Characterization of promoter expression patterns derived from the Pht1 phosphate transporter genes of barley (Hordeum vulgare L.)

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Abstract

By comparison with dicot plant species, relatively little work has been reported on the phosphate transporter (Pht1) gene family from monocot species. Initial studies have shown that barley contains at least eight homologous genes. The promoters of six of these genes were analysed for the presence of regulatory elements potentially associated with expression specificity. In particular, the P1BS-like elements (implicated in phosphorus-regulated expression of genes in plants) was identified in all HvPht1 promoters examined. For two members of the family (HvPht1;1 and HvPht1;2), promoter fusions to β-glucuronidase and green fluorescent protein reporter genes were constructed, transformed into rice, and the expression profiles observed. The inclusion of an intron derived from Adh1 enhanced gene expression approximately 20-fold, but did not appear to affect the specificity of expression. The HvPht1;1 and HvPht1;2 promoters showed minor differences in expression patterns but, in general, expression was observed at high levels in trichoblast cells (root hairs) and stele of the nodal root, throughout secondary roots, and at a relatively low level in leaf tissues. Under phosphorus deficiency, expression was induced by up to 5-fold. These observations are consistent with a primary role for the encoded genes in the uptake of phosphate by root hairs from soil solution and further current understanding of the mechanisms involved. The promoters also have application for providing a new resource for cereal transformation, ideally suited for driving the expression of foreign genes associated with nutrient uptake.

Key words: Gene expression, intron, Pht1 gene family, phosphate transport, phosphorus, plant root, promoter.

Introduction

Despite phosphorus (P) being an important macronutrient required for plant growth and function, many soils throughout the world are deficient in forms of P that are readily available to plants. Plants take up P as phosphate anions (Pi) from soil solution, but most soils contain low concentrations of Pi (1±5 µM) with generally lower levels around plant roots and limited diffusion of the anion toward root surfaces (Sanyal and De Datta, 1991). Unless the Pi pool is readily replenished, the low Pi concentrations in soil solutions limit plant growth (Bieleski, 1973; Schachtman et al., 1998). Plants have evolved a range of strategies that result in both morphological and biochemical changes at the soil–root interface to increase the availability and access to soil P. For example, P-deficient plants typically show increased root growth and branching, proliferation of root hairs and release of root exudates. These processes allow plants greater access to soil P or increase the availability of Pi from otherwise poorly available sources (Raghothama, 1999; Vance et al., 2003). Plant roots also possess membrane-bound transporters specifically involved in the uptake of Pi from the soil solution. The expression of these transporters is induced in P-deficient plants, allowing Pi to be effectively taken up against the large concentration gradient that occurs.
between the soil solution and internal plant tissues (Smith et al., 2000).

Phosphate transporters belonging to two major gene families have now been identified for a range of species (reviewed in Rausch and Bucher, 2002). The Pht1 family was originally identified by sequence homology with the yeast Pi transporter PHO84 and has been extensively studied in Arabidopsis thaliana (Muchhal et al., 1996; Smith et al., 1997; Okumura et al., 1998; Smith et al., 2000). Analysis of the Arabidopsis genome identified nine members of the Pht1 (ARAth;Pht1) gene family (Mudge et al., 2002). At least two of the genes (ARAth;Pht1;1 and Pht1;2) are predominantly expressed in roots and their expression is induced under conditions of P-deprivation (Muchhal et al., 1996; Smith et al., 1997; Okumura et al., 1998; Mudge et al., 2002). When expressed in tobacco suspension culture, ARAth;Pht1;1 showed high affinity kinetics ($K_m \approx 3 \mu M$) for the transport of Pi (Mitsukawa et al., 1997). Using reporter gene fusions, Mudge et al. (2002) further showed that expression of ARAth;Pht1;1 occurs predominantly in the root epidermis and is largely confined to root hair (trichoblast) cells, which are the primary site for Pi uptake by plants from the soil solution (Gahoonia and Nielsen, 1998). Such observations provide strong support for the transporters being the major pathway for the uptake of Pi from soil solutions.

