

TIP3;2 is a Tonoplast Transporter Contributed to Nickel Detoxification in *Arabidopsis thaliana*

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Abstract

Nickel (Ni) is a necessary trace element for plants, but excessive amounts can be harmful. To mitigate Ni toxicity, plants have evolved distinct mechanism, such as storing Ni in the vacuole or confining it to less sensitive root tissues to prevent its translocation to the vulnerable shoot tissues. Despite this, the exact mechanism of Ni immobilization remains unclear. In *Arabidopsis*, sequestration of excess Ni into root vacuoles is crucial for Ni immobilization, facilitated by distinct tonoplast-localized transporters. As some members of the aquaporin superfamily have been implicated in transporting both metal ions and polar, non-charged small molecules, we explored whether *Arabidopsis thaliana* tonoplast intrinsic proteins (TIPs) are involved in Ni immobilization and tolerance. We found that TIP3;2 helps retain excess Ni in the root, restricts its translocation to the shoot, and facilitates its accumulation in the leaf trichome. Furthermore, when TIP3;2 was expressed in yeast, its enhanced Ni resistance, suggesting that TIP3;2 plays a vital role in Ni detoxification. In addition, partial expression of TIP3;2 at yeast plasma membrane demonstrated its capability to facilitate the uptake of Ni-PC complexes into yeast cells. The results reveal a dual function for TIP3;2 in Ni detoxification and underscore the expand substrate specificity of aquaporins to include heavy-metal complexes, uncovering a new aspect of plant adaptive responses to metal stress.

Introduction

Nickel (Ni) is an essential trace element for plant growth and development at low concentrations. Ni deficiency can inhibit plant growth, disrupt nitrogen and iron uptake, and lead to chlorosis and plant senescence [1, 2]. On the other hand, Ni is harmful at high concentrations [3]. Excess Ni results in decreased germination, cell division, biomass accumulation, nutrients absorption, and metabolic disorders [4-6]. As a result, all adverse effects of Ni toxicity reduce crop production and quality [7-9]. Ni is also toxic to humans, which is associated with lung cancer, kidney ailments and cardiovascular problems [10, 11]. With the increasing industrialization and urbanization, heavy metal pollution in soil, including Ni, become a worldwide environmental issue [12]. Plants are more frequently exposed to Ni toxicity than Ni deficit [13].

Some hyperaccumulator plants can thrive on Ni contaminated soils, and accumulate more Ni in their tissues, especially in leaves, without no toxic effects [14, 15]. This ability highlights the existence of specialized Ni transport and detoxification system in plants. Phytoremediation using hyperaccumulators represents an eco-friendly strategy for soil decontamination [16-18]. However, most known hyperaccumulators are photosynthesis C3 plants, which typically produce lower biomass than C4 species [19, 20]. This limitation extends the time required for effective phytoextraction [21]. Identifying key heavy metal transporters and leveraging molecular biology tools [22, 23] could enable the development of engineered “super plants” with desirable traits such as deep roots, fast growth, high biomass, and efficient metal translocation and accumulation. Such advancements would make phytoremediation a more viable and faster solution to the growing challenge of heavy metal pollution.

Plant aquaporins (AQPs) are integral membrane proteins that form a large gene family [24, 25]. They are characterized by two conserved asparagine-proline-alanine (Asn-Pro-Ala, NPA) motifs embedded in the plasma membrane and correlate with substrate selectivity [26, 27]. Another conserved structural feature of AQP family is the aromatic/arginine (ar/R) constriction site, which contains highly conserved aromatic and arginine residues that acts as a selectivity filter [26, 27]. While traditionally known to transport polar and non-charged small solutes and metalloids across cellular membranes [25, 28], recent evidence indicates that some AQPs can also facilitate the transport of metal ions [29]. For example, our recent studies suggest that *Arabidopsis* AtNIP1;2 transports aluminum-malate (Al-Mal) complexes, thereby alleviating Al toxicity by removing Al³⁺ from the root cell wall – the primary site of Al damage [30-35]. Similarly, the tonoplast-localized AtTIP2;2 plays an important role in Zn immobilization and sequestration into the root vacuole in *Arabidopsis*, enhancing Zn tolerance [36]. In rice, a coordinated system involving plasma-localized OsNIP1;2 and tonoplast-localized OsTIP2;1 facilitates the removal of cell wall Al and its vacuolar sequestration, improving Al detoxification [37, 38].

Building on this foundation, we further investigated the potential role of the tonoplast intrinsic protein TIP3;2 (NCBI accession number: NP_173223.1, <https://www.ncbi.nlm.nih.gov/datasets/gene/838359/#transcripts-and-proteins>) in Ni detoxification and resistance in *Arabidopsis*. We report that *tip3;2* mutants are sensitive to Ni stress, and that TIP3;2 facilitates sequestration of excess Ni into vacuole in roots and is crucial for conferring Ni resistance. Our findings establish TIP3;2 as a key component of the Ni detoxification machinery in *Arabidopsis*.

Materials and Methods

Plant Material and Growth Conditions.

The *Arabidopsis thaliana* T-DNA insertion mutants *tip3;2-1* (At1g17810, SALK_091612) and *tip3;2-2* (At1g17810, SALK_125353) were acquired from the Arabidopsis Biological Resource Center (ABRC) (<https://abrc.osu.edu/>). Wild-type (Col-0), and mutant seeds were surface-sterilized, cold stratified and sown on 250 µm polypropylene meshes floating on hydroponic solutions, at pH6.5, supplemented without or with Ni or other metal ions in Magenta boxes. The components of the hydroponic solution are described in reference [32].

Relative root growth (RRG %) was calculated as the percentage of root growth of individual plants under toxic Ni treatment over the average root growth under the control (0.5 µM Ni) condition. Three biological replicates (Magenta boxes) were conducted for each treatment.

For testing the sensitivity to other metals, WT and two *tip3;2* SALK lines were treated with hydroponic solutions (pH 6.5) containing (in µM) 50, ZnSO₄; 6, CdCl₂; 200, MnCl₂; 20, AlCl₃; or 5, CuSO₄ for 7 days and the primary root length was measured.

GUS Staining Assays and Localization of GUS Expression.

A 1.9 kb TIP3;2 promoter (ATG as +1) was PCR-amplified from Arabidopsis genomic DNAs with primers 5'-GGTTAAGCTTTACGACTCGATGCCTTCTC-3' and 5'-AGCACCATGGCTCTCCCGAATCCATATGC-3' (the underlined sequences are restriction enzyme sites for *Hind* III: and *Nco* I, respectively), and then cloned into the pCAMBIA1305.2 vector. The resulting TIP3;2 promoter:: β -glucuronidase (GUS) construct was transformed into the WT (Col-0) genome through *Agrobacterium tumefaciens* (strain GV3101)-mediated transformation. GUS staining were processed according to the methodology outlined in a previous paper [36].

Subcellular Localization of TIP3;2.

