Journal of Integrative Plant Biology http://www.blackwell-synergy.com
Formerly *Acta Botanica Sinica* 2005, **47** (7): 839–848 http://www.chineseplantscience.com

Nylon Filter Arrays Reveal Differential Expression of Expressed Sequence Tags in Wheat Roots Under Aluminum Stress

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Abstract: To enrich differentially expressed sequence tags (ESTs) for aluminum (Al) tolerance, cDNA subtraction libraries were generated from Al-stressed roots of two wheat (*Triticum aestivum* L.) near-isogenic lines (NILs) contrasting in Al-tolerance gene(s) from the Al-tolerant cultivar Atlas 66, using suppression subtractive hybridization (SSH). Expression patterns of the ESTs were investigated with nylon filter arrays containing 614 cDNA clones from the subtraction library. Gene expression profiles from macroarray analysis indicated that 25 ESTs were upregulated in the tolerant NIL in response to Al stress. The result from Northern analysis of selected upregulated ESTs was similar to that from macroarray analysis. These highly expressed ESTs showed high homology with genes involved in signal transduction, oxidative stress alleviation, membrane structure, Mg^{2+} transportation, and other functions. Under Al stress, the Al-tolerant NIL may possess altered structure or function of the cell wall, plasma membrane, and mitochondrion. The wheat response to Al stress may involve complicated defense-related signaling and metabolic pathways. The present experiment did not detect any induced or activated genes involved in the synthesis of malate and other organic acids in wheat under Al-stress.

Key words: aluminum tolerance; differential gene expression; near-isogenic lines; suppression subtractive hybridization (SSH); wheat.

Aluminum (Al^{3+}) toxicity occurs in approximately 30% of arable land worldwide and is a major limiting factor for crop production in acidic soils (Carver *et al*. 1995; Von Uexkull *et al*. 1995). Aluminum may alter the cation-exchange capacity of cell walls (Horst 1995), change the potential of the cell membrane, affect uptake of Ca^{2+} and/or Mg^{2+}, induce oxidative stress via lipid peroxidation, replace Mg^{2+} or Fe^{3+} in cellular reactions, interfere with signal transduction (Jones *et al*. 1995), and directly bind to DNA and/or RNA. These interactions are manifested as inhibited root growth and stunted shoot growth (Carver *et al*. 1995). The exudation of organic compounds, such as malate, oxalate, or citrate, that are capable of chelating Al^{3+} into non-toxic complexes, was proposed as one of the important mechanisms to relieve Al^{3+} toxicity in plants (Delhaize *et al*. 1993; Basu *et al*. 1994; Delhaize and Ryan 1995; Horst 1995; Kochian 1995; Ryan *et al*. 1995; Pellet *et al*. 1996; Zhang *et al*. 2001; Tang *et al*. 2002). Other mechanisms may also exist, especially in light of polygenic control of Al^{3+} tolerance in wheat (Carver *et al*. 1995).

So far, though more than 20 Al-induced genes have been reported from a range of plant species. The molecular mechanism of Al tolerance in plants remains largely unknown. In wheat, those earlier studies on gene expression in response to abiotic stress mostly used differential screening or differential display methods.

Received 8 Oct. 2004 Accepted 21 Feb. 2005
Supported by the Targeted Research Initiative Program of the Oklahoma Agricultural Experimental Station and the Oklahoma Wheat Research Foundation.
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Those studies were often limited to a small number of genes (less than 10) for one study (Snowden *et al*. 1993; Cruz-Ortega *et al*. 1997). Because Al tolerance may involve complicated signal and metabolic pathways (Ezaki *et al*. 2001), quantitative methods for simultaneous genome-wide analysis of gene expression patterns may improve our molecular understanding of the wheat defense response to Al toxicity (Schenk *et al*. 2000). Suppression subtractive hybridization (SSH) is a powerful approach to enrich and isolate differentially expressed sequence tags (ESTs; Diatchenko *et al*. 1996, 1998). Therefore, a high level of enrichment, low background, and normalized abundance of cDNAs in the subtracted library make the method attractive for rapid cloning of cDNAs of differentially expressed genes for Al tolerance.