By contrast with the studies on Pi transporters in dicots, relatively little work has been reported on analogous studies in monocot species despite the fact that cereals represent the major crops of the world and consume a large proportion of the world’s P-fertilizers. In a recent report 13 Pht1 genes were identified in rice, but only the ORYsa;Pht1;11 gene studied in detail and shown to be specifically activated during mycorrhizal symbiosis (Paszkowski et al., 2002). In barley (Hordeum vulgare) at least eight Pht1 homologues have been identified (Rae et al., 2003). The genes were named according to the Commission on Plant Gene Nomenclature (http://mbcservver.rutgers.edu/CPGN/Guide.html) as HORva;Pht1;1 through HORva;Pht1;8, but for simplification will be referred to here as HvPht1;1 through HvPht1;8. In a preliminary study, Smith et al. (1999) showed that HvPht1;1, HvPht1;2, and HvPht1;3 (previously referred to as HVPT1, 2, and 3) were primarily expressed in roots and induced under P-deficiency. More recently, Rae et al. (2003) have verified the root specificity and low-P inducibility of the HvPht1;1 gene and showed that it has high affinity ($K_m \approx 8 \mu M$) for the transport of Pi.

To extend this initial work on monocot Pi transporter genes, the characterization of the promoter regions from two of the genes, HvPht1;1 and HvPht1;2, is reported here and the reporter gene constructs containing GUS ($\beta$-glucuronidase) and GFP (green fluorescent protein) were used to determine the patterns of expression of their gene promoters in transgenic rice plants. Since several studies have shown that introns are commonly important for high level expression of transgenes in monocots (Callis et al., 1989 McElroy et al., 1990; Luerhsen and Walbot, 1991; Cornejo et al., 1993), the effect of the maize Adh1 intron on the specificity of expression controlled by the barley Pht1 promoters was also investigated. The analysis presented here both provides a better understanding of Pht1 genes themselves and the role they play in the Pi uptake mechanism, and also serves to assess the Pht1 promoters as a resource in plant transformation, for driving the expression of foreign genes associated with improved plant nutrition.

**Materials and methods**

**Preparation of HvPht1 promoter constructs**

To analyse promoter activity, the regions upstream from the coding regions of the HvPht1;1 and HvPht1;2 genes (GenBank accession numbers AF543197 and AF187019, respectively) were amplified by PCR using the primers listed in Table 1. The nopaline synthase (Nos) gene terminator was similarly amplified from pWBvec8 (Wang et al., 1998). Promoter and terminator amplification products were digested with NotI and Xhol, and simultaneously cloned into NotI digested pWBvec8. pWBvec8 is a binary vector designed for cereal transformation and incorporates a hygromycin (Hpt) selectable marker gene under the control of the CaMV 35S promoter and Nos terminator (Wang et al., 1998). In all cases the HvPht1-expression constructs were cloned upstream of, and in the same orientation as, the selectable marker cassette. For vectors that contained introns, the first intron of the maize alcohol dehydrogenase gene (Adh1) was amplified from pEmu (Last et al., 1991) using the primers listed in Table 1 and cloned into the AscI/HpaI sites of the generated

