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Increased Nicotianamine Biosynthesis Confers Enhanced Tolerance of High Levels of Metals, in Particular Nickel, to Plants

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Nicotianamine, a plant-derived chelator of metals, is produced by the trimerization of *S*-adenosylmethionine catalyzed by nicotianamine synthase. We established transgenic *Arabidopsis* and tobacco plants that constitutively overexpress the barley nicotianamine synthase gene. Nicotianamine synthase overexpression resulted in increased biosynthesis of nicotianamine in transgenic plants, which conferred enhanced tolerance of high levels of metals, particularly nickel, to plants. Promoter activities of four nicotianamine synthase genes in *Arabidopsis* were all increased in response to excess nickel, suggesting that nicotianamine plays an important role in the detoxification of nickel in plants. Furthermore, transgenic tobacco plants with a high level of nicotianamine grew well in a nickel-enriched serpentine soil without developing any symptoms of nickel toxicity. Our results indicate that nicotianamine plays a critical role in metal detoxification, and this can be a powerful tool for use in phytoremediation.

Keywords: *Arabidopsis* — Metal toxicity — Nickel — Nicotianamine synthase — Phytoremediation — Tobacco.

Abbreviations: CaMV, cauliflower mosaic virus; GUS, β-glucuronidase; NA, nicotianamine; NAS, nicotianamine synthase; NAAT, nicotianamine aminotransferase; NT, non-transgenic; ORF, open reading frame.

Introduction

Heavy metal pollution of soils and waterways causes serious environmental and human health problems. Decontamination of metal-polluted soils using plants, i.e. phytoremediation, is a very attractive technology, the successful use of which requires a better understanding of the mechanisms of metal uptake, translocation and accumulation by plants. Metals are toxic to plants when present in excess or distributed incorrectly within cells, although many metals are essential micronutrients required for metabolism, growth and development (Marschner 1995). Therefore, their concentrations within cells must be controlled carefully. Consequently, plants possess a range of potential mechanisms for metal ion homeostasis and tolerance, including membrane transport processes (Clemens 2001, Hall 2002). A number of genes encoding metal transporters have been identified and characterized, including the heavy metal ATPases (HMAs) (Mills et al. 2003), the Nramps (Williams et al. 2000), the cation diffusion facilitator (CDF) family (Persans et al. 2001), ZRT- and IRT-like proteins (ZIPs) (Grotz et al. 1998, Guerinot 2000) and the cation antiporters (Gaxiola et al. 2002). These transport systems are thought to be involved in metal homeostasis in plants.

One potential mechanism for heavy metal detoxification in plants is the chelation of metal ions by ligands, such as organic acids, amino acids, peptides and polypeptides, and, in some cases, the subsequent compartmentalization of ligand–metal complexes, which could play a role in tolerance (Cobbett 2000, Hall 2002). Organic acids are thought to contribute to the tolerance, transport and accumulation of heavy metals in plants, including Al, Cd, Fe, Zn and Ni (Brooks et al. 1981, Godbold et al. 1984, Krotz et al. 1989, Homer et al. 1991, Yang et al. 1997, Ma et al. 2001, Nigma et al. 2001). Furthermore, the correlation of free nickel and histidine levels in *Alyssum lesbiacum* showed that an Ni–histidine complex was responsible for xylem transport (Krämer et al. 1996). Phytochelatins, a family of heavy metal-inducible peptides, are important in the detoxification of heavy metals, primarily Cd. The gene encoding phytochelatin synthase has been isolated from *Arabidopsis* (Clemens et al. 1999, Ha et al. 1999, Vatamaniuk et al. 1999). Overexpression of phytochelatin synthase is postulated to give plants a genetically enhanced tolerance to heavy metals (Cobbett and Meagher 2002, Li et al. 2004).

The non-proteinogenic amino acid nicotianamine (NA) was first found in tobacco (Noma et al. 1971) and has been found in all plants investigated so far (Noma and Noguchi 1976, Rudolph et al. 1985). In graminaceous plants, NA is the biosynthetic precursor of mugineic acid family phytosiderophores (Shojima et al. 1990), which are secreted from the roots to chelate Fe(III) in the soils (Takagi 1976). The Fe(III)–phytosiderophore complex is taken up by a specific transporter at the root surface. Maize *yellow stripe 1* (*YS1*) is the gene encoding the Fe(III)–phytosiderophore transporter in maize

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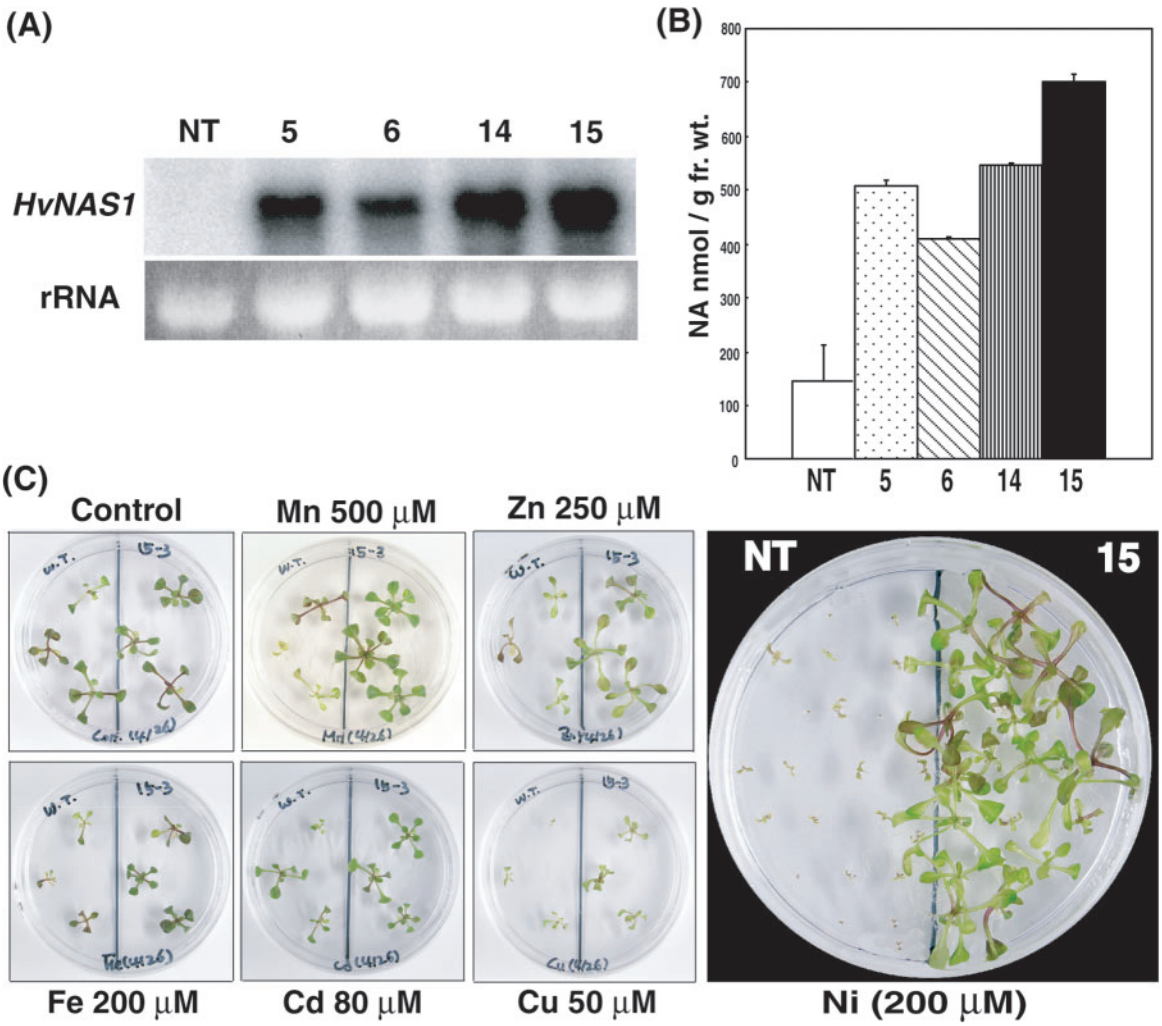


Fig. 1 Transgenic *Arabidopsis* expressing *HvNAS1* are tolerant to excess metals. (A) Northern blot analysis of non-transgenic (NT) and transgenic (lines 5, 6, 14 and 15) plants. Total RNA was prepared from shoots of 14-day-old plants. Each lane was loaded with 10 μg of total RNA. A probe specific to *HvNAS1* was used for hybridization. (B) NA concentration in leaves of NT and transgenic *Arabidopsis* plants (lines 5, 6, 14 and 15). The plant materials used for Northern blot analysis were analyzed by HPLC. Experiments were repeated three times, and each value represents the mean of triplicate experiments. (C) Seeds were germinated and grown for 14 d on MS medium containing the indicated concentrations of Cd, Cu, Fe, Mn, Zn or Ni. The NT and transgenic line 15 were germinated on the left and right half of each plate.

