Microarray analysis of *Arabidopsis* genome response to aluminum stress

S.B. GOODWIN[1] and T.R. SUTTER

W. Harry Feinstone Center for Genomic Research, Department of Biology, University of Memphis, Memphis, TN 38152, USA

Abstract

To better understand the mechanisms involved in aluminum toxicity and tolerance in plants, microarray technology was used to evaluate changes in gene expression in *Arabidopsis thaliana* under Al stress. With the use of *Affymetrix Arabidopsis ATH1 Genechip*, a comparison of RNA expression profiles was made between control and Al-treated *Arabidopsis* seedlings. A total of 256 genes were identified as Al-responsive. Ninety-four genes were shown to be up-regulated and 162 were down-regulated; comprising 1.1 % of the 24 000 *Arabidopsis* genes. Real-time RT-PCR was used to confirm the microarray data. The analysis showed that a large number of transcription factors and several putative signaling components were up-regulated by aluminum. Chloroplast structural and photosynthetic genes were, in general, down-regulated. A number of previously identified Al-responsive genes, *e.g*. *GST*, *Auxin-regulated*, *Peroxidase*, and *Chitinase*, were up-regulated by Al-stress, whereas *Wali 3* and *Wali 4* were down-regulated. We also identified several up-regulated genes involved in vacuolar signaling, sorting and docking. Three genes were also up-regulated by Al-stress, *Ras GTP*-binding protein, *ABC-cassette* binding, and the *AtELP1* receptor genes, have previously been documented as responsive to drought and/or oxidative stress and may play important roles the detoxification of Al ions by transportation and storage into root vacuoles. Ultrastructural changes in the roots tips cells of *Arabidopsis* were evaluated using transmission electron microscopy and energy-dispersive X-ray analysis with scanning electron microscopy and results showed Al accumulation in the root tips of *Arabidopsis.*

Additional key words: gene expression, microarray gene chip.

Introduction

Aluminum is the most abundant metal and the third most abundant element in the earth's crust. In alkaline soil Al is bound as alumino-silicate complexes and is easily released as phytotoxic Al^{3+} ions upon soil acidification (Moffat 1999, Sivaguru *et al*. 2000). Comprising over 50 % of the world's arable lands (Von Uexkull and Mutert 1995), acidic soils and Al toxicity is the primary limiting factor in crop productivity. When grown on strongly acidic soils, plants can accumulate toxic levels of Al which is grossly manifested in severe inhibition of root growth. This inhibition interferes with the ability of plants to uptake, transport, and utilize water and essential nutrients (Foy 1988, Ma and Hiradate 2000), thus impeding the performance of both apoplastic and symplasmic processes at the apical root zone, resulting from the displacement of cations by Al^{3+} for interactions at negatively charged sites in the cell wall, membranes, and cytoplasm (Kochian 1995, Zheng and Yang 2005). Such a toxic environment forced plants to adopt multiple survival strategies to either restrict Al accumulation in the root tips by excluding Al from the root apex or by tolerating Al accumulation in the symplasm. The release of high levels of carboxylic acids, such as malate, oxalate or citrate, into the rhizosphere, effectively excludes Al from root tissue through chelation (Hoekenga *et al*. 2003, Ezaki *et a*l. 2001, Hayes and Ma 2003, Sasaki *et al.* 2002). Recent studies on internal mechanisms of plant response to Al stress have focused on Al accumulators

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Abbreviations: CW - cell wall; EDXMA - energy-dispersive X-ray analysis; EM - electron microscopy; IS - interstitial space; MS - Murashige and Skoog; PCR - polymerize chain reaction; PD - plasmodesmata; SEM - scanning electron microscopy; TEM - transmission electron microscopy; V - vacuole.

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[1] Corresponding author; fax: (+901) 678 2458; e-mail: sgoodwin@memphis.edu

such as buckwheat and *Hydrangea*. The results of the studies have shown that detoxification occurs by cytosolic chelation with carboxylic acids and vacuole compartmentation (Ma *et al.* 1997, Vazquez *et al*. 1999, Shen *et al*. 2004).

Tolerance or susceptibility to Al stress in plants is a complex phenomenon, involving many genes and a number of signaling pathways (Hoekenga *et a*l. 2003). The mechanisms involved in Al tolerance or resistance are not fully understood at the molecular level. Therefore, efforts have been directed toward understanding the molecular basis of plants responses to Al stress and to identify Al-responsive genes whose expression can be manipulated to enable plant growth in acidic soils. Although a host of Al-induced genes have been identified and characterized in several species, including wheat (Snowden and Gardner 1993, Richards *et al*. 1994), soybean (Ragland and Soliman 1997) and *Arabidopsis* (Richards *et al*. 1998, Hoekenga *et al*. 2003, 2006, Larsen *et al.* 2005, 2006), our knowledge of global changes in the expression of Al-responsive genes is still lacking. To broaden this understanding at the molecular level, a molecular genetic approach was undertaken using *Arabidopsis* as a model plant to identify changes in genes expression patterns in response to Al stress. Microarray technology has proven to be a powerful tool for identifying plant genes induced by environmental stimuli or stress and for analyzing their expression profiles in response to such stresses (Brinker *et al.* 2004, Bray 2004, Leonhardt *et al.* 2004).

Plant tolerance also involves the compartmentation of the toxic metal at the cellular or subcellular levels. Measurements of transmembrane uptake of Al ions or cytosolic Al in plant cells can be an important aspect toward understanding how plants survive in acidic soils. The use of *in situ* analytical techniques to quantify Al in plant cells, such as energy-dispersive X-ray analysis (EDXMA), had been used in several studies with range of plant models.

The aim of this paper was to describe global gene expression changes during Al^{3+} stress using microarray *GeneChip* technology and to evaluate changes in the ultrastructure of *Arabidopsis* root tips using EDXMA with SEM.

Materials and methods

Plants, growth conditions and Al treatment: *Arabidopsis thaliana* L. cv Columbia seeds were surface sterilized using 1.5 g dm^{-3} sodium hypochlorite solution containing 2 g dm^{-3} *Tween* for 20 min followed by 3 washes with water. Seeds germinated for 7 d at 22 °C under continuous irradiance (200 μmol m^{-2} s^{-1}) on agar plates containing Murashige and Skoog (MS) salts, 10 g dm^3 sucrose, and 50 kanamycin. Seedlings were then used for Al treatment.

Seedlings with 2 to 3 cm root length (approx 7-d-old) were removed from agar plates gently to avoid damaging the roots, and approximately 50 seedlings were submerged in a 1000 cm^3 flasks containing 500 cm^3 of modified one-sixth Murashige and Skoog liquid medium containing 50 μM $Al_2(SO_4)_3$ and 1 g dm^{-3} sucrose added as a carbon source for 16 h under continuous aeration. The pH was adjusted to 4.0.

For EM studies, seedling were rinsed with distilled water, the primary roots were excised from control and Al-treated. Otherwise, seedlings were frozen with liquid nitrogen and stored at -80 °C for RNA extraction, microarray analysis and real-time PCR studies.

RNA preparation: For RNA extraction, 100 seedlings were harvested at 16 h post-treatment, comprising the pooling of two media flasks per treatment. Tissue was ground to a fine powder in liquid nitrogen using a pestle and mortar and total RNA was extracted using the following method. Each 0.5 to 1 g dm^{-3} of powdered tissue were added to 5 cm^3 of *STAT-60* (*Tel-Test*, *Friendswood*, TX, USA). The mixture was homogenized and stored on ice for 10 min. Chloroform/isoamyl EtOH (24:1, v/v) was added to the homogenate and centrifuged at 1200 *g* for 30 min (4 °C). Following the transfer of the aqueous phase to a fresh tube, RNA was precipitated with isopropanol overnight at -20 °C, pelleted by centrifugation at 1200 *g* for 30 min (4 °C), and resuspended 0.1 cm^3 of RNase-free water. Total RNA was quantified using *Beckman DU640* (Fullerton, USA) spectrophotometer, and qualified by running 1 μg of each sample onto a RNA *Lab-On-A-Chip* using a *Agilent Bioanalyzer 2100* (*Agilent Technologies*, Mountain View, CA, USA).