Atlas 66 is one of a few winter wheat cultivars identified with a high level of Al tolerance and is the most adopted Al-tolerant cultivar to wheat growing environments in the US. Using a back-crossing strategy, Al tolerance genes from Atlas 66 were transferred into wheat cultivar Century, a susceptible cultivar, and near isogenic lines (NILs) contrasting in Al-tolerance were developed (Carver *et al*. 1993). The tolerant NIL (OK91G106) was found to confer a high level of Al tolerance in nutrient solution culture and in the field. These are ideal plant materials for differential gene expression studies. The aims of the present study were to identify differential ESTs between NILs contrasting in Al tolerance and to gain a better understanding of the molecular mechanisms resulting in Al tolerance in wheat.

1 Materials and Methods

1.1 Plant materials

Seeds of the wheat (*Triticum aestivum* L.) cultivar Century and its Al-tolerant NIL OK91G106 were sown in Metro-Mix 360 soil mix (composition including peat, vermiculite, processed bark ash, nutrient charge, and wetting agent; Hummert International, Earth City, MO, USA) in a growth chamber with a 16 h photoperiod at 20 °C and an 8 h dark period at 16 °C. Plants were watered once a day with Hoagland solution. After 9 d of normal growth, seedlings were started on Al treatment by supplying the Hoagland solution plus 0.3 mmol/L $AlK(SO_4)_2$ at pH 4.1 (Carver *et al*. 1988). During Al treatment, fresh Al-added Hoagland solution was supplied three times a day. The pH of the soil solution in Century and OK91G106 was monitored by a glass pH electrode (Metrohm, Herisau, Switzerland) and the continuous measurements were registered with a pen recorder, showing that the pH around the plant roots was constant. After 48 h Al treatment, roots were rinsed gently with tap water and approximately 2-cm tips were harvested and stored immediately at –80 °C for RNA isolation.

1.2 Measurement of root elongation reduction rate

Root elongation rates of both wheat lines were examined daily during a 7-d period of Al treatment. Seedlings with a primary root of approximately 10 mm were transplanted into styrofoam cells floating on the nutrient solution without Al^{3+} in a plastic tray. Seven days after transplanting, seedlings were transferred to a fresh nutrient solution containing 0.3 mmol/L $AlK(SO_4)_2$ at pH 4.1. Control plants were grown in the same nutrient solution without $AlK(SO_4)_2$. During 7 d of Al treatment, the culture solution was replaced fresh daily and 10 of each of the Al-treated and control seedlings were sampled daily to measure primary root lengths. Daily relative reduction rates of root elongation were measured as the root length ratio between Al-treated and control seedlings for each NIL.

1.3 Construction of the SSH library

Total RNA of 48 h Al-treated root tips from each NIL was extracted with Trizol reagent (Gibco, Gaithersburg, MD, USA). MessengerMaker Kit (Gibco) was used to isolate mRNA. The polymerase chain reaction (PCR)-select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) was used for SSH and the Advantage™ PCR Cloning Kit (Clontech) was used to generate the subtractive library. The NIL OK91G106 served as the tester during the cDNA subtraction process.

1.4 Macroarray analysis

A total of 696 clones was obtained from the subtractive library. The plasmid from each clone was isolated by using the Mini plasmid isolation Kit (Qiagen, Hilden, Germany). The inserted DNA fragment in each plasmid was PCR amplified using M13 primers nested in the cloning vector. The PCR products with high quality from 614 clones were ethanol purified, redissolved in double-distilled (dd) H_2O and adjusted to the DNA concentration of 0.3 μg/μL. The purified PCR products were denatured and arrayed directly on a nylon membrane (Amersham Pharmacia, NJ, USA) using a 384-pin replicator (V&P Scientific, San Diego, CA, USA). For data normalization, the PCR-amplified housekeeping gene actin and a plasmid without insert were assigned to various positions on each array as positive and negative controls, respectively. Subtraction screening of cDNA was performed by both forward subtraction with cDNA (from 48 h Al-treated OK91G106) and reverse subtraction with cDNA (from 48 h Al-treated Century) according to the instruction supplied with the Subtracted Library Screen Kit (Clontech).

