

Transcriptional analysis between two wheat near-isogenic lines contrasting in aluminum tolerance under aluminum stress

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Received: 19 July 2006 / Revised: 23 August 2006 / Accepted: 7 September 2006 / Published online: 13 October 2006
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Abstract To understand the mechanisms of aluminum (Al) tolerance in wheat (*Triticum aestivum* L.), suppression subtractive hybridization (SSH) libraries were constructed from Al-stressed roots of two near-isogenic lines (NILs). A total of 1,065 putative genes from the SSH libraries was printed in a cDNA array. Relative expression levels of those genes were compared between two NILs at seven time points of Al stress from 15 min to 7 days. Fifty-seven genes were differentially expressed for at least one time point of Al treatment. Among them, 28 genes including genes for aluminum-activated malate transporter-1, ent-kaurenoic acid oxidase-1, β -glucosidase, lectin, histidine kinase, and phosphoenolpyruvate carboxylase showed

more abundant transcripts in Chisholm-T and therefore may facilitate Al tolerance. In addition, a set of genes related to senescence and starvation of nitrogen, iron, and sulfur, such as copper chaperone homolog, nitrogen regulatory gene-2, yellow stripe-1, and methylthioribose kinase, was highly expressed in Chisholm-S under Al stress. The results suggest that Al tolerance may be co-regulated by multiple genes with diverse functions, and those genes abundantly expressed in Chisholm-T may play important roles in enhancing Al tolerance. The down-regulated genes in Chisholm-S may repress root growth and restrict uptake of essential nutrient elements, and lead to root senescence.

Keywords *Triticum aestivum* L. · Aluminum tolerance · Microarray · Transcription profiles

Communicated by J.-K. Zhu.

Electronic supplementary material Supplementary material is available in the online version of this article at <http://dx.doi.org/10.1007/s00438-006-0169-x> and is accessible for authorized users.

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Introduction

Aluminum (Al) toxicity is the major limiting factor for crop productivity in acid soils, which comprise about 40% of world arable lands (Conner and Meredith 1985). Fortunately, plants with different levels of tolerance to Al toxicity have been found in several species, which provides a useful base for crop improvement and for exploring mechanisms of Al tolerance in plant species (Anoop et al. 2003; Garvin and Carver 2003). Two major mechanisms were previously established for plant tolerance to Al toxicity: internal and exclusion mechanisms (Taylor 1991; Delhaize et al. 1993). The internal mechanism reduces Al toxicity by immobilization, compartmentalization, or detoxification of Al ions that have penetrated into plant cells, whereas the exclusion mechanism enhances plant tolerance to Al

stress by preventing Al ions from entering the root apex cells. Central to the exclusion mechanism is that root tips secrete organic acids such as malate, citrate, or oxalate to chelate Al^{3+} in the rhizosphere (Ryan et al. 2001; Kochian et al. 2004).

A number of over-expressed genes under Al stress were reported from different plant species, including those regulating the organic acid pathway featuring citrate synthase gene (de la Fuente et al. 1997; Anoop et al. 2003; Garvin and Carver 2003), or the anti-oxidant pathway with genes for superoxide dismutase and glutathione peroxidase (Richards et al. 1998; Milla et al. 2002), pathogen defense such as genes for β -1,3-glucanase and phenylalanine ammonia-lyase (Snowden and Gardner 1993; Cruz-Ortega et al. 1997), signal transduction such as cell wall-associated receptor kinase 1 (WAK1) gene (Sivaguru et al. 2003), and the general stress-responsive pathway such as blue copper-binding protein gene (Richards et al. 1998; Milla et al. 2002). However, most of these genes can also be induced by other biotic and abiotic stresses. Furthermore, identification of these genes was based on comparison of gene expression levels of a single genotype under Al-stressed versus non-stressed conditions, or between two genotypes with different genetic backgrounds under Al-stressed conditions.

More recently, a gene encoding a wheat Al-activated malate transporter (ALMT1) has been isolated using the suppression subtractive hybridization (SSH) approach (Sasaki et al. 2004). Transgenic barley plants with this gene conferred Al-activated efflux of malate and showed a high level of Al tolerance (Delhaize et al. 2004). Mapping analysis indicated that *ALMT1* was located on chromosome 4DL (Ma et al. 2005; Raman et al. 2005). However, Al tolerance can assume different forms of resistance, as would be expected for what is considered a complex trait (Pellet et al. 1996). For example, in the well-studied wheat cultivar, Atlas 66, Al permeates the plasma membrane and accumulates in the root tips (Samuels et al. 1997). Beside one major QTL on chromosome 4DL, three additional QTLs located on 5AS, 2DL, and 7AS were identified to contribute to Al-tolerance in Chinese Spring (Ma et al. 2006). Therefore, that malate release may not be the only mechanism for Al tolerance in wheat leaves open the prospect for identifying candidate genes other than ALMT1. The objectives of this study were to identify genes that are differentially expressed between two wheat near-isogenic lines (NILs) contrasting in Al tolerance under Al stress by using SSH and microarray techniques. Based on putative function of the identified genes, we elucidated possible mechanisms of Al tolerance in wheat.

Materials and methods

Growth conditions and Al-tolerance evaluation

Wheat cultivar Atlas 66 (Al-tolerant) and two near isogenic-lines (NILs, Chisholm-S, and Chisholm-T) were used in this study. Chisholm-T (Al-tolerant) and Chisholm-S (Al-sensitive) were developed from selected BC₃-derived NILs of Chisholm (Al-sensitive), with Al tolerance from Atlas 66 (Carver et al. 1993). Wheat seeds were sterilized with 0.5% NaOCl for 10 min, rinsed extensively with deionized water for 10 min, and germinated on wet filter paper in petri dishes at 23°C for 1 day, before they were transferred to polyethylene cups lined with nylon net on the bottom. Seedlings were grown in an aerated hydroponics nutrient-culture system for seedling growth. The cups were allowed to float on the surface of the nutrient solution in a 12-liter plastic container in a controlled growth chamber with a 16/8 h photoperiod regime at 24°C (day) and 20°C (night). The nutrient solution contained 5 mM CaCl_2 , 6.5 mM KNO_3 , 2.5 mM MgCl_2 , 0.1 mM $(\text{NH}_4)_2\text{SO}_4$, and 0.4 mM NH_4NO_3 (Polle et al. 1978) and was replaced with fresh solution daily. Four-day-old seedlings were treated with 0–15 mg/l Al^{3+} by adding $\text{AlK}(\text{SO}_4)_2$ into the culture solution. The pH of the culture solution was adjusted to 4.2 with 0.1 N HCl after addition of $\text{AlK}(\text{SO}_4)_2$.

