

Over-expression of an *Arabidopsis* zinc transporter in *Hordeum vulgare* increases short-term zinc uptake after zinc deprivation and seed zinc content

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Abstract

Increasing the zinc content of cereal grains will be important for improving human nutrition. Improved plant zinc efficiency will lead to increased yields when available zinc is limiting plant growth. The aim of our work was to test how the over-expression of zinc transporters in cereals affects plant growth, seed mineral content, and zinc transport rates. Known zinc transporters from *Arabidopsis* were over-expressed in *Hordeum vulgare* cv. Golden Promise by means of a ubiquitin promoter. Multiple transgenic lines were obtained, and the locus number and expression levels were verified. Transgenic lines were tested in long-term growth and short-term uptake experiments. Seeds from transgenic lines grown in soil had higher zinc and iron contents than controls. Short-term uptake rates were higher in the transgenic lines after zinc deprivation. Resupply of zinc after a period of deprivation resulted in the rapid decrease in zinc uptake even in transgenic lines in which a zinc transporter gene was constitutively expressed. Similar to processes in yeast and *Arabidopsis*, we hypothesize that this rapid decrease in zinc transport activity may be caused by the degradation of transporters in response to zinc-sufficient conditions. In the long-term growth experiments, there were no significant differences between transgenic and control lines in leaf zinc content or shoot biomass under zinc-sufficient or -deficient conditions. However, root-to-shoot ratios were higher in the transgenic plants grown under low-zinc conditions; this could impact zinc acquisition under field conditions. Increased seed zinc and iron content by over-expression of a zinc transporter provides a new strategy for increasing the micronutrient content of cereals.

Introduction

Micronutrients are essential for balanced nutrition in plants and animals (Welch and Graham, 1999; Graham *et al.*, 2001). Zinc, iron and iodine are the mineral micronutrients most frequently deficient in humans. Micronutrient malnutrition now afflicts over two billion people worldwide. It results in overall poor health and higher rates of mortality, and it permanently impairs the cognitive abilities of infants. Recent evidence suggests that zinc is

one of the nutrients most frequently deficient in human nutrition; further, zinc has been shown to be of great importance to human development and immune function (Hambidge, 2000).

Zinc plays multiple important roles in the various physiological and metabolic processes of plants and may limit plant growth. In many soils, either zinc is not present (Cakmak *et al.*, 1996b), or the soil's physiochemical composition does not allow for uptake by plant roots. Plants grown in soils where zinc is not available yield less and have

a lower nutritional quality (Kochian and Garvin, 1999). Previous studies have shown that in certain plant species, some genotypes are more zinc-efficient than others (Streeter *et al.*, 2001; Genc *et al.*, 2002). The basis of zinc efficiency, which can be defined as the ability to grow and maintain yields in soils with low zinc, is poorly understood. Comparisons of zinc-efficient and zinc-inefficient genotypes have found no differences in zinc uptake (Hacisalihoglu *et al.*, 2001) or zinc content in leaves. The release of phytosiderophores has in some cases been correlated to increased Zn^{2+} efficiency (Cakmak *et al.*, 1996a; Erenoglu *et al.*, 1996; Rengel *et al.*, 1998). In one study, efficiency was correlated to increased activity of a superoxide dismutase and carbonic anhydrase. It was suggested that biochemical utilization of Zn^{2+} may be important for the zinc efficiency of wheat (Hacisalihoglu *et al.*, 2003). Genetic engineering approaches have not been tested for increasing plant zinc efficiency or for increasing the zinc content of seeds. However, this technology has been applied to increasing plant tolerance of low iron (Takahashi *et al.*, 2001).

Much progress has recently been made towards identifying the molecular mechanisms of zinc transport in plants (Gaither and Eide, 2001). As a result, several possible targets are now available for engineering zinc efficiency in plants and for increasing the zinc content of edible parts. Among these targets are divalent cation transporters from the plasma membrane and the vacuole. The plasma membrane cation transporters include the ZIP family of zinc and iron transporters (Guerinot, 2000; Maser *et al.*, 2001). Another class of transporters located in the vacuolar membrane includes ZAT and MHX (Shaul *et al.*, 1999; van der Zaal *et al.*, 1999; Assunção *et al.*, 2001; Shaul, 2002). Testing the over-expression and engineering of these transporters *in planta* will determine if this approach will lead to solutions for increasing zinc uptake when this micronutrient is available for uptake.

Over-expression of plant membrane transport proteins can be used to determine if the number of molecules in the membrane is rate limiting for mineral uptake and compartmentalization. In whole-plant studies with *Thlaspi caerulescens* and *T. arvense* (Lasat *et al.*, 1996), it has been shown that increased expression of genes encoding zinc transporters can increase the plant's zinc uptake.

The high expression of zinc transporters in *T. caerulescens* has been suggested to be the main reason for its enhanced zinc uptake (Lasat *et al.*, 2000; Pence *et al.*, 2000; Assunção *et al.*, 2001). The ZAT gene (van der Zaal *et al.*, 1999) from *A. thaliana* is homologous to mammalian zinc transporters (Palmiter *et al.*, 1996). Transgenic *Arabidopsis* that over-expressed a ZAT gene exhibited enhanced zinc resistance and increased zinc content in the roots of plants grown in a high external zinc environment. Measurable phenotypic changes have also been observed in tests that over-expressed a few other types of plant membrane transporters (Mitsukawa *et al.*, 1997; Curie *et al.*, 2000; Hausler *et al.*, 2000; Pence *et al.*, 2000; Thomine *et al.*, 2000; Aharon *et al.*, 2003).