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Incorporated restriction sites</th>
</tr>
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<tbody>
<tr>
<td>Pht1:1 5’</td>
<td>ctcctagaGGGCCCCGTTAATTTAAGAGCTCCCGACTACCCCGG</td>
<td>NotI, PacI</td>
</tr>
<tr>
<td>Pht1:1 3’</td>
<td>gatctccGTCGAGGTACCTAAGCTTAAACGCGCCGTGCGCCGATCTC</td>
<td>Ascl, HpaI, AAvII, KpnI, Xhol</td>
</tr>
<tr>
<td>Pht1:2 5’</td>
<td>ctcctagaGGGCCCCGTTAATTTAAGAGCTCCCGACTACCCCGG</td>
<td>NotI, PacI</td>
</tr>
<tr>
<td>Pht1:2 3’</td>
<td>gatctccGTCGAGGTACCTAAGCTTAAACGCGCCGTGCGCCGATCTC</td>
<td>Ascl, HpaI, AAvII, KpnI, Xhol</td>
</tr>
<tr>
<td>Nos 5’</td>
<td>ctcctagaGGGCCCCGTTAATTTAAGAGCTCCCGACTACCCCGG</td>
<td>Xhol, SmaI, BstZ171</td>
</tr>
<tr>
<td>Nos 3’</td>
<td>gatctccGTCGAGGTACCTAAGCTTAAACGCGCCGTGCGCCGATCTC</td>
<td>SdaI, NotI</td>
</tr>
<tr>
<td>Adh1 5’</td>
<td>ctcctagaGGGCCCCGTTAATTTAAGAGCTCCCGACTACCCCGG</td>
<td>Ascl</td>
</tr>
<tr>
<td>Adh1 3’</td>
<td>gatctccGTCGAGGTACCTAAGCTTAAACGCGCCGTGCGCCGATCTC</td>
<td>HpaI</td>
</tr>
</tbody>
</table>
promoter construct. The generated HvPh1-derived expression construct is given in Fig. 1. For GUS-containing constructs, the GUS gene was excised (Smal/XbaI) from pBl101 (Jefferson et al., 1987) and cloned into compatible HpaI/AvrII sites of the HvPh1-construct polylinker. For GFP-containing constructs, the GFP gene (sgpS65T) was excised from pMNG1004 (Upadhyaya et al., 1998) using XbaI and EcoRI, and cloned into compatible AavII/Smal sites. A GUS control vector, pWBvec10a, was used that has the GUS gene driven by the ubiquitin promoter and Nos terminator (Wang et al., 1998). A control GFP construct, pPSUbiGFP (containing the ubiquitin promoter and Nos terminator), was prepared by excising the GFP gene from pMNG1004 (Upadhyaya et al., 1998), cloning the gene into adjacent EcoRI/BamHI sites of pubi.tml (Wang and Waterhouse, 2000), subsequent excision with HindIII, and cloning of the expression cassette into pWBvec8. Expression constructs were transferred to Agrobacterium tumefaciens strain AGL1 by tri-parental mating (Lazo et al., 1991). Rice (Oryza sativa cv. Taipei 309) was transformed as described by Upadhyaya et al. (2000). All PCR products were sequenced to confirm that errors had not been inadvertently introduced.

Plant growth conditions

Plants were grown hydroponically in 10 l containers, 12 plants per container, with 29/22 °C day/night temperatures and a 16 h light period. The nutrient solution was changed twice weekly and contained 0.5 mM KNO₃, 0.5 mM Ca(NO₃)₂, 0.25 mM NH₄NO₃, 0.5 mM KNO₃, 0.25 mM NH₄NO₃, 0.15 mM MgSO₄, 0.1 mM KH₂PO₄, 10 mM FeEDTA, 12 μM Na₂EDTA, 44 μM H₂BO₃, 8 μM MnCl₂, 1.4 μM ZnCl₂, and 0.8 μM CuCl₂. Duplicate sets of clonal plants were prepared by separating tillers from single primary (T₀) transformants. This enabled plants deprived of P to be compared with P-fed controls of the same T₀ line. After approximately 3 weeks growth, the clonal plant sets were transferred to 2× strength nutrient solution with or without the inclusion of KH₂PO₄. Plants were grown for a further 3 weeks.

Measurement of Pi in leaf tissue

Leaf tissue (25 mg), taken 5 cm from the tip of the first mature leaf (leaf 1), was ground in 100 μl of 5M H₂SO₄. After adding 900 μl of H₂O, samples were centrifuged for 5 min. To 50 μl of the supernatant solution was added 950 μl of H₂O and 500 μl of malachite green reagent (Irving and McLaughlin, 1990). After 30 min incubation, absorbance at 650 nm was measured and the amount of Pi quantified relative to Pi standards.