Ten representative seedlings of Atlas 66, Chisholm-S, and Chisholm-T were selected daily to measure the elongation rate of the primary root under different Al treatments during a 7-day period. Relative root growth (RRG) was calculated as the ratio of mean root length in the Al treatment to mean root length in the control (0 mM Al) treatment.

RNA isolation and SSH cDNA library construction

Total RNA and mRNA were isolated and purified from wheat root tips by using Trizol reagent and the MessageMaker kit (Invitrogen, Carlsbad, CA, USA), respectively. The SSH cDNA libraries were constructed from root tips of Chisholm-T and Chisholm-S, receiving the 10 mg/l Al^{3+} treatment for 6 h and for 1 day using the PCR-select cDNA subtraction kit (Clontech Laboratories Inc., Palo Alto, CA, USA) according to the manufacturer's instruction. Forward subtraction was conducted by using the tester (the mRNA of Chisholm-T) subtracted against the driver (mRNA of Chisholm-S) and vice versa for the reverse subtraction. Subtracted cDNAs were subjected to two rounds of PCR to normalize cDNA populations. The PCR products were ligated into pGEM-T Easy Vector

(Promega, Madison, WI, USA) and transferred into *Escherichia coli* DH5 α competent cells. The positive clones were regrown and stored at a -80°C freezer. The analysis of unigenes and function categories for all sequences were carried out through PipeOnline 2.0 (Ayoubi et al. 2002).

cDNA microarray preparation

Universal M13 primers were used to amplify the inserts of selected clones by using bacterial cultures as templates with a PCR profile of 40 cycles of 45 s at 94°C, 45 s at 53°C, and 1 min at 72°C. The PCR products were examined in an agarose gel, and clones with only a single band were selected. PCR products from selected clones were purified by ethanol precipitation and dissolved in 3 \times SSC buffer for printing. A total of 1,628 cDNAs from the SSH libraries were arrayed onto Corning GAPS II slides coated with amino-silane (Corning Inc. Corning, NY, USA) using the Generation III Array Spotter (Amersham Biosciences Corp., Piscataway, NJ, USA). Wheat actin gene and pGEM-T easy vector (Promega) were also printed onto slides as an internal quality control. Actin gene was amplified with forward primer (5'-CTGGACTCACACCTTCTACAACGAGCTCCGTGT-3') and reverse primer (5'-ATCCAGACACTGTACTTCCTT-3') according to Xu et al. (2002). After printing, the slides were baked at 80°C for 3 h and stored in a desiccator at room temperature.

Microarray analysis

RNA was isolated from the root tips of Chisholm-S and Chisholm-T at 0, 15 min, 1, 3, 6 h, 1, 3, and 7 days after 10 mg/l of Al³⁺ exposure by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The 3DNA Array 50™ Expression Array Detection Kit (Genisphere, Hatfield, PA, USA) was used to detect differentially expressed genes between Chisholm-S and Chisholm-T after Al stress according to the manufacturer's protocol. Microarray probes were synthesized from equal amounts of total RNA isolated from the roots of Chisholm-S and Chisholm-T at different time points. Biological replications were made for all time points, and dye swap analysis was applied between two repeated samples to avoid bias in dye intensity detection. cDNA hybridizations were conducted at 48°C overnight in a Corning hybridization chamber (Corning Inc.). After washing, 3DNA hybridization was performed at 48°C for 3 h to incorporate Cy5 or Cy3 fluorochrome. The slide was washed for 12 min with pre-warmed buffer containing 2 \times SSC and 0.1% SDS,

followed by 10 min with 2 \times SSC and 10 min with 0.2 \times SSC at room temperature. The slide was dried by low-speed centrifuge and stored in a light proof box until scanned. The fluorescent signals of Cy5 and Cy3 were captured through ScanArray™ Express scanner (PerkinElmer Inc., Boston, MA, USA) at a resolution of 10 μ m per pixel. Scanning parameters were adjusted to obtain balanced signal intensities in both channels using the line-scan function. Signal intensity data were quantified using GenePix Pro 4.0 (Axon Instrument, Union city, CA, USA), and were normalized with Loess within-print-tip-group intensity-dependent normalization using R-project statistical environment (<http://www.r-project.org>) and Bioconductor (<http://www.bioconductor.org>). Any spot with poor quality data and signal intensity less than 200 in both channels was filtered out. After normalization, the signal ratios of log₂-fold changes for the actin gene and pGEM-T vector were around zero or not detectable, indicating that the results from the microarray experiment were reliable. However, a stronger signal for Cy5 was found tightly associated with several genes in this study as shown in the self-versus-self and dye swap hybridizations (Supplemental Table 2). This phenomenon could result from specific sequences bound by 3DNA dendrimer (<http://www.genisphere.com>); thus, those genes were excluded from further analysis.

Differential fold-cutoff was determined by self-versus-self hybridization, in which the same RNA was labeled with both Cy3 and Cy5 and hybridized to the same cDNA array. After normalization, the ratios of log₂-fold changes ranged from -0.595 to 0.595 (Supplemental Fig. 1). Thus, differential expression was considered to be significant if an average ratio of log₂-fold was greater than 0.595 or less than -0.595. Selected differentially expressed genes were subjected to KMC clustering analysis using Genesis 1.5 (Graz University of Technology, <http://www.genome.tugraz.at>).

