Supporting Information

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SI Materials and Methods

Plant Materials and Growth Conditions. Seeds were sterilized in 10% NaClO for 15 min, washed three times with deionized water, and then rolled into wet germination paper, followed by germination at 26~30 °C for 4 d in the dark. The seminal roots from the seedlings were inserted through the mesh bottoms of polyethylene cups placed into polyethylene containers filled with modified Magnavaca nutrient solution that we had previously altered to eliminate Al precipitation due to the very high Al tolerance of rice (1). Plants were grown in this nutrient solution at pH 4.2 with or without an Al^{3+} activity of 160 μM for 3 d. *Arabidopsis thaliana* plants were grown in the growth chamber at 22 °C with 16-h light/8-h dark cycles. For Al tolerance analyses, *Arabidopsis* seedlings were grown for 4–5 d on half-strength Murashige and Skoog medium with 0.8% (wt/vol) sucrose and 1.0% Bacto agar and then transferred to agar (0.8%) plates containing 1/2 strength nutrients supplemented with different concentrations of Al^{3+}. Plants were grown vertically for an additional 5–7 d, at which point root elongation was measured. Relative root growth (RRG %) was calculated according to the following formula: RRG% = root growth of individual plants under Al treatment/mean root growth under control (−Al) conditions.

Preparation of Constructs and Transformation. For preparing the *Arabidopsis* transformation constructs, the full-length Nramp aluminum transporter (*NRAT1*) coding sequences for the tolerant and sensitive alleles were PCR amplified (forward, 5′-CGGTACCATGGAAGGGACTGGTGAGATGA-3′; reverse, 5′- GGTCGACCTACATGGAAGCATCGGCAA-3′) and subcloned into the plant binary vector, pCAMBIA1300S (Cambia), that was predigested with *Kpn I* and *Sal I*. The constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and transferred into *Arabidopsis* Col-0 by Agrobacterium-mediated transformation as previously described (2). T2 seeds with a 3:1 segregation with regards to growth on hygromycin plates indicated a single copy insertion of the transgene in the transgenic lines. The hygromycin-resistant T2 lines were allowed to self to the T3 generation. Segregation analysis of individual T3 lines allowed us to identify T2 lines homozygous for the transgene, with all of the T3 plants derived from a single homozygous T2 line exhibiting hygromycin resistance. Homozygous T3 lines were then allowed to self to produce homozygous T4 lines with increased number of seeds used for the studies conducted here.

RNA Extraction and Quantitative Real-Time PCR. Total RNA was extracted from rice root tips (0 ~ 1 cm), or *Arabidopsis* leaves and roots using the RNeasy Mini Kit (GIAGEN) following the manufacturer's instructions. First-strand cDNA was synthesized from 5 μg DNaseI-digested total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen). PCR was performed on a Bio-Rad MyCycler C1000 using GO-Taq DNA polymerase (Promega); quantitative real-time RT-PCR was performed on a 7500 Fast Real-Time PCR System with Power SYBR Green PCR Master Mix according to manufacturers' protocols (Applied Biosystems, Inc.). The relative expression levels of the target genes were referred to an endogenous calibrator gene, *OsHistoneH3:5′*, in each single real-time qRT-PCR experiment. The real-time primers were as follows: *OsNRAT1*, 5′-TATCATCACACCATACAA-3′ and 5′-AGACTACACTCAATAAGG-3′; *OsHistoneH3*, 5′-AGATCCGCAAGTACCAGAAGAGCA-3′and 5′-AAGCGGAGGTCGGTCTTGAAGT-3′; *OsALS1*, 5′-CTGTTGCTTCATCCATAG-3′ and 5′-ACCATATCGTAAGACTTCA-3′; *AtALS1*, 5′-ATAGGTTCAACAACTAATCTC-3′ and 5′-TCTAACAGCAATCAAGGA-3′; *AtMATE*, 5′-ATTCTGTGTAACTCTCTCCGC-3′ and 5′-TCCTGTTCCTGTCCCAAT-3′; *AtACTIN2*, 5′-CGTGACCTTACTGATTAC and 5′-TTCTCCTTGATGTCTCTT-3′; and *AtALMT1*, 5′-GATTGGAGGAGTCAGTTG-3′ and 5′-AGTTAGAGGCAAGGAGAG-3′.

