Supporting Information

S1 Materials and Methods

Plant Material and Growth Conditions. The *Arabidopsis thaliana* T-DNA insertion mutants *nip1;2*-1 (SALK_126593), *nip1;2*-2 (SALK_147353) and *nip1;2*-3(SALK_076128) and for other *NIP*s (Table S1) as well as *almt1* (SALK_009629C) were acquired from the Arabidopsis Biological Resource Center (ABRC) (https://abrc.osu.edu/). Homozygosity of each line was confirmed by PCR-based sequencing. Wild-type (*Col-0* and *Ler-0*) and mutant seeds were surface-sterilized and cold stratified in dark at 4^{o}C for 3 days before sown on 250 μm polypropylene meshes floating on hydroponic growth solutions supplemented without or with Al or other metal ions in Magenta boxes. The hydroponic solution consisted of the following macronutrients in mM: $MgCl_2$, 3.0; $(NH_4)_2SO_4$, 0.25; $Ca(NO_3)_2$, 1.0; KCl, 2.0; $CaCl_2$, 2.75; KH_2PO_4, 0.18; and the following micronutrients in μM: H_3BO_3, 5.0; $MnSO_4$, 1.0; $CuSO_4$, 0.05; $ZnSO_4$, 0.2; Na_2MoO_4, 0.02; $CoCl_2$, 0.001. Two milimolar (mM) of Homo-PIPES were included in the solutions to maintain the pH at 4.2. Plants were grown in a plant growth chamber with a setting of 16/8 h day/night at 22^{o}C.

Relative root growth (RRG %) was calculated as the percentage of root growth of individual plants under Al treatment over the average root growth under the control (−Al) condition. In detail, ~30-40 seeds (technical replicates) of the WT or the mutant lines were germinated in a hydroponic solution in a Magenta box supplemented with or without 30 μM $AlCl_3$ at pH4.2. Three biological replicates (Magenta boxes) were conducted for each treatment each line. Primary root lengths of 10 randomly selected 7-d-old seedlings from a biological replicate were measured manually and the means of the primary root length of three biological replicates were calculated for the control of each line. The RRG% of a randomly selected 7-d-old seedling under treatment condition was calculated as individual primary root length divided by the mean root length of the same line of the control condition. Ten seedlings from each biological replicate were randomly selected for RRG% calculation. The presented RRG% data were the means of three biological replicates.

For testing the sensitivity to other metals, WT and two T-DNA insertion lines (*nip1;2*-1 and *nip1;2*-2) were treated with hydroponic solutions (pH 4.2) containing (in μM) 20, $AlCl_3$; 10, $CdCl_2$; 5, $LaCl_3$; 10, $ZnSO_4$; or 5, $CuSO_4$ for 8 days and the primary root length was measured.

For gene expression analysis, ~ 500 seeds (~10 mg) were germinated in control hydroponic solution (-Al) for 5 days. Then seedlings were transferred to hydroponic solutions (pH 4.2) containing 20 μM $AlCl_3$ for 0, 1, 2, 4, 6, 8, 12, 24h; or containing (in μM) 20, $AlCl_3$; 10, $CdCl_2$; 5, $LaCl_3$; 10, $ZnSO_4$; or 5, $CuSO_4$ at pH 4.2 or to the control solution at pH 5.5 for 6h before collection of the root samples for RNA extraction. Three replicates (Magenta boxes) were included for each treatment.

GUS Staining Assays and Localization of GUS Expression. A 1.89 kb *NIP1;2* promoter (ATG as +1) was PCR-amplified from *Arabidopsis* genomic DNAs with primers 5′-TAACctgcagGGTCTGAGCCAATCGTGATA-3′ and 5′-ACAGccatggCTCTAGCATCACCACCGTT -3′ (the underlined sequences are restriction enzyme sites for *Pst*I and *Nco*I, respectively), and then cloned into the pCAMBIA1305.2 vector. The resulting *NIP1;2promoter*::*β-glucuronidase* (GUS) construct was transformed into the WT (*Col-0*) genome through *Agrobacterium tumefaciens* (strain GV3101)-mediated transformation. Seven-day-old seedlings of T2 or T3 generations were stained with the GUS staining solution containing 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.5% Triton X-100, 0.5mg/L 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 50 mM sodium phosphate (pH7.0) at 37^{o}C, followed by incubated in 70% ethanol to remove chlorophyll at room temperature.

The histochemical analysis was performed as described previously (1). The stained tissues were fixed in formalin: acetic acid: 50% ethanol (1:1:18) for 24h at room temperature. After fixation, the tissues were dehydrated in ethanol and tert-butanol with gradient concentrations and embedded in paraffin, and then sectioned by Leica microtome (RM2255). The 10μm thick sections were transferred onto slide and observed with Leica 5500 stereomicroscope with a color CCD camera.

Subcellular Localization of NIP1;2. The coding sequence of *NIP1;2*, excluding the stop codon, was amplified from the *NIP1;2* cDNA using the primers 5′-CTACggatccAAAATGGCGGAGATCTCGGGAAA-3′ and 5′- CGGGctcgag ACGAGAGCTACCGTTTCGCA -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 (2). The resulting *NIP1;2-GFP* construct was then transformed into *Agrobacterium tumefaciens* strain GV3101, followed by transient

transformation into the tobacco leaves by infiltration (3) or by stable transformation into *Arabidopsis* (Col-0). The plasma membrane marker *35S::PIP2;1-RFP* (pm-rk-CD3-1007) was as described in Nelson et al., (2007) (4). GFP signals were observed with a Leica SP5 confocal laser microscope. To visualize nuclei, leaves were stained with 15mg/ml DAPI for 30 min prior to microscopic observations.

RNA Isolation and Quantitative Real-time RT-qPCR. Total RNAs were extracted from *Arabidopsis* shoot or root tissues using the RNeasy Mini Kit (Qiagen) following the manufacturer's instruction. First-strand cDNAs were synthesized from 5 μg DNaseI-digested total RNAs using the SuperScript III First-Strand Synthesis System (Invitrogen). Real-time RT-qPCR was performed with a 7500 Fast Real-Time PCR System according to manufacturers' protocols (Applied Biosystems, Inc.). 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 *NIP1;2* are: GGTTCGATATACTGATAAGCCA and GATACAACTTAACCTCCGATGAC.

