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Transcriptional profiling of aluminum toxicity and tolerance responses in maize roots

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Summary

• Aluminum (Al) toxicity is a major factor limiting crop yields on acid soils. There is considerable genotypic variation for Al tolerance in most common plant species. In maize (*Zea mays*), Al tolerance is a complex phenomenon involving multiple genes and physiological mechanisms yet uncharacterized.

• To begin elucidating the molecular basis of maize Al toxicity and tolerance, a detailed temporal analysis of root gene expression under Al stress was performed using microarrays with Al-tolerant and Al-sensitive genotypes.

• Al altered the expression of significantly more genes in the Al-sensitive genotype, presumably as a result of more severe Al toxicity. Nevertheless, several Al-regulated genes exhibited higher expression in the Al-tolerant genotype. Cell wall-related genes, as well as low phosphate-responsive genes, were found to be regulated by Al. In addition, the expression patterns of genes related to Al-activated citrate release indicated that in maize this mechanism is probably regulated by the expression level and/or function of the citrate transporter.

• This study is the first comprehensive survey of global transcriptional regulation under Al stress. The results described here will help to further our understanding of how mechanisms of Al toxicity and tolerance in maize are regulated at the transcriptional level.

Key words: aluminum (Al), cell wall, gene expression, maize (*Zea mays*), microarrays, organic acids, tolerance, toxicity.

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Introduction

Aluminum (Al) toxicity is the primary factor limiting crop production on strongly acidic soils. At soil pH values at or below 5, toxic forms of Al are solubilized into the soil solution, inhibiting root growth and function and consequently reducing crop yields. In most plant species there is considerable genotypic variation for the ability to withstand Al toxicity. Plants have evolved different mechanisms to overcome Al stress, either by preventing Al^{3+} from entering the root ('exclusion' mechanisms) or by being able to neutralize toxic Al^{3+} absorbed by the root system (true 'tolerance' mechanisms). The basis of these mechanisms has been the focus of intense research (reviewed by Kochian *et al.*, 2004). Nevertheless, so far the only well-documented mechanism of Al resistance is the exclusion of Al from the root tip based on the release of organic acids, which chelate Al^{3+} forming stable, nontoxic complexes. Release of malate, citrate and/or oxalate from roots upon exposure to Al has been correlated with differential Al tolerance in a large number of monocot and dicot species. In maize (*Zea mays*),

although Al tolerance is strongly associated with high rates of root citrate release (Pellet *et al.*, 1995; Jorge & Arruda, 1997; Piñeros *et al.*, 2002; Mariano & Keltjens, 2003), it appears that, in contrast to other species, Al tolerance in maize is a rather complex phenomenon involving multiple genes and probably multiple physiological mechanisms. For example, Piñeros *et al.* (2005) observed a clear correlation between root tip Al exclusion and Al tolerance across a panel of six maize genotypes. However, Al-activated root citrate release was not as well correlated with Al tolerance, suggesting that, although Al-activated root citrate release plays an important role, it is likely that other tolerance mechanisms are also operating in maize roots. These physiological observations are supported by several genetic studies that have described maize Al tolerance as a quantitative trait, subject to additive gene effects (Magnavaca *et al.*, 1987; Pandey *et al.*, 1994; Borrero *et al.*, 1995). A recent study using quantitative trait locus (QTL) mapping identified five distinct genomic regions with importance for Al tolerance in maize (Ninamango-Cárdenas *et al.*, 2003).

Temporal changes in gene expression are major determinants of normal metabolic and physiological processes, and are also the primary mediators of altered cellular properties that define various stress- and disease-related states (Jiang *et al.*, 2000). The identification of genes and the determination of their expression patterns in response to stress should improve our understanding of their functions and provide the basis for effective strategies to improve stress adaptation. In particular, a number of genes have been shown to be differentially regulated by Al stress in different plant species (Ezaki *et al.*, 1995; Hamel *et al.*, 1998; Richards *et al.*, 1998; Mao *et al.*, 2004). However, genes identified as Al-responsive so far have turned out to be mostly related to a general stress response resulting from the toxic effects of Al, and are unlikely to play a significant role in Al tolerance (Kochian *et al.*, 2004). The use of more sensitive and high-throughput gene expression profiling techniques and their application in comparative studies will be crucial to reveal the role of differential gene regulation in Al toxicity and tolerance. Here, we present a detailed comparative investigation of the changes in gene expression that take place in roots of an Al-tolerant and an Al-sensitive genotype of maize under short-term Al stress using microarrays. The present work substantially extends our current knowledge of transcriptional regulation by Al stress in maize roots, and will provide valuable insights into which aspects of Al toxicity and tolerance should be the focus of further investigation.

Materials and Methods

Plant material and plant growth

Seeds from the tropical maize (*Zea mays* L.) inbred lines C100-6 and L53 were provided by Centro de Biologia Molecular e Engenharia Genética (University of Campinas, Campinas, Brazil) and by EMBRAPA Maize and Sorghum Research Center (Sete Lagoas, Brazil), respectively. Seeds were germinated for 3 d, and then seedlings were transferred to a full nutrient solution as previously described (Magnavaca *et al.*, 1987; Piñeros *et al.*, 2002). The pH of the nutrient solution was adjusted to 4.0 with HCl. Plants were grown in a growth chamber at 26°C:24°C (light:dark, 16:8 h). Aluminum treatment of 4-d-old seedlings was initiated after a 24-h adaptation period by replacing the nutrient solution with the same solution containing the concentration of Al indicated in the text, supplied as $KAl(SO_4)_2$. Free Al^{3+} activities were calculated using GEOCHEM-PC speciation software (Parker *et al.*, 1995).

Root growth measurements

Root measurements after 2 and 6 h of exposure to Al were obtained using high-resolution digital photography; measurements at 24 h were obtained manually with a ruler. For the 2- and 6-h time-points, each root was photographed with a calibration grid in the background, fixed on the bottom of 150-mm Petri dishes filled with nutrient solution. The digital camera (Nikon/Kodak Professional DCS 760; Kodak, Rochester, NY, USA) was placed at a fixed height above the dish. Digital images were converted to TIFF format and processed using ImageJ software (http://rsb.info.nih.gov/ij/). Measured lengths were imported into Microsoft Excel for data analysis. Root growth (RG) was calculated as the length (L) at a given time-point minus the initial length (e.g. $RG_{2h} = L_{2h} - L_{0h}$). Relative root growth (RRG) was calculated as the mean of the RG in Al-treated plants divided by the mean of the RG in control (–Al) plants ($RRG_{2h} = RG_{2h\ (Al)}/RG_{2h\ (control)}$).

