Zinc Deficiency Up-Regulates Expression of High-Affinity Phosphate Transporter Genes in Both Phosphate-Sufficient and -Deficient Barley Roots[1]

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Phosphate (P) is taken up by plants through high-affinity P transporter proteins embedded in the plasma membrane of certain cell types in plant roots. Expression of the genes that encode these transporters responds to the P status of the plants, and their transcription is normally tightly controlled. However, this tight control of P uptake is lost under Zn deficiency, leading to very high accumulation of P in plants. We examined the effect of plant Zn status on the expression of the genes encoding the HVPT1 and HVPT2 high-affinity P transporters in barley (*Hordeum vulgare* L. cv Weeah) roots. The results show that the expression of these genes is intimately linked to the Zn status of the plants. Zn deficiency induced the expression of genes encoding these P transporters in plants grown in either P-sufficient or -deficient conditions. Moreover, the role of Zn in the regulation of these genes is specific in that it cannot be replaced by manganese (a divalent cation similar to Zn). It appears that Zn plays a specific role in the signal transduction pathway responsible for the regulation of genes encoding high-affinity P transporters in plant roots. The significance of Zn involvement in the regulation of genes involved in P uptake is discussed.

Although relatively large amounts of phosphate (P) are essential to plant growth, forms that can be taken up directly by plants are only found in low concentrations (0.01–3.0 μM) in most soil solutions (Barber, 1995). Low availability of P in soils limits crop yields. Under normal growing conditions the uptake of P by plants is tightly controlled. Plants normally moderate their capacity to take up P to maintain the P concentration in their tissues within physiological limits (Mimura, 1999). They therefore restrict their capacity to take up P when grown under high-P conditions but enhance this capacity when grown under low-P conditions (Clarkson and Scattergood, 1982; Jungk et al., 1990). However, under Zn-deficient conditions, high levels of P accumulate in the tissues of both dicotyledon and monocotyledon plant species (Welch et al., 1982; Cakmak and Marschner, 1986; Webb and Loneragan, 1988; Welch and Norvell, 1993) and can reach levels that are toxic to the plants if high concentrations of P are supplied (Loneragan et al., 1982; Welch et al., 1982; Cakmak and Marschner, 1986; Norvell and Welch, 1993). This suggests that Zn-deficient plants somehow lose control over the P absorption mechanism (Safaya and Gupta, 1979; Marschner and Cakmak, 1986).

The kinetic characterization of the P uptake system by whole plants indicates a high-affinity transporter activity operating at the micromolar range (Raghothama, 1999). The high-affinity transporters are a key component of P uptake by plants at the very low concentrations of P found in many soil solutions. When the P concentration is low in the root growth medium, expression of genes encoding high-affinity P transporters is up-regulated in plant roots (Muchhal et al., 1996; Smith et al., 1997, 1999; Leggewie et al., 1997; Liu et al., 1998; Smith et al., 1997, 1999; Dong et al., 1999; Muchhal and Raghothama, 1999), and a concurrent increase in the transporter protein is observed (Muchhal and Raghothama, 1999). When the P concentration is high in the root growth medium, expression of genes encoding high-affinity P transporters is repressed, and the accumulation of the corresponding protein is decreased (Muchhal et al., 1996; Leggewie et al., 1997; Smith et al., 1997, 1999; Liu et al., 1998; Dong et al., 1999; Muchhal and Raghothama, 1999). Available data indicate that transcription of these genes is regulated by systemic signals that respond to the internal P status of the plant (Smith et al., 2000). Accumulation of very high levels

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of P in the tissues of Zn-deficient plants suggests that this regulatory process may be impaired under Zn deficiency. Recent progress in cloning and characterization of genes encoding high-affinity P transporters from higher plants (Muchhal et al., 1996; Leggewie et al., 1997; Smith et al., 1997, 1999; Liu et al., 1998) now enables this hypothesis to be tested. We have used a probe that is specific to transcripts from genes encoding high-affinity P transporters in barley (*Hordeum vulgare* L. cv Weeah) (Smith et al., 1999) to examine expression of these genes in barley plants of different Zn status. In this paper, we report on the involvement of Zn in the regulation of genes encoding P transporters in barley roots.