(Curie et al. 2001). Non-graminaceous plants produce NA, but lack a pathway to synthesize phytosiderophores. NA is synthesized by the trimerization of three molecules of *S*-adenosyl methionine, a reaction catalyzed by nicotianamine synthase (NAS) in both graminaceous and non-graminaceous plants (Shojima et al. 1989, Shojima et al. 1990, Higuchi et al. 1995). In graminaceous plants, NAS activities are markedly increased to produce a higher amount of phytosiderophores in response to Fe deficiency. Since Higuchi et al. (1999) isolated the first *NAS* gene from *Hordeum vulgare*, *NAS* genes have been isolated from *H. vulgare* again (Herbik et al. 1999), and from *Lycopersicon esculentum* (Ling et al. 1999), *Arabidopsis thaliana* (Suzuki et al. 1999, Ushio et al. 2003), *Oryza sativa* (Higuchi et al. 2001) and *Zea mays* (Mizuno et al. 2003). NA chelates metal cations, including Fe(II) and Fe(III) (Benes et al. 1983, von Wirén et al. 1999). Unlike phytosiderophores, NA is not secreted, and is thought to have a role in metal homeostasis in non-graminaceous plants. The NA synthesis-defective tomato mutant *chloronerva* (Rudolph et al. 1985, Higuchi et al. 1996) has a phenotype indicative of Fe deficiency (Pich and Scholz 1996). Recently, Takahashi et al. (2003) showed that transgenic tobacco plants constitutively expressing the barley NA aminotransferase (NAAT) gene had young leaves with interveinal chlorosis and abnormally shaped flowers. NAAT catalyzes the amino transfer of NA in phytosiderophore synthesis in graminaceous plants. Endogenous NA was consumed as a result of NAAT overproduction in transgenic tobacco plants, and both the concentration and distribution of metals in leaves

and flowers were changed in these plants. A shortage of NA caused disorders in internal metal transport, leading to these abnormal phenotypes. The results suggest that a shortage of NA also impairs the functions of metal-requiring proteins, including transcription factors. Conversely, increasing the amount of NA by overexpression of *HvNAS1* led to a increase in Fe and Zn concentrations in leaves and flowers.

In the hyperaccumulator species *Thlaspi caerulescens*, NA was found to chelate Ni in the xylem as a response to toxic levels of external Ni (Vacchina et al. 2003). This suggests that the NAS activity in *T. caerulescens* may be induced to tolerate toxic levels of external Ni. Weber et al. (2004) reported that high NA synthesis activity appears to be a key factor for metal hyperaccumulation in *Arabidopsis halleri*. They also showed that overexpression of the *Arabidopsis NAS* gene (*AtNAS2*) in *Schizosaccharomyces pombe* cells confers increased Zn tolerance in the medium.

In this study, we established transgenic *Arabidopsis* and tobacco plants that constitutively overexpress the barley NAS gene, and showed that increased NA biosynthesis confers enhanced tolerance of high levels of metals, particularly Ni, to plants. Furthermore, promoter activities of all four *NAS* genes in *Arabidopsis* were increased in response to excess Ni in the medium. Our results indicate that NA plays a critical role in metal detoxification and, therefore, can be a powerful tool for use in phytoremediation.

Results

Arabidopsis overexpressing HvNAS1 tolerates excess metals

The *HvNAS1* gene, which encodes a major barley NAS, was introduced into *Arabidopsis* under the control of the *35S* promoter. Thirty-four independent transgenic *Arabidopsis* lines were obtained, and the expression of the transgene in each transgenic line was analyzed using Northern blot hybridization. Four lines (5, 6, 14 and 15) were used for further experiments. Of these lines, line 15 had the highest level of *HvNAS1* transcripts (Fig. 1A). To determine whether the high *HvNAS1* expression in transgenic plants results in increased NA concentration, the amount of endogenous NA was measured in leaves of non-transgenic (NT) and transgenic lines (5, 6, 14 and 15) using HPLC (Fig. 1B). As expected, the NA concentrations of transgenic plants overexpressing *HvNAS1* were increased and the NA concentrations in the four lines differed in proportion to the level of *HvNAS1* transcripts (Fig. 1B).

Seedlings of the NT line and four transgenic lines were evaluated for tolerance to excess metals by exposing them to various concentrations of Cd, Cu, Fe, Mn, Zn or Ni in MS medium. All of the transgenic plants exhibited improved tolerance to all of these metals, particularly Ni (Fig. 1C). Although Ni is an essential micronutrient for higher plants (Brown et al. 1987), excess Ni is toxic to plants. Seedlings of lines 14 and 15, which express high levels of *HvNAS1* and consequently contain higher amount of NA, showed no marked symptoms of Ni toxicity and grew more rapidly than the NT plants on medium containing 200 μM $NiSO_4$. On this medium, seeds of the transgenic plants germinated and continued to grow, but NT seeds either did not germinate or died during the early seedling stage (Fig. 1C).