Quantitative real-time PCR

To validate the results from the microarray experiment, specific primers (Supplemental Table 3) for selected differentially expressed genes were designed for a 100 bp amplicon with T_m at 58–59°C. First-strand cDNA was synthesized from 2.0 μ g of total RNA using SuperScript II RNase H⁻ Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time RT PCR was performed in an ABI Prism 7700 DNA analyzer (Applied Biosystems, Foster, CA, USA) using the QuantiTect SYBR green PCR kit (Qiagen, Valencia, CA, USA) for signal detection. To normalize the total amount of cDNAs present in each reaction, a housekeeping gene of 40S ribosomal

protein S8 was co-amplified. Its expression between Chisholm-T and Chisholm-S was similar during various time courses of Al stress. The RT-PCR primers designed for all genes used in this study were evaluated for PCR amplification efficiencies by performing real-time PCR using a series dilution of each cDNA at rates of 1/2, 1/5, 1/10, 1/20, 1/50, and 1/100 as described in Applied Biosystems User Bulletin No. 2 (<http://www.docs.appliedbiosystems.com/pebiiodocs/04303859.pdf>). The efficiency test showed that all the primers amplified the genes with approximately the same efficiency as that of the normalizer 40S ribosomal protein S8. The $\Delta\Delta C_T$ method of relative gene quantification recommended by Applied Biosystems was used to calculate the expression level of Chisholm-T relative to Chisholm-S.

Results

Effect of Al on root growth

Inhibition of root growth is a major symptom of Al toxicity. The two NILs, Chisholm-S and Chisholm-T, and Atlas 66, the donor of Al tolerance in these lines, showed significant differences in root growth across Al concentrations (Fig. 1). In general, inhibition of root growth, as a proportion of the control, gradually increased with increasing Al^{3+} concentrations in the growth medium. RRG was lowest in Chisholm-S (Fig. 1a), equaling 15, 9, and 4% at 5, 10, and 15 mg/l of Al^{3+} , respectively. Chisholm-T showed slightly lower RRG than Atlas 66 at the highest Al concentration but substantially higher than Chisholm-S at all Al concentrations.

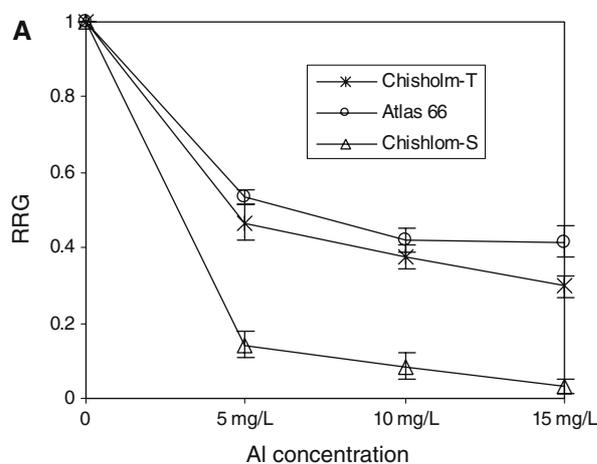


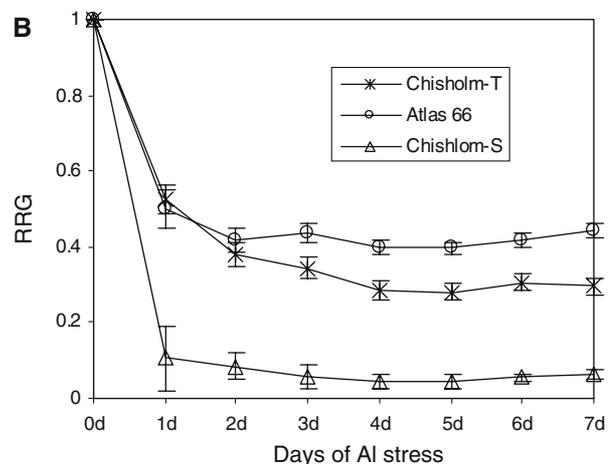
Fig. 1 Effect of Al^{3+} on root growth of Atlas 66, Chisholm-S, and Chisholm-T. Values are the means \pm SD for ten replicate seedlings from two independent experiments. **a** Four-day-old seedlings were exposed to varying concentrations of Al^{3+} . The length of the primary root from ten seedlings was measured after 2 days of growth in

For all genotypes, root elongation dramatically decreased on the first day of exposure to 10 mg/l Al^{3+} (Fig. 1b). Root elongation of Atlas 66 and Chisholm-T was about 50% of the control on the first day of Al exposure, and then about 40% on the second day. Root elongation remained constant in Atlas 66 with subsequent exposure, but Chisholm-T was not able to maintain the same growth as Atlas 66, decreasing to 30% RRG on day 4 of Al exposure and thereafter. On the other hand, root elongation of Chisholm-S was only about 10% of the control at 1 day of Al^{3+} exposure, decreasing to about 5% of the control by day 4 of Al^{3+} exposure (Fig. 1b). High-Al concentration and extended exposure significantly limited root growth of both NILs compared with Atlas 66, but the growth-rate reduction was different between the NILs.

Identification of differentially expressed genes

After subtractive hybridization of root cDNAs between the NILs at 6 and 24 h of Al exposure, a total of 1,628 cDNA clones (788 from Chisholm-T and 840 from Chisholm-S) were isolated with an average fragment size of 587 bp ranging from 400 to 700 bp. After contig analysis for all the ESTs using PipeOnline 2.0 (Ayoubi et al. 2002), 1,065 putatively unique genes were identified. Their sequences were compared with the NCBI database using Blastx, then grouped based on putative function using PipeOnline 2.0.

Approximately 63% of the genes were either unclassified or had no match (“no hit”) in the database (Table 1). The largest group (17%) of genes with known function was involved with intermediate metabolism and bioenergetics (e.g., carbohydrate, lipid,



the control and at different Al^{3+} concentrations (0–15 mg/l). The RRG was calculated as $100 \times (\text{root growth of Al-treated seedlings} / \text{root growth of control seedlings})$. **b** Root length for seedlings was measured daily over a 7-day period of exposure to 10 mg/l Al^{3+} . Data are presented as RRG compared to the control

Table 1 Functional classification of the transcripts from two SSH cDNA libraries

Functional categories	Number of gene	%
Electron transport	52	4.88
Information pathways	80	7.51
Intermediate metabolism and bioenergetics	177	16.62
Signal transduction	43	4.04
Structure and function of the cells	8	0.75
Transmembrane transport	30	2.82
Unclassified proteins	266	24.98
No hit	409	38.40
Total	1,065	100

protein, and nucleic acids metabolism), and the second most abundant group (8%) of genes was involved in information pathways crucial to DNA replication, mRNA transcription, protein biosynthesis, and stress response. A small proportion of genes was involved in transmembrane transport, signal transduction, electron transport, cell structure, and cell function.

