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Constitutively High Expression of the Histidine Biosynthetic Pathway Contributes to Nickel Tolerance in Hyperaccumulator Plants [W]

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Plants that hyperaccumulate Ni exhibit an exceptional degree of Ni tolerance and the ability to translocate Ni in large amounts from root to shoot. In hyperaccumulator plants in the genus Alyssum, free His is an important Ni binding ligand that increases in the xylem proportionately to root Ni uptake. To determine the molecular basis of the His response and its contribution to Ni tolerance, transcripts representing seven of the eight enzymes involved in His biosynthesis were investigated in the hyperaccumulator species *Alyssum lesbiacum* by RNA gel blot analysis. None of the transcripts changed in abundance in either root or shoot tissue when plants were exposed to Ni, but transcript levels were constitutively higher in *A. lesbiacum* than in the congeneric nonaccumulator *A. montanum*, especially for the first enzyme in the biosynthetic pathway, ATP-phosphoribosyltransferase (ATP-PRT). Comparison with the weak hyperaccumulator *A. serpyllifolium* revealed a close correlation between Ni tolerance, root His concentration, and *ATP-PRT* transcript abundance. Overexpression of an *A. lesbiacum ATP-PRT* cDNA in transgenic *Arabidopsis thaliana* increased the pool of free His up to 15-fold in shoot tissue, without affecting the concentration of any other amino acid. His-overproducing lines also displayed elevated tolerance to Ni but did not exhibit increased Ni concentrations in either xylem sap or shoot tissue, suggesting that additional factors are necessary to recapitulate the complete hyperaccumulator phenotype. These results suggest that *ATP-PRT* expression plays a major role in regulating the pool of free His and contributes to the exceptional Ni tolerance of hyperaccumulator Alyssum species.

INTRODUCTION

Plants that hyperaccumulate metallic elements to exceptional concentrations in their tissues have attracted considerable interest in recent years from biologists, geochemists, and environmental scientists. These plants may provide valuable insights into fundamental mechanisms of metal-ion uptake, chelation, translocation, and sequestration in plant cells. The term ''hyperaccumulator'' was introduced by Jaffré et al. (1976) to describe plants exhibiting shoot Ni concentrations two to three orders of magnitude higher than those in normal plants. Although a comparatively rare trait, metal hyperaccumulation has now been described for several hundred species of vascular plants that can exhibit elemental concentrations exceeding 1% of shoot dry mass for Zn and Mn, 0.1% for Ni, Co, Cu, and Se, and 0.01% for Cd and As (Baker and Brooks, 1989; Reeves and Baker, 2000). Taxonomically, hyperaccumulators of Ni are the most numerous, accounting for three-quarters of all known hyperaccumulator species (Baker et al., 2000; Reeves and Baker, 2000). Amongst these, the largest genus is Alyssum (family Brassicaceae), which contains 48 taxa capable of accumulating Ni to concentrations as high as 3% of shoot dry biomass (Brooks et al., 1979; Reeves and Baker, 2000). The adaptive significance of metal hyperaccumulation remains controversial, with a defensive function against herbivores or pathogens currently being the most favored hypothesis (Boyd, 1998; Pollard et al., 2002; Macnair, 2003).

To avoid growth impairment by potentially toxic metals, hyperaccumulator plants must possess highly effective biochemical tolerance mechanisms at the cellular level (Baker et al., 2000; Clemens et al., 2002; Pollard et al., 2002). A crucial component of tolerance is the buffering of free metal ions in the cytoplasm via chelation with high-affinity ligands. Still and Williams (1980) first proposed that Ni hyperaccumulation might involve a critical ligand containing two nitrogen donor centers and one oxygen donor center because this would exhibit a sufficiently high affinity for Ni and could account for the observed preference for Ni over Co in these plants. This proposal was later vindicated by Krämer et al. (1996), who observed a linear relationship between the concentrations of Ni and free His appearing in the xylem of hyperaccumulating species of Alyssum exposed to a range of Ni concentrations. Furthermore, application of exogenous His to

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the congeneric nonaccumulator plant *Alyssum montanum* ameliorated the toxic effect of Ni on this species and led to greatly increased flux of Ni through the root into the xylem (Krämer et al., 1996). These observations suggested that His plays an important role in Ni hyperaccumulation, both through detoxification of the metal ion by chelation as well as by facilitating the export of Ni from root to shoot in the xylem. Formation of a Ni:His complex also seems to be involved in Ni detoxification in *Saccharomyces cerevisiae* (Joho et al., 1990), and *S. cerevisiae his* mutants display elevated sensitivity to Ni, Co, and Cu (Pearce and Sherman, 1999). However, at the molecular–biochemical level, it is not yet understood how Ni elicits the His response in hyperaccumulator plants.

His biosynthesis was the last metabolic pathway for a proteinogenic amino acid to be characterized in plants, and little is known about the way in which this pathway is regulated (Ward and Ohta, 1999). His biosynthesis shares close links with nucleotide metabolism, both via the precursors ATP and 5′-phosphoribosyl 1′-pyrophosphate (PRPP) and through a purine intermediate released at a branch point in the pathway (Alifano et al., 1996; Ward and Ohta, 1999; Boldt and Zrenner, 2003). The His pathway (Figure 1) consists of nine reactions catalyzed by eight proteins (Winkler, 1987; Alifano et al., 1996), beginning with the condensation of ATP and PRPP catalyzed by ATP-phosphoribosyltransferase (ATP-PRT). The activity of three enzymes involved in His biosynthesis—ATP-PRT, histidinol-phosphate phosphatase (HPP), and histidinol dehydrogenase (HDH)—was first demonstrated in plants by Wiater et al. (1971), but it has only recently been established that the pathway proceeds via the same route as in bacteria and fungi (Ohta et al., 2000). Genes encoding all the His biosynthetic enzymes except for HPP have now been cloned from *Arabidopsis thaliana* (as well as several from other plant species), partly in an effort to develop selective herbicides that block the pathway (Mori et al., 1995). All plant His biosynthetic genes isolated to date contain a predicted N-terminal plastid transit peptide coding sequence, and two

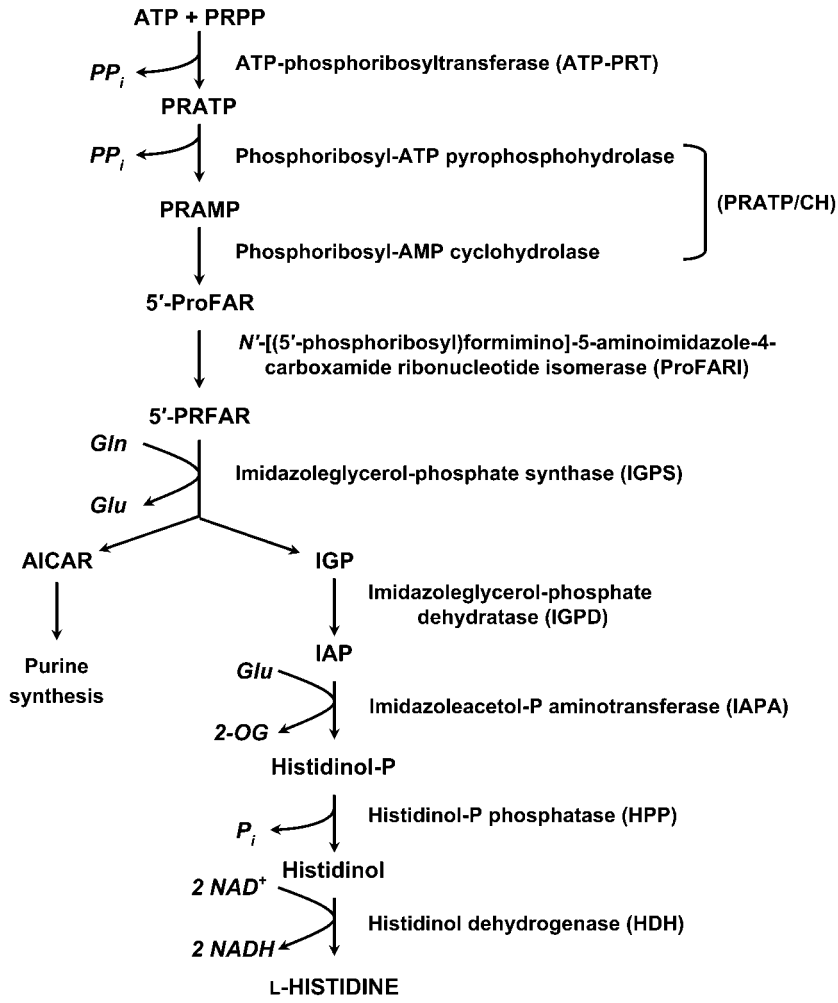

Figure 1. Pathway of His Biosynthesis in Plants.

Abbreviations used for enzyme names in this work are shown in parentheses. *2-OG*, 2-oxoglutarate. Modified after Ward and Ohta (1999) and Ohta et al. (2000).

enzymes—imidazole glycerol phosphate dehydratase (IGPD) and HDH (Nagai et al., 1992; Tada et al., 1995)—have been immunolocalized to the chloroplast, suggesting that His biosynthesis in plants, like nucleotide biosynthesis, occurs in the plastid (Fujimori and Ohta, 1998; Fujimori et al., 1998; Ohta et al., 2000; Boldt and Zrenner, 2003).

In bacteria and fungi, His biosynthesis is controlled in part via feedback inhibition of ATP-PRT activity by His (Ames et al., 1961; Alifano et al., 1996). Inhibition by His of the catalytic activity of recombinant ATP-PRT from *A. thaliana* has been demonstrated in vitro (Ohta et al., 2000), but it is not known to what extent this feedback mechanism regulates flux through the pathway in vivo. A general nitrogen control mechanism analogous to that operating in *S. cerevisiae* (Hinnebusch, 1992) has also been proposed to occur in plants because inhibition of His biosynthesis in *A. thaliana* with IRL 1803 (an inhibitor of IGPD activity) results in increased expression of several genes involved in the synthesis of aromatic amino acids, Lys, and purines (Guyer et al., 1995). However, this may be a consequence of His limitation rather than general nitrogen control (Denby and Last, 1999), and the way in which His biosynthesis in plants responds to changes in metabolic demand or cellular signals remains largely unknown.

Here, we have investigated the molecular basis of Ni-induced His production in the genus Alyssum to determine how regulation of His biosynthesis contributes to metal tolerance in hyperaccumulator plants. Concentrations of free His in roots of the hyperaccumulator species *A. lesbiacum* are severalfold higher than in the nonaccumulator *Brassica juncea*, even in the absence of Ni (Kerkeb and Krämer, 2003). Similarly, Persans et al. (1999) observed that root His concentration is 17-fold higher in the Ni hyperaccumulator *Thlaspi goesingense* than in the nonaccumulator *T. arvense*. However, the underlying biochemical cause of such species differences in His content, and their significance for plant metal tolerance and hyperaccumulation, is not yet understood. We have thus explored the relationship between His production, gene expression, and Ni hyperaccumulation for three congeneric species of Alyssum differing markedly in their degree of Ni tolerance. Furthermore, we have used a transgenic approach to test the hypothesis that the first enzyme in the His biosynthesis pathway, ATP-PRT, plays an important role in controlling the steady state pool of free His in plants and have investigated the consequences of altered His content for plant Ni tolerance. Our findings suggest that ATP-PRT exerts a large influence on His biosynthesis in plants and that the high capacity of this biosynthetic pathway is a major determinant of Ni tolerance within the genus Alyssum.