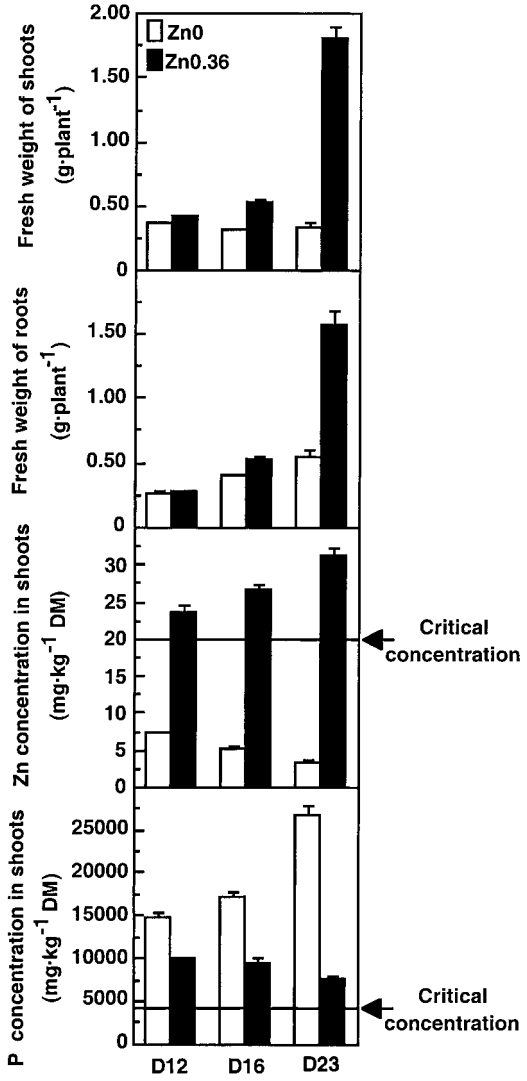

Figure 1. Plant growth, Zn and P concentrations of shoots at two rates of Zn addition in a Zn-deficient soil. SEs (n = 4) are shown as vertical bars. Zn 0 is nil Zn addition in the soil. Zn 0.36 is 0.36 mg Zn kg^{-1} soil addition in the soil. Plants were harvested at D12, D16, and D23.

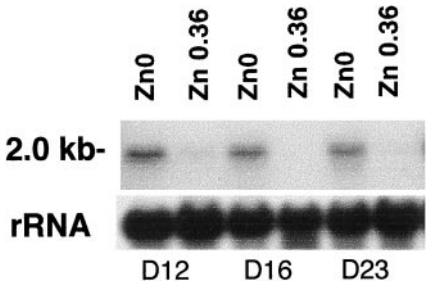

Figure 2. Accumulation of transcripts corresponding to genes encoding high-affinity P transporters in barley roots at two rates of Zn addition in a Zn-deficient soil. Zn treatments and harvest time are the same as those in Figure 1. A ^{32}P-labeled fragment of the HVPT1 cDNA that encodes a high-affinity P transporter expressed in barley roots was used as a hybridization probe. The panel labeled 2.0 kb is the hybridizing P transporter mRNA. The rRNA panel is the loading control, obtained by reprobing the stripped blot with [^{32}P]18S rDNA.

RESULTS

Effect of Zn Supply on Plant Growth, Zn and P Nutrition, and P Transporter Transcripts in Zn-Deficient Soil

Shoot growth, expressed as fresh weight, was slightly reduced at nil Zn addition by the 12th d (D12; Dn, where D stands for days and n = no. of days after soaking), but no difference in fresh weight of roots was found between nil Zn and 0.36 mg Zn kg^{-1} addition to soil (Fig. 1). A large reduction of fresh weight in shoots and roots occurred at later harvests. Zn concentration of shoots at nil Zn addition was well below the critical concentration range of 20 to 25 mg kg^{-1} dry matter (DM) (Welch and Norvell, 1993), even at D12, and continued to fall afterward (Fig. 1). At 0.36 mg Zn kg^{-1} soil addition, Zn concentrations of shoots were adequate throughout the experiment. P concentrations in shoots were above the critical concentration for both rates of Zn at all three harvests (Fig. 1). Zn deficiency dramatically increased P concentrations in shoots so that the concentration of P in shoots at D23 reached a level that can be considered as P toxicity (Loneragan et al., 1982). The K concentrations of shoots were adequate in the two rates of Zn at all three harvests. The range of K concentrations was 4.53% to 5.95% DM.

