surface area (Dinkelaker *et al.*, 1989; Marschner, 1995). Genes coding for phosphate transporters (PTs) and APs are among the major P-deficiency-induced genes isolated and

characterized from a variety of plant species (Del Pozo *et al.*, 1999; Leggewie *et al.*, 1997; Liu *et al.*, 1998, 2001; Miller *et al.*, 2001; Muchhal *et al.*, 1996; Smith *et al.*, 1997). As demonstrated by 'split root' experiments, P-deficiency-induced genes appear to be regulated systemically by P status of the whole plant instead of local P concentration and supply (Burleigh and Harrison, 1999; Liu *et al.*, 1998; Shane *et al.*, 2003).

The molecular mechanism of P-deficiency response has been extensively studied in unicellular model organisms. The existence of a P-deficiency-responsive *pho* regulon in the yeast *Saccharomyces cerevisiae* has been well documented (Oshima, 1997). A major positive transcription

regulator, PHO4, activates gene expression upon P stress by binding to the conserved element (5'-CACGTG/T-3') in the promoters of a set of genes, including the phosphatase and phosphate transporter genes of the yeast *pho* regulon (Ogawa *et al.*, 1995). Wykoff *et al.* (1999) reported the isolation of a regulatory gene *psr1* controlling P-deficiency response in the green alga *Chlamydomonas reinhardtii*, a unicellular photosynthetic model. Mutation of the *psr1* gene leads to a reduced response of various P-deficiency-induced genes such as those coding for secreted APs. In higher plants, identification of an Arabidopsis mutant, *phr1*, and the cloning of a PHR1 MYB-like transcription activator have shed more light on the regulatory network of P-deficiency-induced genes (Rubio *et al.*, 2001). Mutation of the *phr1* gene caused impaired response of several P-deficiency-induced genes and other changes typical of P-deficiency such as anthocyanin biosynthesis. PSR1 from green algae and PHR1 from Arabidopsis share a high amount of homology in MYB domains, both domains belonging to the MYB-coiled coil (MYB-cc) family of transcription factors. A similarity between the two regulatory genes from green algae and higher plants suggests that there is a partly conserved positive regulatory mechanism among the unicellular and multicellular photosynthetic organisms in response to P-deficiency (Wykoff *et al.*, 1999). However, it is expected that the temporal and spatial expression of P-deficiency-induced genes in higher plants will be much more complex because of multiple metabolic changes occurring in different parts of the plant. The mechanism for generating such a systemic P-deficiency signal in the shoot of P-stressed plants remains largely unknown.

We have used white lupin, a nitrogen-fixing legume, as a model to assess crop plant adaptations to P-deficiency. White lupin has high tolerance to P-deficiency and striking morphological changes in its root architecture under P-deficient conditions, namely the formation of proteoid (cluster) roots. Concomitant with the development of cluster roots is enhanced synthesis of organic acids, P-transporters, secreted acid phosphatase, and H⁺-ATPase (Vance *et al.*, 2003). We previously reported the cloning and characterization of a P-deficiency-induced phosphate transporter gene (*LaPT1*) and a secreted acid phosphatase (*LaSAP1*) gene from white lupin (*Lupinus albus*) (Liu *et al.*, 2001; Miller *et al.*, 2001). Both *LaPT1* and *LaSAP1* promoters contain a short sequence with the consensus 5'-GNAT-ATNC-3' which was identical to the binding site for AtPHR1, an MYB-cc transcription activator for P-deficiency-induced genes in Arabidopsis (Rubio *et al.*, 2001). While *LaSAP1* is mainly induced in cluster roots, *LaPT1* is also expressed in aerial parts in addition to roots of white lupin. We postulate that the uptake and redistribution of phosphate under P-deficiency is crucial for plants to develop tolerance and survive P-deficient conditions and the spatial expression pattern of *LaPT1* and *LaSAP1* may reflect this dynamic

process. To test this hypothesis and to obtain further details of the spatial and temporal pattern of both promoter activities, we introduced *LaPT1-GUS* and *LaSAP1-GUS* reporter genes into a heterologous legume – alfalfa (*Medicago sativa*). In this study, we not only confirmed the specific induction of both *LaPT1* and *LaSAP1* promoters by P-deficiency, but also extended our understanding of the developmental regulation in reproductive organs and in nitrogen-fixing nodules.

As the induction of both *LaPT1* and *LaSAP1* proved to be highly specific to P stress in alfalfa as well as in white lupin, we decided to further investigate how P stress treatment is transduced into a systemic –P signal in white lupin. There is evidence that nitrogen signaling in higher plants involves crosstalk between carbon and nitrogen metabolisms. For example, NO₃⁻ uptake is regulated by photosynthesis and sugar supply (Lejay *et al.*, 2003). An Arabidopsis gene, *GLB1*, involved in nitrogen sensing, is also regulated by light and sucrose (Hsieh *et al.*, 1998). Therefore, we hypothesized that sugars may be involved in transduction of P-deficiency-induced gene expression. We found that the expression of genes induced in response to P-deficiency could be induced by exogenous sugars in P-sufficient white lupin seedlings. We also discovered that light and phloem transport were required for P-deficiency-induced gene expression in cluster roots of intact white lupin. These results show that P-deficiency response is mediated at least in part by sugars and photosynthates in higher plants.

Results

Construction of promoter-GUS reporter genes and regeneration of transgenic alfalfa

Because white lupin is not readily transformed via *Agrobacterium tumefaciens* we transformed alfalfa with *LaPT1-GUS* and *LaSAP1-GUS* reporter gene constructs. The 5'-untranslated intron in the *LaPT1* gene was included in the *LaPT1-GUS* reporter gene to achieve a GUS activity pattern most representative of the *LaPT1* expression pattern in white lupin, because the 5'-untranslated intron in some plant genes contributes to the intrinsic expression pattern and/or expression level. Both *LaSAP1-GUS* and *LaPT1-GUS* translational fusions were introduced into alfalfa via *Agrobacterium*-mediated transformation. Plants regenerated from kanamycin-containing medium were initially tested by PCR of genomic DNA to identify transgenics. More than 80% of the regenerated plants carried the reporter gene. To further confirm the integration of reporter genes, six plants were randomly chosen among the *LaSAP1-GUS* and *LaPT1-GUS* transgenic plants and analyzed by DNA blot hybridization. *EcoRI* cuts only at the left border of the T-DNA region and not internally within the reporter gene, so each band observed

represents a different integration site of the reporter gene in the alfalfa genome. Transgenic plants harbored from one to four copies of the promoter-GUS reporter genes (data not shown). The equal intensity among individual bands suggests that each integration site represented single-copy instead of multi-copy reporter genes. Regenerated but non-transgenic plants were used as controls and did not show hybridization signals.

