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Characterization of the high-affinity phosphate transporter *PHT1;4* gene promoter of *Arabidopsis thaliana* in transgenic wheat

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Abstract

The root specificity and phosphate (Pi) deficiency responsiveness of high-affinity phosphate transporter (*PHT1*) genes point to their promoters as a sustainable system to drive Pi acquisition-related transgenes in plants. In this study, a 3-kb promoter of the *AtPHT1;4* gene from *Arabidopsis thaliana* fused to the β-glucuronidase (*GUS*) reporter gene was biolistically introduced into wheat (*Triticum aestivum* L.) and functionally characterized in transgenic plants grown in hydroponics and in pots with soil under various Pi supply rates. From among 27 T_1 progeny derived from 250 T_0, four transgenic lines reached T_3, with two of them showing detectable GUS activity in the roots of T_4 plants. An unusually high number of transgene insertions characterized these transgenic lines, along with an irregular pattern of histochemical GUS staining and weak GUS activity. *GUS* expression driven by *AtPHT1;4* was consistently higher under most assay conditions, as it was unaffected by 0 to 0.5 mM Pi in hydroponically grown plants, as well as by 16 to 20 mg(P) kg^{-1}(soil) in potted plants. Raising the soil P up to or above 40 mg kg^{-1} significantly down-regulated the quantity of *GUS* transcripts. These results show that the responsiveness of the *AtPHT1;4* promoter to Pi availability in transgenic wheat was restricted to soil-grown plants, which highlighted the relevance of the substrate and Pi supply rates in assessing molecular responses to Pi deficiency.

Additional key words: *GUS* reporter gene, phosphate deficiency, *Triticum aestivum*.

Introduction

High-affinity phosphate (Pi) transporters are membrane-associated proteins that operate at low Pi concentration by moving Pi from the external medium to the cytosol. These proteins are encoded by genes grouped into the PHT1 family of H^+/Pi co-transporters to differentiate them from those belonging to the PHT2, PHT3, and PHT4 families of low-affinity Pi transporters in *Arabidopsis thaliana* (Rausch and Bucher 2002). Of the nine *PHT1* genes identified in this species, four drive reporter gene expression in roots and are induced under Pi deficiency (Mudge *et al.* 2002). Similarly, partial sequences of seven *PHT1* genes, along with the full-length *TaPHT1:*2 (formerly known as *TaPT2*), were isolated from wheat (*Triticum aestivum* L.) (Davies *et al.* 2002). Histochemical analyses showed that reporter genes driven by the *TaPHT1;2* promoter were expressed in roots of Pi-deficient *Arabidopsis* and wheat (Tittarelli *et al.* 2007, Miao *et al.* 2009). The *PHT1* genes have also been identified in rice, barley, and maize (Paszlowski *et al.* 2002, Rae *et al.* 2003, Schünmann *et al.* 2004a, Nagy *et al.* 2006), with most *PHT1* genes exhibiting expression predominantly in roots under Pi-deficient conditions.

In plants, *PHT1* genes are part of the multiple responses to Pi deficiency, in which the genes involved share a common regulatory system. Evidence has

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Abbreviations: DAT - days after transplantation; GUS - β-glucuronidase; PAE - Pi acquisition efficiency; PHT1 - high affinity phosphate transporter 1; Pi - phosphate; RT-qPCR - reverse transcription quantitative polymerase chain reaction.
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indicated that the P1BS motif, which is the binding site of PHR1 (Rubio *et al.* 2001), miRNA399, and the E2 ubiquitin conjugase PHO2 are major determinants coordinating the transcriptional response of Pi homeostasis in *Arabidopsis* (Bari *et al*. 2006). The P1BS-like motifs, or PHR1 homologs, have been found in *PHT1* promoters of barley (Schünmann *et al.* 2004b), wheat (Tittarelli *et al.* 2007, Miao *et al*. 2009), and rice (Zhou *et al*. 2008). Similarly, transcribed sequences encoding miRNA399 or potential orthologs of PHO2 have also been identified in wheat and rice (Bari *et al*. 2006, Zhou *et al.* 2008, Oono *et al.* 2013), which implies that signaling molecules controlling Pi homeostasis may be conserved across species.

The role that PHT1 transporters play in Pi uptake lead to the use of *PHT1* overexpression as a means to improve Pi acquisition efficiency (PAE) in plants. However, evidence from barley (Rae *et al.* 2004) and rice (Jia *et al.* 2011) suggests that *PHT1* overexpression may not be a robust strategy to improve PAE in most species. Nevertheless, *PHT1* promoters have successfully been used to drive PAE-related transgenes in *Arabidopsis* (Mudge *et al.* 2003), as well as reporter genes in tobacco (Karthikeyan *et al.* 2002), rice (Schünmann *et al.* 2004ab, Koyama *et al.* 2005), *Arabidopsis* (Tittarelli *et al.* 2007, Miao *et al.* 2009), maize (Coehlo *et al.* 2010), and wheat (Tittarelli *et al.* 2007). Most studies using reporter genes have relied on heterologous promoters, the exception being wheat, in which it was demonstrated that a short 579 bp *TaPHT1;2* promoter was sufficient to drive Pi-deficiency-induced *GUS* expression in this species, albeit at low levels (Tittarelli *et al.* 2007).