The coding sequence of TIP3;2, excluding the stop codon, was amplified from the TIP3;2 cDNA using the primers 5'-TCGCGGATCCAAAATGGCTACATCTGCTAGAAG-3' and 5'-ATGGCTCGAGGTAATCTTCCGGAGCCAAT-3' (the underlined sequences are restriction enzyme sites for *Bam*HI and *Xho*I, respectively), and then cloned in frame with the 5' end of the GFP coding region in the pGPTV.GFP.Bar vector. The resulting TIP3;2-GFP construct was then transformed into *Agrobacterium tumefaciens* strain GV3101, followed by transiently transformed into the tobacco leaves by infiltration. The vacuolar membrane marker 35S::TIP1;1-RFP (vac-rk-CD3-975) was as described in Nelson et al. [39]. GFP signals were observed with a Leica SP5 confocal laser microscope.

RNA Isolation and Quantitative Real-time RT-qPCR.

Total RNAs were extracted from Arabidopsis root, stem, shoot or seed using the RNeasy Mini Kit (Qiagen) following the manufacturer's instruction and according to previously described [32]. The relative expression levels of the target genes were referred to an endogenous calibrator gene, 18S rRNA, for each RT-qPCR experiment. The sequences of real-time primers for *TIP2;2* are: 5'-AGTACATGATCATCCCCAG-3' and 5'-CATAGGAAATGGCAGGAAAAC-3'.

Root and Shoot Ni Content Measurement.

Seven-day-old seedlings of the indicated lines were cultured in a hydroponic solution (pH 6.5) with or without the supplement of 0.5 or 50 μ M NiCl₂ for 24h. Plant samples were harvested and processed according to previous paper [36]. Elements of each sample were analyzed with ICP-MS (inductively coupled plasma mass spectrometry). Three biological replicates for each treatment were made.

Cell Sap and Cell Wall Preparation and Ni Content Determination.

Following a 24-h treatment with 0.5 or 50 μ M NiCl₂ (pH 6.5), roots or shoots were excised. The collection and analysis procedures were detailed by Wang et al [36]. Nickel contents in the samples were analyzed by ICP-MS.

Collection and Analysis of Xylem Sap.

Six weeks old seedlings were subjected to 0.5 or 50 μM NiCl_2 treatment for 24h. Xylem sap collection followed the previous procedures [36]. ICP-MS was used to determine the Ni and K concentrations.

Trichome Isolation

Trichomes were collected and processed according to the methodology outlined in a previous paper [36]. Ni content in the trichomes were analyzed by ICP-MS.

Yeast Ni Sensitivity Analysis.

The coding sequence of TIP3;2 was amplified with the primers 5'-TCGCGGATCCAAAATGGCTACATCTGCTAGAAG-3' and 5'-ATCCGCGGCCGCGTAATCTTCCGGAGCCAAT-3' (the underlined sequences are restriction enzyme sites for *Bam*H I and *Not* I, respectively). The PCR fragments were ligated to the corresponding restriction sites of the pYES2-GFP vector [37]. Yeast cells expressing the pYES2-TIP3;2-GFP construct were observed under a Leica SP5 confocal laser microscope.

For Ni sensitivity evaluation, the TIP3;2 CDS fragment was obtained through PCR with primers: 5'-TCGCGGATCCAAAATGGCTACATCTGCTAGAAG-3' and 5'-ATCCGCGGCCGCGCTAGTAATCTTCCGGAGCCAAT-3', and subcloned into the pYES2 vector at *Bam*H I and *Not* I sites. The pYES2-TIP3;2 plasmid was transformed into yeast BY4741 cells. Three independent yeast BY4741 lines, containing the pYES2 empty vector, or pYES2-TIP3;2 plasmid, were selected and cultivated overnight in an SD-Ura medium (+2% glucose). Then, the cultures were centrifuged for 5 min (at 5000 g), and resuspended in the SD-Ura medium with 2% galactose and cultured overnight. After sequential 10-fold dilutions, 10 μl of cell suspensions were spotted on SD-Ura plates with different Ni concentration, and incubated at 30 $^{\circ}\text{C}$ for 3 days.

Creation of NIP1;2¹⁻³⁰::TIP3;2³¹⁻²⁶⁸ fusion protein and uptake assays in yeast.

To create NIP1;2¹⁻³⁰::TIP3;2³¹⁻²⁶⁸ fusion protein, the 1-150 nucleotide from ATG of TIP3;2 gene was replaced by NIP1;2 gene's 1-150 nucleotide with infusion PCR, the first PCR primer pairs are (1) 5'-CTACggatccAAAATGGCGGAGATCTCGGGAAA-3' and 5'-GTGAGCCGCCGTGTCCCAATAGAAAGGGACAGAGATAGAGAG-3' and (2) 5'-TATTGGGACACGGCGGCTCAC-3' and 5'-ATCCgcggccgcGTAATCTTCCGGAGCCAAT-3', using plasmid as template which contains NIP1;2 or TIP3;2 CDS respectively. The second PCR was performed with primer pair: 5'-CTACGGATCCAAAATGGCGGAGATCTCGGGAAA-3' and 5'-ATCCGCGGCCGCGTAATCTTCCGGAGCCAAT-3', (the underlined sequences are restriction enzyme sites for *Bam*H I and *Not* I, respectively), the first PCR products are as template. Then, the individual PCR fragments were sub-cloned into the *Bam*H I and the *Not* I restriction sites in frame with the 5' end of the GFP coding region in the pYES2-GFP construct. The resulting pYES2- NIP1;2¹⁻³⁰::TIP3;2³¹⁻²⁶⁸-GFP construct was transformed into yeast strain BY4741, and observed with a Leica SP5 confocal laser microscope.

The yeast lines with *pYES2-GFP* and *pYES2-NIP1;2¹⁻³⁰::TIP3;2³¹⁻²⁶⁸-GFP* were inoculated in an SC-Ura medium containing 2% galactose and grown at 30 °C to a mid-exponential phase. Then, the precultured yeast cells were resuspended at OD600 = 3.0 in the uptake medium, i.e., the SC-Ura medium supplemented with different possible transport substrates at final concentrations of 0 or 20 µM NiCl₂: 40 µM ligands. The Ni-ligands tested included Ni-Citrate, Ni-Malate, Ni-Nicotinamide, Ni-glutathione, Ni-phytochelatin, Ni- metallothionein.

After incubation in the uptake media for 4 h, yeast cells were harvested and washed three times with deionized water and dried in a 55°C oven for 2 days.. The Ni concentrations were determined by ICP-MS. Three biological replicates for each line and each treatment were conducted.

Results

***tip3;2* Mutants Are Sensitive Specifically to Ni Stress.**

Given the reported involvement of aquaporins in the transport of B, As, Sb, and Al, we investigated a potential role in Ni tolerance. We screened T-DNA insertion mutant lines for nine Arabidopsis *TIP* genes under Ni stress, obtained from the Arabidopsis Biological Resource Center (ABRC). Two independent *TIP3;2* (At1g17810) T-DNA lines, *tip3;2-1* (SALK_091612, intronic insertion) and *tip3;2-2* (SALK_125353, exonic insertion), exhibited significantly reduced *TIP3;2* transcript levels by real-time quantitative RT-PCR (qRT-PCR) (Figure 1D), and showed hypersensitivity to a range of Ni concentrations (Figure 1 A–C). These results suggest that *TIP3;2* contributes to Ni tolerance in *Arabidopsis*.

To assess specificity, we compared the sensitivity of wild-type and *tip3;2* mutants to other toxic metals, including Cd, Al, Zn, Cu and Mn. No difference in sensitivity were observed for Cd (6 µM), Al (20 µM), Zn (60 µM), Cu (5 µM) or Mn (200 µM) between WT and mutants (Supplemental Figure 1). These results indicate that the mutant phenotype is specific to Ni stress.

