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Transcriptional and physiological changes of alfalfa in response to aluminium stress

Q. CHEN[1], X. D. ZHANG[1], S. S. WANG[1], Q. F. WANG[1], G. Q. WANG[1], H. J. NIAN[1], K. Z. LI[1], Y. X. YU[2] AND L. M. CHEN[1]*

[1] *Biotechnology Research Center, Bailong Campus, Faculty of Environmental Science and Engineering, Kunming University of Science and Technology, Bailong Road, Kunming, 650224, China*
[2] *College of Zoological Science and Technology, Southwest University, Chongqing, 400715, China*

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SUMMARY

Medicago sativa is an excellent pasture legume, but it is very sensitive to aluminium (Al) toxicity. To better understand the mechanism of *M. sativa* sensitivity to Al, a forward suppression subtractive hybridization (SSH) cDNA library for an Al-sensitive cultivar, *M. sativa* L. cv. Yumu No. 1 (YM1), under 5 μM Al stress over a 24 h period was constructed to analyse changes in its gene expression in response to Al stress. Sequence analysis for the SSH cDNA library generated 291 high-quantity expression sequence tags (ESTs). Of these, 229 were known as functional ESTs, 137 of which have already been reported as Al response genes, whereas the other 92 were potentially novel Al-associated genes. The up-regulation of known Al resistance-associated genes encoding the transcription factor sensitive to proton rhizotoxicity 1 (*STOP1*) and malate transporter *MsALMT1* (Al-activated malate transporter) as well as genes for antioxidant enzymes was observed. Reverse transcription polymerase chain reaction analysis validated the reliability of the SSH data and confirmed the up-regulated expression of *STOP1* and *MsALMT1* under 5 μM Al stress. The analysis of physiological changes indicated that hydrogen peroxide (H_2O_2) and malondialdehyde levels were elevated rapidly under 5 μM Al stress, suggesting that severe oxidative stress occurred in the YM1 roots. The up-regulation of antioxidant-related genes might be an important protective mechanism for YM1 in response to the oxidative stress induced by 5 μM Al toxicity. Al-induced malate exudation was increased drastically during the early period after Al treatment, which might have been due to the up-regulation and function of *MsALMT* and *STOP1*. However, malate exudation from the YM1 roots declined quickly during the subsequent period, and a gradual decrease in malate content was simultaneously observed in the YM1 roots. This result is in agreement with the observation that organic acid metabolism-associated enzymes such as phosphoenolpyruvate carboxylase, citrate synthase and malate dehydrogenase were not present in the SSH library. This might be a major reason for the YM1 sensitivity to Al.

INTRODUCTION

Aluminium (Al) toxicity is a major limiting factor for crop development and production in acidic soils, which cover >0·30 of the world's arable land (Sasaki *et al.* 2004). After oxygen and silicon, Al is the third most abundant element and the most abundant metal, making up 0·07 of the earth's crust (Tesfaye *et al.* 2001). Under normal conditions, most Al combines efficiently with oxygen and silicon to form insoluble oxides and aluminosilicates, which are harmless to plants (Ma & Furukawa 2003). However, in acidic soils, where the pH values are lower than 5·50, Al is released into soils in the form of the aluminium ion Al^{3+}, which is toxic to plants. Al toxicity first inhibits plant root growth, even at micromolar concentrations (Barcelo & Poschenrieder 2002). Consequently, the plant's water and nutrient uptake decreases, which results in poor growth and biomass production (Ma & Furukawa 2003). Al has been shown to be capable of affecting the plasma membrane structure, inhibiting the intracellular Ca^{2+} signalling pathway (Ryan & Kochian 1993), eliciting reactive oxygen species

* To whom all correspondence should be addressed. Email: chenlimeikm@126.com

(ROS) (Richards *et al.* 1998; Watt 2003) and arresting cell division by down-regulation of cell cycle genes (Chandran *et al.* 2008*a*).

