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Enhancing the first enzymatic step in the histidine biosynthesis pathway increases the free histidine pool and nickel tolerance in *Arabidopsis thaliana*

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Abstract **Naturally selected nickel (Ni) tolerance in *Alyssum lesbiacum* has been proposed to involve constitutively high levels of endogenous free histidine. Transgenic *Arabidopsis thaliana* expressing a *Salmonella typhimurium* ATP phosphoribosyl transferase enzyme (*St*HisG) resistant to feedback inhibition by histidine contained approximately 2-fold higher histidine concentrations than wild type plants. Under exposure to a toxic Ni concentration, biomass production in *St*HisG expressing lines was between 14- and 40-fold higher than in wild-type plants. This suggested that enhancing the first step in the histidine biosynthesis pathway is sufficient to increase the endogenous free histidine pool and Ni tolerance in *A. thaliana*.**

Keywords: Hyperaccumulation; Nickel; *HisG*; Chelation; Phytoremediation

1. Introduction

Worldwide large areas of soil contaminated with toxic heavy metals represent an environmental hazard through wind and water erosion. In addition, plants cultivated on polluted soils are a major route for the entry of toxic heavy metals into the food chain. It has been proposed to use high-biomass non-food crops for phytoremediation, defined as the stabilization or clean-up of metal-contaminated soils using plants [1,2]. Expressing microbial genes of known function, mercury tolerance [3], arsenic tolerance and substantial shoot arsenic accumulation [4], as well as cadmium tolerance [5–7], have recently been engineered in plants.

Among the plants found on nickel (Ni)-rich soils and possessing naturally selected Ni tolerance, about 350 taxa of so-called Ni hyperaccumulators are known to accumulate very high concentrations of between 1000 and 38 000 mg kg^{-1} Ni in dry leaf biomass [8]. In contrast, a majority of non-accumulator taxa in the same habitat accumulate between below 1 and 200 mg kg^{-1} Ni [9]. Metal hyperaccumulators are rare and generally slow-growing plants unsuitable for phytoremediation, but may serve as models to engineer or breed high biomass plants for this purpose.

In the Ni tolerant Ni hyperaccumulator *Alyssum lesbiacum* (Candárgy) Rech.f., root free histidine concentrations are constitutively high, and Ni exposure induces a large and proportional increase in the concentration of free histidine in the xylem sap [10]. Histidine acts as a low-molecular weight chelator for Ni^{2+} ions in the xylem sap and in root and shoot tissues of *A. lesbiacum* [10]. In the closely related Ni sensitive non-accumulator plants *Brassica juncea* L. cv Vitasso and *Alyssum montanum* L., supplying exogenous free histidine increases Ni tolerance of whole plants, as well as the rate of Ni translocation from the roots into xylem exudates of de-topped root systems [11]. This supports the hypothesis that increasing the pool of endogenous free histidine in plants can confer Ni tolerance, and may increase the accumulation of Ni in the shoot by enhancing the rate of Ni loading into the xylem.

In plants, other known low-molecular-weight metal chelators are phytochelatins [12] and nicotianamine [13]. Phytochelatins, which are synthesised from glutathione by the enzyme phytochelatin synthase [14–16], have a role in arsenic tolerance [17], and in basal tolerance to cadmium and copper. Interestingly, phytochelatins have apparently not been selected as chelators in metal hypertolerance of hyperaccumulator plants, such as *Thlaspi caerulescens* J. & C. Presl [18]. In accordance with this, thiol group-containing molecules, such as phytochelatins or glutathione, are not involved in Ni binding in the Ni hyperaccumulators *A. lesbiacum* [10] and *T. goesingense* Hálácsy [19]. Nicotianamine is synthesized by the enzyme nicotianamine synthase from three molecules of *S*-adenosylmethionine, whereby three molecules of 5′-*S*-methyl-5′-thioadenosine are released. Nicotianamine is involved in iron homeostasis and in naturally selected zinc [20,21] and Ni tolerance [22]. The question why histidine has been selected as a major chelator in Ni hyperaccumulators of the genus *Alyssum* cannot be answered with any certainty. However, it is interesting to note that chelation of metal ions with histidine, which

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Abbreviations: ATP-PRT, adenosine 5′-triphosphate phosphoribosyl transferase; bp, base pairs; Col, accession Columbia; gDNA, genomic DNA; Ni, nickel; RT-PCR, reverse transcriptase-polymerase chain reaction; S.D., standard deviation; *St*HisG, ATP-PRT of *Salmonella typhimurium*

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contains six C and three N atoms, is of relatively low metabolic cost because its biosynthesis does not involve the assimilation of sulfate into thiol groups, and because less carbon and nitrogen atoms are committed for the chelation of one metal ion, when compared to nicotianamine (12 C and 3 N) or phytochelatins (approximately 18 or 36 C, 5 or 10 N, and 2 or 4 S).