In addition to the structural differences related to the requirement of foreign introns to enhance transgene expression (Schünmann *et al.* 2004ab), *PHT1* promoters may exhibit different sensitivities to changing Pi concentrations in the medium. Varying sensitivities to Pi have been reported for the *AtPHT1;2* and *AtPHT1;4* genes of *Arabidopsis* (formerly known as *AtPT1* and *AtPT2*) (Mukatira *et al.* 2001), which implies that their promoters would provide additional control of transgene expression. To further extend our study on *PHT1* promoters (Tittarelli *et al.* 2007), we generated transgenic wheat plants expressing the *ß-glucuronidase* (*GUS*) reporter gene under the regulation of the *AtPHT1;4* gene promoter of *Arabidopsis*. This paper reports on the characterization of this promoter in transgenic wheat grown in a broad range of Pi concentrations and in two different substrates.

Materials and methods

Construction of the *AtPHT1;4* promoter-reporter gene fusion: DNA from *Arabidopsis thaliana* L. ecotype Columbia was used as a template to amplify a 3-kb fragment of the *AtPHT1;4* promoter upstream of the start codon, with the primers shown in Table 1 Suppl. DNA was amplified by PCR using *Pfu High-Fidelity* DNA polymerase (*Stratagene*, La Jolla, CA, USA), according to the manufacturer's instructions. The amplified product was cloned into *pGEM-T* (*Promega,* Madison, WI, USA), and sub cloned into *Sal*I and *Sma*I-digested pBI101.3 binary vector (*Clontech,* Palo Alto, CA, USA) upstream of the *GUS* reporter gene to generate pCN2 (*AtPHT1; 4-GUS*).

Plant transformation: Wheat (*Triticum aestivum* L. cv. Bobwhite) was grown in a greenhouse under a 16-h photoperiod with an irradiance of 250 μmol m^{-2} s^{-1}, a temperature of 25 ºC, and a relative humidity of 40 %. Immature embryos were cultured in solidified Murashige and Skoog (1962; MS) medium supplemented with 150 mg dm^{-3} L-asparagine, 2.5 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D), and 150 g dm^{-3} maltose. The gene construct pCN2 was introduced into embryos by microprojectile bombardment using a *PDS-1000/He* particle delivery system (*Bio-Rad,* Hercules, CA, USA) along with plasmid pAL51. The construct pAL51 contains the *Bar* gene as a selectable marker driven by the maize *Ubiquitin* promoter (Lonsdale *et al.* 1995). After bombardment, explants were cultured on MS medium containing L-asparagine, 2,4-D, and 2 % sucrose. Cultures were then moved to shoot regeneration medium composed of MS supplemented with L-asparagine, 3 % (m/v) sucrose, and 5 mg dm^{-3} glufosinate. After 2 - 3 weeks, plantlets were transferred to soil and grown to maturity.

Molecular characterization of transgenic plants: To visualize the expression of the *Bar* gene in T_0 plants, a 0.2 % (v/v) glufosinate solution containing 0.1 % (v/v) *Tween 20* was applied on the upper portion of the second youngest leaf using a cotton plug. The reaction to the herbicide was visually assessed 6 - 7 d after painting.

Transgenic plants harboring the *AtPHT1;4*, *GUS,* and *Bar* genes were identified by PCR of genomic DNA from T_1 and T_2 progenies; the primers used are shown in Table 1 Suppl. Expression of the *GUS* reporter gene was detected by semi-quantitative reverse transcription PCR (RT-PCR) on total RNA from roots of T_3 plants grown hydroponically as described in the next section, and sampled at 0, 3, and 6 d after Pi withdrawal. To amplify the *GUS* transcripts, a forward primer at the 3´ end of the *GUS* gene and a reverse primer anchored at the 5´ end of the *Nos* terminator were used (Table 1 Suppl.). The *18S rRNA* gene was included as an internal control for RT-PCR, which was amplified with the primers described by Davies *et al.* (2002).

For Southern blotting, genomic DNA from each transgenic line (10 μg) was digested with *Eco*RI and electrophoresed in a 1 % (m/v) agarose gel. DNA was denatured and capillary blotted onto a *GeneScreen Plus*

nylon membrane (*NEN*) and fixed by UV-crosslinking. A 670-bp digoxigenin-labeled *GUS* probe was prepared by PCR according to the *DIG* probe synthesis kit manual (*Roche*); the primers are listed in Table 1 Suppl. Hybridization, washing, and detection were carried out according to the *DIG* application manual for *in situ* hybridization (*Roche*). The transgene copy number was also estimated by real-time PCR following the protocol of Ahmad *et al*. (2005), on a serial 10-fold dilution of the plasmid pCN2. Primers for amplifying the transgene of plasmid pCN2 and transgenic lines are shown in Table 1 Suppl.

Functional analyses: Seedlings from transgenic plants were grown hydroponically under the greenhouse conditions as previously described. The nutrient solution contained 1.4 mM NH_4NO_3, 2 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1.4 mM $MgSO_4$, 0.1 mM KH_2PO_4, 0.04 µM $(NH_4)_6Mo_7O_{24}$, 5 µM H_3BO_3, 1.5 µM $ZnSO_4$, 0.4 µM $CuSO_4$, 2 µM $MnSO_4$, and 35 µM FeNa-EDTA. The nutrient solution was changed twice a week, and the pH was adjusted to 5.8. Plants were maintained with 0.1 mM Pi for 7 d and then with either 0.1 or 0.5 mM Pi or without Pi and sampled at different time points depending on the experiment. Transgenic plants were also evaluated in plastic pots containing 0.5 kg of acidic soil characterized by a pH of 5.6 and extractable Olsen-P of 11 mg kg^{-1}(soil). Phosphorus was supplied as KH_2PO_4 at 0, 80, 250, 500, and 1000 mg(P) kg^{-1}(soil) depending on the experiment, using KCl to maintain a constant K rate. Unless stated otherwise, plants were harvested when they reached the five-leaf stage in the Pi unfertilized treatment. At harvest, roots were rinsed thoroughly with distilled water, blot-dried, frozen in liquid nitrogen, and stored at -80 ºC. Soil P content was determined at the end of experiments.