Activity of LaSAP1 and LaPT1 promoters in roots of transgenic plants

To test whether alfalfa plants transformed with *LaSAP1-GUS* and *LaPT1-GUS* showed P-deficiency-induced reporter gene activity, vegetative cuttings were made from each of 20 independent transformants and grown on +P (1.0 mM calcium phosphate) or -P conditions for 4 weeks. Root segments were excised and stained for GUS activity. Roots of both *LaSAP1-GUS* and *LaPT1-GUS* plants grown under -P conditions displayed strong GUS activity (Figure 1). In contrast, no GUS activity was detected under +P conditions for both the *LaSAP1-GUS* and *LaPT1-GUS* root tissues (Figure 1). These data show that the 5'-upstream promoter region for *LaSAP1* and *LaPT1* directs P-deficiency-induced reporter gene activity in alfalfa roots, similar to that of the endogenous *LaSAP1* and *LaPT1* expression in P-deficient white lupin (Liu *et al.*, 2001; Miller *et al.*, 2001). It is noteworthy that the *LaSAP1* promoter is active not only in P-deficient mature roots but also early in the development of lateral roots and in root hairs (Figure 1a,b). In comparison, expression of the *LaPT1-GUS* reporter gene was most intense in root tips and the root stele (Figure 1i,j). Slight GUS staining was also detected in vascular bundles of P-deficient leaves (data not shown).

P-deficiency-dependent promoter activity was further verified by measuring GUS enzyme activity in roots of each *LaSAP1-GUS* and *LaPT1-GUS* line showing GUS staining in roots (a minimum of eight independent transformants). GUS enzyme activity in roots of P-sufficient *LaSAP1-GUS* and *LaPT1-GUS* plants was not detectable while that of P-deficient plants was 1539 ± 574 and 202 ± 162 pmol $4 \text{ MU min}^{-1} \text{ mg}^{-1}$ protein, respectively. Both the *LaSAP1* and *LaPT1* promoters directed reporter gene activity in P-deficient alfalfa roots and the GUS activity in plants containing the *LaSAP1-GUS* gene was about eightfold greater than that of activity in plants with *LaPT1-GUS*.

Specificity of LaPT1 and LaSAP1 promoter response to P-deficiency

Crosstalk between signaling pathways of various abiotic stresses has been a topic of growing interest in recent years. For instance, iron (-Fe) and nitrate (-NO₃) deficiency induce developmental changes similar to those induced by

P-deficiency, including higher root hair density (by -Fe) and higher root-to-shoot ratio (by -NO₃) (Rogers and Guerinot, 2002; Zhang *et al.*, 1999). To test whether the *LaPT1* and *LaSAP1* promoters are also subject to induction by stresses other than P-deficiency, 16 cuttings were prepared from transgenic lines having high reporter gene activity, *PTGUS-2* and *APGUS-28*, for testing the effect of Fe-deficiency, NO₃-deficiency, +Al toxicity and +NAA in +P plants. In these experiments, -P plants and +P plants without additional treatments were included as controls to demonstrate the presence and absence of GUS activity in roots, respectively. GUS activity was detected only in roots under -P conditions and no GUS activity was detected in roots of plants from all other treatments under +P conditions (data not shown). These results indicate that both the *LaPT1* and *LaSAP1* promoters are specifically responsive to P-deficiency in transgenic alfalfa.

Induction of both LaSAP1 and LaPT1 promoter activity by P-deficiency in nitrogen-fixing nodules

Symbiotic nitrogen fixation is among the most distinctive traits of legume plants and P-deficiency is one of the major constraints for efficient nitrogen fixation (Vance, 2001; Vance *et al.*, 2000). Roots have been the primary target of interest for studying plant response to nutrient stress and the regulation of -P-induced genes in Arabidopsis and many other non-legumes. By examining the response of both promoters in effective nitrogen fixing versus ineffective non-N₂-fixing nodules, we expected to obtain novel information about the interaction between phosphorus stress and N₂ fixation/metabolism in root nodules. Both the *LaSAP1* and *LaPT1* promoters directed GUS activity in nitrogen-fixing nodules of -P transgenic plants and showed differential activity in different cell types of mature nodules (Figure 1c,k). Under +P conditions, *LaSAP1* promoter GUS activity was detected mainly in the nitrogen-fixing zone, showing much less GUS activity in the infection zone and the meristematic zone (Figure 1g). In nodules, the *LaPT1* promoter is preferentially active in vascular tissues (Figure 1k). To further explore the relationship between *LaPT1* and *LaSAP1* regulation and N₂ fixation, we inoculated both -P and +P plants with a mutant *fix⁻ Sinorhizobium meliloti* strain (Rm1491) to evaluate the promoter activity in ineffective nodules. In ineffective nodules, neither *LaPT1* nor *LaSAP1* promoter activity was induced by P-deficiency (Figure 1d,l). These findings are consistent with the interpretation that *LaPT1* plays a role in facilitating P transport into nodules under P-deficiency to accommodate the well-known high demand of P for nitrogen fixation. The ready induction of promoter activity by P-deficiency in N₂-fixing nodules reflects an apparent interrelationship between P-regulated gene expression and N₂ and/or N assimilation in nodules.