Target preparation/processing for *GeneChip* hybridization: Isolated total RNA samples were processed as recommended by *Affymetrix GeneChip* expression analysis technical manual. Briefly, total RNA (8 μg) was synthesized to cDNA using the superscript double-stranded cDNA synthesis kit (*Invitrogen*, Carlsbad, CA, USA) and poly dT-nucleotide primers that contain a sequence recognized by T7 RNA polymerase. The newly synthesized cDNA was used as a template to generate biotin-labeled *in vitro* transcribed (IVT) cRNA using the bio-array high yield RNA transcript labeling kit (*Enzo Diagnostics*, Farmingdale, NY, USA). Twenty micrograms of the cRNA was fragmented to strands of 35 to 200 bases in length. The fragment cRNA was hybridized to and *Affymetrix ATH1* array *GeneChip* at 45 °C with rotation for 16 h (*Affymetrix GeneChip Hybridization Oven 320*). The *GeneChip* arrays were washed and stained (streptavidin phycoerythrin) on an *Affymetrix Fluidics Station 400*, followed by scanning.

***GeneChip* data analysis:** The mRNA contents were

measured using *Affymetrix ATH1* chips (containing 22 810 probe sets) according to standard *GeneChip* expression assay protocol. Total RNA was extracted from 100 pooled *Arabidopsis* seedlings for each *GeneChip*, control and Al-treated. A total of four chips were used in this experiment, representing two biological replicates to measure expression of whole plant material. After hybridization, the chips were scanned using a *GeneChip* scanner and raw hybridization data from microarray experiments were imported directly into *Affymetrix GeneChip* operating software (*GCOS*) *v1.2* for normalization and to determine the probe intensities and "present" (P) or "absent" (A) calls for each chip. The scanned image results for all 4 chips were quantified and analyzed using *dChip* (Li and Wong 2001) and *RMA* software (Irizarry *et al*. 2003). The gene list generated in dchip was uploaded into gene ontology consortium website (http://www.geneontology.org) to further determine the functional categories of up- and down-regulated gene expression. The microarray data has been submitted to *NCBI's Gene Expression Omnibus* repository (series accession number GSE7334).

Real-time PCR: Template cDNA samples were prepared using 2.5 μg of total RNA, oligo-dT and Superscript II reverse transcriptase (*Invitrogen*). Gene specific primers to test expression levels by quantitative PCR were as follows: *Ras GTP* forward primer (FP) (5'-AAGCC AAAGCCACATAGAA-3') and reverse primer (RP) (5'GTGCGAATTTTAAAGTCAACA-3'); *pEARL* forward primer (5'CCGGCCGGTTATCCTCCAG-3') and reverse primer (5'AGCGCAGCCAAACATCCTTCA-3'); MDR *ABC binding* forward primer (5'TCTTGCCT CGCTGTTGTTCCTCA-3') and reverse primer (5'ACGGTTACGCCTTTGCATTCACTC-3'); *ATELP* forward primer (5'GGGGCAGTGCTTAAGGCTATGT-3') and reverse primer (5'AGAGGCTTTGCAGTGAGT GTAACC-3'); *CBL1* forward primer (5'GTTTTCCACC CCAATGCTTCTCTA-3') and reverse primer (5'CTCGTGGCAATCTACTCGGTCTTA-3').

The housekeeping gene was tubulin: forward primer (5'-GTGGGCACAATGTTCAGGTTCC-3') and reverse primer (5'-TGTGCATTCCCAAAGTCGTAAGC-3'). Real-time PCR was performed with the *Bio-Rad iCycler* using *iQ SYBR Green Supermix* (*Bio-Rad Laboratories*, Hercules, CA, USA). Each PCR reaction contained 100 ng cDNA and 25 μM cm^3 of each of the primers. Amplification was carried out as follows: 40 cycles, 95 °C for 30 s, 50 - 62 °C for 30 s and 72 °C for 30 s. Relative gene expression was quantitated by comparative Ct method (Pfaffi 2001).

Sample fixation, electron microscopy and EDXMA: For EM studies, the root tips (0 - 5 mm) were fixed in 2.5 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) overnight at 4 °C, followed by rinsing with same buffer 3 times, 10 min. Samples were postfixed in 1 g dm^{-3} osmium tetroxide in the same buffer for 1 h then rinsed 3 times in the glutaraldehyde buffer for 10 min, dehydrated in a series of ethylalcohol, and embedded in Spurr's resin. Longitudinal serial sections, between 0 and 1.5 mm from apex were studied by electron microscopy, and the elemental distribution in vacuoles was determined by EDXMA on dry-cut sections. Dry-cut sections of approximately 1.5 μm were mounted onto gold grids. The microanalytical determinations were performed on an electron microscope operated at 100 kV in the scanning TEM mode using the energy-dispersive detector and a *Delta class 4460* analyzer. The counts were made over a 100 s period and spectra were recorded. Gaussian deconvolution was applied to the data and after background correction the results were expressed as the counts to second ratio. No Al signals were detectable by EDXMA in translucent cell areas and all data shown are from electron-dense deposits. Blank resin was analyzed to check for contaminants.

Results and discussion

Functional classification of genes: When comparisons were made between control and Al-treated plants, 256 genes were identified as Al responsive. Some genes were assigned to categories independent of the *Arabidopsis* system due to unclear or unknown classification. The Al-regulated genes were placed into gene ontology (GO) functional categories (http://www.geneontology.org/). Overall, 70 % of the 256 genes identified as Al responsive were shown to be involved in some type of metabolism, which includes: cell wall biosynthesis, cell wall weakening and modification, membrane biosynthesis, and cellular metabolism. Interestingly, 20 % of the expressed genes had some function related to protein metabolism, 19 % responded to external and internal stimulus; whereas 10 % were stress-related. Overall, the largest proportion of genes was characterized into metabolic pathways (Table 1). Out of the 256 genes, 27 (11 %) genes were categorized as unclassified because the sequences displayed no similarity to known proteins or were homologs to hypothetical proteins. The up- and down-regulated genes were further classified based on their predicted biological function using categorization developed for *Arabidopsis* using both *PEDANT* (http://pedant.gsf.de), and *TAIR* (http://www.arabidopsis.org) (Fig. 1). The functional categories included transcription/transcription factors, cell wall and membrane metabolism, oxidative burst/plant defense, pathogen resistance, signal transduction, protein synthesis/modification, cellular transport, other stress related, and vascular function. There were a total of 94 (36 %) up-regulated and 162 down-regulated genes. Notably, the majority of genes showing differential expression in response to Al stress were down-regulated (64 %). The bulk of the differentially expressed genes

Table 1 Functional grouping of aluminum responsive genes in *Arabidopsis*. A total of 256 gene differentially expressed during Al^{3+} treatment were grouped into functional categories based on the categorization developed by the Gene Onthology (GO) Consortium and TAIR for *Arabidopsis*. Each category was given a percentage of all genes differentially expressed in that group. Each gene can belong to more than one category. All the data has been submitted to NCBI's Gene Expression Omnibus repository (series accession number GSE7334).

Functional category	Gene [%]
Cell growth/maintenance	21.00
Protein metabolism	20.00
Biosynthesis	16.05
External stimulus response	16.05
Signal transduction	12.35
Energy pathways	12.35
Nucleic acid metabolism	11.11
Photosynthesis	11.11
Response to stress	9.88
Sugar metabolism	9.88
Regulation of cell death	7.41
Regulation of metabolism	6.17
Catabolism	6.17
Electron transport	4.94
Lipid metabolism	4.90
Aromatic compound metabolism	3.70
Carbon utilization	3.70
Alcohol metabolism	2.47
Response to endogenous stimulus	2.47
AA and derivative metabolism	2.47
Oxygen and ROS metabolism	2.47
Organic acid metabolism	2.47

were involved in protein synthesis (41 %) and cell wall/cell membrane metabolism (22 %).