In *Escherichia coli*, *Saccharomyces cerevisiae* and plants, histidine is synthesised from ATP and phosphoribosyl pyrophosphate in 11 catalytic steps (Fig. 1). In plants, cDNAs have been identified which encode seven out of presumably eight enzymes involved in histidine biosynthesis, mostly by functional complementation of yeast and *E. coli* mutants [23–32]. The enzyme ATP phosphoribosyl transferase (ATP-PRT) catalyses the first committed step in histidine biosynthesis in bacteria (for example, *E. coli* and *Salmonella typhimurium* HisG proteins), yeast (*Sc*His1p) and in plants (Fig. 1) [27,33]. Yeast, bacterial and plant ATP-PRT enzymes are subject to allosteric feedback inhibition by free histidine [27,34]. Thiazolealanine and triazolealanine are toxic false feedback inhibitors of ATP-PRT and have been used to screen for feedback-resistant mutants in bacteria and yeast, respectively [35,36]. Most of the mutations conferring feedback resistance were later mapped to the genes encoding ATP-PRT. However, sequence information has so far only been published for mutations in the *HisG* gene of *S. typhimurium* [37]. Resistance of the encoded *S. typhimurium* ATP-PRT enzyme to the histidine analogue thiazolealanine was conferred by small deletions of between one and two amino acids including the residue Q_{207}. Mutants possessing feedback-resistant forms of ATP-PRT were found to secrete histidine, suggesting that they overproduced this amino acid [34,38]. Thus the reaction catalysed by ATP-PRT appears to be rate-limiting for histidine biosynthesis in bacteria and yeast. In analogy, it can be hypothesised that the expression of a feedback-resistant microbial ATP-PRT in plants may result in an increased rate of histidine biosynthesis and in a larger pool of free histidine. A larger pool of endogenous free histidine may in turn increase Ni tolerance in plants.

Reaction		Plants	NCBI	*S. cerevisiae*	*E. coli*
ATP + PRPP					
PP_i	ATP phosphoribosyl transferase (2.4.2.17)	*At*ATP-PRT1[1] *At*ATP-PRT2 *Tg*THG1[2]	AB025251 AB025250 AF003347	HIS1	HisG
PR-ATP					
PP_i	Phosphoribosyl-ATP pyrophosphohydrolase (2.6.1.31)	*At*IE[3]	AB006082	HIS4	HisE
PR-AMP					
	Phosphoribosyl-AMP cyclohydrolase (3.5.4.19)	*At*IE	AB006082	HIS4	HisI
BBMII		*At* BBMII			
	BBMII isomerase (5.3.1.16)	isomerase[4]	AB006139	HIS6	HisA
BBMIII					
Gln Glu	Glutamine amidotransferase (2.4.2-)	*At*HF[5]	AB006210	HIS7	HisH
AICAR	Imidazoleglycerol-phosphate synthase cyclase (4.1.3.-)	*At*HF	AB006210	HIS7	HisF
Imidazoleglycerolphosphate					
H_2O	Imidazoleglycerol phosphate dehydratase (4.2.1.19)	*At*IGPD1[6] *At*IGPD2 *Tg*THB1[2]	U02689 O23346 AF023140	HIS3	HisB
Imidazoleacetolphosphate					
Glu 2-Ogl	Histidinol phosphate aminotransferase (2.6.1.9)	*Nt*HPA[7]	Y09204	HIS5	HisC
L-Histidinolphosphate					
P_i	Histidinol phosphate phosphatase (3.1.3.15)	n.i.		HIS2	HisB
L-Histidinol					
NAD^+ NADH	Histidinol dehydrogenase (1.1.1.23)	*Bo*HDH[8] *Tg*THD1[2]	M60466 AF023141	HIS4	HisD
L-Histidinal					
NAD^+ NADH	Histidinol dehydrogenase	*Bo*HDH *Tg*THD1[2]	M60466 AF023141	HIS4	HisD
L-Histidine					

[1][27]; [2][32]; [3][28]; [4][30]; [5][29]; [6][26]; [7][31]; [8][24,25].

