Comparative Genome-Wide Transcriptional Analysis of Al-Responsive Genes Reveals Novel Al Tolerance Mechanisms in Rice

Tomokazu Tsutsui¹, Naoki Yamaji¹, Chao Feng Huang¹, Ritsuko Motoyama², Yoshiaki Nagamura², Jian Feng Ma¹*

¹ Institute of Plant Science and Resources, Okayama University, Kurashiki, Japan, ² Genome Resource Center, Division of Genome and Biodiversity Research, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan

Abstract

Rice (Oryza sativa) is the most aluminium (Al)-tolerant crop among small-grain cereals, but the mechanism underlying its high Al resistance is still not well understood. To understand the mechanisms underlying high Al-tolerance, we performed a comparative genome-wide transcriptional analysis by comparing expression profiling between the Al-tolerance cultivar (Koshihikari) and an Al-sensitive mutant star1 (SENSITIVE TO AL RHIZOTOXICITY 1) in both the root tips and the basal roots. Exposure to 20 μM AlCl₃ for 6 h resulted in up-regulation (higher than 3-fold) of 213 and 2015 genes including 185 common genes in the root tips of wild-type and the mutant, respectively. On the other hand, in the basal root, genes up-regulated by Al were 126 and 2419 including 76 common genes in the wild-type and the mutant, respectively. These results indicate that Al-response genes are not only restricted to the root tips, but also in the basal root region. Analysis with genes up- or down-regulated only in the wild-type reveals that there are other mechanisms for Al-tolerance except for a known transcription factor ART1-regulated one in rice. These mechanisms are related to nitrogen assimilation, secondary metabolite synthesis, cell-wall synthesis and ethylene synthesis. Although the exact roles of these putative tolerance genes remain to be examined, our data provide a platform for further work on Al-tolerance in rice.

Introduction

Aluminum (Al) toxicity is a major factor limiting crop production on acid soils, which comprise approximately 40% of the world’s arable soils and up to 70% of potentially arable land [1]. At soil pH below 5.0, toxic forms of Al (mainly Al³⁺) are solubilized into the soil solution, which inhibit root growth and function, consequently reducing crop yields [2,3]. However, there is a great variation for the ability to withstand Al-toxicity between plant species and cultivars within a species. To survive on acidic soils, some plant species or cultivars have evolved mechanisms to tolerate high levels of toxic Al. Many mechanisms for both Al-tolerance and -toxicity have been proposed [3].

Rice (Oryza sativa) is the most Al-tolerant crop among small-grain cereals [4]. A number of quantitative trait loci (QTLs) for Al-tolerance have been identified in rice by using different populations [5], but responsible QTL genes have not been isolated. Recently, through genome-wide association analysis and QTL mapping, 48 loci associated with Al³⁺ tolerance have been identified [6] in rice. On the other hand, mutant approaches have revealed an ART1-regulated Al-tolerance mechanism in rice [7]. ART1 (AL³⁺ RESISTANCE TRANSCRIPTION FACTOR 1) is a Cys2His2-type zinc-finger transcription factor [8]. ART1 is constitutively expressed in the roots and its expression is not induced by Al³⁺ treatment. ART1 regulates the expression of at least 31 genes with a cis-element [GGN(T/g/a/C)V(C/A/g)S(C/G)] (ART1-binding affinity of nucleotides with small characters is weaker than those with large characters) [9]. Among them, only six genes have been functionally characterized. OsaSTAR1 and OsaSTAR2 (SENSITIVE TO ALUMINUM RHIZOTOXICITY 1 & 2) encode a ATP-binding domain and a transmembrane domain, respectively, of a bacterial-type ATP binding cassette (ABC) transporter, which transports UDP-glucose [10]. The complex is implicated in cell wall modification [10]. OsaFRDL4 (FERRIC REDUCTASE DEFECTIVE LIKE 4) encodes a citrate transporter, which secretes citrate from the roots to chelate Al in the rhizosphere [11]. On the other hand, OsaNat1 (NRAMP ALUMINUM TRANSPORTER 1) encodes an Al transporter localized at the plasma membrane, which transports Al into the cells [12], while OsALS1 (ALUMINUM SENSITIVE 1) encodes a tonoplast-localized transporter for Al, which sequestrates Al into the vacuoles [13]. Recently, up-regulation of a Mg transporter, OsMGT1 (MAGNESIUM TRANSPORTER 1), is reported to be required for conferring Al-tolerance in rice [14]. All of these genes are specifically induced by Al and knockout of either gene results in decreased Al-tolerance, indicating their important roles.
in Al-tolerance. However, the mechanisms underlying high Al-tolerance in rice are not fully understood at the molecular level.

In the present study, we performed a genome-wide transcriptional analysis of Al-responsive genes in rice. By comparing transcriptional profiling between a wild-type rice and an Al-sensitive rice mutant star1, we found that rice possesses novel mechanisms of Al-tolerance in addition to ART1-regulated mechanism in rice.

Materials and Methods

Plant Materials and Growth Conditions

Seeds of wild-type rice (Oryza sativa cv. Koshikihari) and an Al-sensitive mutant, star1 [10], were germinated for 2 days at 30°C. The seedlings were then transferred to a plastic net floating on a 0.5 mM CaCl2 solution in a 1.5 L plastic box. At day 4, the seedlings were exposed to a 0.5 mM CaCl2 solution (pH 4.5) containing 0 or 20 μM AlCl3. Root length was measured with a ruler before and after 6 h treatments. Ten seedlings were used for each treatment.