Isolation of NIP1;2 cDNAs from the *WT*, *almt1* and *mate* backgrounds. Approximate 500 seeds (~10 mg) of the *WT*, *almt1* and *mate* lines were germinated in hydroponic solutions for 6 days. Then roots were used for total RNAs extraction using the RNeasy Mini Kit (Qiagen) following the manufacturer's instruction. First-strand cDNAs were synthesized from 5 μg DNaseI-digested total RNAs using the SuperScript III First-Strand Synthesis System (Invitrogen). *NIP1;2* cDNAs were PCR amplified with primers: GAGAAATACAGTTATGGCGGAGATCT and CCGATGACTAGTACAACATAGTCATG. The PCR amplified cDNAs were then subjected to sequencing.

Yeast Al Sensitivity, Influx and Efflux Transport Analysis. The GFP coding sequence was amplified by PCR from the *pGPTV.GFP.Bar* vector (2) with primer 5'- ATCCgcggccgcC ATGAGTAAAGGAGAAGAACTTTTC -3' and 5'-: TCGCtctagaTTTGTATAGTTCATCCATGCCATG -3' (the underlined sequences are restriction enzyme sites for *Not*I, and *Xba*I, respectively). Then the PCR fragment was sub-cloned into the *Not*I, and *Xba*I restriction sites in the expression vector, *pYES2*, and assigned as *pYES2-GFP*.

The coding sequence of *NIP1;2* was PCR amplified from the *Arabidopsis* cDNAs with primers 5'- CTACggatccAAAATGGCGGAGATCTCGGGAAA-3' and 5'-ATCCgcggccgc

ACGAGAGCTACCGTTTCGCA -3' (the underlined sequences are restriction enzyme sites for *Bam*HI and *Not*I, respectively). Individual PCR fragments were sub-cloned into the *BamH*I and the *Not*I restriction sites in frame with the 5' end of the GFP coding region in the *pYES2-GFP* construct. For Al sensitivity evaluation, the resulting *pYES2-NIP1;2-GFP* and the control *pYES2-GFP* constructs were transformed into the yeast strain BY4741. Three independently transformed yeast colonies from each of the transformation events were selected to represent 3 biological replicates for the following experiments. Individual yeast colonies were first cultured in the SD-Ura medium to the stationary phase. Cells of each culture were collected by centrifuge at 5000g for 5 min, followed by washes 3 times with ddH_2O and with low pH, low magnesium (LPM) medium buffered with 5 mM Succinic acid to pH 4.2 for 3 times. The LPM medium consisted of the following macronutrients in mM: $(NH_4)_2SO_4$, 40; KCl, 5; NaCl, 2; $CaCl_2$, 0.1; KH_2P0_4, 0.01; $MgSO_4$, 0.25; and the following micronutrients (in μM): $FeCl_3$,1; H_3BO_3,10; KI, 0.5; $MnSO_4$, 2.5; Na_2MoO_4, 1; $ZnSO_4$, 1.5; and the following amino acid in mg/L: Tyr, 0.03; Glu, 0.075; Ade, 0.02; Ura, 0.02; Phe, 0.05; Val, 0.15; Ser, 0.4; Leu, 0.03; Ile, 0.03; Lys, 0.03; Trp, 0.02; Arg, 0.02; His, 0.02; Met, 0.02; Asp, 0.0625; Thr, 0.2; and 2% Galactose, and following Vitamins in ng/L: Folic acid, 0.2; Biotin, 0.2; p-aminobenzoic acid, 20; Riboflavin, 20; Calcium pantothenate, 40; Niacin, 40; Pyridoxine hydrochloride, 40; Thiamine hydrochloride, 40; Inositol, 200.

For measurement of Al uptake (influx), yeast cells at mid-exponential phase were harvested from liquid LPM culture by centrifuge at 5000 g for 5 min, followed by 3 time washes with the LPM medium, then transferred to a LPM medium containing 2% galactose for induction of the GAL promoter with pH adjusted to 4.2 by 5 mM succinic acid. The pre-cultured yeast cells were re-suspended in the same medium to reach an OD_{600} value at 3.0. $AlCl_3$, Al-Malate, Al-Citrate, Al-oxalate, Al-succinate, Al-fumarate, Al-aconite, Al-histidine, Al-glutathione, Al-phytochelatin or Al-metallothionein complex were added to the cell culture at a final concentration of 0, 50 μM at pH 4.2 or 7.0. After 1h incubation with gentle shaking, cells were harvested by centrifuge at 5000× g for 5 min and washed 3 times with deionized water (ddH_2O) (MilliQ; Millipore), dried and then digested with 2N HCl. For the time-course Al uptake experiment, yeast cells were collected at the indicated time points after exposure to 50 μM Al-Malate complex. For the 0 h Al treatment, immediately after Al-Malate complex was added to the yeast cell culture at final

concentration of 50 μM, the yeast cells were washed 3 times with ddH_2O, harvested, dried and digested with 2N HCl. The Al contents of each digested sample were determined by inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 7500 Series ICP mass spectrometer. Three biological replicates for each line and each treatment were conducted.

For measurement of Al efflux, yeast cells at mid-exponential phase were harvested from liquid LPM culture by centrifuge at 5000 g for 5 min, then re-suspended in 10 ml LPM medium containing 2% galactose with pH adjusted to 4.2 by 5 mM succinic acid and cell density at OD_{600} 3.0. Al-Malate complex were added to the cell culture at a final concentration of 50 μM $AlCl_3$ and 150 μM malate at pH 4.2. After 8 h incubation with gentle shaking, cells were harvested by centrifuge at 5000× g for 5 min and washed 3 times with LPM, then re-suspended in 10 ml fresh LPM (pH 4.2 or 7.0) containing 0 or 5 μM $AgNO_3$. Then, yeast cells were collected by centrifuging at the indicated time points, washed 3 times with ddH_2O, dried and digested with 2N HCl. The Al contents of each digested sample were determined by inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 7500 Series ICP mass spectrometer. Three biological replicates for each line and each treatment were conducted.

Hematoxylin Staining. After grown in hydroponic solution (pH 4.2) in a Magenta box for 7 days, ~50 seedlings of a biological replicate from a Magenta box were transferred to a hydroponic solution (pH4.2) supplemented with 50 μM $AlCl_3$ for 8h, followed by staining with 0.2% hematoxylin containing 0.02% KIO_4 for 30 min and washing twice with deionized water before microscopic observations. Three biological replicates for each treatment and each line were made and representative samples were chosen of photo images.