Up-regulated gene expression: In this study at least 19 % of the Al responsive gene transcripts identified encoded proteins associated with cell wall metabolism. The majority (approximately 22 %) of these genes were induced rather than repressed in response to Al treatment (Fig. 1). Gene profile analysis revealed that Al induces the expression of various genes encoding proteins involved in cell wall biosynthesis, and organization, as well as genes known to be involved in different metabolic pathways other than cell wall synthesis such as oxidative stress, pathogen defense, and the phenylpropanoid pathway (Table 2). Al stress up-regulates transcripts for enzymes such as sucrose-UDP glycosyltransferase, glycosyltransferase family (14 and 20), xyloglucan endo-transglycosylase, pectinacetylesterase, sucrose synthase, and β-1,3-glucanase-like protein (callose). These enzymes are involved directly or indirectly in pathways synthesizing structural components of cell walls such as cellulose, hemicellulose and pectin.

The enzymes glycosyltransferase (GT) and glycoside hydrolases (GHs) are superfamilies of sugar-active enzymes. They catalyze similar biochemical reactions, namely the transfer of sugar moieties to acceptor molecules (Bowles 2002). *GTs* are large multigene families involved in multiple plant processes such as secondary metabolites, abiotic and biotic defense, and conjugating hormones. Alternatively, GHs break bonds existing between sugar moieties and other molecules. Glycoside hydrolase (chitinase) specifically hydrolyses

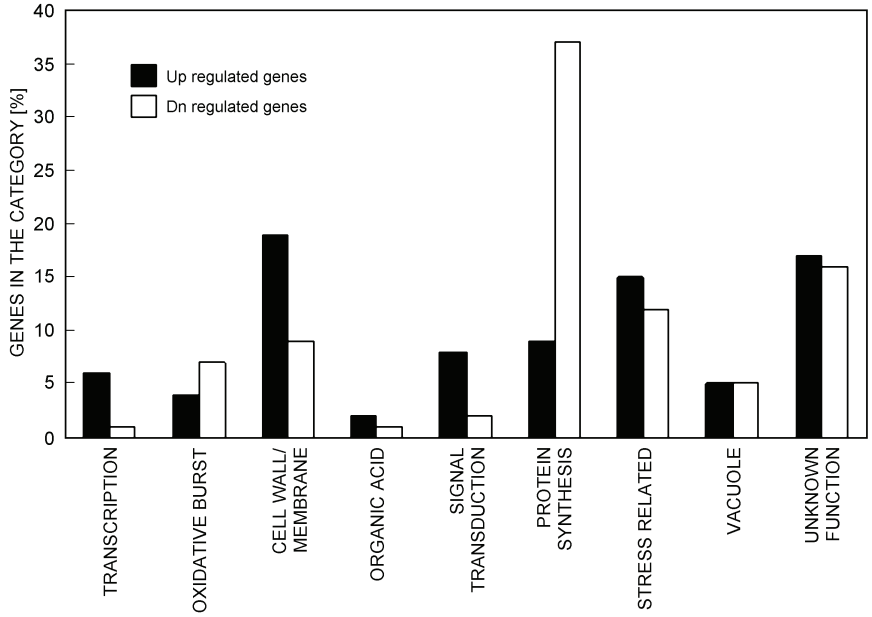

Fig. 1. Biological Function classification of Al-responsive genes according to TAIR and PEDANT. The 256 expressed gene transcripts (differentially expressed following 16 h Al treatment in *Arabidopsis*) were separated into two groups according to up- or down-regulation. *Dark columns* - genes that were upregulated by Al (total 94); *white columns* - genes that were downregulated by Al (total 164).

the β-1,4-N-acetyl-D-glucosamine linkages in chitin polymers and functions in plant defense against fungal and insect pathogens by destroying their chitin-containing cell walls. Thus, this gene is probably induced under Al-stress as defense mechanism. One major member of the GH 16 family, xyloglucan endotransglycosylase (XTH14) was previously shown to be expressed in the base of elongating roots (Vissenberg *et al.* 2005). GH 16 family is comprised largely of xyloglucanases, which are often associated with the apoplast and the cell wall. XTHs modify a major component of the plant cell well, xyloglucan, therefore may play critical roles in influencing cell wall metabolism.

Cell wall structural proteins have been previously identified in *Arabidopsis* as Al-responsive. Richards *et al.* (1998) detected two cell wall proteins, pEARLI (pro-rich hydrophobic protein) and pEARLI4 (pro-rich hydrophilic protein) in *Arabidopsis* during the first hours of Al exposure. Both genes showed both up-regulation and down-regulation following Al treatment, similar to our results. Al stress up-regulates glycine-proline rich protein (GPRP) and a putative hydroxyproline-rich glycoprotein. Wall proteins are typically glycoproteins (polypeptide backbone with sugar side-chains) particularly rich in the amino acids hydroxyproline (hydroxyproline-rich glycoprotein, HPRG), proline (proline-rich protein, PRP), and glycine (glycine-rich protein, GRP). There is strong evidence that these proteins function in strengthening cell wall the plant defense (Keller 1993). Extensin, a well-studied HRGP, was down regulated. HRGP is induced by wounding and pathogen attack. Structural proteins, such as proline-rich protein, appear to be cross-linked to pectic substances and may have sites for lignification. The expression of the β-1,3-glucanase gene is often associated with pathogen infection, particularly by fungi. Although its role in Al toxicity is unknown, Cruz-Ortega *et al.* (1997) suggested that this protein is synthesized as a protective response, because during Al stress roots are more susceptible to pathogen attack. This hypothesis is also a possible explanation for the induction by Al of other genes related to pathogen response.

Al treatment, like various environmental stresses, generates reactive oxygen species (ROS; Kochian 1995, Mittler *et al.* 2004), which are thought to be primarily activated by peroxidation of membrane lipids, but can also be triggered by stress perception such as Al-toxicity. Plants use the production of ROS as signals in response to various types of stress and during development. ROS species and their by-products can however, be toxic to plant cells. Plants therefore have evolved large gene families for detoxification of ROS components, such as glutathione reductase (GR), catalase, and peroxidases (POX). In *Arabidopsis* there are at least 152 defined enzymes identified as part of the ROS scavenging network. This ROS network encodes both ROS-scavenging and ROS-producing proteins (Mittler *et al.* 2004). Richards *et al*. (1998) reported that Al induced the expression of oxidative stress genes in *Arabidopsis thaliana*, thereby indicating that stress caused by this metal is closely related to oxidative stress. We found three putative oxidative stress genes induced by Al, glutathione transferase, peroxidase, GR. Glutathione transferase, POX, and cytochrome P_{450} (ROS-producing enzyme) have previously been shown to be induced upon Al exposure (Richards *et al.* 1998, Milla *et al*. 2002). Wang *et al*. (2007) showed that exogenous GSH counteracted the root growth inhibition in rice unon Al exposures by preventing the decrease in H_2O_2 and thus decreasing lignin content in the roots. GR catalyzes the reduction of the oxidized form of glutatione (GSSG) to its reduced form (GSH) and has a central role in the cell response during stress. However, there is no report in the literature of the expression of genes encoding this enzyme in response to Al. A cytochrome P_{450} (CYP81D8) transcript was expressed in response to Al. The *Arabidopsis* genome contains 272 P_{450} (246 genes and 26 pseudogenes), attributing to a range of mixed reactions that they perform. For example, CYP81D8 belongs to the CYP81 family, which forms a small cluster in the *Arabidopsis* genome. The function and expression of these genes has so far not been investigated (Werck-Reichhart *et al*. 2002). There role of CYP81D8 in Al-stress is unclear but there may be a possible role in the detoxification of xenobiotic compounds (such as Al^{3+}) in both the cytoplasm and endoplasmic reticulum (Ekman *et al*. 2003).

