Proteome changes induced by aluminium stress in tomato roots

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Abstract

Growth inhibition in acid soils due to Al stress affects crop production worldwide. To understand mechanisms in sensitive crops that are affected by Al stress, a proteomic analysis of primary tomato root tissue, grown in Al-amended and non-amended liquid cultures, was performed. DIGE-SDS-MALDI-TOF-TOF analysis of these tissues resulted in the identification of 49 proteins that were differentially accumulated. Dehydroascorbate reductase, glutathione reductase, and catalase enzymes associated with antioxidant activities were induced in Al-treated roots. Induced enzyme proteins associated with detoxification were mitochondrial aldehyde dehydrogenase, catechol oxidase, quinone reductase, and lactoylglutathione lyase. The germin-like (oxalate oxidase) proteins, the malate dehydrogenase, wali7 and heavy-metal associated domain-containing proteins were suppressed. VHA-ATP that encodes for the catalytic subunit A of the vacuolar ATP synthase was induced and two ATPase subunit 1 isoforms were suppressed. Several proteins in the active methyl cycle, including SAMS, quercetin 3-O-methyltransferase and AdoHcyase, were induced by Al stress. Other induced proteins were isovaleryl-CoA dehydrogenase and the GDSL-motif lipase hydrolase family protein. NADPH-dependent flavin reductase and β-hydroxyacyl-ACP dehydratase were suppressed.

Key words: Acidic pH, aluminium toxicity, DIGE-MALDI-TOF-TOF, proteomics, tomato root.

Introduction

In acid soils, toxic levels of aluminium (Al) inhibit plant development and cause substantial yield reductions in sensitive crops. The first line of protection against aluminium toxicity resides in the root system since it controls the absorption and transportation of toxic metals to above-ground tissues. Following long-term exposure to Al, roots of tomato thicken and growth is retarded (Blancaflor et al., 1998). Root tips swell and branching ceases (Zhou et al., unpublished data). Auxin transportation in the root cortex is impeded, root elongation stops (Kollmeier et al., 2000), and the cytoskeleton of elongating cells deforms due to the depolymerization of microtubules (Sivaguru et al., 2003). Cell division is reduced due to cell distortion and vacuolization associated with rigidification, or fragmentation of the actin network (Horst et al., 1999; Vazquez et al., 1999; Frantzios et al., 2005). On exposure to toxic Al levels, sensitive wheat cultivars produce and deposit higher amounts of callose (β-1,3-glucan) on the external face of the plasma membranes of root cap, epidermal, and outer cortical root tip cells than on tolerant cultivars (Schreiner et al., 1994). Peroxidation of phospholipids on the membrane network can block intercellular signal transduction causing mitochondria to malfunction (Illes et al., 2006). This triggers the production of reactive oxygen species resulting in oxidative stress (Achary et al., 2007, and references therewith).

All cellular, physiological, and biochemical disorders induced by Al stress thwart root development and root growth (Kochian, 1995; Yamamoto et al., 2002). These changes are controlled by alterations in gene expression at
transcriptional, post-transcriptional, translational, and post-translational levels. Comparative proteomics analysis coupled with bioinformatics techniques allow for the identification of expressed proteins under specific stress conditions (Pandey and Mann, 2000; Qureshi et al., 2007). Studies with an Al-resistant soybean cultivar (Baxi 10) showed that Al induced the production of heat shock proteins, glutathione S-transferase, chalcone-related synthetase, GTP-binding protein, and ABC transporter, ATP-binding protein (Zhen et al., 2007), copper/zinc superoxide dismutase, S-adenosylmethionine synthetase, cysteine synthase, 1-aminocyclopropane-1-carboxylic acid oxidase, and other abiotic and biotic stress-induced proteins (Yang et al., 2007).

The impact of Al stress on root development can be divided into two phases: the immediate inhibition of root cell elongation that occurs within 30–60 min after exposure (Horst, 1995), and the reduction in root tip cell proliferation that occurs hours to days after exposure (Doncheva et al., 2005). The objective of this study was to identify molecular mechanisms associated with long-term survival (10 d) of tomato plants to Al toxicity by comparing quantitative and qualitative changes in proteomes that occurred in roots of Al-treated and non-treated plants.