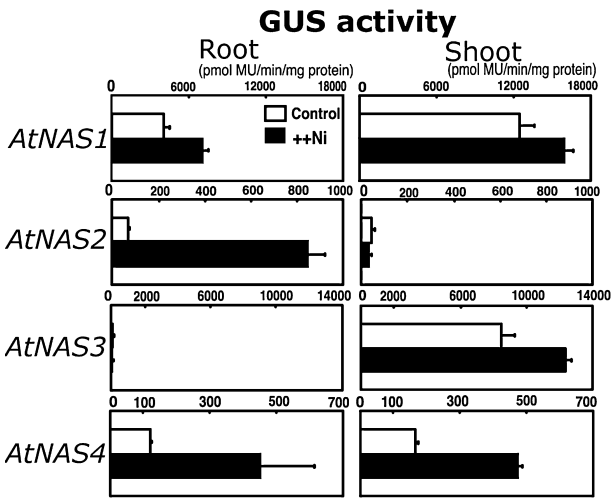


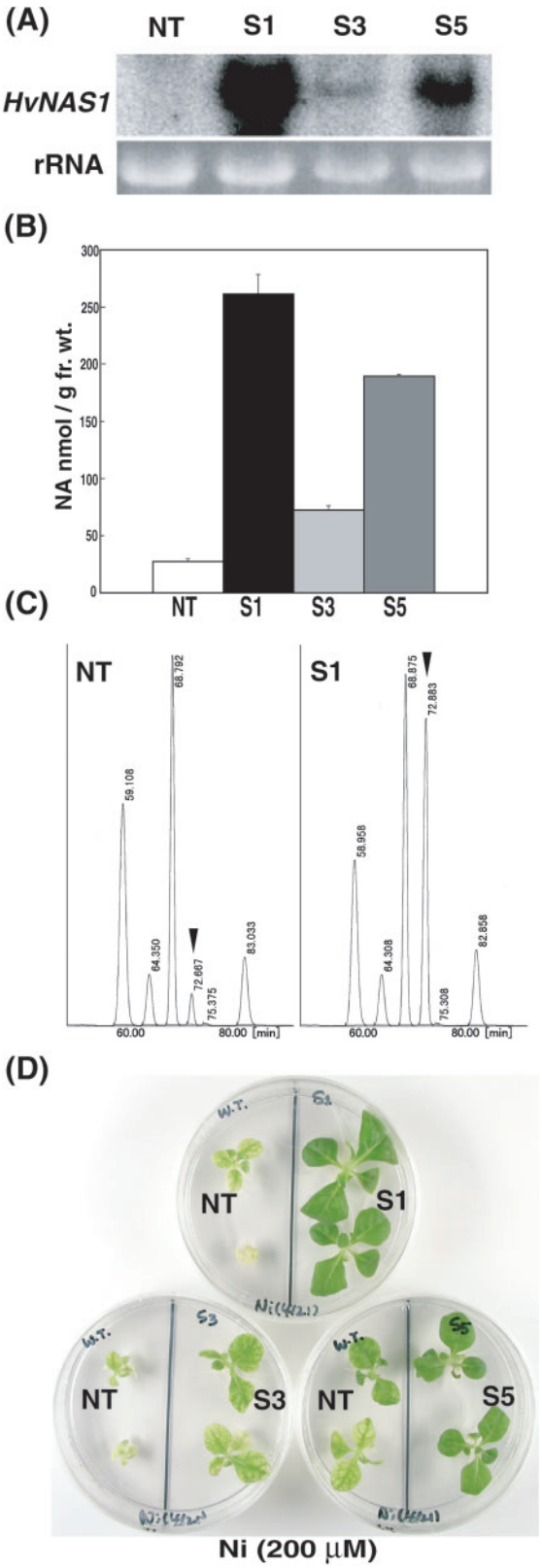
Fig. 2 Promoter activities of four *AtNAS* genes in *Arabidopsis* are enhanced in response to excess Ni. GUS activities in the roots and shoots of transgenic *Arabidopsis* plant grown on MS (control) or MS medium containing 200 μM $NiSO_4$ (++Ni). Experiments were repeated three times.

Promoter activities of four Arabidopsis NAS genes were enhanced by excess Ni

Since the transgenic *Arabidopsis* plants overexpressing *HvNAS1* tolerate excess Ni, we examined whether the expression of native *NAS* genes in *Arabidopsis* is changed in response to excess Ni by promoter-β-glucuronidase (GUS) analysis (Fig. 2). *Arabidopsis* has four *NAS* genes, *AtNAS1*, *AtNAS2*, *AtNAS3* and *AtNAS4* (Suzuki et al. 1999, Ushio et al. 2003). The promoter activities of four *AtNAS* genes were different in their strength in the roots and shoots on MS medium. However, the expression of all four *AtNAS* genes was increased on medium containing 200 μM $NiSO_4$. This result indicates that *Arabidopsis* plants exposed to excess Ni produced a higher amount of NA with enhanced activities of native AtNAS to tolerate Ni toxicity.

Tobacco overexpressing HvNAS1 tolerates excess Ni

To elucidate the effect of *HvNAS1* expression on heavy metal stress, we introduced the *HvNAS1* gene into tobacco and obtained five independent transgenic lines. Three lines (S1, S3 and S5) showing various levels of *HvNAS1* expression as determined by Northern blot hybridization were used for further experiments. *HvNAS1* expression was very low in line S3, intermediate in line S5 and high in line S1 (Fig. 3A). The amount of endogenous NA was measured in leaves of NT and three transgenic lines (line S1, S3 and S5) using HPLC (Fig.



3B, C). Similarly to the transgenic *Arabidopsis* plants, NA concentrations of transgenic tobacco plants overexpressing *HvNAS1* were increased and differed in proportion to the level of *HvNAS1* expression. The NA concentration of line S1, which has the highest NAS expression, was nine times higher.

The NT and transgenic lines (S1, S3 and S5) were germinated and grown on MS medium containing excess amounts of Fe, Mn, Zn, Cd, Cu or Ni. There was no clear difference in tolerance between NT and the transgenic lines to high levels of Fe, Mn, Zn, Cd or Cu (data not shown). Interestingly, however, transgenic tobacco plants overexpressing *HvNAS1* were clearly more tolerant than NT plants to 200 μM $NiSO_4$ (Fig. 3D), with leaves of the NT tobacco plants showing severe chlorosis and retarded development after a 20 d exposure to 200 μM $NiSO_4$. Interveinal chlorosis and inhibited root elongation are symptoms of Ni toxicity in plants (Brune and Dietz 1995, Marschner 1995). In contrast, the leaves of S1 transgenic tobacco plants were green and continued to grow on medium containing 200 μM $NiSO_4$. The three transgenic tobacco lines (S1, S3 and S5) differed in tolerance to high Ni in proportion to the level of NA concentrations (Fig. 3D). Therefore, the enhanced tolerance of the transgenic tobacco to excess Ni resulted from the increased NA concentration in transgenic tobacco. Under normal conditions, the growth of S1 tobacco was slower than that of the NT plants (data not shown). In the presence of 200 μM $NiSO_4$, however, the fresh weight of the transgenic plants exceeded that of the NT plants. Indeed, the fresh weights of both the shoots and roots of S1 tobacco were 3–4 times those of the NT plants (Fig. 4A). This indicates that line S1, which has the highest NAS expression and consequently contains the highest amount of NA, showed significant tolerance to Ni toxicity.

To determine whether *HvNAS1* overexpression and increased NA biosynthesis altered metal accumulation, the concentrations of metals in shoots of NT and S1 transgenic plants

Fig. 3 Transgenic tobacco plants expressing *HvNAS1* are tolerant to 200 μM $NiSO_4$. (A) Transcripts level of *HvNAS1* expression in tobacco plants detected by Northern blot analysis. Total RNA was extracted from the leaves of the NT and transgenic (S1, S3 and S5) tobacco lines. Each lane was loaded with 10 μg of total RNA. A probe specific to *HvNAS1* was used for hybridization. (B) NA concentration of leaves of NT and transgenic tobacco plants (lines S1, S3 and S5). The plant materials used for Northern blot analysis were extracted and analyzed by HPLC. Experiments were repeated three times, and each value represents the mean of triplicate experiments. (C) HPLC profiles of the extract of NT and transgenic tobacco (S1). Arrowheads indicate the HPLC peaks corresponding to NA. (D) NT and three transgenic tobacco lines overexpressing *HvNAS1* were grown on MS medium in the presence of 200 μM $NiSO_4$ for 20 d. The three transgenic tobacco lines (S1, S3 and S5) differed in tolerance to high Ni in proportion to the level of NA concentrations. Transgenic tobacco line S1, which has the highest HvNAS1 expression, shows significant tolerance to Ni toxicity after 20 d growth on MS medium containing 200 μM $NiSO_4$. However, transgenic tobacco line S3, which has the lowest HvNAS1 expression, shows interveinal chlorosis in leaves.