The shikimate pathway is a major pathway in primary and secondary plant metabolism. It provides substrates for the synthesis of the aromatic amino acids Phe, Trp, and Tyr, which serve, as precursors for a variety of aromatic substances and metabolic pathways. Isochorismate synthase I (ICS) which catalyzes the conversion of chorismate into isochorismate, the first committed step in this pathway, was induced by Al. Isochorismate is an important metabolite formed at the end of the shikimate pathway. Literature suggests that isochorismate can act as an alternative pathway for salicylic acid in plants when the phenylpropanoid pathway is repressed (Wildermuth *et al*. 2002, Von Rad *et al.* 2005). The role of ICS and salicylic acid in response to Al exposure however is unclear. Yang *et al*. (2003) showed that in *Cassia tora* salicylic acid induced Al tolerance by increasing citrate efflux from root tips.

Al stress induced the expression of three transcripts involved in the organic acid pathway, malate dehydrogenase, 2-oxoglutarate dehydrogenase, and alanine aminotransferase. Malate dehydrogenase and 2-oxoglutarate involvement in Al-stress has not been identified as Al responsive. All three enzymes play important roles in the TCA cycle. In the TCA pathway, malate dehydrogenase, 2-oxoglutarate dehydrogenase catalyzes the reaction from oxaloacetate to malate to oxaloacetate and from 2-oxoglutarate (2-OG) to succinyl-CoA, respectfully.

There is also strong evidence that secretion of organic acids from the roots is one of the mechanisms involved in Al tolerance (Kochian *et al.* 2005). Organic acids are able to form strong complexes with Al which acting in both

Table 2 Up-regulated transcripts in *Arabidopsis* seedlings in response to 50 μM Al treatment. [a] - *Affymetrix* probe ID number; [b] - *Arabidopsis* genome initiative number; [c] - gene function predicted by *Affymetrix* annotation, TAIR definition, and PEDANT database; [d] - relative gene transcript level compared with the same gene in control-treated plants.

	Probe ID[a]	AGI ID[b]	Gene description[c]	Fold-change[d]
Transcription factors	246962_s_at	At5g24800	bZIP protein	2.46
	247707_at	At5g59450	scarecrow transcription factor (SCL 11)	2.47
	253105_at	At4g35840	zinc finger family protein	8.69
	254531_at	At4g19650	mitochondrial transcription termination factor	7.38
	259626_at	At1g42990	bZIP family transcription factor 60	3.27
	267028_at	At2g38470	WRKY transcription factor 33	2.58
Cell wall metabolism/	253046_at	At4g37370	cytochrome P450, putative (CYP81D8)	3.72
oxidative burst/defense	250798_at	At5g05340	peroxidase, putative	2.73
	257252_at	At3g24170	glutathione reductase, putative	2.23
	266181_at	At2g02390	glutathione transferase, (GST 18)	2.37
	245996_at	At5g20830	sucrose-UDP glycosyltransferase	2.50
	245998_at	At5g20830	sucrose synthase (SUS 1)	2.26
	248968_at	At5g22850	pectinacetylesterase, putative	2.15
	254044_at	At4g25820	xyloglucan endotransglycosylase (XTH14)	2.83
	262117_at	At1g74710	isochorismate synthase 1 (ICS1)	2.41
	256776_at	At3g13772	endomembrane protein 70, putative	2.27
	264844_at	At1g03520	glycosyltransferase family 14 protein	4.56
	266072_at	At2g18700	glycosyltransferase family 20	2.67
	254188_at	At4g23920	UDP-glucose 4-epimerase, putative	6.20
	247009_at	At5g67600	pEARLl-11/14	3.13
	256235_at	At3g12490	cysteine protease inhibitor	2.72
	247268_at	At5g64080	protease inhibitor (LTP) family	2.56
	260568_at	At2g43570	glycosyl hydrolase (chitinase)	4.90
	246440_at	At5g17650	glycine/proline rich protein (GPRP)	5.62
	253284_at	At4g34150	hydroxyproline-rich glycoprotein putative	5.20
	248100_at	At5g55180	β-1,3-glucanase-like protein	5.30
	260421_at	At1g69640	purple acid phosphatase	2.42
	264953_at	At1g77120	ADH-alcohol dehydrogenase (ADH)	4.15
	264005_at	At2g22470	arabinogalactan-like protein (AGP2)	4.83
Organic acid pathway	251787_at	At3g55410	2-oxoglutarate dehydrogenase,	2.44
	266457_at	At2g22780	malate dehydrogenase	2.76
Signal transduction	248313_at	At5g52590	RabGAP/TBC domain-like protein	4.40
	250545_at	At5g08160	serine/threonine protein kinase	2.32
	258463_at	At3g17410	serine/threonine protein kinase (RLCK8)	2.60
	245251_at	At4g17615	calcineurin B-like protein 1 (CBL1)	3.40
	257087_at	At3g20500	calcineurin-like phosphoesterase (PAP)	2.46
	262401_at	At1g49300	Ras family GTP-binding protein (RAB 7C)	2.39
	248792_at	At5g47200	Ras-related GTP-binding protein (RAB 1C)	2.93
	249832_at	At5g23400	leucine-rich repeat protein (LRR)	3.76
Vacuolar function	250816_at	At5g05010	clathrin adaptor complexes(coatomer)	2.43
	252027_at	At3g52850	AtELP1 (vacuolar sorting receptor)	3.75
	263743_at	At2g21390	coatomer protein complexes (alpha-COP)	2.57
	260932_s_at	At1g02530	mulitdrug resistance P-glycoprotein ATP-binding cassette (PGP12)	3.44
	255498_at	At4g02620	vacuolar ATPase subunit F family	2.20
Protein degradation	249914_at	At5g22850	aspartyl protease family protein	3.05
	247433_at	At5g62540	ubiquitin-conjugating enzyme 3 (UBC3)	3.21
	248615_at	At5g49570	transglutaminase-like family	3.34
	246147_s_at	At5g20000	26S proteasome AAA (ATPase subunit)	2.13
	246219_at	At4g36760	aminopeptidase-related protein	2.16
	255569_at	At4g01320	CAAX protease	2.34
	251930_at	At3g53780	rhomboid family protein	2.38
	256235_at	At3g12490	cysteine protease inhibitor	2.72
	262686_at	At1g75990	26S proteasome (regulatory subunit S3)	2.32
Al-stress related	249922_at	At5g19140	aluminum-induced protein	2.20
	249719_at	At5g35735	auxin-induced protein family	2.42
	246219_at	At4g36760	aminopeptidase-related protein	2.16
	265194_at	At1g05010	1-aminocyclopropane-1-carboxylate oxidase	2.36

table continued

	259105_at	At3g05510	rubber elongation factor/stress related	4.90
	246335_at	At3g44880	rieske (1Fe-2S) domain	2.31
	251721_s_at	At3g56190	alpha-soluble NSF attachment protein	2.55
	263517_at	At2g21620	universal stress protein	2.11
	266851_at	At2g26820	avRpt2-induced protein-1	2.44
	263948_at	At2g35980	hairpin-induced protein-1 family	3.11
	265221_s_at	At2g02010	glutamate decarboxylase	2.42
	267280_at	At2g19450	diacylglycerol O-acyltransferase	2.44
	264042_at	At2g03760	flavonol sulfotransferase	4.10
	264219_at	At1g60420	DC1 domain containing protein	2.50
	264506_at	At1g09560	germin-like protein (GLP)	2.28
	261410_at	At1g07610	metallothionein-like protein	2.30
Cellular transport	257543_at	At3g28960	amino acid transporter family	3.00
	260410_at	At1g69870	proton-dependent oligopeptide transport	3.56
Cell metabolism	251150_at	At3g63120	cyclin family	2.57
and cell cycle	258649_at	At3g09840	cell division cycle protein 48	2.46
	245791_at	At1g32210	defender against cell death (DAD1)	2.28
	260847_s_at	At1g17290	alanine aminotransferase	2.15
	245951_at	At5g19550	aspartate aminotransferase (ASP2)	2.77
Unclassified proteins	254227_at	At4g23630	reticulon family protein	2.35
	259198_at	At3g03610	phagocytosis/cell motility protein	2.38
	257654_at	At3g13310	DNAJ heat shock N-terminal	2.76
	260287_at	At1g80440	kelch repeat-containing F-box	2.75

major mechanisms Al tolerance by either by chelating Al ions present in the apoplast or by the internal detoxification of this metal by reversing its toxic effects inside the cell (Ma 2000). Studies have shown that increased sugar exudation does not correspond with increased induction of enzymes which catalyzes sugar synthesis and metabolism (malate dehydrogenase, 2-oxoglutarate dehydrogenase) (Hayes and Ma 2003). An overexpression of malate dehydrogenase in transgenic alfalfa produced a 7.1 fold increase in root exudation of organic acids (Tesfaye *et al*. 2001). However, the transcript, alanine aminotransferase, a phosphate-dependent enzyme operating in a wide range of metabolic pathways that catalyzes the reversible transfer of an amino group from alanine to 2-oxoglutarate to form pyruvate and glutamate, has previously been induced in response to Al exposure. It has been suggested by Richards *et al.* (1998) that the shutdown of these transcripts might reflect the response of a central metabolic pathway to Al stress. In our study, all three transcripts were induced by Al, rather than repressed.