Here we report how the over-expression of the *Arabidopsis* zinc transporter *AtZIP1* in *Hordeum vulgare* cv. Golden Promise affects short-term zinc uptake, long-term growth under zinc-sufficient and zinc-deficient conditions, and seed mineral content.

Materials and methods

Transformation of AtZIP1 cDNA into Hordeum vulgare cv. Golden Promise

The *AtZIP1* cDNA from *Arabidopsis thaliana* (Grotz *et al.*, 1998) was directionally cloned into pWUbi.tml vector downstream of the ubiquitin promoter (Wang *et al.*, 1997). The cassette containing the ubiquitin promoter (Christensen and Quail, 1996), *AtZIP1* cDNA, and tm1 terminator was excised from the vector and directionally cloned into the binary vector pWVec8 (Wang *et al.*, 1997). This vector contained the 35S promoter to drive the expression of the selectable marker gene encoding hygromycin resistance.

H. vulgare cv. Golden Promise plants were grown under controlled conditions in growth chambers at 18 °C during a 16 h light period and 13 °C during the 8 h dark period. Seeds were sterilized, and the immature embryos (1–2 mm) were excised (Tingay *et al.*, 1997). Immature embryos were transformed with the binary constructs according to the protocol of Tingay *et al.* (1997). Transformed lines were selected with 50 µg/ml hygromycin. After they developed a root system, the plantlets were transferred to soil, acclimatized,

grown to maturity, and self-pollinated (18 °C/16 h day and 13 °C/8 h night cycle). The soil used for propagation of the transgenic barley was comprised of composted pine bark supplemented with Osmocote (3–4 months of high P), ammonium nitrate, micronutrients, iron sulfate, and agricultural lime. Seeds were harvested from mature plants and used for further analysis.

Molecular analysis of transgenic plants

Southern analysis was performed to determine the number of sites where the t-DNA integrated. Genomic DNA was extracted from the youngest expanded leaves of T₀ barley plants by a standard phenol-chloroform method. The genomic DNA from putative transgenic lines of barley (10 µg) was used for Southern analysis. The genomic DNA from the putative *AtZIP1* lines was digested with restriction enzyme *Bgl*III because the integrated fragment had one *Bgl*III site, and the digested samples were size-fractionated on a 1% agarose gel by electrophoresis. The DNA was transferred onto Hybond N⁺ membrane (Amersham) according to the manufacturer's instructions. DNA was fixed with UV light. The membranes containing the genomic DNA of *AtZIP1*-transformed plants were probed with a labelled *AtZIP1* cDNA probe, which was synthesized with the Gigaprime DNA labelling kit (Geneworks, Adelaide, Australia) according to the manufacturer's instructions. The membranes were hybridized for 16–18 h in buffer containing 2.5× Denhardt's reagent, 2.5% dextran sulfate, 5× SSPE, 0.5% SDS, 4.5 ml deionized formamide, and 0.5 ml salmon sperm DNA (5 mg/ml) at 42 °C after the addition of the denatured probe. Membranes were washed twice in 2× SSC (150 mM NaCl and 15 mM tri-sodium citrate, pH 7.0) at 65 °C, once in 1× SSC at 65 °C, and twice in 0.1× SSC and 0.1% SDS at 65 °C; they were then exposed to Kodak film (Biomax) with intensifying screens at –80 °C. Autoradiographs were developed after 3–5 days.

Northern analysis was performed to determine whether transcript levels had increased due to the integration of the *Arabidopsis ZIP1* cDNA driven by the ubiquitin promoter. Total RNA (10 µg per sample) was loaded onto a 1.2% denaturing agarose gel after heat denaturation in loading buffer. After electrophoresis, RNA was transferred onto a

Hybond N⁺ membrane (Amersham, UK), and RNA was fixed to the membrane by UV light. Purified *AtZIP1* cDNA was used for synthesizing a probe, as described above. The membranes containing the RNA samples were hybridized and washed under the same conditions as Southern blots. Membranes were exposed to Kodak film (Biomax) with intensifying screens at –80 °C. Autoradiographs were developed after 5 days.

Growth of transgenic barley plants and RNA extraction

Multiple putative transgenic lines of barley transformed with the *AtZIP1* binary construct were selected for northern analysis. Twenty plants per sibling per line from the T₂ generation were grown in a glasshouse under conditions similar to those described for the growth of the *H. vulgare* cv. Golden Promise plants. Twenty plants of each sibling were bulked. Total RNA was extracted from the bulked roots and shoots by a standard phenol-chloroform method. Northern analysis allowed us to determine which siblings lacked the transgene but did not allow us to distinguish between heterozygous and homozygous plants that contained the transgene.

Hygromycin resistance

The transmission of the transgenes in the T₂ progeny was tested using a leaf antibiotic resistance assay method (Wang and Waterhouse, 1997). A 1 cm piece of leaf from the growing axis was excised and placed on plates with MS medium supplemented with 50 mg/l hygromycin (Sigma) and 1 mg/l BAP (Sigma). The plates were incubated at 25 °C for seven days under fluorescent light (50 µmol m⁻² s⁻¹) and then scored for resistance/sensitivity to hygromycin. Bleached leaf sections were scored as being sensitive to hygromycin. To determine whether the lines were homozygous or heterozygous, the segregation ratios were calculated based on the resistance/sensitivity ratios of the siblings in a line (10 siblings per line).