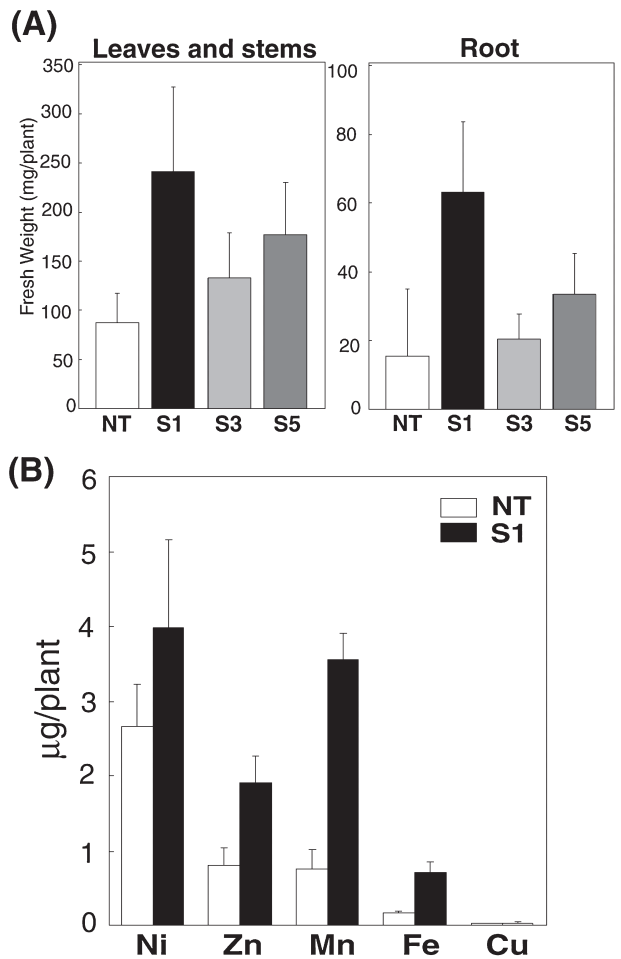


Fig. 4 NT and three transgenic tobacco lines grown on MS medium containing 200 μM $NiSO_4$ for 20 d. (A) Fresh weights of the shoots (leaves and stems) and roots of tobacco plants grown for 20 d on MS medium containing 200 μM $NiSO_4$. Error bars indicate the standard error of the means ($n = 21$). (B) Total amount of metals in the shoots of NT or S1 tobacco plants grown in the presence of 200 μM $NiSO_4$ in the medium. The absorbed and accumulated amounts of metals in the shoots (μg per plant) are shown here. The amounts of metals are calculated based on metal concentrations in the shoots of NT or S1 tobacco plants and dry weights of shoots. Error bars indicate the standard errors of the means ($n = 21$).

were measured after 20 d of growth on MS medium containing 200 μM $NiSO_4$ (data not shown). Then the total amount of metals in shoots of NT and S1 transgenic plant was calculated based on the concentartions of metals and the dry weights of each NT and S1 transgenic plant (Fig. 4B). The largest differences were evident in the contents of Mn, Fe and Zn (2- to 4-fold increases), with a 33% increase in Ni content. Therefore, transgenic plants have greater tolerance of 200 μM $NiSO_4$ in the medium and accumulate more metals in the shoot than NT. This indicates that plants overexpressing NAS take up a large amount of metals from the contaminated soils and therefore can be used for phytoremediation purposes.

Tobacco plants with increased NA biosynthesis tolerated an Ni-enriched serpentine soil

When plants were grown on medium supplemented with 200 μM $NiSO_4$, transgenic plants overexpressing *HvNAS1* showed a greater tolerance to excess Ni. Consequently, the plants were cultured in an Ni-enriched serpentine soil to examine whether the transformants have enhanced tolerance to excess Ni. As expected, the S1 plants were remarkably tolerant of high Ni in soil, and grew better than the NT plants (Fig. 5). During the early stages of development, the NT plants were smaller and had necrotic patches in the leaves, whereas the transgenic plants did not develop any sign of Ni stress (Fig. 5A, B). The control plants showed reduced growth, and their old leaves displayed necrosis caused by excess Ni (Fig. 5D). In contrast, the transformant plants had green leaves and shoots (Fig. 5C). For example, the average SPAD-502 value measured using a chlorophyll meter was 36 in the leaves of the transformants and 20 for control plants at the flowering stage (Fig. 5E). Measurements of plant height (Fig. 5F) showed that the transformants grew much better than the control plants. Metal concentrations (Ni, Zn, Mn, Fe and Cu) were measured in the leaves, stems and seeds of mature NT and transgenic plants grown in Ni-enriched soil and the total amount of metals was calculated based on the concentrations of metals and the dry weights (Fig. 6). The metal content of the transgenic plants did not differ significantly from that of the NT plants in leaves (Fig. 6A), but transgenic plants accumulated more than twice as much Zn and Fe in their stems (Fig. 6B). Transgenic plants contained more Ni (1.3-fold) in their shoots (leaves and stems). Nevertheless, symptoms of Ni toxicity were observed only in NT plants. Therefore, increased NA biosynthesis resulted in enhanced tolerance of a high level of Ni in the shoots. As shown in Fig. 6C, seeds of plants overexpressing *HvNAS1* contained more Fe (6.6-fold), Zn (3.2-fold), Cu (2.0-fold) and Mn (1.8-fold), whereas the amount of Ni was similar to those in the NT plants. This and previous studies (Takahashi et al. 2003) suggest that NA promotes the transport of Fe, in particular, and Zn to the seeds.

Discussion

In recent years, the hyperaccumulation of certain metals and metalloids by some plant species has received increased attention with respect to the development of technologies aimed at the decontamination of metal-polluted soils using plants (Chaney 1983, Salt et al. 1995). These plant species, called hyperaccumulators, are endemic to metal-rich soils and can accumulate and tolerate high levels of heavy metals in their aboveground tissues (Baker and Brooks 1989). However, these plants are usually low-growing weeds with little biomass. Therefore, the search for hyperaccumulator genes has begun. Hyperaccumulators have been identified for the heavy metals Ni, Zn, Co and Cd, as well as for the metalloids As and Se. Krämer et al. (1996) have reported that enhanced production of