Root organic acid exudation

Whole-root organic acid exudation measurements were performed as previously described (Piñeros *et al.*, 2002, 2005), with some modifications. In order to examine root organic acid release under short-term Al stress, the plants were not pre-exposed to Al before the collection of root exudates. Instead, after a 24-h growth period in full nutrient solution, roots were rinsed with deionized water and placed in 4.3 mM $CaCl_2$ (pH 4.5). Plants were allowed to acclimate to the new medium for 4 h. The solution was then replaced with the same medium containing 0 (control) or 140 μM $AlCl_3$ (39 μM Al^{3+} activity). Root exudates were collected after 2, 6 and 24 h. Samples were passed through an OnGuard-Ag chromatography column to remove excess Cl^- (Dionex, Sunnyvale, CA, USA). Excess Al^{3+} was removed using cationic exchange resin (Dowex 50W X 8, H^+ form; Fluka Chemie, Seelze, Germany). Samples were then lyophilized and resuspended in deionized water. Organic acids in root exudates were analyzed with a capillary electrophoresis system (P/ACE 5510; Beckman Instruments, Fullerton, CA, USA) as described previously (Piñeros *et al.*, 2002). The identity of the peaks was confirmed by spiking samples with organic acid standards.

Root tip Al content

Al content was measured from the same roots used for organic acid exudation analysis. Roots were rinsed in deionized water to remove the nutrient solution, and the first centimeter of each root was collected. Root samples were dried at 55°C, and dry weights were determined using an MT2 microgram balance (Mettler, Greifensee, Switzerland). Samples were digested with 70% perchloric acid, resuspended in 0.5% nitric acid and analyzed using an inductively coupled plasma mass spectrometer (Agilent ICP-MS 7500cs; Agilent Technologies, Wilmington, DE, USA).

RNA isolation and target preparation

Total RNA was isolated from roots (first 2 cm) using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA yield and purity were determined spectrophotometrically and verified by electrophoresis in 3-(N-Morpholino)-propanesulfonic acid (MOPS)/formaldehyde agarose gels. First-strand cDNA synthesis was carried out using Superscript III (Invitrogen), followed by labeling with Cy3/Cy5 (Amersham Biosciences, Piscataway, NJ, USA) via the aminoallyl method (Hegde *et al.*, 2000).

Microarray hybridization

The maize microarrays were provided by the Maize Oligonucleotide Array Project as part of a beta-testing study (www.maizearray.org). The array platform used (version 1.3) contained approx. 58 000 spotted 70-mer oligonucleotide probes. Probe rehydration and immobilization were performed as recommended in www.maizearray.org/maize_protocols.shtml, using a Stratalinker (Stratagene, La Jolla, CA, USA). Hybridization and washes were performed according to Hegde *et al.* (2000). Slides were scanned in a ScanArray Express microarray scanner (PerkinElmer, Boston, MA, USA) and images were processed using ScanArray Express software. Signal intensity measurements are available in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (accession number GSE10308).

Experimental design and statistical analysis

The experimental design consisted of two interconnected loops (Supplementary Material Fig. S1), and four independent biological replicates were utilized. For both genotypes, each time-point was contrasted with the previous and subsequent time-points in a loop. Additionally, the loops were interconnected so that the two genotypes were contrasted directly at each time-point. The design was balanced for dye distribution throughout samples and biological replicates. Raw signal intensity values were $\log_2$-transformed and analyzed using two interconnected ANOVA mixed models (Jin *et al.*, 2001; Wolfinger *et al.*, 2001) via PROC MIXED in SAS (SAS Institute, Cary, NC, USA). The normalization model $y_{ij} = \mu + A_i + D_j + (A \times D)_{ij} + \varepsilon_{ij}$ was applied to account for experiment-wide sources of variation associated with array (A_i, random effect), dye (D_j, fixed effect), and their interactions. The residuals (ε_{ij}) were treated as normalized values and analyzed using the following ANOVA (gene model), where effects were evaluated for each gene individually: $r_{ikl} = \mu + A_i + G_k + T_l + (G \times T)_{kl} + \varepsilon_{kl}$. G_k represents the kth genotype (i.e. C100-6 or L53), T_l represents the lth treatment (i.e. 0, 2, 6 or 24 h of Al treatment), and $(G \times T)_{kl}$ represents the interactions between genotype and treatment. Array (A_i) was included as a random effect to control for spot effects (Jin *et al.*, 2001). Least-square means were generated, and estimates of differential expression were calculated as the difference between least-square means for each of the terms in the model. False discovery rate (FDR) was applied to control for Type I errors (Benjamini & Hochberg, 1995). Q values were calculated from P values using the R-based software QVALUE (Storey & Tibshirani, 2003). FDR was set to 0.15.

Quantitative real-time PCR

First-strand cDNA was synthesized from total RNA collected from an independent biological replicate using SuperScript III reverse transcriptase (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed with an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR green I PCR master mix (Applied Biosystems). Primers were designed using PRIMER EXPRESS 2.0 software (Applied Biosystems) and are listed in Supplementary Material Table S4. The maize 18S rRNA was used as an endogenous control. Relative expression levels were calculated by the comparative C_T method. For each gene, expression values were normalized against sample C100-6 (time zero), which was set to 1. Each data point is the average ± SD of three independent replicates.

Results and Discussion

Al tolerance of maize genotypes used in the study

Most studies to date examined Al tolerance in maize over long periods of exposure, documenting root growth inhibition after 24–96 h (Jorge & Arruda, 1997; Piñeros *et al.*, 2005). These studies demonstrated that in many instances root growth of Al-sensitive genotypes is almost entirely abolished after 24 h of Al treatment. Because physiological and metabolic parameters measured under these long treatment periods will probably be distorted by the severe toxicity effects of Al, we chose to compare the genotypes used in this study under short-term Al stress. For this purpose, root growth was monitored after 2, 6 and 24 h of exposure to 39 μM Al^{3+} activity (Fig. 1a). A small but significant difference in relative