The abundance of transcripts corresponding to high-affinity P transporters in the whole roots was high at the nil Zn addition throughout the experiment (Fig. 2) despite extremely high concentrations of P in shoots. In contrast, the transcripts of the P transporter genes at the high rate of Zn addition were barely detectable at all three harvests.

Effect of Mn and P Supply on Plant Growth, Mn Nutrition, and P Transporter Transcripts in Mn-Deficient Soil

As shown in Figure 3, at sufficient P supply (65 mg P kg^{-1} soil), plant growth was reduced by a low rate

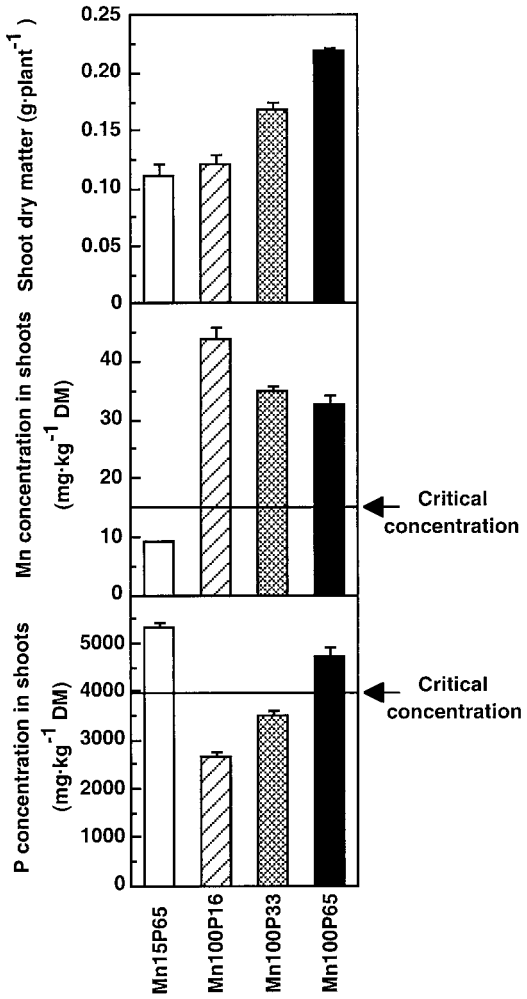

Figure 3. Plant growth, Mn and P concentrations of shoots at two rates of Mn, and three rates of P in a Mn-deficient soil. Plants were harvested after 28 d. SES ($n = 4$) are shown as vertical bars. Mn15P65 is plants grown at 15 mg Mn plus 65 mg P kg^{-1} soil. Mn100P16 is plants grown at 100 mg Mn plus 16 mg P kg^{-1} soil. Mn100P33 or Mn100P65 is plants grown at 100 mg Mn plus 33 mg P kg^{-1} soil or 65 mg P kg^{-1} soil.

of Mn addition (15 mg Mn kg^{-1} soil) in comparison with that occurring at an adequate rate of Mn addition (100 mg Mn kg^{-1} soil). Plant growth also was decreased with lower rates of P supply at the adequate rate of Mn addition. The Mn concentration of shoots was less than 10 mg kg^{-1} DM at the low-Mn addition, which was below the critical concentration (15 mg kg^{-1} DM; Reuter and Robinson, 1997). At the same time, the Mn concentrations of shoots at the adequate Mn addition were well above the critical concentration regardless of P supply. P concentrations of shoots were adequate for plants grown at the adequate P supply with either rate of Mn addition (Mn15P65 and Mn100P65) (Reuter and Robinson, 1997). However, P concentrations of shoots were below the critical concentration for plants grown at the lower rates of P supply (Mn100P33 and Mn100P16). P concentrations of roots were not measured in this experiment, all roots having been used for RNA analysis. However, in several other growth chamber experiments that we previously conducted in the same soil, P concentrations of roots were similar in Mn-deficient and -sufficient barley plants (e.g. 3,712 ± 130 mg P kg^{-1} DM at Mn15P33 and 3,779 ± 32 at Mn100P33).