RESULTS

Changes in Free His Concentrations in *A. lesbiacum* on Exposure to Ni

To determine how Ni induces a proportional increase in xylem His concentrations in hyperaccumulator plants in the genus Alyssum (Krämer et al., 1996; Kerkeb and Krämer, 2003), we first sought to establish whether this response can be accounted for by mobilization of His from a preexisting pool within the root or instead requires de novo His biosynthesis. Exposure of the roots of hydroponically grown *A. lesbiacum* plants to 30 or 300 μM Ni for 48 h led to a 5- or 15-fold increase, respectively, in shoot free His concentration; root free His concentration was 10-fold higher than shoot His in the absence of Ni but did not change significantly in response to Ni over the 48-h period (Table 1). Longer-term (21 d) exposure of *A. lesbiacum* to 300 μM Ni led to a further increase in His concentration in both shoot and root tissue to approximately double their concentration after 48 h of exposure (Table 1). These results suggested that Ni causes an increase in the rate of net His biosynthesis in *A. lesbiacum*, rather than merely a release of His from a preexisting pool. Other amino acids showed little or no change in their free pool size when plants were exposed to Ni, with the exception of a transient increase in shoot Arg concentration after short-term (48 h), but not long-term (21 d), exposure to both 30 and 300 μM Ni (data not shown).

Effect of Ni on Transcript Abundance of His Biosynthetic Genes in *A. lesbiacum*

We next tested whether the increase in free His concentrations in *A. lesbiacum* caused by Ni was associated with increased transcript abundance for genes in the His biosynthesis pathway. Partial cDNA clones were generated for all the His genes in *A. lesbiacum*, with the exception of HPP, for use as homologous probes in RNA gel blot analysis. To be able to assay transcript levels for both isoforms of ATP-PRT, specific probes were generated with primers designed to the respective 3′-untranslated region (UTR) sequences of *ATP-PRT1* and *ATP-PRT2*. RNA gel blot analysis in four independent experiments revealed no

Table 1. Concentrations of Free His in *A. lesbiacum* after Exposure to Ni

		His Concentration (mmol kg^{-1} Dry Biomass)	
Exposure	Ni Treatment (μM)	Shoot	Root
Short term (48 h)	0	0.58 ± 0.01	6.01 ± 0.33
	30	2.81 ± 0.29**	6.40 ± 0.41
	300	8.48 ± 0.39***	7.10 ± 0.56
Long term (21 d)	0	1.85 ± 0.17	9.14 ± 0.85
	300	15.0 ± 0.89***	16.6 ± 0.39**

Values are means ± SE ($n = 3$ independent replicates); each replicate consisted of tissue pooled from 10 5-week-old plants. Values significantly different from the respective control plants not exposed to Ni are indicated with two asterisks for $P < 0.01$ and with three asterisks for $P < 0.001$.

Ni-dependent increase in transcript levels for any of the His biosynthesis genes in root tissue of *A. lesbiacum* within the first 48 h of exposure to 300 μM Ni (Figure 2). Similarly, there were no Ni-dependent changes in His transcript levels in shoot tissue within this time period (data not shown). No regular day–night fluctuations in transcript levels were observed in either root (Figure 2) or shoot tissue (data not shown).

Analysis of Free His Concentrations within the Genus Alyssum

Although exposure to Ni increases the rate of net His biosynthesis in *A. lesbiacum*, root concentrations of free His may be constitutively higher in Ni hyperaccumulator plants than in nonaccumulator plants, even in the absence of Ni (Persans et al., 1999; Kerkeb and Krämer, 2003). To test this hypothesis more systematically, steady state concentrations of free His were measured in three congeneric species of Alyssum exhibiting differing degrees of Ni tolerance. The species selected, *A. lesbiacum*, *A. serpyllifolium*, and *A. montanum*, differed markedly in their responses to Ni under these conditions, showing no impairment of growth at concentrations of Ni up to ~300, 30, and 3 μM, respectively (Figure 3; cf. Brooks et al., 1979; Morrison et al., 1980; Krämer et al., 1996).

For plants grown in the absence of Ni, shoot concentrations of free His were similar in the three Alyssum species, although His was one of the least abundant amino acids, accounting for only ~1% of the total pool (Table 2). In the roots, free His concentration in *A. montanum* was similar to that in shoot tissue but was progressively higher in the root tissue of *A. serpyllifolium* and *A. lesbiacum*. His also constituted a larger proportion of total free amino acids in roots of the more Ni-tolerant species, being the fourth most abundant amino acid and accounting for ~5% of

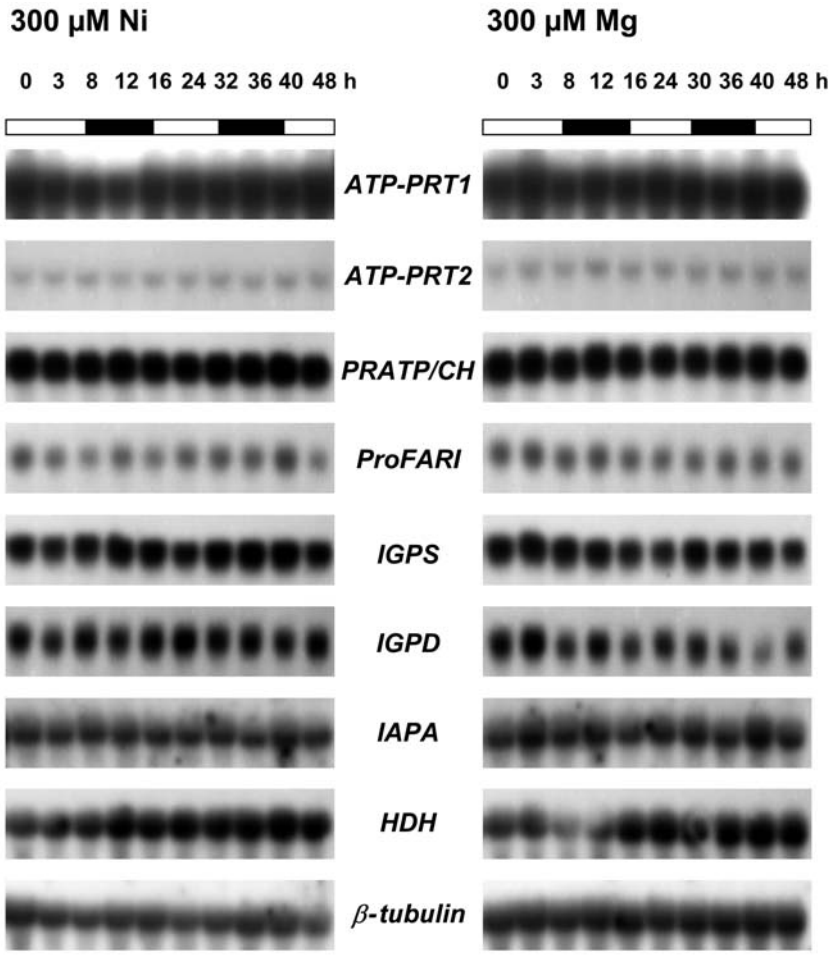

Figure 2. Transcript Abundance of His Biosynthetic Genes in Root Tissue of *A. lesbiacum* Is Unaffected by Ni.

Five-week-old hydroponically grown plants, cultivated in standard nutrient solution not containing Ni, were exposed at the start of the experiment to either 300 μM $NiSO_4$ (Ni) or an additional 300 μM $MgSO_4$ (Mg). Four plants were harvested for analysis at various times between 0 and 48 h after exposure to Ni or Mg. Tissue was pooled and total RNA extracted for RNA gel blot analysis using ^{32}P-labeled partial cDNA probes for each His gene amplified from *A. lesbiacum*. Expression of β-tubulin transcript (using an *A. lesbiacum* cDNA probe) was used as a control for equal loading of RNA. Time points of sampling are shown, together with horizontal bar indicating the 16-h-light (white bar)/8-h-dark (black bar) cycle in the growth chamber. The results shown are for one experiment representative of four.

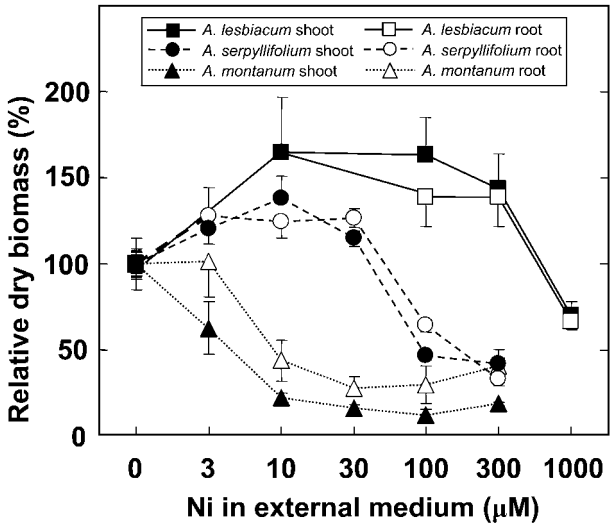

Figure 3. Nickel Tolerance of Three Species of Alyssum.

Plants were cultivated for 6 weeks in standard hydroponic medium supplemented with various concentrations of $NiSO_4$ before harvesting for dry biomass determination of shoots and roots. Values are means ± SE ($n = 8$ plants). Absolute total plant biomass for control plants grown in the absence of Ni were as follows: *A. lesbiacum*, 104.0 ± 9.7 mg; *A. serpyllifolium*, 53.7 ± 2.5 mg; *A. montanum*, 108.9 ± 7.7 mg.

the total pool in *A. lesbiacum* (Table 2). Thus, there was a good correlation between the intrinsic degree of Ni tolerance and steady state pool of free His in the roots of these Alyssum spp, even in the absence of Ni.

Transcript Levels of *ATP-PRT* Are Correlated with Steady State His Concentrations in Alyssum Species

To determine whether differences in root His concentrations in Alyssum are associated with differences in gene expression, transcript levels of His biosynthesis genes were compared in the hyperaccumulator *A. lesbiacum* and the nonaccumulator *A. montanum*. In plants cultivated in the absence of Ni, transcript levels of *ATP-PRT1* and *ATP-PRT2* were considerably higher in *A. lesbiacum* than *A. montanum* for both root and shoot tissues (Figure 4). *ATP-PRT1* transcript was ~9-fold higher in roots and 5-fold higher in shoots of *A. lesbiacum* compared with *A. montanum*, whereas *ATP-PRT2* transcript was detectable only in *A. lesbiacum*. By contrast, transcript levels of the other His biosynthetic genes examined were at most 2.5-fold higher (for HDH) in *A. lesbiacum* than *A. montanum* in either root or shoot tissue (Figure 4). The same expression patterns were observed for these genes using semiquantitative RT-PCR (data not shown).