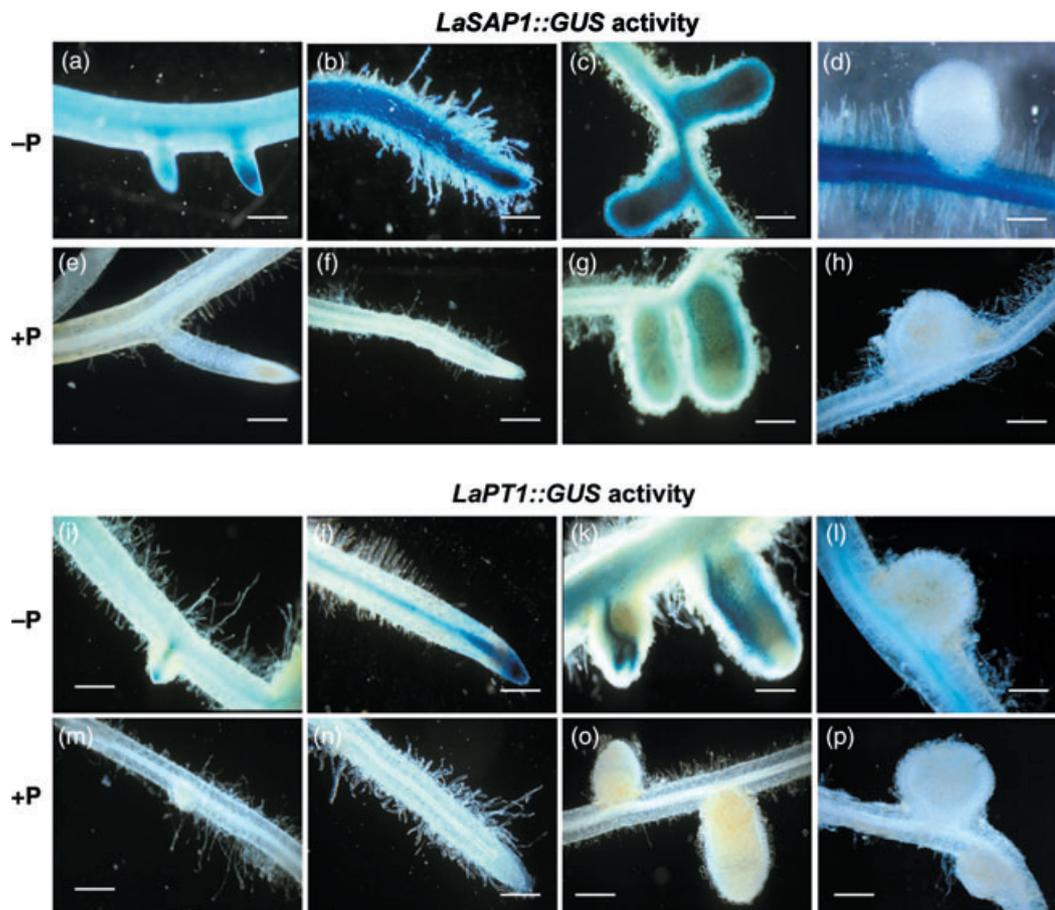


Figure 1. Histochemical localization of GUS activity in roots and nodules of transgenic alfalfa plants carrying *LaSAP1-GUS* and *LaPT1-GUS* fusions.

- (a) *LaSAP1-GUS* expression in $-P$ -emerging lateral roots.
 (b) *LaSAP1-GUS* expression in $-P$ -elongated lateral roots.
 (c) *LaSAP1-GUS* expression in $-P$ nitrogen-fixing nodules.
 (d) *LaSAP1-GUS* expression in $-P$ -ineffective nodules.
 (e) *LaSAP1-GUS* expression in $+P$ -emerging lateral roots.
 (f) *LaSAP1-GUS* expression in $+P$ -elongated lateral roots.
 (g) *LaSAP1-GUS* expression in $+P$ nitrogen-fixing nodules.
 (h) *LaSAP1-GUS* expression in $+P$ -ineffective nodules.
 (i) *LaPT1-GUS* expression in $-P$ -emerging lateral roots.
 (j) *LaPT1-GUS* expression in $-P$ -elongated lateral roots.
 (k) *LaPT1-GUS* expression in $-P$ nitrogen-fixing nodules.
 (l) *LaPT1-GUS* expression in $-P$ -ineffective nodules.
 (m) *LaPT1-GUS* expression in $+P$ -emerging lateral roots.
 (n) *LaPT1-GUS* expression in $+P$ -elongated lateral roots.
 (o) *LaPT1-GUS* expression in $+P$ nitrogen-fixing nodules.
 (p) *LaPT1-GUS* expression in $+P$ -ineffective nodules.

Scale bars = 0.5 mm.

LaSAP1 and *LaPT1* gene promoter activity in reproductive organs

Because P-deficiency has a profound effect on overall plant growth and development, we were interested in the expression pattern of $-P$ -induced reporter genes in reproductive organs. To obtain flowering plants under $+P$ and $-P$ conditions, two sets of cuttings were both grown under P-sufficient conditions until 2–3 weeks before flowering.

Following this initial growth period, one set of cuttings was treated with $-P$ nutrient solution and the other maintained on the original $+P$ solution. Both mature flowers and young flower buds were examined for GUS activity. No apparent qualitative difference in GUS activity was observed in either flower buds or mature flowers between $-P$ and $+P$ *LaPT1-GUS* plants. GUS activity was found in the pedicle, sepals, petals, filaments, and stigma of mature flowers (Figure 2a). Highly localized *LaPT1* promoter activity was also observed

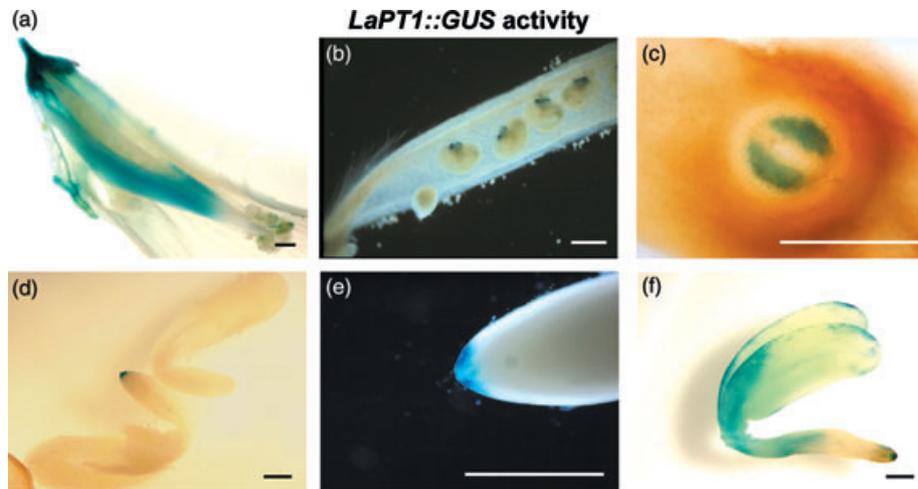


Figure 2. Histochemical localization of *LaPT1-GUS* activity in floral tissues and young alfalfa seedlings irrespective of P treatment.

- (a) *LaPT1-GUS* expression in a flower.
 (b) *LaPT1-GUS* expression in ovule funiculus.
 (c) *LaPT1-GUS* expression in hilum of a developing seed.
 (d, e) *LaPT1-GUS* expression in seedling root tip.
 (f) *LaPT1-GUS* expression in expanded green cotyledons, hypocotyl, and root tip of a young alfalfa seedling.
 Scale bars = 0.5 mm.

in the ovule funiculus and the hilum of developing seeds, suggesting a putative role for *LaPT1* in P import into developing seeds (Figure 2b,c). In particular, the GUS activity pattern in the hilum provides evidence that the *LaPT1* gene can be regulated by developmental signals and/or local demand for P in a cell-specific manner. Unlike the *LaPT1* promoter, the *LaSAP1* promoter was not active in floral tissues.