***TIP3;2* Expression is Induced by Ni.**

Specificity of *TIP3;2* expression was examined via *TIP3;2* promoter β-glucuronidase (GUS) analysis in transgenic Arabidopsis plants. GUS activity was consistently observed in roots, trichomes of the leaf and stem, and hydathode (Figure 2A-E). qRT-PCR analyses found *TIP3;2* expression mainly in the root, stem, shoot, but barely in the seed (Figure 3A). Time-course qRT-PCR analyses indicated that *TIP3;2* expression in the root was up-regulated rapidly and peaked with a 3.8-fold increase after 6 h of Ni treatment (Figure 3B). After 6 h treatments with different Ni concentrations, *TIP3;2* expression showed a concentration-dependent pattern (Figure 3C). In addition, *TIP3;2* expression was explicitly induced by Ni stress, but not by other toxic metal ions, including Cd²⁺, Al³⁺, Zn²⁺, Cu²⁺ and Mn²⁺ (Figure 3D).

***TIP3;2* is Localized to the Tonoplast**

To determine subcellular localization, the *TIP3;2* cDNA was fused with the GFP gene and the TIP3;2::GFP protein was transiently expressed in *Nicotiana benthamiana* epidermal cells. Fluorescence was consistently observed on the inner side of the chloroplast autofluorescence, and associated with transvacuolar strands (indicated by the blue arrows in Figure 4), which are cytoplasmic tunnels bounded by the tonoplast [40, 41], suggesting that TIP3;2 was confined to the vacuolar membrane. The tonoplast localization was confirmed by co-expression of the TIP3;2::GFP fusion protein in *N. benthamiana* with a vacuolar membrane marker protein, TIP1;1-RFP (vac-rk-CD3-975) [42]. The vac-rk protein was tagged with the mCherry fluorophore, which allows signals from both fluorophores to be detected in the same cell. Fluorescence from the vac-rk control was detected at the tonoplast of *N. benthamiana* cells and overlapped with signals from TIP3;2::GFP (Figure 4).

TIP3;2 Affects Ni Homoeostasis in Arabidopsis

Ni accumulation in WT and *tip3;2* mutants was examined by inductively coupled plasma mass spectrometry (ICP-MS). When plants were grown in control medium (containing 0.5 μ M Ni), no difference in Ni content was observed between *tip3;2* mutants and wild type. However, under 50 μ M Ni stress, while the root cell wall Ni content was similar between genotypes (Figure 5A), the Ni concentrations in the root cell sap (primarily vacuolar contents [43]) was significantly lower in the mutants (Figure 5B). Given the tonoplast localization of TIP3;2, these results suggest its involvement in sequestration Ni from cytoplasm into the vacuole.

Given the loss of TIP3;2 function, Ni in root is less sequestered in the vacuole and may be translocated to the shoot via transpiration flow. We further tested the Ni concentration in xylem sap, roots and shoots. The results suggested that TIP3;2 knockout increased Ni concentration in the xylem sap (Figure 5E), and mutant lines contained more Ni in shoots (Figure 5D), and less Ni in roots (Figure 5C) than WT. There are no differences in Fe, Mn, Cu, and Zn content between WT and mutant lines (Supplemental Figure 2).

It is well known that the trichomes play an important role in plant ion and metal homeostasis, one of the important functions is sequestration and compartmentalization of heavy metals. As *TIP3;2* is also expressed in trichome and hydathodes, the *tip3;2* mutants contained obviously less Ni in trichomes under excess Ni condition than that in WT (Figure 5F). Collectively, these results indicate that TIP3;2 functions to sequester Ni into root vacuoles, limiting its translocation to shoots, and may also facilitate Ni sequestration into trichomes or its secretion via hydathodes for detoxification.

Heterologous Expression of TIP3;2 enhanced Yeast Ni tolerance

When expressed in yeast, a TIP3;2::GFP fusion protein was localized to the vacuolar membrane, as observed under a confocal microscope (Figure 6A). To test the function of TIP3;2 to Ni tolerance, we transferred *pYES2* empty vector and *pYES2-TIP3;2* to wild type (BY4741) yeast strains. We then compared the Ni stress tolerance of the transformed yeast lines using a drop assay. In the absence of Ni, the growth of yeast cells expressing TIP3;2 was similar to that of the vector control (Figure 6B). However, in the presence of Ni, growth of both the vector control and TIP3;2-expressing yeast cells was inhibited,

with the vector control showing greater inhibition than the TIP3;2-expressing yeast cells, across all tested Ni concentration (Figure 6B). These results suggest that TIP3;2 functions to sequester Ni into the vacuoles, thereby enhancing Ni tolerance in yeast.

TIP3;2 Facilitates the Uptake of Nickel-Phytochelatin Complexes

In the cytosol (pH 7.0–7.5), ionic metal is mostly chelated by ligands such as malate, citrate, GSH, MT, etc. [44]. Therefore, TIP3;2 might transport Ni in complexed form from the cytosol to the vacuole. However, since TIP3;2 is not localized to the plasma membrane in the yeast expression system, determining its transport form is challenging. To address this, we engineered an NIP1;2¹⁻³⁰::TIP3;2³¹⁻²⁶⁸ fusion protein by replacing the first 30 amino acids of TIP3;2 with those of NIP1;2, which is known to be localized to the plasma membrane [30]. The initial 30 amino acids may contain the signal peptide to the plasma membrane and lack the key NPA and ar/R motifs of aquaporin. When such a fusion protein was expressed in yeast cells, the GFP signal partially colocalized with red CellMask PM Staining (Thermo Fisher Scientific) at the plasma membrane (Figure 7A), allowing us to determine the transport form of TIP3;2.

We conducted a short-term (4 h) Ni uptake assay on yeast lines (BY4741) carrying the *pYES2-GFP* or *pYES2-NIP1;2¹⁻³⁰::TIP3;2³¹⁻²⁶⁸-GFP* construct in the presence of Ni²⁺ or Ni²⁺ conjugated with different cellular ligands, such as citrate (Cit), malate (Mal), nicotinamide (NA), glutathione (GSH), phytochelatin (PC), and metallothionein (MT) (Figure 7B). Compared with the control line (*pYES2-GFP*), the *pYES2-NIP1;2¹⁻³⁰::TIP3;2³¹⁻²⁶⁸-GFP* line showed significantly enhanced Ni uptake activities in the presence of the Ni-PC conjugated complex, but not with other Ni-ligands (Figure 7B). This result suggests that the Ni-PC complex could be a transport substrate for TIP3;2.

Discussion

TIP3;2 is Probably a Nickel Transporter

Aquaporins have evolved beyond their canonical role in water transport to facilitate the movement of various small, neutral solutes and, as increasingly evidenced, charged metal complexes, such as B, As, glycerol, Al-malate complexes, and Zn complexes [29, 30, 36, 45]. Our study adds significant weight to this paradigm shift by implicating TIP3;2, a member of the tonoplast intrinsic protein (TIP) subfamily [46], in Ni transport. The hypersensitivity of two independent *tip3;2* mutants to Ni stress (Figure 1A–C), coupled with the specific Ni-induced upregulation of TIP3;2 expression (Figure 3B, D), provides strong genetic evidence for its dedicated role in Ni detoxification, distinct from responses to other heavy metals like Cd, Al, or Zn.