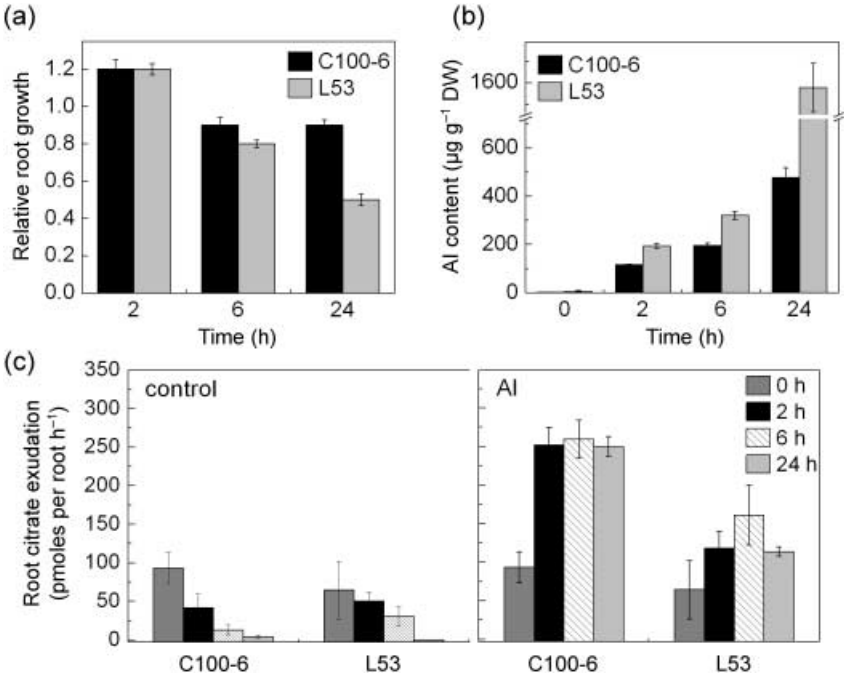

Fig. 1 Physiological characterization of maize genotypes C100-6 (aluminum (Al)-tolerant) and L53 (Al-sensitive). (a) Al tolerance as determined by relative root growth monitored after 2, 6 and 24 h of treatment with 39 μM Al^{3+} activity. Means and standard errors were calculated from 10 replicates per treatment. (b) Root tip Al content determined after 0, 2, 6 and 24 h of treatment with 39 μM Al^{3+} activity. Means and standard errors were calculated from five replicate measurements (each from a pool of eight root tips). (c) Citrate exudation rates from whole roots were measured after 0, 2, 6 and 24 h of exposure to 0 (control) or 39 μM Al^{3+} activity. Means and standard errors were calculated from five replicate measurements (each from a pool of five roots).

root growth was observed between the Al-tolerant (C100-6) and Al-sensitive (L53) genotypes as early as 6 h after exposure to Al. After 24 h this difference increased significantly (50% relative root growth in L53 versus 90% in C100-6).

To investigate whether the difference in Al tolerance observed between the two genotypes correlated with the levels of Al accumulated in the roots, we examined root tip Al content in plants subjected to 39 μM Al^{3+} activity for 2, 6 and 24 h (Fig. 1b). The Al-tolerant genotype C100-6 accumulated significantly less Al in the root tip than the Al-sensitive L53. A clear differentiation between the tolerant and sensitive genotypes on the basis of root tip Al accumulation could be observed as early as 2 h after Al exposure; this difference increased continuously after 6 and 24 h.

Because the Al-sensitive genotype accumulated significantly more Al in the root tip than the Al-tolerant genotype, it is likely that a mechanism of Al exclusion is operating in roots of the Al-tolerant genotype. Therefore, we also examined the rates of citrate release from intact roots of plants exposed to short-term Al stress (Fig. 1c). Exposure to Al resulted in an increase in the rates of root citrate release in both genotypes when compared with the control. In plants grown in Al-containing medium, citrate exudation rates exhibited a significant increase at the earliest Al exposure time (2 h) and remained relatively constant for the remainder of the treatment. The rates of Al-activated citrate release in C100-6 roots were nearly twofold higher than those of L53. Low rates of root citrate release were detected at the 0-h time-point in plants grown in control (–Al) medium, which then decreased over time in both genotypes. This observation is probably a result of handling of the plants and/or the change in medium, as in order to perform a short-term, time-course analysis of Al-activated organic acid release the plants could not be acclimated to the Al-containing medium before root exudate collection (see the Materials and Methods). Nonetheless, Al-induced citrate exudation rates were significantly higher than those caused by the change in medium (in particular in the Al-tolerant genotype), and correlated well with citrate exudation rates reported in other studies (Piñeros *et al.*, 2005).

Microarray experiment: analysis of the estimated effects

The gene model applied to the microarray data yielded estimates of the effects of genotype (G_k), treatment (T_l) and genotype × treatment interactions ($(G \times T)_{kl}$) on differential gene expression. To illustrate the contribution of each effect to the overall variation, the estimates generated for each effect were plotted against their corresponding Q values (i.e. P values corrected for FDR; see the Materials and Methods) in volcano plots (Fig. 2a). Genotype effects displayed a wide distribution of estimates with very low Q values, that is, high significance levels (as shown by the large number of data points above the FDR threshold). This indicates that large differences in constitutive gene expression exist between the two genotypes regardless of the treatment, a likely consequence of their divergent genetic backgrounds. By contrast, treatment effects showed a much smaller number of significant estimates, indicating that, when averaged across the two genotypes, only a small set of genes showed significant differential expression