Yeast Al Tolerance and Uptake Analysis. The *NRAT1* coding sequences were amplified by PCR with forward primer (5′-GGTACCAAA ATGGAAGGGACTGGTGAGATGA-3′) and reverse primer (5′-CTACATGGAAGCATCGGCAA -3′) from cDNAs generated from each of the 24 rice lines selected from the rice diversity panel and cloned into the yeast expression vector, pYES2. Single amino acid NRAT1 mutants were generated via partial DNA fragment substitution using *Msc I*, *Nar I*, and *SrgA I*. The resulting constructs and pYES2 were then transformed into the yeast DY4741 cell line following the small-scale yeast transformation protocol (Invitrogen). For examining the Al tolerance of different yeast transformants, yeast cells in midexponential phase were diluted to an OD_{600} value of 0.1 with low pH, low magnesium (LPM) medium containing 2% galactose for induction of the GAL promoter. For the time course experiment, cell concentrations were measured at different time points after exposure to 50 μM Al^{3+}. For the Al concentration experiment, yeast cell numbers were measured after growth in the LPM medium containing 0, 100, 200, or 500 μM Al for 15 h. For the measurement of Al accumulation, the yeast cells were harvested by centrifugation and washed three times with deionized water (MilliQ; Millipore) and then digested with 2 N HCl. The concentration of Al in the digest solution was determined by inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 7500 Series ICP mass spectrometer.

Root Cell Sap Preparation and Al Determination. Rice lines were grown in the modified Magnavaca nutrient solution for 3 d as described above and then transferred to hydroponic solution containing 160 active μM Al^{3+} (pH 4.2) for 3 d. After the treatment, the first 1 cm of root tip segments were cut and washed three times with deionized water and then centrifuged at 3,000 × *g* for 10 min at 4 °C using Ultra Free-MC Centrifugal filter units (Millipore) to remove apoplastic solution. The samples were then frozen at −80 °C overnight. The root cell sap solution was obtained by thawing the samples at room temperature and then centrifuging at 20,600 × *g* for 10 min. The residual cell wall was washed with 70% ethanol three times and then digested in 0.5–1 mL of 2 N HCl for at least 24 h with occasional vortexing. Al content in the symplastic solution and cell wall extracts was determined by ICP-MS.

Sequence and Haplotype Analysis. The *NRAT1* coding sequences were amplified from cDNA and promoter sequences were amplified from genomic DNA by PCR using AccuPrime Pfx DNA Polymerase (Invitrogen). The primers for the *NRAT1* coding sequence are 5′-GGTACCAAAATGGAAGGGACTGGTGAGATGA-3′ and 5′-CTACATGGAAGCATCGGCAA-3′. The primers for the *NRAT1* promoter sequence are 5′-AACACGTCTGACGCTTGTT -3′ and 5′- ATTCTATGTTGCTAATGCACCTTGT-3′. The DNA fragments were sequenced using BigDye Terminators V3.1 cycle sequencing kit on an Applied Biosystems Automated 3730 DNA Analyzer. The haplotypes were obtained by multiple sequences alignment using ClustalX 2.1 (3).

1. Famoso AN, et al. (2010) Development of a novel aluminum tolerance phenotyping platform used for comparisons of cereal aluminum tolerance and investigations into rice aluminum tolerance mechanisms. *Plant Physiol* 153(4):1678–1691.
2. Clough SJ, Bent AF (1998) Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J* 16(6):735–743.
3. Larkin MA, et al. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* 23(21):2947–2948.