The tonoplast localization of TIP3;2 (Figure 4) directs its function to a critical cellular compartment for metal homeostasis. The central vacuole, occupying 30–90% of a plant cell's volume [47], serves as a primary storage and detoxification site, sequestering excess heavy metals, including Ni, to protect the cytosolic machinery [48]. Our physiological data align perfectly with this model: *tip3;2* mutants exhibit

reduced Ni accumulation in root cell sap (vacuolar content) but increased Ni in the xylem and shoots (Figure 5B-E). Supporting this hypothesis, heterologous expression of TIP3;2 in yeast resulted in enhanced Ni resistance (Figure 6B), with the protein correctly localizing to the tonoplast (Figure 6A). This phenotype mirrors that of other tonoplast metal transporters, such as AtTIP2;2 for Zn [36], suggesting a conserved functional logic within the TIP subfamily for vacuolar metal sequestration.

Crucially, our heterologous expression studies in yeast provide direct functional support. The enhanced Ni tolerance conferred by TIP3;2 (Figure 6B) confirms its capacity to mediate cellular detoxification. Furthermore, the innovative plasma membrane-targeting strategy via the NIP1;2¹⁻³⁰::TIP3;2³¹⁻²⁶⁸ fusion was instrumental in identifying the putative transport substrate. The specific enhancement of Ni uptake only in the presence of Ni-phytochelatin (Ni-PC) complexes (Figure 7B) suggests that TIP3;2 likely transports Ni in a chelated form. This result is physiologically highly plausible, as PCs are rapidly synthesized under heavy metal stress and are key ligands for vacuolar sequestration [49, 50]. This finding positions TIP3;2 as a potential missing link in the PC-dependent detoxification pathway, specifically responsible for the tonoplast transport step of the Ni-PC complex into the vacuole.

The Integrated Role of TIP3;2 in Nickel Tolerance: from Root Sequestration to Shoot Detoxification

Our results paint a comprehensive picture of how TIP3;2 orchestrates Ni tolerance at the whole-plant level. Its function begins in the root, where high expression in the elongation and hair zones (Figure 2) positions it to intercept Ni entering the symplast. By actively pumping Ni into root vacuoles, TIP3;2 performs a dual function: it directly protects root cell metabolism and acts as a “checkpoint” that limits the amount of Ni entering the xylem stream for shoot translocation. This strategy explains the classic tolerance mechanism of reduced translocation observed in many non-hyperaccumulators [51, 52].

However, TIP3;2's role extends beyond root containment. Its expression in leaf and stem trichomes (Figure 2B-D) and the concomitant reduction of Ni in *tip3;2* mutant trichomes (Figure 5F) reveal a secondary, shoot-based detoxification layer. Trichomes function as external metabolic sinks, and their ability to sequester metals is a well-documented tolerance trait [53]. TIP3;2 may facilitate the loading of Ni (likely as a complex) into trichome vacuoles, providing a final line of defense by isolating the metal from mesophyll cells. This role is particularly significant, given that the mutants had higher total shoot Ni but lower trichome Ni, indicating that TIP3;2 is essential for redirecting and safely storing the Ni that does reach the shoot.

The expression of *TIP3;2* in hydathodes adds a fascinating potential third mechanism (Figure 2B-C). Guttation fluid can contain heavy metals, suggesting a secretory detoxification route. TIP3;2 could be involved in loading Ni into this fluid, effectively excreting it from the plant. This multi-compartmental strategy—root vacuole sequestration, trichome storage, and possible hydathode secretion—underscores the sophisticated, tiered adaptation plants employ against metal stress, with TIP3;2 emerging as a central player in this network.

Broader Implications and Future Perspectives

The identification of TIP3;2 has important implications. First, it challenges the strict definition of aquaporin substrates and encourages re-examination of other TIPs, and indeed NIPs, for roles in heavy metal transport. The mechanistic parallel with Al detoxification via AtNIP1;2 (Al-malate) and OsNIP1;2/OsTIP2;1 [30, 37, 38] suggests a potential common theme where aquaporins handle metal-ligand complexes.

Second, from a phytoremediation perspective, our findings open new avenues. While hyperaccumulators like *Alyssum* species use specific ligands (e.g., histidine) and transporters (e.g., YSL families) for Ni xylem loading and shoot accumulation [52], our work identifies a key vacuolar transporter for Ni in a non-hyperaccumulator. Engineering or overexpressing *TIP3;2* in high-biomass plants, perhaps in conjunction with enhanced PC synthesis, could improve root storage capacity, reducing food chain contamination. Conversely, silencing homologous genes in hyperaccumulators might alter their metal partitioning.

Future work should focus on several key questions: (1) Directly confirming the Ni-PC transport activity of TIP3;2 in planta using tonoplast vesicle transport assays. (2) Identifying the protein interactors that might regulate TIP3;2 activity or target it to specific membrane microdomains. (3) Exploring whether TIP3;2 orthologs in crop species like rice or soybean share this function, which would be critical for breeding or engineering metal-tolerant cultivars. By elucidating the role of TIP3;2, this study not only advances our understanding of fundamental plant metal homeostasis but also provides a promising genetic tool for addressing agricultural and environmental challenges posed by nickel pollution.

Conclusion

This study establishes a critical role for the tonoplast-localized aquaporin TIP3;2 in nickel detoxification in *Arabidopsis*. We demonstrate that TIP3;2 expression is specifically induced by Ni stress. Functional loss in *tip3;2* mutants results in hypersensitivity to Ni, disrupted root-to-shoot partitioning (with reduced root vacuolar sequestration and increased shoot accumulation), and impaired Ni deposition in leaf trichomes. The enhanced Ni tolerance conferred by heterologous TIP3;2 expression in yeast, coupled with evidence suggesting it transports Ni-phytochelatin complexes, reinforces its direct role in Ni transport. Overall, our findings identify TIP3;2 as a key component of the cellular machinery that regulates Ni homeostasis and tolerance in plants.

Abbreviations

Ni : Nickel ; TIP: Tonoplast Intrinsic Protein ; AQP : Aquaporin ; NPA : Asparagine-Proline-Alanine ; ar/R : Aromatic/arginine ; PC: Phytochelatin ; Cit : Citrate ; Mal : Malate; Nic: Nicotinamide; Glu: Glutathione ; MT : Metallothionein ; GFP: Green Fluorescent Protein; GUS: β -Glucuronidase.

Declarations

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Author's contributions

Y.W. conceived and supervised the research. Y.W. and K.X. designed the experiments. Y.-M.F., W.-Y.Z., W.-J.Z., M.-Y.W., and B.-X.L. performed the experiments and analyzed the results. Y.W. and K.X. wrote the paper. All authors have read and approved the final manuscript.