Fortunately, Al-resistant and Al-tolerant plant species and cultivars have evolved external and internal Al-detoxification mechanisms (Kochian 1995; Furukawa & Ma 2003). The Al-resistant plants with external detoxification mechanisms can exude large amounts of organic acids such as citrate, malate and oxalate (Pellet *et al.* 1996; Ryan *et al.* 2001) to chelate Al and decrease the uptake of free Al^{3+} by plant roots. Al-tolerant plants that have internal detoxification mechanisms accumulate high concentrations of organic acids that can bind with Al^{3+} in cells, thus reducing or eliminating Al toxicity (Ma *et al.* 1997, 1998; Ma & Hiradate 2000). The Al-activated release of organic acid anions from root tips has been characterized as the most important Al-resistant mechanism in plants (Sasaki *et al.* 2004; Hoekenga *et al.* 2006). Differential display reverse transcription-polymerase chain reaction (DDRT-PCR), cDNA-amplified fragment length polymorphisms (cDNA-AFLP), suppression subtractive hybridization (SSH), DNA microarray and proteomic approaches have been used widely to analyse plant responses to Al stress. Using these powerful tools, Al-responsive genes have been identified in the roots of *Arabidopsis thaliana* (Richards *et al.* 1998; Kumari *et al.* 2008; Goodwin & Sutter 2009), sugarcane (Watt 2003), rye (Milla *et al.* 2002), soybean (Ermolayev *et al.* 2003), rice (Mao *et al.* 2004) and *Medicago truncatula* (Chandran *et al.* 2008*a*). Combining these approaches with the isolation of Al-hypersensitive mutants and positional cloning techniques, a number of Al-resistance genes from several Al-resistant or Al-tolerant species, such as wheat (Sasaki *et al.* 2004), rape (Ligaba *et al.* 2006), *Arabidopsis* (Kobayashi *et al.* 2007) and sorghum (Magalhaes *et al.* 2007) have been identified.

It is well known that both *M. truncatula* and *M. sativa* are sensitive to soil acidity and Al toxicity (Sledge *et al.* 2005). Recently, a DNA microarray approach was used to analyse Al-responsive genes in the roots of *M. truncatula* cultivar Jemalong genotype A17 (Chandran *et al.* 2008*a*). A multi-drug and toxin efflux protein (*MATE*) gene showing high homology to the citrate efflux transporter from other plant species was found to be highly up-regulated under Al stress conditions. However, up-regulated *MATE* expression did not confer Al-resistance on *M. truncatula*.

M. sativa is not only the world's major forage, producing more protein per hectare than grain and oilseed crops (Sledge *et al.* 2002), but it is also a model plant for genetic engineering studies (Dragiiska *et al.* 1996). However, the sensitivity of *M. sativa* to Al (Kamp-Glass *et al.* 1993) limits its breeding and growth in acidic soils. Recently, a study performed by Tesfaye *et al.* (2001) tried to improve its Al-resistance by over-expressing a nodule-enhanced malate dehydrogenase (*neMDH*). Nevertheless, the results were not sufficient to justify its use on an agricultural scale. To better decipher the mechanism for the sensitivity of *M. sativa* to Al, the present study investigated the changes in gene expression (using the SSH method) and the physiological characters of an Al-sensitive *M. sativa* cultivar, Yumu No. 1, in response to Al stress. The results are useful for suggestions of more effective and feasible strategies for genetic engineering to enhance its Al-resistance and support sustainable land use (Humphreys 2005).