To ascertain the effect of the substrate on *AtPHT1;4* strength, transgenic wheat was simultaneously grown hydroponically and potted in soil, along with a wheat line harboring the *TaPHT1;2-GUS* construct referred herein as line IIIA35. Line IIIA35 was developed by the same procedure and in the same genetic background as transgenic lines hosting the *AtPHT1;4-GUS* construct. Line IIIA35 bears a 579-bp *TaPHT1;2* promoter from wheat fused to the *GUS* reporter gene (Tittarelli *et al*. 2007). This line was included as a control for substrate and Pi treatments because it was previously shown that neither of the variables modulated *TaPHT1;2*-driven *GUS* expression. The endogenous *TaPHT1;2* served as a marker gene for Pi deficiency in these experiments.

Genes encoding actin *(TaACT)***,** 26S rRNA *(Ta26S)*, glyceraldehyde-3-phosphate dehydrogenase (*TaGPDH*), and α-tubulin (*TaTUB*) were evaluated as candidate reference genes for quantitating *GUS* and *TaPHT1;2* expression. Primer pairs to amplify the four reference genes, along with *GUS* and *TaPHT1;2* transcripts, are shown in Table 1 Suppl. Selection of the best reference gene was based on the cycle threshold (Ct) values (Mane *et al.* 2008), from transcripts of hydroponically grown wheat supplied with 0 and 0.5 mM P and exposed at 37 and 22 ºC for 2 h. Under these conditions, *TaACT* was identified as the most stable gene for expression analyses in transgenic wheat (Fig. 1 Suppl.).

Arabidopsis seedlings were grown in complete nutrient solution for two weeks at day/night temperatures of 22/18 °C, a 8-h photoperiod and an irradiance of 100 µmol m^{-2} s^{-1} to delay flowering. Plants were then transferred into small centrifuge tubes with a 3-mm hole at the bottom. Tubes were set on perforated *Styrofoam* lying on the top of the nutrient solution such that a portion of roots was allowed to grow through the 3-mm hole down to the nutrient solution, whereas the remaining roots were forced to grow within the tube to induce mechanical constraints. Plants with roots growing unaltered into the nutrient solution served as controls. Roots were sampled two weeks after the mechanical stress was initiated. The ethylene responsive factor (*AtERF1*) was used as a marker gene for mechanical impedance, whereas the elongation factor 1α (*AtEF1α*) served as a reference gene for this stress (Okamoto *et al.* 2008). Primer pairs for the amplification of transcripts of *AtPHT1;4*, *AtERF1* and *AtEF1α* are shown in Table 1 Suppl.

GUS assays: Frozen tissue was ground in liquid nitrogen and homogenized in GUS extraction buffer (Jefferson 1987). Samples were centrifuged at 4 ºC, and the crude supernatant was used for GUS assays. The GUS activity was measured in undiluted extracts by monitoring the cleavage of the ß-glucuronidase substrate 4-methyl-umbelliferyl ß-D-glucuronide (MUG) as described by Jefferson (1987). Data are expressed as nmol of 4-methylumbelliferone (4-MU) mg^{-1}(extracted protein) min^{-1}. Background fluorescence from plant tissues that did not contain the *GUS* transgene was subtracted from all fluorometric measurements.

Histochemical staining was performed as described by Tittarelli *et al.* (2007). Micrographs were obtained from 10 µm-thick transversal root sections, which were embedded in paraffin and examined using a light microscope. A positive staining control consisted of *Arabidopsis* plants expressing the *GUS* reporter gene under the control of the 35S cauliflower mosaic virus promoter.

RT-qPCR: First-strand cDNA synthesis was performed in 0.5 µg of DNase-treated total RNA primed with 50 µM oligo(dT) using an *Affinity Script QPCR* cDNA synthesis kit (*Stratagene*). Quantitative PCR was carried out in 96-well plates with *SYBR-Green Master Mix* using 6 ng of cDNA to detect *GUS, TaACT, Ta26S*, *TaGPDH, TaTUB*, and *TaPHT1;2* transcripts from *T. aestivum*, whereas 1.5 ng of cDNA was used to detect *AtPHT1:4*, *AtERF1*, and *AtEF1α* from *A. thaliana*. The thermal profile for determination of all genes was 95 ºC for 10 min, followed by 40 cycles at 95 ºC for 30 s, 60 ºC for 30 s, and 72 ºC for 40 s. The fold change in gene expression was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Soluble root Pi was extracted with 1 % (v/v) acetic acid from pre-weighed frozen root tissue ground to fine powder. The homogenate was centrifuged, and the supernatant was used for the quantification of Pi by the ascorbic acid-molybdate method (Ames 1966). Olsen-P was extracted with sodium bicarbonate and the P content determined colorimetrically (Olsen *et al*. 1954).

Statistical analysis: Data were subjected to one- or two-way *ANOVA* after being checked for normality and homogeneity. Multiple comparison procedures were performed with Tukey's HSD test at $P < 0.05$ for normally distributed data. The non-parametric Kruskal-Wallis one-way *ANOVA* was used when the assumption of normality or variance equality was not met. Statistical analyses were performed with *InfoStat v. 2008* (Di Rienzo *et al.* 2008).