The ability of cells to sense Al-stress and transduce that signal to downstream events is a poorly understood process. Within our study there were a total of seven genes induced by Al that could possibly shed light on the signaling pathway involvement with Al-stress (Table 2). Transcripts for two serine/threonine protein kinases, two calcineurin-like phosphatase, and three Ras family GTP-binding proteins were induced by Al. Kinases and phosphatases are key signal molecules in the perception of Al stress and appear to play pivotal roles in mediating stress responses. Osawa and Matsumoto (2001) demonstrated that protein phosphorylation may be required for the signal transduction in Al-activated malate efflux. In their study, a 48 kDa protein kinase was rapidly and transiently activated after Al treatment, which was then followed by malate efflux. This suggests that the protein kinase might be involved in the phosphorylation of an anion channel leading to the modification of that channel and malate efflux (Osawa and Matsumoto 2001). Two serine/threonine protein kinases (STK) were induced in our study. One putative serine/threonine transcript RLCK8, encodes for a receptor like cytoplasmic kinase which contains the kinase catalytic domain but lack a predicted transmembrane region and the other putative serine/threonine transcript contained the consensus N-myristylation signal sequence suggesting that this protein may be membrane bound (Tchieu *et al.* 2003), however, its function is unknown. Recently a serine/threonine protein kinase (STK) was identified whose activity and expression are induced by ROS. Oxidative signal-inducible 1 (OXI1) was shown to play a central role in ROS sensing and activation of mitogen-activated-protein kinases (MAPK) downstream by Ca^{2+} (Rentel *et al.* 2004, Mittler *et al.* 2004). In addition, OXI1 expression was seen in the root, which is consistent with the known role for ROS in root development. There were no related MAPK gene transcripts expressed in this study. However, further research is needed to identify and characterize the proteins associated with signal transduction and their role in Al stress.

Arabidopsis genomes contain approximately 93 small GTP-binding proteins, which act as molecular switches in a variety of important regulatory processes (Vernoud *et al.* 2003). The members of this superfamily have been structurally and functionally classified into five sub-families: Ras, Rho, Rab, Arf and Ran. Three subfamily members, Ras-related Rab GTP-binding protein, were induced in our study. Rab GTPases function in vacuolar and vesicle trafficking (Nahm *et al.* 2003, Sohn *et al.* 2003). Interestingly, a Rab/GAP/TBC domain-like protein was also induced by Al. TBC (Tre-2/Bub2/Cdc16) is

Table 3. Down-regulated transcripts in *Arabidopsis* seedlings in response to 50 μM aluminum treatment (for [a-d] see Table 2).

	Probe ID[a]	AGI ID[b]	Gene description[c]	Fold-change[d]
Transcription	245746_at	At1g51070	basic helix-loop-helix (bHLH)	-2.27
Cell wall modification/	264436_at	At1g10370	glutathione S-transferase (ERD9)	-3.04
oxidative burst/defense	246145_at	At5g19880	peroxidase, putative	-2.75
	247327_at	At5g64120	peroxidase, putative	-2.71
	254687_at	At4g13770	cytochrome P450 family protein CYP83B1)	-3.19
	253174_at	At4g35090	catalase	-2.10
	266118_at	At2g02130	plant defense-fusion protein	-2.60
	266123_at	At2g45180	protease inhibitor/ lipid transfer protein	-8.62
	258821_at	At3g09500	wound-responsive protein S19	-2.66
	256772_at	At3g13750	β-galactosidase	-2.69
	264371_at	At1g12090	protease inhibitor/lipid transfer protein	-2.98
	254832_at	At4g12490	pEARL1-like protein (PRP)	-3.11
	264371_at	At1g12090	pEARLI 1-like protein (PRP)	-16.0
	247251_at	At5g64740	cellulose synthase catalytic unit	-2.30
	247450_at	At5g62350	invertase/pectin methylesterase inhibitor	-4.09
	245325_at	At4g14130	xyloglucan endotransglycosylase (XTH15)	-6.09
	255433_at	At4g03210	xyloglucan:xyloglucosyl transferase (XTH9)	-7.92
	252971_at	At4g38770	extensin-like protein (PRP4)	-2.25
	252997_at	At4g38499	expansin protein family (EXPL2)	-3.28
	257203_at	At3g23730	xyloglucan endotransglycosylase (XTH16)	-8.80
	257008_at	At3g14210	myrosinase-associated protein	-3.03
	266215_at	At2g06850	endoxyloglucan glycosyltransferase (XTH4)	-5.70
	264371_at	At1g12090	expansin-like protein (ELP)	-2.98
	263016_at	At1g23410	ubiquitin extension protein	-2.31
	252115_at	At3g51600	nonspecific lipid transfer protein 5 (LPT5)	-3.49
Phenylpropanoid pathway	248200_at	At5g54160	*O*-methyltransferase	-2.10
	251984_at	At3g53260	phenylalanine ammonia-lyase 2 (PAL2)	-5.06
	263845_at	At2g37040	phenylalanine ammonia-lyase 1 (PAL1)	-2.90
	261899_at	At1g80820	cinnamoyl-CoA reductase	-4.32
Organic acid pathway	250278_at	At5g12860	2-oxoglutarate/malate translocator	-2.10
Signal transduction	249430_at	At5g39900	GTP-binding protein LepA	-4.01
	256274_at	At3g12080	GTP-binding family protein	-4.11
	257974_at	At3g20820	leucine-rich repeat family protein	-2.23
Vacuolar function	258054_at	At3g16240	δ-tonoplast integral protein (delta-TIP)	-3.96
	265444_at	At2g37180	plasma membrane intrinsic protein 2C	-3.16
	251962_at	At3g53420	plasma membrane intrinsic protein 2A	-3.42
	257313_at	At3g26520	γ-tonoplast intrinsic protein (δ-TIP)	-2.80
	251324_at	At3g61430	plasma membrane intrinsic protein 1A	-2.90
	256145_at	At1g48750	δ-adaptin, putative lipid transfer protein	-3.00
Cell metabolism and cell cycle	248128_at	At5g54770	thiazole biosynthetic enzyme (ARA6)	-2.44
	254740_s_at	At4g13890	glycine hydroxymethyltransferase	-2.94
	263371_at	At2g20490	adenylysulfate kinase 1	-2.35
	264188_at	At1g54690	histone H2A	-2.46
	263689_at	At1g26820	ribonuclease 3	-2.47
	248768_at	At5g47700	60S acidic ribosomal protein P1	-2.09
	261362_s_at	At1g41880	60S ribosomal protein L35a	-3.51
	266256_at	At2g27710	60S acidic ribosomal protein P2	-2.57
	266699_at	At2g19730	60S ribosomal protein L28	-2.25
	266981_at	At2g39460	60S ribosomal protein L23	-2.54
	261578_at	At1g01100	60S acidic ribosomal protein P1	-2.73
	262985_s_at	At1g23290	60S ribosomal protein L27A	-2.68
	263131_at	At1g78630	ribosomal protein L13	-2.14
	263665_at	At1g04489	60S ribosomal protein L23	-3.13
	263667_at	At1g04270	40S ribosomal protein S15	-2.14
Stress-related	245928_s_at	At5g24780	VSP 1 and VSP 2	-6.08
	246004_at	At5g20630	germin-like protein	-3.64
	246548_at	At5g14910	heavy-metal-associated domain	-2.24
	254059_at	At4g25200	23.6 kDa mitrochondrial sm heat shock	-3.45
	254384_at	At4g21870	26.5 kDa class P-related heat shock	-2.45
	258821_at	At3g7230	wound-responsive protein S19	-2.66