LaPT1 and *LaSAP1* expression in seedlings

To determine whether the *LaPT1-GUS* and *LaSAP1-GUS* reporter genes were active in seedlings, seeds derived from selfing of each transgenic line were germinated and stained for reporter gene activity (Figure 2d–f). While *LaPT1-GUS* reporter gene activity was restricted to the tip of the radicle in imbibed alfalfa seeds, much stronger GUS staining was observed in the elongated primary root, hypocotyl, and fully expanded green cotyledons of young seedlings 2 days after

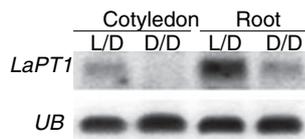


Figure 3. Regulation of the endogenous *LaPT1* gene expression in white lupin seedlings. *LaPT1* expression was enhanced in seedlings grown in the light (under a normal light/dark cycle, i.e., 16/8 h L/D) compared with that of seedlings grown in continuous dark (D/D) for 5 days. Twenty micrograms of total RNA extracted from both cotyledons and roots was subjected to RNA blot hybridization. A polyubiquitin cDNA (*UB*) probe was used as a control for RNA loading and quality.

germination (DAG). The *LaPT1* promoter activity in young seedlings is similar to that of *AtPht1;5* with a putative function in P remobilization during seed germination (Mudge *et al.*, 2002). Unlike *LaPT1* reporter gene activity, no GUS staining was detected in *LaSAP1-GUS* seedlings (data not shown).

Having evaluated reporter gene expression in a heterologous system, we thought it important to determine the abundance of *LaPT1* and *LaSAP1* transcripts in young seedlings of white lupin. Total RNA isolated from white lupin seedlings 5 DAG was separated on gels and the abundance of *LaPT1* and *LaSAP1* mRNA determined by RNA blot hybridization. *LaPT1* transcripts were readily detectable in seedlings, grown under a normal photoperiod (16/8 h: L/D), at 5 DAG (Figure 3). By comparison, dark-grown seedlings had strikingly reduced *LaPT1* transcript accumulation. *LaPT1* transcript accumulation was greater in roots than in cotyledons. Similar results were obtained with both +P and –P grown seedlings. Because P concentration at these early stages of growth were nearly identical for +P and –P (1.00 and 0.96%, respectively), changes in *LaPT1* transcript accumulation must be attributed to differences in exposure to light. In contrast, *LaSAP1* transcripts were not detectable in 5 DAG white lupin seedlings. The expression patterns for *LaPT1* and *LaSAP1* endogenous transcripts in white lupin seedlings are similar to the promoter-GUS activity patterns seen in transgenic alfalfa seedlings. The reduced endogenous *LaPT1* transcript accumulation in dark-grown lupin seedlings led us to suspect a link between *LaPT1* gene expression and either photosynthates or sugars.

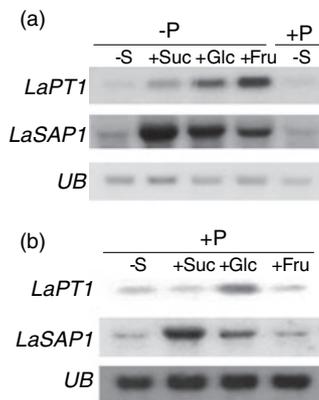


Figure 4. Influence of exogenous sugars on *LaPT1* and *LaSAP1* expression in roots of dark-grown white lupin seedlings. (a) Sugar effect under $-P$. (b) Sugar effect under $+P$. Total RNA was isolated from 5-day-old seedlings grown with 1/2 strength Hoagland's solution in continuous dark. $-P$, no phosphate; $+P$, 1.5 mM $\text{Ca}(\text{H}_2\text{PO}_4)_2$. $-S$, no sugar supplements; $+Suc$, 3% sucrose; $+Glc$, 3% glucose; $+Fru$, 3% fructose.

Effect of exogenous sugar supply on the expression of *LaPT1* and *LaSAP1* in white lupin seedlings

To further investigate whether products of photosynthesis were involved in the striking differences in *LaPT1* transcript abundance observed in roots between light-grown (16/8 h:L/D) and dark-grown (0/24 h:L/D) seedlings, we germinated white lupin seedlings in $-P$ nutrient solution (1/2 Hoagland's) supplemented with sucrose, glucose, or fructose, respectively. Similar to the results shown in Figure 3, seedlings grown in darkness without exogenous supplemental sugars ($-P-S$; $+P-S$) had low *LaPT1* transcript accumulation (Figure 4a). Low transcript abundance was also noted for *LaSAP1* in the absence of supplemental sugar. However, the presence of either sucrose, glucose, or fructose in the $-P$ germination medium stimulated not only *LaPT1* transcript accumulation but also that of *LaSAP1* (Figure 4a). Similar although less dramatic results were seen in seedlings grown on $+P$ medium (Figure 4b). In light-grown seedlings, exogenous supplemental sugar had little effect on the expression of *LaPT1* or *LaSAP1* in roots (data not shown). The effect of sugars on *LaPT1* and *LaSAP1* expression implies that carbohydrates may be involved in mediating or signaling aspects of the P-deficiency stress response similar to the proposed role of sugars in the N response cascade (Moore *et al.*, 2003).

Reversible light/dark regulation of P-deficiency-induced genes

As shown for promoter-GUS reporter experiments displayed in Figures 1 and 2, and in previously published reports from our laboratory (Liu *et al.*, 2001; Miller *et al.*, 2001) both *LaPT1* and *LaSAP1* are highly expressed in P-deficiency-stressed roots of alfalfa and cluster roots of

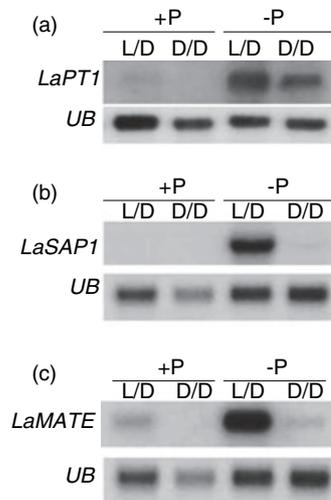


Figure 5. Regulation of *LaPT1* and *LaSAP1* gene expression in proteoid roots by light/dark conditions and P-deficiency.

(a) RNA gel blot of *LaPT1* expression.

(b) RNA gel blot of *LaSAP1* expression.

(c) RNA gel blot of *LaMATE* expression.

Total RNA was isolated from proteoid roots of white lupin plants 14 DAE grown under 16/8 h photoperiod (L/D) or transferred to continuous dark for 24 h (D/D) before the collection of proteoid roots. A labeled polyubiquitin (*UB*) cDNA was used as control.