Uptake experiments with ⁶⁵Zn

Seeds of untransformed *Hordeum vulgare* cv. Golden Promise (GP) and the over-expressing

transgenic lines 51-2 (sibling 7), 51-2 (sibling 8), 91-3 (sibling 3), 91-3 (sibling 6), and null 91-3 (sibling 9) were surface sterilized and germinated on filter paper in petri plates in darkness. A null line is one in which the transgene has been lost due to segregation. Seedlings with emerging plumules and radicles were placed in foam rubber plugs, and roots were immersed in 50-litre tanks containing modified Hoagland solution. The modified Hoagland solution contained 0.25 mM KNO₃, 0.25 mM Ca(NO₃)₂, 0.125 mM MgSO₄, 0.05 mM NaH₂PO₄, 0.03 g/L FeEDTA, 46 μM H₃BO₃, 10.7 μM MnCl₂, 0.8 μM ZnSO₄, 0.3 μM CuSO₄ and 0.2 μM Na₂MoO₄. All stock solutions were made with deionized water, and the tanks contained reverse-osmosis water. The modified nutrient solutions were changed every 5 days. The plants were grown under a 16 h light/8 h dark cycle. After 12–15 days, barley plants that were grown on the modified Hoagland solution were subjected to zinc deprivation. Plants were deprived of zinc for 0, 0.5, 1, 3, 12, and 24 h in modified Hoagland solution minus zinc. The minus-zinc solutions were made with deionized water and groups of 18 plants were deprived of zinc in 2-litre containers. For resupply experiments, over-expressing line 51-2 (sibling 7) and null line 91-3 (sibling 9) were deprived of zinc for 3 h in modified Hoagland solution minus zinc; zinc was resupplied to the plants for 0, 0.5, 1, and 2 h by placing them in modified Hoagland solution containing 0.8 μM ZnCl₂. Zinc uptake was measured with ⁶⁵Zn isotope for 5 min after the addition of the isotope.

Zinc uptake assays followed the protocol by Hart *et al.* (1998) with a slight modification: all the uptake experiments were carried out under a light intensity of 400 μmol m⁻² s⁻¹. During the course of the experiments, the solutions were agitated gently at 5 min intervals. Zinc-deprived plants were placed in an uptake assay buffer containing 2 mM MES-Tris pH 6.0, 0.05 mM CaCl₂ minus zinc for 9 min. ZnCl₂ (10 μM) was added 1 min before the addition of 0.069 μM ⁶⁵Zn isotope. Uptake in the accumulation experiments was measured for 20 min after the addition of 0.069 μM ⁶⁵Zn (NEN Life Science Products), whereas in the resupply experiments, zinc uptake was measured for 5 min after the addition of the isotope. At the end of the uptake period, plants were transferred to a container with desorption solution (100 μM ZnCl₂, 5 mM CaCl₂, 2 mM

MES-Tris pH 6.0) for 15 min. All solutions were at 22 °C. Plants were harvested after desorption, roots were blotted dry, and the fresh weights of the roots and shoots were measured. Roots and shoots were placed in scintillation vials; scintillation fluid was added, and ⁶⁵Zn in the tissue was measured with a Beckman Scintillation Counter (Beckman, LS 380). Three replicate plants per line were used in the experiment, and experiments were repeated on two separate days. Results represent the average of six plants per treatment.

Growth and ion accumulation of transgenic plants under zinc-deficient and zinc-sufficient conditions

For studies of whole-plant ion accumulation and growth over a long period of time, plants were established in a zinc-deficient sand. Different lines of transgenic plants were used for the long-term experiments because quantities of T₂ seed were limited. The transgenic lines over-expressing the *AtZIP1* cDNA were all tested by Southern and northern analysis, and lines 38, 16, and 72 were determined to be over-expressing the transgene. These lines were compared to wild-type Golden Promise and two null siblings from lines 38 and 72. A null line is one in which the transgene has been lost due to segregation. Seeds were planted in sand on April 2 and plants were harvested on May 16 and 17. Plants were grown in a glasshouse in Adelaide, South Australia, where temperatures in the glasshouse were 20 °C day and 16 °C night.

Zinc-deficient sand was collected from a specific location in South Australia by removing the upper soil layer and collecting the sand underneath. The sand was washed thoroughly with deionized water to remove organic matter and minerals. The sand was then allowed to dry thoroughly. Prior to planting, 3 g of CaCO₃ was hand-mixed into each kilogram of sand. After the CaCO₃ was added, nutrients were added to the sand in five solutions. For each kilogram of sand, 5 ml of solution 1 and 2 ml of solutions 2–4 were used for each kilogram of sand. For solution 5, which contained Zn²⁺, 300 μl was added for the low-zinc treatment mix, and 15 ml was added for the high-zinc treatment. Solution 1 contained (per litre) 24 g K₂SO₄, 18 g MgSO₄·7H₂O, 1.4 g MnSO₄·4H₂O, 0.1 g H₂MoO₄·H₂O, 0.2 g CoSO₄·7H₂O, 0.028 g NiSO₄·6H₂O, 0.4 g CuSO₄·5H₂O, and 0.02 g H₃BO₃. Solution 2

contained 175 g/l NH_4NO_3 . Solution 3 contained 75 g/l KH_2PO_4 . Solution 4 contained 0.7 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. Solution 5 contained 0.44 g/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. Water was added to the sand to obtain a 12–14% water content. Half a kilogram of sand was added to each pot, which was made from a one-litre milk container lined with a plastic bag. Three pots were planted for each sibling; each pot contained three plants. After 30 days of plant growth, 3 ml of NH_4NO_3 (44 g/l) and KH_2PO_4 (19 g/l) were added to every pot.