RNA Isolation, Microarray and Data Analysis

Root tips (0–1 cm) and basal region (1–2 cm) of the roots (20–30 plants per sample) were excised from the seedlings of both wild-type rice and star1 mutant which had been exposed to 20 μM AlCl3 for 6 h and immediately frozen in liquid nitrogen. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Germany). The RNA quality was assessed on agarose gels and with the Nanodrop ND-1000 (Thermo Fisher Scientific, USA). Microarray analysis was performed according to Agilent Oligo DNA Microarray Hybridization protocols using the Agilent 44 K Rice Oligo DNA Microarray RAP-DB (Agilent Technologies, USA; G2519F#15241) [15] with three biological replicates (Agilent Technologies, USA; G2519F#15241) [15]. The hybridized slides were scanned using a DNA microarray scanner (Agilent Technologies, USA). Signal intensities were extracted by Feature Extraction software (Agilent Technologies, USA). For statistical analysis, we excluded genes with low signal intensities less than 500 (sum of Cy3 and Cy5) and normalized the microarray data and quantatitive RT-PCR data. Twelve genes randomly selected were subjected to quantitative real-time PCR analysis. OsHistone H3 was used as an internal standard. Microarray data (fold change of gene expression) were plotted against data (fold change of gene expression) from quantitative real-time PCR. Both x- and y-axes are shown in log2 scale. t indicates correlation coefficient. doi:10.1371/journal.pone.0048197.g002

Figure 1. Al-induced inhibition of root elongation. Seedlings (6-d-old) of both wild-type rice (WT) and an Al-sensitive mutant (star1) were exposed to a 0.5 mM CaCl2 solution (pH 4.5) containing 0, 20 μM Al for 6 h. The root length was measured with a ruler before and after Al treatment. Error bars represent ± SD (n = 10). Different letters indicate significant differences at P<0.05 by Tukey’s Honestly Significantly Different test. doi:10.1371/journal.pone.0048197.g001

Figure 2. Correlation of gene expression ratio between microarray data and quantitative RT-PCR data. Twelve genes randomly selected were subjected to quantitative real-time PCR analysis. OsHistone H3 was used as an internal standard. Microarray data (fold change of gene expression) were plotted against data (fold change of gene expression) from quantitative real-time PCR. Both x- and y-axes are shown in log2 scale. t indicates correlation coefficient. doi:10.1371/journal.pone.0048197.g002

Quantitative Real-time PCR

To validate microarray data, 12 genes were randomly selected for quantitative real-time PCR (qRT-PCR) (Table S1). Total RNA was prepared from the root tips and basal root regions of wild-type and star1 mutant using RNeasy Plant Mini Kit (Qiagen, Germany) and reversely transcribed using SuperScript™ II Reverse Transcriptase (Invitrogen, USA) and Oligo(dT) primers. The qRT-PCR was performed on an Eppendorf MasterCycler ep realplex real-time PCR (Eppendorf, Germany) using the specific primers described in Table S1.

One-fifth dilutions of the cDNAs were used as a template for the qRT-PCR in a total volume of 20 μL as follows; 10 μL SYBR Premix Ex Taq™ Perfect Real Time (TaKaRa Biol Inc., Japan), 0.4 μL ROX Reference Dye, 0.8 μL primer mix (50:50 mix of forward and reverse primers at 10 pmol μL−1 each), 6.8 μL distilled water and 2 μL template. The reaction conditions were: 90 s at 95°C followed by 40 cycles of 30 s at 95°C, 20 s at 60°C and 35 s at 72°C. The rice Histone H3 was used as an internal control. Relative expression levels were calculated by the

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comparative Ct method. Three independent biological replicates were made for each gene.

Results and Discussion

Tolerance and toxicity of Al stress are a complicated phenomenon, involving many genes and a number of signaling pathways [19]. However, microarray technique has provided a useful tool for investigation of genome-wide changes in transcripts. So far, microarray analysis for Al response has been reported in Arabidopsis [20–22], maize [23,24], Medicago truncatula [25,26], and wheat [27]. Since the mechanisms for Al-tolerance differ with plant species, in the present study, we performed a microarray analysis with rice, a well-known Al-tolerant species, to understand genes involved in high Al-tolerance at genome-wide scale.

Al-toxicity is characterized by inhibition of root elongation, which occurs within a few hours after exposure to Al [3]. Therefore, to exclude genes associated with Al-toxicity, we sampled the roots exposed to Al solution for 6 h for microarray analysis. Furthermore, to extract genes related to Al-tolerance, we compared the transcriptional profiling between the wild-type rice and an Al-sensitive mutant, star1 [10]. Moreover, we selected a concentration of 20 \( \mu \text{M} \) for Al treatment. At this concentration, the root elongation of the wild-type rice was hardly inhibited, whereas that of the mutant was inhibited by 75% (Figure 1), which make possible to extract genes possibly associated with Al-tolerance.

Verification of Microarray Results by Quantitative Real-time PCR

To validate the reliability of the microarray data, we randomly selected 12 genes from root tips and basal root regions for the quantitative real-time PCR (qRT-PCR) analysis. There was a good correlation (\( r = 0.84 \)) between the microarray data and the

Table 1. Functional classification of Al-responsive genes in the roots of the wild-type rice and star1 mutant.

|                         | Up-regulated* | Down-regulated* |
|-------------------------|---------------|-----------------|
|                         | Root tips     | Basal roots     | Root tips | Basal roots |
|                         | WT (%)        | star1 (%)       | WT (%)    | star1 (%)   |
| Transport               | 19 (8.8)      | 125 (6.2)       | 10 (7.9)  | 113 (4.7)   |
| Metabolism              | 31 (14.4)     | 251 (12.5)      | 18 (14.3) | 340 (14.1)  |
| Protein synthesis and processing | 14 (6.5)     | 129 (6.4)       | 7 (5.6)   | 125 (5.2)   |
| Signal transduction     | 6 (2.8)       | 147 (7.3)       | 2 (1.6)   | 140 (5.8)   |
| Translation initiation or transcription factors | 8 (3.7)       | 113 (5.6)       | 3 (2.4)   | 182 (7.5)   |
| Abiotic or biotic stress response | 39 (18.1)    | 276 (13.7)      | 13 (10.3) | 269 (11.1)  |
| Cell-wall, cell cycle, cell growth and cell cytoskeleton modification or metabolism | 14 (6.5)      | 67 (3.3)        | 13 (10.3) | 133 (5.5)   |
| DNA/RNA binding or metabolism | 1 (0.5)       | 26 (1.3)        | 1 (0.8)   | 60 (2.5)    |
| Phytohormone metabolism and response | 2 (0.9)       | 21 (1.0)        | 3 (2.4)   | 26 (1.1)    |
| Mitochondria or plastid | 3 (1.4)       | 26 (1.3)        | 1 (0.8)   | 27 (1.1)    |
| Other                   | 0 (0.0)       | 19 (0.9)        | 2 (1.6)   | 31 (1.3)    |
| Unknown molecular function protein | 76 (35.2)     | 815 (40.4)      | 53 (42.1) | 973 (40.2)  |
| Total                   | 213           | 2015            | 126       | 2419        | 21 | 1521 | 112 | 1663 |