$-P$ -stressed white lupin, respectively. Based on the corresponding effect that exogenous supplemental sugars and light have on *LaPT1* and *LaSAP1* in rootlets of white lupin seedlings, we postulated that photosynthates may be playing a key role in $-P$ -stress-induced gene expression in cluster roots of white lupin. To test whether light played a role in $-P$ -stress-induced gene expression in cluster roots, expression of *LaPT1* and *LaSAP1* was evaluated in cluster roots of both $+P$ and $-P$ -treated white lupin grown under a normal photoperiod (16/8 h; L/D) for 12–13 days after emergence (DAE) (Miller *et al.*, 2001) or under dark-adapted conditions (24 h continuous dark) after 12 days in the normal photoperiod. As expected, *LaPT1* and *LaSAP1* transcripts were low to not detectable in cluster roots of $+P$ plants grown under either light condition. In addition, transcripts of *LaPT1* and *LaSAP1* were highly enhanced in $-P$ -stress-induced cluster roots of white lupin plants grown in a normal photoperiod (Figure 5a,b). In comparison, transcript accumulation for *LaPT1* and *LaSAP1* in $-P$ -stressed cluster roots of dark-adapted plants was strikingly diminished (Figure 5a,b), showing an apparent light requirement to achieve high expression of $-P$ -stress-controlled genes in cluster roots.

To further assess whether light was involved in regulating expression of $-P$ -stress-induced genes in white lupin cluster roots we evaluated transcript accumulation of *LaMATE*, a multidrug and toxin efflux gene that is also highly upregulated in P-deficient white lupin proteoid roots (Uhde-Stone *et al.*, 2003b). As shown in Figure 5(c), *LaMATE* transcript

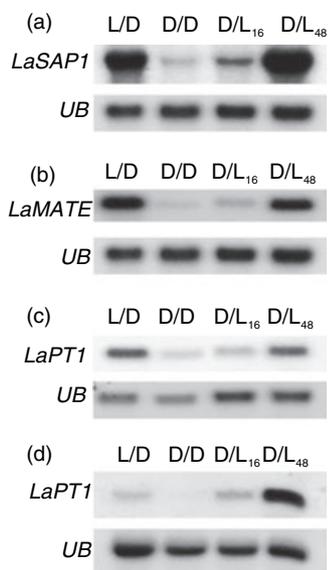


Figure 6. RNA gel blots demonstrating reversible light/dark regulation of *LaSAP1* and *LaPT1* expression in proteoid roots and leaves of P-deficient white lupin plants.

- (a) *LaSAP1* expression in proteoid roots.
 (b) *LaMATE* expression in proteoid roots.
 (c) *LaPT1* expression in proteoid roots.
 (d) *LaPT1* expression in leaves.

Total RNA from proteoid roots and leaves of white lupin plants was separated on agarose gel. A polyubiquitin cDNA probe (*UB*) was used as control for the loading and quality of total RNA. Light/dark conditions: L/D, under 16/8 h photoperiod; D/D, transferred to continuous dark for 24 h before collection; D/L₁₆ or D/L₄₈, D/D plants transferred to continuous light for 16 or 48 h, respectively.

accumulation and response to light mirrored that of *LaPT1* and *LaSAP1*. Thus the expression of three white lupin genes coordinately enhanced in -P-stress-induced cluster roots was integrally related to light.

Furthermore, to test whether dark suppression of gene expression could be reversed, dark-adapted P-deficient plants (D/D) were returned to continuous light for either 16 or 48 h in the same growth chamber and labeled as D/L₁₆ or D/L₄₈, respectively. RNA blot hybridization with both *LaSAP1* and *LaMATE* probes showed that the accumulation of both transcripts in cluster roots began to recover after 16 h (D/L₁₆) in the light. By 48 h (D/L₄₈), transcript abundance equaled or even surpassed that in the control plants (L/D) (Figure 6a,b). These results indicate that the light/dark regulation of both *LaSAP1* and *LaMATE* expression is reversible.

While *LaSAP1* and *LaMATE* gene expression is fairly specific for -P-stress-induced cluster roots (Liu *et al.*, 2001; Uhde-Stone *et al.*, 2003b), *LaPT1* is expressed in both leaves and roots of -P-stressed white lupin plants (Liu *et al.*, 2001). Because *LaPT1* expression is enhanced in both shoots and roots, we were able to evaluate whether light impacted systemic expression of a P-responsive gene. We therefore evaluated *LaPT1* transcript abundance in leaves and cluster

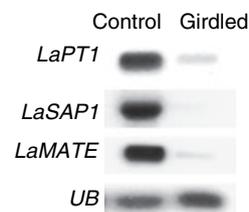


Figure 7. Effect of stem girdling on the expression of P-deficiency-induced genes in proteoid roots. Total RNA from proteoid roots of (14 DAE) P-deficient white lupin plants (control) or 24 h after stem girdling (girdled) was separated on agarose gel and transferred onto nylon membrane. Blot was hybridized with *LaPT1*, *LaSAP1*, *LaMATE*, and a *UB* cDNA probe.

roots of -P-stressed plants. As with *LaSAP1* and *LaMATE*, -P stress resulted in enhanced *LaPT1* transcript abundance in cluster roots and leaves (Figure 6c,d). Dark adaptation of plants resulted in a diminished amount of *LaPT1* transcript with subsequent recovery of expression when dark-adapted plants were returned to the light.

These data show that the highly synchronized expression of three genes in cluster roots of -P-stressed white lupin plants is mediated in part by light and that the effect of light on gene expression appears to be systemic.

Expression of P-deficiency-induced genes in roots is reduced by stem girdling

Phloem translocation is the primary route for transport of leaf photosynthates (sugars) to roots. To determine whether phloem translocation of photosynthates was required for the expression of P-deficiency-induced genes in lupin cluster roots we stem-girdled white lupin plants at 13 DAE. After 24 h of stem girdling *LaPT1*, *LaSAP1*, and *LaMATE* transcript accumulation was determined in cluster roots of girdled and non-girdled P-deficient plants. As shown in Figure 7, transcript accumulation of all three genes was dramatically reduced within 24 h in stem-girdled P-deficient plants. By comparison, and consistent with data presented elsewhere in this report, *LaPT1*, *LaSAP1*, and *LaMATE* transcripts were quite abundant in cluster roots of non-girdled P-deficient plants.

To ensure phloem transport to roots was blocked by stem girdling, 1 μ Ci of ¹⁴C-sucrose was applied to a 4 mm diameter slightly abraded area on a single leaflet of each girdled and control plant. At 24 h after girdling and concomitant with collection of cluster roots for RNA isolation, radioactivity was measured from a subsample of cluster roots collected from each plant. In stem-girdled plants translocation of ¹⁴C-sucrose to cluster roots was reduced by 95%. Radioactivity detected in cluster roots of stem-girdled plants was 1.3 dpm mg⁻¹ while that in non-girdled plants was 48.6 dpm mg⁻¹. These data further support a striking link between leaf photosynthate and the expression of P-deficiency-induced genes in cluster roots.