A comparison of total *ATP-PRT* transcript levels in tissues of the three Alyssum species and in *A. thaliana* was performed using a 415-bp internal fragment of the *A. thaliana ATP-PRT2* cDNA. This revealed transcript abundance in roots to be in the order *A. lesbiacum* ≫ *A. serpyllifolium* > *A. montanum* > *A. thaliana* (Figure 5), which correlated closely with both the degree of Ni tolerance observed in these Alyssum species (Figure 3) and the size of the steady state pool of root free His (Table 2). In shoot tissue, *ATP-PRT* transcript abundance was again highest in *A. lesbiacum* but was similar in the other three species (Figure 5).

The two *ATP-PRT* genes found in the *A. thaliana* genome, *ATP-PRT1* (At1g58080) and *ATP-PRT2* (At1g09795), are derived from a segmental duplication associated with the most recent polyploidization event in this lineage (Ohta et al., 2000; Haberer et al., 2004). Expression analysis by massively parallel signature sequencing has shown that, as in *A. lesbiacum* (Figure 4), *ATP-PRT1* transcript is more highly expressed than *ATP-PRT2* in vegetative tissues of *A. thaliana* and that this difference is particularly pronounced in roots (Meyers et al., 2004).

Ni-Induced Release of Preexisting His from Roots of *A. lesbiacum*

To test whether Ni can induce release of His into the xylem from a preexisting pool in the root, a pulse–chase experiment was performed with *A. lesbiacum* using radiolabeled His. Plants grown in the absence of Ni were labeled with [^{3}H]His for 4 h before transfer to fresh hydroponic medium lacking [^{3}H]His but supplemented with 300 μM Ni, 300 μM Zn, or no addition (control). [^{3}H]His was detected in xylem root-pressure exudate

Table 2. Concentration of Free His in Shoot and Root Tissues of Three Alyssum Species Grown in the Absence of Ni

His Concentration	*A. lesbiacum*	*A. serpyllifolium*	*A. montanum*
Shoot			
His (mmol kg^{-1} dry biomass)	1.85 ± 0.12	2.28 ± 0.06	2.52 ± 0.17**
His (% of total free pool)	0.97 ± 0.07	1.00 ± 0.03	1.11 ± 0.05
Relative abundance of His	18/19	18/19	15/19
Root			
His (mmol kg^{-1} dry biomass)	9.12 ± 0.84	6.55 ± 0.22*	1.91 ± 0.04***
His (% of total free pool)	5.16 ± 0.36	2.99 ± 0.08**	1.33 ± 0.03***
Relative abundance of His	4/19	14/19	17/19

His concentrations are expressed both in units of mmol kg^{-1} dry biomass and as a percentage of total free amino acids. Values are means ± SE ($n = 3$ independent replicates); each replicate consisted of tissue pooled from four 7-week-old plants cultivated hydroponically without added Ni. Values significantly different from those of *A. lesbiacum* are indicated with one asterisk for $P < 0.05$, with two asterisks for $P < 0.01$, and with three asterisks for $P < 0.001$. The relative abundance of His ranked out of the 19 amino acids detected (Trp not detected) is also indicated.

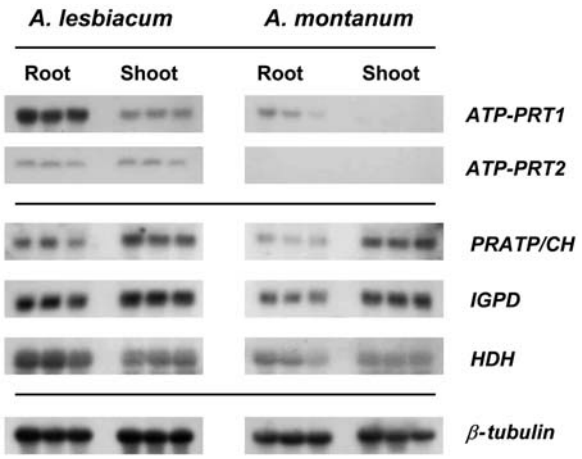

Figure 4. RNA Gel Blot Analysis of His Gene Expression in *A. lesbiacum* and *A. montanum*.

Total RNA was extracted from three independent groups of pooled tissue (four 6-week-old plants per group) from plants grown hydroponically at different times without added Ni. Gene-specific ^{32}P-labeled 3′-UTR probes for the two Alyssum species were used to detect *ATP-PRT1* and *ATP-PRT2* transcripts and heterologous *A. thaliana* cDNA probes to detect all other His biosynthesis genes. The specific radioactivity of the two *ATP-PRT* 3′-UTR probes was determined and for both genes was found to be slightly higher for the *A. montanum* probes (data not shown). Expression of β-tubulin transcript was used as a control for equal loading of RNA. The results shown are from one experiment representative of three for the *ATP-PRT* transcripts or representative of two for other transcripts.

collected over 10 h regardless of treatment, indicating that xylem loading of His was occurring (Figure 6). Exposure to Zn did not affect [^{3}H]His concentration in the xylem exudate, but Ni caused a significant increase in xylem [^{3}H]His concentration (Figure 6), showing that there is a Ni-specific component to the mobilization of root His and its loading into the xylem in *A. lesbiacum*.

Isolation of Full-Length *ATP-PRT1* and *ATP-PRT2* cDNAs from *A. lesbiacum*

Because *ATP-PRT1* and *ATP-PRT2* transcripts showed the largest difference in expression level between the hyperaccumulator species (*A. lesbiacum*) and nonaccumulator species (*A. montanum*), further analysis of these genes was performed. Full-length *ATP-PRT1* and *ATP-PRT2* cDNAs were isolated from *A. lesbiacum* by rapid amplification of cDNA ends (RACE) using primers designed to the partial cDNA clones obtained previously. The full-length *ATP-PRT1* cDNA contained an open reading frame of 1182 bp encoding a predicted 42.9-kD polypeptide of 394 amino acids. Analysis of the amino acid sequence using TargetP (Emanuelsson et al., 2000) indicated the presence of a 52–amino acid putative plastid transit peptide at the N terminus, consistent with the localization of His biosynthesis in plants to the plastid. The *ATP-PRT2* cDNA contained an open reading frame of 1218 bp encoding a predicted 43.9-kD polypeptide of 406 amino acids with a putative plastid transit peptide of 48 amino acids at the N terminus. Both predicted polypeptides contained the ATP-PRT signature sequence E-(x5)-G-x-[SAG]-(x2)-[IV]-x-D-[LIV]-(x2)-[ST]-G-x-T-[LM] found in all ATP-PRT proteins identified to date.

DNA gel blot analysis of genomic DNA was performed to confirm that the two *ATP-PRT* cDNAs isolated from *A. lesbiacum* represented the entire gene family in this species. The simple banding pattern observed in three restriction digests (Figure 7) was consistent with the presence of single copies of the two *ATP-PRT* genes in the *A. lesbiacum* genome, as is the case in *A. thaliana* (Ohta et al., 2000). This indicates that the high *ATP-PRT* transcript levels in *A. lesbiacum* are not the result of increased gene copy number.

Phylogenetic Analysis of Plant *ATP-PRT* Sequences

Analysis of *ATP-PRT* nucleotide sequences from *A. lesbiacum*, *A. montanum*, and *A. thaliana* revealed a higher degree of sequence identity between the putatively orthologous genes in the three species than between the paralogous genes within the same species (see Supplemental Table 1 online). This suggests that the gene duplication event giving rise to the two forms of *ATP-PRT* in these species occurred before their divergence from a common ancestor. In another Ni-hyperaccumulating member of the Brassicaceae, *T. goesingense*, a single *ATP-PRT* sequence has been cloned to date, but DNA gel blot analysis suggests the presence of at least one further *ATP-PRT* gene in this species (Persans et al., 1999). By contrast, BLAST analysis (Altschul et al., 1997) indicates the presence of only one *ATP-PRT* gene in *Oryza sativa* (AC099399), and only a single gene has been identified to date in *Zea mays* (AY112299).

Examination of the plant EST databases yielded partial putative *ATP-PRT* cDNA sequences for several other species. These ESTs were assembled into contigs to generate putative full-length *ATP-PRT* sequences for phylogenetic reconstruction.

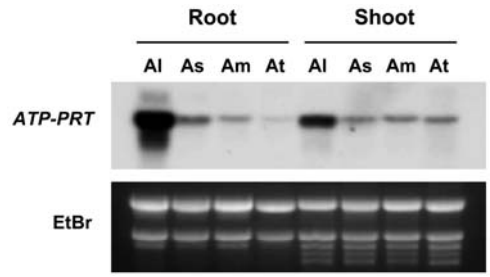

Figure 5. Elevated *ATP-PRT* Expression Is Correlated with Ni Tolerance in the Genus Alyssum.

RNA gel blot analysis of *ATP-PRT* transcript abundance in root and shoot tissue of *A. lesbiacum* (Al), *A. serpyllifolium* (As), *A. montanum* (Am), and *A. thaliana* (At). Tissue was pooled from 10 6-week-old plants cultivated hydroponically without added Ni. Ten micrograms of total RNA was probed with an internal 415-bp fragment of the *A. thaliana ATP-PRT2* cDNA. Equal loading of RNA was checked by ethidium bromide (EtBr) staining. The same expression pattern was observed when the corresponding *A. thaliana ATP-PRT1* probe was used (data not shown).

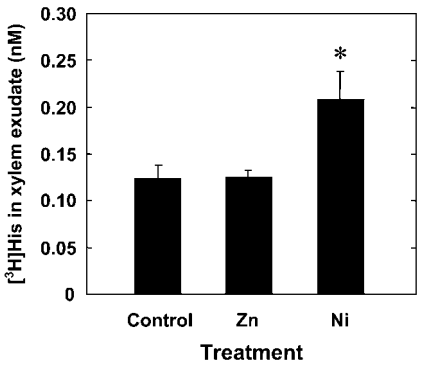

Figure 6. Ni-Induced Release of His from Roots into the Xylem of *A. lesbiacum*.

Concentration of [^{3}H]His in xylem exudates from 8-week-old hydroponically grown *A. lesbiacum* plants, cultivated in standard nutrient solution not containing Ni and then transferred to 300 μM $NiSO_4$ (Ni), 300 μM $ZnSO_4$ (Zn), or no addition (control) for 16 h. Xylem sap exudation rates were not significantly different under the three treatments (data not shown). Bars are means + SE ($n = 4$ individual plants). The asterisk indicates significance at $P < 0.05$.

Analysis of the aligned *ATP-PRT* cDNA sequences (see Supplemental Figure 1 online) using the maximum likelihood algorithm, with the bryophyte *Physcomitrella patens* as outgroup, suggested that a monocot–eudicot split was followed by at least two independent *ATP-PRT* gene duplication events (Figure 8). This model provided a significantly ($P < 0.001$) better fit to the data by the Shimodaira–Hasegawa test than an alternative one invoking an ancient gene duplication before the monocot–eudicot split, with subsequent loss of one of the duplicated *ATP-PRT* genes in monocots. The occurrence of an *ATP-PRT* gene duplication before divergence of the four species of Brassicaceae from a common ancestor is consistent with the proposal of a polyploidization event before the Arabidopsis–Brassica split, perhaps around the time of the early diversification of crucifers (Blanc et al., 2003; Bowers et al., 2003; Blanc and Wolfe, 2004). Furthermore, because *A. lesbiacum* and *T. goesingense* were not resolved as sister taxa in the phylogenetic reconstruction in Figure 8, it is likely that the Ni hyperaccumulation trait evolved independently in these two lineages.