table continued

	260546_at	At2g43520	trypsin inhibitor	-5.07
	266118_at	At2g02130	plant defense-fusion protein	-2.60
	267126_s_at	At2g23590	hydrolase, α/β-fold family	-2.91
	260714_at	At1g14980	10 kDa chaperonin	-3.21
	261572_at	At1g01170	ozone-responsive stress related protein	-2.12
	262911_s_at	At1g59860	17.6 kDa class 1 heat shock protein	-2.90
	264195_at	At1g22690	gibberellin-responsive protein	-2.88
	247660_at	At5g60070	ankyrin repeat family protein	-6.20
	267233_s_at	At2g43920	thiol methyltransferase	-3.37
Cellular transport	246003_at	At5g20720	20 kDa chaperonin	-2.28
	254102_at	At4g26230	acyl carrier family protein	-2.33
	252115_at	At3g51600	nonspecific lipid transfer protein 5	-3.49
	252479_at	At3g46560	mitochondria membrane translocase	-2.22
	266239_at	At2g29530	mitochondrial membrane translocase	-2.87
	260693_at	At1g32450	proton-dependent oligopeptide transporter	-4.73
Chloroplast metabolism	247320_at	At5g64040	photosystem I reaction center subunit	-2.39
	248151_at	At5g54270	chlorophyll *a,b*-binding protein	-2.75
	248197_at	At5g54190	protochlorophyllide reductase A	-2.82
	245306_at	At3g22840	chlorophyll *a,b*-binding family	-2.69
	258993_at	At3g08940	chlorophyll *a,b*-binding protein	-2.18
	258055_at	At3g16250	ferredoxin-related	-3.66
	263345_s_at	At2g05070	chlorophyll *a,b*-binding protein	-3.58
	260481_at	At1g10960	ferredoxin, chloroplast	-2.27
Unclassified proteins	249847_at	At5g23210	serine carboxypeptidase S10	-2.70
	246154_at	At5g19940	plastid-lipid associated protein	-2.25
	247109_at	At5g65870	phytosulfokines 5	-2.50
	253236_at	At4g34370	IBR domain-containing protein	-5.66
	259102_at	At3g11660	hairpin-induced family protein	-2.27
	259161_at	At3g01500	carbonic anhydrase 1	-2.58
	266353_at	At2g01529	major latex protein-related	-4.13
	260523_at	At2g41720	pentatricopeptide repeat-containg	-5.28
	260876_at	At1g21460	nodulin MtN3 family protein	-3.50
	262539_at	At1g17200	integral membrane family protein	-3.03
	259788_at	At1g29670	GDSL-motif lipase/hydrolase family	-3.31
	260704_at	At1g32470	glycine cleavage system H protein	-2.10

predicted to encode GTPase activating proteins (GAPs) for the Rab family G proteins. The Ras GTPases are located on the inner surface of the plasma membrane and play a crucial role in initialing the kinase cascade that relays signals from RTKs (receptor tyrosine kinases) to the nucleus in animal cells. In plants our understanding of the Ras-related GTPases and their role in signaling is relatively new. To date, the Ras family is regarded as absent in plants (Vernoud *et al*. 2003, Sormo *et al.* 2006).

Gene transcripts involved in vacuolar sorting, binding and transport (sorting receptor (AtELP1) and MRP-ATP-binding cassette proteins) were up-regulated (Table 2). The regulated transport of molecules across the vacuolar membranes is a well characterized response to abiotic stress such as Al stress. Sequestration of toxic compounds into vacuoles is an important mechanism used by plant to avoid harm. Members of the ABC family of transporters are used to transport toxic chemicals from the cytoplasm into the vacuoles. ABC proteins comprise a large family of transporters that bind ATP during transport of a wide range of organic and inorganic solutes (Kochian 2005). There are approximately 120 ABC transporters in *Arabidopsis*, which function not only in transport, but act as channel regulators and molecular switches as well (Sugiyama *et al*. 2006). One ABC transporter, MRP (multidrug resistance protein) which translocate GS-conjugates was upregulated in response to Al. Gaedeke *et al.* (2001) isolated an ABC transporter AtMRP5 from *Arabidopsis* roots and determined that the gene was expressed mainly in vascular tissues. In wheat, a gene encoding MDR-like protein was induced by Al as well as inhibitors of calcium flux (Sasaki *et al*. 2002). Recently, genes encoding a putative ABC transporter (ALS3, ALS1) was found to be contributing to an Al tolerance mechanism in *Arabidopsis* possibly either by facilitating the redistribution of absorbed Al away from the sensitive root tissues (Larsen *et al.* 2005) or by transporting chelated Al into the vacuoles as part of the Al sequestering mechanism (Larsen *et al.* 2006). Determining the roles ABC transporters play in Al stress, to either allow plants to tolerate Al accumulation or by excluding Al from enter sensitive regions of the plant root, will be vital to our understanding of Al tolerance. *Arabidopsis* epidermial growth factor-like protein (AtELP) is a vacuolar sorting receptor protein located at the trans-Golgi of *Arabidopsis* root cells (Sanderfoot *et al.* 1998). The role that it plays in Al stress is unclear. However, it may be involved in trafficking proteins to the vacuoles.

Down-regulated gene expression: Transcripts implicated in the remodeling of the cellulose/xyloglucan network in which xyloglucan functions as a major load-bearing cross-links between cellulose microfibrils were identified (Table 3). Enzymes such as expansin, ubiquitin extensin, xyloglucan endotransglycosylase/hydrolase (XTH) showed decrease expression patterns. Inhibition of root elongation is a primary characteristic of Al toxicity (Kochian 1995). Therefore, repressed expression of these genes would be consistent with the decreased in cell wall flexibility observed in response to Al-stress in plants. Expansins are classified as cell wall loosening proteins capable of causing cell wall expansion under acidic conditions (Cosgrove 2000). They function by reversibly disrupting the hydrogen bonding between cellulose microfibrils and xyloglucan resulting in cell wall loosening which allows turgor-driven deformation of cellulose/xyloglucan framework (Sampedro and Cosgrove 2005). Four genes encoding for xyloglucan endotransglycosylase/hydrolases were down-regulated. Xyloglucan endotransglycosylase/hydrolase (XTH) is a member of the GH 16 family and often associated with cell wall metabolism and modification. XTHs are a group of enzymes responsible for the reorganization of the cellulose-xyloglucan framework (Shin *et al.* 2006). XTHs mediate both the splitting and/or reconnection of the xyloglucan chains function in loosening and rearrangement of the cell wall. The repressed expression of these genes can trigger a reduction in cellular growth during Al stress.