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Availability of data and materials

The datasets used and analyzed during the current study are included in this article and its supplementary files. The TIP3;2 (AT1G17810) DNA sequence data referenced in this study were available at the NCBI (National Center for Biotechnology Information) GenBank database, the Gene ID is 838359, Transcript accession number is NM_101644.3, and Protein accession number is NP_173223.1.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

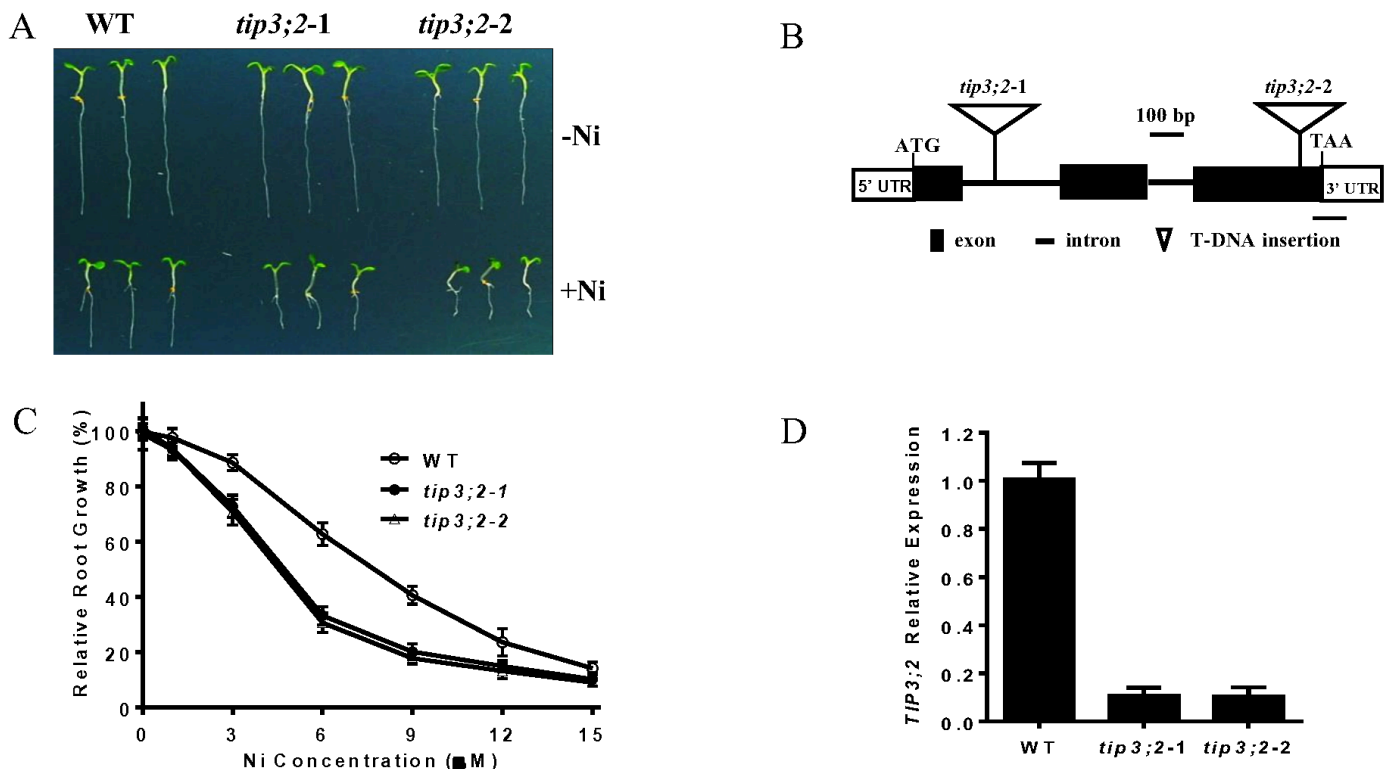


Figure 1

TIP3;2 mutations sensitize mutant plants to Ni stress. (A) Ni sensitivity of WT and two *tip3;2* T-DNA lines. Seedlings were grown in hydroponic solution containing 0 or 7 μM NiCl_2 for 7 days. (B) Gene structure of *TIP3;2*. Boxes, exons; horizontal lines, introns; closed boxes, the coding sequences; triangles, T-DNA insertions. (C) Relative root growth (RRG%) of WT and *tip3;2* mutants under Ni stress. (D) Real-time RT-qPCR analyses of *TIP3;2* expression in roots. Data in C and D are means $\pm$ SD of three biological replicates.

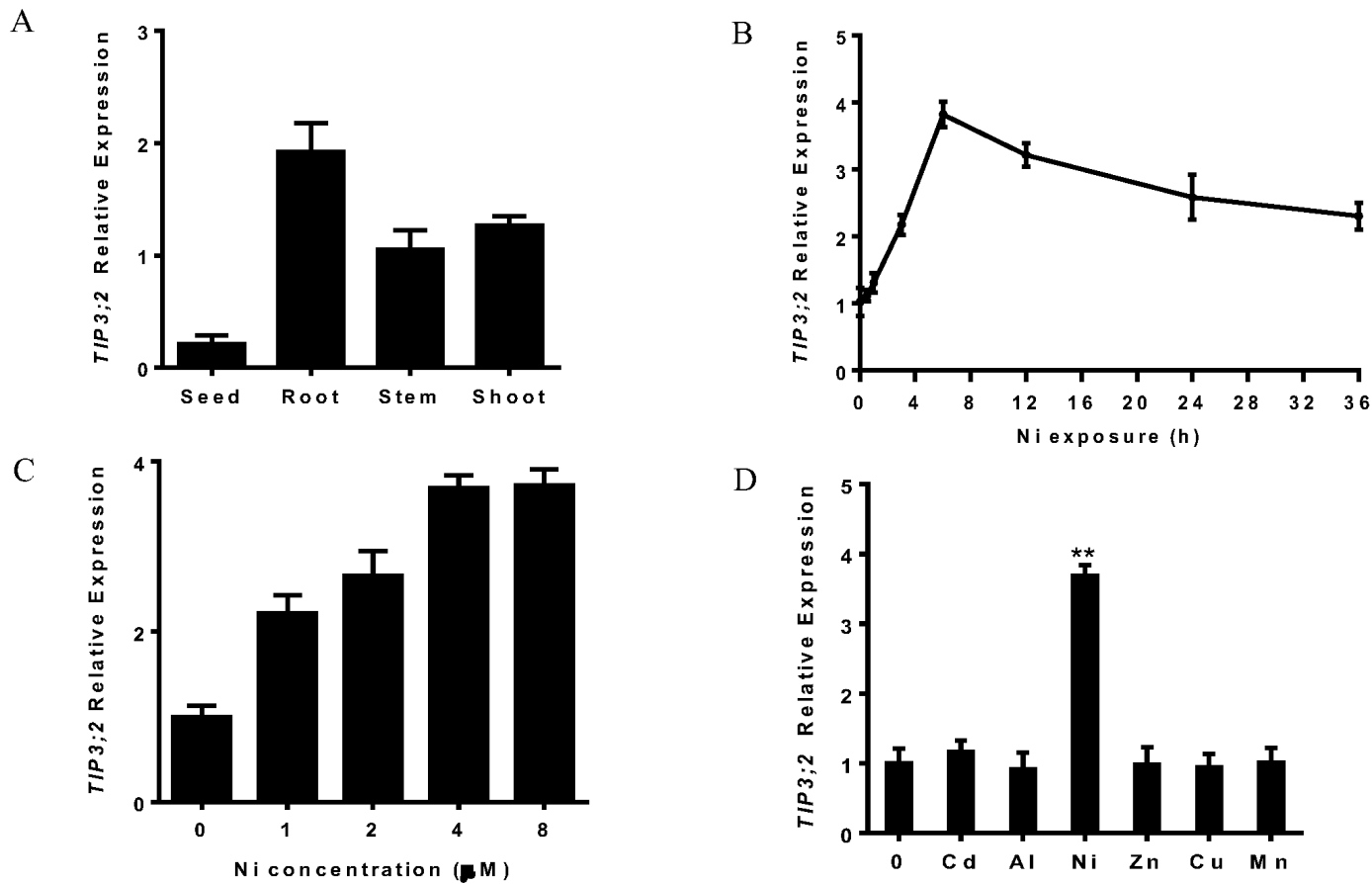


Figure 2

Tissue-specific expression of *TIP3;2*. GUS activity driven by the *TIP3;2* promoter in the whole seedling (A) Leaf (B) partial magnified leaf (C) Stem (D) and Root tip (E). Scale bars: 1mm in A, B, D; 100 μm in C, E.