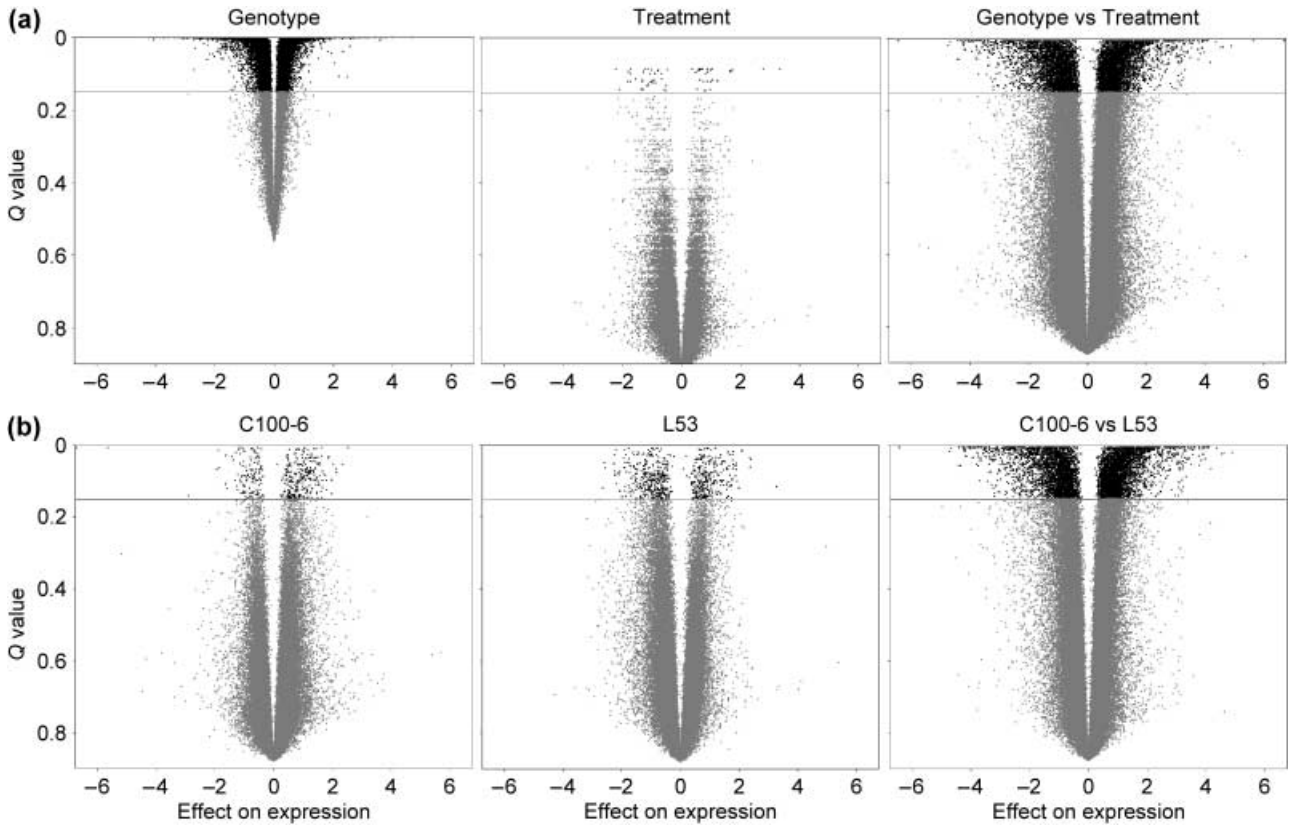

Fig. 2 Volcano plots. Estimates of differential expression were calculated as the difference between least-square means for each indicated effect (*x*-axis: effect on expression), and were plotted against the corresponding *Q* values (*y*-axis). (a) Distribution of each effect considered in the gene model (see the Materials and Methods). (b) Distribution of each component of the genotype vs treatment effects. An estimate equal to zero means no change in gene expression, whereas estimates away from zero indicate differential expression. The horizontal line represents a 15% false discovery rate. Data points marked in black (above the horizontal line) represent significant observations (*Q* value ≤ 0.15).

attributable to the Al treatments. A much larger number of significant estimates (i.e. with low *Q* values) were observed in the genotype × treatment interaction effects in comparison to those observed in treatment effects alone. In order to further dissect this observation, we subdivided the genotype × treatment effects into three volcano plots (Fig. 2b): 'within genotypes' comparisons (up- or down-regulation of genes within each genotype) and 'between genotypes' comparisons (genes differentially expressed between C100-6 and L53 at any given time-point). A substantially larger number of genes showed significant differential expression in response to Al when each genotype was looked at individually (Fig. 2b; left and central panels for C100-6 and L53, respectively) than when these responses were averaged across the two genotypes (see treatment effects above: Fig. 2a, central panel). This indicates that a somewhat different set of genes is being affected by Al in each genotype and that the experimental conditions used in this study were efficient in isolating genotype-specific responses to the treatment. Finally, the genotype × treatment estimates 'between genotypes' (Fig. 2b; right panel) displayed a wide distribution of data points showing high significance, indicating that large differences in gene expression between the two genotypes also exist when looking at each time-point individually. The same observation was made when looking at the average across treatments (Fig. 2a), suggesting that the large inherent differences in gene expression observed between the genotypes are not greatly affected by the Al treatments.

Validation of microarray results by quantitative real-time PCR

Microarray data were independently verified by qRT-PCR. Nineteen expressed sequence tags (ESTs) from various functional categories and displaying diverse expression profiles were chosen among all differentially regulated genes. Fig. 3 displays a linear regression analysis comparing the microarray and qRT-PCR results. A significant correlation was observed between the two data sets, with an R^2 of 0.7174. Expression differences measured by qRT-PCR appeared to be greater than those measured by microarray for most of the genes tested, causing the regression line to intercept the *y*-axis at values higher than zero (Fig. 3). This tendency results from

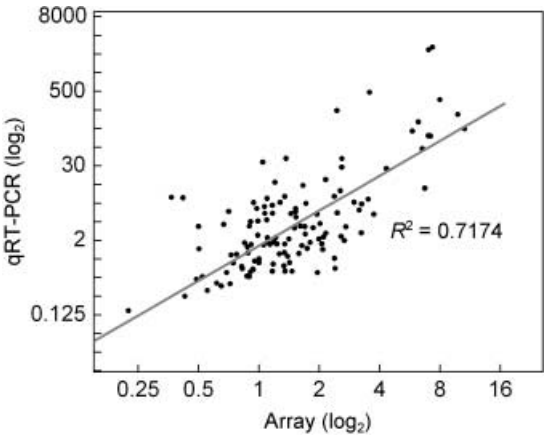

Fig. 3 Validation of microarray results via quantitative real-time PCR (qRT-PCR). Quantitative real-time PCR analysis was performed for 19 genes in both genotypes, under the same conditions used for microarray analysis (0, 2, 6 and 24 h of treatment with 39 μM Al^{3+} activity). Microarray data (least-square means) were plotted against data from qRT-PCR and fit into a linear regression. Both *x*- and *y*-axes are shown in $\log_2$ scale.

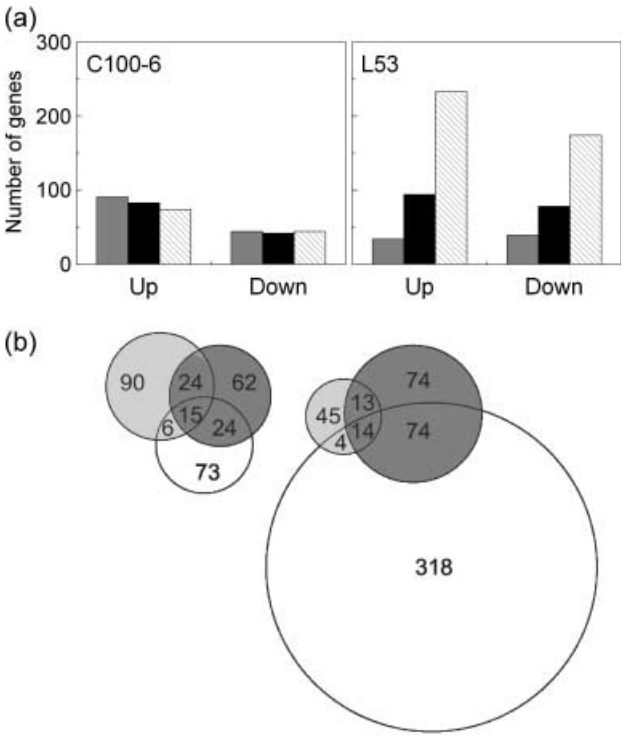

Fig. 4 Genes differentially regulated in roots of C100-6 and L53 under aluminum (Al) stress. (a) Number of genes up- and down-regulated by Al after 2 (gray bars), 6 (black bars) and 24 h (hatched bars) of treatment with 39 μM Al^{3+} activity. (b) Venn diagrams illustrating the genes differentially regulated by Al in C100-6 (left) and L53 (right) roots. The 2-h time-point, light gray circles; 6-h time-point, dark gray circles; 24-h time-point, white circles.

the fact that qRT-PCR has a much wider dynamic range than microarrays (Wang *et al.*, 2006), and is a feature that has also been observed in other studies (Kolotilin *et al.*, 2007).