1.5 Sequencing and blast search

Complementary DNA inserts from differential clones were sequenced by using the Thermo Sequenase™ DyEnamic Direct Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Pharmacia) on the Li-Cor IR^2 4200 DNA Analyzer. Putative functions of the cDNA were identified using the BLAST/X alogorithm by comparing the target sequences to those genes with known function in GenBank for similarity.

1.6 Northern blotting analysis

Northern blotting analysis of differential ESTs was conducted using NorthernMax Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Hybridization was performed at 42 °C overnight and the membrane was washed twice at 42 °C in a 2×standard saline citrate (SSC) buffer with 0.1% sodium dodecyl sulfate (SDS) for 10 min each and twice in a 0.1×SSC buffer with 0.1% SDS for 15 min each. The membrane was exposed to an X-ray film for approximately 48 h at – 80 °C and developed. Autoradiographs were scanned in a GS-710 Imaging Densitometer (Bio-Rad, Hercules, CA, USA) and the gel images were quantified with associated software. Membranes spotted with cDNA were re-used three to five times by striping the membranes and reprobing with different probes according to the Strip-EZ DNA protocol (Ambion).

2 Results

2.1 Aluminum tolerance of two wheat NILs

Although Al stress significantly reduced root elongation rates of both Century and Al-tolerant NIL OK91G106, a higher reduction rate was observed in Century than in OK91G106 after Al treatment (Fig. 1). Within 4 d of Al-stress, the relative root elongation rate of OK91G106 decreased slower than that of Century. The reduction in elongation rate was less than 20% in OK91G106, whereas it was approximately 50% in Century. After 7 d of Al-treatment, the elongation rate reduced to 58% in OK91G106 and 75% in Century compared with the untreated controls. In addition, a marked decrease of root elongation rate in Century started 2 d after Al-treatment, whereas the decrease in OK91G106 started 2 d later than that of Century (Fig. 1). The significant differences in root elongation rates between these two NILs in response to Al stress are

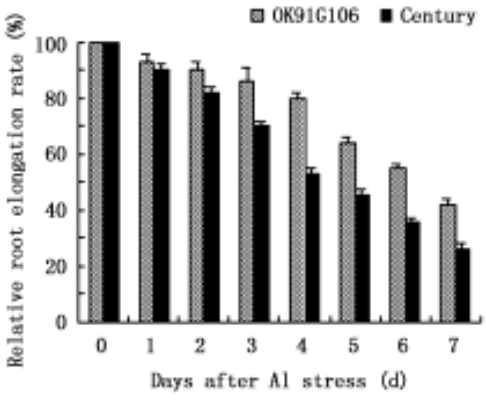

Fig. 1. Relative root elongation rate (%) of aluminum-treated wheat compared with non-treated control observed in near-isogenic lines Century and OK91G106. Error bars represent standard deviations of mean relative root elongation rate in four replicates.

consistent with previous results from root tip hematoxylin staining (Carver *et al*. 1993; Tang *et al*. 2002).