MATERIALS AND METHODS

Plant cultivation and Al treatments

M. sativa L. cv. Yumu No. 1 (henceforth referred to as YM1), developed by Professor Yu at the Southwest University of China, was used as the plant material throughout the experiments. The seeds of YM1 were placed in tap water overnight for germination under dark at 25 °C. Seedlings with roots *c.* 10 mm long were sown on a floating mesh in a polypropylene pot with full nutrient solution (5 litres) as previously described (Lipton *et al.* 1987), and the solution was renewed every other day. The seedlings were grown in a tissue culture room at 25 °C under constant light (100 μmol/m^2/s) for 5 days. Before Al treatment, the 5-day-old seedlings were pre-grown overnight in a 0·5 mM $CaCl_2$ solution (pH 4·2) at 25 °C under constant light as described above.

For the Al sensitivity experiment, the seedlings were then transferred into a 0·5 mM $CaCl_2$ solution containing either 0 (control), 3, 5, 10, 20, 30, 50 or 100 μM $AlCl_3$ (pH 4·2), respectively, and grown at 25 °C under constant light as described above. The root length was measured with a ruler before Al treatment (0 h) and after 24 h of Al treatment (24 h). The relative root growth (RRG) was defined as the ratio of the net root growth in plants treated with Al compared with the control. As chrome azurol sulphonate (CAS) can bind with Al efficiently, it has been widely used in the determination of Al concentration (Sombra *et al.* 2001) and the CAS staining method was used as an indicator of the Al bound to the YM1 root tips. After Al treatment, the roots of YM1 were stained with CAS solution for 30 min, washed thoroughly with distilled water and photographed. Spectrophotometric determination of Al content in roots is described later.

For SSH analysis, the plants were grown in a full nutrient solution for 3 weeks under glasshouse conditions at 30/25 °C day/night temperatures and 12 h of light (1200 μmol/m^2/s). The nutrient solution was renewed every other day. After the roots reached a length of 50–100 mm, the plants were treated with Al as follows: the seedlings were pre-grown overnight in a 0·5 mM $CaCl_2$ solution (pH 4·2) at 25 °C under

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Table 1. *Primer sequences used in the RT-PCR analysis*

Gene	Accession no.	Forward (5′–3′)	Reverse (5′–3′)	Size (bp)
AAT	GW316816	ACCTTATTATGCAATTGATCCTG	ACCGATATTTTCCTCTTCATATTC	448
ANN	GW316837	AAGGCAATGAAAGGTCTG	ACATATTACTTACAGAGCACTGC	412
GEP	GW316771	TTAGACTCCAAAGAAAACAAC	TGTGTTCCCAATTGGTTTAATTC	359
MCA2	GW342448	ACCTTGACATTGAATCAAATGAG	GTACACATTGCAAGTATCATCTC	389
MsALMT1	GW316815	AGGGTCATTCATTCTTTG	TGGAAGAAACTACATCAAGC	270
PPCK	GW316812	GATCGGTCGTGGCCGTTTCGGTAC	ACAAAAGCACACCACAGCTCCAC	548
PR 10	GW316767	TGTATTCAACTTTGAGGATGAAAC	ACATATGACTTACAATCTCACAC	610
SQS	GW316807	ACATGATCTGTGTATTTAAATAAC	AACAAAGACTATGGCTGATGTC	550
STOP1	GW316742	ACAAAACTCCAGCAGCCCTTGC	CAGATTTAGGAGCATAATTAAAC	654
18S rRNA	Z99245.1	TCACTAAGACACCAAAGGA	ATGCATGGCTTAATCTTTGAGAC	492

AAT, cationic amionic acid transporter; *ANN*, annexin-like protein; *GEP*, germin-like protein; *MCA*, type IIB calcium ATPase (MCA2) gene; *MsALMT1*, Al-activated malate transporter; *PPCK*, phosphoenolpyruvate carboxykinase; *PR10*, class 10 PR protein; *SQS*, farnesyl-diphosphate farnesyltransferase; *STOP1*, sensitive to proton rhizotoxicity 1.

constant light as described above. Then, the seedlings were transferred into a 0·5 mM $CaCl_2$ solution containing 5 μM $AlCl_3$ (pH 4·2) for Al treatment at 25 °C under constant light as described above. The roots of the plants treated in the $CaCl_2$ solution without $AlCl_3$ were used as the control; these plants were maintained in the $CaCl_2$ after the others were transferred to $AlCl_3$ and remained there for the same length of time as those in $AlCl_3$. The root tips were sampled at 10 and 30 min, 2, 4, 6, 8, 10 and 24 h after treatment. The samples were immediately frozen in liquid nitrogen and stored at –80 °C for RNA extraction.