Root and Shoot Al Content Measurement. For total Al and other elements quantification, ~150 seeds (3 mg) of the WT, *nip1;2-1* and *nip1;2-2* were planted hydroponically in a Magenta box (one biological replicate) at pH 4.2. After seven days growth, seedlings were transferred to a fresh hydroponic solution (pH 4.2) with or without the supplement of 30 μM $AlCl_3$ for 8h. After treatment, seedlings were washed three times with 0.5 mM $CaCl_2$ and two times with deionized water, then the roots and shoots were harvested, dried, and digested in pure nitric acid at 110°C. Elements of each sample were analyzed with ICP-MS. Three biological replicates for each treatment were made.

Cell Sap and Cell Wall Preparation and Al Content Determination. Around 150 seeds (3 mg) of the wild-type or the two *NIP1;2* knockout lines were germinated on a mesh floating on the hydroponic solution at (pH 4.2) in a Magenta box. Then, 7-d-old seedlings were exposed to the hydroponic solution (pH 4.2) containing 30 μM $AlCl_3$ for 0, 0.5, 1, 2, 4, or 8 h.

To analyze the Al accumulation caused by externally supplied Al^{3+} or Al-Mal, ~150 7-d-old seedlings of the *WT*, *mate*, *almt1* and *nip1;2-1* lines were treated with hydroponic solution (pH 4.2) supplemented with 50 μM $AlCl_3$ or 50 μM $AlCl_3$ + 150 μM malate for 8 h. For testing the effects of sequential Al^{3+} and malate treatment on Al accumulation, ~150 7-d-old seedlings of the *WT*, *almt1* and *nip1;2-1* lines were pretreated with hydroponic solution (pH 4.2) supplemented with 50 μM $AlCl_3$ for 8 h, washed three times with 0.5 mM $CaCl_2$, then transferred to hydroponic solution (pH 4.2) supplemented with or without 200 μM malate for 8h. Three biological replicates (Magenta boxes) with the same setting were prepared for each plant line and each treatment.

In the study of Ryan et al.1995, a rather simple solution was used to test Al toxicity, which contained 200 μM $CaCl_2$ and 3 μM $AlCl_3$ at pH 4.3 (5). The corresponding Al activity (free Al^{3+} concentration) for this solution as predicted by the GEOCHEM-EZ program (6) was 1.93 μM. Also, 20 μM malate was required to completely detoxify Al^{3+} in this solution (5). Therefore, when Al activity is considered, the formation constant required for completely detoxify Al^{3+} was ~ 1:10 (1.93 μM free Al^{3+}: 20 μM malate). For our hydroponic solution, when Al activity is considered, the ratio of total concentrations of 50 μM $AlCl_3$ vs. 150 μM will be translated to a ratio of free Al^{3+} vs. malate of ~1:26, which means that more than sufficient malate was present in our hydroponic solution for complete detoxification of Al^{3+} in the solution.

After the treatment, roots were excised from the ~150 seedlings, washed 3 times with 0.5 mM $CaCl_2$ and 2 times with ddH_2O and then put into an Ultra free-MC Centrifugal filter units (Millipore) and centrifuged at 3,000 × g for 10 min at 4°C to remove apoplastic solution. The root samples were then frozen in a -80°C freezer overnight. The cell sap solutions were obtained by thawing the samples at room temperature and then centrifuging at 20,600 × g for 10 min. The residual cell walls were washed with 70% ethanol three times and then digested in 1 mL of 2 N

HCl for at least 24h with occasional shaking. Al contents in the symplastic solutions and cell wall extracts were determined by ICP-MS.

Collection and Analysis of Xylem Sap. Wild-type and *nip1;2* mutant plants were grown in hydroponic media for 6 weeks until the late vegetative stage. Plants were then subjected to 30 μM $AlCl_3$ treatment for 8h. To collect xylem sap, all rosette leaves were removed with scissors, and the inflorescence stem was cut with a razor blade. Xylem sap exudation was facilitated through a high humidity environment by covering the plants with a plastic dome. The first droplets were excluded from the collection to avoid contamination and then xylem sap was collected quantitatively with a micropipette. Each sample was collected five plants of the same line and same treatment. Aluminum concentrations in the root sap were analyzed by ICP-MS and normalized per volume of collected sample.

Phylogenetic Analyses. The evolutionary history was inferred using the Neighbor-Joining method (7). The optimal tree with the sum of branch length = 4.00853896 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (8) and are in the units of the number of amino acid substitutions per site. The analysis involved 10 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 258 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.06 (9).

References

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Table S1. List of Acquired T-DNA Insertion Lines of *NIP*s

NIPs	Gene Name	T-DNA Insertion Lines Tested
NIP1;1	At4g19030	SALK_016617; SALK_017916
NIP1;2	At4g18910	SALK_126593; SALK_147353; SALK_076128
NIP2;1	At2g34390	SALK_023890; SALK_100513; CS820193
NIP3;1	At1g31880	SAIL_161_E04; SAIL_610_H06; SAIL_341_A01
NIP4;1	At5g37810	SALK_007730; SALK_013924; SALK_038278
NIP4;2	At5g37820	CS83576; SALK_142789
NIP5;1	At4g10380	SALK_012572; SALK_035508
NIP6;1	At1g80760	SALK_038761; SALK_046323; SALK_097969
NIP7;1	At3g06100	SAIL_1164_G05; SALK_042756; SAIL_1164_G05