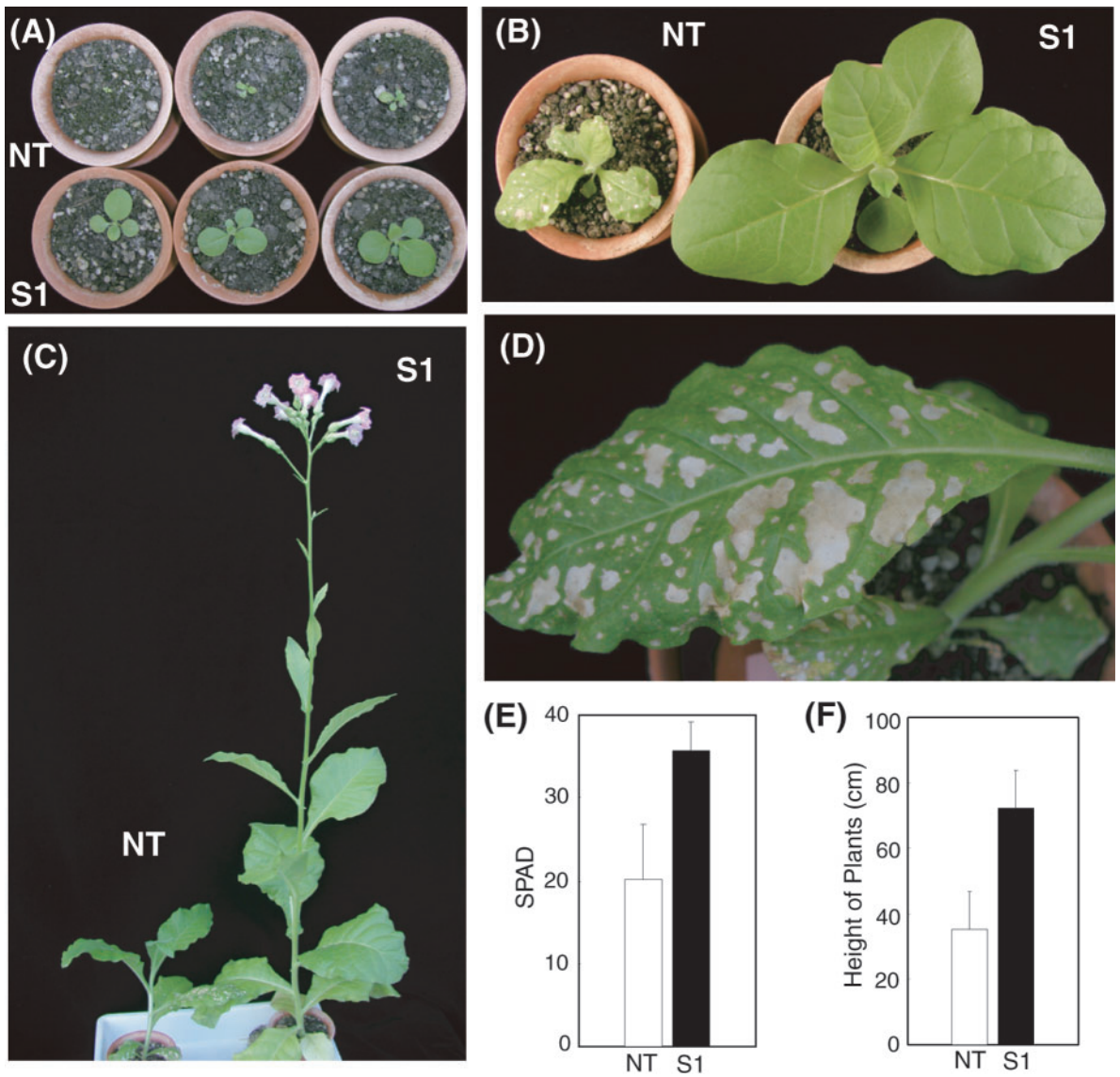


Fig. 5 Growth of NT and transgenic (line S1) tobacco plants grown in an Ni-enriched serpentine soil. (A) NT (upper three pots) and transgenic S1 tobacco plants (lower three pots) grown in Ni-enriched serpentine soil for 25 d. (B) Leaves of NT (left) and S1 tobacco (right) plants grown for 35 d. During the early stages of development, the NT plants are smaller and have necrotic patches in the leaves, whereas the transgenic S1 plants do not develop any sign of Ni toxicity. (C) Transgenic S1 tobacco plants at the flowering stage grown for 2 months in a serpentine soil. The NT plant shows reduced growth. (D) An old leaf of an NT plant displays necrosis caused by excess Ni. (E) SPAD values of NT and S1 tobacco plants. (F) Plant heights after 45 d grown in a serpentine soil. Error bars represent standard errors ($n = 17$).

histidine, which acts as a metal chelator, is responsible for the Ni hyperaccumulation by *Alyssum* and *Brassica* spp. (Kerkeb and Krämer 2003). Freeman et al. (2004) have reported that increased glutathione biosynthesis plays a role in Ni tolerance in a *Thlaspi* Ni hyperaccumulator.

NA is a promising candidate for chelating metals and transporting metals within plants (Koike et al. 2004). The constitutive overproduction of NA and high expression of NAS have been reported in the Zn hyperaccumulator *A. halleri* (Weber et al. 2004). Furthermore, Takahashi et al. (2003) reported that transgenic tobacco plants overexpressing *HvNAS1* contained increased Fe and Zn concentrations in the young leaves and flowers. The recent identification of Ni^{2+} complexed to NA in the Ni and Zn hyperaccumulator *T. caerulescens* (Vacchina et al. 2003) suggests that NA plays an important role in the detoxification of Ni in hyperaccumulating plants. Douchkov et al. (2005) reported that ectopic expression of *AtNAS1* in tobacco results in enhanced tolerance of seedlings on medium containing excess Ni.

In this study, the transgenic plants with increased NA biosynthesis showed improved tolerance to various metal stresses. In particular, the transgenic lines were much more tolerant of

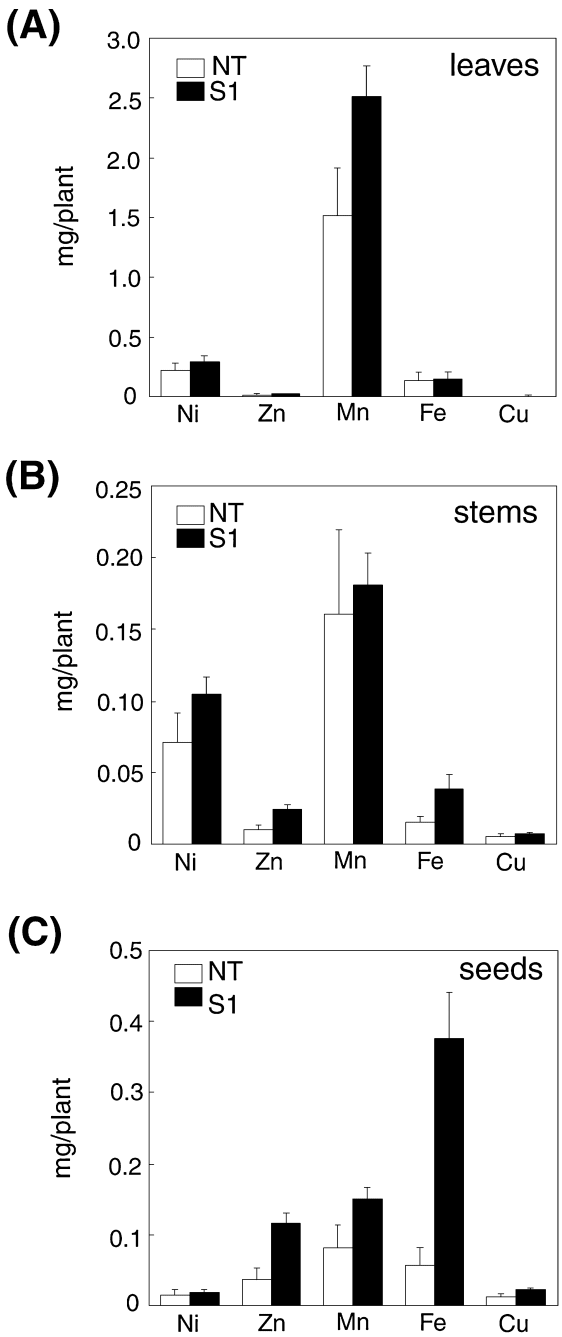


Fig. 6 The amount of metals in NT or S1 tobacco plants grown in a serpentine soil for 45 d. (A) The amount of metals in the leaves of NT and transgenic (line S1) tobacco plants. (B) The amount of metals in the stems of NT and transgenic (line S1) tobacco plants. (C) The amount of metals in the seeds of NT and transgenic (line S1) tobacco plants. Error bars indicate the standard errors of the mean (n = 17).

high concentrations of Ni than the NT plants, confirming that the overexpression of *HvNAS1* in transgenic plants effectively enhances their tolerance to Ni stress. Interestingly, promoter activities of four *AtNAS* genes in *Arabidopsis* were all enhanced in response to excess Ni in the medium, although their expression patterns were different in roots and shoots. This suggests that *Arabidopsis* plants may increase the expression of all four *AtNAS* genes to enhance NA biosynthesis and tolerate excess Ni. It is interesting why the transgenic plants were particularly tolerant to excess Ni, although NA can form stable complexes with Zn, Mn, Cu and Fe in addition to Ni. The log stability constants of metal–NA complexes are as follows: Mn(II), 8.8; Fe(II), 12.1; Zn, 14.7; Ni, 16.1; Cu(II), 18.6; and Fe(III), 20.6 (Benes et al. 1983, von Wirén et al. 1999). Since NA is a strong chelator of Ni, the chelation of Ni by NA in the xylem or phloem may be an important factor in the tolerance of plants to Ni toxicity. It may also be possible that NA chelates excess Ni in the cytoplasm and Ni–NA complexes are sequestrated into the vacuole within the cell. Both NA and the transporters of metal–NA complexes play a major role in metal homeostasis, in removing high concentrations of metal ions from sensitive sites within a cell.

In graminaceous plants, we showed that one of the ZmYS1 homologs in rice, OsYSL2, is an Fe-regulated metal–NA transporter and is involved in the long-distance transport and accumulation of Fe in grains (Koike et al. 2004). Schaaf et al. (2004) showed that ZmYS1 encodes a proton-coupled broad-range metal–phytosiderophore transporter that also transports Fe–NA and Ni–NA in maize. Roberts et al. (2004) also reported that ZmYS1 transports Fe–NA. YS1 homologs in *Arabidopsis* (AtYSL) are postulated to be metal–NA transporters in non-graminaceous plants. DiDonato et al. (2004) reported that AtYSL2 transports Cu–NA and Fe(II)–NA. Although no YSL family protein that transports Ni–NA in *Arabidopsis* or tobacco has been identified yet, presumably one of the YSL proteins transports Ni–NA and plays a role in Ni transport and the detoxification of excess Ni in these plants.