Protein extraction and GUS assays

GUS activities were assayed in 0–2 cm, 3–5 cm, and 0–8 cm sections of the nodal root, and in leaf samples taken 5 cm from the tip. Plant tissues were ground with fine sand in sodium phosphate buffer (50 mM, pH 7.0, 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Triton X-100). After centrifugation, the soluble protein was assayed (Bradford, 1976) and GUS activity was measured using a modification of the method of Breney et al. (1993). In brief, 10 μg of protein was analysed in 240 μl of sodium phosphate buffer containing 2.3 mM 4-methylumbelliferyl β-D-glucuronide (MUG). Measurements were recorded at 1–2 min intervals over a 10 min period using a micro-plate fluorometer (Fluoroskan II: Labsystems) with dedicated software (Deltasoft II: BioMetallics), and standardized against known concentrations of methylumbelliferone.

Histochemical localization of GUS expression

Histochemical analysis of GUS activity was performed essentially as described by Stomp (1992). Excised root tissues were incubated at 25 °C for 4–12 h in a 100 mM sodium phosphate buffer (pH 7.2. 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% Triton X-100) containing 0.1–1 mM 5-bromo-4-chloro-3-indoyl glucuronide. After fixation, roots were sectioned and visualized under dark-field microscopy.

Confocal microscopy

For analysis of gene expression in plants transformed with GFP-containing constructs, confocal microscopy images of cross-sections of nodal roots, intact secondary roots, and cross-sections of leaf tissue, were captured and processed using an upright Leica DMRXE microscope and Leica TCS SP2 confocal software.

Results

Identification of motifs in HvPh1 promoters

The isolation of genomic clones of the barley Pi transporter genes has been described previously (Smith et al., 1999; Rae et al., 2003). To identify motifs potentially involved in root and P-responsive expression, the available sequence immediately upstream of the translation start were compared for six of the Ph1 genes. GenBank accession numbers (promoter region and coding sequence) are as follows: HvPh1;1 (AF543197), HvPh1;2 (AY187019), HvPh1;4 (AY187024), HvPh1;5 (AY187021), HvPh1;6 (AF543198), and HvPh1;7 (AY187022). No promoter region was available for the HvPh1;3 gene (AY187026), and only a short region for HvPh1;8 (AY220455), therefore these genes were excluded from the investigation.

Nucleotide sequence lengths (in bases) used in the analysis were 1400 for HvPh1;1, 1295 for HvPh1;2, 4838 for HvPh1;4, 1318 for HvPh1;5, 916 for HvPh1;6, and 1347 for HvPh1;7 (Fig. 2), with the sizes reflecting the length of sequence available from the genomic clones. Nucleotide sequence identity between the six promoter regions was low, ranging from 36% to 45% along the full length for any promoter pair. Since the sequences differed widely in length, identities were also investigated for the 120 nucleotides immediately upstream of the translation start, which included the 5′ untranslated region of the encoded genes. HvPh1;1 and HvPh1;2 were 90% identical over this region, contrasting markedly with their full-length identity of 45%. None of the other promoters showed significant identity to one another over the same region (29–48%). The HvPh1;4 promoter was unusual in

Fig. 1. Generalized structure of the intron-containing HvPh1 expression cassette. The cassette is bordered by NotI sites within pWBvec8 (Wang et al., 1998), and is designed for convenient manipulation directly within the binary vector.
that it included a 1.48 kb region with high homology (>90% in three consecutive regions, Fig. 2) to BARE-1, a barley copia-like retroelement (Manninen and Schulman, 1993), and it is therefore likely that the region originated from integration of a retrovirus.

The HvPht1 promoter sequences were analysed to identify promoter elements for which there is evidence of a functional role in plants. Four unrelated monocot promoters (the maize ubiquitin promoter (Christensen and Quail, 1996); the rice actin promoter (McElroy et al., 1990); and promoters derived from the barley genes hordein (Sainova et al., 1993) and dihydroflavonol reductase (Kristiansen and Rohde, 1991)) were also examined to identify only those motifs which might be specifically related to the HvPht1 promoters.