*Genes which expression was changed higher than 3-fold (fluorescence signal more than 500) in the root tips and the basal roots were categorized.

*Genes which expression was changed lower than 3-fold (fluorescence signal more than 500) in the root tips and the basal roots were categorized.

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Figure 3. Genes up- and down-regulated by Al in the wild-type and star1 mutant roots. Numbers of Al-responsive genes up-regulated (higher than 3-fold) (A) and down-regulated (lower than 3-fold) (B) are extracted. Wild-type (gray circle) and star1 mutant (black circle) are shown in both the root tips and basal root region.

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regions (black circle) was shown in the wild-type and responsive genes up-regulated (higher than 3-fold) (A) and down-regulated (lower than 3-fold) (B) are extracted. Root tips (gray circle) and basal root

star1 rice, but severely inhibited in the stress response' were mostly affected by Al stress in both the wild-type (Table 1). Genes related to 'Metabolism' and 'Abiotic or biotic stress response' were mostly affected by Al stress in both the wild-type rice and star1 mutant.

Functional category analysis showed that 27.0–42.8% of the up- and down-regulated genes are assigned to unknown function (Table 1). Genes related to 'Metabolism' and 'Abiotic or biotic stress response' were mostly affected by Al stress in both the wild-type rice and star1 mutant (Figure 3A and 3B).

Since the root elongation was hardly inhibited in the wild-type rice, but severely inhibited in the star1 mutant, three different groups for Al-responsive genes could be divided by comparing expression profiling between wild-type rice and star1 mutant. Group 1 includes genes which are up- or down-regulated by Al only in the wild-type rice. These genes are probably involved in Al-tolerance. Twenty eight up-regulated and three down-regulated genes in the root tips, 50 up-regulated and 43 down-regulated genes in the basal root region, belong to this group (Figure 3A and 3B). Group 2 includes genes which are up- or down-regulated by Al in both the wild-type rice and star1 mutant. These genes are probably involved in Al-tolerance or -toxicity. There are 185 up-regulated and 52 down-regulated genes in this group in the root tip, 76 up-regulated and 69 down-regulated genes in the basal root region (Figure 3A and 3B, Table S2–S5). Genes in Group 3 are those up- or down-regulated only in the mutant. These genes are related to Al-toxicity and included 1830 up-regulated genes and 1503 down-regulated genes in the root tip, 2343 up-regulated and 1594 down-regulated genes in the basal root region (Figure 3A and 3B). Most genes in this group are also response to general stresses and found in microarray data of other plant species such as Arabidopsis [20], maize [23,24], M. truncatula [25,26] and wheat [27]. For example, the genes encoding a NADPH oxidase, peroxidase, oxalate oxidase, which are reactive oxygen species (ROS; O$_2^-$, H$_2$O$_2$) generators, were up-regulated (Table S2). Most types of abiotic stresses disrupt the metabolic balance of cells, resulting in enhanced production of ROS [28]. The accumulation of ROS such as $^{1}$O$_2$, O$_2^-$, H$_2$O$_2$ and HO$_2^-$, during abiotic stresses was considered to be a by-product of stress metabolism as well as an overall unwelcome by-product of aerobic metabolism [29]. These findings indicate that these genes are involved in arrest of plant root elongation in response to general stress.

Spatial Profiling of Al-responsive Genes

Root tip has been considered as the target of Al-toxicity [3] based on root elongation inhibition, however, surprisingly, similar numbers of genes were up- and down-regulated by Al in the root tips and mature regions of both wild-type rice and star1 mutant (Figure 4A and 4B). This result raises a question on whether the root tip is only the target of Al-toxicity. Among genes affected, 49 up-regulated and 7 down-regulated genes were the same between root tip and basal root region in the wild-type rice (Figure 4A and 4B), but most Al-responsive genes were different between the root tip and basal root region. This was the same in the star1 mutant; 1385 up-regulated and 522 down-regulated genes were the same between the root tip and basal root region, whereas other genes showed root region-dependent (Figure 4A and 4B). These results suggest that basal root region is also a target of Al-toxicity in addition to the root tip. In M. truncatula, Al-induced gene expression is also found not to be restricted to the root tip [25]. In fact, some genes identified from rice were expressed in both the root tips and basal root region. For example, OsFRDL4 was expressed in both the root tip and the mature root zone [11]. The expression of OsSTAR1 and OsSTAR2 was also induced in both regions [10]. These findings suggest that the basal root region is also involved in Al-tolerance and -toxicity.