The transgenic tobacco plants with increased NA biosynthesis showed improved tolerance to excess Ni and accumulated a large amount of Ni in the shoots (Fig. 3, 4, 6). This suggests a possible use for transgenic plants overexpressing the *NAS* gene in Ni phytoextraction. The greater tolerance to high Ni and the greater biomass of transgenic tobacco plants support the possible use of *HvNAS1* overexpression, as in S1 plants, as a strategy for Ni phytoremediation. Furthermore, the amounts of Fe, Mn and Zn were higher in S1 shoots than in the NT, indicating that NA promotes the transport of these metals to the shoots.

In conclusion, our results show that *Arabidopsis* plants exposed to excess Ni produce a higher amount of NA with enhanced activities of native AtNAS to tolerate Ni toxicity. Furthermore, we showed that increased NA biosynthesis caused by barley NAS genes in plants contributes to the detoxification of Ni. This suggests a means whereby plants can be developed for phytoremediation purposes, particularly for soils highly contaminated with Ni.

Materials and Methods

Construction of binary vectors and transformation

A cauliflower mosaic virus (CaMV) 35S–*HvNAS1* open reading frame (ORF)–nopaline synthase 3′-untranslated region (UTR) cassette was cloned between the *Hin*dIII and *Not*I sites of pBIGRZ1 (Akiyama et al. 1997), and this vector, designated 35S-*HvNAS1* by Higuchi et al. (2001), was introduced into *Arabidopsis* [ecotype Columbia (Col-0)] and tobacco (*Nicotiana tabacum* cv. SR1) by *Agrobacterium*-mediated transformation. For GUS assay of *Arabidopsis*, four vectors were constructed as follows. Genomic sequences containing putative promoter regions of *AtNAS1*, *AtNAS2* and *AtNAS3* (–3,000 to –1 bp from the translational initiation codon) were amplified by PCR from genomic DNA. PCR amplification was conducted by dividing each promoter region into two or three parts. Both ends of each DNA fragment amplified by PCR were digested by restriction enzymes and then ligated. Each DNA fragment of the total promoter region was subcloned into the upstream area of the ORF of the *uid A* gene, which encodes GUS in the plasmid pCB308 (Xiang et al. 1999). For *AtNAS1*, six primers were used: 5′-GAGAGAGAGCCGGCCGCTAGCTTGATTTTACGCGGCACAAATATATATT-3′, 5′-GAGAGAGACTGCAGTGACGTAAAACATTTTGCTAAAAGTATAAG-3′, 5′-GAGAGAGAGGATCCTTATAGATCAGTTAAATATATAGAGTTAAA-3′, 5′-CCATGCTTTGTTGGGGATAAATCTCAATTG-3′, 5′-TTAAATATAGCGGAAGTCTAGAGCCCGACG-3′ and 5′-GAGAGAGAGTCGACGCTAGCTCGACACTATGAGGTTATTTCAGGGGAGA-3′. The restriction enzymes used for digesting the three DNA fragments were as follows: *Not*I, *Nhe*I, *Pst*I, *Bam*HI, *Eco*RI, *Sal*I and *Bcl*I. For *AtNAS2*, four primers were used: 5′-GAGAGAGAGGTACCTCTAGACAAATCCCAAAACACAAACAGCATTTTCA-3′, 5′-GAGAGAGAGAGCTCTGAGTGCGACGATCCACGTAGTCATATACT-3′, 5′-GAGAGAGACTCGAGACCATACTAAATCTCCAGAAATTTTGATAC-3′ and 5′-GAGAGAGATCTAGAGTCGACACTATGAAGAGAGAATATTGAGTT-3′. The restriction enzymes used for digesting the two DNA fragments were as follows: *Kpn*I, *Sac*I, *Xba*I, *Xho*I and *Nde*I. For *AtNAS3*, four primers were used: 5′-GAGAGAGACTCGAGGATCCTCTCTGTACTTGTAGAACCTTGCCAAGAA-3′, 5′-GAGAGAGACTGCAGAGATACACACACACAACAACTTGAAAGG-3′, 5′-GAGAGAGACTGCAGAAATATGATGCGTCATTTACATAAAAGAAT-3′ and 5′-GAGAGAGAGGATTCGTCGACACTATGAGGTTATTGATTTTTCTC-3′. The restriction enzymes for digesting two DNA fragments are as follows: *Xho*I, *Pst*I, *Bam*HI, *Cla*I and *Spe*I. For *AtNAS4*, two primers were used: 5′-GAGAGATCTAGATAACGTACAAGATCAGACAGTGTTT-3′ and 5′-GAGAGAACTAGTGTCGACACTATGAGGTTTTACGAGA-3′. The DNA fragment was digested by *Spe*I. Transformation and regeneration were performed in tobacco using the standard leaf disc transformation method (Helmer et al. 1984). The transformation procedures were performed in *Arabidopsis* using two transformation methods: the floral dip method (Clough and Bent 1998) and the infiltration method (Bechtold et al. 1993).

Northern blot analysis

Total RNA was isolated from the shoots of transgenic and non-transformant plants using RNeasy Plant Mini kits (Qiagen, Tokyo, Japan). RNA (10 μg) was denatured and electrophoresed on 1.2% (w/v) agarose gels containing 0.66 M formaldehyde and transferred to Hybond-N^+ membranes (Amersham Biosciences Corp., Piscataway, NJ, USA). The blotted membrane was hybridized at 65°C with an α-^{32}P-labeled probe specific to *HvNAS1*.

Plant material, growth conditions and heavy metal treatments

Transgenic plants transformed with the pBIGRZ1 vector carrying the CaMV *35S* promoter-*HvNAS1* gene were examined for overexpression of *NAS*. Seeds were surface sterilized and sown on MS medium (Murashige and Skoog 1962) supplemented with metals at the following concentrations: Fe (150–300 μM Fe-EDTA), Mn (0.50–2 mM $MnSO_4$), Zn (0.25–1 mM $ZnSO_4$), Cu (50–65 μM $CuSO_4$), Cd (50–60 μM $CdSO_4$) and Ni (100–350 μM $NiSO_4$). *Arabidopsis* seedlings were grown in a growth chamber for 14 d with a 16 h/8 h day/night cycle at 23°C. Tobacco seedlings were grown with a 16 h/8 h day/night cycle for 20 d at 25°C.

GUS assay

The GUS activity was assayed using the method described by Jefferson (1987). The roots and leaves of each transformant were homogenized with GUS extraction buffer [50 mM sodium phosphate, 10 mM β-mercaptoethanol, 10 mM Na_2-EDTA, 0.1% sodium lauryl sarcosine and 0.1% (v/v), Triton X-100] and were centrifuged at 15,100×*g* for 5 min. The supernatants were mixed with the assay buffer (1 mM 4-methylumbelliferyl-D-glucuronide in extraction buffer) and incubated at 37°C for 60 min. The concentration of the reaction product (methylumbelliferone; MU) was determined using a spectrofluorometer (F2500, Hitachi, Tokyo, Japan) with excitation at 365 nm and emission at 455 nm.

Determining metal concentrations

Shoots were harvested and dried at 80°C for 3 d. Dry shoot samples weighing 20–50 mg were mixed with 0.5 ml of nitric acid in a sealed polytetrafluoroethylene vessel with a stainless steel jacket, and heated in an oven at 150°C for 5 h. After cooling to room temperature, the samples were dissolved in 3 ml of deionized water and the metal concentrations measured using inductively coupled plasma atomic emission spectrometry (SPS1200VR; Seiko, Tokyo, Japan).