The P1BS motif (GNATATNC), associated with the Pi starvation response in Arabidopsis (Rubio et al., 2001), was identified in all of the HvPht1 promoters investigated, with multiple copies being present for the HvPht1;1, HvPht1;2, and HvPht1;7 promoters (Table 2; Fig. 2). This suggests that the motif may also have a functional role in cereals. The motif was present in only one of the unrelated promoters (ubiquitin), albeit in four copies. Additional motifs identified using the PLACE algorithm (Higo et al., 1999) as being specific to several of the HvPht1 promoters were diverse in putative function (Table 2), being associated with circadian expression (CIAACDIANNLHC, Piechulla et al., 1998), photosynthesis and/or phytochrome regulation (-10PEHVPSB, Thum et al., 2001; REBETALGLHC821, Degenhardt and Tobin, 1996; and S1FBXSORPS1L21, Lagrange et al., 1993), and the low temperature response (LTR1HVBLT49, Dunn et al., 1998).

**Functional analysis of the HvPht1 promoters**

The HvPht1;1 and HvPht1;2 promoters were selected for further study as they had previously been shown to be predominantly expressed in the roots (Smith et al., 1999). The expression cassette was designed that allowed for convenient alterations to be made in order to assess promoter function (Fig. 1). Both GUS (Jefferson et al., 1987) and GFP (Haseloff et al., 1997) were used as reporter genes: GUS to provide quantitative analysis of promoter function, and GFP to enable the spatial distribution of expression to be visualized directly in living tissues.

**Quantitative assessment of promoter activity**

Rice plants were grown in hydroponic culture with high levels of initial P supply. After 3 weeks, one set of T0 clones (derived from separating tillers) was transferred to P-free nutrient solution. Plants deprived of P showed a substantial reduction in leaf Pi concentrations over the subsequent 21 d following transfer (Table 3) during which period the GUS activity observed increased steadily (Fig. 3), to a maximum of 4-fold for the HvPht1;1 promoter and approximately 2-fold for HvPht1;2 (Fig. 4). The inclusion of the Adh1 intron in the constructs increased GUS expression significantly, averaging 19-fold and 23-fold for the HvPht1;1 promoter and approximately 2-fold for HvPht1;2 promoters, respectively, but appeared to have very little effect on the specificity of expression. Following P-deprivation, root expression levels for the intron-contain-
Motifs shown are those which (i) are present in at least four of the HvPht1 promoters, (ii) are present in no more than one of the unrelated promoters investigated (ubiquitin, actin, hordein and dhfr), and (iii) have at least six defined nucleotides.

Table 2. Motif analysis for promoter regions of the six HvPht1 genes investigated

<table>
<thead>
<tr>
<th>Motif identity</th>
<th>Motif sequence</th>
<th>Frequency of motif occurrence (either orientation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Phl1:1 Phl1:2 Phl1:4 Phl1:5 Phl1:6 Phl1:7</td>
</tr>
<tr>
<td>P1BS</td>
<td>GNATATNC</td>
<td>3 2 1 1 1 4</td>
</tr>
<tr>
<td>-10PEHVPSBD</td>
<td>TATTC</td>
<td>0 2 4 1 0 4</td>
</tr>
<tr>
<td>C1ACADIANLLEHC</td>
<td>CAANNNATC</td>
<td>1 1 6 0 4 2</td>
</tr>
<tr>
<td>LTR1HVBLT49</td>
<td>CCGAAA</td>
<td>2 1 3 0 1 0</td>
</tr>
<tr>
<td>REBETALGHC2B1</td>
<td>CGGATA</td>
<td>1 0 1 1 1 0</td>
</tr>
<tr>
<td>S1FOXSORPS1L21</td>
<td>ATGGTA</td>
<td>0 1 2 0 1 1</td>
</tr>
</tbody>
</table>

Table 3. Inorganic P levels in transgenic rice leaves grown for up to 21 d without P

Values shown are means (±SE) for 12 independent transformants across different transgenic lines.