At 44 days after emergence, plants were harvested. Roots and shoots were washed in deionized water and oven-dried at 60 °C. Roots, shoots, and the youngest expanded leaf were separated and weighed. Roots and the youngest expanded leaf blades (YELB) were finely chopped with a stainless steel scissors, digested in nitric/hydrochloric acid, and analysed for ion content by ICP-AES.

Results

Transformation of Golden Promise immature embryos with the *AtZIP1* cDNA resulted in 55 different transgenic lines. DNA was extracted from these lines, and the number of loci containing the transgene was assessed by Southern blot analysis. Two bands were detected in the wild-type Golden Promise under these hybridization conditions indicating the presence of a homologous gene in barley. These same bands were also present in the transformed lines as well as additional bands. The Southern blots of the transformed lines had additional bands indicating the presence of the transgene. Three lines were chosen for short-term radioisotope uptake experiments. Two of these lines, 25 and 51, had multiple loci that contained the *AtZIP1* cDNA; the other, 91, had a single locus containing the *AtZIP1* cDNA (Figure 1). These three lines were further characterized using northern blot analysis to determine whether the *AtZIP1* cDNA was over-expressed. In all three lines, levels of *AtZIP1* transcript found in both roots and shoots of the plants grown in soil were higher than the homologous gene found in the untransformed Golden Promise control (Figure 2). In a few of the lines tested, barely detectable levels of transcript were found (e.g. Figure 2B, sibling 9). These low levels were either due to silencing of the transgene and endogenous

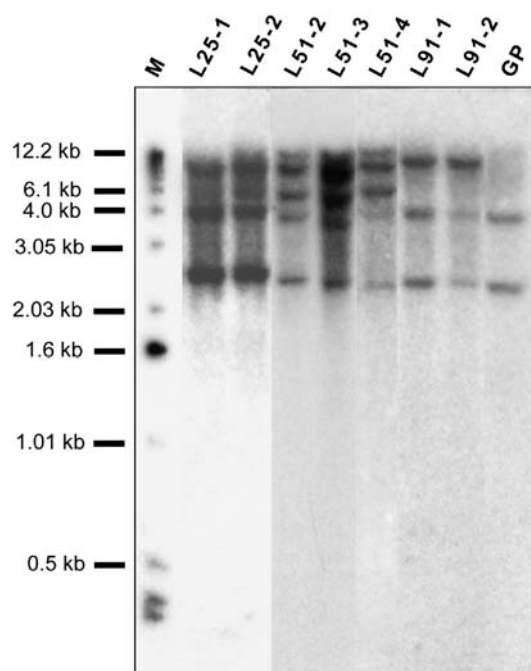


Figure 1. Southern blot analysis of genomic DNA extracted from transgenic barley lines (L25, L51, L91) and from the Golden Promise control (GP). Blots were probed with *AtZIP1* probe. M is the molecular weight marker. Two to three siblings from each line are shown.

gene or due to the loss of the transgene from segregation in the T_2 generation. Hygromycin testing of leaves from transgenic siblings confirmed the Southern blot analysis (data not shown). All leaves from the untransformed *H. vulgare* cv. Golden Promise tested were susceptible to the antibiotic.

It has been shown that the ZIP transporters from yeast and an *Arabidopsis* ZIP transporter involved primarily in iron uptake are post-translationally down-regulated under zinc-sufficient conditions (Gitan and Eide, 2000; Connolly *et al.*, 2002). Therefore, we measured zinc uptake and translocation in the transgenic lines over-expressing *AtZIP1* and in non-transgenic lines before and after zinc deprivation to avoid any possible post-translational down-regulation. Uptake and translocation rates were measured in zinc-deprived plants 3, 12, and 24 h after zinc deprivation and in plants grown under zinc-sufficient conditions (Figure 3). Under zinc-sufficient conditions, no differences between Golden Promise, the null line, and the transgenic lines over-expressing the transgene could be detected in zinc uptake or

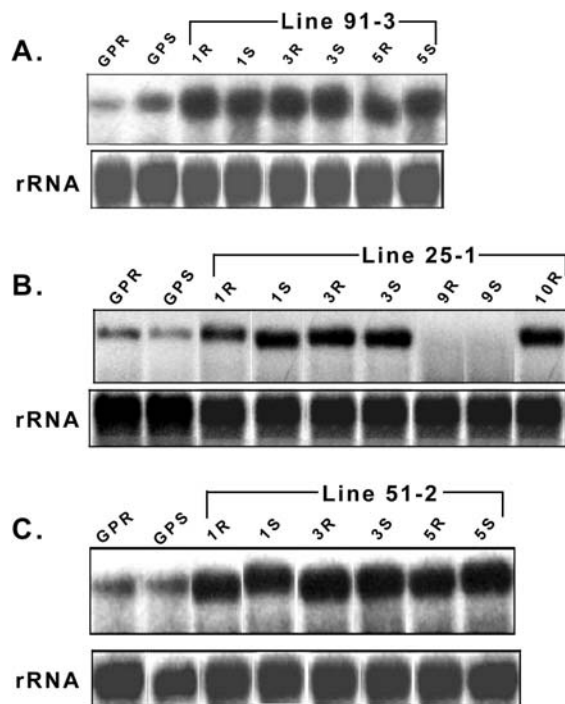


Figure 2. Northern blot analysis of total RNA extracted from roots (R) and shoots (S) of T₂ transgenic barley lines and the Golden Promise control (GP). Blots were probed with the full-length *AtZIP1* cDNA probe. The numbers before the R or S refer to each sibling in a line.