Results

Approximately 250 T_0 putative transgenic wheat plantlets derived from about 3 000 embryos co-transformed with plasmids pCN2 and pAL51 were screened by the leaf-painting assay. Plantlets that showed the Bar phenotype were self-pollinated and harvested. The PCR performed on the T_1 progeny identified 27 lines, from 10 to 12 plants analyzed for each line, containing the *GUS*, *AtPHT1;4*, and *Bar* genes. Further, PCR on T_2 progeny identified five lines for the *AtPHT1;4-GUS* construct, which were self-pollinated to obtain the next generation. These T_3 lines were grown hydroponically and analyzed for *GUS* expression by RT-PCR. Four of these lines

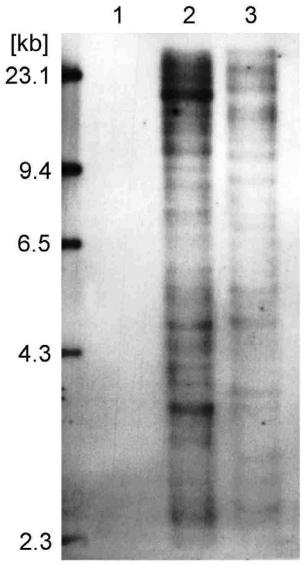

Fig. 1. Southern blot of transgenic wheat lines. Total DNA (10 µg) was digested with *Eco*RI and hybridized with a 0.7 kb DIG-labeled *GUS* probe. 1 - untransformed cv. Bobwhite, 2 - line IIA5, 3 - line IIA292. DNA size markers are indicated on the left.

showed transcripts of the *GUS* gene, which were detected even at the highest root Pi content (Fig. 2*A,B* Suppl.). From these four transgenic lines, only lines IIA5 and IIA292 reached T_4 with noticeable GUS activity, both being characterized by an unusually high number of transgene insertions (Fig. 1). Using the real-time PCR approach of Ahmad *et al.* (2005), the estimated copy number per haploid genome was determined to be 41 ± 11 for IIA5 and 53 ± 15 for IIA292 lines (Table 2 Suppl.).

Fluorometric assays detected a near-background weak GUS activity in roots of transgenic lines grown hydroponically (Fig. 2*A*). This GUS activity was not consistent with strong but unevenly distributed histochemical GUS staining (Fig. 3*A,B*). Both GUS activity and GUS staining were unaffected by Pi treatments (0 and 0.1 mM Pi), despite the 80 % reduction in root Pi observed seven days after Pi withdrawal (Fig. 2*B*). Transgenic lines were then moved to soil supplemented with 0 or 80 mg(P) kg^{-1}(soil). In this substrate, GUS activity was 30- to 40-fold higher than in hydroponically grown plants, without a significant ($P > 0.05$) response to the Pi supply (Fig. 2*C*). Averaged

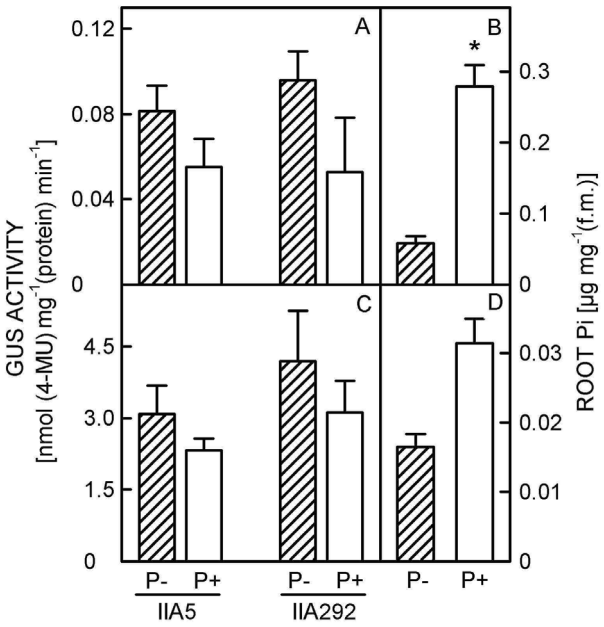

Fig. 2. GUS activity in roots of transgenic wheat grown hydroponically (*A*) and in soil (*C*). In (*A*), 0.1 mM P was maintained throughout the experiment (P+) or withdrawn at day seven (P-). Means ± SEs of four biological replicates sampled 7 d after P was removed. In (*C*), plants were grown in an acidic soil supplemented with 0 (P-) or 80 (P+) mg(P) kg^{-1}(soil). Means ± SEs of three biological replicates sampled at the five-leaf stage. Root Pi (means ± SEs) averaged over both lines is shown in (*B*) and (*D*) for hydroponically and soil grown plants, respectively. Significant differences between Pi treatments are indicated by an *asterisk*, as determined by Tukey's test at $P \leq 0.05$.

over the Pi treatments and genotypes, the GUS activity in shoots was 0.025 ± 0.015 nmol(4-MU) mg^{-1}(protein) min^{-1} (n = 12), which showed that the *AtPHT1;4* promoter directed transgene expression primarily in the roots of transgenic wheat.

Compared to hydroponically grown plants, *GUS* expression in potted plants was distributed in a patchy pattern, ranging from strong to mild levels throughout the roots and across both lines, irrespective of the Pi treatments (Fig. 3*C,D*). *GUS* expression in root hairs was detectable only in trichoblasts arising from stained root epidermal zones (Fig. 3*E*-*G*), whereas expression was not observed in root apices of unstained epidermal tissue (Fig. 3*H*). Micrographs of representative root sections showed varying degrees of GUS staining from line IIA292, and reporter gene expression was localized to pith and cortical cells of strong GUS-stained zones, whereas reporter gene expression was mostly detected through the cortex of GUS-unstained epidermal tissue (Fig. 3*I,J*).