Of the 1,065 genes, microarray analysis identified 57 genes that were differentially expressed between the NILs for at least one of seven time points of Al exposure (Table 2). Among them, 28 genes each showed a higher expression level in either of the two NILs and only one gene (non-symbiotic hemoglobin) showed differential expression alternately in the NILs. The difference in relative gene expression level between the two NILs varied from -2.31 to 2.34 of log₂-fold among time points (Supplemental Table 1). Interestingly, abundant transcripts of *ALMT1* and jasmonate-responsive gene (*JRGI*) were detected in Chisholm-T and Chisholm-S, respectively, before Al stress; therefore (Table 2, Supplemental Table 1), *ALMT1* and *JRGI* were most likely expressed constitutively.

To validate the microarray results, ten differentially expressed genes were selected for real-time RT PCR analysis. Although the microarray log₂-fold values were lower than the corresponding values from the real-time RT PCR values, their high concordance ($r^2 = 0.95$) indicated that cDNA microarray analysis effectively identified differentially expressed transcripts with sufficient sensitivity (Fig. 2).

Differentially expressed genes in response to Al stress

The 57 differentially expressed genes identified from microarray analysis were grouped into six clusters according to their NIL × Al time course expression patterns (Table 2; Fig. 3). Clusters A, B, and C represented

groups of genes that were highly expressed in Chisholm-T for at least one time point of Al exposure. Genes in clusters D, E, and F were abundantly expressed in Chisholm-S. Hence, genes in clusters A, B, and C may serve to enhance Al tolerance, whereas genes in cluster D, E, and F may associate with sensitive reactions to Al toxicity.

Genes in cluster A, which encode *ALMT1*, putative lectin (HL2), β-glucosidase aggregating factor (BGAF), ent-kaurenoic acid oxidase 1 (KAO1), and β-glucosidase, were abundantly expressed in Chisholm-T at most time points of Al exposure. These five genes appear to have a predominant and consistent presence in enhancing Al tolerance in Chisholm-T. Most of the 15 genes in cluster B showed abundant transcripts in Chisholm-T after 1–3 h Al exposure. Genes with known function in this cluster are related to signal transduction, H⁺-efflux/influx of membrane, defense response, and organic acid metabolism. Differential expression of genes in cluster C occurred with prolonged Al exposure (1–7 days). These genes encode several proteins such as lipid transfer protein-like proteins, δ-tonoplast intrinsic protein (δ-TIP), cytochrome P450-like protein, and sucrose synthase 1 (SuSy 1).

Genes in cluster D mainly showed abundant transcripts in Chisholm-S at 6 h or 7 days of Al stress. Half of those genes had no match in the NCBI and TIGR databases (The Institute for Genomic Research), and the others encode NADP-specific isocitrate dehydrogenase, isovaleryl-CoA dehydrogenase (IVD), putative transporter and formin homology protein interacting protein 1. Most of the genes in cluster E again were abundantly expressed in Chisholm-S, but during early time points (from 15 min to 6 h) of Al exposure. Genes in cluster F showed more abundant transcripts in Chisholm-S at most time points. Genes in the last two clusters mainly encode several ABC transporters, acyl-CoA oxidase (ACX1), subtilisin-chymotrypsin inhibitor 2, β-amylase, or they relate to uptake and metabolism of nitrogen, sulfur, iron, and copper.

Discussion

Genes related to organic acid exudation

The exudation of organic acids from root apices to chelate Al³⁺ is one means for plants to protect themselves against Al damage (Ryan et al. 2001; Kochian et al. 2004). ABC transporters were suggested as an anion channel for organic acid exudation (Ryan et al. 2001). In this study, several ion transporter genes were identified.

Table 2 Clone ID, accession number, annotation, and similarity with known genes in the GeneBank and TIGR database for differential expressed transcripts between Chisholm-T and Chisholm-S under AI stress