Al treatment led to distinct changes in phenylpropanoid and monolignol metabolism. Several genes coding for key enzymes in both pathways where repressed, *e.g.*, phenylalanine ammonia lyase (PAL1 and 2), cinnamoyl-CoA reductase (CCR) and *O*-methyltransferase (OMT). These enzymes could play beneficial roles in detoxifying Al that has entered the symplasm (Hamel *et al.* 1998, Milla *et al.* 2002, Mao *et al.* 2006). In *Oryza sativa* PAL accumulation increased more in the Al-sensitive cultivar than in the Al-tolerant cultivar (Mao *et al*. 2006). Peixoto *et al.* (1999) found that PAL activity in the roots of *Sorghum bicolor* decreased in the presence of Al, suggesting that reduction in the enzyme activity may contribute to lowering lipid peroxidation in membranes and possibly reducing lignification in cell wall. PAL is a well-known defense protein as well that has been shown to accumulate in several different incompatible plant-pathogen combinations and in response to elicitors (Ebel and Cosio 1994). This is the first report indicating PAL mRNA response in *Arabidopsis* upon exposure to Al. This is also the first report indicating that Al exposure affects the expression of cinnamoyl-CoA reductase (CCR) in *Arabidopsis*. This enzyme is the first enzyme of the monolignol-specific part of the lignin biosynthesis pathway. CCR catalyzes the reduction of cinnamic acid CoA esters into their corresponding aldehydes, the first step of the phenylpropanoid pathway specifically dedicated to monolignol biosynthesis. However, the role of this pathway in Al stress is unclear, but the down-regulation of CCR could suggest a decrease in lignin formation. Decreased expression in PAL and CCR might cause decreased biosynthesis of lignin intermediates thus improving root growth (Hossain *et al.* 2005). Various stresses can induce the deposition of lignin in cell walls, such as pathogen infection and wounding. Reduction in lignin deposition can cause the wall to become less rigid and thus more susceptible to injury. Hamel *et al*. (1998) reported that PAL, cysteine proteinase**,** oxalate oxidase and a peroxidase were up-regulated in wheat in proportion to the level of Al, suggesting that these proteins may provide protection against Al by strengthening the cell wall of root cells. However, in our study, PAL was exclusively down-regulated, cysteine proteinase was up-regulated, and both peroxidases (ATPOX58 and AtPOX7) was down-regulated in response to Al treatment suggesting that these genes may have unique roles in Al stress-response not necessarily related to lignification but may play a role inhibiting root growth and cellular elongation (Le Van *et al*. 1994). Šimonovičová *et al.* (2004) reported that, in barley, peroxidases activity and root inhibition increased with increase Al toxicity. Interestingly, the peroxidases expressed in our study belong to the type III (heme-containing) peroxidases, which are not usually considered to be part of the ROS scavenging network and may have more diverse roles including generating active oxygen species (AOS) for cell wall modification (Passardi *et al*. 2005).

Al-responsive aquaporins detected by microarray analysis were almost exclusively repressed (Table 3). Because Al toxicity also triggers drought stress in plants, it was interesting that aquaporins were exclusively down-regulated. Aquaporins are membrane proteins that belong to the major intrinsic protein (MIP) family (Javot and Maurel 2002). MIPs are further divided into two subgroups, the plasma membrane intrinsic proteins (PIPs) and the tonoplast intrinsic proteins (TIPs). Studies have also shown that these genes are highly expressed in elongating cells (Chaumont *et al.* 1998, Ferguson *et al.* 1997). In our study both groups were down-regulated in response to Al. Two different genes encoding tonoplast intrinsic proteins (TIP) had decreased expression in response to Al. In *Secale cereale*, decreased transcript expression of γ-TIP and δ-TIP were reported in response to Al (Milla *et al*. 2002). In *Zea mays*, γ-TIP were highly expressed in the apical meristem and the elongation zone of root tips, consistent with the γ-TIPs permitting the rapid influx of water into the vacuole, generating the pressure potential that drives cell elongation (Chaumont *et al*. 1998). Karlsson *et al.* (2000) suggested that the expression of a δ-TIP gene in *Spinacia oleracea* might be induced during the formation of the large vacuole of elongating cells. Therefore, down-regulation of TIPs in response to Al stress would generate a lower pressure potential in the cell elongation zone, resulting in reduced root growth. Factors leading to the reduced expression of TIPs are unclear, but Smart *et al* (2001), reports that in *Nicotiana glauca* TIPs genes were down-regulated in

response to drought stress and it is possible that Al-induced changes to the cell wall (*e.g*. binding of Al, lignification and callose accumulation) may lead to a reduced permeability and as a result to water stress.

Three subunits of plasma membrane intrinsic proteins (PIPs) (1*A*, 2*B*,*C*) were down-regulated. PIPs are a subfamily of aquaporins that enable fast and controlled translocation of water across the membrane. There is a strong relationship between Al stress and drought stress due in part to the reduction in root growth, a characteristic of Al toxic affects causing plants to respond similarly as to drought stress. The repression to PIPs transcripts, will not only affect the influx of water transport but also nutrient uptake by the roots (Tamas *et al*. 2006).

Validation of microarray results: To validate the reliability of the microarray data generated from the *Arabidopsis ATH1 GeneChip*, five target genes were selected for validation by real-time PCR (Fig. 2). Two of the five Al responsive genes were found to be associated with vacuole function: mulitdrug resistance protein (ABC transporter, *At1g02530*) and AtELP (vasuolar sorting receptor, *At3g52850*). Transcripts for Ras GTPase protein (*At1g493000*) and pEARLI4 (*At5g67600*) (Richards *et al*. 1998) have been reported to be induced within a few hours of Al-stress. CBL1 (*At4g17615*) showed enhanced expression in *Arabidopsis* during drought, temperature, and wounding stresses (Cheong *et al.* 2003). All five genes expression data was consistent for both microarray and PCR (Fig. 1). Most of the genes transcripts expressed in response to Al stress are considered to be general stress related (Ezaki *et al*. 1995, Richards *et al.* 1998, Hamilton *et al.* 2001). The expression analysis of the selected genes indicated that all were induced by exposure to Al, based on *GeneChip* profiles. The results of the *GeneChip* expression data obtained from microarray analysis were consistent with real-time PCR.

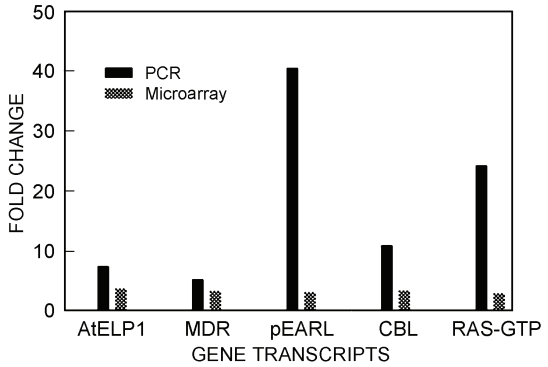

Fig. 2. Quantitative real-time PCR analysis of total RNA from *Arabidopsis* seedlings. Genes representing a variety of functional categories were selected from the microarray dataset for further validation using quantitative RT-PCR. Values used are the mean of at least 2 independent measurements from microarray data and real-time PCR.

Previously documented genes in response to aluminum: To date, over 50 genes have been documented as Al-responsive in a variety of plant species. 54 % of those genes documented were discovered using the model plant *Arabidopsis thaliana* (Table 4). In our study approximately 10 % of responsive genes had previously been identified as Al responsive. Transcript abundance of these genes, as expected, were mostly up-regulated in response to Al, however, three previously reported genes showed differing expression patterns in *Arabidopsis*. Transcripts for leucine-rich repeated protein (Savenstrand *et al.* 2000) and PAL (Snowden and Gardner *et al.* 1993) were reported to be up-regulated in response to Al, both genes showed decreased expression in our study. The gene for alanine aminotransferase was up-regulated in our study while, Richards *et al*. (1998) reported the down-regulation of this gene in *Arabidopsis* in response to Al. Two copies of the gene *pEARLI1*, detected in *Arabidopsis* within 2 h of Al treatment (Richards *et al.* 1998) were down-regulated and a transcript encoding *pEARLI4* was induced in our study following the 16 h Al treatment. This was perhaps due to the differences in time and concentrations of Al exposure. Additionally, down-regulation of reported early Al responsive genes (expression within minutes of Al exposure) would be expected following 16 h Al treatment. Overall, genes representing a wide mix of cellular functions were present indicating that the microarray *GeneChip* is a useful tool for the discovery of Al-regulated genes.