<table>
<thead>
<tr>
<th>Period of growth (d) with/without P</th>
<th>P-fed plants (µg P mg⁻¹ leaf FW)</th>
<th>P-deprived plants (µg P mg⁻¹ leaf FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0.277±0.028</td>
<td>0.092±0.005</td>
</tr>
<tr>
<td>14</td>
<td>0.229±0.013</td>
<td>0.072±0.004</td>
</tr>
<tr>
<td>21</td>
<td>0.286±0.026</td>
<td>0.060±0.006</td>
</tr>
</tbody>
</table>

Spatial distribution of HvPht1 expression

Roots from P-deprived plants containing the HvPht1 promoter::GUS constructs were stained for GUS activity (Fig. 6). Expression patterns observed for the intronless HvPht1;1 and HvPht1;2 promoters were indistinguishable from corresponding intron-containing lines but, consistent with results from the enzyme assay (Fig. 3), absolute expression levels were considerably lower (data not shown).

Nodal roots showed maximal GUS staining behind the root apex and primarily in the epidermal and vascular tissues (Fig. 6A, B). A few transgenic lines for both HvPht1;1 and HvPht1;2 had root cap expression (Fig. 6A), which may be attributable to the specific site of gene insertion. Reduced GUS levels were observed in more mature root sections (Fig. 6C–E). In the epidermal layer, expression was strong within the trichoblast (root hair) cells (Fig. 6F, G) and absent from atrichoblast (non-root hair) cells. This contrasted with the constitutive control promoter, ubiquitin, which showed high levels of expression in a proportion of both trichoblast and atrichoblast cells (Fig. 6J). Although the general pattern of expression driven by the HvPht1;1 and HvPht1;2 promoters was similar, for HvPht1;2 the degree of vascular expression was greater, particularly near the root apex (Fig. 6B), and high levels of expression were observed at the site of secondary root emergence (Fig. 6A, B). Consistent with the GUS results, analyses using the GFP reporter gene identified the HvPht1 promoters as having high levels of expression in trichoblast cells and much lower levels in the cortex (Fig. 7B, C). By contrast, the ubiquitin promoter was expressed in most cell types (Fig. 7A). Both HvPht1 promoters had reduced expression in the apical region compared with ubiquitin (Fig. 7D, shown for secondary roots but applicable to all roots examined). In secondary roots, GFP expression was apparent in most cell types but frequently at a low level in the outer epidermal layer compared with other cell types, even in very young roots (Fig. 7E–G). The level of induction following P-deprivation was quantified for the

Characterization of promoter expression patterns

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...continued.
secondary roots using the TCS SP2 confocal software and
determined to be 4–5-fold (data not shown). No difference
in spatial distribution of expression was observed between
plants grown under P-sufficiency or P-deficiency for either
of the *HvPht1;1* or *HvPht1;2* promoters.

Both of the *HvPht1* promoters expressed GFP in leaves
(Fig. 7), again with a distribution pattern unaffected by P-
nutrition. For the *HvPht1;1* promoter, expression was
observed in the leaf diaphragm and parenchyma cells of
the mid-rib, in the bulliform cells and vascular bundles of
the leaf blade, and at a relatively low level in mesophyll
cells (Fig. 7I, L). Expression was also observed in the
ligule and auricle (data not shown). The *HvPht1;2*
promoter showed a similar distribution of gene expression
in leaves, although at a slightly higher level in the
mesophyll compared with other cell-types (Fig. 7J, M).
Distribution patterns in leaves for the intronless *HvPht1;1*
and *HvPht1;2* promoters were indistinguishable from
corresponding intron-containing lines, although absolute
expression levels were greatly reduced (data not shown).
The ubiquitin promoter resulted in a constant high level of
expression in most cell types of the leaf (Fig. 7H, K).