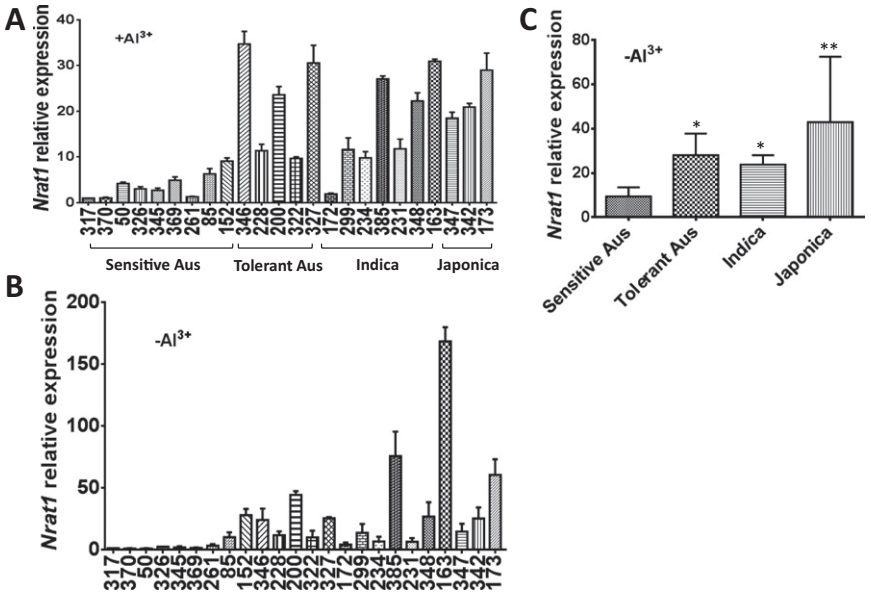


Fig. S1. *NRAT1* relative expression in 24 diverse rice lines. (*A*) *NRAT1* expression determined using quantitative real-time PCR with RNA from roots of the 24 diverse rice lines grown for 3 d on nutrient solution with an Al^{3+} activity of 160 μM. *NRAT1* expression for each line is presented in relation to *NRAT1* expression in the most sensitive *aus* line, NSF-317, whose expression was set to 1. The rice gene *OsHistoneH3:5′* was used as the endogenous calibrator gene for each single real-time qRT-PCR experiment. (*B*) Relative *NRAT1* expression for the same 24 rice lines in *A* for plants grown under control (−Al) conditions. (*C*) The *NRAT1* expression data from *B* averaged for the lines classified into the following categories: sensitive *aus*, tolerant *aus*, *indica*, and *japonica*. Asterisks indicate significant differences in expression between the Al-sensitive *aus* lines expressing *NRAT1.2* and the Al-tolerant *aus*, *indica*, and *japonica* lines expressing *NRAT1.1*. Significant differences determined by *t* tests (*$P < 0.05$, **$P < 0.01$). Values are means ± SE.