Materials and methods

Tomato root culture and Al treatment

Tomato (Solanum esculentum Mill cv. Money Maker) seeds (Seedman, USA) were surface-sterilized by submersion in 2% (the solution consisted of a 40% delution of a 5% hypochloride solution) sodium hypochlorite solution for 10 min followed by three washes in sterile distilled water. For seed germination, six clean seeds were placed in a 500 ml flask containing 300 ml Modified Magnavaca’s solution (Magnavaca et al., 1987) adjusted to pH 4.5 and incubated on a shaker (150 rpm) at 25 °C for 4 d. Following germination, seedlings were transferred to a fresh Modified Magnavaca’s solution supplemented with 20 μM AlK (SO₄)₂ (Fisher Scientific, St Louis, MO, USA) (Zhou et al., 2008). Seedlings serving as controls were placed in Magnavaca’s solution without the Al. All cultures were maintained for 12 h. Following protocol: 16 h at 20 mA for 30 min then at 50 mA for 5–6 h until the bromephenol blue front marker reached the bottom of the gel.

Preparation of protein samples, protein quantification and DIGE (Difference Gel Electrophoresis) analysis

Protein mixtures were centrifuged at 15 500 RCF (G) for 10 min at 4 °C to remove cell debris. Proteins in supernatants were precipitated overnight in a 20% trichloroacetic acid (TCA) solution at –20 °C, and pelleted at 15 500 g. Pellets were washed five times in prechilled acetone to remove residual TCA and air-dried at 4 °C. After reconstitution in a buffer that contained 7 M urea, 2 M thiourea, 4% CHAPS (Sigma) in 25 mM TRIS, pH 8.6, the concentration of total protein in each sample was quantified using the Bradford protein assay (Bio-Rad; Hercules, CA). Bovine serum albumin ranging in concentrations from 0–1 mg ml⁻¹ was used as the standard.

For allow for detectable differences in CyDye (GE Healthcare; Piscataway, NJ, USA) intensities, each biological repeat protein sample was labelled with Cy 3 and Cy 5, and an internal standard containing equal amounts of protein from all samples was labelled with Cy 2. A total of six gels were run, each having the Cy2-labelled internal standard, and one biological replicate from the untreated control and Al-treated samples with dye swaps.

Two-dimension (2-D) gel electrophoresis

Samples used for 2-D gel electrophoresis were initially fractionated using a Multiphor II system (GE Healthcare). Immobilized pH gradient IPG strips (GE Healthcare) of 24 cm in length with non-linear pH 3.0–10.0 gradients were used for this first-dimension separation. Labelled proteins from non-treated (50 μg), treated plants (50 μg), and internal standard (50 μg) samples (150 μg in total) were mixed with the IPG strip rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT, all from Sigma), and 2% IPG strip buffer (GE Healthcare). Four hundred and fifty micro litres of this solution was added to each lane of each rehydration tray and allowed to rehydrate at room temperature for 12 h. Isoelectric focusing (IEF) was carried out at 20 °C with voltage increasing from 500 V (4 h) to 1000 V (1 h), then ramping to 8000 V where it was maintained for approximately 16 h to reach a total of 85 000 V h. At the completion of the IEF run, proteins were reduced and alkylated (Zhang et al., 2003).

Strips were transferred and pressed on to 24×21 cm 12% precast SDS-polyacrylamide gels (Jule Biotechnologies, New Haven, CT, USA). The gels were run on a DALT Six electrophoresis apparatus (GE Healthcare) using the following protocol: 16°C at 20 mA for 30 min then at 50 mA for 5–6 h until the bromphenol blue front marker reached the bottom of the gel.

For expression analysis, proteins were labelled with Cy dyes as previously described. Digital gel images were obtained using a Typhoon 9400 laser scanner (GE Healthcare) and raw image files were analysed using the Progenesis...
Samespots (Nonlinear Dynamics, Newcastle-upon-Tyne, UK). Protein spots that showed a change of at least 1.2-fold between control and treated samples with \( P \leq 0.5 \) were selected for digestion and identification by MS analysis. For protein identification, separate picking gels were run. The picking gels were stained with colloidal Coomassie blue (NOVEX CCB staining kit, Invitrogen; Carlsbad, CA, USA) and the protein spots were picked manually.