Fig. 1. The pathway of histidine biosynthesis in plants, *S. cerevisiae* and *E. coli*. Each reaction (EC number) is listed on the left. The enzymes catalysing each reaction are listed on the right. Please note that some enzymes are bi- or trifunctional. For the plant enzymes, NCBI (National Center for Biotechnology Information) Accession Numbers are given of the cDNAs encoding them. Abbreviations are as follows: PRPP, phosphoribosyl pyrophosphate; AICAR, 5′-phosphoribosyl-5-amino-4-imidazolecarboxamide; BBMII, *N*′-[(5′-phosphoribosyl)-formimino]-5-aminoimidazole-4-carboxamide ribonucleotide; BBMIII, *N*′-[(5′-phosphoribulosyl)-formimino]-5-aminoimidazole-4- carboxamide ribonucleotide; 2-Ogl, 2-oxoglutarate; n.i. not identified; *At*, *Arabidopsis thaliana*; *Bo*, *Brassica oleracea*; *Nt*, *Nicotiana tabacum*; *Tg*, *Thlaspi goesingense*.

Here we report the generation of transgenic *A. thaliana* plants engineered to express wild-type and feedback-resistant variants of the *S. typhimurium* ATP-PRT, HisG. In order to assess the potential of this metabolic engineering approach for the phytoremediation of soils contaminated with Ni, free histidine concentrations, Ni tolerance and Ni accumulation were analysed in the transgenic lines.

2. Materials and methods

2.1. Plant cultivation

All plants were cultivated in a climate-controlled growth chamber at a photon flux density of 120 μmol m^{-2} s^{-1} during the day, a 14-h photoperiod, day and night temperatures of 20 and 16 °C, respectively, and 75% constant relative humidity.

2.2. Cloning and generation of transgenic lines

Cloning and DNA manipulations were performed according to standard protocols [39]. All kits and enzymes were used according to the manufacturers recommendations. A 991 bp long DNA fragment including the coding sequence of *S. typhimurium HisG* (Genbank Accession No. X13464) encoding an ATP-PRT enzyme was amplified by PCR in a total reaction volume of 50 μL from pAQ1 DNA containing the wild-type *StHisG* sequence [37] (kindly provided by the laboratory of B. N. Ames, University of California, Berkeley, USA), using 0.5 μL of a proofreading DNA polymerase and 5 μL of the accompanying 10× reaction buffer (Pfu Turbo, Stratagene, La Jolla, CA, USA), 0.4 μM of each primer 5′-GCGCGCGATACAGACCGGTTCAG-ACA-3′ and 5′-AACGCATTACGTAGGCCTGAT-3′, and 0.2 μM dNTPs using the following PCR programme: 94 °C (240 s), 36 cycles of 94 °C (60 s), 57 °C (45 s), and 72 °C (180 s), and 72 °C (600 s). The PCR reaction product was purified in a 0.8% (w/v) TAE-agarose gel, the excised fragment purified using silica [40], and eluted in 20 μL of ultrapure H_2O. Ten microliters of the eluate was used for addition of single 3′ A overhangs and cloning into the vector pCRIITOPO (TOPO TA Cloning kit, version E, Invitrogen, Carlsbad, CA, USA). The ΔQ_{207}-E_{208} deletion was introduced by site-directed mutagenesis using the primer 5′-GGCACAGAGCAAGCTGATCGATAAAT-TGC-3′, which contains a deletion of six nucleotides corresponding to nucleotides 619–624 of the *HisG* coding sequence, and 5′-CTCGGTACCAAGTTTGATGCATAGC-3′, which removes a unique *Hin*dIII site in the pCRIITOPO backbone [39]. *StHisG* and *StHisG* ΔQ_{207}-E_{208} were subcloned into the binary vector pMON530 [41] using *Eco*RI. *Agrobacterium tumefaciens* strain GV3101(pMP90) was used for transformation of *Arabidopsis thaliana* (L.) Heynhold (accession Columbia, Col) using the floral dip method [42]. In the T2 generation, lines were selected that exhibited a 25:75 segregation ratio of kanamycin-sensitive to tolerant plants on agar plates containing 0.5× Murashige & Skoog salts (Duchefa, Haarlem, The Netherlands), 0.5% (w/v) sucrose and 50 mg L^{-1} kanamycin. The selected lines were propagated into the T3 generation, and homozygous seed batches were used in all experiments.