2.2 Expression profiles and function classifications of ESTs

Partial results of macroarray analysis are given in Fig. 2. A representation of the signal intensity ratio of the ESTs of two Al-treated NILs is depicted in Fig. 3 based on macroarray data. The expression signal intensity of most ESTs appeared to be similar between the two NILs treated with 0.3 mmol/L $AlK(SO_4)_2$ for 48 h (Fig. 3). Twenty-five non-redundant ESTs were upregulated and the expression signal intensity of these ESTs in OK91G106 was 1.5-fold greater than that in Century in response to Al treatment (Fig. 3). In our experiments, 614 ESTs on the array derived from the forward SSH library in which the Al-tolerant NIL OK91G106 served as the tester, so the ratio of differentially upregulated ESTs to total tested ESTs reached 4.07%, suggesting that the SSH approach could

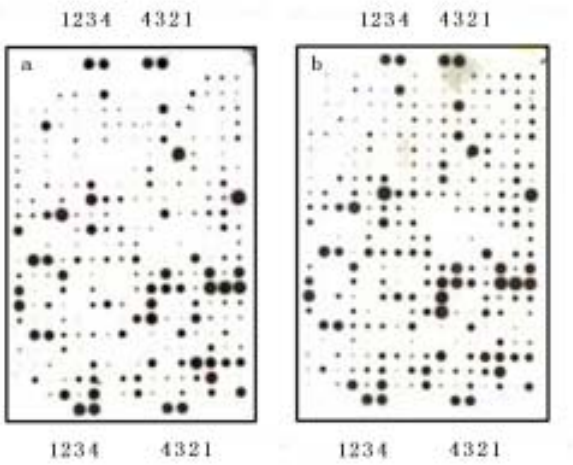

Fig. 2. Macroarray blotting analysis for differentially expressed sequence tags by suppression subtractive hybridization (SSH). PCR-amplified products from bacterial clones of SSH-derived subtracted libraries were dot-blotted onto nylon membranes and were probed with cDNA from 48 h Al-treated OK91G106 (**a**) or 48 h Al-treated Century (**b**). For data normalization, two sets of control were placed in the top and bottom row of each membrane, where lanes 3 and 4 are the housekeeping gene actin, as a positive control, and lanes 1 and 2 are the plasmid without insert as a negative controls.

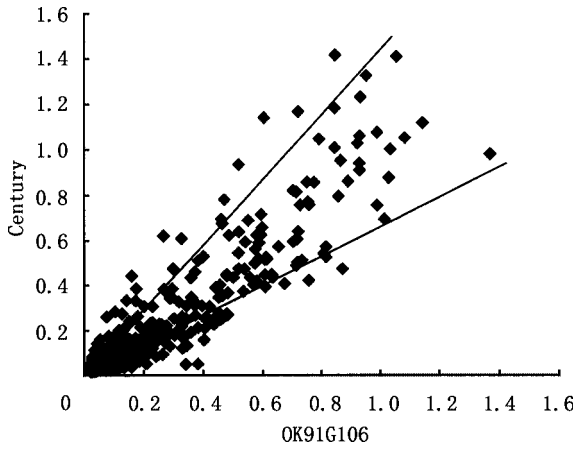

Fig. 3. Scatter plot of signal intensities for all expressed sequence tags (ESTs) on the macroarray. Signal intensities derived from aluminum-treated near-isogenic lines OK91G106 and Century were normalized for each clone on the macroarray and are plotted on the *x*- and *y*-axes, respectively. The diagonal lines represent 1.5-fold ratio cutoffs. The data in scatter plot are based on the results from two replicates.

effectively enrich the differentially expressed genes between the wheat NILs.

2.3 Putative differentially expressed ESTs for Al tolerance

Several previously reported Al-induced or -regulated genes, including *wali5*, *ubiquitin*, and *glutathione S-transferase*, were among those identified in the present study. Table 1 lists differentially expressed ESTs associated with Al tolerance from Al-tolerant NIL OK91G106 according to EST expression analysis and their putative functions based on their sequence homologies with known genes from wheat and other organisms. For most of these ESTs, their expression levels in Al-tolerant NIL OK91G106 were approximately 2.5-fold higher than those in Al-sensitive NIL Century based on macroarray data. These differentially upregulated ESTs in OK91G106 included those genes that play a significant role in cellular signal transduction (genes for ABA cluster, calcium-dependent protein kinases (CDPKs), and serine/threonine phosphatase), cell wall structure (genes for cell wall protein, proline-rich protein, and cellulose synthase), membrane structure