Clone ID ^a	Accession number ^b	Annotation	TIGR EST No. ^c	<i>E</i> -value ^d	SIM ^e (%)	IDV ^f (%)	DE ^g
Cluster A							
A33	DT045032	Aluminum-activated malate transporter (<i>Almt1</i>)	TC275842	2.40E–147	99	99.78	T
C828	DT045033	High-light-induced mRNA for putative lectin	TC259678	2.30E–74	84	57.58	T
A653	DT045034	Putative β -glucosidase aggregating factor	TC245345	1.50E–66	99	62.5	T
C592	DT045048	ent-kaurenoic acid oxidase (<i>KAO1</i>)	Rice TC253095	2.10E–31	73	61.7	T
C430	DT045035	β -glucosidase	TC262778	9.00E–148	99	92.01	T
Cluster B							
A187	DT045037	ATP6V1G2 protein	TC247938	2.40E–43	83	100	T
A208	DT045038	prx63 gene for class III peroxidase 63 precursor	TC251690	4.40E–66	95	78.23	T
C273	DT045039	O-methyltransferase	TC250624	1.60E–122	90	88.66	T
A198	DT045040	Unkown protein	–	–	–	–	T
A598	DT045041	Signal transduction histidine kinase	TC257791	5.30E–31	98	92.86	T
A253	DT045042	Stress-related peroxidase, prx7	TC237958	1.60E–66	92	77.98	T
A614	DT045043	Abscisic acid- and stress-inducible protein (<i>Asr1</i>)	TC234057	3.30E–34	99	76.54	T
A134	DT045044	Unkown protein	–	–	–	–	T
A427	DT045050	Hydrophobic protein OSR8	TC265602	1.10E–10	62	87.04	T
C633	DT045055	Putative Glucan 1,3- β -glucosidase precursor	TC235234	2.40E–74	96	71.11	T
C293	DT045056	Unknown protein	CA641035	4.20E–54	95	–	T
A91	DT045047	Unknown protein	CV066510	0.0098	60	–	T
C832	DT045059	Putative B12D protein	TC266548	1.70E–23	99	66.27	T
C72	DT045060	Phospenolpyruvate carboxylase	TC234827	2.10E–142	98	99.46	T
C37	DT045062	Plasma membrane H ⁺ -ATPase (<i>ha1</i>)	TC246826	1.30E–126	99	99.37	T
Cluster C							
AC11-8	DT045046	Delta tonoplast intrinsic protein TIP2;1	TC234572	1.10E–55	99	99.6	T
AC10-61	DT045025	Non-symbiotic hemoglobin	TC266279	1.40E–115	97	99.38	S, T
A65	DT045036	Unknown protein	CK216118	5.70E–113	97	–	T
C55	DT045049	Predicted transcriptional regulator	TC271107	3.60E–71	95	100	T
AC5-35	DT045051	Unknown protein	BE403101	1.30E–26	76	–	T
A313	DT045052	Cytochrome P450-like protein	BQ161768	2.40E–38	73	73.79	T
C752	DT045053	Lipid transfer protein-like protein (<i>LTP1</i>)	TC246871	2.30E–74	98	75.25	T
AC10-67	DT045054	Lipid transfer protein-like protein	TC247774	6.40E–43	94	77.66	T
C758	DT045063	Sucrose synthase 1	TC232520	4.40E–82	97	98.39	T
Cluster D							
C225	DT045003	Putative transporter	TC248079	1.60E–98	99	90.05	S
C553	DT045004	Unknown protein	BQ236901	6.30E–22	86	–	S
A26	DT045005	Unkown protein	–	–	–	–	S
A295	DT045006	Unkown protein	–	–	–	–	S
A374	DT045009	NADP-specific isocitrate dehydrogenase	TC233152	2.80E–107	99	94.13	S
A390	DT045007	Unknown protein	TC254341	1.40E–52	99	–	S
C761	DT045010	FH protein interacting protein FIP1	TC255887	6.40E–102	100	88.03	S
C375	DT045011	Putative isovaleryl-CoA dehydrogenase	TC236485	6.10E–46	96	87.53	S
Cluster E							
A306	DT045013	IDI1 mRNA	TC264733	4.10E–134	95	99.49	S
C310	DT045014	Methylthioribose kinase (<i>MTK1</i>)	TC267394	3.10E–61	98	87.84	S
C150	DT045012	Subtilisin-chymotrypsin inhibitor 2	TC246697	2.50E–101	95	90	S
C69	DT045008	PDR-like ABC transporter	CA682753	1.30E–74	97	67.92	S
A526	DT045015	Putative alternative oxidase 1	BQ165963	1.20E–22	85	78.12	S
C540	DT045016	VHS2 protein	TC254366	3.00E–116	94	76.99	S

Table 2 continued

Clone ID ^a	Accession number ^b	Annotation	TIGR EST No. ^c	<i>E</i> -value ^d	SIM ^e (%)	IDV ^f (%)	DE ^g
C589	DT045017	Unkown protein	–	–	–	–	S
A493	DT045018	Iron-phytosiderophore transporter yellow stripe1	CA615901	1.00E–53	94	83.95	S
C253	DT045019	Putative nit protein 2	TC267413	1.20E–104	98	73.04	S
C374	DT045021	ABC-type transport-like protein	TC238486	6.70E–118	95	76.4	S
C518	DT045022	Unknown protein	BQ743738	5.40E–39	97	–	S
C29	DT045023	ABC transporter-like protein	Rice TC268201	4.80E–68	81	72.33	S
C349	DT045024	Putative ABC transporter	Rice TC262863	3.10E–63	86	72.39	S
C619	DT045020	Putative pyruvate orthophosphate dikinase	TC268825	1.20E–100	98	91.3	S
Cluster F							
C689	DT045026	JRG1, a 32 kDa protein	TC273477	9.20E–72	98	61.9	S
C36	DT045027	Acyl-CoA oxidase (<i>ACX1</i>)	TC247119	6.20E–31	86	77.4	S
C701	DT045028	β-amylase	TC265191	4.30E–19	97	100	S
C597	DT045029	Copper chaperone homolog (<i>cch gene</i>)	TC266251	3.40E–44	93	95	S
C62	DT045030	P450 monooxygenase CYP72A26	CK196379	1.20E–75	90	75.66	S
C345	DT045031	Putative MRP-like ABC transporter, mrp3	TC237753	1.50E–74	99	90.8	S

^a Clone ID: Clone identification nos in the libraries of this study. cDNA sequences of all clones listed in Table 2 have been submitted to the GenBank database

^b Accession No: Gene accession no. in GenBank

^c TIGR EST No: EST number in TIGR EST database of wheat; EST with “rice” present rice EST

^d *E*-value: *E*-value in BLAST between EST in this study with EST in TIGR database

^e SIM: The similarity value between EST sequence in this study and EST in TIGR database

^f IDV: Identity value of EST sequence in TIGR with known gene

^g DE: Differentially expressed genes in comparison of expression level between Chisholm-T and Chisholm-S. “T” means more abundant mRNA level in Chisholm-T, “S” vice versa in Chisholm-S

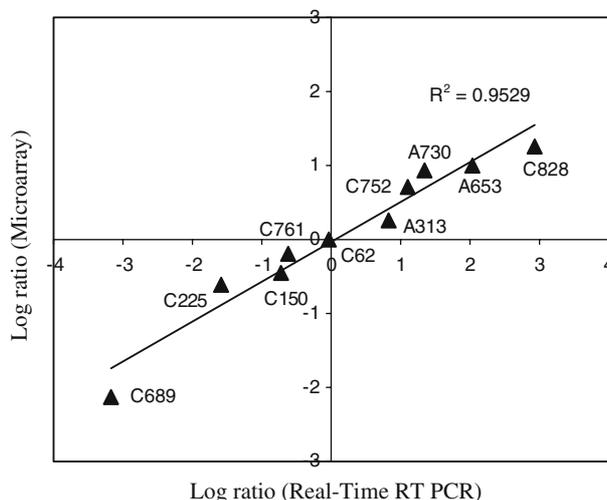


Fig. 2 Confirmation of microarray data by real-time RT-PCR. Correlation analysis of selected data between microarray and real-time RT-PCR experiments. The fold changes in gene expression in response to Al stress were log₂ transformed. The microarray data log₂-values were plotted against the real-time RT-PCR log₂ values. C689, A313 et al. are the clone names