Genes differentially expressed under Al stress

The number of genes up- and down-regulated by Al at different time-points in C100-6 and L53 roots is displayed in Fig. 4. In C100-6 (Al-tolerant), the number of genes up- and down-regulated by Al remained nearly constant over the course of the treatment, while in L53 (Al-sensitive) it increased dramatically over time (Fig. 4a). A similar, although not as striking, trend can be observed in the case of down-regulated genes. In addition, the number of genes up-regulated early in the treatment (2 h) was substantially larger in C100-6 than in L53. This difference could possibly be attributable to the early activation, in the Al-tolerant genotype C100-6, of genes underlying tolerance mechanisms upon exposure to Al. After 24 h of Al exposure C100-6 plants show practically no inhibition of root growth, while Al-sensitive L53 roots show greatly inhibited growth, as well as visible signs of damage. The large number of differentially regulated genes in L53 roots after 24 h of Al treatment is likely to result from strong toxicity caused by Al, a hypothesis reinforced by the analysis of the biological processes influenced by Al stress in L53 roots (Fig. 5). Genes up-regulated (Fig. 5a) and down-regulated (Fig. 5b) in L53 roots after 24 h of Al treatment were assigned functional categories based on gene ontology. Nearly half (49%) of the up-regulated and 34% of the down-regulated genes could not be assigned to any functional category. Analysis of the genes with assigned functional categories revealed the up-regulation of several metabolic processes linked to energy generation. Genes involved in proteolytic pathways were up-regulated by Al, as were genes encoding DNA repair and heat shock proteins, along with other genes involved in general stress and defense responses. Several transcription factors were up-regulated, which is consistent with a large number of genes undergoing transcriptional regulation in response to the stress. A large number of genes involved in transcription and translation displayed down-regulation, including several ribosomal proteins (Fig. 5b). The nature of the biological processes down-regulated after 24 h of Al treatment suggests an inhibition of cell growth and division by Al in roots of the Al-sensitive genotype L53.

The complete list of genes differentially regulated by Al in each genotype can be found in Supplementary Table S1. In an attempt to identify genes potentially involved in the differential Al tolerance observed between the two genotypes, we focused our attention on genes that displayed different patterns of expression in response to Al between the Al-tolerant and Al-sensitive genotypes.

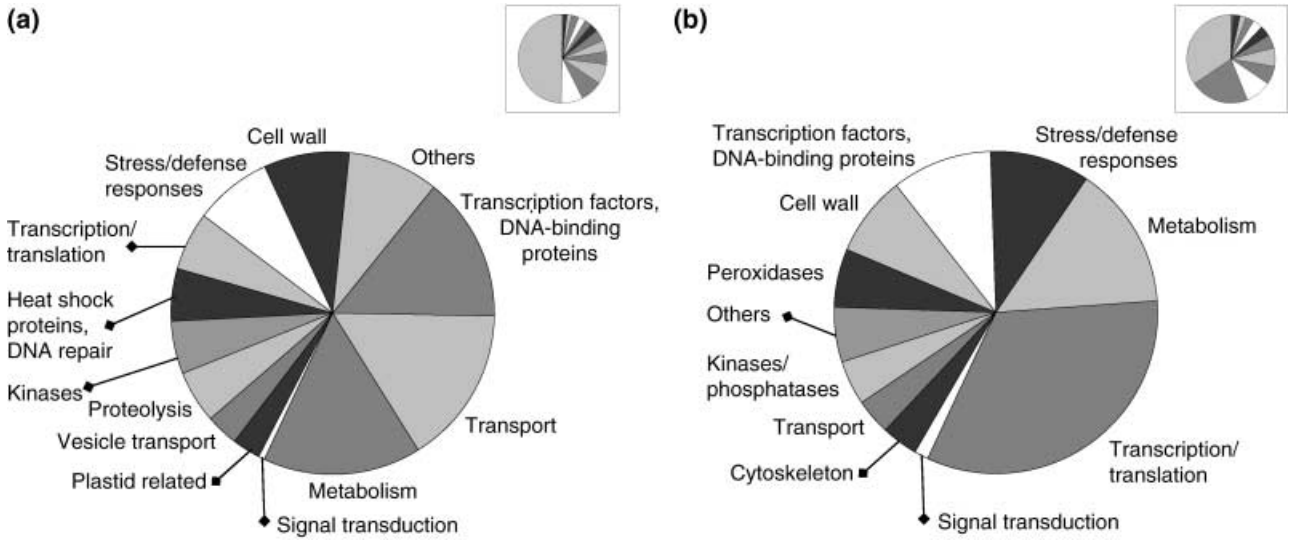

Fig. 5 Functional classification of genes (a) up-regulated and (b) down-regulated by aluminum (Al) in L53 (Al-sensitive) roots after 24 h of treatment with 39 μM Al^{3+} activity. For clarity, the genes classified as function 'unknown' were not included in the pie charts. As a reference, the insets on the right of each panel display the same pie charts including the genes with unknown function.

Cell wall-related genes

A number of studies have suggested a role for the cell wall both as a primary target for Al toxicity and as a possible site for Al tolerance mechanisms. Al binds rapidly to the cell wall, and as much as 90% of the Al absorbed in root tissues can be localized to the apoplast (Kochian, 1995). Evidence of a role for cell wall polysaccharides in Al exclusion is starting to emerge in different plant species (Eticha *et al.*, 2005; Yang *et al.*, 2008). We identified several genes implicated in cell wall structure and composition exhibiting differential expression under Al stress. Pectin methylesterase (PME), the enzyme responsible for the demethylation of pectin in the apoplast, was up-regulated in both genotypes by 2 h of treatment. PME expression was significantly higher and its up-regulation was more pronounced in the Al-sensitive genotype L53 (Fig. 6a).

Methyl esterification of the pectin carboxylic groups controls the negative charge it carries. The amount of pectin and its degree of methylation largely determine the cation exchange capacity (CEC) of the cell wall, which in turn determines how tightly Al^{3+} binds within the cell wall. Higher levels of apoplastic PME, leading to a higher degree of pectin de-methylesterification, might result in a higher capacity to accumulate Al^{3+} in the wall. Therefore, it is possible that the higher levels of PME expression observed in L53 roots may contribute to its increased sensitivity to Al stress. In fact, differences in pectin content and degree of methylation have been linked to genotypic differences in Al tolerance in maize (Eticha *et al.*, 2005). Recent work by Yang *et al.* (2008) in rice (*Oryza sativa*), which is one of the most Al-tolerant cereals and which shows no apparent correlation between root organic acid release and Al tolerance, also supports this hypothesis. When comparing two rice cultivars with contrasting Al tolerance, the authors demonstrated that root tips of the Al-sensitive cultivar had higher PME activity and a higher degree of pectin de-methylesterification, and that Al was bound more tightly to the cell walls when compared with the Al-tolerant cultivar.