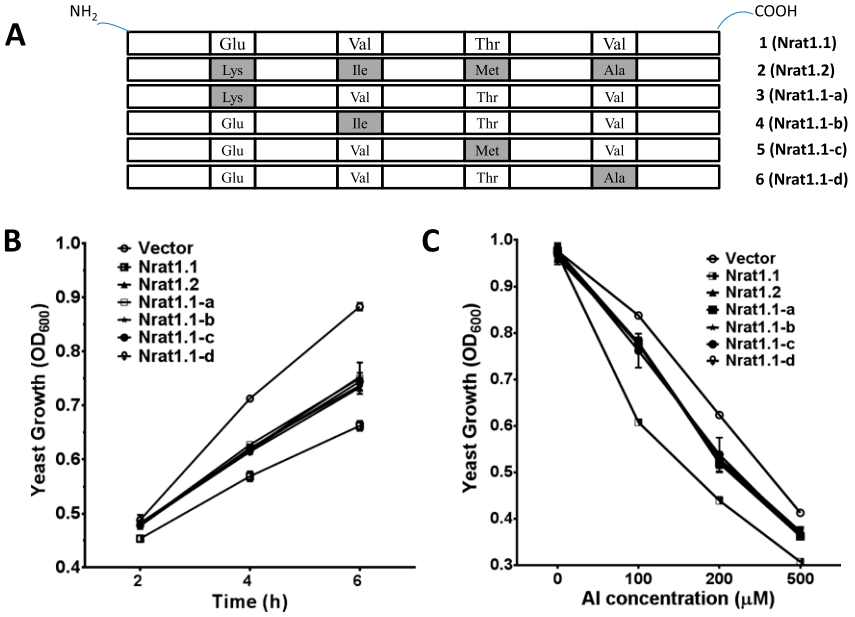


Fig. S2. Functional analysis of the *NRAT1* missense mutations. (*A*) Diagrammatic representation of the four amino acid differences between the *NRAT1.1* and *NRAT1.2* alleles (1 and 2) and then the maps for the single amino acid mutants that were generated (3–6). Gray boxes represent amino acid alterations compared with the amino acid sequence from Nipponbare, which harbors the more tolerant *NRAT1.1* allele. (*B*) Time-dependent kinetics for yeast growth on a toxic level of Al (50 μM Al^{3+}) for yeast cells expressing the empty expression vector, the tolerant *NRAT1.1* allele, the sensitive *NRAT1.2* allele, or each of the single amino acid mutants (3–6 in *A*) grown on LPM medium + Al for 2, 4, and 6 h. (*C*) Yeast growth on media without Al or increasing concentrations of Al (100, 200, and 500 μM Al^{3+}) for yeast cells expressing the empty expression vector, the tolerant *NRAT1.1* allele, the sensitive *NRAT1.2* allele, or each of the single amino acid mutants (3–6 in *A*) grown on LPM media ±Al for 6 h. In *B* and *C*, values are means ± SE ($P < 0.01$).

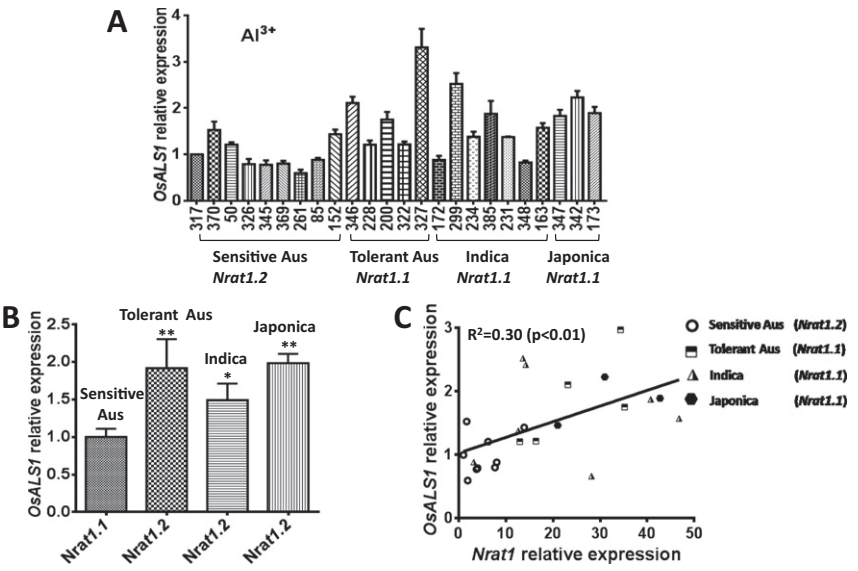


Fig. S3. *OsALS1* and *NRAT1* expression in roots of the 24 diverse rice lines. (*A*) Quantitative real-time PCR analysis of root *OsALS1* expression in plants grown on Al^{3+} for the 24 diverse rice lines. *OsALS1* relative expression in each line was determined by quantitative real-time PCR; the relative expression of each line was in relation to the *OsALS1* expression in the most sensitive *aus* line, NSF-317, whose *OsALS1* expression was set to 1. The expression values are the means ± SE. (*B*) The mean *OsALS1* expression under +Al growth conditions for the four different categories of the 24 rice lines. Values are the mean ± SE. Asterisks indicate significant differences by *t* tests between the sensitive *aus* lines carrying the *NRAT1.2* allele and the more tolerant *aus*, *indica*, and *japonica* lines carrying the tolerant *NRAT1.1* allele (*$P < 0.05$, **$P < 0.01$). (*C*) Regression analysis for the relationship between *OsALS1* relative expression under Al^{3+} treatment for the 24 rice lines.

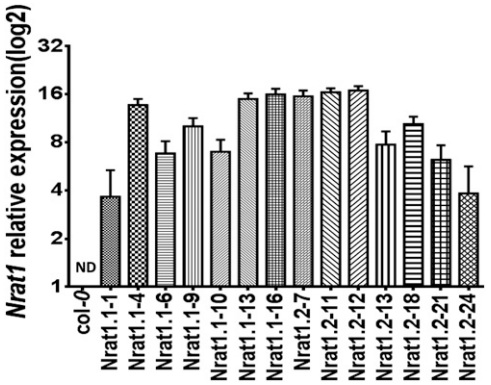


Fig. S4. *NRAT1* expression analysis in the *NRAT1*-expressing transgenic *Arabidopsis* lines. Quantitative real-time PCR analysis of *NRAT1* relative expression in *NRAT1*-expressing transgenic *Arabidopsis* lines (relative to wild type). The *actin2* gene was used as an endogenous internal standard. Values are the mean ± the SE.