Extracting and measuring endogenous NA

Freshly harvested plant leaves were frozen and stored at –80°C. Frozen plant materials were homogenized with a mortar and pestle in liquid nitrogen and then thawed by mixing with 20 vols of deionized water (w/v). The sample was heated to 80°C for 20 min and centrifuged. The supernatants were applied to a DOWEX column (Muromachi Technos, Tokyo, Japan). After washing the column with 0.2 M pyridine, the fraction was eluted with 0.5 M AcOH and concentrated in a rotary evaporator at 40°C. HPLC was used for quantitative analysis of NA (Takahashi et al. 2003).

Plant growth in Ni enriched soil

Seeds were germinated and grown at a constant temperature of 25°C under natural light (106 μmol m^{-2} s^{-1}) in pots containing an Ni-enriched serpentine soil (soil taxonomy: Typic Hydraquents, ultramafic rock) supplied with coated fertilizers. The soil is collected from Yaunnai, Horokanai-cho, Uryugun, Hokkaido, Japan. The ultramafic rock in this area contains 3,000 mg^{-1} kg^{-1} Ni. The concentration of $CH_3CO_2NH_4$-soluble Ni is 9.8 mg^{-1} kg^{-1} (167 μM) at pH 4.9. The plants were watered every day with tap water. The heights of the plants were measured and the degree of chlorosis of the youngest leaves was determined using an SPAD-502 chlorophyll meter (Minolta, Tokyo, Japan).

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References

Akiyama, K., Nakamura, S., Suzuki, T., Wisniewska, I., Sasaki, N. and Kawasaki, S. (1997) Development of a system of rice transformation with long genome inserts for their functional analysis for potisional cloning. *Plant Cell Physiol.* 38 [Suppl]: s94.

Baker, A.J.M. and Brooks, R.R. (1989) Terrestrial higher plants which hyperaccumulate metallic elements—a review of their distribution, ecology and phytochemistry. *Biorecovery* 1: 81–126.

Bechtold, N., Ellis, J. and Pelletier, G. (1993) In planta agrobacterium mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C. R. Acad. Sci. Paris Life Sci.* 316: 1194–1199.

Benes, I., Schreiber, K., Ripperger, H. and Kircheiss, A. (1983) Metal complex formation by nicotianamine, a possible phytosiderophore. *Experientia* 39: 261–262.

Brooks, R.R., Shaw, S. and Asensi Marfil, A. (1981) The chemical form and physiological function of nickel in some Iberian *Alyssum species*. *Physiol. Plant.* 51: 167–70.

Brown, P.H., Welch, R.M. and Cary, E.E. (1987) Nickel: a micronutrient essential for higher plants. *Plant Physiol.* 85: 801–803.

Brune, A. and Dietz, K.-J. (1995) A comparative analysis of element composition of roots and leaves of barley seedlings grown in the presence of toxic cadmium, molybdenum, nickel and zinc concentrations. *J. Plant Nutr.* 18: 853–868.

Chaney, R.L. (1983) Plant uptake of inorganic waste constituents. *In* Land Treatment of Hazardous Wastes. Edited by Parr, J.F., Marsh, P.B. and Kla, J.M. pp. 50–76. Noyes Data, Park Ridge, NJ.

Clemens, S. (2001) Molecular mechanisms of plant metal tolerance and homeostasis. *Planta* 212: 475–486.

Clemens, S., Kim, E.J., Neumann, D. and Schroeder, J.I. (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. *EMBO J.* 18: 3325–3333.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16: 735–743.

Cobbett, C.S. (2000) Phytochelatin biosynthesis and function in heavy-metal detoxification. *Curr. Opin. Plant Biol.* 3: 211–216.

Cobbett, C. and Meagher, R. (2002) Phytoremediation and the *Arabidopsis* proteome. *In Arabidopsis*. Edited by Somerville, C. pp. 1–22. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Curie, C., Panaviene, Z., Loulergue, C., Dellaporta, S.L., Briat, J.-F. and Walker, E.L. (2001) Maize *yellow stripe1* encodes a membrane protein directly involved in Fe(III) uptake. *Nature* 409: 346–349.

DiDonato, R.J., Jr, Roberts, L.A., Sanderson, T., Eisley, R.B. and Walker, E.L. (2004) Arabidopsis Yellow Stripe-Like2 (YSL2): a metal-regulated gene encoding a plasma membrane transporter of nicotianamine–metal complexes. *Plant J.* 39: 403–414.

Douchkov, D., Gryczka, C., Stephan, U.W., Hell, R. and Bäumlein, H. (2005) Ectopic expression of nicotianamine synthase genes results in improved iron accumulation and increased nickel tolerance in transgenic tobacco. *Plant Cell Environ.* 28: 365–374.

Freeman, J.L., Persans, M.W., Nieman, K., Albrecht, C., Peer, W., Pickering, I.J. and Salt, D.E. (2004) Increased glutathione biosynthesis plays a role in nickel tolerance in thlaspi nickel hyperaccumulators. *Plant Cell* 16: 2176–2191.

Gaxiola, R.A., Fink, G.R. and Hirschi, K.D. (2002) Genetic manipulation of vacuolar proton pumps and transporters. *Plant Physiol.* 129: 967–973.

Godbold, D.L., Horst, W.J., Collins, J.C., Thurman, D.A. and Marschner, H. (1984) Accumulation of zinc and organic acid in roots of zinc tolerant and non-tolerant ecotypes of *Deshampsia caespitosa*. *J. Plant Physiol.* 116: 59–69.

Grotz, N., Fox, T., Connolly, E., Park, W., Guerinot, M.L. and Eide, D. (1998) Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency. *Proc. Natl Acad. Sci. USA* 95: 7220–7224.

Guerinot, M.L. (2000) The ZIP family of metal transporters. *Biochim. Biophys. Acta* 1465: 190–198.

Ha, S.B., Smith, A.P., Howden, R., Dietrich, W.M., Bugg, S., O'Connell, M.J., Goldsbrough, P.B. and Cobbett, C.S. (1999) Phytochelatin synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe*. *Plant Cell* 11: 1153–1163.

Hall, J.L. (2002) Cellular mechanisms for heavy metal detoxification and tolerance. *J. Exp. Bot.* 366: 1–11.

Helmer, G., Casadaban, M., Bevan, M., Kayes, L. and Chilton, M.D. (1984) A new chimeric gene as a marker for plant transformation: the expression of *Escherichia coli* β-galactosidase in sunflower and tobacco cells. *Biotechnology* 2: 520–527.

Herbik, A., Koch, G., Mock, H.-P., Dushkov, D., Czihal, A., Thielmann, J., Stephan, U.W. and Bäumlein, H. (1999) Isolation, characterization and cDNA cloning of nicotianamine synthase from barley. *Eur. J. Biochem.* 265: 231–239.

Higuchi, K., Nishizawa, N., Römheld, V., Marschner, H. and Mori, S. (1996) Absence of nicotianamine synthase activity in the tomato mutant '*chloronerva*'. *J. Plant Nutr.* 19: 1235–1239.

Higuchi, K., Nishizawa, N.-K., Yamaguchi, H., Röheld, H., Marschner, H. and Mori, S. (1995) Response of nicotianamine synthase activity to Fe-deficiency in tobacco plants as compared with barley. *J. Exp. Bot.* 46: 1061–1063.

Higuchi, K., Suzuki, K., Nakanishi, H., Yamaguchi, H., Nishizawa, N.K. and Mori, S. (1999) Cloning of nicotianamine synthase genes, novel genes involved in the biosynthesis of phytosiderophores. *Plant Physiol.* 119: 471–480.

Higuchi, K., Watanabe, S., Takahashi, M., Kawasaki, S., Nakanishi, H., Nishizawa, N.K. and Mori, S. (2001) Nicotianamine synthase gene expression differs in barley and rice under Fe-deficient conditions. *Plant J.* 25: 159–167.

Homer, F.A., Reeves, R.D., Brooks, R.R. and Baker, A.J.M. (1991) Characterization of the nickel-rich extract from the nickel hyperaccumulator *Dichapetalum gelonioides*. *Phytochemistry* 30: 2141–2145.