Another class of cell wall-related enzymes up-regulated by Al to a greater degree in roots of the Al-sensitive genotype were laccases, or diphenol oxidases (Fig. 6b,c). In plants, laccases are thought to be involved in lignin biosynthesis, because of their capacity to oxidize lignin precursors and their localization in lignifying cell walls, although their role in lignification is still controversial (Gavnholt & Larsen, 2002). Other genes involved in lignin biosynthesis have also been shown to be up-regulated by Al (Mao *et al.*, 2004). In wheat (*Triticum aestivum*), root growth inhibition by Al has been correlated with the amount of lignin deposited in root tips (Sasaki *et al.*, 1996). These results suggest that lignin deposition may also play a role in Al toxicity as a potential cause of root growth inhibition.

Genes encoding oxalate oxidases (OXOs) and peroxidases were also transcriptionally regulated under Al stress. Oxalate oxidases (or germins) catalyze the conversion of oxalate and O_2 to CO_2 and H_2O_2. Extracellular OXOs are thought to participate in cell wall remodeling related to developmental processes and stress responses (Bernier & Berna, 2001). Expression of OXOs was constitutively higher and was up-regulated by Al only in roots of the Al-tolerant genotype C100-6 (Fig. 6d,e). Several peroxidases also exhibited differential expression under Al stress; however, their patterns of expression varied considerably. Some peroxidases appeared to be up-regulated exclusively in the Al-tolerant genotype, while

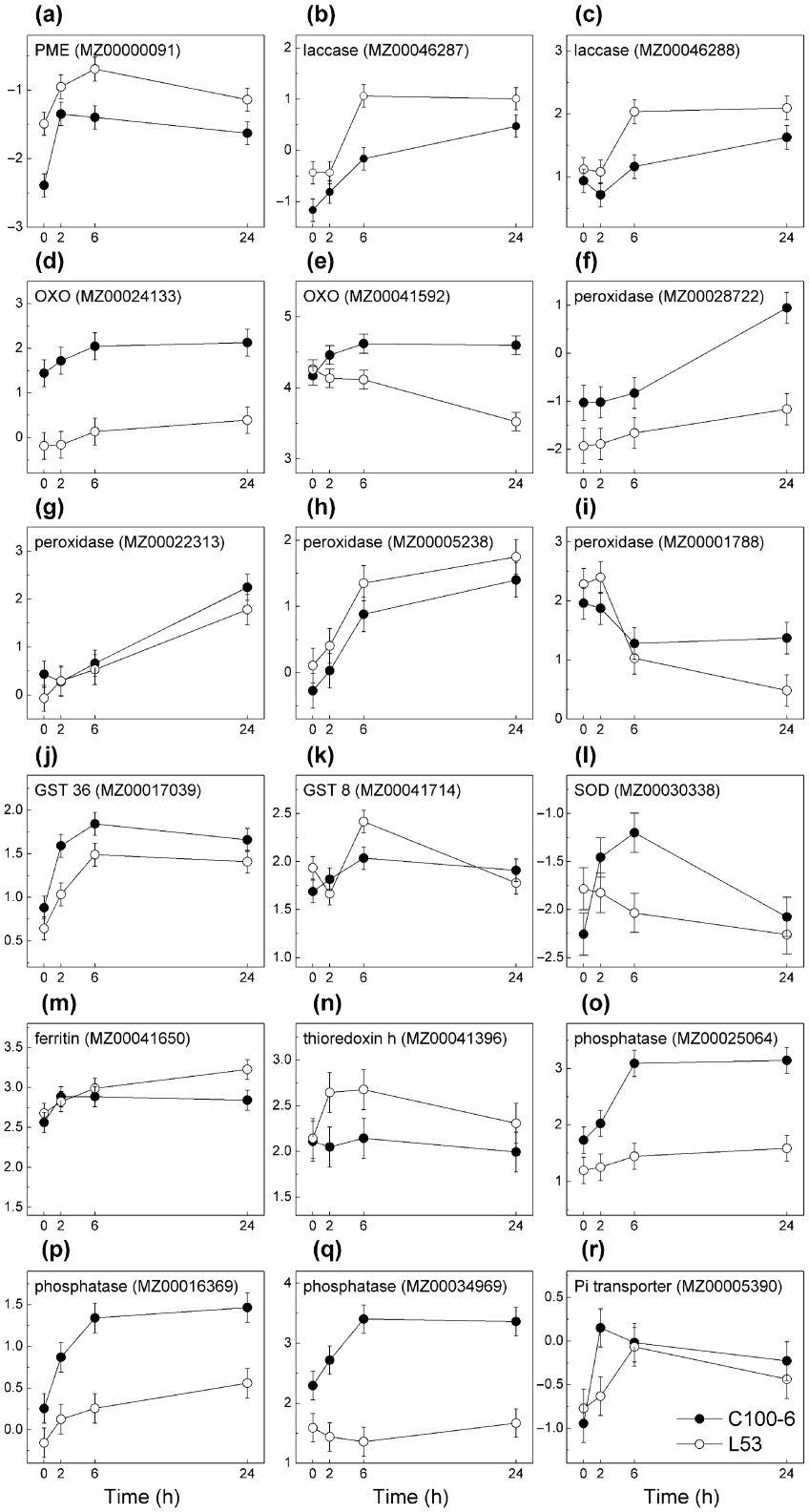

Fig. 6 Expression profiles of selected aluminum (Al)-regulated genes determined by microarray analysis. Gene expression levels are represented by their estimated least-square means (*y*-axis) in genotypes C100-6 (closed circles) and L53 (open circles). Note that the *y*-axis scale is not the same across the plots. Expression profiles of the genes shown in (d), (g), (j), (k), (o) and (q) were confirmed by quantitative real-time PCR (qRT-PCR). GST, glutathione S-transferase; OXO, oxalate oxidase; Pi, phosphate; SOD, superoxide dismutase.

others were up-regulated in both genotypes, and others were down-regulated under Al stress (Fig. 6f–i).

Peroxidases catalyze the reduction of H_2O_2 by transferring electrons from several different donor molecules, and in rice are encoded by a multigene family of at least 138 predicted members (Passardi *et al.*, 2004). The diversity of reactions catalyzed by plant peroxidases is indicative of their implication in a wide range of physiological processes such as auxin metabolism, lignin formation, cross-linking of cell wall components, defense against pathogens and cell elongation. Perhaps due in part to their diversity of roles, peroxidases have also been implicated in mechanisms of both Al toxicity and Al tolerance. Therefore, it is not surprising that the expression patterns of peroxidases in response to Al stress were also so diverse.

Peroxidases have a well-established role in cross-linking of cell wall components to reduce wall extensibility, which has been correlated with Al toxicity. Al stress has been shown to lead to increased amounts of wall-bound ferulic and diferulic acids in wheat roots (Tabuchi & Matsumoto, 2001). Peroxidase-catalyzed cross-linking of arabinoxylans by diferulic acid bridges is believed to reduce cell wall extensibility, and therefore could be a primary cause of inhibition of root elongation. However, peroxidase-driven cross-linking has also been suggested as a potential mechanism of Al tolerance. Apoplastic oxalate oxidases are believed to generate locally the H_2O_2 required for these reactions (Lane, 1994), and their expression is up-regulated in response to fungal infection leading to the lignification of infectious sites through peroxidative cross-linking (Dumas *et al.*, 1995). Delisle *et al.* (2001) suggested that OXOs and peroxidases may function together in a similar fashion to reduce cell wall porosity in response to Al in wheat. Our results show that OXO and peroxidases can be coordinately up-regulated by Al in C100-6 roots, indicating that a similar mechanism could potentially be operating in this maize genotype.