The level of transcripts corresponding to high-affinity P transporters in the whole roots was barely detected in either Mn-deficient or Mn-adequate plants when P supply was adequate but was enhanced in roots of P-deficient plants at the adequate Mn supply (Fig. 4).

Effect of Zn Supply on Plant Growth, Zn and P Nutrition, and P Transporter Transcripts in a Chelate-Buffered Nutrient Solution

Because of complex nature of interactions between plant and soil, a further experiment was conducted in which plants were exposed to controlled Zn and P by using solution culture. As shown in Figure 5, plant growth was not affected by Zn concentrations in a chelate-buffered nutrient solution at D12, but by D20 the dry matter was reduced by low concentrations of Zn supply. Zn concentrations in shoots were adequate at D12 for all three rates of Zn, whereas by D20, the Zn concentration of youngest emerged leaf blade (YEB) was adequate only at 10 μM Zn *N*-(2-hydroxyethyl) ethylenediaminetriacetic acid (ZnHEDTA). The YEBs of plants in the nil ZnHEDTA and 1 μM ZnHEDTA treatments fell below the critical concentration range (Welch and Norvell, 1993), although the Zn concentrations of whole shoots remained in the critical concentration range. The Zn concentration of YEB is a better indicator for Zn status than that of shoots at D20, and the reduced growth in shoots also is in agreement with this conclusion. P concentrations in shoots at D12 were adequate at all three rates of Zn

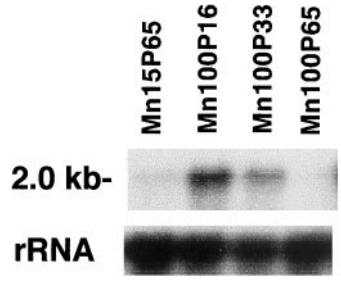

Figure 4. Accumulation of transcripts corresponding to genes encoding high-affinity P transporters in barley roots grown at two rates of Mn and three rates of P addition in a Mn-deficient soil. Plants were harvested after 28 d. Mn and P treatments are the same as those in Figure 3. A ^{32}P-labeled fragment of the HVPT1 cDNA that encodes a high-affinity P transporter expressed in barley roots was used as a hybridization probe. The panel labeled 2.0 kb is the hybridizing P transporter mRNA. The rRNA panel is the loading control, obtained by reprobing the stripped blot with [^{32}P]18S rDNA.