2.3. Nickel tolerance experiments

Seeds of eight independent lines per construct were sterilised, and ten seeds were plated in a horizontal line on a plastic petri plate containing 20 mL of a sterile minimal medium composed of 2.5 mM $Ca(NO_3)_2$, 1 mM H_3PO_4, 5 mM KNO_3, 2 mM $MgSO_4$, 0.2 μM $CuSO_4$, 0.1 mM Fe(III)NaEDTA, 7 μM H_3BO_3, 1.4 μM $MnSO_4$, 0.01 μM MoO_3, 3.8 μM NaCl, 1 μM $ZnSO_4$, 5 mM MES, 0.5% (w/v) sucrose (pH 5.7), 0.8% (w/v) agarose (Seakem LE, BMA, Rockland, ME, USA), with or without 120 μM added $NiSO_4$. Two replicate plates were set up per line and Ni concentration in each experiment. Plates were kept at 4 °C for 5 d, then incubated in a vertical position in a climate-controlled growth chamber for 12 d. Final root length was measured for each seedling. Where indicated, seedlings were taken from plates and pooled in groups of five. After drying at 60 °C for 3 d, dry biomass was determined. Seven independent replicate experiments were performed for root length and four for biomass determination.

2.4. Determination of Ni accumulation and histidine concentrations

Plants were grown in a hydroponic system as described earlier [20]. Hydroponic solutions were exchanged weekly. After a four-week preculture of 60 seedlings in a 2-L culture vessel, two plants were transferred into one vessel filled with 500 mL of the hydroponic solution and cultivated for 1 w. Subsequently, the hydroponic medium of three replicate vessels per line was supplemented with 3 μM $NiSO_4$ for two weeks before harvest. At harvest, one leaf was taken from each plant and frozen immediately in liquid nitrogen for the determination of histidine concentrations. The remainder of the rosette leaves was harvested and rinsed in ultrapure water. To remove apoplastically bound Ni^{2+}, roots were desorbed in 5 mM $Ca(NO_3)_2$ for 20 min, with one exchange of solutions, and then rinsed in ultrapure water. Roots and shoots were dried at 60 °C for 3 d. Plant tissues were ashed [11] and analysed by inductively-coupled atomic emission spectrometry as described previously [20]. Extraction of frozen leaves, derivatisation and amino acid analysis were performed as described [11].

2.5. Protein extraction and immunoblot analyses

Total soluble proteins extracted from leaves of soil-grown four-week-old plants were separated in a denaturing SDS gel and blotted using a semidry procedure onto a polyvinylidine fluoride membrane (Millipore Corp., Bedford, MA, USA) according to standard protocols [43,44]. Membranes were incubated in affinity-purified polyclonal anti-HisG antibody (Pineda Antikörper-Service, Berlin, Germany), followed by biotinylated anti-rabbit-IgG and streptavidin alkaline phosphatase conjugate (Amersham Pharmacia Biotech, Little Chalfont, UK), with colour detection using nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Roche Molecular Biochemicals, Mannheim, Germany). Anti-HisG antibodies were raised against a synthetic peptide designed according to the ATP-PRT of *S. typhimurium* (*St*HisG) protein sequence (E_{80}LLNRRAQ-GEDPRYL$_{94}$), which was coupled to keyhole limpet haemocyanin prior to immunization of two rabbits. Antisera were affinity purified against the peptide before use.

2.6. Transcript analysis by RT-PCR

Total RNA was isolated from leaves of *A. thaliana* grown on soil using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Two μg of total RNA were treated with 0.2 U of RNase-free DNase (Promega, Madison, WI, USA) with 1.5 μl 5× SuperScriptII-RT-Buffer in a total volume of 11 μl for 10 min at 37 °C to remove residual genomic DNA (gDNA), followed by 10 min at 70 °C to inactivate the DNase. The DNase treated sample was directly used for first strand cDNA synthesis using the SuperScript^{TM}II RNase H^- Reverse Transcriptase kit (Invitrogen) in a total volume of 20 μL. Aliquots of the cDNA or of gDNA, isolated according to [45], were amplified using either *StHisG* -specific primers 5′-TATCGGCGAAAACGTGCT-3′ and 5′-TAAC-CTCTTCCAGGCGTTCA-3′, which are designed to generate a product of 512 bp from *StHisG* and 506 bp from *StHisG*ΔQ_{207}-E_{208}, or primers specific for the *A. thaliana actin 8* cDNA (*ACT8*; At1g49240; NCBI: AY087348), namely 5′-AGCTGCAGGGATCCACGAGA-3′ and 5′-TGCCTGGACCTGCTTCATCA-3′, designed to generate a product of 296 bp from cDNA and a product of 403 bp from gDNA. Thirty cycles of amplification were performed at 94 °C (60 s), 64 °C (30 s), and 72 °C (30 s) using a *Taq* polymerase kit (Invitrogen). The reaction products were resolved electrophoretically using ethidium bromide-containing agarose gels, and visualised in ultraviolet light. The experiment was repeated 3 times with similar results.