Oxidative stress-responsive genes

Al stress has been shown to elicit the production of reactive oxygen species (Yamamoto *et al.*, 2002), although the possible role of oxidative stress in Al toxicity is not clear. The up-regulation by Al of oxidative stress-responsive genes has been shown in a number of plant species, including maize (Cançado *et al.*, 2005), Arabidopsis (Richards *et al.*, 1998), tobacco (*Nicotiana tabacum*; Ezaki *et al.*, 1995) and rye (*Secale cereale*; Milla *et al.*, 2002). In this study, a number of genes related to oxidative stress were shown to be up-regulated by Al stress. Genes encoding glutathione S-transferases (GSTs) showed up-regulation by Al either in both genotypes or transiently only in L53 (Fig. 6j,k). A gene encoding superoxide dismutase (SOD) appeared also to be transiently up-regulated only in roots of C100-6 (Fig. 6l). In addition, two oxidative stress-related genes not previously shown to be Al-inducible were up-regulated in the Al-sensitive genotype. Expression of ferritin 1, believed to play a role in protecting cells from oxidative damage, increased steadily over the course of the treatment in L53 roots (Fig. 6m). Furthermore, expression of a gene encoding a cytoplasmic thioredoxin *h* was strongly up-regulated in L53 roots after 2 h of Al treatment (Fig. 6n). Thioredoxins are small ubiquitous proteins involved in cellular redox regulation, although the function of cytoplasmic thioredoxins is still not clear.

It is important to note that a larger number of oxidative stress-related genes were up-regulated by Al in the Al-sensitive genotype L53 than in the Al-tolerant C100-6. This suggests that rather than a component of the Al tolerance response, the up-regulation of oxidative stress-responsive genes appears to be merely a consequence of Al toxicity. Hence, root cells from the Al-tolerant genotype are probably suffering less oxidative damage than those of the Al-sensitive genotype. This suggests that the Al tolerance response of C100-6 acts before the onset of oxidative damage, a supposition that correlates well with a tolerance mechanism(s) based on Al exclusion from the root tip.

Low phosphate status-responsive genes

In conjunction with Al toxicity, low phosphate (Pi) availability is another major limiting factor to plant growth on acid soils, particularly because of its fixation with Al and iron (Fe) oxides on the surface of clay minerals. Plants have evolved a number of adaptive mechanisms to acquire Pi from the soil, including changes in root architecture, association with mycorrhizas, root exudation of compounds that can increase phosphorus (P) availability in the soil, and up-regulation of Pi transporters (Kochian *et al.*, 2004). In the present study, plants were submitted to Al stress under conditions of sufficient Pi availability (see Piñeros *et al.*, 2002 for composition of the nutrient solution). According to chemical speciation calculations, enough PO_4 was supplied so that, upon addition of Al to the medium, the plants would still have a substantial amount of available P and would not undergo P deficiency (results not shown). Nevertheless, we observed up-regulation of genes known to be responsive to Pi starvation under these conditions. Three genes encoding purple acid phosphatases were strongly up-regulated by Al exclusively in C100-6 (Fig. 6o–q). The synthesis and exudation of acid phosphatases, which presumably catalyze the hydrolysis of P fixed in organic compounds in the soil, are universal plant responses to P deficiency. In addition, a high-affinity Pi transporter was transiently up-regulated by Al stress in both genotypes (Fig. 6r). This transient up-regulation occurred earlier (2 h) and was significantly more pronounced in the Al-tolerant genotype C100-6.

The immobilization of Al by phosphate in the root has been suggested as a potential mechanism of plant Al tolerance. Vázquez *et al.* (1999) observed the accumulation of Al and P in the vacuoles of root tip cells of an Al-tolerant maize variety under Al stress, and proposed that the compartmentation of

Al-P complexes in the vacuole might serve as an internal mechanism of Al detoxification. It has also been suggested that the immobilization of Al via formation of insoluble Al-P precipitates at the root surface or in the root tissue may contribute to Al tolerance (Gaume *et al.*, 2001). Evidence of the role of Al immobilization by P was also recently reported in the highly Al-tolerant species buckwheat (*Fagopyrum esculentum*; Zheng *et al.*, 2005).

Results from an analysis of root tip mineral content indicated a substantial increase in P content in root tips of C100-6 under Al exposure, which was not as pronounced in L53 root tips (results not shown). These results suggest that Al immobilization by P may in fact be taking place in roots of the Al-tolerant genotype, at least under conditions in which sufficient P is readily available. In addition, although the Al-sensitive genotype L53 accumulated considerably more Al in the root tip than C100-6, a substantial amount of Al could still be detected in the root tips of C100-6 (Fig. 1b). Therefore, it is possible that an additional tolerance mechanism that does not rely on Al exclusion – such as immobilization by P – could also be operating in C100-6 roots.

Organic acid release-related genes

The Al-activated release of citrate from roots is a well-characterized mechanism of Al tolerance in maize. In this study, the Al-tolerant genotype C100-6 exhibited significantly higher Al-activated citrate release rates than the Al-sensitive L53 (Fig. 1c). We therefore examined the effects of Al stress on the expression of genes involved in organic acid synthesis and metabolism. Only small changes in the expression of genes involved in organic acid synthesis were observed (results not shown). No correlation was observed between gene expression and differences in the magnitude of citrate exudation observed between the maize genotypes. In fact, to date there is no evidence implicating changes in key enzymes catalyzing organic acid synthesis and metabolism in the Al-activated root exudation response (Ryan *et al.*, 1995; Hayes & Ma, 2003). No correlation has been observed between changes in root tip internal organic acid concentration and Al-activated transport activity (i.e. exudation rates) exhibited by Al-tolerant and Al-sensitive genotypes of either maize or wheat (Delhaize *et al.*, 1993; Piñeros *et al.*, 2005). Therefore, the expression of enzymes involved in organic acid synthesis is not likely to be implicated in regulating the Al-induced citrate release response in maize roots.

Transporters

The above results contribute to the emerging body of evidence indicating that activation of transport is the main regulatory step in the root organic acid exudation response. This idea has been significantly strengthened by the recent cloning of Al tolerance genes in other plant species. The wheat aluminium-activated malate transporter gene (*TaALMT1*) was the first Al tolerance gene to be cloned (Sasaki *et al.*, 2004), and encodes a root-specific plasma membrane transporter that facilitates malate efflux. A member of this novel family of transporters has also been identified as the transporter mediating Al-activated malate release in Arabidopsis (Hoekenga *et al.*, 2006). Magalhaes *et al.* (2007) subsequently identified the Al tolerance gene in sorghum (*Sorghum bicolor*) as a member of the multidrug and toxin extrusion (MATE) family of transporters, responsible for Al-activated citrate release in sorghum roots. Evidence of a role for these two families of transporters in Al-activated organic acid release is also starting to emerge in other plant species, such as *Brassica napus* (Ligaba *et al.*, 2006) and barley (*Hordeum vulgare*; Furukawa *et al.*, 2007).