**In-gel digestion with trypsin**

In-gel digestions and trypptic peptide extractions were performed following the protocol of Shevchenko et al. (1996) with slight modifications. Gel pieces were washed and destained using a series of washes consisting of 50 \( \mu l \) of water, 50 \( \mu l \) of 100\% acetonitrile (Fisher Scientific; Fair Lawn, NJ, USA) /50\% 50 mM ammonium bicarbonate (Sigma) pH 7.8, and 50 \( \mu l \) of 100\% acetonitrile. Once samples were completely dried down, 0.2 \( \mu l \) of modified trypsin (Promega; Madison, WI, USA) in 20 \( \mu l \) of 50 mM ammonium bicarbonate pH 7.8/10\% acetonitrile was added to each tube. Samples were kept on ice for 15 min and incubated overnight at 37 °C.

The supernatant from each sample was recovered and the remaining peptides were then sequentially extracted from the gel using a series of solutions. The first one contained 50 \( \mu l \) of 50\% acetonitrile with 2% formic acid (FA) and the second 50 \( \mu l \) of 90\% acetonitrile with 0.5% FA. Each sample was sonicated for 10 min before removing the supernatant. All supernatants were combined and dried in a Speedvac (Thermo Savant; Holbrook, NY, USA).

**Protein identification by MALDI-TOF-TOF mass spectrometry**

Samples were reconstituted in 3 \( \mu l \) of 50\% acetonitrile (ACN) (Fisher Scientific) with 0.1% trifluoroacetic acid (TFA) prior to MS analysis. One \( \mu l \) was spotted on a MALDI target plate and immediately spotted on top with 0.5 \( \mu l \) of saturated matrix (10 mg ml\(^{-1}\) \( \alpha \)-CHCA (\( \alpha \)-cyano-4-hydroxy cinnamic acid) purchased from Sigma and crystallized prior to use in 50\% ACN with 0.1\% TFA and 1 mM ammonium phosphate) and allowed to dry completely. Samples were then subjected to MALDI MS/MS analysis using a 4700 Proteomics Analyser equipped with TOF-TOF ion optics (Applied Biosystem; Framingham, MA, USA) with 4700 Explorer version 3.5. This instrument was operated in 1 kV reflector positive ion mode and calibrated with a calibration kit (Applied Biosystems) containing a mixture of six standard peptides as a default calibration for spectra acquisition. The laser power was set to 4600 for MS and 5200 for MS/MS with CID off. MS spectra were acquired across the mass range of 800-4000 Da with a minimum S/N filter at 25 for precursor ion selection. MS/MS spectra were acquired for the 20 most abundant precursor ions with a total accumulation of 2000 laser shots.

The combined MS and MS/MS data from the MALDI-TOF/TOF analysis were submitted to Mascot 2.2 using GPS Explorer 3.65 for a search against the SGN annotated database (downloaded on 08/07/2007). The search parameters allowed for one missed tryptic cleavage and variable modifications of methionine oxidation and cysteine carboxymethylation. The precursor mass tolerance was 75 ppm and 0.15 Da was used for fragment ions. Only proteins with a >95\% confidence interval (CI) were listed as identified.