Transcriptional Profiling of ART1-regulated Genes in the Wild-type and the star1 Mutant Roots

ART1-regulated Al-tolerance has been identified as a major mechanism responsible for high Al-tolerance in rice [7,8]. We compared expression profiling of ART1-regulated downstream genes between wild-type rice and star1 mutant. Among 31 downstream genes, 11 genes showed higher fold changes in the expression in the mutant than in the wild-type rice (Table 2), whereas 13 genes showed higher fold changes in the wild-type rice than in the mutant (Table 2). Seven genes showed similar fold changes in the expression between wild-type rice and mutant
Table 2. Expression changes of ART1-regulated genes in the roots of wild-type rice and star1 mutant.

| RAP ID       | Accession | Annotation                                      | Root tips |           |           | Basal roots |           |           |
|--------------|-----------|-------------------------------------------------|-----------|-----------|-----------|-------------|-----------|-----------|
|              |           |                                                  | WT        | star1     | WT        | star1       | WT        | star1     |
|              |           |                                                  | Fold      | ≤SD*      | Fold      | ≤SD         | Fold      | ≤SD       | Fold      | ≤SD       | Fold      | ≤SD       |
|              |           |                                                  | change (+Al/-Al) |         | change (+Al/-Al) |            | change (+Al/-Al) |         | change (+Al/-Al) |            |
|              |           |                                                  |           |           |           |             |           |           |           |           |           |           |
| **Cell wall maintenance and Root elongation** |           |                                                  |           |           |           |             |           |           |           |           |           |           |
| Os01g0178300 | AK062450  | OsCDT3                                          | 7.43      | 1.58      | 24.67     | 3.28        | 11.72     | 3.85      | 9.82      | 2.66      |           |           |
| Os01g06652100| AK069291  | Protein of unknown function DUF231 domain       | 3.31      | 0.40      | 2.55      | 0.41        | 3.97      | 0.61      | 1.58      | 0.83      |           |           |
| Os01g0860500 | AK069860  | Chitinase                                        | 10.32     | 4.51      | 12.09     | 2.34        | 3.67      | 0.84      | 7.41      | 4.20      |           |           |
| Os03g0760800 | AK121316  | Gibberellin regulated protein family protein     | 4.91      | 1.29      | 21.94     | 6.13        | 7.54      | 2.07      | 9.13      | 5.14      |           |           |
| Os04g0583500 | AK062225  | Expansin 4                                      | 5.28      | 1.23      | 1.36      | 0.42        | 1.01      | 0.19      | 1.47      | 0.84      |           |           |
| Os09g0479900 | C269495   | Peptidase S8 and S53, subtilisin, kexin, sedolisin domain containing protein | 3.43 | 0.25 | 6.40 | 0.78 | 1.40 | 0.19 | 9.83 | 5.61 |           |           |
| Os10g0524600 | AK069238  | Peptidase S8 and S53, subtilisin, kexin, sedolisin domain containing protein | 2.26 | 0.15 | 3.46 | 0.76 | 10.59 | 2.88 | 25.71 | 11.89 |           |           |
| **Membrane protein** |           |                                                  |           |           |           |             |           |           |           |           |           |           |
| Os01g0869200 | AK073453  | Mg^{2+} transporter/OsMGT1                       | 4.43      | 0.77      | 2.12      | 0.42        | 3.24      | 0.59      | 1.78      | 0.48      |           |           |
| Os02g0131800 | AK102180  | OsNamp4/OsNrat1                                  | 7.85      | 0.31      | 0.74      | 0.08        | 5.53      | 0.18      | 0.27      | 0.08      |           |           |
| Os02g0755900 | AK104985  | UDP-glucuronosyl/UDP-glucosyltransferase family protein | 5.91 | 0.28 | 23.35 | 1.40 | 1.21 | 0.29 | 44.24 | 6.83 |           |           |
| Os03g0755100 | AK066049  | Tonoplast-localized half-size ATP binding cassette (ABC) transporter/OsALS1 | 3.43 | 0.15 | 4.21 | 0.18 | 2.57 | 0.09 | 2.63 | 0.74 |           |           |
| Os05g0119000 | AK069359  | Bacterial-type ATP binding cassette (ABC) transporter/OsSTAR2 | 6.75 | 1.86 | 3.62 | 0.85 | 3.68 | 0.90 | 1.63 | 0.47 |           |           |
| Os06g0695800 | AK109450  | Bacterial-type ATP binding cassette (ABC) transporter/OsSTAR1 | 4.39 | 0.18 | 4.60 | 0.33 | 3.88 | 0.19 | 2.41 | 0.15 |           |           |
| Os09g0426800 | AK060786  | GI1 protein                                      | 1.97      | 0.38      | 4.67      | 0.53        | 5.34      | 0.71      | 0.99      | 0.22      |           |           |
| Os10g0206800 | AK072077  | Multidrug and toxic compound extrusion (MATE) family protein/OsFRDL2 | 5.76 | 0.21 | 3.44 | 0.30 | 5.89 | 0.44 | 1.58 | 0.29 |           |           |
| Os10g0578800 | AK065815  | Lgr8-like protein family protein                 | 7.89      | 0.49      | 2.36      | 0.15        | 5.22      | 0.24      | 0.74      | 0.47      |           |           |
| **Metabolism and Detoxification** |           |                                                  |           |           |           |             |           |           |           |           |           |           |
| Os01g0716500 | AK101454  | SAM (and some other nucleotide) binding motif domain containing protein | 1.90 | 0.10 | 42.65 | 4.84 | 1.99 | 0.30 | 13.98 | 12.53 |           |           |
| Os02g0186800 | NM_001052658 | Cytochrome P450 family protein                 | 12.48     | 4.64      | 5.23      | 0.59        | 5.80      | 0.90      | 1.99      | 0.22      |           |           |
| Os02g0770800 | AK102178  | Nitrate reductase                               | 8.63      | 1.34      | 10.99     | 0.45        | 30.19     | 4.29      | 59.40     | 7.64      |           |           |
| Os12g0227400 | CI50939   | Allyl alcohol dehydrogenase                     | 16.06     | 0.78      | 2.64      | 0.20        | 8.94      | 0.15      | 0.48      | 0.23      |           |           |
| RAP ID* | Accessionb | Annotationc | Root tips | Basal roots |
|---|---|---|---|---|
| | | | WT | star1 |
| | | | WT | star1 |
| **Fold change (+AI/−AI)d ±SD** | **Fold change (+AI/−AI) ±SD** | **Fold change (+AI/−AI) ±SD** | **Fold change (+AI/−AI) ±SD** |
| **Unknown** | | | | |
| Os01g0731600 | NM_001050684 | Conserved hypothetical protein | 18.18 | 4.39 | 23.14 | 5.08 | 1.68 | 0.31 | 14.15 | 13.58 |
| Os01g0766300 | NM_001050890 | Conserved hypothetical protein | 6.23 | 1.82 | 30.97 | 3.95 | 5.94 | 0.63 | 12.56 | 3.48 |
| Os01g0919200 | AK071325 | Cell division protein FtsZ family protein | 4.24 | 0.80 | 11.58 | 2.03 | 2.24 | 0.51 | 18.69 | 16.31 |
| Os01g02126900 | AK109217 | Conserved hypothetical protein | 7.32 | 0.67 | 4.11 | 0.24 | 7.84 | 0.47 | 2.11 | 0.08 |
| Os01g02304100 | AK111121 | Hypothetical protein | 10.73 | 0.49 | 34.63 | 11.22 | 4.04 | 0.49 | 0.78 | 0.57 |
| Os04g0419100 | AK107777 | Hypothetical protein | 16.41 | 0.44 | 5.38 | 0.39 | 1.09 | 0.23 | 7.96 | 5.25 |
| Os04g0494900 | AK073892 | Protein of unknown function DUF642 family protein | 15.05 | 0.86 | 3.64 | 0.74 | 2.98 | 0.16 | 99.56 | 31.86 |
| Os07g0493100 | AK068708 | Non-protein coding transcript, uncharacterized transcript | 26.08 | 11.10 | 7.18 | 0.87 | 13.41 | 4.30 | 6.97 | 2.12 |
| Os07g0587300 | CI285201 | Hypothetical protein | 6.62 | 1.76 | 208.44 | 71.70 | 6.36 | 1.10 | 104.49 | 46.28 |
| Os11g0488100 | CI197875 | Hypothetical protein | 4.50 | 0.16 | 7.00 | 0.56 | 2.02 | 0.12 | 1.96 | 0.13 |
| Os11g0490100 | AK108872 | Uncharacterized plant-specific domain 01627 containing protein | 6.10 | 0.83 | 47.98 | 7.57 | 7.25 | 0.70 | 64.89 | 24.08 |