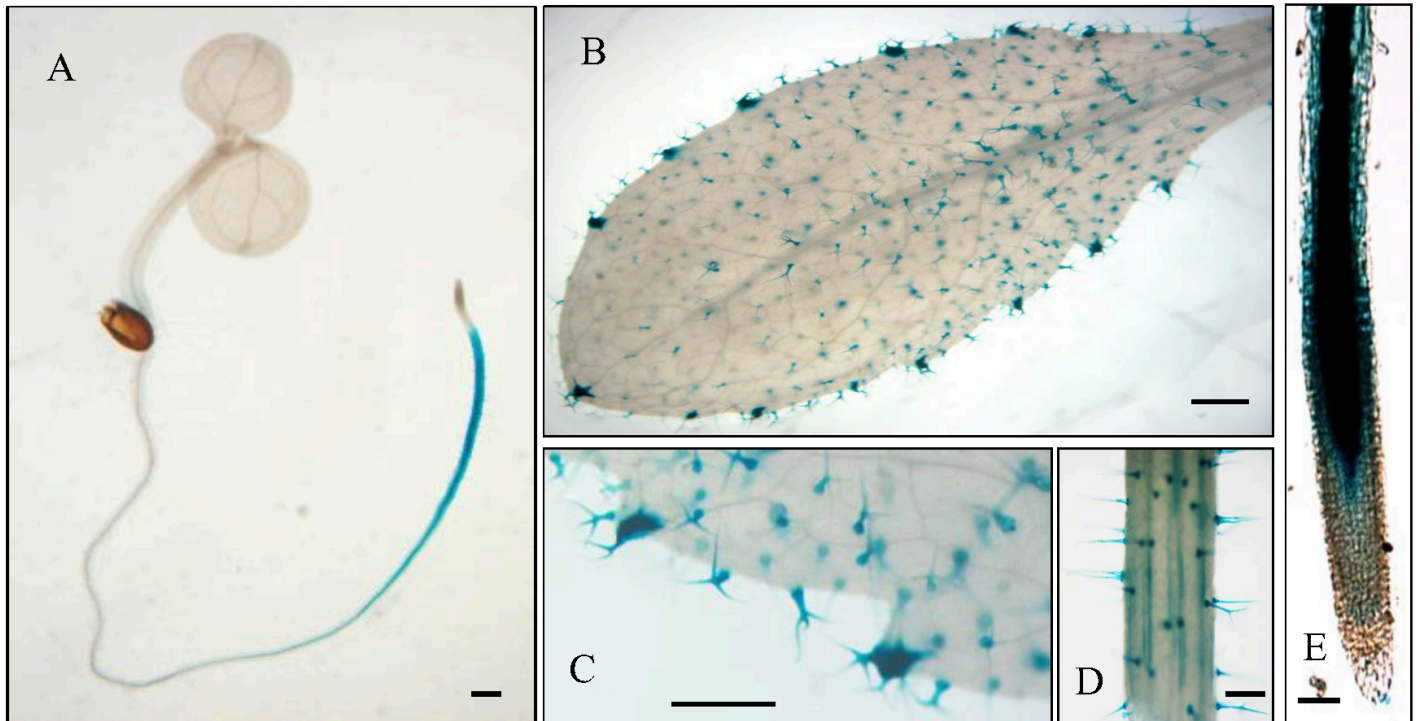


Figure 3

TIP3;2 expression patterns. (A) Detection of *TIP3;2* expression in seed, root, stem and shoot of 7-d-old WT(Col-0) by qRT-PCR analysis. (B) Time course qRT-PCR analysis of *TIP3;2* gene expression in WT roots treated with 8 μM NiCl_2 . (C) Concentration-dependent expression of *TIP3;2* in WT root treated with different Ni concentration. (D) qRT-PCR analysis of *TIP3;2* expression in WT roots in response to different metal ions. Here 7-d-old WT plants were treated with 6 μM CdCl_2 , 20 μM AlCl_3 , 8 μM NiCl_2 , 50 μM ZnSO_4 , 5 μM CuSO_4 , or 200 μM MnCl_2 for 6 h. Asterisks indicate significant differences between 0 and 8 μM NiCl_2 treatments (** $P < 0.01$).