**Results and discussion**

**Proteins affected by Al stress**

Eighty-eight protein spots showed significant different intensities (1.2–2.3-fold, ANOVA \( P < 0.05 \)) between the Al-treated and control root proteomes (Fig. 1). Sixty-one spots contained induced proteins and 27 suppressed ones. The use of proteomics analysis resulted in the identification of 49 (56\%) proteins (Table 1). Spots containing induced protein were: two spots for lactoylglutathione lyase/glyoxalase, spot no. 125, 1.5-fold and spot no. 282, 1.3-fold; monodehydroascorbate reductase (NADH), spot no. 18, 2.1-fold; polyphenol oxidase precursor catechol oxidase, spot no. 48, 1.8-fold; glycolysyl hydrolase family 79, spot no. 93, 1.6-fold; aldehyde dehydrogenase (ALDH2), spot no. 128, 1.5-fold; 20S proteasome alpha subunit A2 (PAA2), spot no. 132, 1.5-fold; GDSL-motif lipase hydrolase family protein, spot no. 148, 1.5-fold; quinone reductase family protein, spot no. 153, 1.5-fold; alcohol dehydrogenase class III, spot no. 220, 1.4-fold; glutathione reductase, spot no. 221, 1.4-fold; isovaleryl-CoA-dehydrogenase (IVD), spot no. 231, 1.3-fold; quercetin 3-O-methyltransferase, spot no. 245, 1.3-fold; cytosolic aminopeptidase family protein, spot no. 268, 1.3-fold; enolase, spot no. 285, 1.3-fold; cytosolic UDP-glucose pyrophosphorylase, spot no. 328, 1.2-fold; SAMS proteins, spot no. 79, 1.7-fold, and spot no. 262, 1.3-fold. Two spots for pkB-type carbohydrate kinase family protein were induced, spot no. 380, 1.2-fold and spot no. 218, 1.4-fold. Spot no. 55 coding for VHA-ATPase was induced by 1.8-fold.

Spots with suppressed proteins were: four germin-like protein (oxalate oxidase) isoforms, such as spot no. 14, −2.1-fold, spot no. 39, −1.9-fold, spot no. 40, −1.9-fold, and spot no. 42, 1.8-fold. Other proteins included malate dehydrogenase (NAD), spot no. 6, −2.3-fold; the NADPH-dependent FMN reductase family protein, spot no. 11, 1.2-fold; quercetin 3-O-methyltransferase, spot no. 245, 1.3-fold; cytosolic aminopeptidase family protein, spot no. 268, 1.3-fold; enolase, spot no. 285, 1.3-fold; cytosolic UDP-glucose pyrophosphorylase, spot no. 328, 1.2-fold; SAMS proteins, spot no. 79, 1.7-fold, and spot no. 262, 1.3-fold. Two spots for pkB-type carbohydrate kinase family protein were induced, spot no. 380, 1.2-fold and spot no. 218, 1.4-fold. Spot no. 55 coding for VHA-ATPase was induced by 1.8-fold.

**Protein isoforms with varied changes were four spots for catalase:** spot no. 127, reduced by −1.5-fold, spot no. 84 induced 1.7-fold, spot no. 82 induced 1.7-fold and spot no. 116, 1.6-fold. Four ATPase subunit 1 proteins were
identified, such as spot nos 62 and 203 reduced by –1.7-fold and –1.4-fold, and spot nos 155 and 250 induced by 1.5-fold and 1.3-fold.

Proteins in regulation of antioxidant and detoxification mechanism

Al toxicity triggers the production of reactive oxygen species through the activation of various enzymatic and non-enzymatic reactions. Oxalate oxidase (OXO) degrades oxalate to hydrogen peroxide (H₂O₂), which is the major component of reactive oxygen molecules and is very toxic to cells. Tamás et al. (2004) found that elevated OXO activities induced by Al stress were correlated with plasma membrane injury in young barley roots, and the resultant oxidative burst could cause the cell death of root border cells (Tamás et al., 2005, 2006). Removal of the dead epidermal cells would protect deeper meristematic cell layers in the elongation zone and allow for root growth (Delisle et al., 2001).

In tomato roots, Al suppressed the production of four isoforms of oxalate oxidase (OXO), between 1.83–2.14-fold. The same gene was also repressed at transcript level (Zhou et al., 2008). By contrast, a higher abundance of transcript and protein of OXO were observed in salt-treated tomato leaves (Zhou et al., 2007, 2009) and roots (S. Zhou, unpublished data) where no phenotypic damages were shown. These results suggest that suppressed expression of OXO gene could be specific to Al stress in tomato, and such a response may be related to the deformed root tips (swollen and retarded growth; S. Zhou, unpublished data).