transport (Figure 3A and B). At 3–24 h after zinc deprivation, zinc uptake and transport to shoots increased in both the controls and in the lines over-expressing *AtZIP1*. Zinc uptake into the roots by transgenic lines was 1.5–3 times higher than for the controls with the largest differences in uptake measured at 12 and 24 h after zinc deprivation (Figure 3A). Zinc transport to the shoots was also higher after zinc deprivation and followed the same trends as zinc uptake by roots (Figure 3B). The comparison of two siblings from each line provided additional replication to confirm that the transgenic lines had higher zinc uptake rates upon zinc deprivation.

In the absence of an antibody to monitor the *AtZIP1* protein levels, we conducted additional uptake experiments to determine how the resupply of zinc after a period of zinc deprivation affected zinc uptake rates in the null line and one transgenic line 51-2(7). After 3 h of zinc deprivation, uptake rates were measured; they were higher in the transgenic line 51-2(7), as was previously

observed. After zinc was added, the flux of zinc into roots decreased very quickly over 30 min and then remained steady (Figure 4A). The uptake rates decreased in the transgenic line to about 20% of control rates after 30 min and to only 50% of control rates for the null line tested (Figure 4B).

Long-term growth experiments were conducted with several different lines of transgenic barley over-expressing *AtZIP1*. In the long-term experiments we used a different set of lines than were used in the short-term experiments because of the limited quantities of T₂ seed. Southern blot analysis indicated the presence of a single locus or multiple loci containing the *AtZIP1* transgene (data not shown). Northern analysis confirmed that the transgenic lines had levels of expression of *AtZIP1* in roots and shoots that were higher than control levels (Golden Promise and null lines) (data not shown).

The mineral content of the seed used in these long-term experiments was analysed because seed zinc content may affect plant growth (Rengel and Graham, 1995a, b; Grewal and Graham, 1997, 1999). The T₂ plants were grown under standard conditions in a pine bark medium (see Materials and methods). Five seeds per sample were bulked into a single sample; four to ten samples from each line and Golden Promise were analysed. Individual seeds from the transgenic lines were significantly smaller than seeds from Golden Promise (Table 1). However, the transgenic lines had higher zinc concentrations and more zinc per seed overall. Iron and magnesium concentrations were also higher in seeds from transgenic lines, but the calcium concentration was higher only in the seeds from transgenic lines 16 and 72 (Table 1).

After 40 days of growth in deficient and sufficient conditions, plants were harvested. Biomass and ion content were measured. Ion content in the youngest fully extended leaf blade (YELB) was determined for plants growing under zinc-deficient and zinc-sufficient conditions. The mean zinc concentrations in the YELB were lower for the plants grown under zinc deficient conditions than for plants grown under sufficient conditions. There were no significant differences in the zinc concentrations of YELBs between plants over-expressing *AtZIP1* and the non-transgenic lines (Table 2). Phosphorus concentrations in YELBs were higher in the non-transgenic lines than in the transgenic

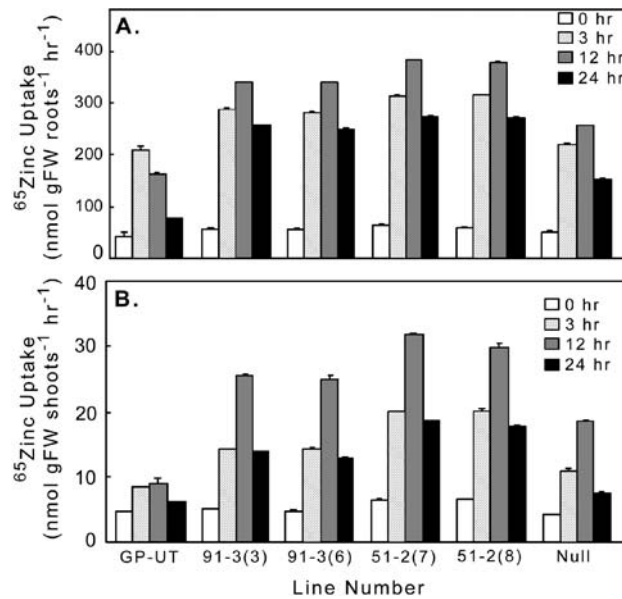


Figure 3. Mean zinc uptake rates (\pm SE) in transgenic barley lines, Golden Promise and in a null line. A null line is one in which the transgene has been lost due to segregation. (A) Uptake by roots after 3, 12 and 24 h of zinc deprivation. (B) Translocation rates of zinc to shoots after 0, 3, 12 and 24 h of zinc deprivation. 91-3 and 51-2 are two different lines. The sibling number from each line is indicated in parenthesis.