**Discussion**

Analysis of the six *HvPht1* promoters identified a range of
previously characterized elements that were common to
the *HvPht1* promoters, yet uncommon in unrelated
promoters (Table 2). Significantly, the P1BS-like element
was found in all of the *HvPht1* promoters examined, and in

![Fig. 3. Activity and P-responsiveness of the *HvPht1;1* and *HvPht1;2* promoters. GUS activity in root segments from plants transformed with (A) *HvPht1;1* promoter ± intron and (B) *HvPht1;2* promoter ± intron constructs is shown for the 0–2 cm and 3–5 cm nodal root sections following 7
and 21 d of P deprivation, and following 14 d and 21 d of P-deprivation for the 0–8 cm nodal root section and leaf 1 samples. Values shown are
mean GUS activities for eight independent transgenic lines (±SE). For significance levels, multifactorial analysis of variance was performed using GenStat v6.1 using data for the plus P and 21 d without P treatments only.](image-url)
some instances as several copies. Rubio et al. (2001) have previously shown that in Arabidopsis the PHR1 protein, a MYB transcription factor, binds to the P1BS element, potentially regulating the P-starvation response. P1BS-like elements are present in the promoters of a range of P-regulated genes in Arabidopsis as well as in other dicot plant species, indicating that it may be a conserved signalling pathway for the P-starvation response in plants. The presence of P1BS-like elements in the HvPht1 promoters suggests that the element may serve a similar role in both monocots and dicots.

Monocot genes usually contain at least one intron and numerous studies have shown that the inclusion of the gene’s intron is often essential for the high-level expression of a foreign gene, including reporter genes (McElroy et al., 1990; Luerhsen and Walbot, 1991; Cornejo et al., 1993; Snowden et al., 1996). Neither the HvPht1;1 nor the HvPht1;2 gene contain any native introns for such use. Whilst a promoter derived from a similar intronless gene, Bx17 (Reddy and Appels, 1993), has previously been shown to activate high levels of expression of a foreign gene in its unmodified form (Schünemann et al., 2002), in their native form, the HvPht1;1 and HvPht1;2 promoters resulted in only low levels of reporter gene expression in transgenic rice. Modification of the promoters by the addition of a foreign intron (derived from the maize Adh1 gene) increased their strength approximately 20-fold (Fig. 3). A deletion of 85 nucleotides from the 3′ end of the HvPht1;1 promoter-intron construct (which included the putative transcription start) resulted in no detectable activity, indicating that inclusion of the Adh1 intron was not in itself activating the observed gene expression (data not shown). Although promoter activity was greatly enhanced by the inclusion of the Adh1 intron, the intron did not appear to alter the cell-type specificity or the low P-response. This was consistent with the results of Xu et al. (1993) who observed no differences in the distribution of gene activity attributable to the addition of the act1 intron to the potato pin2 promoter in rice although the level of gene activity was greatly enhanced, and Dugdale et al. (2001) who showed that the addition of the ubi1 intron to the BBTV BT6.1 promoter significantly enhanced promoter activity in banana without affecting tissue specificity.

Both the HvPht1;1 and HvPht1;2 promoters directed expression to several cell types within the root, with
maximal activity observed in the epidermis, specifically in the trichoblast cells. Root hairs are recognized as being the major site for Pi uptake from soil solution (Gahoonia and Nielsen, 1998) and a similar pattern of expression has been shown for the Arabidopsis ARAth;Pht1;1 and ARAth;Pht1;2 genes (Mudge et al., 2002).

High levels of expression for the ARAth;Pht1;3 and ARAth;Pht1;4 (referred to as AtPT2) promoters have previously been observed in the vascular tissues of differentiated roots (Mudge et al., 2002; Karthikeyan et al., 2002), indicating that the Pht1 transporters are not only involved with the uptake of Pi from the soil, but that they may also be involved with its transfer into the vascular system. This is consistent with the strong activity that was observed here in vascular tissue, particularly for the HvPht1;2 promoter, and suggests that the two genes (HvPht1;1 and HvPht1;2) may serve slightly different, yet complementary, roles in Pi uptake by plant roots.