Previous experiments showed that Pi treatments did not significantly affect the strength of *AtPHT1;4*, so the responsiveness to Pi supply was further assessed in potted plants fed with 0 to 1000 mg(P) kg^{-1}(soil) and sampled 50 d after transplanting (5 - 6 leaf stage). This range of Pi supply provided 18 to 129 mg(P) kg^{-1}(soil) when expressed as available Olsen-P at the end of the experiment (Fig. 4*C*). Within this range of Olsen-P, the highest *GUS* transcript abundance and GUS activity occurred in the non-Pi fertilized treatment as expected, whereas both *GUS* transcripts and GUS activity were reduced at Pi rates above 250 mg(P) kg^{-1}(soil)

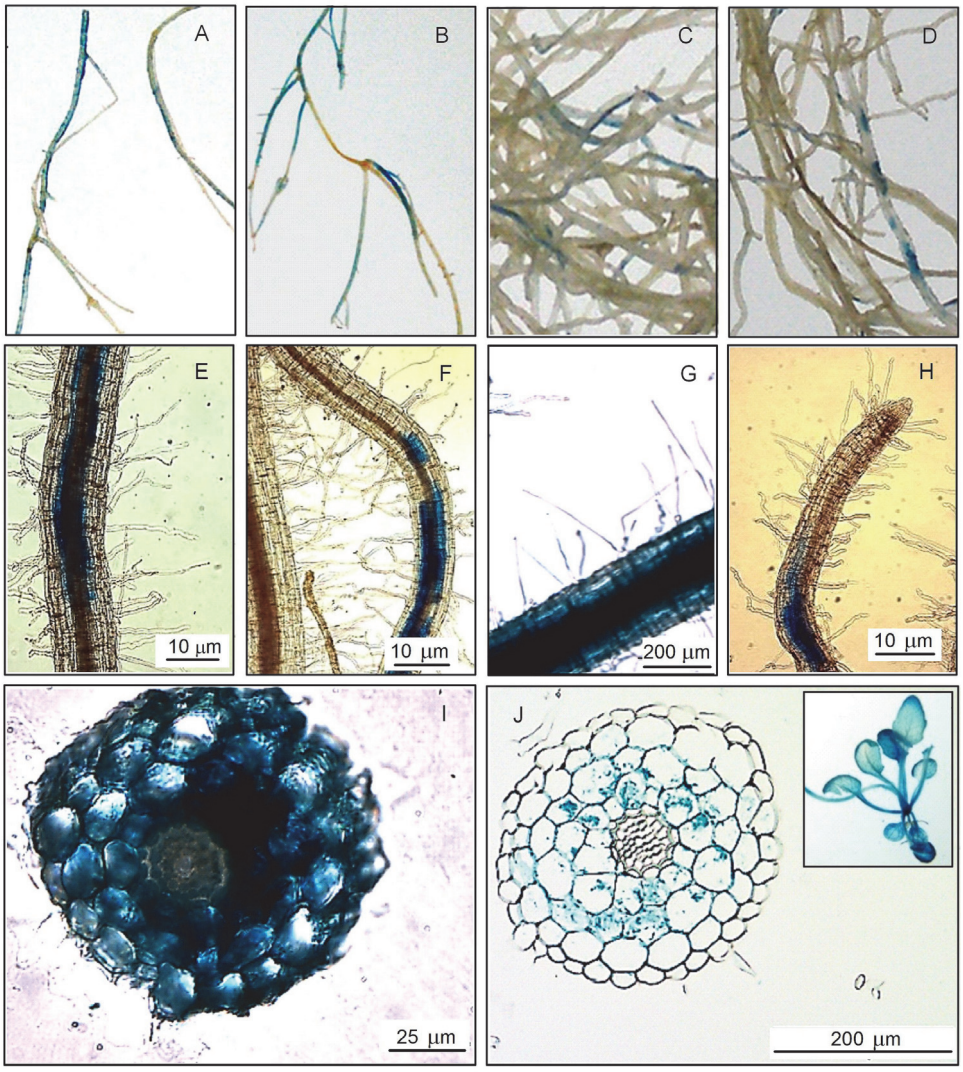

Fig. 3. Histochemical GUS staining in roots of transgenic wheat grown hydroponically (*A*,*B*) and in soil (*C*-*J*). GUS staining is shown in the roots of lines IIA5 (*A*,*C*) and IIA292 (*B*,*D*), root hairs from unstained (*E*,*F*) and stained (*G*) epidermal root zones, and the root apex from unstained epidermal root zones (*H*). Transversal root sections from line IIA292 with strong and mild G*US* expression are shown in *I* and *J*, respectively. The insert in (*J*) is an *Arabidopsis* plant transformed with the *35S CMV-GUS* construct used as a GUS staining control. In both lines, plants from Pi-fed and Pi-unfed treatments showed the same pattern of GUS staining.

(Fig. 4*A,B*). Plants grown in soil with less than 250 mg(P) kg^{-1} were Pi-deficient, as shown by the significant reduction in shoot biomass (Fig. 4*D*). The effect of Pi deficiency was also documented for *Arabidopsis* grown simultaneously in the same acidic soil (Fig. 3 Suppl.).

A key component in understanding the effect of growth media on the *AtPHT1;4* promoter activity was the responsiveness of the endogenous *TaPHT1;2* gene in transgenic wheat grown hydroponically and in soil (Fig. 5). Transcripts of this Pi deficiency-induced marker gene were highly expressed in both substrates, at almost the same level as *Actin*, as measured by the 40-ΔCt values according to Bari *et al.* (2006) (Table 3 Suppl.). In hydroponics, 0.5 mM P significantly down-regulated the transcription of the *TaPHT1;2* gene, whereas 250 mg(P) kg^{-1}(soil) did not affect the quantities of the transcript (Fig. 5*A*). This lack of responsiveness to the Pi supply in soil correlated with a root Pi content that was 20-fold lower than in hydroponically grown plants (Fig. 5*B*).