Figure S1

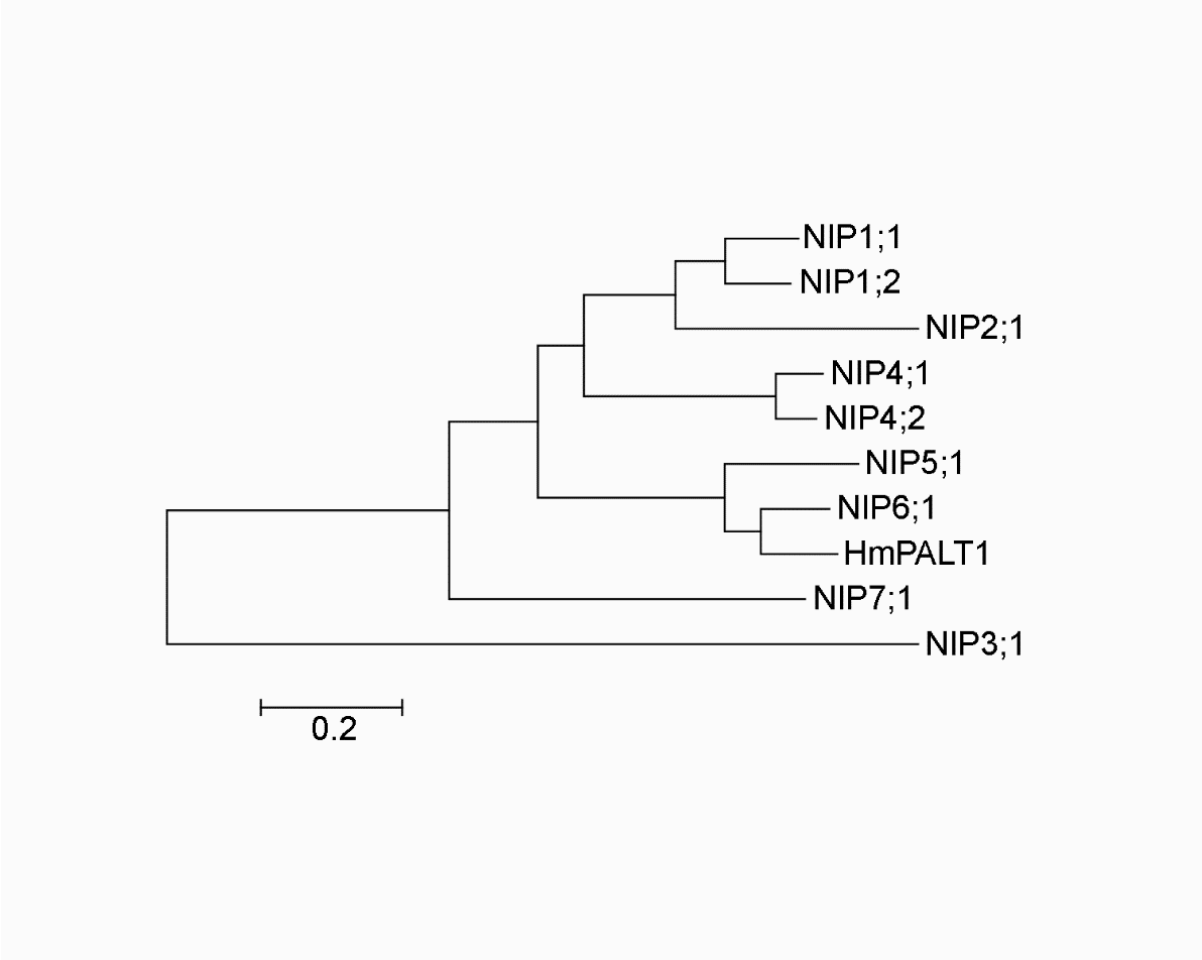


Fig. S1. Phylogenetic Analyses. Amino acid sequences of the NIP subfamily and HmPALT1 were aligned by the ClustalW method with the MEGA 6.06 software. The test neighbor-joining phylogenetic tress was built with the same software.

Figure. S2

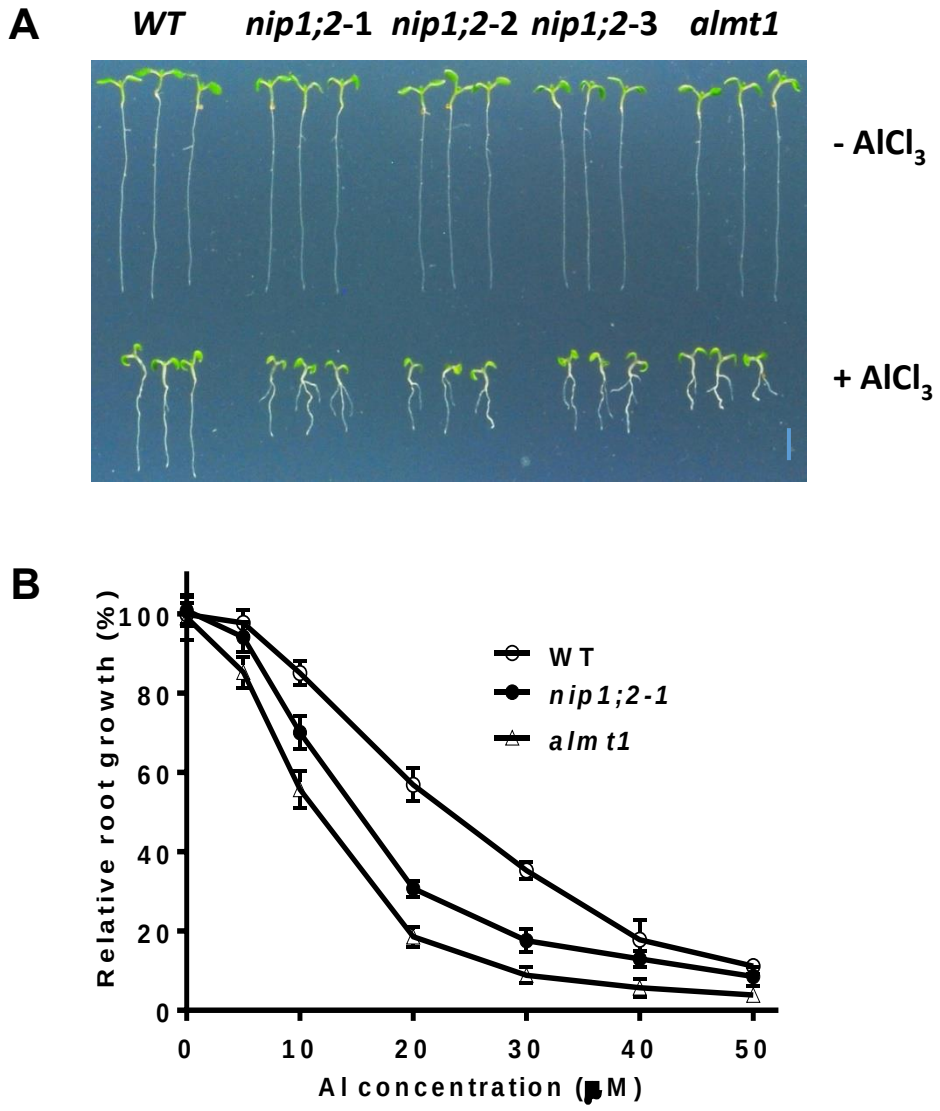


Fig. S2. The *almt1* mutant is more sensitive to Al stress than do the *nip1;2-1* mutant. (*A*) Al sensitivity of *WT* and the *almt1*, *nip1;2* mutant lines. Seedlings were grown in hydroponic solutions containing 0 or 30 μM $AlCl_3$ (pH 4.2) for 7d. (*B*) Relative root growth (RRG%) of *WT* and *almt1*, *nip1;2-1* mutants under Al stress. Data in *B* means ± SD of three biological replicates.