In Arabidopsis, both ALMTs and MATEs are encoded by gene families, with 14 and 56 members, respectively. Although the number of members of these two gene families in maize has yet to be resolved, several *ALMT1*- and *MATE*-like sequences have been identified in maize EST and genomic sequence collections. Among the ESTs represented in the maize oligonucleotide array, we identified 14 putative *ALMT1*-like sequences (Supplementary Material Table S2). These sequences were identified based on annotation as well as via BLAST searches using *ALMT1* sequences from wheat and rice as query (results not shown). Three of the 14 putative *ALMT1*-like sequences present in the array exhibited differential expression under Al treatment; however, their expression patterns suggest that these genes are unlikely to play a role in Al tolerance in maize. One *ALMT1*-like sequence was down-regulated by Al in C100-6. Expression of two other *ALMT1*-like sequences was up-regulated by Al in L53, but not in the Al-tolerant C100-6.

A total of 45 putative *MATE*-like sequences were identified among the ESTs represented in the microarray (Supplementary Material Table S3). In contrast to *ALMT1*-like sequences, some putative *MATE*-like sequences displayed patterns of expression that are consistent with a potential role in Al-activated citrate release. Seven *MATE*-like sequences were differentially regulated by Al, exhibiting various patterns of expression. Among these, four oligos representing putative *MATE*-like sequences exhibited higher constitutive expression levels in C100-6 compared with L53 at all times, and one of these also displayed up-regulation under Al stress.

Although the function of most MATE proteins in plants remains uncharacterized, these transporters are likely to be involved in a number of different cellular processes. Members of this gene family have been shown to function as drug/cation antiporters that remove toxic compounds and secondary metabolites from the cytosol by exporting them out of the cell or into the vacuole (Debeaujon *et al.*, 2001; Diener *et al.*, 2001). Recently, the Arabidopsis MATE transporter ferric reductase defective 3 (FRD3) was shown to be a citrate transporter involved in the loading of iron into the xylem (Durrett *et al.*, 2007). The variety of expression patterns for *MATE*-like sequences observed in our study is in agreement with the apparent multiplicity of roles of these transporters in plants.

To a lesser degree, the *ALMT1*-like sequences present in the array also exhibited somewhat diverse patterns of expression. In contrast to the MATEs, the first member of the ALMT1 family to be identified was a malate transporter with a role in Al tolerance in wheat. Nevertheless, evidence that ALMT transporters are also involved in other processes not related to Al tolerance is quickly emerging. For instance, the recently characterized *ZmALMT1* encodes an ALMT1-type transporter that does not mediate Al-activated organic acid release, but rather is implicated in the selective transport of anions involved in mineral nutrition and ion homeostasis (Piñeros *et al.*, 2008).

The cloning and characterization of organic acid transporters involved in Al tolerance are providing new insights into the regulation of the Al-activated organic acid exudation response. The first and foremost conclusion that can be drawn from these studies (which concurs with physiological evidence) is that this response is regulated mainly at the transport level. Little or no change in the synthesis and metabolism of organic acids is involved in regulating the organic acid release observed upon root exposure to Al. Rather, the presence and/or activation of plasma membrane transporters that facilitate the movement of these anions down their electrochemical gradient is the key step that regulates this response. Another important conclusion from the characterization of these organic acids transporters is that, in all species studied so far, it is the expression level of the gene encoding the transporter that determines Al tolerance. Levels of *TaALMT1* expression are highly correlated with the degree of Al tolerance among a number of wheat genotypes (Raman *et al.*, 2005). Expression of Arabidopsis *AtALMT1* and of rape (*Brassica napus*) *BnALMT1/BnALMT2* is highly up-regulated under Al stress (Hoekenga *et al.*, 2006; Ligaba *et al.*, 2006). In the case of sorghum *SbMATE*, its expression is not only up-regulated by Al, but also constitutively higher in tolerant than in sensitive genotypes. Finally, even though barley is a relatively Al-sensitive species, expression of the *MATE* aluminium-activated citrate transporter gene (*HvAACT1*) is higher in roots of a moderately tolerant genotype than in roots of a sensitive genotype of barley (Furukawa *et al.*, 2007). It has yet to be established whether expression of a gene(s) encoding a citrate transporter(s) is also important for Al tolerance in maize. Nevertheless, the evidence collected so far from other species is overwhelming. In this study, we identified four putative *MATE*-like sequences with patterns of expression that are in agreement with a potential role in Al tolerance. These putative sequences are going to be the subject of further investigation to establish whether they encode the Al-activated citrate transporter(s) responsible for Al tolerance in maize.

Concluding remarks

Although a number of genes differentially regulated by Al have been identified and characterized by traditional expression studies (Ezaki *et al.*, 1995; Hamel *et al.*, 1998; Richards *et al.*, 1998; Mao *et al.*, 2004), knowledge of global changes in gene expression in response to Al is still largely limited. With the application of a sensitive, high-throughput technology such as microarrays, the present study offers the first comprehensive survey of global transcriptional regulation in maize roots under Al stress. The use of short Al exposure periods in a time-course study allowed us to observe the effects of Al on gene expression before the onset of severe toxicity symptoms, as indicated by the increase in the number of differentially regulated genes over time in roots of the Al-sensitive genotype. In addition, using a comparative approach we were able to identify a number of genes displaying different patterns of expression in response to Al between an Al-tolerant and an Al-sensitive maize genotype.

Although Al-activated citrate release is an important mechanism of Al tolerance in maize, other mechanisms are likely to be operating in this species and have yet to be characterized. The recent cloning of the first Al tolerance genes is starting to shed light on the regulation of the Al tolerance response in plants. In the case of organic acid transporters, it is their expression level that plays a key role in differential Al tolerance. It is therefore plausible to assume that differential expression might also play a role in other unknown mechanisms of Al tolerance. Consequently, the results of this study are likely to become a valuable resource to help further our understanding of the mechanisms of Al toxicity and tolerance in maize, and of how these mechanisms are regulated at a transcriptional level.

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Supplementary Material

The following supplementary material is available for this article online:

Fig. S1 Interconnected loop design of the microarray experiment.

Table S1 List of genes differentially regulated under Al treatment in roots of C100-6 (Al-tolerant) and L53 (Al-sensitive)

Table S2 Oligonucleotides in maize microarray representing *ALMT1*-like sequences and their expression patterns in this study

Table S3 Oligonucleotides in maize microarray representing *MATE*-like sequences and their expression patterns in this study

Table S4 Primers used in qRT-PCR

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