Among them, *ALMT1* was expressed more abundantly in both Al-stressed and unstressed root tips of Chisholm-T than Chisholm-S (Table 2, Supplemental

Table 1), suggesting that it most likely expresses constitutively. These results are in good agreement with the finding of Sasaki et al. (2004) and add further support that some wheat cultivars might gain their Al tolerance by activating a pre-existing specific Al-dependant transporter to initiate rapid malate efflux (Ma et al. 2001). However, the level of *ALMT1* expression was also significantly enhanced at 3 and 6 h of Al exposure in comparison with the non-stressed controls (data not shown). This result is in agreement with Li et al. (2000) that malate efflux significantly increased in Atlas 66 from 2 to 6 h of Al exposure, indicating that Al could activate even higher levels of *ALMT1* expression.

Unlike *ALMT1*, several different transporter genes, especially ABC transporter genes identified in cluster E, were abundantly expressed in Chisholm-S under Al stress (Fig. 3, Table 2 and Supplemental Table 1). It was previously reported that ABC transporters were regulated by stress conditions and mediated ion channel activity, detoxification, stomatal movement, and plant development (Alvarado et al. 2004). They are also involved in pathogen defense (Stukkens et al. 2005). However, the ABC transporter genes highly expressed in this study were unique in the susceptible NIL. Thus, they appear not to mediate tolerance but

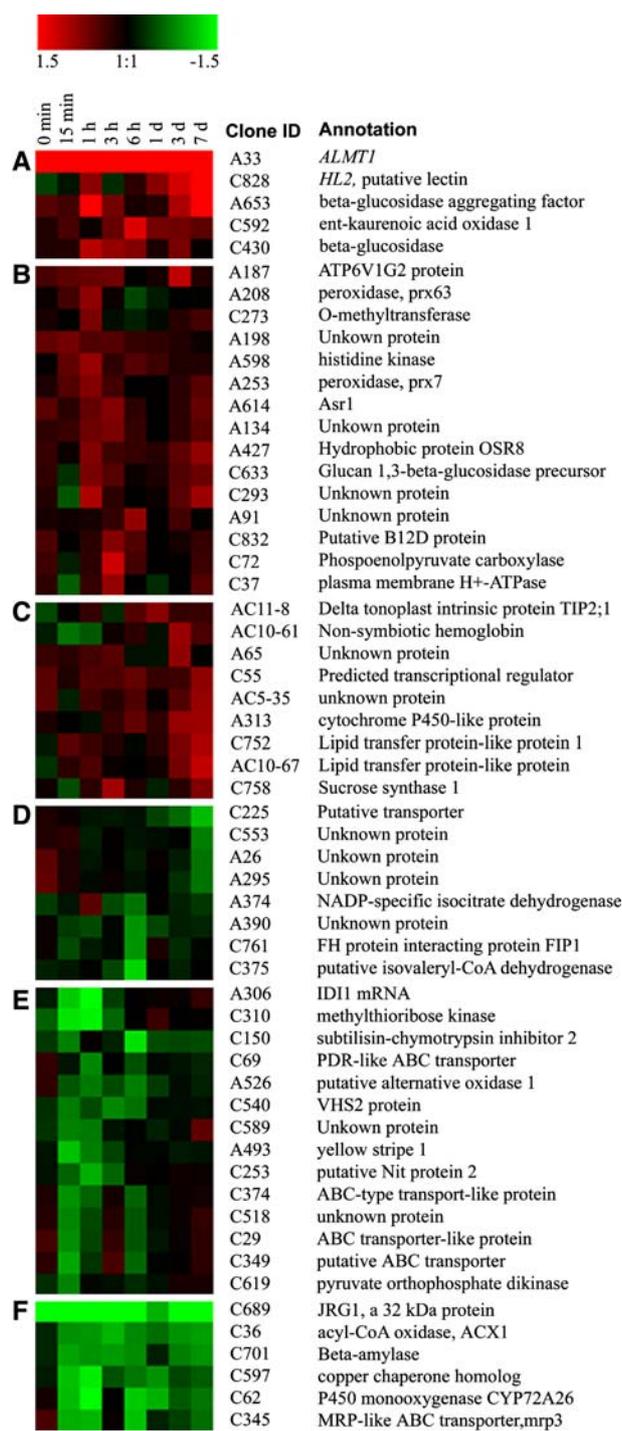


Fig. 3 Cluster analysis of 57 genes that differed significantly in their gene expression levels between Chisholm-T and Chisholm-S after Al stress. Cluster analysis was performed using KMC clustering of Genesis 1.5. Rows and columns represent differentially expressed genes and time courses of Al treatment, respectively. Red and green boxes indicate that the gene expression levels in Chisholm-T are higher and lower than those in Chisholm-S, respectively. Black boxes represent gene expression that is not significantly different between the two genotypes. The clone ID and annotation of each gene are listed at the right of the figure, and the cluster identification is listed on the left

instead to indicate sensitivity to Al stimulation. Their role in transporting other substrates besides malate remains to be investigated.

Precisely how wheat regulates organic acid efflux from root apices in response to Al stress remains a mystery. Takita et al. (1999) reported that organic acid exudation was associated with changes in enzyme activities for organic acid metabolism; however, Li et al. (2000) concluded that activities of phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase, citrate synthase, and NADP-dependant isocitrate dehydrogenase (NADP-ICDH) did not associate with malate efflux of root apices under Al stress. Ma et al. (2001) suggested that enzyme activity for malate biosynthesis might not be the limiting factor; malic acid might be replenished by activating pre-existing enzymes. In this study, *PEPC* transcripts were more abundant in Chisholm-T than in Chisholm-S at 3 h of Al exposure. In contrast, *NADP-ICDH* was highly expressed in Chisholm-S at 6 h of Al stress. Takita et al. (1999) also observed the down-regulation of this gene. These results indicate that Al stress may affect malate biosynthesis through regulating enzyme activities in the organic acid metabolic pathway in wheat, and could be further assumed that the malate level in Chisholm-T might be adjusted by PEPC in biosynthetic pathway.