His Concentrations Are Correlated with Transgene Expression in *35S:PRT2* Plants

If the larger pools of free His in the roots of Ni-hyperaccumulator species are a direct result of high levels of expression of the first enzyme in the biosynthetic pathway, ATP-PRT, overexpression of an *ATP-PRT* cDNA in transgenic plants may lead to an elevation of plant His content, depending on the flux control coefficient associated with this enzyme in vivo. Attempts were therefore made to produce transgenic *A. thaliana* plants expressing an *A. lesbiacum ATP-PRT* cDNA under the control of a *Cauliflower mosaic virus* 35S promoter.

To confirm that the *A. lesbiacum ATP-PRT* cDNAs encode functional ATP-PRT proteins, a complementation experiment was performed using NK5526, an *Escherichia coli hisG*$^-$ mutant strain. NK5526 cells transformed with the empty *pUCmod* vector were able to grow on M9–ampicillin plates only if supplemented with 50 μM His. By contrast, transformation with either *pUCmod*:*PRT1* or *pUCmod*:*PRT2* allowed the *hisG*$^-$ strain to grow on M9–ampicillin plates without added His (Figure 9), confirming that both *A. lesbiacum ATP-PRT* cDNAs encoded functional ATP-PRT proteins.

Twenty-eight independent *35S*:*PRT2* lines were generated by transformation of *A. thaliana* using a construct based on pBI121, from which 17 homozygous T3 lines were obtained and used for further analysis. However, we were unable to generate any viable *35S*:*PRT1* lines using a construct based on either pBI121 or pGreen (Hellens et al., 2000). Concentrations of free His in rosette tissue of the pBI121 empty-vector control line did not differ significantly from those in untransformed wild-type plants (0.83 ± 0.10 versus 0.85 ± 0.20 mmol His kg^{-1} dry biomass, respectively) nor did those of any other amino acid (data not shown). In the *35S*:*PRT2* transgenic lines, His concentrations ranged from 0.48 ± 0.03 mmol kg^{-1} to 10.8 ± 0.69 mmol kg^{-1} dry biomass, a 22-fold variation (Figure 10A). In total, 11 lines exhibited significantly ($P < 0.01$) greater free His concentrations compared with the wild-type and empty-vector control plants. As a percentage of the pool of free amino acids, His concentration was 4.4% ± 0.3% in line 21.2 compared with 0.35% ± 0.0% in empty-control plants. Mirroring the 22-fold variation in His concentration, a 24-fold variation in *ATP-PRT2* transcript level was observed in the *35S*:*PRT2* transgenic plants from the

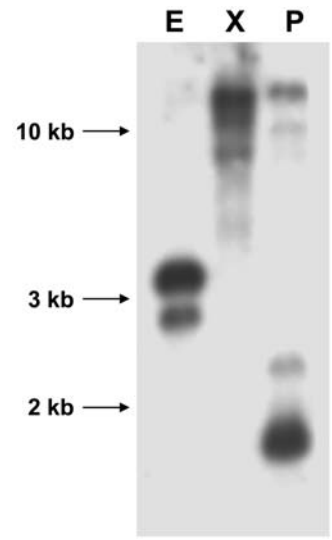

Figure 7. DNA Gel Blot Analysis of *ATP-PRT* Copy Number in *A. lesbiacum*.

Three micrograms of genomic DNA were digested with *Eco*RV (E; no site in either cDNA), *Xho*I (X; one site in *ATP-PRT1* cDNA), or *Pst*I (P; one site in both *ATP-PRT1* and *ATP-PRT2* cDNAs) and probed with a ^{32}P-labeled probe corresponding to the full-length *ATP-PRT2* cDNA (minus predicted plastid transit peptide) under low-stringency conditions. The same banding pattern was observed when the corresponding *ATP-PRT1* probe was used under the same conditions (data not shown).

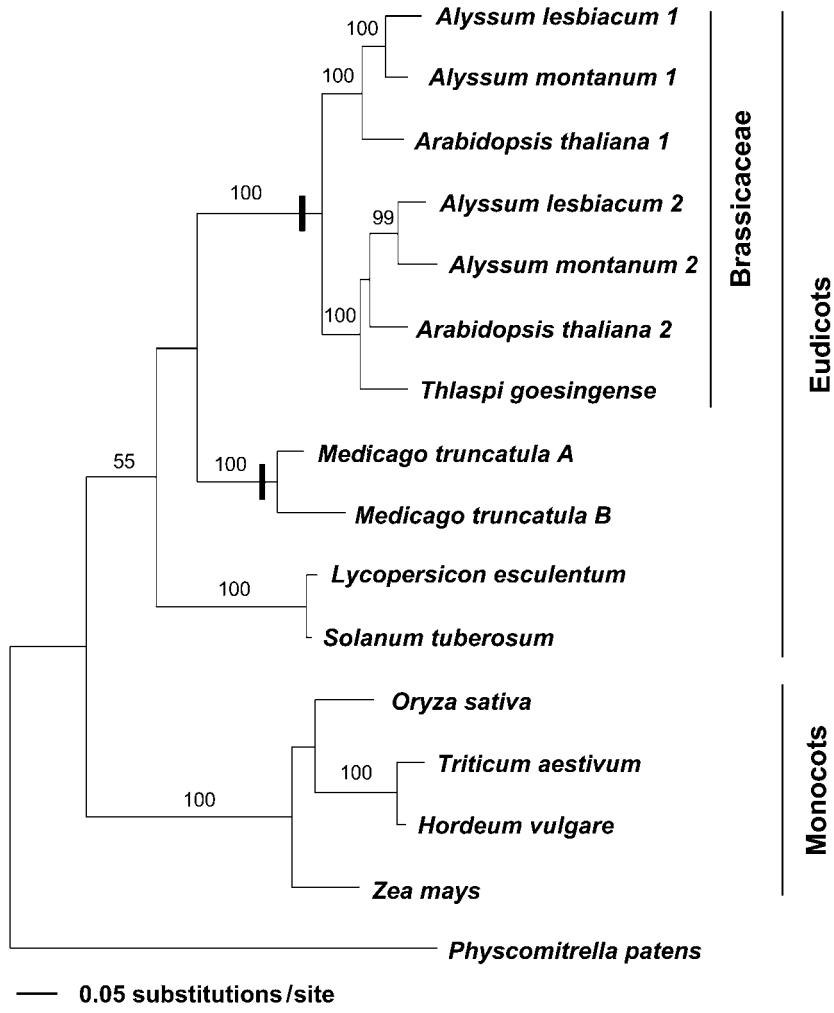

Figure 8. Maximum Likelihood Tree Depicting Phylogenetic Relationships between Different *ATP-PRT* cDNA Sequences in Plants.

cDNA sequences for *A. lesbiacum* and *A. montanum* were obtained in this work. Sequences for *A. thaliana* (*ATP-PRT1*, AB025249; *ATP-PRT2*, AB025250), *T. goesingense* (AF003347), *Oryza sativa* (AC099399), and *Zea mays* (AY112299) were retrieved from GenBank. Full-length cDNA sequences for *Medicago truncatula*, *Lycopersicon esculentum*, *Solanum tuberosum*, *Triticum aestivum*, *Hordeum vulgare*, and *Physcomitrella patens* were assembled from ESTs as described in Methods. Sequences for the open reading frame of *ATP-PRT* were aligned using ClustalW and refined manually, but the sequence encoding the predicted N-terminal plastid transit peptide was excluded from the phylogenetic analysis (see Supplemental Figure 1 online). The angiosperm maximum likelihood tree was rooted on the bryophyte outgroup (*P. patens*). Bootstrap support values (as percentages of 1000 replicates) are given above nodes subtending the relevant branches. Inferred gene duplication events are indicated by vertical bars.

lowest- to highest-expressing line (lines 4.4 and 21.2, respectively) (Figure 10B). Regression analysis showed a highly significant correlation ($r^2 = 0.832$, $P < 0.001$) between transcript levels of the *ATP-PRT2* transgene and free His concentration in rosette tissue of the *35S*:*PRT2* lines.

Concentrations of all other amino acids in the *35S*:*PRT2* lines were generally unaffected by expression of the transgene (see Supplemental Figure 2 online). The only exception was Pro because two lines with high His concentrations (lines 21.2 and 22.1) also had significantly ($P < 0.01$) higher Pro concentrations than empty-vector control plants. However, a significant increase in Pro concentration was also observed in lines 5.3 and 23.3, which did not have elevated His concentrations, whereas Pro concentrations were not significantly different in the other three lines with the highest free His concentrations. This suggested that the increase in Pro concentrations might be unrelated to His concentration within the plant. No obvious phenotypic differences between *35S*:*PRT2* lines with elevated concentrations of free His and wild-type plants were observed.

To analyze the distribution of free His between shoot and root tissue in the transgenic plants, two lines with the highest concentrations of free His in their rosette tissue (lines 21.2 and 30.5) were selected for comparison with wild-type and empty-vector control lines. Free His concentrations did not differ significantly between shoots and roots of 5-week-old wild-type plants and were similar in the empty-vector control line (Table 3).

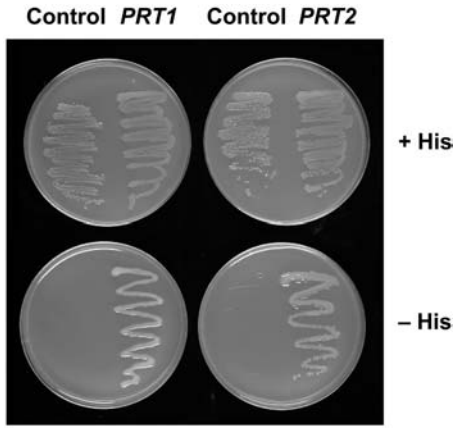

Figure 9. *A. lesbiacum ATP-PRT1* and *ATP-PRT2* Encode Functional ATP-PRT Proteins.

Functional complementation of the *E. coli hisG*$^-$ mutant strain NK5526. Bacteria transformed with *pUCmod*:*PRT1* or *pUCmod*:*PRT2* were able to grow on minimal medium in the absence of supplemental 50 μM His, whereas those transformed with the empty vector (control) were not.

In the *35S*:*PRT2* plants, shoot His concentration was up to 15-fold higher (line 21.2) than in the wild type, but this was ~6-fold higher than root His concentration, which was elevated only 2.3-fold on average in the two lines relative to the wild type. Correspondingly, free His represented 6.9% and 2.4% of the total amino acid pool in shoots and roots, respectively, of the two transgenic lines averaged (cf. with 0.9% and 1.1% for the shoots and roots, respectively, of the two control lines averaged).