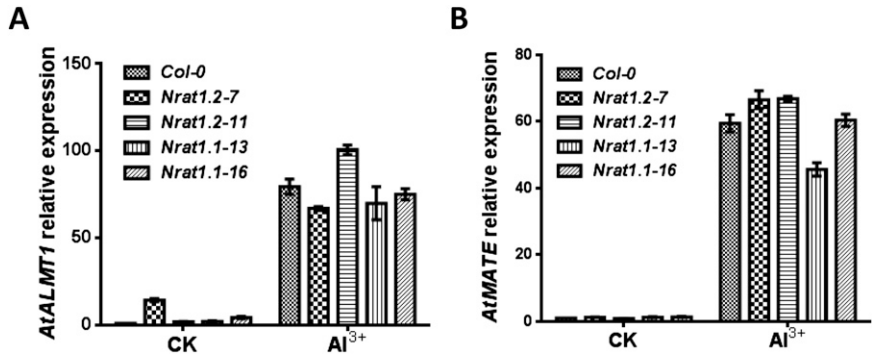
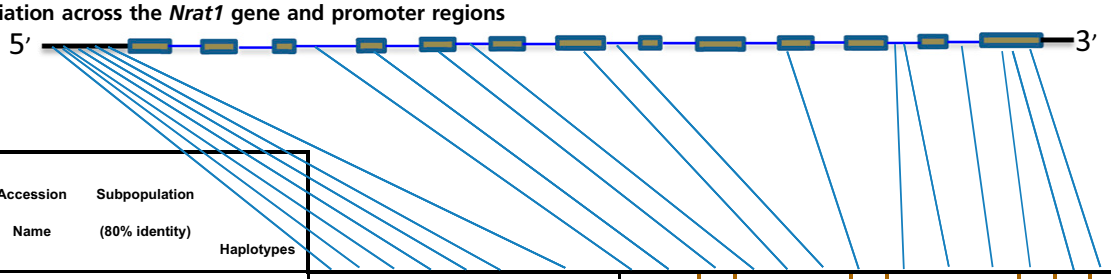


Fig. S5. *AtALMT1* and *AtMATE* expression in *NRAT1*-expressing transgenic *Arabidopsis* lines in response to control (−Al) and +Al treatments. (*A*) Quantitative real-time PCR analysis of *AtALMT1* expression in WT and *NRAT1* expressing transgenic *Arabidopsis* lines in response to control (−Al) and +Al treatments. (*B*) Quantitative real-time PCR analysis of *AtMATE* expression in WT and *NRAT1* expressing transgenic *Arabidopsis* lines in response to control (−Al) and +Al treatments. Values are means ± SE. Asterisks indicate significant differences based on *t* tests (*$P < 0.05$, ***$P < 0.001$).