Table 1 Differentially upregulated expressed sequence tags in aluminium-tolerant near-isogenic lines OK91G106 under aluminum stress for 48 h

Clone no.	Putative gene	Accession no.	Identity (%)	*E*-value	Organism
FDC10	ABA-regulated gene cluster	AF085279	99	1e-167	*Arabidopsis thaliana*
FDC8	Calcium dependent protein kinase (*CDPK1*)	AF072908	83	2e-08	*Nicotiana tabacum*
FDC4	Serine/threonine protein phosphatase (*MOJ9.27*)	AY042854.1	87	3e-22	*A. thaliana*
FDC9	Protein kinase family (At5g18500)	NM-121855.2	94	7e-11	*A. thaliana*
FDC7	ABA and stress-inducible protein (*Asr1*)	AF039573	87	2e-64	*Oryza sativa*
FDC12	Cellulose synthase-2 (*Ces-2*)	AF200526	88	2e-66	*Zea mays*
FDC20	Cell wall invertase (*IVR3*)	AF030421.1	86	9e-63	*Triticum aestivum*
FDC21	Proline-rich protein	X52472.1	82	1e-09	*T. aestivum*
FDC19	Putative proline-rich and glycine protein	AJ24280.1	88	3e-15	*Sporobolus stapfianus*
FDC13	Seven transmembrane protein Mlo8	AY029319.1	87	2e-95	*Z. mays*
FDC1	Superoxide dismutase-Fe	AB014056.1	90	5e-64	*O. sativa*
FDC11	Manganese superoxide dismutase (*SOD-3*)	M33119.1	94	1e-172	*A. thaliana*
FDC2	Glutathione-S-transferase Cla47	AY064480.1	99	0	*T. aestivum*
FDC14	Putative magnesium transporter (*mrs2-2* gene)	ATH297817	81	7e-27	*A. thaliana*
FDC3	*Triticum aestivum* protein of unknown function (*wali5*)	L11882.1	100	1e-153	*T. aestivum*
FDC5	Mitochondrial ATP synthase subunit 9 (*ATP9*)	X15919.1	98	1e-165	*T. aestivum*
FDC15	WHTMT26SRR mitochondrial 26S rRNA	M37474.1	100	0	*T. aestivum*
FDC18	WHTTEF1X TEF1 α-subunit	M90077.1	99	1e-123	*T. aestivum*
FDC16	Polyubiquitin (*RuBQ1*)	AF184279.1	91	1e-108	*O. sativa*
FDC17	Cysteine protease component of protease-inhibitor complex unknown (5)	AB020961.1	85	3e-52	*Z. mays*

Differentially upregulated expressed sequence tags in aluminum (Al)-tolerant near-isogenic lines OK91G106 under Al stress for 48 h have been submitted to GenBank in NCBI. ABA, abscisic acid; CDPK1, calcium-dependent protein kinase.

(transmembrane protein gene), reactive oxygen species (ROS; genes coding superoxide dismutase (SOD)-Fe, SOD-Mn, and glutathione S-transferase), magnesium transport (putative magnesium transporter), mitochondrial function (genes coding for mitochondrial 26S rRNA, mitochondrial ATP synthase), and protein synthesis and processing (genes coding translation elongation factor (TEF) subunit, polyubiquitin, and protease inhibitor). These genes seem to have function in tolerance to Al toxicity in the Al-tolerant line tested.

To validate the result from macroarray analysis, eight upregulated ESTs were selected for further Northern analysis (Fig. 4). The trend of EST expression from Northern blotting was similar to that from macroarray analysis (Fig. 4). The result confirmed that the expression of all eight ESTs tested was Al regulated in the Al-tolerant line OK91G106 and demonstrated that the macroarray analysis was reliable and repeatable.