Ni Tolerance of *35S*:*PRT2* Transgenic *A. thaliana* Plants

To determine whether elevated free His concentrations in the *35S*:*PRT2* transgenic lines resulted in increased tolerance or accumulation of Ni, four lines with elevated concentrations of free His in their rosette tissue (lines 1.1, 22.1, 30.5, and 21.2) were chosen for further study. For comparison of Ni tolerance, plants were precultivated on agar plates for 2 weeks and then transferred to half-strength hydroponic solution supplemented with either 0 or 30 μM $NiSO_4$ for 3 weeks. Exposure to 30 μM Ni caused a reduction in growth of the wild-type and empty-vector control lines to less than half their respective growth rates on Ni-free medium, whereas all four *35S*:*PRT2* transgenic lines displayed significantly reduced sensitivity to Ni (Figure 11). Indeed, lines 22.1, 30.5, and 21.2 showed no reduction in biomass at 30 μM compared with their growth at 0 μM Ni, and the relative degree of Ni tolerance exhibited by these four lines was proportional to their free His content. Toxicity symptoms in the form of Ni-induced leaf necrosis were also pronounced in the wild-type and empty-vector control lines but not in the transgenic lines (see Supplemental Figure 3 online).

Ni Accumulation by *35S*:*PRT2* Transgenic *A. thaliana* Plants

Because free His is also proposed to play a role in root to shoot translocation of Ni, we investigated the Ni content of plants used in the Ni tolerance assays. At the lower concentrations of Ni tested, Ni content of shoot biomass did not differ significantly between the *35S*:*PRT2* transgenic lines and wild-type or empty-vector control lines (Figure 12A). At 50 μM Ni, although transgenic lines exhibited approximately five times greater biomass than control lines, growth of all lines was reduced to <20% of that at 0 μM Ni (data not shown); under these conditions, shoot Ni concentrations became more variable, with evidence in some plants of the high values symptomatic of breakthrough of elements from root to shoot caused by the onset of acute metal toxicity (Roosens et al., 2003). Ni concentrations in root tissue did not differ between transgenic lines and control plants (Figure 12B), and shoot-to-root ratios of Ni content were <1 at all external Ni concentrations, whereas Ni hyperaccumulators, such as *A. lesbiacum*, typically show ratios > 1 (Krämer et al., 1996).

To determine whether lack of enhanced Ni accumulation in the shoot of His-overproducing lines might be related to limited export of Ni from the root, xylem sap was sampled from the plants exposed to different concentrations of Ni. Lines 30.5 and 21.2 showed xylem His concentrations 2.5-fold higher on average than the wild type (Figure 13A), reflecting the difference in root His concentrations of these plants (Table 3). However, none of the lines showed a Ni-induced increase in xylem His concentration. Furthermore, Ni flux in the xylem, calculated as the product of xylem sap Ni concentrations and sap exudation rate, did not differ significantly (within the limits of the relatively large error associated with this estimate) between the *35S*:*PRT2* and control lines in plants exposed to either 1 or 10 μM Ni (Figure 13B), consistent with the similar shoot Ni concentrations observed in these lines. Thus, although the elevated His content of *35S*:*PRT2* transgenic plants contributed to enhanced Ni tolerance, this did not lead to increased export of Ni from root to shoot in the xylem.

DISCUSSION

Metal hyperaccumulator plants exhibit exceptionally high degrees of metal tolerance, suggesting that they possess effective mechanisms for complexation and sequestration of metals at the cellular level. Although there is good evidence that tolerance and hyperaccumulation are genetically independent traits (Assunção et al., 2003; Macnair, 2003; Frérot et al., 2005), a high degree of metal tolerance may be a constitutive feature of populations of hyperaccumulator plants such as Alyssum, species of which are often restricted in their natural distribution to metalliferous soils (Brooks et al., 1979; Baker and Brooks, 1989). At the biochemical level, there are likely to be multiple components to the tolerance phenotype. For example, in addition to high-affinity ligands needed to control the activity of free metal ions in the cell cytoplasm (Baker et al., 2000; Fraústo da Silva and Williams, 2001; Clemens et al., 2002), mechanisms are required to minimize the damaging effects of metal-induced oxidative stress (Boominathan and Doran, 2002; Freeman et al., 2004). Transport processes also represent critical components of the hyperaccumulator phenotype, being responsible for effective export of metals out of the root into the xylem, their long-distance translocation to the shoot, and ultimately their accumulation at high concentrations in the cell vacuole of leaf and stem tissues

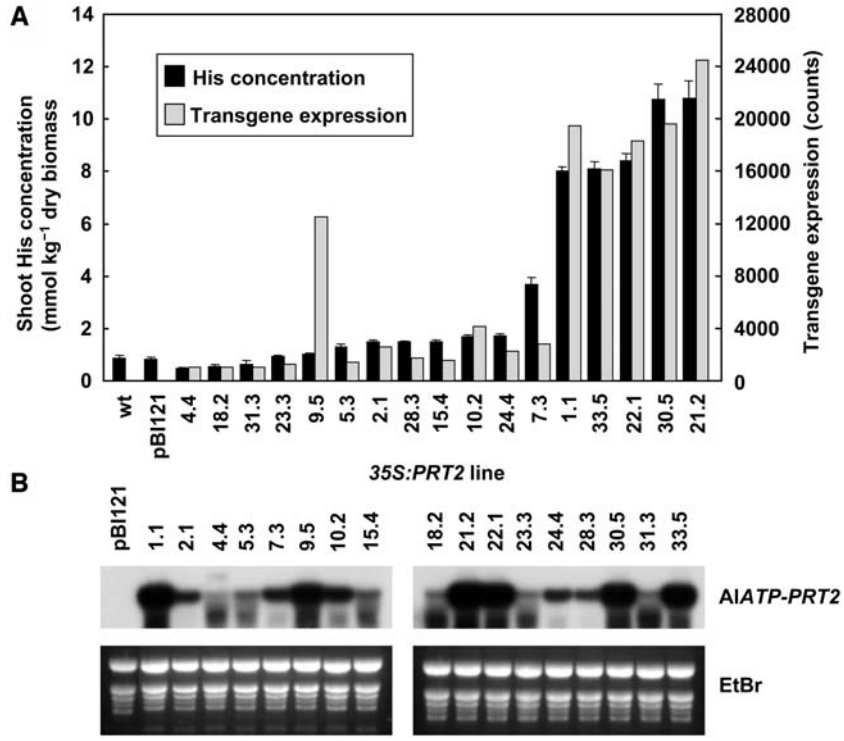

Figure 10. Free His Concentrations in *35S:PRT2* Transgenic *A. thaliana* Are Correlated with Transgene Expression.

(A) Free His concentration in rosette tissue of wild-type, empty-vector control (pBI121), and *35S:PRT2* transgenic lines was determined by HPLC analysis. Bars are means + SE of three independent replicates per line, each consisting of tissue pooled from two 21-d-old plants. The *35S:PRT2* transgenic lines are ranked according to free His concentration. Eleven lines had significantly ($P < 0.01$) higher concentrations of free His than wild-type plants (line 2.1 onwards), as determined using the least significant difference test (Sokal and Rohlf, 1995). *A. lesbiacum ATP-PRT2* transgene expression was quantified as phosphor imager counts.

(B) RNA gel blot analysis of *A. lesbiacum* (Al) *ATP-PRT2* expression in the *35S:PRT2* transgenic lines. Total RNA was isolated from rosette tissue pooled from six 21-d-old plants and probed with a 156-bp cDNA fragment encoding the predicted plastid transit peptide of the *A. lesbiacum* ATP-PRT2 protein. EtBr, ethidium bromide.

(Krämer et al., 1997, 2000; Lasat et al., 2000; Persans et al., 2001; Clemens et al., 2002).

Free His has been implicated as an important Ni binding ligand in hyperaccumulator species of Alyssum because Ni elicits a dose-dependent increase in xylem His concentration, and provision of exogenous His to nontolerant plants can partially mimic the hyperaccumulator phenotype (Krämer et al., 1996; Kerkeb and Krämer, 2003). In addition, coordination of Ni by His has been demonstrated in Ni hyperaccumulator plants by x-ray absorption spectroscopy (Krämer et al., 1996; Persans et al., 1999). Another mixed N/O-donor ligand, nicotianamine, may also play a role in Ni binding in hyperaccumulator plants (Vacchina et al., 2003). These mechanisms of tolerance are of interest because other elements, such as the softer metals Cu, Cd, As, and Hg, are coordinated in plant cells predominantly by S-donor centers present in ligands, such as phytochelatins and metallothioneins (Cobbett and Goldsbrough, 2002). Consequently, there may be a high degree of biochemical selectivity for metal tolerance determinants at the cellular level. Elucidating how the pathway of His biosynthesis is regulated in plants is thus important for a more detailed understanding of Ni hyperaccumulation and metal-ion homeostasis.

Although free His concentrations in the root tissue of *A. lesbiacum* are constitutively high, even in the absence of Ni, shoot His concentrations only increase to comparable levels when plants are exposed to Ni (Table 1). Part of this increase in shoot His can be accounted for by Ni-induced export of His from the root into the xylem (Figure 6; Krämer et al., 1996; Kerkeb and Krämer, 2003), but a mechanism must exist for maintaining the pool of free His in the root. For example, in the presence of 300 μM Ni, export of His to the shoot in the xylem would completely drain the pool of root free His in 1 to 2 d unless it were replenished. However, no evidence was found for any Ni-induced upregulation of the His biosynthesis pathway at the gene expression level because transcripts representing seven enzymatic steps in the pathway were unaltered when plants were exposed to Ni (Figure 2). Persans et al. (1999) also found no evidence for transcriptional regulation of three genes in the His pathway (*ATP-PRT*, *IGPD*, and *HDH*) in the hyperaccumulator *T. goesingense* after exposure to Ni, although in those experiments there was no detectable increase in xylem His concentration after exposure to Ni. How then is the large increase in His transport from root to shoot achieved in Alyssum in response to Ni?

Table 3. Concentrations of Free His in Shoot and Root Tissues of *35S:PRT2* Lines 21.2 and 30.5 Relative to Controls

	His Concentration (mmol kg^{-1} Dry Biomass)	
Line	Shoot	Root
21.2	20.8 ± 3.23***	3.44 ± 0.09*
30.5	18.1 ± 1.14***	3.21 ± 0.22*
pBI121	2.50 ± 0.40	1.51 ± 0.18
Wild type	1.38 ± 0.21	1.46 ± 0.12

Values are means ± SE ($n = 4$ independent replicates); each replicate consisted of tissue pooled from four 5-week-old plants cultivated hydroponically without added Ni. Values significantly different from those of wild-type plants are indicated with three asterisks for $P < 0.001$ and with one asterisk for $P < 0.05$.

Comparison of free His concentrations in three congeneric species of Alyssum differing in Ni tolerance by two orders of magnitude revealed a striking correlation between steady state pool size of free His in the roots and Ni tolerance (Figure 3, Table 2). This suggests that an important component of Ni tolerance in this genus may be the size of the steady state pool of free His available to complex Ni in the cell cytoplasm, which in turn reflects differences in the capacity for His biosynthesis in these species. The most pronounced difference was observed in transcript levels for the first enzyme in the pathway, ATP-PRT, particularly for the *ATP-PRT1* transcript (Figure 4). Thus, the presence of a larger pool of free His in the roots of *A. lesbiacum* appeared to be associated with constitutively high expression of *ATP-PRT* transcript relative to nonaccumulator species (Figure 5).