Under Al stress, various antioxidant mechanisms are activated to alleviate the oxidative stress in both tolerant and sensitive plant species (Lukaszewski and Blevins, 1996; Tamás et al., 2006). The major antioxidant enzymes include glutathione-S-transferase (GST), ascorbate peroxidase, catalase, superoxide dismutase, and so on (Ezaki et al., 2000, 2001). In Al-treated tomato roots, the catalase proteins were induced, together with two enzymes in the ascorbate-glutathione cycle that quench free radicals, monodehydroascorbate reductase (spot no. 18, 2-fold) and glutathione reductase (spot no. 221, 1.4-fold). The GST protein (spot no. 118) was suppressed (~1.56-fold), which is in contrast to Al up-regulation of GST in tolerant species (Ezaki et al., 1995, 2000; Darkó et al., 2004).

Proteins in detoxification mechanisms

Aldehydes, formaldehydes, methylglyoxal, and phenolic compounds are highly toxic to cellular structure and

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**Fig. 1.** Picking gel showing all protein spots that had significant difference ($P < 0.05$) between treated and untreated tomato root samples. The image was generated in Progenesis Samespots.
functions. Enzymes that detoxify these molecules were induced in Al-treated tomato roots. The glutathione-dependent formaldehyde dehydrogenase (GSH-FDH), which is associated with the removal of formaldehyde, was induced by 1.4-fold (spot no. 220; Table 1). The mitochondrial aldehyde dehydrogenase that oxidizes aldehydes to carboxylic acids was induced by 1.5-fold (spot no. 128).

Two enzymes, such as lactoylglutathione lyase and glyoxalase I, are critical in the detoxification of methylglyoxal. Two isoforms of glyoxalase I were induced (spot no. 128 and 129).

### Table 1. Al-regulated proteins in tomato roots identified using MALDI-TOF-TOF analysis coupled with database searches

<table>
<thead>
<tr>
<th>Spot number</th>
<th>Fold (Al-treated/Control)</th>
<th>ANOVA (P value)</th>
<th>SGN-ID</th>
<th>Annotated ID</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>-2.3</td>
<td>2.39E-06</td>
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<td>Malate dehydrogenase (NAD)</td>
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<td>11</td>
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<td>SGN-U317644</td>
<td>NADPH-dependent FMN reductase family protein</td>
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<td>Germin-like protein</td>
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<td>0.0033</td>
<td>SGN-U319533</td>
<td>Actin-depolymerizing factor</td>
</tr>
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<tr>
<td>40</td>
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<td>SGN-U322216</td>
<td>Germin-like</td>
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<td>46</td>
<td>-1.8</td>
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<td>SGN-U312745</td>
<td>Wali7 (aluminium-induced protein, Triticum aestivum, GI:451193)</td>
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<td>-1.7</td>
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<td>Atp1</td>
</tr>
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<td>SGN-U318707</td>
<td>Expressed protein</td>
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<td>1.2</td>
<td>0.00037</td>
<td>SGN-U314753</td>
<td>Actin-2</td>
</tr>
<tr>
<td>382</td>
<td>1.2</td>
<td>0.00117</td>
<td>SGN-313048</td>
<td>Reversibly glycosylated polypeptide-1 (RGP1)</td>
</tr>
</tbody>
</table>
Quinone reductase was induced by 1.5-fold (spot no. 153); this enzyme is involved in the reduction of electrophilic quinones, and plays a key role in cellular antioxidant defence by detoxifying quinine derivatives. The polyphenol oxidase precursor/catechol oxidase (spot no. 48), which catalyses the oxidation of phenols to form benzoquinone, was induced by 1.8-fold. The induction of these detoxification enzyme proteins should increase the capacity for degradation of the toxic compounds that were formed under Al stress.

Organic acid metabolism

When exposed to Al, tolerant plants secrete organic acid (OA) anions (malate, oxalate, citrate, and so on) to chelate and immobilize Al$^{3+}$ at their root surface. This prevents Al from entering their root system (Hoekenga et al., 2003; Kobayashi et al., 2005, 2007). Both malate and citrate are formed in the tricarboxylic acid cycle (TCA), where malate dehydrogenase catalyses the conversion of malate into oxaloacetate which is subsequently converted into citrate.