*RAP-ID based The Rice Annotation Project (RAP) ID numbers.

bAccession based GenBank locus of the National Center of Biotechnology Information (NCBI).

cAnnotation based on the Rice Annotation Project Database (RAP-DB) build 3.0 by the International Rice Genome Sequencing Project (IRGSP).

dfold change, ratio of transcript abundance in Al treatment/transcript abundance in control (−Al) treatment.

eStandard deviation of the mean.

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Table 3. Genes up- and down-regulated only in the root tips of wild-type rice.

| Functional classification | Accession | Annotation | Fold change (±SD) |
|---------------------------|-----------|------------|------------------|
| **Up-regulated**          |           |            |                  |
| ART1-regulated genes      |           |            |                  |
| Os12g0227400              | CI560939  | Allyl alcohol dehydrogenase | 16.06 ± 0.78 |
| Os10g0578800              | AK065615  | LrgB-like protein family protein | 7.89 ± 0.49 |
| Os02g0131800              | AK102180  | OsNramp4/OsNrat1 | 7.85 ± 0.31 |
| Os11g0490100              | AK108872  | Uncharacterized plant-specific domain 01627 containing protein | 5.04 ± 0.72 |
| Os04g0583500              | AF247165  | Expansin 4 | 5.00 ± 1.49 |
| Os01g06869200             | AK073453  | Mg2+ transporter/OsMGT1 | 4.43 ± 0.77 |
| **Other genes**           |           |            |                  |
| Transpot                  |           |            |                  |
| Os05g04109000             | AK119621  | Nitrate transporter/OsNRT1 | 6.91 ± 0.93 |
| Os03g0667500              | AK327039  | Iron-regulated transporter 2/OsIRT2 | 3.16 ± 0.28 |
| Metabolism                |           |            |                  |
| Os08g0468100              | AK101662  | Nitrate reductase [NADH] 1/OsNr | 3.02 ± 0.37 |
| Protein synthesis and processing |       |            |                  |
| Os05g0360400              | AK106046  | Zn-finger, RING domain containing protein | 5.30 ± 0.93 |
| Os04g05355200             | AK060585  | Peptidase aspartic family protein | 3.25 ± 0.33 |
| Translation initiation or transcription factors |       |            |                  |
| Os07g0569100              | AK120160  | Remorin, C-terminal region domain containing protein | 3.17 ± 0.52 |
| Abiotic or biotic stress response |       |            |                  |
| Os03g0804500              | AF072694  | Germin-like protein subfamily T member 1 precursor/OsGLP | 4.68 ± 0.49 |
| Os07g0214900              | NP_001059187 | Chalcone synthase/OsCHS | 4.36 ± 0.24 |
| Os04g0456200              | NP_001052967 | TMV induced protein 1–2 | 3.68 ± 0.23 |
| Cell-wall, cell cycle, cell growth and cell cytoskeleton modification or metabolism |       |            |                  |
| Os04g0664900              | CI550916  | Cell wall invertase | 4.12 ± 0.64 |
| Os04g0507300              | AK119512  | 4-coumarate-CoA ligase-like protein | 3.38 ± 0.16 |
| Hormone metabolism and response |       |            |                  |
| Os07g0568700              | AF466357  | Floral organ regulator 1 | 3.35 ± 1.09 |
| Unknown molecular function protein |       |            |                  |
| Os10g03137300             | NP_001064130 | Conserved hypothetical protein | 7.55 ± 0.91 |
| Os03g0183200              | AK106987  | Conserved hypothetical protein | 4.36 ± 0.98 |
| Os11g0919590              | CI543502  | (No Hit) | 3.67 ± 1.08 |
| Translation initiation or transcription factors |       |            |                  |
| Os07g0558100              | Y11415    | Myb protein (similar to ATMYB102) | 0.27 ± 0.02 |
| Os03g0279700              | AK111338  | ZPT2-12 | 0.33 ± 0.11 |
| Down-regulated genes      |           |            |                  |
| Os10g0391400              | AK107854  | ZIM domain containing protein, (similar to JAZ; JA signaling) | 0.28 ± 0.06 |

*Functional classification based on Table 1.
†RAP-ID based The Rice Annotation Project (RAP) ID numbers.
‡Accession based GenBank locus of the National Center of Biotechnology Information (NCBI).
§Annotation based on the Rice Annotation Project Database (RAP-DB) build 3.0 by the International Rice Genome Sequencing Project (IRGSP).
‖Fold change, ratio of transcript abundance in Al treatment/transcript abundance in control (−Al) treatment.
¶Standard deviation of the mean.