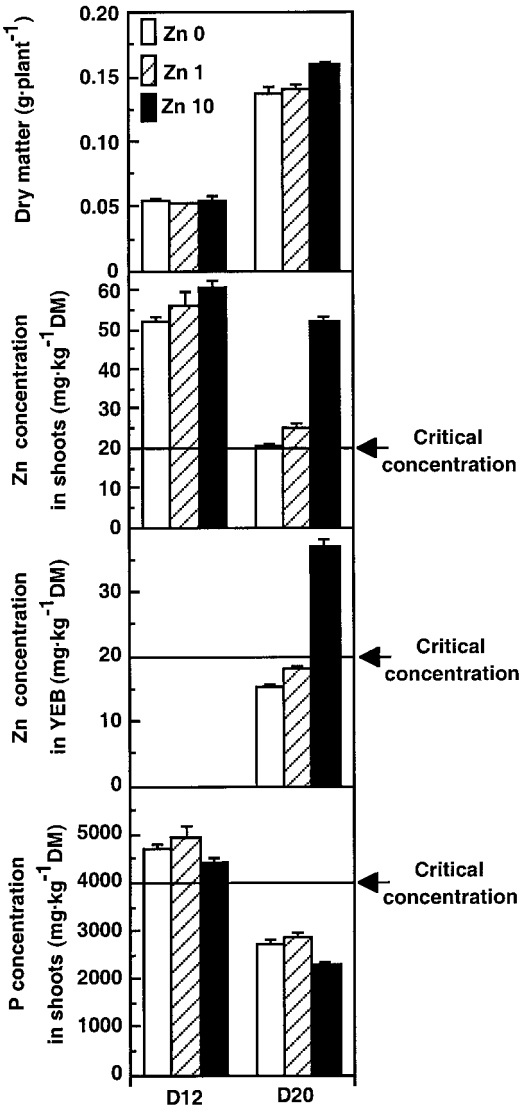

Figure 5. Plant growth and Zn and P concentrations at three rates of ZnHEDTA in a chelate-buffered nutrient solution. SES (n = 4) are shown as vertical bars. Plants were harvested at D12 and D20. Nil ZnHEDTA, 1 μM ZnHEDTA, and 10 μM ZnHEDTA addition in the nutrient solution are shown as Zn 0, Zn 1, and Zn 10, respectively.

but declined to the deficiency range at all three rates of Zn by D20 as low concentrations of P were supplied (Fig. 5). The P concentrations of shoots were slightly higher at nil ZnHEDTA and 1 μM ZnHEDTA addition than at 10 μM ZnHEDTA (Fig. 5). This was also the case for roots (data not shown). The K concentrations of shoots were adequate in all three rates of Zn. At D20, they were 9.8% ± 0.2% DM at nil ZnHEDTA, 10.9 ± 0.2 at 1 μM ZnHEDTA, and 10.9 ± 0.2 at 10 μM ZnHEDTA, respectively.

Low levels of transcripts of genes encoding high-affinity P transporters in the whole roots were found at all rates of Zn supply at D12. At D20 however, much higher levels of these transcripts were detected at all three rates of ZnHEDTA (Fig. 6A). This was expected because the P status of plants grown at all three rates of Zn supply had fallen below the critical concentration (Fig. 5). However, the highest abundance of transcripts was found in roots grown at the nil ZnHEDTA, an intermediate amount at 1 μM ZnHEDTA, and the lowest level was found at 10 μM ZnHEDTA (Fig. 6B). This indicates that, even in P-deficient plants, Zn deficiency moderated the regulation of genes encoding high-affinity P transporters.

DISCUSSION

The phenomenon of specific interactions between Zn and P in nutrition has long been recognized, but the underlying mechanism has not been elucidated (Loneragan and Webb, 1993). The research reported here has provided initial data on the molecular basis for this interaction. The expression of genes encoding high-affinity P transporters in roots is normally tightly controlled at the transcription level (Muchhal

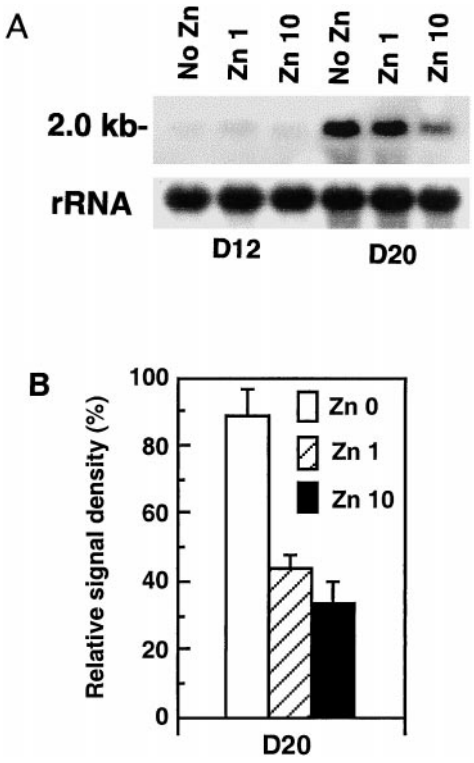

Figure 6. Accumulation of transcripts corresponding to genes encoding high-affinity P transporters in barley roots grown at three concentrations of ZnHEDTA in a chelate-buffered nutrient solution. Plants were harvested at D12 and D20. Zn treatments are the same as those in Figure 5. A, RNA gel-blot analysis. A ^{32}P-labeled fragment of the HVPT1 cDNA that encodes a high-affinity P transporter expressed in barley roots was used as a hybridization probe. The panel labeled 2.0 kb is the hybridizing P transporter mRNA. The rRNA panel is the loading control, obtained by reprobing the stripped blot with [^{32}P]18S rDNA. B, Relative signal density plot. The signal density of the high-affinity P transporters and 18S rRNA was obtained by using a phosphor imager, and relative density was given as percentage. SES (n = 3) are shown as vertical bars.