As expected, the quantities of transcripts for the control line IIIA35 were almost unaffected by the treatments (Table 3 Suppl.). Therefore, an unbiased comparison of *GUS* transcripts across substrates and Pi treatments was possible when normalized to *Actin* and expressed relative to line IIIA35. By following this procedure, *GUS* transcripts driven by *AtPHT1;4* were unaffected by Pi treatments in hydroponics. Similarly, Pi treatments did not alter *GUS* transcripts in plants in soil at 14 days after transplantation (DAT), whereas the addition of 250 mg(P) kg^{-1}(soil) shifted the 40-ΔCt value from 38.0 ± 0.7 down to 31.9 ± 1.0 in line IIA292 at 21 DAT (Fig. 5*C,D*). This Pi supply-induced down-regulation of *GUS* transcripts did not occur in hydroponics, even though hydroponic plants had 10 times higher root Pi than soil grown plants (Fig. 5*B,D*).

To gain insight as to whether the substrate may have any effect on *AtPHT1;4* strength in its own species, a physical constraint was imposed to the roots of hydroponically grown *Arabidopsis* to simulate mechanical impedance, as would occur in compacted or stony soil layers. This physical stimulus up-regulated *AtPHT1;4* transcription in the same direction as transcription of the mechanical impedance-induced marker *AtERF1* gene (Fig. 6*A,B*). Because compressed rolled roots within the centrifuge tube may have also been affected by the oxygen supply, the possibility that *AtERF1* and *AtPHT1;4* expression could be up-regulated by hypoxia or a combination of hypoxia and mechanical stress cannot be ruled out.

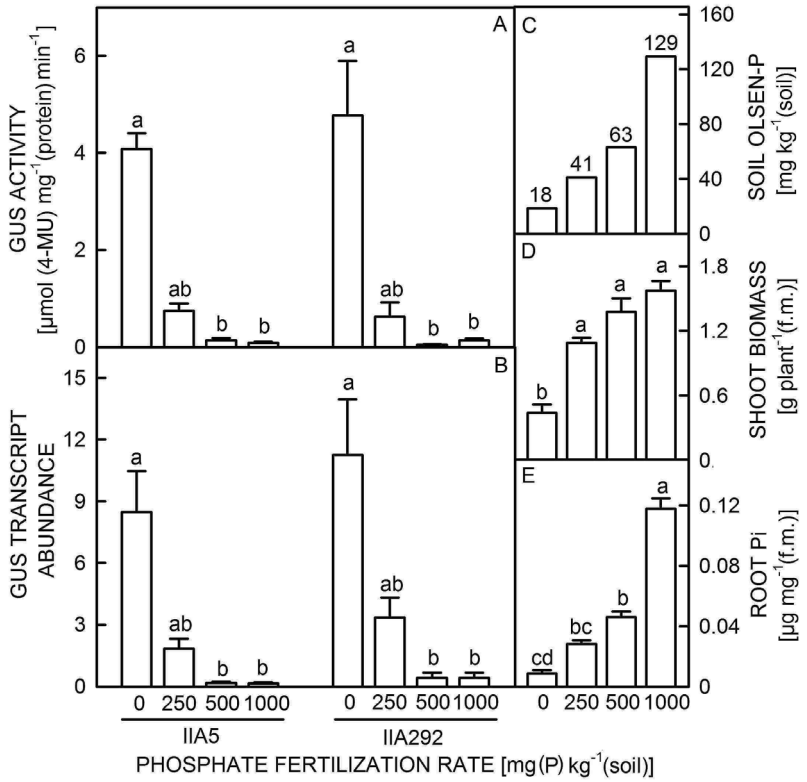

Fig. 4. Expression of the *GUS* reporter gene in transgenic wheat grown in an acidic soil under various phosphate fertilization rates. *A* - Specific GUS activity in roots; *B* - *GUS* transcript abundance in roots using *Actin* as a reference gene and expressed relative to line IIIA35; *C* - Soil Olsen-P at the end of the experiment; *D*, *E* - shoot biomass and soluble root Pi averaged over genotypes, respectively. Means ± SEs of three (*A*,*B*) and six (*D*,*E*) biological replicates. Significant differences between treatments ($P < 0.05$) are indicated by different letters (Kruskal-Wallis test).

Discussion

The *GUS* reporter gene driven by *AtPHT1;4* in roots of hydroponically grown transgenic wheat fed with 0.1 mM P did not show the expected Pi supply-induced down-regulation that was reported for this construct in *Arabidopsis* (Karthikeyan *et al.* 2002). Although this nominal 0.1 mM P may have been reduced due to the un-buffered nature of the source of added Pi (Liu *et al.* 2006), this effect was not of significance because Pi-fed and Pi-unfed plants showed contrasting content of root Pi (Fig. 2*A,B*). Consequently, 0.1 mM P did not allow for the discrimination of responsiveness to the Pi supply in hydroponically grown transgenic wheat hosting the