Discussion

In this report we have extended the fundamental understanding of regulation of plant genes in response to P-deficiency stress by showing: (i) the 5'-upstream putative promoter region of white lupin genes that are highly induced during P-deficiency correctly drive reporter gene activity in P-deficient alfalfa; (ii) the promoters of *LaPT1* and *LaSAP1* direct reporter gene activity in root nodules, and nodules must be effective for reporter gene activity; (iii) the white lupin *LaPT1* promoter is active not only in P-stressed roots but also during reproductive development and seed germination; and (iv) regulation of P-stress-induced gene expression is integrally related to the presence of sugars and photosynthates.

Conservation of P-deficiency response cis-elements

The promoters of different P-deficiency-induced genes in higher plants share common *cis*-acting elements for coordinated regulation of gene expression by P status, as demonstrated in yeast (Raghothama, 1999; Rubio *et al.*, 2001; Wykoff *et al.*, 1999). In this study we analyzed two P-deficiency-induced promoters in transgenic alfalfa and gained further insight into their common characteristics and differential regulation in response to P stress. Both *LaSAP1* and *LaPT1* promoters from white lupin were sufficient to confer GUS reporter gene expression in transgenic alfalfa in response to P-deficiency (Figure 1). In recent preliminary experiments we successfully obtained P-deficiency responsive gene activity in *Arabidopsis* with *LaPT1-GUS* and *LaSAP1-GUS* and hairy roots of white lupin after *Agrobacterium rhizogenes* transformation (K. Zinn, C. Uhde-Stone, D.L. Allan and C.P. Vance, unpublished data). The fact that two different white lupin promoters show a significant response to P-deficiency in alfalfa, lupin and *Arabidopsis* provides strong supporting evidence that the molecular mechanism underlying regulation of -P-induced genes is conserved among dicot plants.

Raghothama (1999) and Rubio *et al.* (2001) have proposed that, like bacteria and yeast, plants have a phosphate (*PHO*) starvation regulon. Correct promoter-reporter gene activity by white lupin P-deficiency-induced genes in heterologous plants strengthens this interpretation. The presence of conserved elements within promoters of P-deficiency-induced genes would provide additional support for a plant *PHO* regulon. Rubio *et al.* (2001) identified an *Arabidopsis* mutant with reduced response to P-starvation. They identified the mutated gene involved in the phosphate starvation response (*phr1*) as a MYB transcription factor. Moreover they found a consensus binding site for PHR1 as 5'-GNAT-ATNC-3'. Mutations in the coiled-coil region of the PHR1 protein block binding to the 5'-GNATATNC-3' motif. This

motif, which is found in many phosphate starvation response genes from *Arabidopsis*, tomato, and *Medicago truncatula*, also occurs in our *LaSAP1* and *LaPT1* promoters that show P-deficiency-induced activity. Whether the PHR1 binding motif is important for expression of *LaSAP1* and *LaPT1* remains to be established, but initial studies of white lupin *LaSAP1* promoter deletion reporter gene constructs in *Arabidopsis* show that the region between -300 and -1 bp from the translation start codon contain elements that affect reporter gene activity (K. Zinn, D.L. Allan, J. Liu and C.P. Vance, unpublished data). A 5'-GNATATNC-3' motif is found at -160 bp in *LaSAP1*. This element is also found at -128 bp in the *LaPT1* promoter. Wykoff *et al.* (1999) identified a MYB-like gene that regulates P-deficiency-induced genes in *Chlamydomonas* but no consensus DNA binding sites were identified in the genes responding to P-deficiency.

We noticed a striking (eightfold) difference in β -glucuronidase activity between the roots of plants containing the *LaSAP1-GUS* and *LaPT1-GUS* constructs grown under -P. These results support previously obtained (Liu *et al.*, 2001; Miller *et al.*, 2001) RNA blot hybridization data indicating *LaSAP1* mRNA is in much greater abundance than *LaPT1* mRNA in P-deficiency-induced white lupin proteoid roots. At this time we cannot explain the difference in *LaSAP1* and *LaPT1* promoter strength, but it is informative that the motif 5'-CACGTG/T-3' is the core *cis*-acting element in -P-induced genes of the *pho* regulon in yeast (Ogawa *et al.*, 1995). This sequence, which is known as a G-box element, commonly functions as an enhancer in plant genes and is common in genes induced by sugars, biotic, and abiotic stress (Bustos *et al.*, 1998; Chattopadhyay *et al.*, 1998; Martinez-Garcia *et al.*, 2000; Pla *et al.*, 1993). A G-box element is found at -195 bp in the *LaSAP1* promoter (30 bp upstream of a putative PHR1 binding site), but is not present in the *LaPT1* promoter. Whether the PHR1 binding site or the G-box motif are critical to the regulation of white lupin P-stress-induced genes awaits the outcome of current studies dissecting these DNA regions.

The noticeable difference in *LaSAP1-GUS* and *LaPT1-GUS* activity in mature roots of transgenic alfalfa versus that in developing seed and young seedlings reflects the divergent function for the respective genes. *LaSAP1* expression is quite specific, the gene is expressed in and the protein exuded from P-deficient white lupin proteoid roots at 12-16 DAE and is thought to be important in releasing P from organic esters in the rhizosphere. By contrast, *LaPT1* expression, although induced by P-deficiency in white lupin proteoid roots, can also be detected in other tissues, albeit at lower levels (Liu *et al.*, 2001), suggesting not only a role in uptake but also in P redistribution. The detection of *LaPT1-GUS* activity in developing seeds and young seedlings further supports a role for *LaPT1* in P redistribution.

LaPT1 and LaSAP1 reporter gene activity in root nodules

Both the *LaPT1* and *LaSAP1* promoters displayed significant reporter gene activity in nitrogen-fixing root nodules of P-deficient transgenic alfalfa (Figure 1c,k). *LaSAP1* also displayed modest activity in root nodules of P-sufficient plants. While APs and PTs are known to occur in nodules (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago), their functional importance is unknown. Because the P content of root nodules tends to be higher than that in other organs, APs and PTs are speculated to play a role in P mobilization across the symbiosome membrane to provide P for synthesis and function of nitrogenase and for the high energy requirements of nitrogen fixation. Surprisingly, when ineffective nodules were produced on transgenic *LaSAP1* and *LaPT1* promoter-GUS plants, no reporter gene activity was detected. This observation suggests that some aspect of effective nodules is required for induction of *LaSAP1* and *LaPT1* promoter activity. The expression of many plant genes involved in nodule function, particularly those related to carbon and nitrogen metabolism, is greatly reduced in ineffective nodules (Egli *et al.*, 1989; Fedorova *et al.*, 1999; Haser *et al.*, 1992). Several authors have implicated the well-documented reduced amounts of carbon and nitrogen metabolites found in ineffective nodules as potential regulators of root nodule gene expression. Recent studies have shown that nitrogen and carbon compounds can act as signals for activating expression of a wide range of genes in plants (Chiou and Bush, 1998; Coruzzi and Bush, 2001; Hsieh *et al.*, 1998). Loss of *LaSAP1* and *LaPT1* reporter gene activity in ineffective nodules suggests that a link exists between either nitrogen and/or carbon metabolism and expression of P-responsive genes.