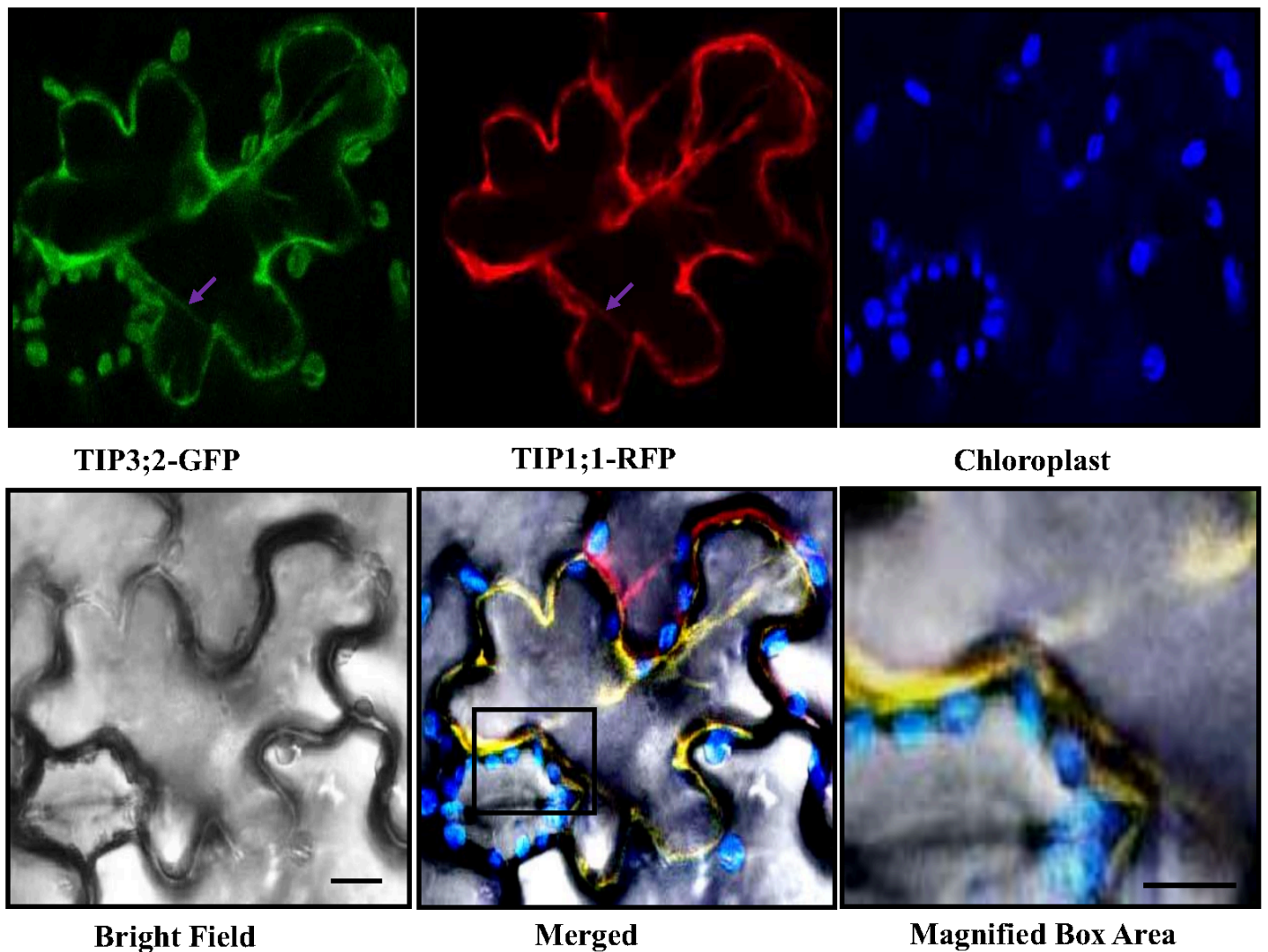


Figure 4

Subcellular localization of TIP3;2 in *Nicotiana benthamiana* epidermal cells. Coexpression of TIP3;2-GFP (green) and the vacuolar membrane marker TIP1;1-RFP (vac-rk-CD3-975) (red) in a tobacco epidermal cell, the overlapped signal (yellow) is on the inside facing side of the chloroplast auto-fluorescent signals (blue), and contained typical transvacuolar strands (indicated by the purple arrows). Boxed area is magnified. Scale bar: 10 μ m.

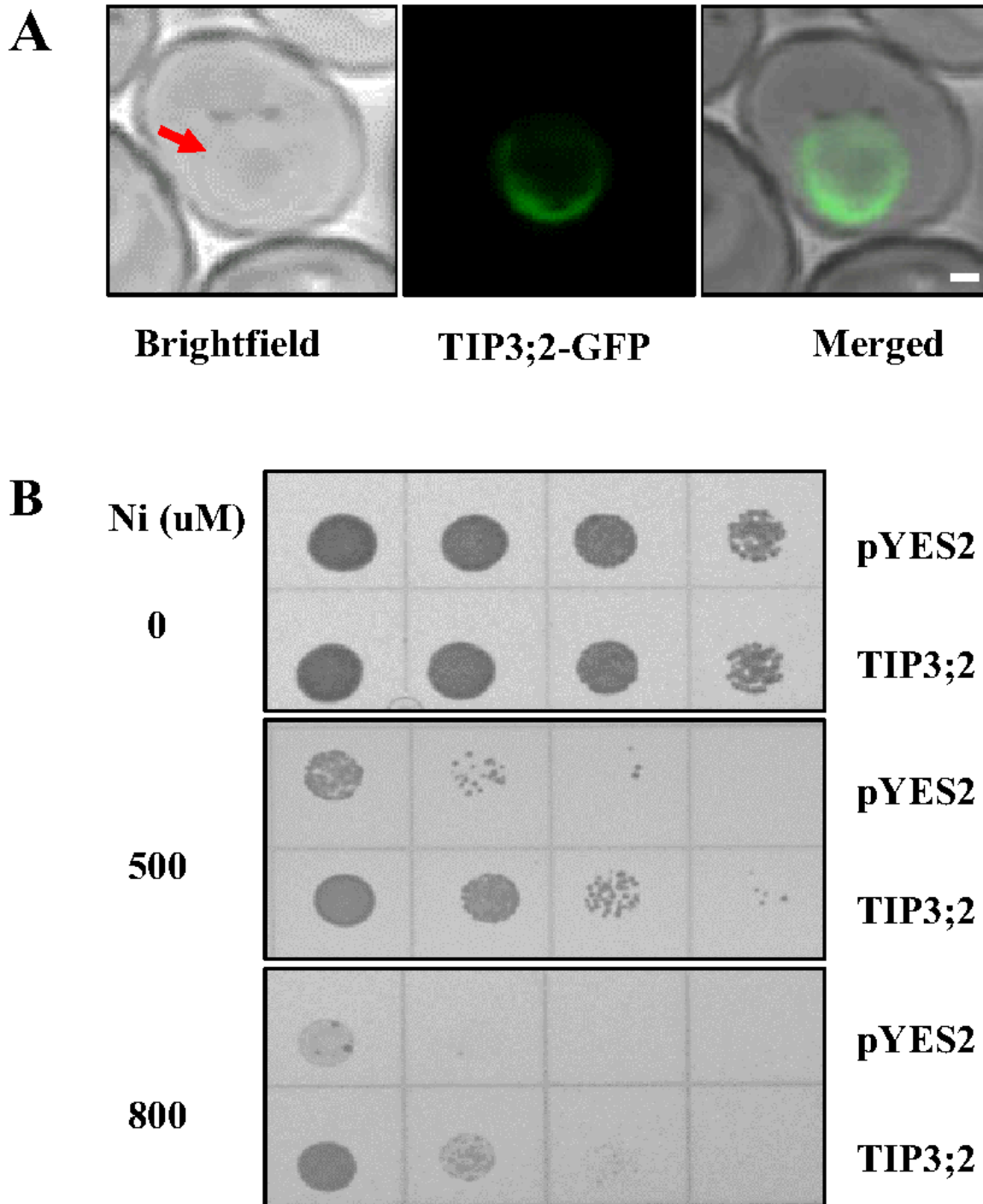


Figure 5

Ni Homoeostasis in Arabidopsis.

The Ni concentration in the root cell wall (A), root cell sap (B), roots (C), shoots (D), xylem sap (E), trichome (F), shoot cell wall (G), shoot cell sap (H). Plants were hydroponically grown in nutrient solution for 7 days and treated with 0.5 or 50 μM NiCl_2 for 24 h, and Ni concentrations were measured using ICP-

MS. Data are mean $\pm$ SD of three biological replicates. ** $P < 0.01$ between WT and individual *tip3;2* lines under the indicated treatment conditions. DW, dry weight.