Aluminum accumulation in roots: Analysis of gene transcripts involved in cell wall and vacuolar function showed both up- and down-expression in response to Al stress. The question arises whether it is likely that the cell wall and membrane, which makes up the ultrastructural framework of the cell and the vacuoles as well would show physiological changes during Al exposure. We addressed this question by examining longitudinal sections or root tips using TEM and EDXMA to identify Al content following Al-stress. We found ultrastructural alterations in cell walls of root tip (1.5 mm) cells were observed after Al exposure (Fig. 3*C*). A higher thickening of the cell walls occurred in the internal (third - sixth) cortex cells (Fig. 3*C*) than in the control roots (3*D*). Cell walls in Al-treated samples appeared more translucent in comparison to control root sections following 16-h Al exposure. Electron dense deposits were detected in the vacuoles of internal cortex cells of root tips treated with Al (Fig. 3A,*B*) whereas the control roots showed no deposits (3*A*). Studies have shown that Al accumulation at the cell wall does occur. Silva *et al.* (2000), using Al-sensitive stain, lumogallion, showed that significant amount of Al accumulated in the symplasm of soybean root tips within 30 min of exposure. Using a rare ^{26}Al isotope, Taylor *et al*. (2000), traced the accumulation of Al ion in the cell wall, and vacuoles in single cells of algae (*Chara corallina*). Although the cell wall was the major site of Al

accumulation, within 30 min Al ions could be detected in the vacuoles.

Small amounts of Al were detected in electron-dense precipitates found in the vacuoles of internal cortex cells of 1.5 mm tips of Al-treated plant after the 16-h Al treatment (Fig. 3*A*,*C*). The relative contents of Al and selected minerals (P, S, C, O, K, Na and Si) was found by EDXMA in electron dense deposits of vacuoles of

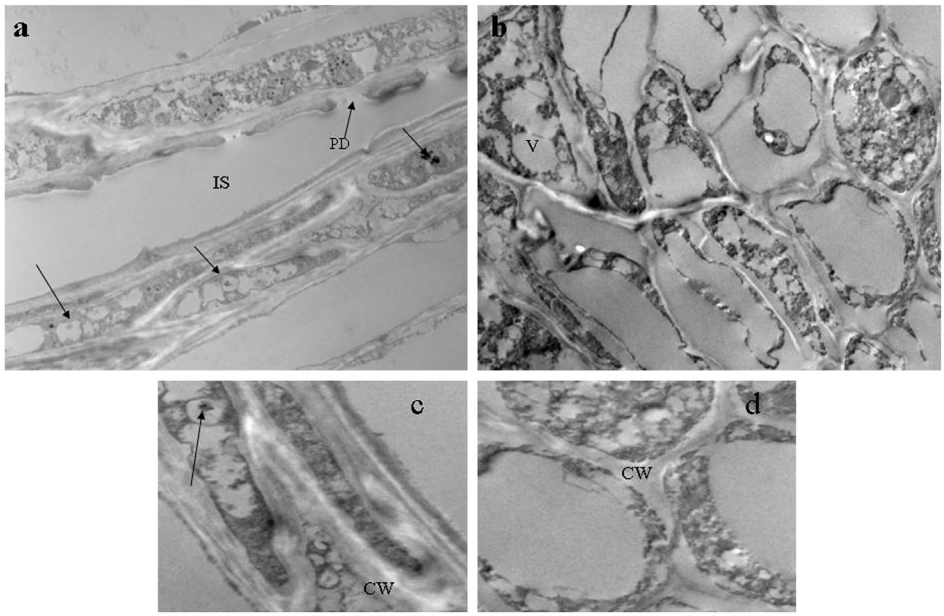

Fig. 3. TEM images from longitudinal sections of root tips of *Arabidopsis* plants exposed for 16 h to 0 μM Al (*b*,*d*) or 50 μM Al (*a*,*c*) in nutrient solution. Samples were fixed with glutaraldehyde prior to TEM. Note cell wall thickening (*a*,*c*) in root samples from Al-treated plants. Note the electron-dense vacuolar deposits (*arrows*) in conventionally fixed samples from plants exposed to Al (*a*,*c*) in comparison with the control (*b*).

Table 4. Previously reported Al-responsive genes in *Arabidopsis.*

Gene	Protein function	Reference	Regulation
PAL111	auxin-regulated protein	Ezaki *et al.* (1995)	up (10 h)
PAL142	glutathion S-transferase	Ezaki *et al.* (1995)	up (10 h)
PAL139	cys-rich domain protein	Ezaki *et al.* (1995)	up (10 h)
PAL141	unknown	Ezaki *et al.* (1995)	up (10 h)
PAL201	anionic peroxidase	Ezaki *et al.* (1996)	up (10 h)
GDI	GDP-dissociation inhibitor	Ezaki *et al.* (1997)	up
pEARLI8	phototropic response transducer	Richards *et al.* (1998)	up/down
pEARLI1	Pro-rich hydrophobic protein	Richards *et al.* (1998)	up/down
pEARLI2	unknown	Richards *et al.* (1998)	up/down
pEARLI4	Pro-rich hydrophilic protein	Richards *et al.* (1998)	up/down
pEARLI5	berberine bridge enzyme	Richards *et al.* (1998)	up (2 h)
ALD	aldolase	Richards *et al.* (1998)	up/down
BCB	blue copper-binding protein	Richards *et al.* (1998)	up (2 h)
PER	peroxidase	Richards *et al.* (1998)	up (1 h)
ALA	Ala aminotransferase	Richards *et al.* (1998)	down (4 h)
CAB	chlorophyll *a*/*b*-binding protein	Richards *et al.* (1998)	down (4 h)
CZSOD	Cu/Zn superoxide dismutase	Richards *et al.* (1998)	up (8 h)
CAT	catalase	Richards *et al.* (1998)	down (2 h)
TaMDR1	multidrug resistence protein	Sasaki *et al.* (2002)	up
WAK1	cell-wall associated Recepror Kinase 1	Sivaguru *et al.* (2003)	up
GST 1	glutathion S-transferase 1	Ezaki *et al.* (2003)	up
GST 11	glutathion S-transferase 11	Ezaki *et al.* (2003)	up
ALS3	ABC Transporter	Larsen *et al.* (2005)	up
ALS1	half-type ABC transporter	Larsen *et al.* (2006)	up
AtALMT1	malate transporter	Hoekenga *et al.* (2006)	up

Al-treated plants (data not shown). The few electron-dense deposits was observed also in the vacuoles of control plants.

The results of both TEM and SEM-EDXMA showed Al accumulattion in the root tip of *Arabidopsis*. These results are consistent with other studies using various methodologies. The results found in this experiment were similar to those obtained by Vazquez *et al*. (1999). Their results showed that Al rapidly (within 4 h) enters cells in *Zea mays* and accumulation increased in the vacuoles. Using TEM with EDXMA, the Vazquez group was able to detect small amounts of Al ions in the vacuoles of internal cortex cells and a significant increase of Al deposits were found after 24 h of Al exposure. Vacuoles are the main storage component of plant cells and there is evidence for vacuolar sequestration of Al ions in plants. In *Fagopyrum esculentum*, an Al-accumulating plant, the intercellular detoxification of Al was achieved by both complexation with oxalate and sequestration into the leaf vacuoles (Shen *et al.* 2002). *Fagopyrum esculentum* was used to investigate Al sequestering as a measure of Al detoxification mechanism. Results of the study showed that Al ions following oxalate complexation, sequestrated in leaf vacuoles (Shen *et al.* 2004). The collective results of these studies indicates that Al is able to penetrated the cell symplasm and accumulate intercellularly within minutes of exposure (Silva *et al*. 2000, Vazquez *et al*. 1999, Lazof *et al.* 1994). Sequestering of Al into the vacuoles can occur within 16 h of treatment and may possibly play a major role in plant ability to tolerate long-term exposure to Al when grown in acidic soils.

Conclusion: The exposure of *Arabidopsis* to toxic Al levels resulted in global changes in gene expression patterns. Our study showed that this microarray technique could be used successfully in the evaluation of global gene expression changes in plant following exposure to Al stress. The first step toward unraveling the complexity of Al resistance mechanism involves understanding at the molecular basis plants responses to Al stress and to identify Al stress-responsive genes whose expression can be manipulated to enable plant growth in acidic soils. Multiple pathway interactions were shown to be induced or repressed by Al exposure, suggesting the connections among several stress-related pathways may have functional implications in the plant survival under Al-stress. Further studies are needed to understand the transcriptional regulation of these Al stress-responsive.

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