2.7. Statistical data analysis

Nickel tolerance datasets were analysed by two-way analysis of variance (ANOVA), using genotype and Ni treatment as factors. Nickel accumulation datasets were analysed by two-way ANOVA, using genotype and tissue type as factors. Tolerance and Ni concentration data were log-transformed for statistical analysis. Where the null hypothesis was rejected, Tukey tests were performed for the comparison of the means obtained for the *HisG* transgenic lines with those of the wild type and the empty vector transformant line, respectively. Free histidine concentration datasets were analysed by one-way ANOVA, followed by the Newman–Keuls test. Results from the ANOVAs and a posteriori tests are given in the figure legends. All analyses were performed using the programme Microsoft Office Excel 2003 (Microsoft Corp., Redmond, WA, USA), according to standard procedures [46].

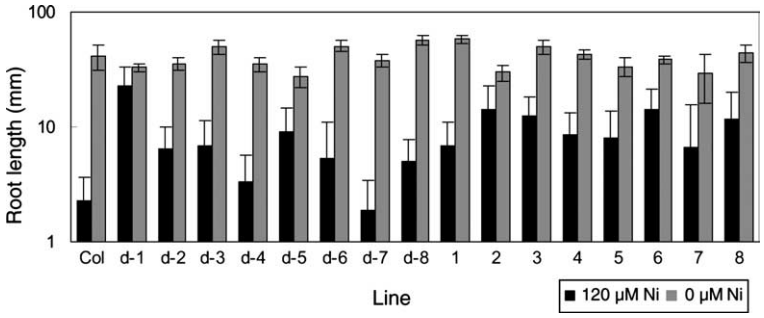

Fig. 2. Nickel tolerance measured as root elongation of wild type *Arabidopsis thaliana* (Col), eight Arabidopsis lines transformed with a *p35S::StHisGΔQ*$_{207}$-*E*$_{208}$ construct (d-1 to d-8) and eight lines transformed with a *p35S::StHisG* construct (1–8). Seeds were germinated on vertically oriented agarose plates containing minimal medium (controls) or the same medium supplemented with 120 µM $NiSO_4$. Values are mean root length ± S.D. of 20 twelve-day-old seedlings (ANOVA: $P < 0.001$ for factors genotype, Ni concentration, and their interaction, respectively). Significant differences of means, when compared to wild type plants ($P < 0.05$, according to a Tukey test), were only detected at 120 µM Ni, for lines d-1, d-2, d-3, d-5, d-6, d-8, 1, 2, 3, 4, 5, 6, 7, and 8.

3. Results and discussion

A. thaliana (Col) were transformed with expression constructs, which comprised either the *StHisG* coding sequence or a mutant *StHisGΔQ*$_{207}$-*E*$_{208}$ sequence, known to encode a thiazolealanine-resistant ATP-PRT enzyme [37], downstream of a CaMV 35S promoter. A number of transformant lines were screened for Ni tolerance using a root growth assay on agarose plates containing 120 µM Ni (Fig. 2). All seedlings exhibited equivalent root growth under control (0 Ni) conditions (Fig. 2). Compared to wild-type seedlings, Ni tolerance was enhanced significantly in seedlings of most *HisG* and *HisGΔ Q*$_{207}$-*E*$_{208}$ lines (Fig. 2). Overall, there was no apparent difference in Ni tolerance between lines transformed with the different constructs (Fig. 2).

Lines d-1 and d-2 (HisGΔ*Q*$_{207}$-*E*$_{208}$) and lines 1 and 2 (*HisG*) were chosen for a detailed characterisation. Transcript analysis by reverse transcriptase-polymerase chain reaction (RT-PCR) suggested that in all four lines stable transcripts were generated from the introduced transgenes (Fig. 3A). In protein extracts an *St*HisG-specific polyclonal antibody detected a single band of the predicted size of *St* HisG (33 kDa) in the transgenic lines, but not in empty vector or wild type controls (Fig. 3B and C). This suggested that the introduced microbial sequences were transcribed and translated into proteins of the correct size in the four *A. thaliana* transformant lines.