Table S1. DNA variation across the *Nrat1* gene and promoter regions



Line I.D. (NSF-TV)	TRG-RRG	Accession Name	Subpopulation (80% identity)	Haplotypes																				
50	0.12	Dz78	AUS	1	G	G	G	T	C	G	TGTGCTT	A	T	A	A	T	C	A	C	A	T	T	C	C
152	0.25	T 1	AUS		G	G	G	T	C	G	TGTGCTT	A	T	A	A	T	C	A	C	A	T	T	C	C
261	0.19	Shim Balte	AUS		G	G	G	T	C	G	TGTGCTT	A	T	A	A	T	C	A	C	A	T	T	C	C
317	0.08	Dj 123	AUS		G	G	G	T	C	G	TGTGCTT	A	T	A	A	T	C	A	C	A	T	T	C	C
326	0.13	Ghorbhai	AUS		G	G	G	T	C	G	TGTGCTT	A	T	A	A	T	C	A	C	A	T	T	C	C
345	0.14	Dz 193	AUS		G	G	G	T	C	G	TGTGCTT	A	T	A	A	T	C	A	C	A	T	T	C	C
369	0.14	Sathi	AUS		G	G	G	T	C	G	TGTGCTT	A	T	A	A	T	C	A	C	A	T	T	C	C
370	0.12	Coarse	AUS		G	G	G	T	C	G	TGTGCTT	A	T	A	A	T	C	A	C	A	T	T	C	C
85	0.2	Kasalath	AUS	2	A	G	G	T	C	G	TGTGCTT	A	T	A	A	A	G	A	A	G	T	T	C	C
228	0.4	Ca 902/B/2/1	AUS	3	G	A	T	A	T	T	TGTGCTT	G	C	G	A	A	G	G	C	G	T	C	T	T
327	0.62	Goria	AUS	4	G	A	T	A	T	T	TGTGCTT	n/a	C	G	n/a	A	G	G	n/a	n/a	n/a	C	T	T
322	0.54	DM 59	AUS	5	G	A	T	A	T	T	deletion	n/a	C	G	n/a	A	G	G	n/a	n/a	n/a	C	C	T
346	0.37	Karkati 87	AUS	6	G	A	T	A	T	T	deletion	G	C	G	A	A	G	G	C	G	T	C	C	T
231	0.43	Hunan Early Dwarf No. 3	IND	7	G	A	T	A	T	T	deletion	G	C	G	G	A	G	G	C	G	T	C	C	T
234	0.31	Aijiaonante	IND		G	A	T	A	T	T	deletion	G	C	G	G	A	G	G	C	G	T	C	C	T
299	0.26	Sml 242	IND		G	A	T	A	T	T	deletion	G	C	G	G	A	G	G	C	G	T	C	C	T
348	0.61	China 1039	IND		G	A	T	A	T	T	deletion	G	C	G	G	A	G	G	C	G	T	C	C	T
385	0.39	Nira	IND		G	A	T	A	T	T	deletion	G	C	G	G	A	G	G	C	G	T	C	C	T
172	0.24	Zhenshan 2	IND		G	A	T	A	T	T	deletion	G	C	G	G	A	G	G	C	G	T	C	C	T
173	0.75	Nipponbare	TEJ	8	G	A	T	A	T	T	deletion	G	C	G	A	A	G	G	C	G	T	C	C	T
342	0.62	Cenit	TRJ		G	A	T	A	T	T	deletion	G	C	G	A	A	G	G	C	G	T	C	C	T
200	0.44	P 737	AUS		G	A	T	A	T	T	deletion	G	C	G	A	A	G	G	C	G	T	C	C	T
163	0.8	Taducan	IND		G	A	T	A	T	T	deletion	G	C	G	A	A	G	G	C	G	T	C	C	T
347	0.42	Creole	TRJ	9	G	A	T	A	T	T	deletion	A	C	G	A	A	G	G	C	G	C	C	C	T

DNA variation was assayed in 24 diverse lines of rice by sequencing the putative promoter region (1 kb upstream of the translational start site) and both the exonic (this study) and intronic sequence (from ref. 1). Twenty variable sites were identified based on comparison with the Nipponbare *Nrat1* sequence, including 19 SNPs and one 7-bp insertion. A total of 9 haplotypes were identified among the 24 diverse rice lines. In the gene model shown above the table, black lines represent 5′ and 3′ UTR, blue lines represent introns, and gray boxes represent exons. Nonsynonymous SNPs are indicated in red, synonymous SNPs and indels are colored blue, and reference (Nipponbare) SNPs and indels are colored brown. n/a indicates missing data where intronic sequence was not available from (1). TRG-RRG indicates the level of Al tolerance for each line, based on the relative root growth (RRG) of the total root system. TRG, total root growth.

1. Famoso AN, et al. (2011) Genetic architecture of aluminum tolerance in rice (O. sativa) determined through genome-wide association analysis and QTL mapping. *PLoS Genetics* 7(8): e1002221.