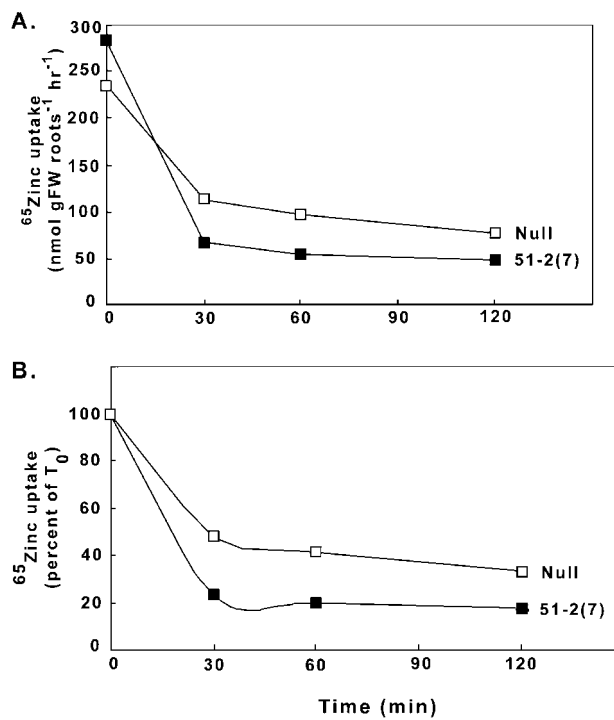


Figure 4. Mean zinc uptake rates (\pm SE) in a null line and in a transgenic barley line after resupply of zinc for 30, 60 and 120 min. Error bars smaller than symbols are not visible. 51-2 is a single line. The sibling number used in these experiments is indicated in parenthesis.

Table 1. Mean cation content (mg/kg) for groups of 5 seeds (4–10 groups), total mean zinc and iron content per seed (μg) and mean individual weights (g) for T₂ seeds used in whole plant growth experiments ($\pm\text{SE}$).

Line	Number of 5-seed samples	Individual seed weight (g)	Zinc (mg/kg)	Zinc per seed (μg)	Iron (mg/kg)	Iron per seed (μg)	Calcium (mg/kg)	Magnesium (g/kg)
GP	4	0.042 \pm 0.002c	31.0 \pm 0.6a	1.3 \pm 0.1a	26 \pm 5.3a	1.09 \pm 0.06a	405 \pm 37a	1.3 \pm 0.06a
16	8	0.030 \pm 0.001a	63.9 \pm 2.7b	1.9 \pm 0.1b	54 \pm 3.8c	1.61 \pm 0.12b	604 \pm 26b	1.7 \pm 0.04bc
38	10	0.035 \pm 0.001b	61.1 \pm 9.6b	2.1 \pm 0.3bc	43 \pm 3.4b	1.52 \pm 0.16ab	445 \pm 23a	1.6 \pm 0.04b
72	8	0.028 \pm 0.001a	84.5 \pm 2.4c	2.4 \pm 0.1c	58 \pm 3.8c	1.64 \pm 0.12b	576 \pm 26b	1.8 \pm 0.04c

Comparison of each pair made with Student's *t*-test. Means followed by different letters in the same column are significantly different ($P < 0.05$).

Table 2. Mean concentrations of selected minerals ($\pm\text{SE}$) in the youngest extended leaf blades of barley lines grown in sand with sufficient and low amounts of zinc.

Line	Zinc level	samples	Fe	Zn	Ca	Mg	P
16	sufficient	15	96 \pm 2	71 \pm 2	9033 \pm 620	2400 \pm 87	11027 \pm 191
38	sufficient	15	98 \pm 3	65 \pm 2	9767 \pm 532	2247 \pm 34	11367 \pm 294
72	sufficient	14	101 \pm 3	66 \pm 3	11571 \pm 630	2500 \pm 66	11479 \pm 304
38 null	sufficient	3	112 \pm 16	57 \pm 17	8067 \pm 1625	2400 \pm 58	11300 \pm 586
72 null	sufficient	3	95 \pm 4	71 \pm 6	8833 \pm 555	2300 \pm 100	10967 \pm 176
GP	sufficient	3	93 \pm 3	59 \pm 1	7133 \pm 1147	2027 \pm 37	10567 \pm 470
16	low	15	125 \pm 6	14 \pm 1	9560 \pm 429	2407 \pm 67	11742 \pm 328
38	low	15	126 \pm 3	14 \pm 1	9680 \pm 644	2119 \pm 60	11353 \pm 337
72	low	14	133 \pm 6	18 \pm 3	11664 \pm 913	2521 \pm 155	10792 \pm 330
38 null	low	3	101 \pm 7	36 \pm 14	11300 \pm 458	2433 \pm 88	12867 \pm 1017
72 null	low	3	148 \pm 4	13 \pm 1	11067 \pm 549	2467 \pm 88	12600 \pm 551
GP	low	3	144 \pm 2	16 \pm 1	12433 \pm 722	2533 \pm 88	14867 \pm 536

All values in mg/kg. A null line is one in which the transgene has been lost due to segregation.

lines under-zinc deficient conditions (Table 2). The analysis of YELBs' mineral content suggests that the plants were specifically deprived of zinc and not other nutrients. Roots of plants grown under zinc deficient conditions were also analysed for iron content. No significant differences were observed between transgenic and non-transgenic lines.

The dry weights of plants were measured at harvest. In all cases, there were no significant differences between the dry weight of shoots from plants grown under zinc-deficient and zinc-sufficient conditions (Table 3). Root biomass of the transgenic plants (lines 16, 38 and 72) was significantly higher under zinc-deficient conditions than the biomass of the non-transgenic plants. Root biomass of the null line 38 was significantly lower under zinc-deficient conditions, whereas the root biomass of 72 null and Golden Promise did not change significantly (Table 3).