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Table 4. Genes up- and down-regulated only in the basal roots of wild-type rice.

| Functional classificationa/RAP IDb | Accessionc | Annotationd | Fold change (+Al/−Al)e ± SDf |
|----------------------------------|------------|-------------|-----------------------------|
| **Up-regulated**                 |            |             |                             |
| ART1-regulated genes             |            |             |                             |
| Os12g0227400                     | CI560939   | Allyl alcohol dehydrogenase | 8.94 0.15                  |
| Os03g0126900                      | AK109217   | Conserved hypothetical protein | 7.84 0.47                  |
| Os10g0206800                      | AK072077   | Multidrug and toxic compound extrusion (MATE) family protein/OsFRDL2 | 5.89 0.44                  |
| Os02g0131800                      | AK102180   | OsNramp4/OsNrat1 | 5.53 0.18                  |
| Os09g0426800                      | AK060786   | GI1 protein | 5.34 0.71                  |
| Os10g0578800                      | AK06615    | LrgB-like protein family protein | 5.22 0.24                  |
| Os03g0304100                      | AK111121   | Hypothetical protein | 4.04 0.49                  |
| Os06g0695800                      | AK064089   | Bacterial-type ATP binding cassette (ABC) transporter/OsSTAR1 | 3.81 0.19                  |
| Os03g0119000                      | AK069359   | Bacterial-type ATP binding cassette (ABC) transporter/OsSTAR2 | 3.68 0.90                  |
| Os1g0869200                      | AK073453   | Mg2+ transporter/OsMTG1 | 3.24 0.59                  |
| **Other genes**                  |            |             |                             |
| **Transport**                    |            |             |                             |
| Os06g0701700                      | AB061311   | HKT-type transporter (Sodium ion transporter) | 2.97 0.83                  |
| **Metabolism**                   |            |             |                             |
| Os08g0547300                      | AK072163   | E-class P450, group I family protein | 7.65 3.42                  |
| Os04g0405300                      | AK110700   | Stem secoisolariciresinol dehydrogenase | 4.04 1.05                  |
| Os06g0500700                      | CI431272   | Cytochrome P450 family protein | 3.48 1.21                  |
| Os02g0176900                      | NP_001046065 | Aldose 1-epimerase family protein | 3.47 0.67                  |
| Os05g0383600                      | AY035554   | Fructose-1,6-bisphosphatase (FBPase) | 3.45 0.16                  |
| Os11g0487600                      | NP_001067918 | Cytochrome P450 family protein | 3.17 0.09                  |
| Os05g0424300                      | AK120987   | Cytochrome P450 family protein | 3.07 0.44                  |
| **Protein synthesis and processing** |            |             |                             |
| Os12g0108500                      | AK122171   | Cyclin-like F-box domain containing protein | 10.16 0.26                 |
| Os04g0535300                      | AK060585   | Peptidase aspartic family protein | 3.10 0.35                  |
| **Translation initiation or transcription factors** |            |             |                             |
| Os01g0286100                      | AK102252   | Basic helix-loop-helix dimerisation region bHLH domain containing protein | 3.34 0.45                  |
| **Abiotic or biotic stress response** |            |             |                             |
| Os09g0361500                      | AK120689   | Isochorismate synthase 1 (ICS1) | 4.08 0.76                  |
| Os05g0223000                      | AK071661   | Calmodulin-related protein 2, touch-induced | 3.32 0.77                  |
| Os04g0635300                      | AK069933   | Wound induced protein | 3.05 0.81                  |
| **Cell-wall, cell cycle, cell growth and cell cytoskeleton modification or metabolism** |            |             |                             |
| Os04g0506800                      | AK070719   | Glycosyl transferase, family 29 protein/OsGT | 5.21 0.77                  |
| Os11g0444000                      | AK099588   | UDP-glucosyltransferase BX8 | 3.50 0.37                  |
| Os02g0802200                      | AK107538   | Glycoside hydrolase, family 79, N-terminal domain containing protein/OsGH | 3.34 0.42                  |
| Os04g0477500                      | AK063950   | Glycosyl transferase, family 17 protein/OsGT | 3.15 0.30                  |
| Os03g0324700                      | AK121618   | Exostosin-like family protein | 3.03 0.25                  |
| **Hormone metabolism and response** |            |             |                             |
| Os04g0667400                      | AK119413   | 2OG-Fe(II) oxygenase domain containing protein | 5.42 1.44                  |
| **Mitochondria or plastid**      |            |             |                             |
| Os07g0469100                      | AK120365   | Thylakoid membrane phosphoprotein 14 kDa | 3.06 0.34                  |
| **Unknown molecular function protein** |            |             |                             |
| Os07g0269000                      | CI251879   | (No Hit) | 3.74 0.10                  |
| Os10g0473200                      | AK105229   | Conserved hypothetical protein | 5.05 0.76                  |
| Os05g0573800                      | CI142713   | (No Hit) | 4.49 0.56                  |
| Os04g0635400                      | CI037812   | Conserved hypothetical protein | 4.38 1.40                  |
| Os04g0603800                      | AK063616   | Hypothetical protein | 4.17 0.13                  |
### Table 4. Cont.