Overexpression of an *ATP-PRT* cDNA from *A. lesbiacum* in transgenic *A. thaliana* provided experimental support for this hypothesis, showing that high levels of expression of this gene could generate an enhanced His pool size. Across 17 independent transgenic lines, there was a strong correlation between transgene expression level and free His concentration in rosette tissue (Figure 10). The highest levels of *ATP-PRT2* expression corresponded to a 15-fold elevation of shoot free His concentration compared with control plants (Table 3). However, concentrations of free His were elevated to a much lesser degree in roots than shoots of the transgenic lines, being approximately twice those observed in roots of control plants. Consistent with the hypothesis that high expression levels of *ATP-PRT* transcript are causally associated with Ni tolerance, four transgenic lines investigated in detail displayed elevated tolerance to Ni, and the degree of Ni tolerance correlated with His concentrations in rosette tissue (Figure 11). Because root free His concentrations were still threefold lower in these lines than in *A. lesbiacum* (Table 2), this may partly explain why the degree of Ni tolerance did not match that seen in hyperaccumulator plants. However, additional mechanisms are likely to be required for Ni hypertolerance besides a larger pool of root free His. For example, constitutively high glutathione levels provide protection against metal-induced oxidative stress in Ni-hyperaccumulating members of the genus Thlaspi (Freeman et al., 2004), and effective transport mechanisms are required to remove Ni from the cytoplasm to sites of sequestration, such as the vacuole (Persans et al., 2001).

A striking finding was that overexpression of *ATP-PRT2* in transgenic plants had little or no effect on the concentration of any amino acid besides His (see Supplemental Figure 2 online). The only exception was the concentration of Pro, which was significantly elevated in four out of 17 transgenic lines, but this increase was not correlated with His concentrations in the plant. His is unique as an amino acid in that its C skeleton, PRPP (derived from ribose 5′-phosphate), is not used in the synthesis of any other amino acid. Thus, the ability to modulate His concentrations in transgenic plants without affecting other amino acids may reflect the relative isolation of the His biosynthetic pathway (Ward and Ohta, 1999). Although His biosynthesis requires both Gln and Glu as amino donors, concentrations of these amino acids remained unaffected in the *35S:PRT2* lines with the highest concentrations of free His. However, increased flux through the His biosynthesis pathway may not have been sufficient to deplete the pools of Gln and Glu, which were the second and third most abundant amino acids in rosette tissue of empty-vector control plants. In shoot tissue of potato (*Solanum tuberosum*), a correlation has been observed between the concentrations of free His and eight other minor amino acids, which might be indicative of coordinated synthesis (Noctor et al., 2002). However, no such correlation was apparent in wheat (*Triticum aestivum*) (Noctor et al., 2002), so it is unclear to what extent His biosynthesis is generally coordinated with metabolism of other amino acids in plants.

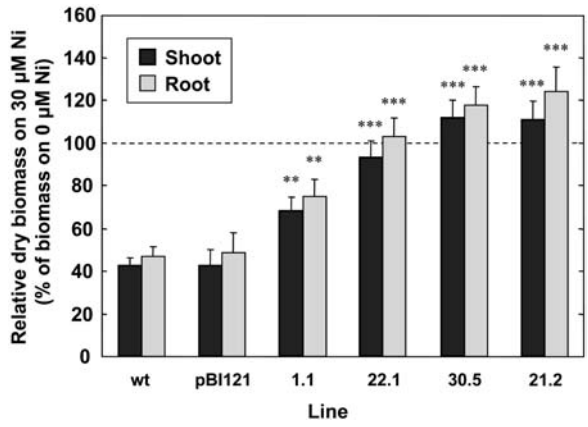

Figure 11. *35S:PRT2* His-Overproducing Lines Show Increased Tolerance to Ni.

Dry biomass at harvest of shoots and roots of 5-week-old plants cultivated hydroponically for 3 weeks on 30 µM $NiSO_4$, expressed as a percentage of biomass of each line grown in the absence of added Ni. Bars are means + SE ($n = 12$ plants, subdivided between four different culture vessels) for each line. Significant differences between wild-type and transgenic lines (i.e., with a probability below the threshold of 0.01 calculated using the Bonferroni method) are indicated with two asterisks for $P < 0.01$ and with three asterisks for $P < 0.001$. For the wild type, empty-vector control (pBI121), and lines 1.1, 22.1, 30.5, and 21.2, absolute shoot biomass in the absence of Ni was 26.7 ± 2.9 mg, 38.7 ± 3.4 mg, 28.8 ± 3.3 mg, 33.8 ± 3.9 mg, 22.6 ± 2.6 mg, and 32.1 ± 3.5 mg, respectively, and root biomass was 7.0 ± 0.7 mg, 8.8 ± 0.8 mg, 7.1 ± 1.0 mg, 7.5 ± 1.2 mg, 5.4 ± 0.6 mg, and 7.0 ± 0.8 mg, respectively. Lines 30.5 and 21.2 were shown to exhibit enhanced Ni tolerance relative to the controls in two additional independent experiments.

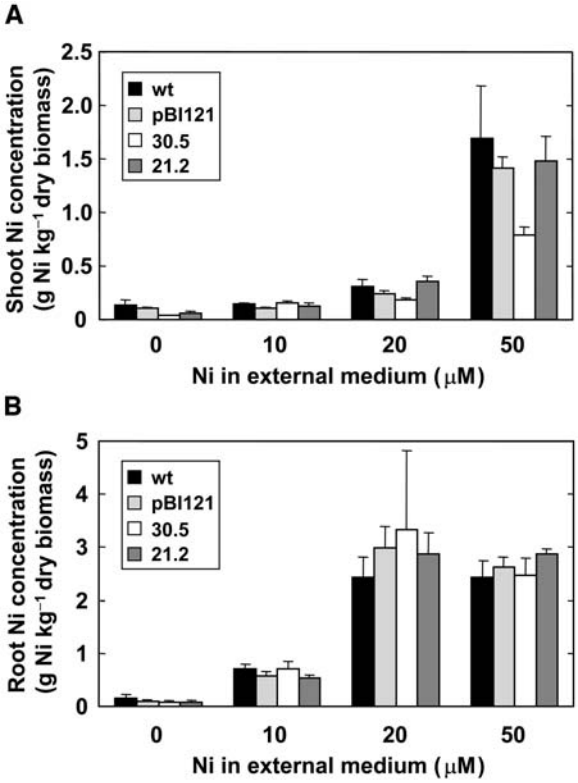

Figure 12. *35S:PRT2* His-Overproducing Lines Do Not Show Elevated Tissue Concentrations of Ni.

Ni content of tissues was determined by atomic absorption spectrophotometry. Plants were exposed to 0, 10, 20, or 50 μM added Ni for 3 weeks before harvesting. Bars are means + SE of four independent replicates per line, each replicate consisting of tissue pooled from four individual plants.
(A) Shoot Ni concentration.
(B) Root Ni concentration.

Constitutively high expression of genes with critical functions in metal chelation, transport, and sequestration may be a characteristic feature of the hyperaccumulation phenotype. For example, transcript levels of the Zn transporter ZNT1 are constitutively high in the Zn hyperaccumulator *T. caerulescens* irrespective of plant Zn status, whereas *ZNT1* transcript can only be detected in the nonaccumulator *T. arvense* under Zn-deficient conditions (Pence et al., 2000; Assunção et al., 2001). Similarly, transcript levels of the putative vacuolar metal transport proteins from the cation diffusion facilitator (CDF) family are constitutively higher in the Ni hyperaccumulator *T. goesingense* compared with *T. arvense* (Persans et al., 2001). Microarray analysis has also demonstrated that several other genes encoding Zn transporters are expressed at constitutively higher levels in the Zn hyperaccumulator *Arabidopsis halleri* compared with *A. thaliana* (Becher et al., 2004; Weber et al., 2004). Indeed, ectopic overexpression of the *A. thaliana* CDF Zn transporter cDNA *ZAT1* in transgenic plants produced lines with elevated tolerance to Zn and higher root Zn content (Van der Zaal et al., 1999). Finally, the elevated levels of glutathione implicated in enhanced oxidative defense in Ni-hyperaccumulating species of Thlaspi are associated with constitutively high activity of Ser acetyltransferase, a key enzyme in the glutathione biosynthesis pathway (Freeman et al., 2004).

Elevated transcript levels for a given gene may be a result of alteration in gene regulation. For example, the yeast mutant *ZAP1-1^{up}* displays constitutive expression of the Zn transporter genes *ZRT1* and *ZRT2*, even under Zn-replete conditions because of a loss in the ability of the ZAP1 transcription factor to respond to changes in Zn status (Zhao and Eide, 1997; Zhao et al., 1998). Alternatively, increased transcript levels may be a consequence of amplification of gene copy number in the genome. For example, DNA gel blot analysis of Cu-tolerant *Silene vulgaris* indicated that elevated expression of the type 2b metallothionein was most likely a result of tandem gene

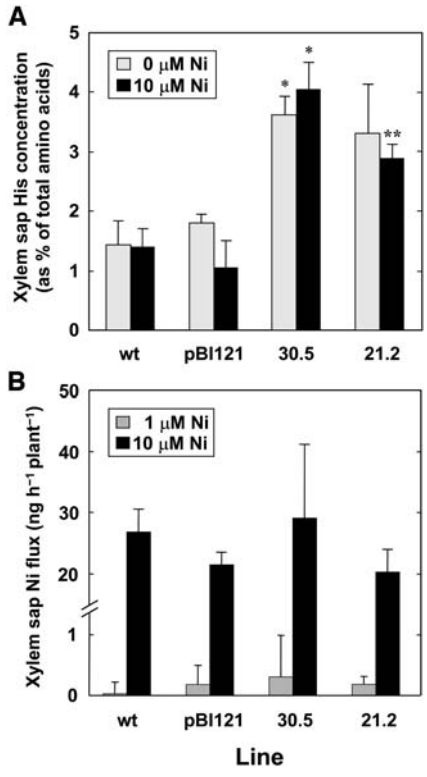

Figure 13. Xylem Sap Concentration of His and Flux of Ni in *35S:PRT2* His-Overproducing Lines.

Xylem sap for analysis was collected over a period of 12 h from detopped root systems.
(A) His concentration in xylem sap of plants exposed to 0 or 10 μM Ni for 3 weeks before sampling. Bars are means + SE ($n = 3$ plants) normalized to the total concentration of free amino acids measured in each sample. Significant differences between the transgenic lines and the wild type are indicated with one asterisk for $P < 0.05$ and with two asterisks for $P < 0.01$.
(B) Ni flux in xylem sap of plants exposed to 1 or 10 μM Ni for 3 weeks before sampling. Values were calculated as the product of measured sap Ni concentration and sap flow rate and are expressed as means + SE ($n = 5$ plants). Note the 10-fold scale expansion for 1 μM Ni treatment. Two-way analysis of variance yielded no evidence for a significant difference between lines or for a line–Ni treatment interaction (both $P > 0.05$).