Table S2. SNPs that distinguish the sensitive *aus Nrat1* haplotype from the tolerant haplotype in *aus*, *indica*, and *japonica* lines

SNP location or rice genotype	Region and SNP									
	Promoter Region					Coding Sequence				
	M2	M3	M4	M5	M6	M8	*M9*	*M12*	*M13*	*M15*
Location (MSU V7)	1656959	1657095	1657260	1657399	1657457	1660011	1660193	1661370	1662323	1662367
Sensitive *aus*	G	G	T	C	G	T	*A*	*A*	*T*	*C*
Tolerant *aus*, *indica*, and *japonica*	A	T	A	T	T	C	*G*	*G*	*C*	*T*

Ten specific SNPs in the *Nrat1* promoter and coding sequences that differentiate the sensitive *aus* lines from the tolerant *aus*, *indica*, and *japonica* lines. The italicized SNPs lead to changes in amino acid sequence in the Nrat1 coding region.

Table S3. Amino acid haplotypes for Nrat1

AA location or rice genotype	SNPs				
	M9	M12	M13	M14	M15
AA mutation positions	120	326	500	502	515
Haplotype 1 (Nrat1.1)	Glu	Val	Thr	Ala	Val
Haplotype 2 (Nrat1.2)	*Lys*	*Ile*	*Met*	Ala	*Ala*
Haplotype 3 (Nrat1.3)	Glu	Val	Thr	*Val*	Val

The identification of three Nrat1 protein coding sequences found in the 24 lines from the rice diversity panel. The three haplotypes based on the three amino acid sequences are named Nrat1.1, Nrat1.2, and Nrat1.3. Amino acids in italics denote amino acids that differ from the AAs in Nrat1.1.

Table S4. Root cell sap and cell wall Al content in the 24 diverse rice lines

Line ID (NSF-TV)	TRG-RRG	Accession name	Subpopulation (80% identity)	Cell wall Al content (ng Al per segment)	Cell sap Al content (ng Al per segment)	Cell wall Al content by populations	Cell sap Al content by populations
85	0.201	Kasalath	AUS	99.5	15.43	112.9 ± 7.75	11.44 ± 1.0
50	0.12	Dz78	AUS	129.83	11.02		
152	0.246	T 1	AUS	75.53	8.2		
261	0.19	Shim Balte	AUS	120.76	7.82		
317	0.08	Dj 123	AUS	117.28	12.48		
326	0.13	Ghorbhai	AUS	87.76	14.54		
345	0.14	Dz 193	AUS	105.26	ND		
369	0.14	Sathi	AUS	129.8	12.62		
370	0.12	Coarse	AUS	150.38	9.38		
228	*0.4*	*Ca 902/B/2/1*	*AUS*	*68.57*	*11.13*	*74.0 ± 6.66*	*14.9 ± 2.24*
327	*0.62*	*Goria*	*AUS*	*56.69*	*10.88*		
346	*0.37*	*Karkati 87*	*AUS*	*93.33*	*18.59*		
322	*0.54*	*DM 59*	*AUS*	*66.28*	*ND*		
200	*0.441*	*P 737*	*AUS*	*85.18*	*18.95*		
231	**0.427**	**Hunan Early Dwarf No. 3**	**IND**	**85.5**	**11.83**	**81.9 ± 5.42**	**14.1 ± 1.29**
234	**0.31**	**Aijiaonante**	**IND**	**97.44**	**10.85**		
299	**0.26**	**Sml 242**	**IND**	**65.29**	**ND**		
348	**0.61**	**China 1039**	**IND**	**86.24**	**18.71**		
385	**0.39**	**Nira**	**IND**	**94.51**	**15.5**		
163	**0.8**	**Taducan**	**IND**	**59.17**	**11.56**		
172	**0.24**	**Zhenshan 2**	**IND**	**81.36**	**16.14**		
173	***0.75***	***Nipponbare***	***TEJ***	***52.6***	***13.06***	***71.8 ± 10.35***	***17.7 ± 3.91***
342	***0.622***	***Cenit***	***TRJ***	***74.67***	***14.56***		
347	***0.42***	***Creole***	***TRJ***	***87.99***	***25.48***		

Al content in the root cell sap and cell wall in the 24 lines from the rice diversity panel, as well as average Al content root cell sap and cell wall of 24 lines. The 24 diverse lines are grouped into the Al-sensitive aus (roman), tolerant *aus* (italicized), *indica* (bold), and *japonica* (bold and italicized). Note that the *indica* and *japonica* lines are much more Al-tolerant than the sensitive *aus* lines and comparable in Al tolerance (and sometimes greater in tolerance) to the tolerant *aus* lines.