In Al-treated tomato roots, malate dehydrogenase (spot no. 6) was strongly inhibited (~2.7-fold). The overexpression of malate dehydrogenase has been reported to improve Al tolerance in transgenic alfalfa (Tesfaye et al., 2001). This suggests that the maintenance of strong TCA activity plays a very important role in Al-tolerance. Suppression of malate dehydrogenase may disturb normal plant metabolism, reduce the biosynthesis of malate and citric acid, and exaggerate Al-toxicity.

Methyl cycling

SAM-dependent methylation is essential for maintaining cellular functions in plants and other organisms (Pereira et al., 2008; Weretilnyk et al., 2001). SAM provides the methyl group for many transmethylation reactions, including phospholipids, protein, DNA, and RNA (Chiang et al.,...
AdoMet (SAM, S-adenosyl-L-methionine) is synthesized by the transfer of the adenosyl moiety from ATP to the sulphur atom of methionine. This reaction is catalysed by AdoMet synthetase (SAMS). In Al-treated tomato root, SAMS 2 (spot no. 262) and SAMS 3 (spot no. 79) were induced by 1.3- and 1.7-fold, respectively. Fukuda et al. (2007) also reported a significant induction of SAMS protein in response to Al stress in rice roots. They proposed that SAM is related to ethylene-mediated inhibition of root growth and/or the alteration of cell wall structures and polymers in roots. In tomato roots, genes in the ethylene biosynthetic pathway were induced by Al (Zhou et al., 2008).

In this study, proteomics analysis resulted in the identification of enzymes, such as quercetin 3-O-methyltransferase (spot no. 245, induced by 1.3-fold) and the AdoHcyase protein (spot no. 325, induced by 1.2-fold), that were induced by Al stress. Enhancement of quercetin 3-O-methyltransferase (spot no. 245, induced by 1.3-fold) would increase the amount of S-adenosyl-L-homocysteine (AdoHcy), which is hydrolysed into homocysteine (Hcy) and adenosine (Ado) by AdoHcyase (S-adenosylhomocysteine hydrolase: EC 3.3.1.1) (Fig. 2). The three induced enzymes, SAMS, quercetin 3-O-methyltransferase, and AdoHcyase, are needed in maintaining flow of the methyl cycle (Rhodes, 2008), which plays a key role in preventing the accumulation of aberrant proteins against oxidative stress (Kunstmann and Osiewacz, 2008).

The direction of the reversible hydrolysis reaction of AdoHcy depends on the concentration of Ado and Hcy. Elevated intracellular accumulation of the two end-products inhibits AdoHcyase activity, resulting in disruption of the methyl cycle, and causing cellular damage and apoptosis (Bergmann et al., 1994; Hultberg et al., 2000; Kruman et al., 2000). However, no enzymes that are needed to recycle Ado back to ADP and Hcy into methionone were found to be affected by Al in tomato, or reported in other plant species.

Other genes

Vacuolar H+-ATPase (V-ATPase), an ATP-dependent proton pump, transports protons across membranes. Similar to other plant species (Kasai et al., 1992, 1993; Oka et al., 1997; Hamilton et al., 2001), the VHA-ATPase (spot no. 55) was induced (by 1.8-fold) in Al-treated tomato roots. The Wali7 gene was an Al-induced gene originally isolated from wheat roots, (Richards et al., 1994), but the homologous protein was suppressed in the treated tomato roots (spot, no. 46, −1.81-fold).

Conclusion

Proteomics analysis indicated that some protein expressions (accumulations) in tomato roots under Al stress differed from that observed in other plant species. Protein expressions were associated with changes in oxidative stress, detoxification, organic acid metabolism, methyl flow, cell proliferation, and cell death. The induction of dehydroascorbate reductase, glutathione reductase, and catalase could enhance the antioxidant activity in Al-treated roots. In order to increase our understanding of molecular mechanisms associated with Al sensitivity in tomato, interesting genes identified in this study are being used in functional studies.

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References