and Raghothama, 1999). When the internal P status of the plant is high as a result of an adequate external P supply, the expression of these genes is normally down-regulated. However, when plants have an inadequate P supply and their internal P status is low, expression of genes encoding high-affinity P transporters in plant roots is up-regulated (Smith et al., 2000). Similar results are shown in plants adequately supplied with Zn in these experiments (Figs. 2, 4, and 6). However, Zn-deficient plants appear to have lost the capacity to down-regulate expression of genes encoding high-affinity P transporters in plant roots (Fig. 2). This results in continued accumulation of high concentrations of P in the plant (Fig. 1) as has been well documented in previous studies (Loneragan et al., 1982; Welch et al., 1982; Cakmak and Marschner, 1986; Norvell and Welch, 1993).

Neither up-regulation of the expression of genes encoding P transporters nor excessive accumulation of P in plant tissues occurred in P adequate plants deficient in Mn, a divalent micronutrient cation similar to Zn (Figs. 3 and 4). It has also been reported that deficiencies in cotton of micronutrients such as Mn, iron, and copper do not enhance the accumulation of P in either roots or shoots (Cakmak and Marschner, 1986), and the deficiencies in tomato of the nutrients, iron, nitrogen, and potassium (Liu et al., 1998), and in barley of nitrogen and sulfur (Smith et al., 1999) do not induce the expression of P transporter genes in roots. An increase in accumulation of high-affinity P transporter transcripts by deficiency of potassium in barley was observed (Smith et al., 1999), although the deficiency of potassium in tomato did not. As potassium concentrations of barley shoots were adequate in our experiments in Zn-deficient soil and in solution culture, they were not the cause of the accumulation of high-affinity P transporter transcripts in these experiments. Taken together these data indicate that Zn ions play a specific role in the regulation of genes encoding high-affinity P transporters in plant roots. This is the first report to demonstrate Zn involvement in the regulation of these genes.

The experiment with defined nutrient solutions indicates that the effect of Zn deficiency on the abundance of transcripts corresponding to high-affinity P transporters also occurs in P-deficient plants (Figs. 5 and 6). The expected up-regulation of expression of these genes in P-deficient plants supplied with adequate Zn (D20Zn10) was further enhanced even under marginal Zn-deficient conditions (D20Zn1, D20Zn0). The data suggest some additive effect related to both the P status and Zn status of the plants.

Little is known about the molecular regulation of P transporters in higher plants (Raghothama, 1999), although the regulation of P transporter genes in yeast is much better understood (Oshima et al., 1996). In yeast, the expression of the P transporter gene *PHO84* is controlled at the transcriptional level by an intricate cascade involving both positive and negative regulatory proteins (Oshima et al., 1996; Persson et al., 1999). Mutations in *pho4*, *pho2*, and *pho81* abolish positive regulation of the *PHO* pathway, leading to no *PHO84* transcripts in yeast cells grown in either high- or low-P medium (Persson et al., 1999). In contrast, a *pho80* mutation diminishes negative regulation of the *PHO* pathway, resulting in constitutive expression of the *PHO84* transcripts (Persson et al., 1999). The effect of Zn deficiency on the accumulation of the high-affinity P transporter transcripts (Figs. 2 and 6) is similar to that of the *pho80* mutation on *PHO84* expression. A regulatory system similar to the yeast *PHO* regulon is expected in higher plants (Raghothama, 1999). However, the complexity of plant morphology and biochemistry suggests existence of additional regulatory mechanisms to those found in yeast (Raghothama, 1999). It appears likely that Zn plays a specific role in the signal transduction pathway involved in the regulation of genes encoding high-affinity P transporters in plant roots. The elucidation of this role of Zn will assist in understanding the regulation of genes involved in P transport in plants and genetic manipulation of P uptake in higher plants.