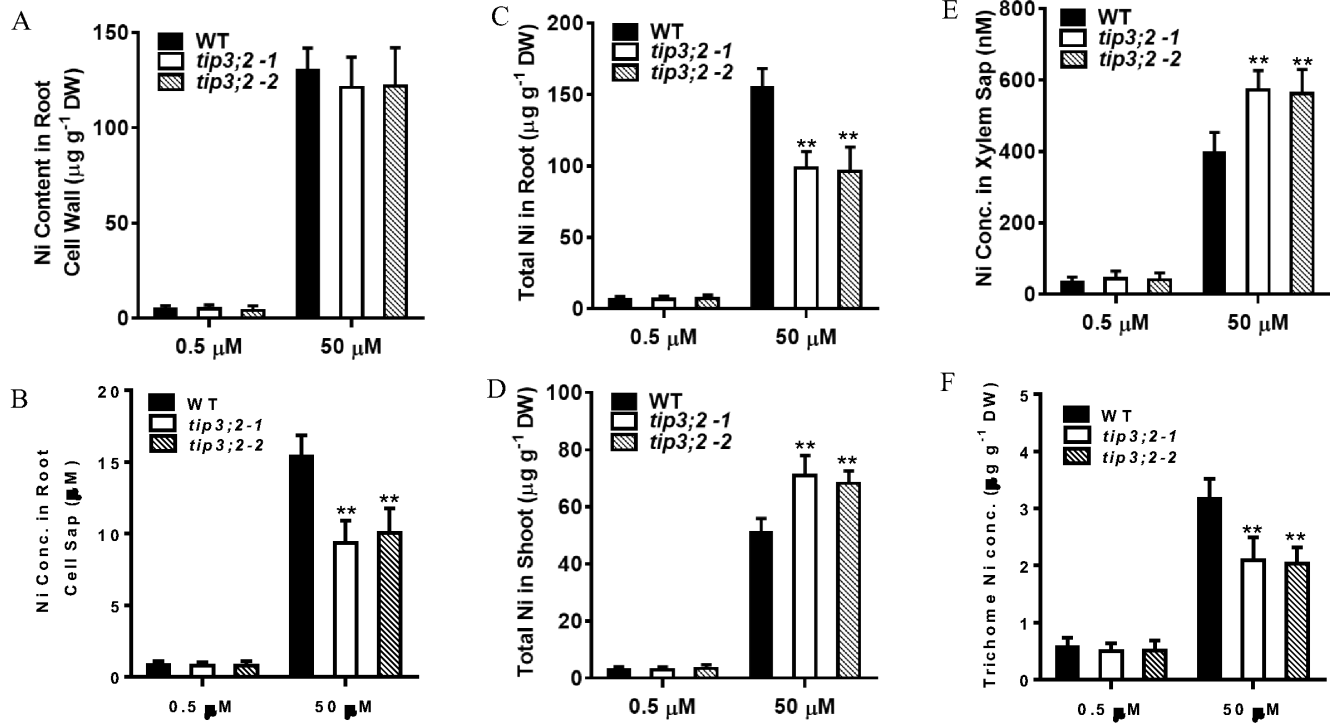


Figure 6

Functional analysis of TIP3;2 in yeast. (A) Subcellular localization of TIP3;2 in yeast. TIP3;2-GFP (green) mainly localized to the tonoplast in a yeast cell carrying pYES2-TIP3;2::GFP. (Scale bar: 1 μ m.) Arrows points to the vacuolar membrane. (B) Expression of TIP3;2 in yeast enhances Ni tolerance. Overnight yeast cell suspension of BY4741 or *cot1* transformed with empty vector pYES2 or TIP3;2 were serially diluted (1:10) and spotted on the media without (Control) or with indicated Ni concentration. Pictures were taken after 3 days growth at 30 °C.

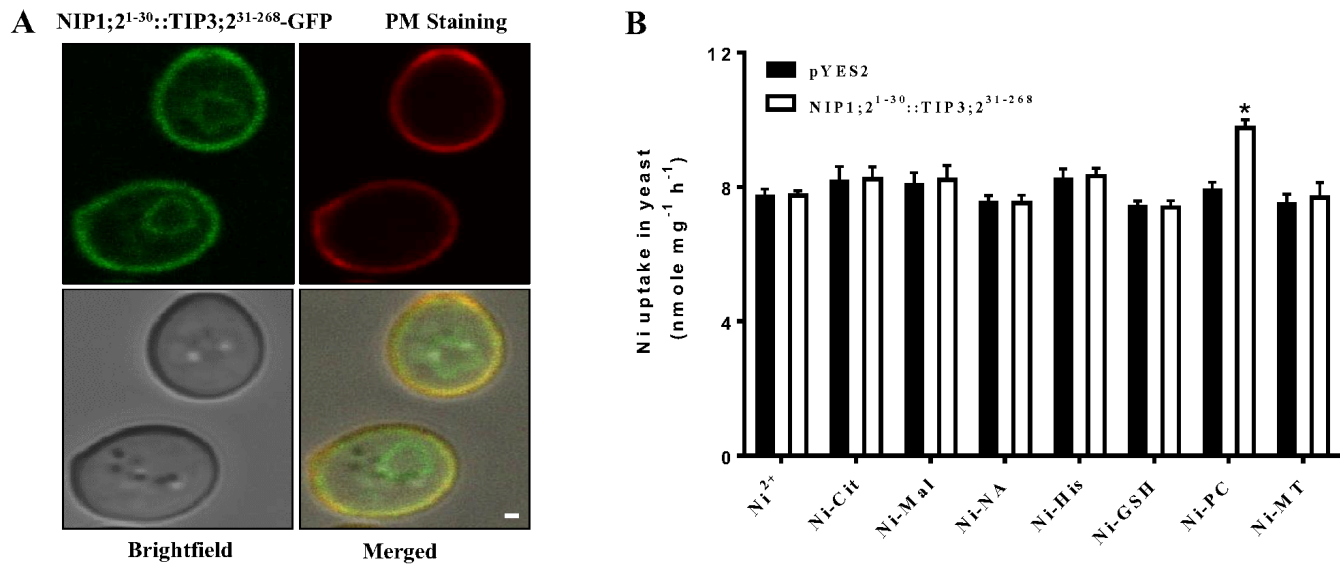


Figure 7

NIP1;2¹⁻³⁰::TIP3;2³¹⁻²⁶⁸ fusion protein facilitates uptake of Ni-PC, but not other AI-ligands, in yeast. (A) Subcellular localization of NIP1;2¹⁻³⁰::TIP3;2³¹⁻²⁶⁸ in yeast. NIP1;2¹⁻³⁰::TIP3;2³¹⁻²⁶⁸-GFP (green) was partially colocalized with red CellMask PM Staining (Thermo Fisher Scientific) to the plasma in a yeast cell carrying pYES2- NIP1;2¹⁻³⁰::TIP3;2³¹⁻²⁶⁸-GFP. (Scale bar: 1 μm). (B) Uptake of Ni²⁺ and different Ni-ligands by NIP1;2¹⁻³⁰::TIP3;2³¹⁻²⁶⁸ fusion protein. Yeast cells carrying pYES2-GFP or pYES2- NIP1;2¹⁻³⁰::TIP3;2³¹⁻²⁶⁸-GFP were exposed to the SC-Ura medium containing NiCl₂ (20 μM), or individual Ni-ligands (20 μM NiCl₂ + 40 μM ligand) for 4 h. The ligands used are citrate (Cit), malate (Mal), nicotinamide (NA), histidine (His), glutathione (GSH), phytochelatin (PC) or metallothionein (MT). Then, Ni concentrations of the yeast cells were determined by ICP-MS. Data are means ± SD of three biological replicates from three independent transformation events. *, significant differences (p<0.05) between two yeast lines under indicated treatment.