Free histidine concentrations analysed by HPLC were approximately 2-fold higher in the transgenic lines d-1, d-2 and 1 than in wild type plants or empty vector transformants (Fig. 4). In line 2 leaf free histidine concentrations appeared to be slightly higher than in the controls, but the difference was not statistically significant (Fig. 4). For comparison, in the Ni tolerant Ni hyperaccumulator *A. lesbiacum*, root free histidine concentrations were constitutively about 4.4-fold higher than in the Ni sensitive plant *B. juncea*. Taken together, the data suggested that the overexpression of the microbial ATP-PRT proteins resulted in increased steady-state free histidine concentrations in *A. thaliana*. Since the Ni hyperaccumulator *A. lesbiacum* is known to contain high concentrations of free histidine constitutively [11], an alternative approach for the engineering of an increased pool of endogenous free histidine might be the expression in *A. thaliana* of an ATP-PRT from a Ni hyperaccumulator plant [32].

A detailed analysis of metal tolerance showed that upon exposure to 120 µM Ni, biomass production in the four lines expressing *S. typhimurium* ATP-PRT was between 14- and 40-fold higher than in wild-type plants and empty-vector

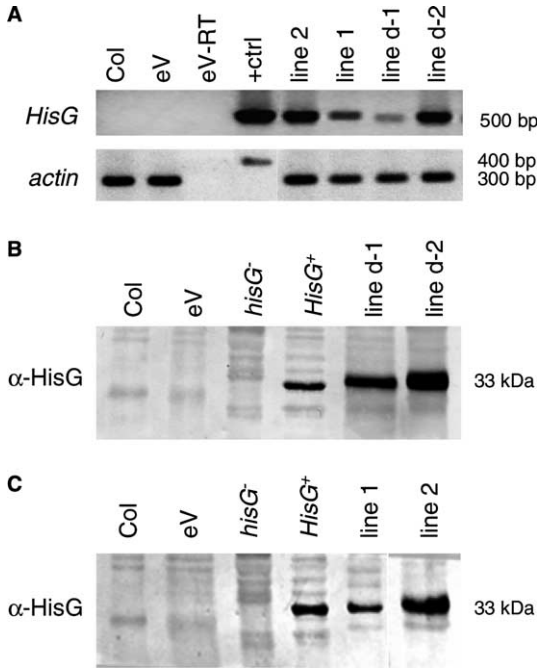

Fig. 3. Expression analysis of microbial ATP-PRT in leaves of transgenic *A. thaliana* lines. Results are shown for two lines transformed with a *p35S::StHisGΔQ*$_{207}$-*E*$_{208}$ construct (d-1, d-2) and two lines transformed with a *p35S::StHisG* construct (1, 2). (A) Analysis of transcript levels by RT-PCR with *StHisG* -specific primers (top panel), and primers specific for the *actin 8* gene used as a constitutive control (lower panel). Lanes are as follows: Col, *A. thaliana* (Col) wild type plants; eV, empty vector transformants; eV-RT, empty vector transformants, control cDNA synthesis performed without added reverse transcriptase; + ctrl, for *HisG*PCR product amplified from pCRIITOPO-*HisG* plasmid DNA, for *actin* PCR product amplified from *A. thaliana* gDNA. (B,C) Analysis of protein levels by Western Blot for (B) lines d-1 and d-2 and (C) lines 1 and 2. Total protein extracts from wild-type (Col) plants, from empty vector transformants (eV), from *E. coli* mutant NK5526 lacking a functional *HisG* gene (*hisG*$^-$, negative control), and from *E. coli* strain DH5α (*HisG*$^+$, positive control) were included as controls.

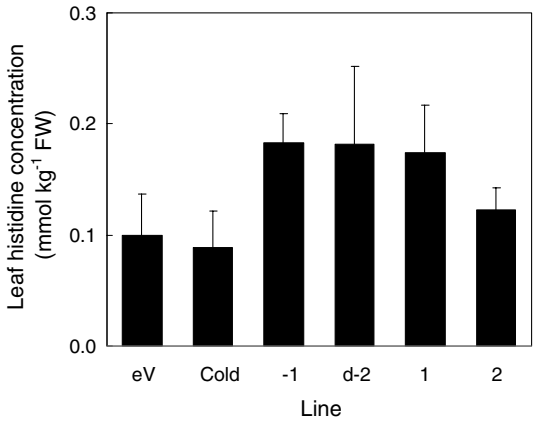

Fig. 4. Total free histidine concentrations in leaves of transgenic *A. thaliana* lines expressing microbial ATP-PRT. Histidine concentrations were analysed in extracts from rosette leaves of seven-week-old hydroponically grown plants of genotypes as described in Fig. 3. Values are means ± S.D. of between 4 and 6 independent replicate samples (ANOVA: $P < 0.01$). Significant differences of means, when compared to the wild type or the empty vector line ($P < 0.05$, according to a Newman–Keuls test), were detected for lines d-1, d-2, and 1.