Discussion

Cereals are important staple foods for human and animal consumption. Little is known about the molecular aspects of zinc transport in cereals (Hacisalihoglu and Kochian, 2003; Ramesh *et al.*, 2003). Therefore, we tested the over-expression of two different zinc transporters (*AtZIP1* and *AtZIP3*) from *Arabidopsis* in barley. The *AtZIP1* and *AtZIP3* genes were transformed into the barley cultivar Golden Promise, but only transformation events with *AtZIP1* resulted in transgenic lines. *AtZIP1* and *AtZIP3* transporters were chosen because they had high zinc transport capacities (Grotz *et al.*, 1998). Also these two transporters were shown to be expressed in roots, and their expression increased upon zinc deprivation (Grotz *et al.*, 1998). Analysis of the *AtZIP1* transgenic lines showed that the ubiquitin pro-

Table 3. Mean dry weight (g) of transgenic and control lines grown in zinc-sufficient and zinc-deficient sand culture.

Line	Tissue	Sufficient zinc DW (g)	Low zinc DW (g)	DW low/sufficient	P value
16	Roots	0.34 ± 0.06	0.51 ± 0.08	1.5	0.06
16	Shoots	0.52 ± 0.03 <i>n</i> = 12	0.48 ± 0.02 <i>n</i> = 15	0.9	ns
38	Roots	0.46 ± 0.06	0.98 ± 0.13	2.1	<0.001
38	Shoots	0.52 ± 0.04 <i>n</i> = 14	0.52 ± 0.03 <i>n</i> = 15	1	ns
72	Roots	0.45 ± 0.08	0.72 ± 0.09	1.6	<0.01
72	Shoots	0.68 ± 0.06 <i>n</i> = 14	0.60 ± 0.05 <i>n</i> = 13	0.9	ns
38 null	Roots	0.81 ± 0.09	0.37 ± 0.08	0.46	<0.01
38 null	Shoots	0.55 ± 0.01 <i>n</i> = 3	0.48 ± 0.02 <i>n</i> = 3	0.87	ns
72 null	Roots	0.91 ± 0.01	0.92 ± 0.13	1	ns
72 null	Shoots	0.60 ± 0.03 <i>n</i> = 3	0.57 ± 0.07 <i>n</i> = 3	0.95	ns
GP	Roots	0.47 ± 0.15	0.28 ± 0.09	0.6	ns
GP	Shoots	0.58 ± 0.02 <i>n</i> = 3	0.48 ± 0.07 <i>n</i> = 3	0.83	ns

Level of significance between mean dry weight (DW) in each row is shown. ns indicates that means are not significantly different. *n* = number of replicate plants. A null line is one in which the transgene has been lost due to segregation.

motor provided high levels of expression of *AtZIP1* in roots and shoots.

Phenotypic changes due to over-expression of *AtZIP1* were measured at the whole-plant level. Analysis of the seed from T₂ plants grown in soil showed that concentrations of zinc and iron were twice as high. The concentration of calcium and magnesium in some of the lines was about 50% higher. The increased mineral concentration of seeds was highly significant, but the seeds from the transgenic plants were smaller. Even though the transgenic lines had smaller seeds, the overall content of zinc and iron per seed was 1.5–2 times higher in the transgenic plants. In the future, these plants must be grown on a range of different soils, and seed zinc and iron content must then be measured (Vansuyt *et al.*, 2000) to determine whether edaphic conditions influence the micro-nutrient content in this transgenic material.

Increasing the zinc content of cereals such as maize would potentially make a valuable contribution to the nutrition of populations in many parts of the world, including Africa (Manary *et al.*, 2002).

Several other groups have studied how iron and zinc content in seed could be increased. A recent report indicates that seed iron and zinc concentrations were increased by the targeted expression of a soybean ferritin in the endosperm (Vasconcelos *et al.*, 2003). Seed size was not considered in that study, so the overall impact of increased seed nutrient content is not known. Several other groups have studied how iron and zinc content in seeds could be increased (Lucca *et al.*, 2001; Murray-Kolb *et al.*, 2002; Zimmermann and Hurrell, 2002).

Increased zinc uptake in response to zinc deficiency is a response that has been observed in many plant species (Nable and Webb, 1993; Rengel and Graham, 1996; Khan *et al.*, 1998). In our studies, uptake rates increased in all lines following zinc deprivation, but the uptake rates of transgenic plants over-expressing *AtZIP1* were higher than those of non-transgenic lines. Previous studies have shown that increased mRNA abundance of a zinc transporter was associated with increased uptake in the root cells of hyperaccumulator *T. caerulescens* (Lasat *et al.*, 2000; Pence *et al.*,

2000). Our studies confirm that increased expression of a zinc transporter increases uptake rates under specific conditions. The transgenic plants tested in our work also showed increased zinc translocation into the shoots. It is not known which of the following is driving the enhanced translocation into the shoots when *AtZIP1* is expressed using a constitutive promoter: increased uptake into the roots, or over-expression of zinc transporters that may occur in specific cells that control xylem unloading. Although the precise steps in zinc transport are not yet understood, over-expression of a *Arabidopsis* zinc transporter with a constitutive promoter leads to increased zinc uptake and translocation in barley in the short term. These transgenic plants provide novel material for further studies of zinc translocation.