In most arable soils, sub-micromolar concentrations of P in soil solution are common. Increased expression of genes encoding high-affinity P transporters can be expected under native soil conditions as was observed under equivalent conditions in this study (Figs. 3 and 4). When plants become P deficient (Fig. 5), any associated mild Zn deficiency could further enhance the abundance of high-affinity P transporters, without risk of accumulating excessive P in plants owing to the low availability of P as in Figures 5 and 6. Therefore, it may be possible to improve P uptake of higher plants in low-P environments by genetic manipulation of the regulatory system of genes encoding P transporters in a manner that mimics the effects of low-Zn status.

MATERIALS AND METHODS

Barley Growing in a Zn-Deficient Soil

A Zn-deficient siliceous sandy soil was used to grow barley (*Hordeum vulgare* L. cv Weeah) plants at high- and low-Zn status. The soil preparation was essentially the same as that described by Rengel and Graham (1995) except that calcium carbonate powder (0.5%, w/w) was added to the soil as it increases the severity of Zn deficiency. Two Zn treatments (nil and 0.36 mg Zn kg^{-1} dry soil) were applied to four replicates. An equivalent of 0.55 kg of dry soil was added to a plastic pot (6.5 cm in diameter $\times$ 15 cm in height, lined with a plastic bag).

Barley seeds with low-Zn content (0.60 $\pm$ 0.01 μg per seed) were used in this experiment to obtain Zn-deficient plants. Low-Zn seeds were obtained from a Zn-deficient field experimental site and had a normal concentration of P (3,696 $\pm$ 34 mg kg^{-1} DM). Surface-sterilized seeds were

soaked overnight and sown three to a pot for harvest at D12, or two to a pot for harvest at D16 or D23. Plants were grown in a controlled environment chamber with a 10-h-light/14-h-dark photoperiod at 15°C day/10°C night. The photon flux density supplied by metal-halide lamps was approximately 300 μmol m^{-2} s^{-1} at the surface of the pot. Pots were watered with high-purity water (18-MΩ resistance) every day; every 2nd or 3rd d they were watered by weight to keep the water content as near as possible to 12% (w/w). At each harvest, shoots were cut off just above the soil surface for element analysis. Roots were carefully washed free from soil with water, gently blotted on laboratory tissues, weighed, immediately frozen in liquid nitrogen, and stored at −80°C for mRNA analysis.

Barley Growing in an Mn-Deficient Soil

The soil used for growing plants of varying Mn status was a calcareous sand of pH 8.3. Soil preparation and basal nutrients have been described elsewhere (Huang et al., 1994), except that here P addition as KH_2PO_4 was 65 mg P kg^{-1} soil.

cv Weeah barley seeds with low-Mn content (0.14 ± 0.01 μg per seed) were surface sterilized, soaked overnight, and sown two to a pot (milk container, 7.0 cm square × 19.5 cm in height, lined with a plastic bag), which contained the equivalent of 0.8 kg of dry soil. Two rates of Mn addition were applied to the soil, adequate Mn (100 mg Mn kg^{-1} soil) and low Mn (15 mg Mn kg^{-1} soil). Two additional treatments of low P (16 mg P kg^{-1}/soil and 33 mg P kg^{-1} soil) were imposed at the adequate Mn level. All treatments were replicated three times. Plant growth conditions and the procedure for harvest were the same as described above for the Zn experiment. Plants were harvested at D28.