Total RNA extraction and mRNA isolation

Frozen root samples were ground in liquid nitrogen, and total RNA was extracted with Trizol reagent (Invitrogen, USA) according to the manufacturer's instruction. The total RNA was treated with DNase I (Promega, USA) for 30 min at 37 °C to remove the residual genomic DNA (gDNA) and then extracted with phenol/chloroform (1/1). The quality and quantity of the extracted total RNA samples were examined by electrophoresis on a 0·02 g/ml agarose/1 × TAE buffer, EtBr-stained gel and by spectrophotometry (Shimadzu, Japan). The total RNA samples showing no gDNA in the EtBr-stained gel and with an A260/280 ratio of 1·9–2·0 (an indication of RNA purity) were used for mRNA isolation. To identify genes differentially expressed over the whole 24-h treatment period but not at a specific time point under the Al stress conditions, the total RNA samples extracted from the roots treated with 5 μM $AlCl_3$ for 10 and 30 min, 2, 4, 6, 8, 10 and 24 h were mixed together and used as the tester. The total RNA samples extracted from the control roots treated with 0·5 mM $CaCl_2$ solution for 10 and 30 min, 2, 4, 6, 8, 10 and 24 h were also mixed together and used as the driver. The mRNA pool of Al-treated roots and control roots was isolated from the total RNA mixtures of tester and driver, respectively, using the Oligotex mRNA Midi Kit (Macherey-Nagel, Germany) according to the protocol described by the manufacturer. The quality and quantity of the isolated mRNA samples were determined by electrophoresis on a 0·02 g/ml agarose/1 × TAE buffer, EtBr-stained gel and by spectrophotometry. The mRNA samples with good quality were used for cDNA synthesis.

Construction of the SSH cDNA library

The cDNA was reverse transcribed from the isolated mRNA samples. The quality of the synthesized cDNA was examined by PCR analysis with alfalfa 18S rRNA primers (see Table 1). The cDNA samples showing only one PCR-amplified band with the expected size of the 18S cDNA was used for SSH by using the Clontech PCR Select-cDNA Subtraction Kit (Clontech, USA) according to the protocol described

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by the manufacturer. The tester cDNA was produced from the mRNA pool of Al-treated roots. The driver cDNA was produced from the mRNA pool of control roots. Briefly, the tester and driver cDNA were digested with *Rsa* I, respectively. The tester cDNA was split into two pools and then ligated with adaptor 1 or adaptor 2R, respectively. After two rounds of hybridization and PCR amplification with adaptor 1 and adaptor 2R as the primers, the differentially expressed cDNAs were normalized and enriched. Then the second PCR products were purified and concentrated. The cDNA fragments for differentially expressed genes were inserted into the pGEM-T easy vector (Promega, USA) and transformed into *Escherichia coli* DH5α competent cells.

Amplification of cDNA inserts

cDNA clones, freshly grown overnight at 37 °C, were used as the PCR templates. The cDNA inserts were amplified using the nested PCR primers 1 and 2R provided in the PCR Select-cDNA Subtraction Kit. The PCR reaction mixtures (20 µl) contained 16·1 µl of sterile water, 2 µl of 10 × Taq reaction buffer (Tiangen, Beijing, China), 0·02 µl of adaptor 1 and adaptor 2R primers (100 µM), 0·2 µl of dNTPs (10 µM each; Tiangen, Beijing, China), 1 unit of Taq DNA polymerase (Tiangen, Beijing, China) and 2 µl of the bacterial culture. The PCR included an initial heating at 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1·5 min and then a final hold at 4 °C. PCR products were run on a 0·01 g/ml agarose/1 × TAE buffer, EtBr-stained gel to validate their quality and quantity.