3 Discussion

3.1 Genes related to organic acid synthesis were not differentially expressed

Exudation of Al-chelating organic acids, such as malate, oxalate, or citrate, into the rhizosphere has been proposed as a tolerance mechanism to avoid Al toxicity in many plants (Delhaize *et al*. 1993; Basu *et al*. 1994; Delhaize and Ryan 1995; Ryan *et al*. 1995; Pellet *et al*. 1996; Zhang *et al*. 2001; Tang *et al*. 2002). It is postulated that organic acid anions can reach the sites of Al binding in the apoplast through anion channels by the negative membrane potential and the deep concentration gradient between the cytosol and apoplast or rhizosphere (Delhaize and Ryan 1995). Although a lot of work has reinforced the concept that Al tolerance in wheat may be based on exudation of malate and its chelation of Al (Delhaize *et al*. 1993; Carver *et al*. 1995;

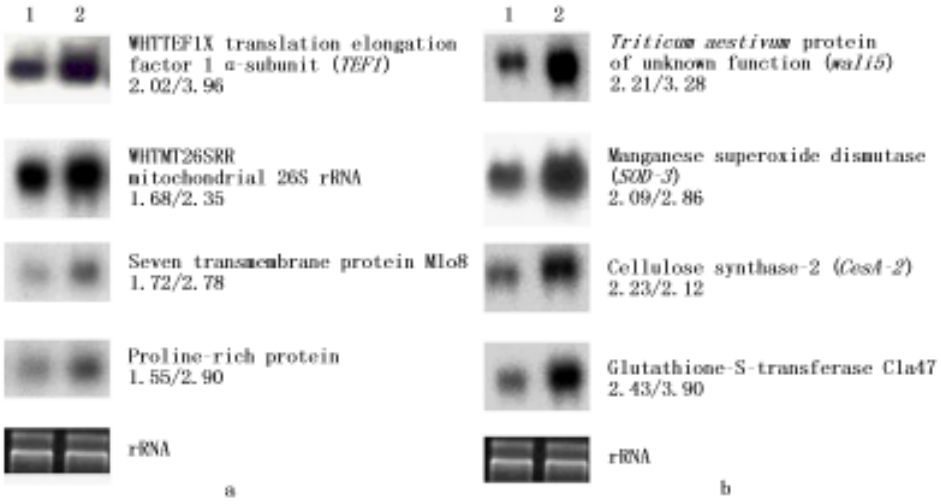

Fig. 4. Northern blotting analysis of randomly selected differentially upregulated expressed sequence tags identified in macroarray analysis. Panel 1 represents 48 h Al-treated Century and panel 2 represents 48 h Al-treated OK91G106. For each cDNA, *a* and *b* in the term *a*/*b* are the ratios of signal intensities between OK91G106 and Century from Northern blotting (**a**) and macroarray analysis (**b**), respectively.

Delhaize and Ryan 1995), differentially upregulated genes directly involved in the malate synthesis pathway were not identified in the Al-tolerant NIL OK91G106 under Al stress. Up to date, approximately 20 Al-induced or regulated genes have been reported (Milla *et al*. 2002), but none of them can be related directly to the biosysthesis of organic acids. Some evidence indicates that Al stress does not affect the activities of isocitrate dehydrogenase, phosphoenolpyruvate carboxylase, and malate dehydrogenase, the important enzymes regulating the synthesis of organic acids in wheat (Li *et al*. 2000). Ma *et al*. (2000) found that Al-induced secretion of malate in wheat and buckwheat has a pattern with no discernible delay between the addition of Al and the onset of the release of malate, which is caused by the activation of an anion channel for organic acids. These results indicate that there seem to be no induced or regulated genes involved in the synthesis of malate and other organic acids in wheat under Al stress. The greater secretion of organic acids in Al-tolerant wheat cultivars under Al stress could be due to the activation of a related anion channel for organic acids (Ma *et al*. 2000) or an aluminum-activated malate transporter (Sasaki *et al*. 2004).