Jefferson, R.A. (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.* 5: 387–405.

Kerkeb, N. and Krämer, U. (2003) The role of free histidine in xylem loading of nickel in *Alyssum lesbiacum* and *Brassica juncea*. *Plant Physiol.* 131: 716–724.

Koike, S., Inoue, H., Mizuno, D., Takahashi, M., Nakanishi, H., Mori S. and Nishizawa, N.K. (2004) OsYSL2 is a rice metal–nicotianamine transporter that is regulated by iron and expressed in the phloem. *Plant J.* 39: 415–24.

Krämer, U., Cotter-Howells, J.D., Charnock, J.M., Baker, A.J.M. and Smith, J.A.C. (1996) Free histidine as a metal chelator in plants that accumulate nickel. *Nature* 379: 635–638.

Krotz, R.M., Evangelou, B.P. and Wagner, G.J. (1989) Relationships between cadmium, zinc, Cd–peptide, and organic acid in tobacco suspension cells. *Plant Physiol.* 91: 780–787.

Li, Y., Dhankher, O.P., Carreira, L., Lee, D., Chen, A., Schroeder, J.I., Balish, R.S. and Meagher, R.B. (2004) Overexpression of phytochelatin synthase in *Arabidopsis* leads to enhanced arsenic tolerance and cadmium hypersensitivity. *Plant Cell Physiol.* 45: 1787–1797.

Ling, H.-Q., Koch, G., Bäumlein, H. and Ganal, M.W. (1999) Map-based cloning of *chloronerva*, a gene involved in iron uptake of higher plants encoding nicotianamine synthase. *Proc. Natl Acad. Sci. USA* 96: 7098–7103.

Ma, J.F., Ryan, P.R. and Delhaize, E. (2001) Aluminium tolerance in plants and the complexing role of organic acids. *Trends Plant Sci.* 6: 273–278.

Marschner, H. (1995) Mineral Nutrition in Higher Plants, 2nd edn. Academic Press, London.

Mills, R.F., Krijger, G.C., Baccarini, P.J., Hall, J.L. and Williams, L.E. (2003) Functional expression of AtHMA4, a P1B-type ATPase in the Zn/Co/Cd/Pb subclass. *Plant J.* 35: 164–175.

Mizuno, D., Higuchi, K., Sakamoto, T., Nakanishi, H., Mori, S. and Nishizawa, N.K. (2003) Three nicotianamine synthase genes isolated from maize are differentially regulated by iron nutritional status. *Plant Physiol.* 132: 1989–1997.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* 15: 473–497.

Nigma, R., Srivastava, S., Prakash, S. and Srivastava, M.M. (2001) Cadmium mobilization and plant availability—the impact of organic acids commonly exuded from roots. *Plant Soil* 230: 107–113.

Noma, M. and Noguchi, M. (1976) Occurrence of nicotianamine in higher plants. *Phytochemistry* 15: 1701–1702.

Noma, M., Noguchi, M. and Tamaki, E. (1971) A new amino acid, nicotianamine, from tobacco leaves. *Tetrahedron Lett.* 22: 2017–2020.

Persans, M.W., Nieman, K. and Salt, D.E. (2001) Functional activity and role of cation-efflux family members in Ni hyperaccumulation in *Thlaspi goesingense*. *Proc. Natl Acad. Sci. USA* 98: 9995–10000.

Pich, A. and Scholz, G. (1996) Translocation of copper and other micronutrients in tomato plants (*Lycopersicon esculentum Mill*.): nicotianamine-stimulated copper transport in the xylem. *J. Exp. Bot.* 294: 41–47.

Roberts, L.A., Pierson, A.J., Panaviene, Z. and Walker, E.L. (2004) Yellow stripe1. Expanded roles for the maize iron–phytosiderophore transporter. *Plant Physiol.* 135: 112–120.

Rudolph, A., Becker, R., Scholz, G., Procházka, Z., Toman, J., Macek, T. and Herout, V. (1985) The occurrence of the amino acid nicotianamine in plants and microorganisms: a reinvestigation. *Biochem. Physiol. Pflanz.* 180: 557–563.

Salt, D.E., Blaylock, M., Kumar, N.P.B.A., Dushenkov, V., Ensley, B.D., Chet, I. and Raskin, I. (1995) Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. *Biotechnology* 13: 468–474.

Schaaf, G., Ludewig, U., Erenoglu, B.E., Mori, S., Kitahara, T. and von Wirén, N. (2004) ZmYS1 functions as a proton-coupled symporter for phytosiderophore- and nicotianamine-chelated metals. *J. Biol. Chem.* 279: 9091–9096.

Shojima, S., Nishizawa, N.K., Fushiya, S., Nozoe, S., Irifune, T. and Mori, S. (1990) Biosynthesis of phytosiderophores. In vitro biosynthesis of 2′-deoxymugineic acid from L-methionine and nicotianamine. *Plant Physiol.* 93: 1497–1503.

Shojima, S., Nishizawa, N.K., Fushiya, S., Nozoe, S., Kumashiro, T., Nagata, T., Ohata, T. and Mori, S. (1989) Biosynthesis of nicotianamine in the suspension-cultured cells of tobacco (*Nicotiana megalosiphon*). *Biol. Metals* 2: 142–145.

Suzuki, K., Higuchi, K., Nakanishi, H., Nishizawa, N.K. and Mori, S. (1999) Cloning of nicotianamine synthase genes from *Arabidopsis thaliana*. *Soil Sci. Plant Nutr.* 45: 993–1002.

Takagi, S. (1976) Naturally occurring iron-chelating compounds in oat- and rice-root washings. I. Activity measurement and preliminary characterization. *Soil Sci. Plant Nutr.* 22: 423–433.

Takahashi, M., Terada, Y., Nakai, I., Nakanishi, H., Yoshimura, E., Mori, S. and Nishizawa N.K. (2003) Role of nicotianamine in the intracellular delivery of metals and plant reproductive development. *Plant Cell* 15: 1263–1280.

Ushio, Y., Mori, S. and Nishizawa, N.K. (2003) Novel nicotianamine synthase and the *AtNAS4* gene in Arabidopsis. Japanese Patent No. 2003–70926.

Vacchina, V., Mari, S., Czernic, P., Marquès, L., Pianelli, K., Schaumlöffel, D., Lebrun, M. and Lobinski, R. (2003) Speciation of nickel in a hyperaccumulating plant by high-performance liquid chromatography-inductively coupled plasma mass spectrometry and electrospray MS/MS assisted by cloning using yeast complementation. *Anal. Chem.* 75: 2740–2745.

Vatamaniuk, O.K., Mari, S., Lu, Y.P. and Rea, P.A. (1999) AtPCS1, a phytochelatin synthase from *Arabidopsis*: isolation and in vitro reconstitution. *Proc. Natl Acad. Sci. USA* 96: 7110–7115.

von Wirén, N., Klair, S., Bansal, S., Briat, J.F., Khodr, H., Shioiri, T., Leigh, B.A. and Hider, R.C. (1999) Nicotianamine chelates both $Fe^{III}$and Fe^{II}: implications for metal transport in plants. *Plant Physiol.* 119: 1107–1114.

Weber, M., Harada, E., Vess, C., v. Roepenack-Lahaye, E. and Clemens, S. (2004) Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. *Plant J.* 37: 269–281.

Williams, L.E., Pittman, J.K. and Hall, J.L. (2000) Emerging mechanisms for heavy metal transport in plants. *Biochim. Biophys. Acta* 1465: 104–126.

Xiang, C., Han, P., Lutziger, I., Wang, K. and Oliver, D.J. (1999) A mini binary vector series for plant transformation. *Plant Mol. Biol.* 40: 711–717.

Yang, X.E., Baligar, V.C., Foster, J.C. and Martens, D. (1997) Accumulation and transport of nickel in relation to organic acids in ryegrass and maize grown with different nickel levels. *Plant Soil* 196: 271–276.

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