Expression sequence tag (EST) sequence analysis

The cDNA clones with inserts ⩾500 bp were sequenced using T7 promoter primer. The low-quality regions and the vector and adaptor sequences were removed to obtain the EST sequences. The ESTs were screened against the National Center for Biotechnology Information (NCBI) non-redundant nucleotide database or protein database by using the BLASTN or BLASTX program, respectively (available from http://www.ncbi.nlm.nih.gov/guide/data-software/#All_; verified 8 Dec 2010). ESTs with ⩾0·70 homology with the nucleotide database or ⩾0·30 homology with the protein database were considered as known function genes. ESTs showing significant homology ($E < 0{\cdot}001$) with sequences of unknown function in the *M. truncatula* genomic database or other databases were defined as unknown functions. The BLAST program identifies similar protein or nucleotide sequences to a given target sequence and provides an S score as a measure of the similarity of the query to the sequence shown. It also provides an *E*-value as a measure of the reliability of the S score. Thus, the definition of the *E*-value is: the probability due to chance that there is another alignment with a similarity greater than the given S score. ESTs showing no significant homology with the *M. truncatula* genomic sequence or other databases were defined as new ESTs. To obtain a higher quality of annotation classification, the ESTs were functionally annotated manually based on comparisons to the Uniprot (http://www.uniprot.org/; verified 8 Dec 2010) and Gene Ontology (GO) databases (http://www.geneontology.org/; verified 8 Dec 2010). All EST sequences were submitted to the EST division of the gene bank to obtain their accession numbers (Tables 2 and 3). Also, see supplementary Tables S1–S3 available at http://journals.cambridge.org/AGS).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

The roots of YM1 were treated with 5 µM $AlCl_3$ for 2, 4, 6, 10 and 24 h at 25 °C under constant light as described above. The roots treated with 0·5 mM $CaCl_2$ without Al for 6 h were used as the control (CK). The total RNA isolated from root samples with Trizol reagent was treated with DNase I (Promega, USA) for 30 min at 37 °C to remove the residual gDNA and then extracted with phenol/chloroform (24/1). First-strand cDNA was synthesized from the total RNA (5 µg) using M-MLV reverse transcriptase (Promega, USA) according to the manual. The primer sequences used in the RT-PCR analysis are shown in Table 1. The PCR reaction mixtures (20 µl) contained 16·1 µl of sterile water, 2 µl of 10 × Taq reaction buffer, 0·02 µl of primers (100 µM), 0·2 µl of dNTPs (10 µM each), 1 unit of Taq DNA polymerase (Tiangen, Beijing, China) and 2 µl of cDNA (200 ng/µl). The PCR products were examined by electrophoresis on a 0·01 g/ml agarose/1 × TAE buffer, EtBr-stained gel. Alfalfa 18S rRNA was used as an internal control. Each PCR analysis was repeated thrice.

Full-length MsALMT1 *cDNA isolation and sequence analysis*

For full-length *MsALMT1* cDNA isolation, the total RNA was extracted using Trizol reagent (Invitrogen, USA) from YM1 roots treated with 5 µM $AlCl_3$ under constant light as described above for 24 h at 25 °C. First-strand cDNA was synthesized from 5 µg of total RNA. The full-length cDNA of *MsALTMT1* was amplified by PCR using the forward primer ALMT1F (ATGGTGTCTGAACCAAATTCAAG) and the reverse primer ALMT1R (TAGTTAATTATAATAACATGTTG). The PCR was performed with Taq plus DNA polymerase (Tiangen, Beijing, China). The cDNA fragment of *MsALTMT1* was subcloned into a TA cloning vector, pMD-18T (TaKaRa, Dalian, China), to generate pMD-*MsALTMT*. The pMD-*MsALTMT* was subjected to sequencing. The amino acid sequence of MsALTMT1 was predicted using