Figure S3

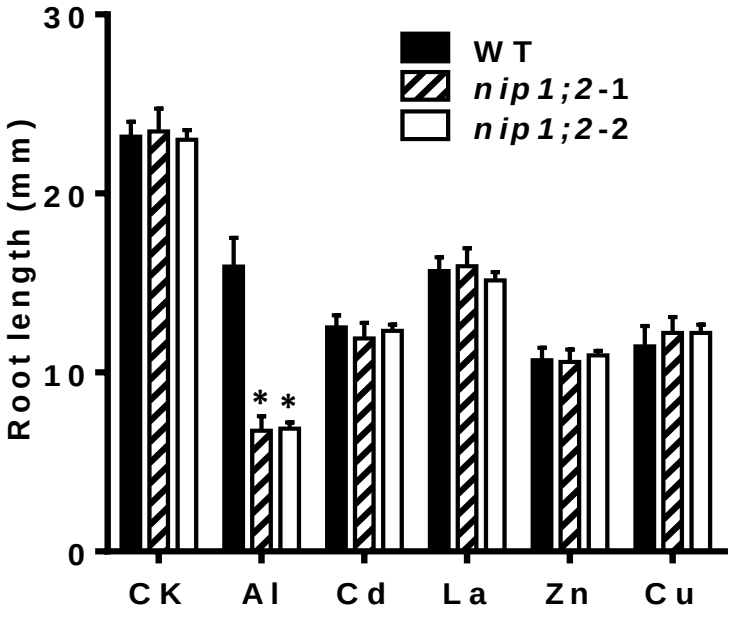


Fig. S3. The *nip1;2* mutants are specifically hypersensitive to Al but not to other metal ions. Seeds of WT, and *nip1;2*-1 and *nip1;2*-2 were germinated in hydroponic solution (pH 4.2) containing 20 μM $AlCl_3$; 10 μM $CdCl_2$; 5 μM $LaCl_3$; 10 μM $ZnSO_4$; or 5 μM $CuSO_4$. Root length was measured at 8d after germination (N = 20). CK, the control hydroponic solution (-Al) pH 4.2. Asterisks indicate significant differences (*, $p<0.05$) between WT and individual nip1;2 lines under indicated treatment conditions.

Figure S4

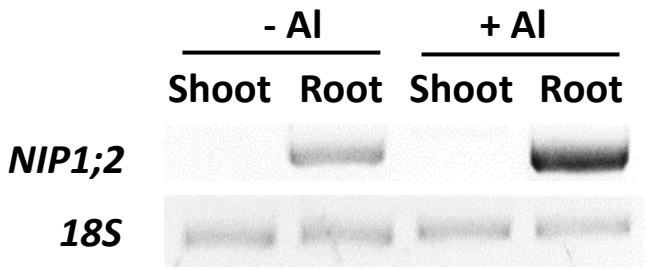


Fig. S4. Detection of *NIP1;2* expression in roots and shoots by RT-PCR. WT plants were treated with 0 or 20 μM Al for 6h.

Figure S5

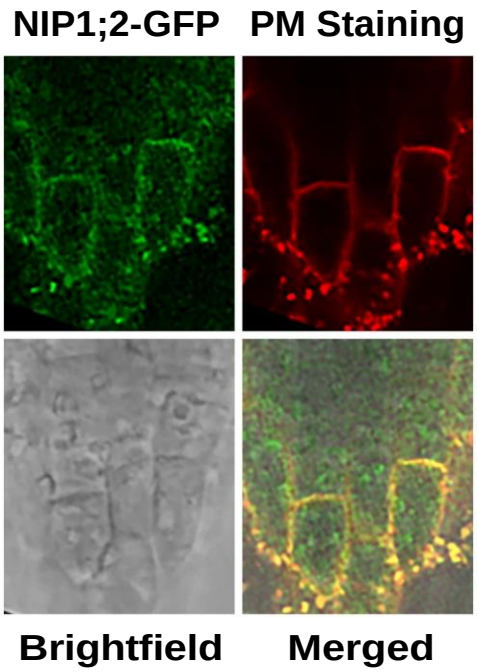


Fig. S5. NIP1;2 is localized to the PM of the *Arabidopsis* root cell. Confocal laser scanning microscopy of the root cells of T3 transgenic *Arabidopsis* plants transformed with *35S$_{pro}$:NIP1;2::GFP*. NIP1;2-GFP (green) was co-localized with the red CellMask^{TM} PM Staining (Thermo Fisher Scientific) in the root cells.

Figure S6

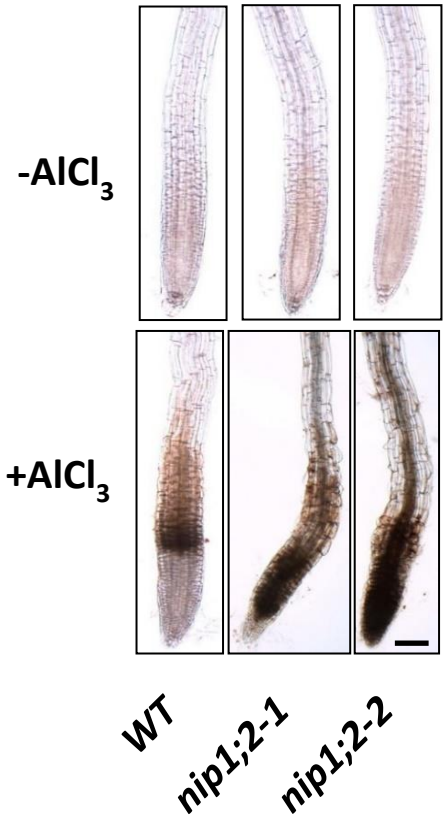


Fig. S6. Aluminum accumulation in cell walls of *WT*, *nip1;2-1* and *nip1;2-2*. Eight-day-old seedlings grown in hydroponic solution (pH4.2) were treated with or without 50 μM $AlCl_3$ for 4h before hematoxylin staining. The dark brown color manifests the hematoxylin-stained Al in the cell walls in the root-tip region. Scale bar, 100 μm.