| Functional classification / RAP ID | Accession | Annotation | Fold change (ΔAI / ΔAl) | ± SD |
|-----------------------------------|-----------|------------|-------------------------|------|
| Os03g0183200                      | AK106987  | Conserved hypothetical protein | 4.10 | 0.75 |
| Os12g0265400                      | CI096837  | Hypothetical protein | 4.05 | 0.23 |
| Os09g0459900                      | AK063208  | Cyclin-dependent kinase inhibitor family protein | 3.92 | 1.21 |
| Os09g0459500                      | AB118006  | Hypothetical protein | 3.87 | 0.49 |
| Os03g0255500                      | AK061620  | Phosphoenolpyruvate carboxykinase | 3.69 | 0.36 |
| Os10g0213500                      | CI426147  | Conserved hypothetical protein | 3.45 | 0.15 |
| Os12g0259100                      | NP_001067644 | Hypothetical protein | 3.43 | 0.60 |
| Os02g0600200                      | AK058978  | IQ calmodulin-binding region domain containing protein | 3.23 | 0.70 |
| Os02g0327000                      | AK073631  | C2 domain containing protein | 3.15 | 0.28 |
| Os06g0535200                      | AK109943  | Zn-finger, RING domain containing protein | 3.10 | 0.17 |
| Os01g0854000                      | AK070440  | Conserved hypothetical protein | 3.10 | 0.72 |
| Os04g0250700                      | AK065832  | Protein of unknown function DUF584 family protein | 3.08 | 0.81 |
| Os03g0113900                      | AK119700  | Protein of unknown function DUF584 family protein | 2.98 | 0.19 |
| Os04g0231800                      | AK068417  | Protein of unknown function DUF1165 family protein | 2.95 | 0.03 |

**Down-regulated**

**Transport**

| Accession | Glyoxalase/bleomycin resistance protein/dioxygenase domain containing protein | 0.29 | 0.10 |
|-----------|------------------------------------------------|------|------|
| Os03g0817200 | Amino acid/polyamine transporter II family protein | 0.31 | 0.03 |
| Os03g0375900 | Amino acid/polyamine transporter I family protein | 0.32 | 0.05 |

**Metabolism**

| Accession | Transferase family protein | 0.05 | 0.02 |
|-----------|-----------------------------|------|------|
| Os06g0185300 | Transferase family protein | 0.15 | 0.04 |
| Os12g0626400 | Squalene/phytoene synthase family protein | 0.22 | 0.03 |
| Os06g0549900 | FAD linked oxidase, N-terminal domain containing protein | 0.23 | 0.07 |
| Os06g0294600 | Cytochrome P450 family protein | 0.24 | 0.01 |
| Os11g0648400 | Tyrosine/nicotianamine aminotransferase family protein | 0.25 | 0.04 |
| Os07g0643400 | Esterase/lipase/thioesterase domain containing protein | 0.31 | 0.02 |

**Protein synthesis and processing**

| Accession | Proteinase inhibitor I12, Bowman-Birk family protein | 0.13 | 0.01 |
|-----------|---------------------------------------------------|------|------|
| Os10g0537800 | Peptidase A1, pepsin family protein | 0.21 | 0.06 |
| Os03g0318400 | Peptidase A1, pepsin family protein | 0.21 | 0.07 |

**Signal transduction**

| Accession | Protein kinase family protein | 0.32 | 0.03 |
|-----------|--------------------------------|------|------|
| Os04g0618700 | Protein kinase domain containing protein | 0.34 | 0.02 |
| Os01g0699600 | Protein kinase domain containing protein | 0.34 | 0.23 |

**Translation initiation or transcription factors**

| Accession | MYB1 protein | 0.11 | 0.09 |
|-----------|--------------|------|------|
| Os11g0702400 | Zn-finger, C2H2 type domain containing protein | 0.26 | 0.03 |

**Abiotic or biotic stress response**

| Accession | Pathogenesis-related protein 1 precursor | 0.07 | 0.02 |
|-----------|----------------------------------------|------|------|
| Os06g0546500 | Peroxidase | 0.16 | 0.03 |
| Os05g0427400 | Phenylalanine ammonia-lyase | 0.19 | 0.01 |
| Os02g027100 | Phenylalanine ammonia-lyase | 0.22 | 0.04 |
| Os09g0417800 | DNA-binding WRKY domain containing protein | 0.24 | 0.07 |
| Os09g0417600 | DNA-binding WRKY domain containing protein | 0.26 | 0.05 |
| Os10g0542900 | Chtinase | 0.28 | 0.06 |
| Os05g0135400 | Plant peroxidase family protein | 0.29 | 0.06 |
| Os05g0149400 | 1-aminoacyclopropane-1-carboxylate oxidase/OsACC | 0.30 | 0.10 |

**Abiotic or biotic stress response**

| Accession | 1-aminoacyclopropane-1-carboxylate oxidase/OsACC | 0.30 | 0.10 |

**Abiotic or biotic stress response**

| Accession | 1-aminoacyclopropane-1-carboxylate oxidase/OsACC | 0.30 | 0.10 |
Table 4. Cont.