Table 4. PCR Primers Used to Generate Partial cDNA Clones for His Genes from *A. lesbiacum*

Target	Size (kb)	Forward Primer Sequence (5′–3′)	Reverse Primer Sequence (5′–3′)
ATP-PRT1	1.5	GGGATAGCTGATGCCATTTTGG	CCGCTCTAAGTTGCTTCACAGA
ATP-PRT2	1.5	GGGGATAGCTGATGCCATTTTAG	CGCTCTCAGTTGCTGCACAGA
PRATP/CH	1.1	ATACGGGAGCAGTATTGATGC	GAGAAGCGTTTCCTAAGAACTTC
ProFARI	1.1	GAATCGGATAAGTCTGCGGAAGA	CTACGCCATGCACCAGAAACTC
IGPS	2.1	GTACCCCACATTGGITGGAA	CCACCTCCAACAGTTAGTGG
IGPD	1.2	CAACTTGCTTCGCATGGCTTG	GCGGCGTGGATCAGACTCTG
IAPA	1.5	GCIAAYGARAAYCCITAYGG	ATGACGAACCATCACICCCAT
HDH	1.6	GCTATAGCTGCCATGGCCTGGGG	GCGTTCTCAATCAGTCCCTCCA

Approximate sizes of mRNA transcripts detected by these partial cDNAs when used as probes in RNA gel blot analysis are indicated. The PCR primers for *IGPD* produced an amplicon orthologous to *IGPD1* (At4g14910) of *A. thaliana*. I, inosine; R, A or G; Y, C or T.

amplification rather than an alteration in gene expression from a single locus (van Hoof et al., 2001). Also, in the Zn-hyperaccumulator *A. halleri*, high transcript levels of the CDF-type transporter *MTP1* are related to a gene duplication event (Dräger et al., 2004). For *A. lesbiacum*, DNA gel blot analysis indicated the presence of only single copies of each of the two *ATP-PRT* genes (Figure 7), showing that high *ATP-PRT* transcript levels relative to the nonaccumulator *A. montanum* are apparently not a result of increased gene copy number. Possibly, the whole-genome polyploidization event that occurred close to the evolutionary origins of the Brassicaceae (Blanc et al., 2003; Bowers et al., 2003) allowed new roles to be acquired by duplicated genes such as *ATP-PRT* (Figure 7; Blanc and Wolfe, 2004). One-quarter of all Ni-hyperaccumulator species are found in the family Brassicaceae, distributed amongst seven different genera (Reeves and Baker, 2000; Broadley et al., 2001), and it seems probable that the hyperaccumulation trait has evolved multiple times independently within this family.

On the basis of these data, a tentative model can be proposed for the molecular basis of the His response in *A. lesbiacum*. Ni entering the root cell cytoplasm (probably as the free Ni^{2+} ion; Kerkeb and Krämer, 2003) would be complexed by the preexisting pool of free His. Ni and His would then be loaded into the xylem, possibly as a Ni:His complex, enhancing the rate of xylem loading of His (Figure 6; Krämer et al., 1996; Kerkeb and Krämer, 2003). Because no reduction in root His content is seen after either short-term (48 h) or long-term (21 d) exposure to Ni (Table 1), increased de novo synthesis of His must occur to replenish the pool. This might occur by relief of end product inhibition at the first step of the pathway catalyzed by ATP-PRT because the enzyme from bacteria, fungi, and *A. thaliana* is known to be sensitive to free His (Alifano et al., 1996; Ohta et al., 2000). A structure for ATP-PRT has now been determined to 1.8-Å resolution for the enzyme from *Mycobacterium tuberculosis* with His bound to the allosteric site: this shows His hydrogen bonded as part of a well-ordered network to six amino acid residues on two adjacent subunits of the homomultimeric enzyme (Cho et al., 2003). We suggest that coordination of His to Ni would reduce the amount of free His available to bind to the allosteric site of the enzyme by a mass action effect and so would alleviate feedback inhibition of ATP-PRT activity even in the absence of any decline in bulk root His concentration. Alternatively (or in addition), chelation of Ni by His in the cell cytosol might increase the rate of export of free His from the plastid, which could similarly alleviate feedback inhibition of His biosynthesis in the plastid. In nonaccumulator plants with a smaller pool of root His and a lower capacity for His biosynthesis, Ni influx might easily overwhelm the binding potential of His and rapidly lead to metal-ion toxicity in the root cells. By contrast, in hyperaccumulator plants, the capacity of the His pathway to respond to alleviation of end product inhibition would be considerably greater leading to maintenance of the His pool and more effective Ni chelation in these cells, even in the face of enhanced rates of His loading into the xylem.

Although *ATP-PRT* transcript levels and free His concentration are constitutively high in roots of *A. lesbiacum*, xylem His concentration is not. In the absence of Ni, there is no difference between xylem His concentration in *A. lesbiacum* compared with the nonaccumulators *A. montanum* and *B. juncea*, and xylem His only increases in the hyperaccumulator species in the presence of Ni (Krämer et al., 1996; Kerkeb and Krämer, 2003). How can these observations be reconciled? One explanation would be the existence of a transporter that can specifically load the Ni:His complex into the xylem. Experiments using the translational inhibitor cycloheximide have shown that Ni stimulation of xylem His loading is dependent on de novo protein synthesis (Kerkeb and Krämer, 2003), which could be explained if this transporter displayed a rapid turnover or was metal inducible. A transporter of this type may not be present in *A. thaliana* because elevated amounts of free His in the roots of *35S:PRT2* transgenic lines, although leading to increased xylem His concentrations, did not result in increased root-to-shoot translocation of Ni, and exposure of these lines to Ni did not further increase xylem His concentration (Figures 12 and 13). Alternatively, loading of a Ni:His complex may not be the principal mechanism involved in Ni transport to the shoot. Recently, elevated His content and increased Ni tolerance have been demonstrated in transgenic *A. thaliana* plants overexpressing a bacterial ATP-PRT from *Salmonella typhimurium*, but these plants also did not show enhanced tissue Ni concentrations (Wycisk et al., 2004). Similarly, transgenic tobacco (*Nicotiana tabacum*) plants overexpressing nicotianamine synthase exhibit enhanced Ni tolerance without any alteration in tissue Ni concentration (Douchkov et al., 2005). Thus, identification of the transporter(s) responsible for

xylem loading of Ni and His is likely to be a key step in attempts to recapitulate the complete hyperaccumulator phenotype.

From this work, it appears that constitutively high expression of *ATP-PRT* in *A. lesbiacum* is an important component of the mechanism underlying the His response in Ni-hyperaccumulator plants. This allows the generation and maintenance of a large pool of free His in the roots, even in the face of enhanced rates of His removal to the xylem in the presence of Ni. Constitutively high levels of His biosynthesis presumably impose a significant metabolic cost on the plant because 41 ATP are required per His synthesized (Alifano et al., 1996). If enhanced His biosynthesis represents a pleiotropic fitness cost, this might be one factor contributing to the restricted ecological occurrence and niche specialization of Alyssum Ni hyperaccumulators, which are typically endemic to serpentine soils (Baker et al., 2000). Indeed, the metal stimulation of growth of some hyperaccumulator species (e.g., of *A. lesbiacum* by Ni [Krämer et al., 1996; Küpper et al., 2001; see also Figure 3] and *T. caerulescens* by Zn [Shen et al., 1997]) may reflect an increased trace element requirement for optimal growth because of constitutively elevated concentrations of high-affinity metal binding ligands (Shen et al., 1997; Pollard et al., 2002). It would be of interest to investigate whether the ATP-PRT proteins in Alyssum hyperaccumulators show any evidence of positive selection in their evolutionary history or whether their elevated expression can be accounted for by *cis*-regulatory elements in noncoding regions of the *ATP-PRT* genes or by differential expression of *trans*-acting regulators. At any rate, the close correlation between His transcript levels and different degrees of Ni tolerance within this genus accords with the notion that quantitative variation in gene expression may be a significant component of major evolutionary change (King and Wilson, 1975; Carroll, 2000; Oleksiak et al., 2002).

METHODS

Plant Material and Culture

Alyssum lesbiacum seeds from Lesvos, Greece were provided by A.J.M. Baker; *A. serpyllifolium* seeds from Morata de Tajuña, Madrid, Spain were provided by A.J. Pollard; *A. montanum* seeds were obtained from Chiltern Seeds (Ulveston, Cumbria, UK); and *Arabidopsis thaliana* seeds, Columbia-0 background, were obtained from the Nottingham Arabidopsis Stock Centre (Loughborough, UK).

For hydroponic culture of Alyssum spp, seeds were germinated on moist autoclaved sand for 7 d and then transferred to either 1.2-liter culture vessels (8 to 10 plants per vessel) or 0.3-liter culture vessels (four plants per vessel) and supplied with modified 0.1-strength Hoagland solution containing 0.5 mM KNO_3, 0.4 mM $Ca(NO_3)_2$, 0.2 mM $MgSO_4$, 0.1 mM KH_2PO_4, 10 μM ferric ethylenediamine-di-(2-hydroxyphenylacetate) (Duchefa Biochemie, Haarlem, The Netherlands), 10 μM H_3BO_3, 2 μM $MnCl_2$, 0.2 μM $CuSO_4$, 0.2 μM $ZnSO_4$, and 0.1 μM Na_2MoO_4. Hydroponic solutions were continuously aerated and replaced every 7 d. Plants were grown in a glasshouse with natural radiation supplemented by sodium-vapor lamps to provide a photoperiod of 16 h; nighttime temperature was maintained at 14 to 18°C and daytime temperature at a minimum of 25°C. For gene expression studies, plants were cultivated in a controlled environment room (BPG98 plant growth room; Sanyo Gallenkamp, Loughborough, Leicestershire, UK) with a photosynthetic photon flux density of ~200 μmol m^{-1} s^{-1} provided by a combination of fluorescent tubes and tungsten lamps on a 16-h-light (22°C)/8-h-dark (18°C) cycle.

Measurement of Free Amino Acids

Amino acids were extracted from 0.05 g of finely ground, freeze-dried shoot or root material in 5 mL of ultrapure water at 80°C for 45 min. Samples were then centrifuged at 3000*g* for 10 min and the supernatant decanted. Xylem sap was collected as root pressure exudate from detopped plants as described by Roosens et al. (2003). Amino acid concentrations were measured by reverse-phase HPLC after precolumn derivatization with phenylisothiocyanate as described by Krämer et al. (1996).

Pulse–Chase Experiments

Hydroponically grown *A. lesbiacum* plants were exposed to 2.78 nM [2,5-^{3}H]L-His (1.11 to 2.22 TBq $mmol^{-1}$; ICN Biomedical, Basingstoke, Hampshire, UK) in the root medium for 4 h, before rinsing of roots and transfer to fresh hydroponic media lacking [^{3}H]His but supplemented with either 300 μM $NiSO_4$, 300 μM $ZnSO_4$, or no addition (control). After 6 h, plants were detopped and xylem exudate collected for 10 h. The concentration of [^{3}H]His in the xylem exudate was determined by liquid scintillation counting. Sampling of hydroponic media before and after incubation with *A. lesbiacum* indicated that ~50% of the radiolabeled His was taken up by the plants during the 4-h exposure period.

Isolation and Analysis of Nucleic Acids

Genomic DNA was isolated from *A. lesbiacum* using the cetyltrimethylammonium bromide–based extraction procedure of Doyle and Doyle (1990). For PCR screening of transgenic *A. thaliana* plants, DNA was isolated using the protocol of Edwards et al. (1991). Total RNA was isolated from Alyssum species using Trizol (Invitrogen, Paisley, UK) and from *A. thaliana* using the RNeasy system (Qiagen, Crawley, West Sussex, UK).