Regulation of endogenous LaPT1 and LaSAP1 genes by sugars and/or photosynthates

Several observations lead us to propose that photosynthates/sugars play a role in mediating enhanced expression of plant genes under P-deficiency conditions. First, both *LaPT1* and *LaSAP1* transcript accumulation was markedly enhanced by addition of exogenous sugars to dark-grown lupin seedlings (Figure 4). Secondly, the abundance of both transcripts strikingly decreased in cluster roots when light-grown plants were dark-adapted (Figures 5 and 6). This effect of light was also seen in *LaMATE*, a third gene that is highly induced in P-deficient cluster roots (Figures 5c and 6b). Moreover, the decreased transcript abundance seen under dark adaptation is reversed upon reexposure of white lupin shoots to light (Figure 6). Thirdly, within 24 h stem girdling resulted in a striking decrease in expression of genes induced in cluster roots by P-deficiency (Figure 7). The observation that sugar stimulation of *LaPT1* and *LaSAP1* transcript accumulation in seedlings was not completely

alleviated by addition of phosphate (Figure 4b) supports the interpretation that the effect of sugar on gene expression is not merely the result of sequestration of inorganic P by conversion of sugar to sugar-PO₄. Instead, sensing of altered sugar or sugar metabolites is probably involved in mediating the regulation of *LaPT1* and *LaSAP1* expression. Additional preliminary studies with wild-type Arabidopsis seedlings also bolster our proposal for a crosstalk between P and sugar. We germinated Arabidopsis seeds in the presence of 2% glucose and as expected (Moore *et al.*, 2003), we noted suppression of cotyledon expansion and greening. However, glucose inhibition was partially relieved by adding as little as 1 μM phosphate to the germination medium (J. Liu and C.P. Vance, unpublished data). Relief of sugar inhibition by added P, however, was not as complete as that by nitrate.

Sugars and sugar metabolites are known to regulate genes involved in N acquisition, metabolism, and N status sensing (Coruzzi and Bush, 2001). NO₃⁻ and NH₄⁺-transporter genes are upregulated by exogenous sucrose and light in Arabidopsis (Lejay *et al.*, 1999, 2003). An Arabidopsis gene, *GLB1*, involved in nitrogen sensing was regulated in a similar pattern by light and sucrose (Hsieh *et al.*, 1998). Sucrose, together with auxin, has been proposed to be the 'long-range' signal molecule transported from shoot to root to trigger plant responses to nitrate availability, including changes in gene expression patterns and lateral root growth in Arabidopsis (Forde, 2002). These reports provide several lines of evidence for extensive crosstalk between C and N metabolic and signaling pathways (reviewed by Coruzzi and Bush, 2001). Our findings extend the role of sugars in nutrient acquisition to affecting not only genes involved in N but also those related to P acquisition.

Moreover, the differing expression patterns for *LaPT1* and *LaSAP1* in response to different sugars (Figure 4) are a further indication of the complexity of the crosstalk between sugar signaling and P-deficiency-induced gene expression. Gene induction by exogenous sugars is a multi-step process that involves sugar transport, sensing and/or metabolism (Price *et al.*, 2004). Glucose and fructose have different levels of gene induction in dark-grown white lupin seedlings. This may be partly due to the fact that both hexose transporters and hexose sensors have varied affinity to different hexoses. The mechanism of sucrose-induced gene expression is quite distinct from that of hexoses in that sucrose-specific transporters (some of which may also work as sucrose sensors) are the crucial components. While the mechanism of sucrose induction of P-deficiency-related genes is yet to be fully elucidated, our data indicate that genes involved in P uptake, redistribution, and mobilization are differentially regulated by sucrose and hexoses. Considering the regulatory role of sugars in biomass partitioning between shoot and root and in lateral root branching, we propose that exogenous or endogenous sugars transported through the

phloem play a crucial role in regulating both morphological adaptation (higher root-to-shoot ratio and enhanced cluster root formation) and P-deficiency-induced gene expression in response to P limitation. Such an extensive involvement of sugars in P-deficiency response is consistent with the notion that plants have evolved a highly coordinated system to cope with nutrient limitation.

Recently, there has been significant progress on the studies of hexokinase (HXK) as a sugar sensor in sugar metabolism and signaling in Arabidopsis (Moore *et al.*, 2003; Rolland *et al.*, 2002). For instance, HXK1, as an enzyme, catalyzes the conversion of hexose to hexose phosphate. Nevertheless, its role as a sugar sensor is entirely independent of its enzymatic activity, as mutant HXK1 without kinase activity can still function as a sugar sensor. Furthermore, a glucose-insensitive mutant, *gin2-1*, with a mutation in *HXK1*, is also insensitive to auxin but hypersensitive to cytokinin. The suppression effect of glucose on seed germination and expression of photosynthesis-related genes can be effectively antagonized by nitrate. Thus, by sensing both exogenous and endogenous sugars (and/or photosynthates), HXK1 has been proved to play a central role in an extensive regulatory network integrating sugars, nutrients, light, and phytohormones (Moore *et al.*, 2003). Whether the effect of sugar on P-responsive gene expression in white lupin is mediated through sensing of sugars or photosynthate by HXKs remains to be determined. It is, however, worthwhile to note that expression of an HXK mRNA along with that of several other genes involved in carbon metabolism was obviously enhanced in P-deficiency cluster roots of white lupin (Uhde-Stone *et al.*, 2003a,b).

Taken together, the molecular evidence in this report reveals a putative interplay between P stress signaling and sugar metabolism and/or sensing. The systemic signal for P status in the shoot and the subsequent regulation of P-responsive genes seems to be transduced, at least in part, by the altered levels of sugars and/or sugar metabolites in different parts of the plant. Future transcript profiling analysis of -P white lupin plants grown in extended dark versus light may further reveal a more extensive network of interactions between C and P signaling pathways.