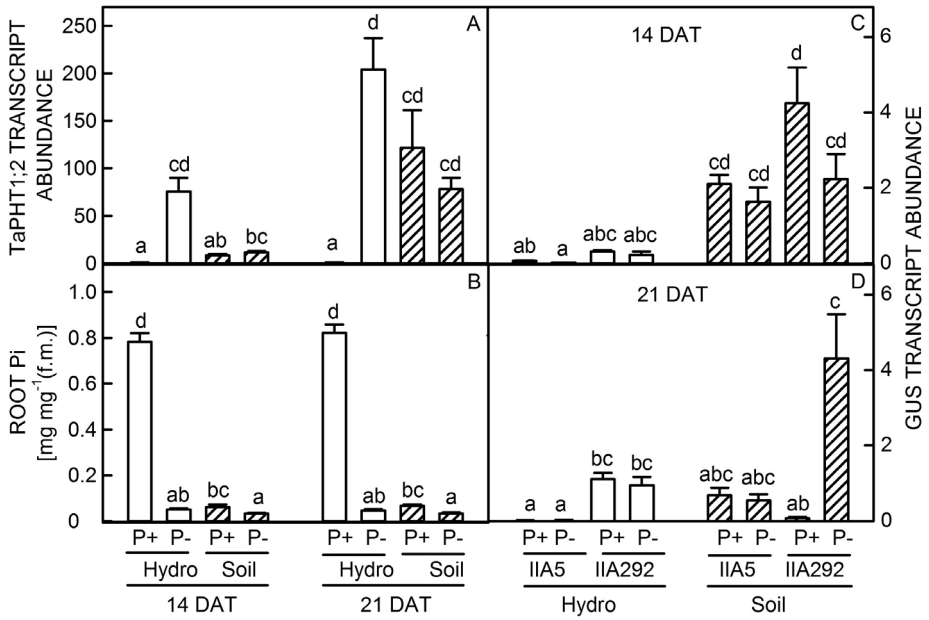

Fig. 5. The effect of the substrate, Pi treatment, and sampling time on the expression of the *GUS* reporter gene in transgenic wheat. Plants were grown in hydroponics (Hydro) or in soil (Soil) with 0.5 mM P (P+) or without P (P-) and sampled at 14 and 21 d after transplantation (DAT). *A* - Expression of the Pi deficiency-induced marker *TaPHT1;2* gene averaged over transgenic lines; *B* - Soluble root Pi averaged over transgenic lines; *C* and *D* - expression of the *GUS* reporter gene at 14 and 21 DAT, respectively. Transcript abundance was determined by RT-qPCR using *Actin* as a reference gene and expressed relative to line IIIA35. Means ± SEs of three biological replicates. Significant differences between treatments ($P < 0.05$) are indicated by different letters (Kruskal-Wallis test).

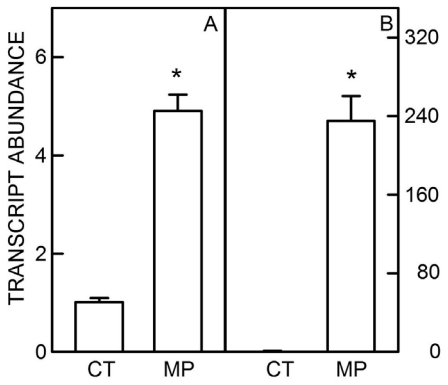

Fig. 6. The effect of mechanical impedance on the expression of *AtPHT1;4* (*A*) and the marker gene *AtERF1* (*B*) in the roots of *A. thaliana* grown hydroponically. For both genes, transcript abundance was calculated by normalizing against the *AtEF1α* gene. CT - untreated root control, MP - mechanically stressed roots. Means ± SEs of four biological replicates. Significant differences between treatments ($P < 0.05$) are indicated by an *asterisks* (Kruskal-Wallis test).

AtPHT1;4-GUS construct, despite its successful use for this purpose in other model systems (Peñaloza *et al.* 2005).

Moving plants from hydroponics to soil enhanced GUS activity by 30- to 40-fold, even though hydroponically grown and soil grown plants showed similar patterns of histochemical GUS staining (Fig. 3*A-D*). This result indicates that the low GUS activity in root extracts of hydroponically grown plants is largely responsible for this discrepancy in GUS performance between substrates. Low GUS activity, which has often been reported in transgenic wheat harboring the *uidA* gene, is attributed to non-proteinaceous inhibitors of the β-glucuronidase that are released once cell membranes are disrupted (Bahieldin *et al.* 2005, Ramadan *et al.* 2011). Therefore, it was hypothesized that inhibitors of this enzyme were synthesized preferentially in hydroponically grown plants, which resulted in root extracts with very low GUS activity.

Histochemical analyses showed that the *AtPHT1;4* promoter directed *GUS* reporter gene in the root

epidermis, cortex, and piths of transgenic wheat, as described by Karthikeyan *et al.* (2002) and Misson *et al.* (2004) in *Arabidopsis*. However, the irregular pattern of *GUS* expression in wheat differed from that reported in *Arabidopsis*, in which the entire root became strongly stained. This irregular pattern of *GUS* expression would explain lack of detectable GUS staining in root tips because the 3-kb *AtPHT1;4* promoter bears its complete 5'UTR sequence that is essential for *AtPHT1;4* expression in the root tip (Karthikeyan *et al.* 2009). The same holds true for root hairs, in which GUS staining was notable but only in trichoblast cells arising from stained epidermal root zones (Fig. 3*E-G*). Irregular patterns of *GUS* expression were also observed in roots of transgenic wheat harboring the *TaPHT1;2-GUS* (line IIIA35) and *AtPHT1;2-GUS* constructs (not shown), which implied that reasons other than the promoter itself would explain this phenotype.