The exudation of organic acids from root apices should be accompanied by the release of cations or uptake of other anions to balance membrane potential. Citrate secretion in soybean under Al stress was associated with H⁺-ATPase activity, and proton efflux via H⁺-ATPase was suggested to play a role in balancing anion release (Shen et al. 2005). A high level of expression of vacuolar ATPase (V-ATPase) was also associated with Al tolerance in wheat cultivar “PT741”, and a homeostatic mechanism was suggested for V-ATPase to maintain the cytoplasmic pH-value during Al stress (Hamilton et al. 2001). In this study, a gene encoding plasma membrane H⁺-ATPase was highly expressed in Chisholm-T 3 h after Al stress, and *ATP6VIG2* encoding a component of V-ATPase was more abundant in Chisholm-T at 1, 3 h, and 3 days after Al stress. Therefore, H⁺-ATPase and V-ATPase may equilibrate membrane potential during peak periods of malate efflux in Chisholm-T under Al stress.

Genes involved in root growth

Cytochrome P450s may serve as monooxygenases in the biosynthetic pathways for lignin, defense compounds, hormones, pigments, fatty acids, and signaling

molecules, and in the detoxification pathway to catalyze numerous endogenous and exogenous toxic compounds encountered in the environment (Schuler and Werck-Reichhart 2003). In this study, transcripts for two P450s, *KAOI* and cytochrome P450-like protein gene, were more abundantly expressed in Chisholm-T throughout Al exposure (Fig. 3 and Supplemental Table 1). *KAOI* is a cytochrome P450 monooxygenase from the subfamily CYP88A, and it catalyzes the biosynthesis of gibberellin (GA) from ent-kaurenoic acid to GA₁₂ (Davidson et al. 2003). In pea, mutant plants without *KAOI* had dwarfed stature with reduced taproot length, and the reduction in growth corresponded with a decrease in GA levels in the shoot and root (Yaxley et al. 2001; Davidson et al. 2003). Similarly, *KAOI* could facilitate root elongation of Chisholm-T under Al stress. The gene encoding cytochrome P450-like protein was also highly expressed in Chisholm-T at 6 h, 3, and 7 days of Al stress, but its function is still unknown.

Genes for β -glucosidase and BGAF were abundantly expressed in Chisholm-T with almost the same expression pattern throughout Al exposure. β -glucosidase (β -D-glucoside glucohydrolase, EC 3.2.1.21) catalyzes the hydrolysis of aryl and alkyl β -D-glucosides as well as glucosides with a carbohydrate moiety (Brzobohatý et al. 1993). It appears abundant in primordial tissues, affects cell wall catabolism, and influences phytohormone activation and root elongation by releasing active cytokinins, gibberellins, and indole acetic acid from their inert precursors of glucoconjugates (Nagahashi et al. 1990; Brzobohatý et al. 1993). Therefore, a high-expression level of β -glucosidase may help maintain a high rate of root growth in Chisholm-T under Al stress. In addition, many phenolic compounds appear as β -D-glucosyl conjugates in plants. These glucosides belong to several chemical families such as benzoic or hydroxycinnamic acids, and flavonoids (Duroux et al. 1998). The hydrolysis of these glycosides catalyzed by the glucosidase may release physiologically active phenolic compounds (Brzobohatý et al. 1993), which may detoxify Al³⁺ in plants by forming a strong complex with Al³⁺ intracellularly at near-neutral pH (Kochian et al. 2004). Active flavonoid-type phenolics released from their inactive form could be exuded from root apexes to the rhizosphere for Al³⁺ chelation (Kidd et al. 2001). Thus, β -glucosidase could play a protective role by releasing active phenolics to chelate toxic Al³⁺ in both the symplast and apoplast of roots. The protein BGAF binds directly to β -glucosidase, but its physiological function in plants is still unknown (Esen and Blanchard 2000). However, co-expression of *BGAF* and β -glucosidase

suggests that *BGAF* may ameliorate physiological activity of β -glucosidase to maintain root growth and detoxify Al toxicity in Chisholm-T.

The gene δ -*TIP2:1*, encoding a TIP, was highly expressed in Chisholm-T at 1 day of Al stress. In rye, Al stress repressed *TIP* expression, which led to a reduction in root growth rate under Al stress by generating a lower turgor pressure in the cell elongation zone (Milla et al. 2002). Therefore, a high level of expression of δ -*TIP2:1* in Chisholm-T may facilitate more rapid root elongation in Chisholm-T than in Chisholm-S after Al stress.

Defense responsive genes

Lectins are a group of carbohydrate-binding proteins abundant in plants. They bind glycans of glycoproteins, glycolipids, or polysaccharides with high affinity (Etzler 1985), and serve in plant defense, carbohydrate metabolism, and packaging of storage proteins (Peumans and Van Damme 1995). In this study, a high-light-induced gene (*HL2*) in barley, putatively encoding lectin, showed abundant transcripts in Chisholm-T at 1 h, 1, 3, and 7 days (Fig. 3). This result may suggest that this lectin functions as a passive-defense protein and its high expression in Chisholm-T may enable adaptation to Al-stressed environments.

Lipid transfer proteins (LTPs) facilitate transportation of lipids to the cell wall for biosynthesis of cutin layers and surface waxes of leaves as a defense mechanism in response to pathogen attack (Kader 1997). They can be induced by various abiotic stresses (Kader 1997; Trevino and O'Connell 1998) to increase cuticle thickness and prevent water loss of cells under stress (Trevino and O'Connell 1998). In this study, two genes encoding LTP-like proteins were highly expressed in Chisholm-T from 3 to 7 days of Al exposure. Increased expression of LTPs in Chisholm-T may increase cutin layer thickness and thereby protect root cells from Al damage.