| Functional classification | Accession | Annotation | Fold change (±SD) |
|---------------------------|-----------|------------|------------------|
| Cell-wall, cell cycle, cell growth and cell cytoskeleton modification or metabolism | Os02g0267200 | CI377660 Alpha-expansin OsEXPA13 | 0.32 ± 0.04 |
| Unknown molecular function protein | Os04g0368000 | CI447876 (No Hit) | 0.15 ± 0.04 |
| | Os06g0587300 | AK121885 Conserved hypothetical protein | 0.05 ± 0.03 |
| | Os06g0586000 | AK063903 Conserved hypothetical protein | 0.07 ± 0.03 |
| | Os12g0437800 | AK063833 CI2E | 0.08 ± 0.02 |
| | Os10g0796000 | CI08923 (No Hit) | 0.12 ± 0.04 |
| | Os10g0391400 | AK107854 ZIM domain containing protein | 0.18 ± 0.15 |
| | Os05g0368000 | NP_001055341 Conserved hypothetical protein | 0.20 ± 0.03 |
| | Os06g0282000 | CI563293 (No Hit) | 0.21 ± 0.03 |
| | Os06g0292400 | CI409636 Embryogenesis transmembrane protein | 0.27 ± 0.02 |
| | Os02g0520100 | AK072610 NUDIX hydrolase domain containing protein | 0.28 ± 0.01 |
| | Os03g0188700 | AK105532 Protein of unknown function DUF250 domain containing protein | 0.30 ± 0.05 |
| | Os06g0155400 | NP_001056850 Hypothetical protein | 0.33 ± 0.06 |

(Rice Al Tolerance Genes)

(Table 2). Six genes were only up-regulated in the wild-type rice, including genes encoding Expanin (Os04g0583500), Mg²⁺ transporter/OsMGT1 (Os01g0809200), OsNramp4/OsNrat1 (Os02g0131800), LrgB-like protein family protein (Os10g0578000), Allyl alcohol dehydrogenase (Os12g0227400) and uncharacterized plant-specific domain 01627 containing protein (Os11g0592000). Among them, OsNrat1 (Al³⁺ transporter) and OsMGT1 (Mg²⁺ transporter) have been demonstrated to be involved in Al-tolerance [12,14]. Interestingly, only IRT2, but not OsIRT1 was up-regulated by Al. This up-regulation seems to be distinct in rice since its homolog is not induced by Al in Arabidopsis, maize, M. truncatula, and wheat roots [20–27]. Al inhibits Fe uptake [34], and Fe uptake is proposed to be mediated through OsIRT1 and OsIRT2 [33]. Interestingly, only IRT2, but not OsIRT1 was up-regulated by Al. Furthermore, this up-regulation seems to be distinct in rice since its homolog is not induced by Al in Arabidopsis, maize, M. truncatula, and wheat roots [20–27]. Al inhibits Fe uptake [34], therefore up-regulation of OsIRT2 is necessary for increasing Fe uptake.

Genes related with secondary metabolism were also up-regulated by Al. Chalcone synthase (CHS, EC 2.3.1.74) is a key enzyme of the flavonoid/isoflavonoid biosynthesis pathway. A gene encoding this enzyme was up-regulated by 4.4-fold (Table 3). CHS is quite commonly induced in different plant species under different forms of stress like UV, wounding, herbivory and microbial pathogens, resulting in the production of compounds that have e.g. antimicrobial activity [phytoalexins], insecticidal activity, and antioxidant activity or quench UV light directly or indirectly [35]. CHS expression causes accumulation of flavonoid and isoflavonoid. On the other hand, 4-Coumarate:CoA ligase has
a pivotal role in the biosynthesis of plant secondary compounds at the divergence point from general phenylpropanoid metabolism to several major branch pathways [36]. Al is known to induce peroxidation and ROS formation in rice roots [37,38]. Increased secondary metabolites such as flavonoids may increase antioxidative capacity, in turn alleviating Al-toxicity. In line with this aspect, a gene encoding germin-like protein (OsGLP; Os03g0804500) was also up-regulated (Table 3). Germin-like proteins (GLPs) constitute a diverse family of ubiquitous plant glycoproteins [39]. Many GLPs have manganese-containing superoxide dismutase (SOD) activity [40,41]. The SOD activities catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. In this study, OsSOD was not up-regulated in rice roots after short exposure to Al stress (Table 3, Table 4, Table S2, S3), indicating OsSOD might not function in rice root after short exposure to Al stress. Thus, OsGLP might function as SOD. Furthermore, the H$_2$O$_2$ produced by OsGLPs is detoxicated by peroxiredoxin (PrxR) and thioredoxin (Trx) because they are only up-regulated antioxidant genes in rice root tips (Table S2). These results suggested that OsGLP, OsPrxR and OsTrx function as major ROS-scavenging enzymes in the rice roots after short exposure to Al stress.

Among genes up-regulated by Al only in the basal region of wild-type rice, some are related to polysaccharide/cell wall metabolism, including genes encoding glycoside hydrolase (GH; Os02g0802200) and glycosyl transferases (GTs; Os04g0506800, Os04g0477500) (Table 4). Glycoside hydrolases (GHS) catalyze the hydrolysis of the glycosidic linkage to release smaller sugars [42]. Glycosyl transferases (GTs) catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, thereby forming glycosidic bonds [43]. Al causes the thickening and rigification of cell walls [44]. Increased expression of OsGH and OsGTs may contribute to the cell wall synthesis, hence alleviating the Al-induced inhibition of longitudinal cell expansion. Gene encoding 1-aminocyclopentane-1-carboxylic acid oxidase (OsACC; Os05g0149400) was down-regulated in the basal root (Table 4). OsACC is related to biosynthesis of ethylene. Ethylene production is associated with inhibition of root elongation in _Lotus japonicus_ and _M. truncatula_ [45]. Down-regulation of OsACC may prevent further inhibition of root growth caused by Al. The association between other genes and Al-tolerance remain to be examined in future.

**Supporting Information**

| Table S1 | Primer sequences used for quantitative real-time PCR. (XLS) |
| Table S2 | Genes up-regulated in the root tips of both the wild-type and _star1_ mutant. (XLS) |
| Table S3 | Genes up-regulated in the basal root regions of both the wild-type and _star1_ mutant. (XLS) |
| Table S4 | Genes down-regulated in the root tips of both the wild-type and _star1_ mutant. (XLS) |
| Table S5 | Genes down-regulated in the basal roots of both the wild-type and _star1_ mutant. (XLS) |

**Author Contributions**

Conceived and designed the experiments: JFM. Performed the experiments: TT NY RM CFH YN. Analyzed the data: TT NY YN JFM. Wrote the paper: TT JFM.
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