Figure S7

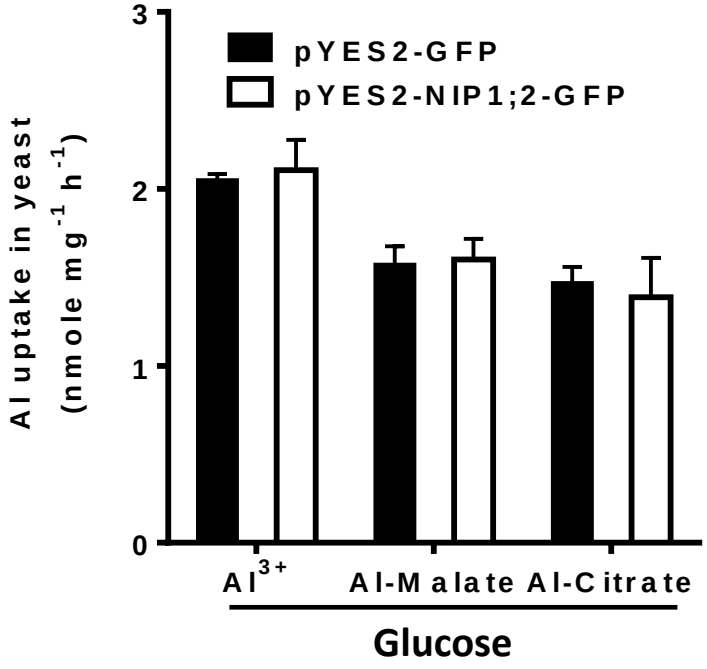


Fig. S7. Aluminum uptakes by yeast lines when glucose, but galactose, was included in the growth media. Yeast cells carrying *pYES2-GFP* or *pYES2-NIP1;2-GFP* were exposed to the low pH, low magnesium (LPM) solution (pH 4.2) containing $AlCl_3$ (50 μM), Al-Mal (50 μM $AlCl_3$ + 150 μM malate) or Al-Cit (50 μM $AlCl_3$ + 150 μM citrate) for 2h. The Al uptakes were measured by ICP-MS.

Figure S8

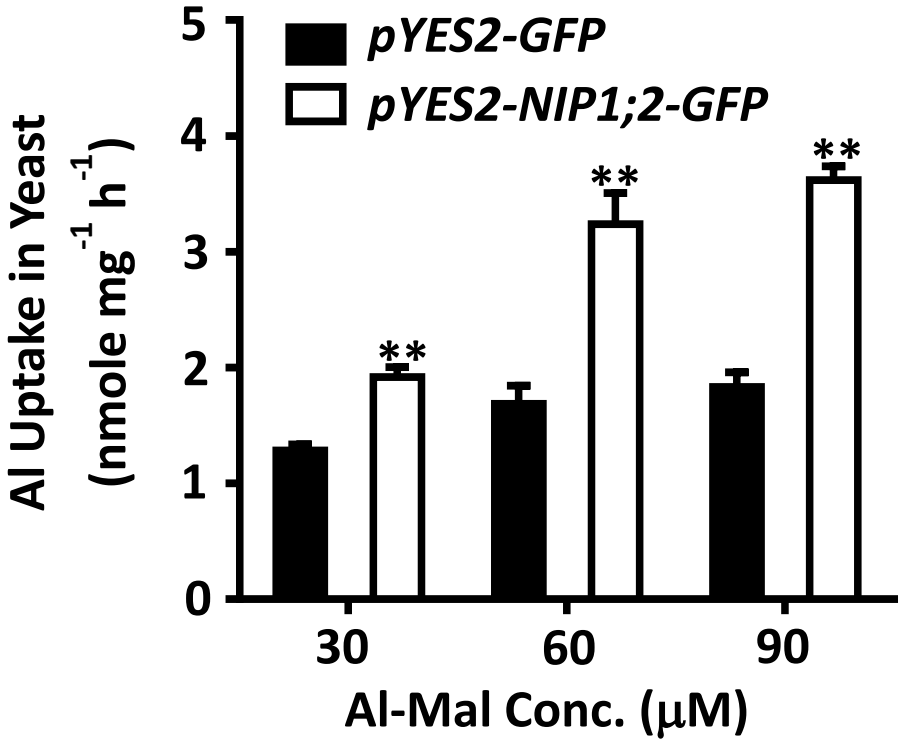


Fig. S8. Concentration-dependent Al-Mal uptake by NIP1;2. Yeast lines carrying *pYES2-GFP* or *pYES2-NIP1;2-GFP* were treated Al-Mal (50 μM $AlCl_3$ + 150 μM malate) at pH 4.2 for 1h. Al uptake was determined by ICP-MS. Data are means ± SD of three biological replicates from three independent transformation events. **, significant differences ($p<0.01$) between two yeast lines under indicated treatment conditions.