3.2 A signal pathway response to Al stress in Al-tolerant NIL

Several signal transduction genes were differentially expressed in the Al-tolerant NIL after Al stress, including an abscisic acid (ABA) cluster gene, a *CDPK* gene, and a serine/threonine phosphatase gene. This result indicates that a signaling pathway may be activated when Al-tolerant wheat is challenged with Al stress. The plant hormone ABA mediates many vital processes in plant growth and development (Hagenbeek *et al*. 2000). Several ABA-signaling genes have been cloned and shown to encode protein phosphatases and transcription factors (Finkelstein *et al*. 1998; Luerssen *et al*. 1998). Meanwhile, as a class of Ca^{2+}-binding sensor proteins, CDPKs can bind directly calcium to act as a ubiquitous second messenger in eukaryotic signal transduction cascades (Cheng *et al*. 2002). Several lines of evidence suggest that CDPKs may mediate abiotic stress-signaling pathways (Sheen 1996; Sajio *et al*. 2000, 2001). These results indicate that the differentially expressed wheat *ABA* cluster genes and *CDPK* genes in Al-tolerant NIL OK91G106 may play a major role in intercepting

the Al-stress signal and regulate the activity of some protein kinases through eliciting the differentially expressed serine/threonine phosphatase gene, which regulates the phosphorylation status of biochemical enzymes in the resistant NIL.

3.3 Genes for cell wall structure and function are involved in Al tolerance

Several ESTs related to cell wall structure and function were differentially expressed in Al-tolerant NIL. These ESTs included a cell wall protein, a proline-rich protein, and a cellulose synthase. Some results indicated that Al could rapidly binds to the walls of root cells when applied externally (Delhaize *et al*. 1993). The Al^{3+} ion binds mainly to the negative-charged carboxylic groups of the pectic matrix in the cell wall; as a result, it reduces the movement of water and mineral nutrients through cell wall interstices and impairs the physical properties of the cell wall, resulting in low extensibility and permeability of the cell wall (Pritchard 1994). The differential expression of genes encoding cell wall and proline-rich proteins in the Al-tolerant line may increase wheat tolerance to Al toxicity through improving cell wall functions, such as improving the extensibility and permeability of the cell wall and increasing the movement of water and mineral nutrients through cell wall interstices. In addition, it has been reported that Al-stressed wheat roots typically appear shorter and thicker, which may elicit secondary responses potentially detrimental to overall growth and development of a wheat plant (Carver *et al*. 1995). In the present study, relative improvement of root growth rate in the Al-tolerant NIL OK91G106 (Fig. 1) than in Century may be attributed, to some extent, to overexpression of the cellulose synthase gene *CesA3*, a key enzyme promoting cell elongation.

3.4 Improvement of membrane stability under Al stress may contribute to Al tolerance in Al-tolerant NIL

Under Al stress, the outer surface of the plasma membrane and cell wall pectin are considered to be two major targets of Al toxicity because Al ions preferentially form electrostatic bonds with oxygen donor ligands, such as carboxylate and phosphate groups (Yamamoto *et al*. 2001). The plasmalemma is a vulnerable site for binding Al ions (Carver *et al*. 1995). The binding of Al to the membrane can cause rigidification of the plasma membrane (Deleers *et al*. 1986), peroxidation of membrane lipids, and an increase in membrane permeability by increasing the ratio of phosphatidylcholine to phosphatidylethanolamine (Lindberg *et al*. 1993). Hence, metabolism in the plasma membrane can be significantly altered under Al stress. However, transgenic *Arabidopsis* plants overexpressing Al-induced tobacco glutathione S-transferase gene (*parB*) and anionic peroxidase gene (*NtPox*) significantly restricted lipid peroxidation in cell membrane regions, showed much lower induction of malondialdehyde (MDA), and maintained normal membrane function under Al stress (Ezaki *et al*. 2001). In the present study, the genes encoding an SOD-Fe, an SOD-Mn, and a glutathione S-transferase were involved scavenging of ROS and have been identified to be upregulated in the Al-tolerant NIL, suggesting that differential expression of these genes may play a role in stabilizing plasma membrane structure and maintaining membrane function under Al stress.