The evidence from the literature points at transgene silencing, or partial cell-to-cell gene silencing, as a major determinant of the uneven and patchy patterns of transgene expression in plants (Day *et al*. 2000, De Buck *et al*. 2007, Marjanac *et al*. 2009). Whereas this phenotype can result from the insertion of a single copy transgene (Day *et al.* 2000), irregular patterns of transgene expression have also been linked to transgenes in multiple copies (Reddy *et al*. 2003, Marenkova *et al.* 2012). Multiple copy transgenes have consistently been reported in transgenic wheat generated by particle bombardment when using supercoiled (as in the present study) or linearized whole plasmid (Sivamani *et al.* 2002, Rooke *et al.* 2003), as compared to gene constructs lacking vector backbone sequences (Yao *et al.* 2006). The vector backbone sequences often have a negative effect on transgene expression, silencing being one of them (Reddy *et al.* 2003). In addition, multi-copy transgenes may produce complex transgenic loci that are prone to further recombination and instability (Altpeter *et al.* 2005). Accordingly, multi-copies in our transgenic wheat, probably derived from concatemerization events, could have contributed to lowering both the transformation efficiency and the overall expression of the transgene that was reflected in uneven and patchy phenotypes.

Most reports have shown that *AtPHT1;4* expression is strongly up-regulated by Pi deficiency in *Arabidopsis*. However, Pi deficiency-induced up-regulation of the *GUS* reporter gene was not significant in hydroponically grown wheat fed 0 to 0.5 mM P, which implies that the transgene was expressed constitutively within this range of Pi supply. Up-regulation by Pi deficiency was noticeable in soil-grown plants exposed to an ample range of soil P and over a time span long enough for plants to reach the 5 - 6 leaf stage. Under these conditions, the highest responsiveness to Pi deficiency occurred at 18 mg(P) kg^{-1}(soil), which lies within the range for most responses of Pi deficiency marker genes in field-grown wheat (Teng *et al.* 2013). *GUS* transcripts were still abundant at 41 mg(P) kg^{-1}(soil), a soil Olsen-P high enough to grow wheat in acidic soils such that no benefits from Pi fertilization are expected (Campillo *et al*. 2011, Teng *et al.* 2013). These results indicate that the *AtPHT1;4* promoter would be relevant in soils with a Pi content well above the plant need. This result provides further evidence to support the role that AtPHT1;4 transporters play in Pi acquisition from both low- and high-Pi soils (Shin *et al.* 2004).

The simultaneous growth of transgenic wheat in hydroponics and soil showed that the endogenous *TaPHT1;2* gene is highly expressed in cv. Bobwhite regardless of the presence of Pi, which is a characteristic of Pi deficiency-tolerant wheat genotypes (Davies *et al.* 2002). This result suggests that the significant modulation of *TaPHT1;2* expression in hydroponics was triggered by 0.5 mM P and not by Pi deficiency (Fig. 5*A*) and that the down-regulation of the *GUS* reporter gene in hydroponics required a Pi supply higher than 0.5 mM P. Nevertheless, down-regulation of *GUS* transcription by Pi supply was stimulated in soil grown plants with root Pi 10-fold lower than their hydroponic counterpart (Fig. 5*B,D*). This inconsistency between root Pi and *GUS* expression suggests the involvement of local signaling in the *AtPHT1.4*-mediated transcriptional response across substrates. The fact that local signaling is regulated by the external Pi concentration and not by the Pi content in the plant as occurs in long-distance signaling (Burleigh and Harrison 1999, Thibaud *et al.* 2010, Chiou and Lin 2011) supports this suggestion. A decline in extracellular Pi initiates local signaling in that high-affinity transporters themselves probably act as sensor of Pi (Nagarajan and Smith 2012). Furthermore, Pi deficiency-induced local sensing enhances ethylene biosynthesis and responsiveness, which is involved in regulating the expression of *PHT1* genes (Lei *et al.* 2011, Nagarajan and Smith 2012). Collectively, these results describe the role of the substrate in the responsiveness of the *AtPHT1;4* promoter to the Pi supply. In addition, they show that there are different thresholds at which *AtPHT1;4* and *TaPHT1;2* genes respond to Pi availability. Importantly, the simultaneous assessment of transgenic plants in hydroponics and soil suggest that results from artificial substrates may be misleading and that this factor should be taken into account when interpreting transcriptional responses to Pi deficiency.

Compared to hydroponics, plants grown in soils frequently experience the effect of mechanical impedance in the rooting media. The evidence that mechanical impedance stimulates ethylene response (He *et al*. 1996, Okamoto *et al*. 2008) and that ethylene up-regulates *AtPHT1;4* expression (Lei *et al.* 2011) suggests that physical-touch stimuli play a role in modulating *AtPHT1;4* strength under soil conditions. Therefore, it is tempting to speculate that ethylene is involved in the increase of *GUS* transcript levels in soil-grown wheat at 14 DAT when Pi deficiency is still not severe enough (Fig. 5*C*), and that a cross-talk between ethylene and low Pi-induced signals plays a role in the enhancement of *AtPHT1;4* strength in potted soil at 21 DAT as plants age and Pi demand increases (Fig. 5*D*). Accordingly, it was

inferred that the soil-induced mechanical impedance may have contributed to the patchy phenotype in transgenic wheat by enhancing *GUS* expression as roots encountered physical barriers when growing through the soil profile. Additional support for the role that mechanical stimuli might have on *PHT1* expression comes from studies showing that the *OsPHT1;2* gene is down-regulated by mechanical impedance in rice (Brown *et al.* 2006). Together, these results indicate that not only Pi deficiency but also other stimuli from the substrate would contribute to the modulation of the *AtPHT1;4* promoter activity.

In conclusion, *AtPHT1;4* performed as a weak promoter in both transgenic wheat lines. This promoter directed reporter gene expression in roots of transgenic wheat within a broad range of external Pi supply in hydroponics. In soil potted plants, the *AtPHT1;4* promoter was constitutive within 16 to 20 mg(P) kg^{-1}(soil), which is a soil P content often found in most wheat-growing areas of acidic soils. In addition, the results show the relevance of the substrate and Pi supply rates when characterizing the transcriptional response of *AtPHT1;4* to Pi deficiency.

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