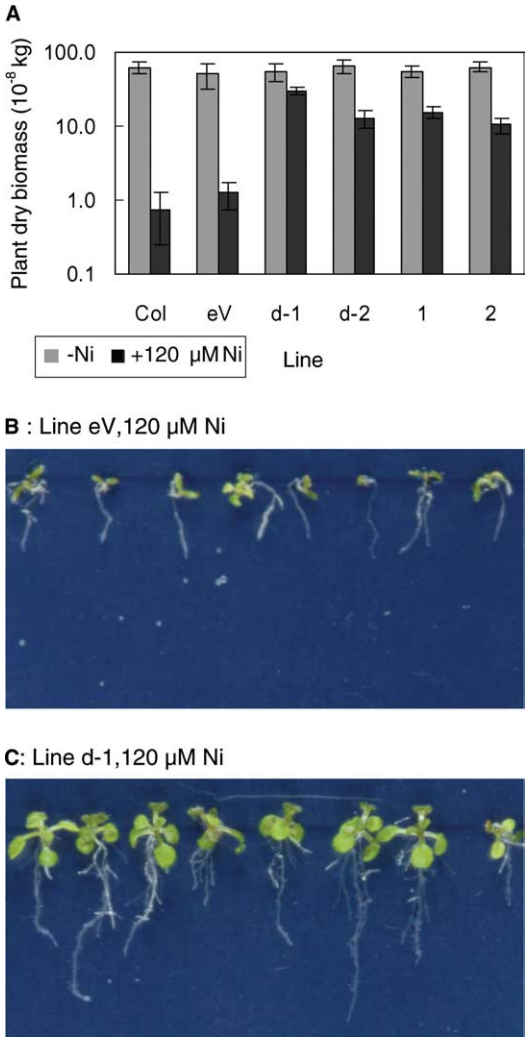

Fig. 5. Detailed characterization of Ni tolerance in selected lines of *A. thaliana* expressing microbial ATP-PRT. Results are from genotypes as described in Fig. 3. (A) Biomass of 12-day-old seedlings after growth on minimal medium without (control) or with 120 µM Ni. Values are mean seedling dry biomass ± S.D. of 4 replicate pools, each containing five seedlings (ANOVA: $P < 0.001$ for factors genotype, Ni concentration, and their interaction, respectively). Significant differences of means, when compared to the wild type or the empty vector line ($P < 0.05$, according to a Tukey test), were only detected at 120 µM Ni, for lines d-1, d-2, 1 and 2. (B) Photograph showing plants of the empty vector transformant line on plates containing 120 µM Ni. (C) Photograph showing plants of line d-1 on plates containing 120 µM Ni.

transformants (Fig. 5A). In agreement with this, growth of empty vector transformant seedlings was arrested under these conditions, whereas seedlings expressing *St*HisG or *St*HisGΔQ_{207}-E_{208} (shown as an example) continued to grow and develop at 120 µM Ni (Fig. 5 B and C). Taken together, the results presented in Figs. 2–5 confirmed our model of an involvement of the endogenous free histidine pool in Ni detoxification, presumably by chelation of Ni^{2+} cations [10].

Lines expressing the feedback-resistant *St*HisGΔQ_{207}-E_{208} were expected to contain a larger free histidine pool and thus to exhibit a higher degree of Ni tolerance than lines expressing the wild type *St*HisG, but the observed differences were only minor (see Figs. 2, 4, and 5). In addition to ATP-PRT activities, there could be other factors limiting the extent of accumulation of free histidine in *Arabidopsis*. Tobacco plants, transformed with a construct encoding a plastid-targeted feedback-insensitive *S. cerevisiae* His1 protein, were very difficult to regenerate, developed necrosis and died (Danuta Maria Antosiewicz, personal communication). *Arabidopsis* lines transformed with the same construct were less Ni tolerant than *StHisG*ΔQ_{207}-E_{208} line d-1 (Ute Krämer, unpublished observations). The histidine concentrations resulting in an inhibition of ATP-PRT enzyme activity by 50% (IC_{50}) were reported to be about 10 µM in crude protein extracts from pea and oat, about 60 µM in crude extracts from *S. typhimurium* and 75 µM for the purified *St*HisG protein [33,47]. It is thus possible that in the metabolic context of a plant and its endogenous ATP-PRT, an expressed wild-type *St*HisG is partially feedback-insensitive.