Pathogen attack and wounding can induce *Prx7*, a barley stress-related peroxidase gene. This gene, which leads to the production of hordatines that in turn may have a role in cell wall biosynthesis (Kristensen et al. 1999), generated abundant transcripts in Chisholm-T after 1 h of Al exposure. The same expression pattern was observed for *Prx63*, another peroxidase gene that functions in disease resistance and wound response by lignification (Sottomayor et al. 2004). In addition, a gene encoding O-methyltransferase also had more transcripts in Chisholm-T after 1 h of Al exposure. This enzyme facilitates modulation under stress (NDong et al. 2003). SuSy is believed to function in channeling UDP-glucose

from sucrose to various β -glucan synthases for cellulose biosynthesis. Its activity was positively correlated with cell wall biosynthesis (Klinge et al. 1997) and with cell wall thickening by cellulose deposition in roots in response to stress (Albrecht and Muströph 2003). In this study, *SuSy 1* was identified with high expression in Chisholm-T after 3 h and 7 days of Al exposure. These genes, combined, appear to offer protection to root cells against Al toxicity in Chisholm-T.

Genes for signal transduction

Although Sivaguru et al. (2003) reported that a cell WAK1 was rapidly induced by Al^{3+} stress and enhanced root growth, evidence to demonstrate that this gene is involved in Al tolerance is still limited (Kochian et al. 2004). Histidine kinase was reported to be involved in the perception and signal transduction of phosphate starvation, nickel toxicity, and high osmotic and salt stresses (Marin et al. 2003; Motoyama et al. 2005). In Chisholm-T, a spike in histidine kinase expression was detected at 1 h of Al exposure, but sharply declined thereafter (Fig. 3 and supplemental Table 1). Expression of this gene in Chisholm-T fits a typical “on-off” pattern; therefore, it may play an important role in perceiving Al^{3+} and switching on expression of other Al-tolerance related genes in Chisholm-T.

The *ASRI* is an ABA- and stress-inducible gene that encodes a small plant-specific protein with a zinc-dependent DNA-binding activity (Kalifa et al. 2004). A high level of expression of this gene was reported when tomato plants were subjected to water and salt stresses (Kalifa et al. 2004). In this study, *ASRI* was highly expressed in Chisholm-T only at 3 h of Al exposure, and may serve a signaling function in response to Al stress in Chisholm-T.

Al-responsive genes in Chisholm-S

The *CYP72A26* belongs to the P450 family and its expression pattern was different from the other two P450 genes discussed previously. Abundant transcripts were instantaneously detected with Al exposure and maintained over various time points in Chisholm-S, indicating that it may be a sensitive-response gene to Al toxicity.

Genes encoding ACX1 and β -amylase were highly expressed in Chisholm-S across almost all time points of Al exposure (Fig. 3, Supplemental Table 1). ACX1 is an essential enzyme for fatty acid degradation in plants (Grossi et al. 2003). β -amylases are believed to be involved in starch degradation through hydrolysis of α -1,4-glucosidic linkages from the reducing ends of

starch molecules (Beck and Ziegler 1989; Taiz and Zeiger 2002). In addition, more transcripts of a putative IVD were detected in Chisholm-S at 6 h of Al exposure. This enzyme catalyzes degradation of leucine and valine for energy metabolism following carbohydrate depletion, and it can be induced in carbohydrate-starved maize root (Faivre-Nitschke et al. 2001). These results imply that Al stress may set off a cascade of degradation reactions of fatty acids, amino acids, and starch in an Al-sensitive genotype.

A high level of expression of alternative oxidase 1 gene (*AOX1*) was detected in Chisholm-S at 1 and 6 h of Al stress. AOX is a quinol-oxygen oxidoreductase that can transfer electrons from ubiquinone to oxygen without proton translocation. The activity and content of AOX increased in aged roots of pea, or in plants under various stresses that inhibit mitochondrial respiration (Millar et al. 1998; Taiz and Zeiger 2002). This finding indicates that Al may inhibit the phosphorylating pathway of electron transport in roots of an Al-sensitive genotype.

High-expression levels of iron deficient induced gene 1 (*IDI 1*) and yellow stripe 1 gene were observed in Chisholm-S at 15 min and 1 h after Al exposure (Fig. 3 and Supplemental Table 1). *IDI 1* is an enzyme involved in the Met cycle and is induced by Fe deficiency in barley roots (Negishi et al. 2002). Yellow stripe 1 is a transporter of an iron-phytosiderophore for iron uptake, and its transcripts were increased in roots under iron deficit (Schaaf et al. 2004). In rye, Milla et al. (2002) reported that Al caused rapid inhibition of Fe uptake and induced high levels of expression of iron deficiency-specific genes, *Ids1*, *Ids3a*, and *Ids3b*, which are responsible for phytosiderophore synthesis and iron uptake.

Similar effects of Al on metabolism of nitrogen, sulfur, and copper was also observed in this study. A nitrogen regulatory gene (*nit-2*) and a methylthioribose kinase gene (*MTK1*) were highly expressed in Chisholm-S during early exposure (15 min to 3 h) to Al (Fig. 3, Supplemental Table 1). *MTK1* is involved in recycling of Met via the MTA cycle and is linked specifically to sulfur metabolism and strongly induced in roots and shoots under sulfur deficit (Sauter et al. 2004); *nit-2* can elicit expression of various unlinked specific structure genes related to enzymes for nitrogen catabolism under nitrogen deficiency (Fu and Marzluf 1990). A copper chaperone homolog (*CCH*) gene was also detected with more abundant transcripts in Chisholm-S than in Chisholm-T at 15 min, 1, 6 h, and 1 d of Al stress (Fig. 3, Supplemental Table 1). *CCH* is a copper-dependant protein and may mediate intracellular copper homeostasis and mobilize copper from decaying

tissues toward reproductive structures (Mira et al. 2001; Lee et al. 2005). It is induced under copper deprivation or during organ senescence (Lee et al. 2005). Combining these results, we can postulate that Al stress on Al-sensitive Chisholm-S may lead to deprivation of essential nutrient elements such as Fe, N, S, and Cu by inhibiting their uptake and utilization, leading to root senescence and inhibition.

Acknowledgments We gratefully acknowledge Drs. Patricia Ayoubi and Hua Wen for the technical assistance in array printing and data analysis. This paper reports the results of research only. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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