Figure S9

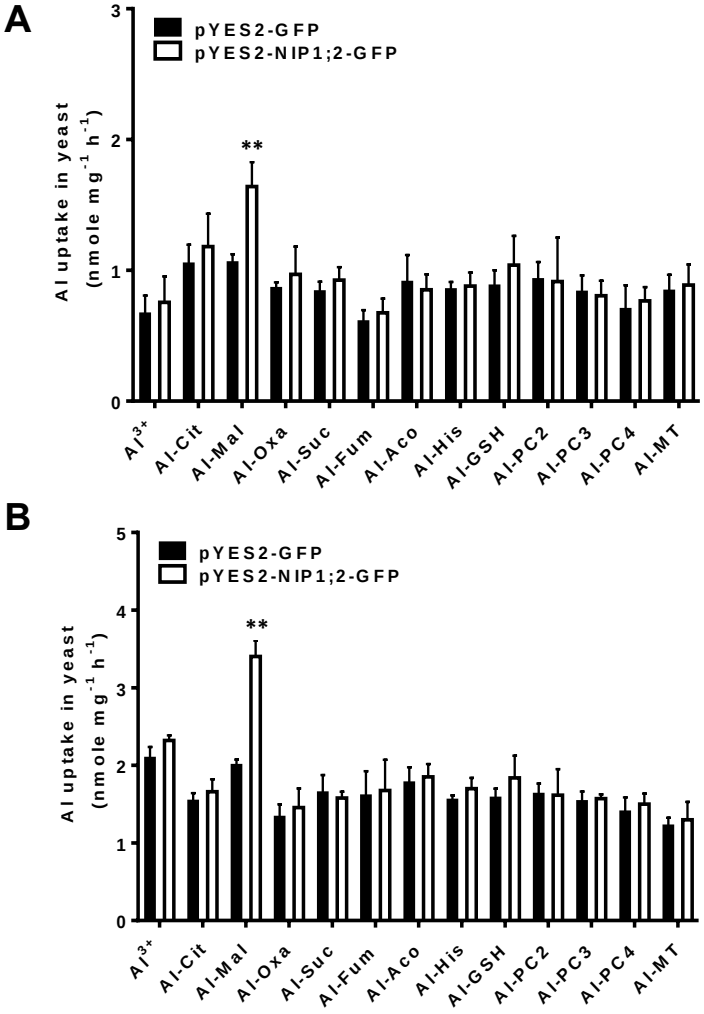


Fig. S9. NIP1;2 facilitates uptake of Al-malate, but not other Al-ligands, in yeast. Uptake of Al^{3+} and different Al-ligands by NIP1;2 at pH 4.2 (*A*) and 7.0 (*B*). Yeast cells carrying *pYES2-GFP* or *pYES2-NIP1;2-GFP* were exposed to the low pH, low magnesium (LPM) solution (pH 4.2) containing $AlCl_3$ (50 µM), or individual Al-ligands (50 µM $AlCl_3$ + 150 µM ligand) for 1h. The ligands used are citrate (Cit), malate (Mal), oxalate (Oxa), succinate (Suc), fumarate (Fum), aconite (Aco), histidine (His), glutathione (GSH), phytochelatin (PC) or metallothionein (MT). Then, Al 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 ($p<0.01$) between two yeast lines under indicated treatment conditions.

Figure S10

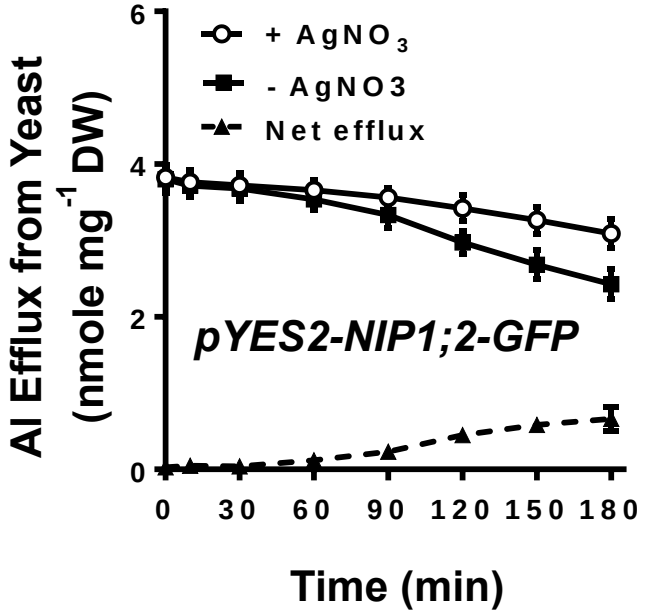


Fig. S10. Time-dependent Al efflux by NIP1;2. Yeast cells carrying *pYES2-NIP1;2-GFP* were pre-treated with Al-Mal for 8h, then transferred to fresh LPM medium supplemented with 0 or 5 μM $AgNO_3$ at pH 4.2 for indicated time. Yeast Al concentration was determined by ICP-MS. Net Al efflux at each time point was the difference between the 2 treatments. Data are means ± SD of three biological replicates from three independent transformation events.

Figure S11

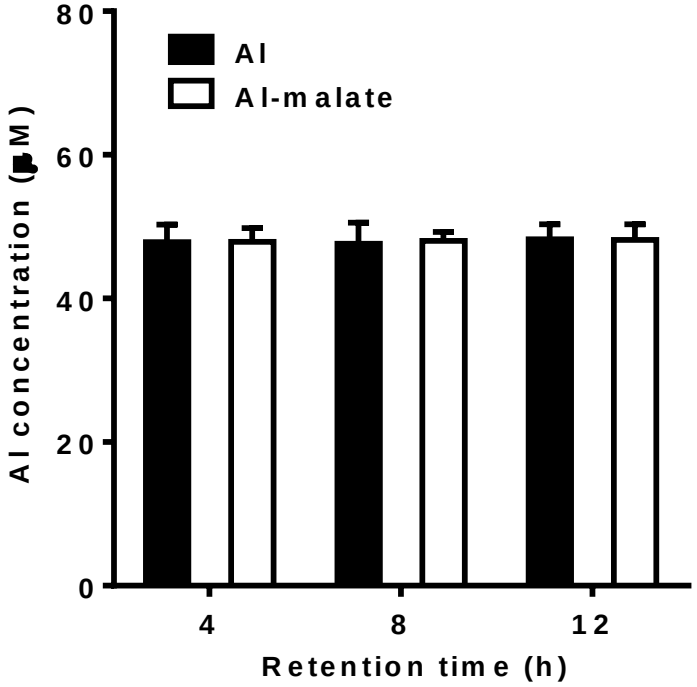
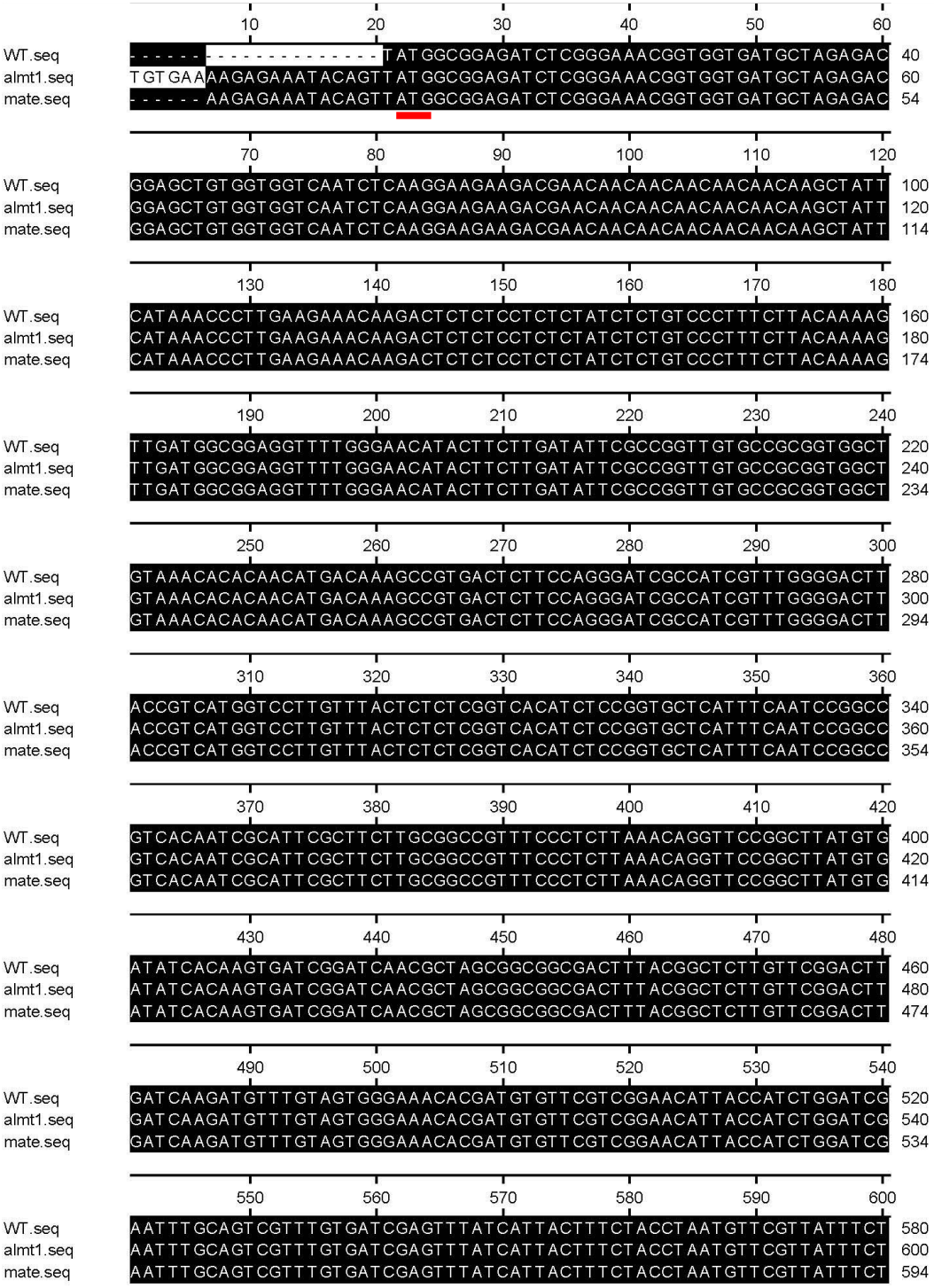