3.5 Magnesium transporter seems to affect Al tolerance in wheat

Magnesium (Mg^{2+}) is the most abundant divalent cation in a living plant cell. In higher plants, Mg^{2+} stabilizes membranes, regulates many cellular enzymes, and acts as an essential component of chlorophyll molecules (Li *et al*. 2001). Meanwhile, the concentration of free ionized Mg can change rapidly in response to environmental stimuli and plays an important role in cell division, growth, and development (MacDiarmid *et al*. 1998). When a plant grows in soil with a high concentration of Al at a low pH, one common result is the perturbation of ionic steady state, leading to an imbalance of ion homoestasis in cells and a deficiency of essential nutrients, like Mg^{2+}, Ca^{2+}, or Mo^{2+} (Carver *et al*. 1995). In a family such as Poaceae, long-term exposure of plants to Al^{3+} causes significant Mg^{2+} deficiency and, therefore, leads to poor plant growth and

low yield (Tan *et al*. 1991). Conversely, increasing Mg^{2+} uptake can ameliorate Al^{3+} toxicity (Tan *et al*. 1991; Matsumoto 2000). Increased activity of the Mg^{2+}-transport system confers yeast resistance to Al^{3+} and also overcomes the inhibition of cation uptake by Al (MacDiarmid *et al*. 1998). In the present study, the putative magnesium transporter was identified in the Al-tolerant NIL and its upregulated expression increases wheat tolerance to Al toxicity. Therefore, this gene may play an important role in enhancing wheat tolerance to Al toxicity by increasing Mg^{2+} uptake and improving ion homeostasis in Al-stressed cells. However, most studies on Mg^{2+} transporters have been performed in yeast. Further investigation of Mg^{2+} transporters in wheat may shed light on the mechanisms of wheat tolerance to Al toxicity.

In summary, our results indicate that wheat tolerance to Al stress involves complicated defense-related signaling and metabolic pathways. Genes related to primary reactions (such as signal transduction and oxidative stress alleviation) and secondary reactions (such as membrane structure, function, and ion transport) were differentially upregulated in the Al-tolerant NIL in response to Al. Under Al stress, genes directly related to the synthesis of malate and other organic acids were not detected.

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(Managing editor: Ping HE)

Appendix I: Expressed sequence tags submitted to GenBank in NCBI

dbEST_Id	User_Id	GenBank_Accn	Clone No.
19875630	EST001	CF569140	FDC1
19875631	EST002	CF569141	FDC2
19875632	EST003	CF569142	FDC3
19875633	EST004	CF569143	FDC4
19875634	EST005	CF569144	FDC5
19875636	EST007	CF569146	FDC7
19875637	EST008	CF569147	FDC8
19875638	EST009	CF569148	FDC9
19875639	EST010	CF569149	FDC10
19875640	EST011	CF569150	FDC11
19875641	EST012	CF569151	FDC12
19875642	EST013	CF569152	FDC13
19875643	EST014	CF569153	FDC14
19875644	EST015	CF569154	FDC15
19875645	EST016	CF569155	FDC16
19875646	EST017	CF569156	FDC17
19875647	EST018	CF569157	FDC18
19875648	EST019	CF569158	FDC19
19875649	EST020	CF569159	FDC20
19875650	EST021	CF569160	FDC21
19875696	EST067	CF569206	FDC67 (unknown function)
19875697	EST068	CF569207	FDC68 (unknown function)
19875698	EST069	CF569208	FDC69 (unknown function)
19875699	EST070	CF569209	FDC70 (unknown function)
19875700	EST071	CF569210	FDC71 (unknown function)