DNA and RNA gel blot analyses were performed using a modified version of the protocol developed by Church and Gilbert (1984). DNA gel blot analysis was performed on 3 μg of genomic DNA digested with the appropriate restriction enzyme and RNA gel blot analysis on 10 μg of total RNA. Blots were prehybridized for 1 h in 20 mL of Church buffer (0.5 M NaH_2PO_4, pH 7.2, 7% [w/v] SDS, and 1 mM EDTA) and probed overnight with [α-^{32}P]-labeled cDNA probes prepared in a random-priming reaction using the DECAprime II kit (Ambion, Huntingdon, Cambridgeshire, UK). Blots were washed for 2 × 20 min in Church wash buffer (20 mM NaH_2PO_4, pH 7.2, 1% [w/v] SDS, and 1 mM EDTA). Hybridization and washes were performed at 65°C for RNA gel blot analysis with homologous probes and at 58°C for heterologous probes and for DNA gel blot analysis. Quantification of transcript levels was performed using a GS-525 phosphor imaging system (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) with Multi-Analyst software (version 1.0).

Generation of Partial cDNA Clones for His Biosynthetic Genes

First-strand cDNA was synthesized from 2 μg of total RNA using a SuperScript II reverse transcriptase system (Invitrogen). One microliter of the cDNA produced was then used as the template in a standard 30-μL PCR reaction. PCR primers (Table 4) were designed to conserved regions with low amino acid codon degeneracy based on known and putative plant His genes identified in the public databases. The resulting PCR products were cloned into the pGEM-T Easy vector (Promega, Southampton, UK) and sequenced using BigDye terminator chemistry (version 3; Applied Biosystems, Warrington, UK). These partial cDNAs were used as probes in RNA gel blot analyses to determine the expression level of the corresponding His biosynthetic gene. The size of the corresponding transcript detected is indicated in Table 4.

Gene-specific probes for the two *A. lesbiacum ATP-PRT* genes were obtained by amplifying regions of the 3′-UTR with the following primers:

ATP-PRT1, 5′-ATACTCCAAGATGGGGTCA-3′ and 5′-CAACAATACTTAGGATCCATACA-3′; *ATP-PRT2*, 5′-CAAGTCTGTGCAGCAACTGAGAG-3′ and 5′-AGATTGTCCACCAAACCCACG-3′. These probes were of similar length (~200 bp), percentage of GC content, and annealing temperature.

Isolation of Full-Length *ATP-PRT* Sequences

The RACE system (Invitrogen) was used to isolate full-length cDNA sequences of *A. lesbiacum ATP-PRT1* and *ATP-PRT2* according to the manufacturer's instructions. For 5′ RACE, the gene-specific primers used were as follows: PRT1GSPI (5′-CTAAGTTGCTTCACAGAGC-3′) and PRT1GSPII (5′-TGCCAGTGATGGTTGATTCAT-3′), and PRT2GSPI (5′-CAGTTGCTGCACAGACT-3′) and PRT2GSPII (5′-CCTGTTAATGATGGTTGAGTTTG-3′). For 3′ RACE, the gene-specific primers used were 5′-GGGATAGCTGATGCCATTTTGG-3′ (*ATP-PRT1*) and 5′-GGGGATAGCTGATGCCATTTTAG-3′ (*ATP-PRT2*). The resulting PCR products were cloned into pGEM-T Easy and sequenced. The sequence information generated by the RACE–PCR experiments allowed the design of PCR primers to amplify the entire coding region of the *A. lesbiacum ATP-PRT* cDNAs. The primers used were PRT1F (5′-ATGTCTCTTCTTCTCCCCACTAA-3′) and PRT1R (5′-CTAAAGCCTGAGGTTTCTTAGGA-3′) at an annealing temperature of 58°C and PRT2F (5′-ATGTCAATCTCAGTTCCGCTCA-3′) and PRT2R (5′-TCAAAGGCCGAGGTTCCTCAG-3′) at an annealing temperature of 63°C. These primers were also used to isolate full-length *ATP-PRT1* and *ATP-PRT2* cDNAs from *A. montanum*.

Phylogenetic Analysis

Sequences for phylogenetic analysis were retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) by similarity searches using the BLAST algorithm (Altschul et al., 1997). *ATP-PRT* cDNA sequences were obtained for six plant species by searching the EST database using the *A. lesbiacum ATP-PRT2* cDNA sequence as query and assembling the following ESTs: *Lycopersicon esculentum* (BI929566, BI924062, and BM535922); *Solanum tuberosum* (BQ116167 and BI434057); *Medicago truncatula* (A: AL384853, BE248607, and BQ138420; B: CB893316 and BF645396); *Triticum aestivum* (BJ221844, BQ841615, and BF292817); *Hordeum vulgare* (BU970273, BQ462458, AJ485188, and BG415672); and *Physcomitrella patens* (BU052061, BQ827580, and BQ826788). cDNA sequences for the *ATP-PRT* open reading frames were aligned using ClustalW with default parameters (Thompson et al., 1994) and adjusted manually using Se-Al v2.0a11 (http://evolve.zoo.ox.ac.uk/), but DNA sequence encoding the predicted N-terminal plastid transit peptide was excluded from the phylogenetic analysis.

Phylogenetic analysis was performed with PAUP 4.0b10 (Swofford, 1998) using the maximum likelihood program. Maximum likelihood trees were found using tree bisection–reconnection branch swapping and the heuristic search option, but with estimates of nucleotide substitutions, invariant sites, and γ-parameters initially calculated from a neighbor-joining tree. Alternative tree topologies were statistically compared using the test of Shimodaira and Hasegawa (1999).

Complementation of *Escherichia coli* His Auxotroph

Full-length open reading frames of *A. lesbiacum ATP-PRT1* and *ATP-PRT2* (minus plastid transit peptide sequence) were amplified by PCR and cloned into the *E. coli* expression vector pUCmod via the *Xba*I-*Eco*RI sites in the multiple cloning site. The primers used were 5′-GCTCTAGAGCAGGAGGATTACAAAATGGCCGTCATGGTGGAGCGAGA-3′ and 5′-CCGGAATTCCCGCTAAAGCCTGAGGTTTCTTAGGA-3′ for *ATP-PRT1* and 5′-GCTCTAGAGCAGGAGGATTACAAAATGTCCTCCGCTCAGAGCTCCG-3′ and 5′-CCGGAATTCCCGTCAAAGGCCGAGGTTCCTCAG-3′ for *ATP-PRT2*. The forward primers contained the Shine–Dalgarno sequence (AGGAGG) upstream of the translational initiation codon (Shine and Dalgarno, 1974). The *hisG*$^-$ mutant strain NK5526, which lacks ATP-PRT activity and is thus a His auxotroph, was obtained from the *E. coli* Genetic Stock Center (Yale University, New Haven, CT). NK5526 cells were transformed with *pUCmod*:*PRT1*, *pUCmod*:*PRT2*, or empty vector (control) and were examined for the ability to grow on M9 minimal media supplemented with 100 μg mL^{-1} ampicillin and 25 μg mL^{-1} amino acids (except His).

Generation of *35S*:*PRT* Transgenic *A. thaliana* Plants

Full-length open reading frames of *A. lesbiacum ATP-PRT1* and *ATP-PRT2* were amplified by PCR and cloned into the binary vector pBI121 via the *Xba*I and *Sal*I sites in the multiple cloning site, the vector having previously been modified to introduce a *Sal*I site. The primers used were 5′-GCTCTAGAGCAAAAATGTCTCTTCTTCTCCCCACTAA-3′ and 5′-CCGGTCGACCTAAAGCCTGAGGTTTCTTAGGA-3′ for *ATP-PRT1,* and 5′-GCTCTAGA-GCAAAAATGTCAATCTCAGTTCCGCTCA-3′ and 5′-CCGGTCGACTCAAAGGCCGAGGTTCCTCAG-3′ for *ATP-PRT2*. The *35S*:*PRT1* and *35S*:*PRT2* constructs were introduced into *Agrobacterium tumefaciens* C58C1 (GV3101) and *A. thaliana* Columbia-0 plants transformed by vacuum infiltration using the floral-dip method (Clough and Bent, 1998). The resulting seed was sterilized and sown on MS plates supplemented with 30 μg mL^{-1} kanamycin. Putative transgenic plants were transferred to soil and the presence of the transgene confirmed by PCR screening. The T1 progeny were self-fertilized to obtain homozygous lines, and all analyses were performed on T3 homozygous lines.

Nickel Tolerance Assays with *A. thaliana*

A. thaliana seeds were germinated on plates containing 0.8% (w/v) agar made up in half-strength Hoagland solution. After stratification in the dark for 48 h at 4°C, plants were transferred to the growth room on a 16-h-light (22°C)/8-h-dark (18°C) cycle. After 14 d, seedlings were transferred to 0.3-liter culture vessels (four plants per vessel) containing half-strength Hoagland solution supplemented with 0 or 30 μM $NiSO_4$ for a period of 3 weeks (hydroponic solutions were replaced every 7 d but were not aerated). Plants were then harvested and split into root and shoot material. Roots were washed in 50 mM EDTA for 2 min and then rinsed twice in double-distilled water to remove Ni adsorbed to the roots. Material was oven dried at 70°C for 72 h for determination of shoot and root dry biomass.

Nickel Determinations

Approximately 20 mg of ground, freeze-dried plant material was digested in 5 mL of 69% (v/v) nitric acid (AnalaR; Merck, Poole, Dorset, UK) in CEM 50 mL advanced composite pressure vessels using a CEM MDS 2000 microwave digestion system (CEM Microwave Technology, Buckingham, UK). Ni was quantified in diluted solutions containing 5% (v/v) nitric acid with an atomic absorption spectrophotometer in graphite furnace mode using a double-beam optical system with deuterium arc background correction (AAnalyst 100; Perkin-Elmer, Beaconsfield, Buckinghamshire, UK). Ni content of xylem sap was determined after dilution of sap samples in 5% (v/v) nitric acid.

Statistical Analysis

Analysis of variance techniques were used to test for the presence of significant differences within experiments. For tests of Ni tolerance in

different *A. thaliana* lines, planned pairwise comparisons of relative plant biomass were made with a two-tailed *t* test, after square-root transformation to homogenize the variances, using the Bonferroni method to limit the overall experiment-wise error rate (Sokal and Rohlf, 1995). Calculations were performed using MINITAB Release 12.1 (Minitab, State College, PA).

Sequence data for the full-length cDNA clones from *A. lesbiacum* and *A. montanum* have been deposited with the EMBL/GenBank data libraries under the following accession numbers: *A. lesbiacum ATP-PRT1* (AY570528), *A. lesbiacum ATP-PRT2* (AY570529), *A. montanum ATP-PRT1* (AY570536), and *A. montanum ATP-PRT2* (AY570537). Accession numbers for the partial cDNA clones from *A. lesbiacum* are as follows: *PRATP/CH* (AY570530), *ProFARI* (AY570531), *IGPS* (AY570532), *IGPD* (AY570533), *IAPA* (AY570534), and *HDH* (AY570535).

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