Experimental procedures

Construction of chimeric reporter genes and alfalfa transformation

Both the phosphate transporter (*LaPT1*) genomic clone and the gene for a secreted acid phosphatase (*LaSAP1*) have been described previously by Liu *et al.* (2001) and Miller *et al.* (2001), respectively. The 2.3 kbp *LaSAP1* promoter was amplified from an acid phosphatase genomic clone with two gene-specific primers. An *Sall* site (underlined) was introduced in the primer APd-6 (sense) 5'-GGCGTCGACTAGGAGTGAGCATGCATGAGTT-3' and an *Xba*I site

(underlined) was included in primer APd-0 (antisense) 5'-GCCTCAGATAACCCATTTTCATTTCTG-3'. The resulting PCR product was purified using the GeneClean II KIT (Bio 101, Carlsbad, CA, USA) and digested with *Sall* and *Xba*I to create a 5' *Sall*/*Xba*I 3' promoter fragment for directional cloning into the plant transformation vector pBI101.2 (Clontech, Palo Alto, CA, USA). Translational fusion with the β -glucuronidase (GUS) coding region was confirmed by sequencing the *LaSAP1*-GUS junction. The 2.3 kbp *LaPT1*-GUS translational fusion was constructed with a similar procedure as for constructing the *LaSAP1*-GUS reporter gene. The 5'-untranslated intron was included in the promoter fragment amplified by PCR with two primers: LaPTd6 (sense) 5'GGCGTCGACAAATCAATGAA-CATACTA-3' and LaPTd-0 (antisense) 5'-GCCTCTAGACTAGCCATTAGTTGAAGCCA-3'. The resulting recombinant plasmids pBIAP-GUS and pBIPT-GUS were introduced into *Agrobacterium* LBA4404 by electroporation and positive clones were selected on YEB plates supplemented with rifampicin (25 μ g ml⁻¹) and kanamycin (50 μ g ml⁻¹) and further confirmed by colony filter hybridization with random-labeled *LaSAP1* and *LaPT1* promoters, respectively. *Agrobacterium*-mediated transformation of alfalfa (*M. sativa* cv Regen SY) was performed by co-cultivation of leaf disks with *Agrobacterium* essentially as described by Austin *et al.* (1996). The regenerated plants were initially screened by PCR of genomic DNA to identify transgenics. Randomly selected transgenic plants were further analyzed by Southern blot hybridization to reveal the copy numbers of the promoter-GUS reporter gene in the genome of independent transgenic alfalfa plants.

Plant materials and growth conditions

The regenerated alfalfa plants were initially grown in the growth chamber and then transferred to a greenhouse. For P-deficiency analysis, two sets of vegetative cuttings were prepared from each of the primary transgenic plants. After rooting, the cuttings were watered daily with 1/2 strength Hoagland's solution supplemented with 1.0 mM Ca(H₂PO₄)₂ for the +P treatment or without Ca(H₂PO₄)₂ for -P treatment for 4 weeks. Both -P and +P plants were inoculated with *Sinorhizobium meliloti* 102F51 (or with a *fix*⁻ mutant strain *S. meliloti* Rm1491) and nodules at different developmental stages as well as young roots were collected for GUS histochemical staining. To obtain flowers from transgenic alfalfa plants, two sets of cuttings were watered with the +P nutrient solution to maintain healthy growth until 2–3 weeks before flowering, then either +P or -P nutrient solution was applied daily to the plants. Mature flowers or flower buds at different stages were collected from -P and +P treatments derived from the same primary transgenic plant for comparison of GUS staining patterns. AlK(SO₄)₂ (450 μ M) was added to the nutrient solution for the aluminum stress treatment. Foliar application of +P nutrient solution to the aluminum-treated plants was carried out to avoid Al-phosphate precipitation from the nutrient solution. The effect of 1-naphthaleneacetic acid (NAA) was tested by foliar application of 1 μ M NAA. Iron deficiency and nitrate deficiency were achieved by withholding the corresponding nutrient component for 4 weeks, respectively. To evaluate *LaSAP1*-GUS and *LaPT1*-GUS expression in young white lupin seedlings, seeds were imbibed for 2 days in the dark and then subjected to continuous dark or 16/8 h photoperiod in the growth chamber. Seeds were soaked with 1/2 strength Hoagland's solution (-P or +P) supplemented with 3% sucrose, 3% glucose, or 3% fructose. The medium was replaced daily until 5 DAG and shoots and roots were harvested for total RNA extraction. For light/dark treatment, lupin plants were grown in potted sand in the growth chamber until 12–14 DAE as described (Johnson *et al.*, 1996). Plants were shaded from light for 24 or 48 h and then re-exposed to continuous light for

16 or 48 h. Control plants were grown under 16/8 h (L/D) photoperiod in the growth chamber.

DNA and RNA gel blot analysis

Alfalfa genomic DNA was isolated from young leaves with a procedure modified from Junghans and Metzloff (1990). Fifteen micrograms genomic DNA was digested with *EcoRI*, extracted once with phenol/chloroform, precipitated and redissolved in 1x TE buffer. DNA gel electrophoresis and capillary transfer to Immobilon N⁺ nylon membrane (Millipore, Bedford, MA, USA) were performed following standard protocols (Sambrook *et al.*, 1989). DNA blots were hybridized overnight at 68°C in 0.5 M Na₂HPO₄, 7% SDS with probes prepared by random primer-labeled *LaSAP1* or *LaPT1* promoters. High stringency washing was carried out using 0.1x SSC, 0.1% SDS as a final wash at 68°C. RNA isolated using the RNeasy kit (Qiagen, Valencia, CA, USA) was separated on 1.5% formaldehyde/agarose gels. RNA transfer, hybridization, and washing stringency were performed as described for the Zeta-Probe membrane by the manufacturer (Bio-Rad, Hercules, CA, USA).

β-Glucuronidase assays

For protein extraction, frozen tissues were ground into a fine powder with liquid nitrogen and resuspended in phosphate buffer (0.1 M KH₂PO₄, pH 7.8, 1 mM EDTA, 10 mM DTT, 5% glycerol, 0.1% Triton X-100). After a 5-min centrifugation, a 10 μl aliquot of the supernatant was used for determining the protein concentration with the Bio-Rad protein assay reagent and another 10 μl aliquot was measured for GUS activity with the substrate 4-methyl umbelliferyl β-D-glucuronide (Jefferson, 1987). The 4-methylumbelliferone (MU) produced in duplicate reactions from each protein sample was measured over 30 min with a FL600 Microtiter Fluorescence Reader (Bio-Tek Instruments, Winooski, VT, USA) equipped with a 360/40 nm excitation filter and 450/50 nm detection filter. For histochemical analysis, plant tissues were vacuum infiltrated for 20 min in GUS staining solution (1 mM 5-bromo-4-chloro-3-indolyl glucuronide, 0.1 M phosphate buffer, pH 7.0, 0.5 mM potassium ferrous and ferricyanide, 5 mM EDTA) followed by an overnight incubation at 37°C. Materials were then transferred to 70% ethanol to remove chlorophyll and examined under a dissecting microscope and photographed.

Acknowledgements

The authors wish to thank Sue Miller for critical reading of the manuscript and Melinda Dornbusch for production of transgenic alfalfa. This work was supported in part by USDA, Agricultural Research Service, CRIS no. 3640-21000-009-00D and USDA/CSREES/2002-35100-12206. This paper is a joint contribution from the Plant Science Research Unit, USDA-ARS, and the Minnesota Agricultural Experiment Station. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the USDA, and does not imply its approval or the exclusion of other products and vendors that might also be suitable.

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