Fig. S11. Aluminum concentrations in hydroponic solutions. Aluminum concentrations in the freshly made hydroponic growth media supplemented with 50 μM $AlCl_3$ or Al-malate complex (50 μM $AlCl_3$ + 150 μM malate) at indicated time after the solutions being made. The Al concentrations in the solutions were measured by ICP-MS.

Figure S12. Sequence Alignments of *NIP1;2* cDNAs



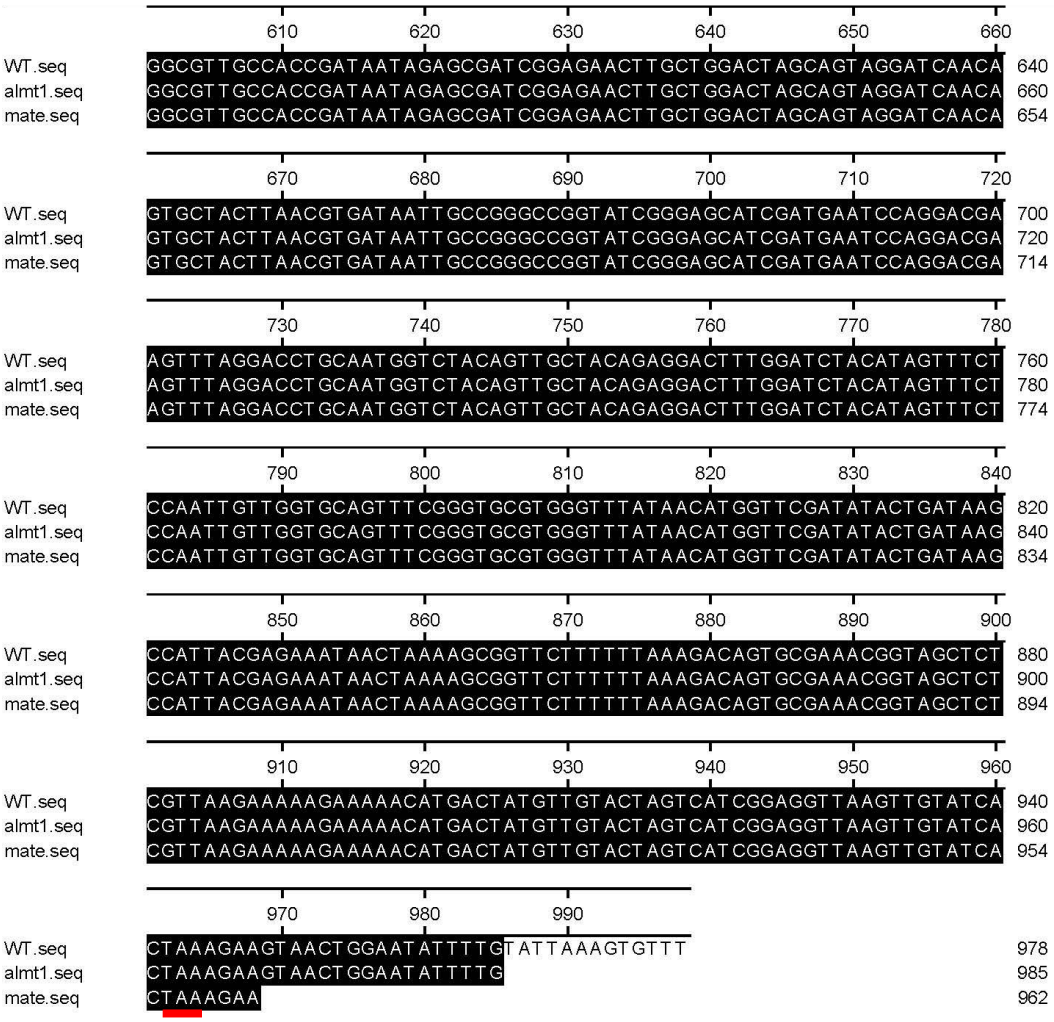


Fig. S12. Nucleotide sequence alignments of the *NIP1;2* cDNAs from the *WT* (*Col-0*), *almt1* and *mate*. The start codon, ATG, and the stop codon, TAA, are highlighted by red underlines.

Figure S13

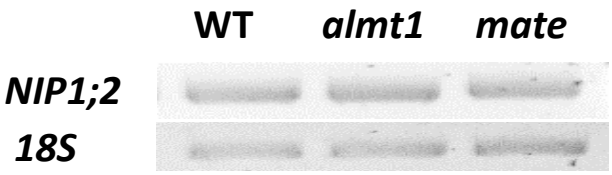


Fig. S13. RT-PCR analysis of *NIP1;2* expression in roots of 7-d-old *WT* (*Col-0*), *almt1* and *mate* treated with 20 μM $AlCl_3$ for 6h.