In several Ni hyperaccumulators of the genus *Alyssum*, a tight correlation has been observed between Ni and histidine concentrations in xylem sap collected as root-pressure exudate from de-topped root systems of Ni-exposed plants [10]. Transport of Ni into the xylem exudates collected from de-topped root systems of two non-accumulator *Brassicaceae* species was enhanced when Ni was supplied in combination with exogenous free histidine, at high concentrations of 300 µM and for short periods of time of several hours [10,11]. In order to test whether the increased pool of free histidine in the transgenic *A. thaliana* lines resulted in increased Ni accumulation in the leaves, five-week-old plants were exposed to a Ni concentration of 3 µM Ni in hydroponic culture for 2 weeks. Whereas agarose-based media contain rather high concentrations of the nutrients Fe and Ca, which are known to alleviate Ni toxicity, hydroponic solutions approximate the composition of the soil solution and contain considerably lower nutrient concentrations. A concentration of 3 µM Ni was used in the hydroponic experiments, because it was the maximum non-inhibitory concentration for wild-type *A. thaliana* plants (M. Becher, A.N. Chardonnens and

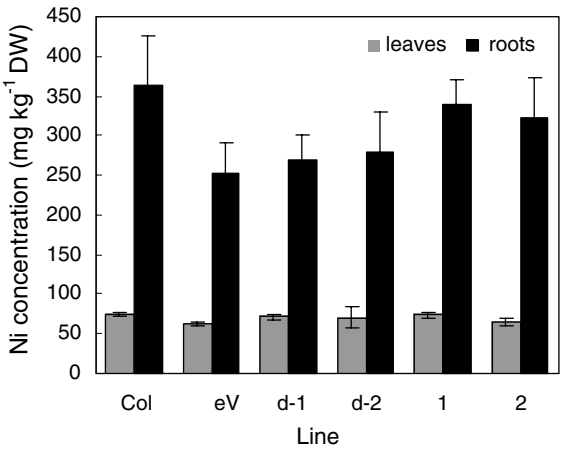

Fig. 6. Nickel concentrations in leaves and roots of transgenic *A. thaliana* lines expressing microbial ATP-PRT. Results are from genotypes as described in Fig. 3. Nickel concentrations were analysed in leaves and roots of seven-week-old hydroponically grown plants after two weeks of growth in a hydroponic nutrient medium supplemented with 3 μM Ni. Values are means ± S.D. of three independent replicates, each consisting of pooled material from two plants grown in one culture vessel (ANOVA: $P < 0.05$ for the factor genotype, $P < 0.001$ for the factor tissue type, $P = 0.311$ for interaction). Significant differences of means, when compared to the wild type or the empty vector line ($P < 0.05$, according to a Tukey test), were not found in either roots or shoots for any of the transgenic lines.

U. Krämer, unpublished data), and thus allowed the analysis of Ni accumulation in wild type and transgenic plants in comparable physiological and developmental states. After 2 weeks of exposure to Ni, neither root nor leaf Ni concentrations in the transgenic lines differed significantly from those found in empty vector or wild type control plants (Fig. 6). Similar results were obtained when plants were grown on a soil contaminated with Ni (data not shown). Increasing the endogenous free histidine pool is thus not sufficient to enhance steady-state Ni accumulation in shoots of *A. thaliana* after two weeks of Ni exposure, suggesting that further modifications are needed in a transgenic plant used for phytoextraction of metals from contaminated soils [2].

The results obtained in our long-term experiments employing intact plants (Fig. 6), are somewhat different from the implications of earlier studies [10,11]. Earlier, the choice of high Ni concentrations may have permitted low-affinity transport processes. It is possible that these transport routes are not available in the transgenic *Arabidopsis* plants under the experimental conditions used (Fig. 6), or that local concentrations of Ni or Ni and histidine are not of sufficient magnitude to allow an increased rate of root-to-shoot translocation of Ni. It may be possible to increase the rate of Ni influx into the root symplasm at low external Ni concentrations by co-expressing a high-affinity cellular Ni uptake system, such as *Schizosaccharomyces pombe* Nic1p [48]. The long-term nature and the use of intact plants in the Ni accumulation experiment (Fig. 6) may add additional levels of control over the translocation of Ni into the xylem, which were not present in the earlier experiments [10,11]. For example, a shoot-derived signal is involved in the control of root iron-deficiency responses in pea plants [49].

In summary, we report here that expression in *A. thaliana* of a microbial enzyme catalysing the first, rate-limiting step in histidine biosynthesis increased the endogenous pool of the low-molecular-weight chelator free histidine. This resulted in enhanced tolerance of the transgenic plants to Ni^{2+} ions, partially recapitulating naturally selected Ni hypertolerance of *A. lesbiacum* and other Ni hyperaccumulators [10].

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