Barley Growing in a Chelate-Buffered Nutrient Solution

A chelate-buffered nutrient solution was used to obtain mildly Zn-deficient plants. The procedures for growing Zn-deficient barley plants in this nutrient solution were similar to those described by Huang et al. (1994). For each pot, seeds with emerging radicles were placed in four seed cups containing five, five, four, and four seeds, respectively. The basal nutrient solution contained macronutrients: 1.5 mM KNO_3, 1.0 mM $Ca(NO_3)_2$, 0.25 mM $MgSO_4$, and a low concentration of 1 to 5 μM $NH_4H_2PO_4$ (see below for details), and the micronutrients: 50 μM KCl, 12.5 μM H_3BO_3, 0.1 μM H_2MoO_4, 20 μM FeHEDTA, 0.4 μM MnHEDTA, 1.0 μM CuHEDTA, 0.1 μM NiHEDTA, and 25 μM K_3HEDTA. MES (2-[*N*-morpholino]-ethanesulfonic acid) buffer was used at 2 mM to buffer the nutrient solution to pH 6.0. Three concentrations of ZnHEDTA (0, 1, and 10 μM) were applied as treatments. The free activities of these three concentrations of Zn in the chelate-buffered nutrient solution were checked with the computer program GEOCHEM-PC (Parker et al., 1995), which covered the range from deficiency to adequacy for barley grown in an equivalent system (Norvell and Welch, 1993). Each treatment was replicated four times. Plants were grown in a controlled environment chamber at 20°C day/15°C night with the same photoperiod and photon flux density as described above for the soil-grown plants.

Germinated seeds of cv Weeah barley with high-Zn content (3.07 ± 0.07 μg Zn per seed; 4,282 ± 61 mg P kg^{-1} DM) were initially grown in a one-half-strength nutrient solution from D1 until D6. The full-strength nutrient solution was applied from D7 to D20. Low, variable concentrations of P (1–5 μM) as $NH_4H_2PO_4$ were added to the nutrient solutions according to plant age: 4.5 μmol per pot was added at D1 for the period of D1 to D6, 0.9 μmol per pot added daily from D7 to D10, 1.8 μmol per pot added daily from D11 to D13, 2.7 μmol per pot added daily from D14 to D16, and 3.6 μmol per pot added daily from D17 to D20. Low concentrations of P were applied in the chelate-buffered nutrient solution to prevent toxic P accumulation in Zn-deficient plants (Norvell and Welch, 1993). At D7, seedlings in each pot were thinned to two cups containing four seedlings for harvest at D12 and the other two cups containing three seedlings for harvest at D20. To minimize depletion of nutrients, plants were transferred to fresh solution at D7, D11, D14, and D17. At each harvest, two cups were removed from each pot. One cup of plants was for mineral element analysis, the roots of which were washed in high-purity water for approximately 10 s, excess water was blotted on fresh laboratory tissues, and then the plants were separated into different parts as required. Another cup of plants was separated into roots and shoots, immediately frozen in liquid nitrogen, and stored at −80°C for mRNA analysis.

Mineral Element Analysis

Plant samples were oven-dried (80°C), digested in 70% (v/v) nitric acid, and analyzed for mineral elements by inductively coupled plasma emission spectrometry (Zarcinas et al., 1987).

RNA Isolation and Gel-Blot Analysis

RNA preparation and gel-blot analysis were similar to those described by Huang et al. (1996). A ^{32}P-labeled probe was prepared by randomly labeling a 388-bp fragment from the 3′ end of the HVPT1 cDNA. HVPT1 encodes a high-affinity P transporter and is expressed in barley roots (Smith et al., 1999). Its sequence has a very high level of homology to HVPT2, another gene expressed in barley roots that encodes a second high-affinity P transporter. The probe used in this study therefore hybridizes to both the HVPT1 and HVPT2 high-affinity transporters. The membranes were hybridized at 42°C for 48 h with the ^{32}P-labeled probe, and then washed sequentially in 2× SSC plus 0.1% (w/v) SDS, 1× SSC plus 0.1% (w/v) SDS, and 0.5× SSC plus 0.1% (w/v) SDS at 65°C for 15 min for each wash. The equal loading of total RNA was assessed by subsequently hybridizing the stripped membranes with the [^{32}P]rDNA probe prepared from the *Bam*HI-*Eco*RI fragment of plasmid PHA1 (Jorgensen et al., 1987) to detect the

18S rRNA. Phosphor image analysis was applied to quantify signal density of high-affinity P transporters and 18S rRNA with Storm 860 scanner and ImageQuanNT software (Molecular Dynamics, Sunnyvale, CA).

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