In yeast, ZRT1 and ZRT2 zinc transporters are regulated at the level of transcription by the ZAP1 transcription factor (Zhao and Eide, 1996a, b, 1997; Zhao *et al.*, 1998). ZRT1 activity is reduced rapidly in response to increased zinc availability by removal of the ZRT1 protein from the plasma membrane (Gitan *et al.*, 1998). This process is mediated by ubiquitination, which subsequently results in the endocytosis and degradation of the transporter (Gitan and Eide, 2000). Recent studies in yeast have also described post-translational regulation of several other plasma membrane transporters (Ooi *et al.*, 1996; Liu and Culotta, 1999; Sharp *et al.*, 2002). In plants, it has been shown that *IRT1*, another member of the ZIP family, is regulated at the transcriptional and post-translational level in response to iron nutrition (Connolly *et al.*, 2002). Levels of both mRNA and protein decreased rapidly in wild type plants, whereas only protein levels decreased in plants over-expressing *AtIRT1* after resupply of iron. The authors suggested that the decline in protein abundance in response to iron and zinc is mediated via a post-translational mechanism similar to the mechanism controlling *ZRT1* abundance in yeast.

Our results show a rapid decrease in zinc uptake activity after transfer from zinc-deficient to zinc-sufficient conditions. Based on this, we hypothesize that the rapid reduction in zinc uptake observed in barley plants over-expressing *Arabidopsis AtZIP1* may be due to post-translational modification. Since the expression of *AtZIP1* is constitutive in the transgenic plants, the decreased transport activity under zinc-sufficient conditions

might be regulated by a post-translational modification. We do not have an antibody for *AtZIP1*, and we are therefore only able to speculate that protein levels were reduced rapidly in response to resupply of zinc. However, it will be necessary in the future to monitor the protein levels via antibodies in over-expressing transgenic barley plants to determine whether the decline in uptake rates correlates with a decline in protein abundance.

Studies with ZRT1 suggested that one of the mechanisms of zinc sensing might involve direct binding of zinc to the transporter at the potential histidine-rich binding domain HDHTHEED located in the variable region; this binding could induce endocytosis of the protein (Gitan *et al.*, 1998). Subsequent studies have shown that the histidines are not required for endocytosis, but rather a region termed a metal-response domain (MRD) elsewhere in the same loop is necessary for inactivation (Gitan *et al.*, 2003). The MRD is located in the same inter-membranous loop as the ubiquitinated lysine residue (Gitan and Eide, 2000) that is involved in the process of endocytosis. The histidine-rich domain was however shown to be important to membrane localization and subsequent function. *AtZIP1* also has a histidine-rich domain, HGHTHG, in the same inter-membranous loop in which lies a conserved lysine residue in ZRT1, but does not appear to contain the same MRD identified in ZRT1 (Gitan *et al.*, 2003).

By studying the zinc uptake in barley plants over-expressing a zinc transporter via a constitutive promoter, we showed that *AtZIP1* activity was down-regulated in response to zinc-sufficient conditions even when expressed in a monocot species. This control over zinc uptake rates may be very important for successful over-expression. If this mechanism of control occurs in the transgenic plants, even under zinc sufficient conditions, the over-expressing lines will not accumulate high levels of zinc transporter proteins under zinc-sufficient conditions. If control over zinc uptake activity is exerted at the post-translational level, this would suggest that the regulatory mechanisms for zinc uptake operating in yeast may be evolutionarily conserved. Thus, over-expression studies can also be used to understand the process of metal regulation and homeostasis in plants.

In contrast to the increased short-term uptake rates after zinc deprivation, there were no significant differences in the zinc content in roots or

YELBs of the transgenic plants vs. non-transgenic lines grown in the long-term experiments. However there were large differences in the zinc content of the YELBs in the plants grown under zinc-sufficient vs. zinc-deficient conditions. In long-term experiments, there were no significant differences in shoot biomass, but root biomass of plants grown at low zinc supply differed between the lines. Under the conditions used in these experiments, the small pots may have restricted the growth of the plants grown under zinc-sufficient conditions; this may be why the differences in shoot biomass were not significant. In contrast to other studies conducted to measure zinc efficiency, we did not see the expected differences in shoot biomass between plants grown with sufficient amounts of zinc and low amounts of zinc. In the plants over-expressing *AtZIP1*, root weights under zinc-deficient conditions increased. In contrast, root weights of the null lines and the Golden Promise control remained the same or decreased. This shift in root-to-shoot ratio between sufficient and deficient conditions may have significant impacts on zinc uptake when transgenic plants are grown under field conditions. Roots of the plants over-expressing *AtZIP1* may be able to explore a greater soil volume and thereby increase zinc uptake of plants grown at low zinc supply. The ability of roots to forage for additional nutrients due to changes in root architecture are particularly important for acquisition of minerals that move in the soil by diffusion, such as in the case of phosphate and zinc (Robinson, 1994; Neumann and Martinoia, 2002; Lopez-Bucio *et al.*, 2003).

Thus, our study provides an example of how over-expression of a zinc transporter can be used to increase short-term zinc uptake and seed cation content. Our results also suggest that the activity of the *Arabidopsis* zinc transporter might be regulated *in planta* at the post-translational level. The over-expression of ion transporters in plants provides new opportunities to understand the processes of metal regulation and homeostasis in plants as well as potential biotechnological avenues for increasing the nutritional qualities of foods.

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