Both HvPht1;1 and HvPht1;2 promoters had low, but detectable, levels of leaf expression, particularly in the younger leaves. Similar results were observed following transformation of the HvPht1;1::GFP constructs into barley (data not shown). It is unlikely that this activity can be attributed to an artefact of the design of the binary plasmids as the HvPht1;1-driven reporter genes were cloned upstream and in the same orientation as the 35S-driven...
selectable marker, thus avoiding the possibility of the 35S promoter influencing expression of the HvPht1 promoters-intron::GUS constructs. Similarly, while some of the Arabidopsis ARAth;Pht1 genes are expressed primarily in roots, they also possess a low level of activity in specific shoot tissues (Mudge et al., 2002). Previous studies based on RNA hybridization in barley identified no expression for either of the HvPht1;1 and HvPht1;2 genes in shoots (Smith et al., 1999; Rae et al., 2003). Although it is possible that not all regulatory elements controlling expression specificities were contained within the promoter region investigated (being either further upstream or

Fig. 7. Tissue specificity of the HvPht1 promoter constructs analysed by confocal microscopy for GFP expression. Shown are: transverse sections of P-deficient nodal roots from plants transformed with (A) ubiquitin promoter::GFP (cell walls stained red with propidium iodide to highlight epidermal cell layer), (B) HvPht1;1 promoter-intron::GFP and (C) HvPht1;2 promoter-intron::GFP. (D) Secondary root-tips for plants transformed with GFP under control of the (top to bottom) ubiquitin promoter, HvPht1;1 promoter-intron and HvPht1;2 promoter-intron. Longitudinal section through secondary roots from plants transformed with a HvPht1;1 promoter-intron::GFP gene following growth at (E) high P and (F) following P deprivation. (G) Expression of HvPht1;1 promoter-intron::GFP in emerging secondary roots. (H–M) GFP expression in leaf tissue. Cross-section through leaf blade (H–J, chlorophyll autofluorescence not shown) or across leaf mid-rib showing leaf diaphragm (K–M, chlorophyll autofluorescence shown in red), for (H, K) ubiquitin promoter::GFP, (I, L) HvPht1;1 promoter-intron::GFP and (J, M) HvPht1;2 promoter-intron::GFP transformed plants. Leaf cell-types showing GFP fluorescence include mesophyll (M), parenchyma (P), bulliform (BF), vascular bundles (VB), mid-vein (MV), and star-shaped diaphragm (DM) cells. Scale bars are indicated in μm.
intragenic), the existence of promoter motifs associated with leaf expression in the HvPht1;1 and HvPht1;2 promoters is consistent with observations of shoot activity, and it is possible that the earlier studies were insufficiently sensitive for the detection of gene expression.

Previous studies based on RNA hybridizations indicated that expression of the HvPht1;1 and HvPht1;2 genes was induced under conditions of P-deficiency (Smith et al., 1999). Results from the present study support this observation with levels of protein induction in roots averaging 3.5- and 2-fold for the HvPht1;1 and HvPht1;2 promoters, respectively. The induction response occurred over 21 d of P deprivation, however, because of their relatively large size, the plants used in the present study had high internal Pi contents prior to P deprivation, and this may have contributed to a delayed response.

To conclude, the HvPht1;1 and HvPht1;2 promoters activate trichoblast-specific expression in the root epidermis, consistent with a primary role for the associated genes in the uptake of Pi from the soil. In addition to furthering an understanding of the role of the HvPht1 genes play in the P-nutrition of plants, the Adh1-intron enhanced HvPht1;1 and HvPht1;2-derived gene expression vectors provide a new resource for cereal transformation, with application for controlling the expression of foreign genes associated with plant nutrition. The promoter constructs described (Fig. 1) represent the first availability of such for use in cereals.

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References


Degenhardt J, Tobin EM. 1996. A DNA binding activity for one of two closely defined phytochrome regulatory elements in an Lhcb promoter is more abundant in etiolated than in green plants. The Plant Cell 8, 31–41.


Muchhal US, Pardo JM, Raghothama KG. 1996. Phosphate
transports from the higher plant *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences, USA 96, 5868–5872.