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Table 2. *Selected known Al-responsive genes*

Accession no.	Putative function	Identity*†	*E*-value	Size (bp)	Reference
Signal transduction and transcription					
GW316735	14-3-3-like protein	0·92*	$1{\cdot}00\times E^{-126}$	393	Milla *et al.* (2002)
GW316736	Abicisic acid responsive element-binding factor 2	0·77*	$7{\cdot}00\times E^{-38}$	636	Milla *et al.* (2002)
GW316739	Calcineurin B-like-interacting protein kinase	0·87*	$9{\cdot}00\times E^{-109}$	330	Sawaki *et al.* (2009)
GW316741	Calcium and calcium/calmodulin-dependent serine/threonine-protein kinase DMI-3	0·94*	$1{\cdot}00\times E^{-32}$	262	Zhang *et al.* (2010)
GW316742	C2-H2 zinc finger protein (STOP1) gene	0·87*	0·00	752	Iuchi *et al.* (2007)
GW316748	MYB transcription factor	0·84*	$2{\cdot}00\times E^{-79}$	292	Zhang *et al.* (2010)
GW316749	Protein phosphatase 2C (pp2C gene)	0·75*	$3{\cdot}00\times E^{-114}$	1007	Milla *et al.* (2002)
GW316765	Zinc finger DNA-binding protein	0·55†	$3{\cdot}00\times E^{-29}$	569	Eticha *et al.* (2010)
Defence, stress and cell death					
GW316788	Cysteine protease	0·88*	0·00	1084	Milla *et al.* (2002)
GW316772	GST	1·00*	0·00	1029	Milla *et al.* (2002)
GW316775	Pathogenesis-related protein	0·85*	$5{\cdot}00\times E^{-90}$	621	Zhang *et al.* (2010)
GW316777	POD	0·80†	$5{\cdot}00\times E^{-54}$	610	Milla *et al.* (2002)
GW316778	Putative senescence-associated protein	0·67†	$3{\cdot}00\times E^{-41}$	565	Eticha *et al.* (2010)
GW316780	Superoxide dismutase	0·88*	0·00	537	Milla *et al.* (2002)
GW316781	Thioredoxin h	1·00*	$2{\cdot}00\times E^{-60}$	517	Eticha *et al.* (2010)
GW316782	Type 1 metallothionein (MET1)	0·95*	$1{\cdot}00\times E^{-120}$	370	Zhang *et al.* (2010)
GW316791	Heat shock protein 70 (HSP70-1)	0·98*	0·00	727	Milla *et al.* (2002)
Metabolism and energy					
GW342430	Chalcone isomerase	0·99*	0·00	536	Maron *et al.* (2008)
GW342431	Chalcone reductase	0·93*	0·00	696	Maron *et al.* (2008)
GW316811	Putative chalcone synthase	0·90*	$4{\cdot}00\times E^{-114}$	364	Maron *et al.* (2008)
GW316807	Farnesyl-diphosphate Farnesyltransferase	0·95*	$0{\cdot}00\times E^{+00}$	527	Maron *et al.* (2008)
GW342434	Fatty acid desaturase 1-like	0·70*	0·003	531	Maron *et al.* (2008)
GW342435	Glycoside hydrolase family 1 protein	0·60†	$3{\cdot}00\times E^{-61}$	602	Maron *et al.* (2008)
GW342436	Glycosyl hydrolase family 1 protein	0·56†	$1{\cdot}00\times E^{-59}$	602	Maron *et al.* (2008)
GW316809	Glycosyltransferase	0·96*	$1{\cdot}00\times E^{-111}$	248	Eticha *et al.* (2010)
GW316813	S-adenosylmethionine decarboxylase	0·85*	$4{\cdot}00\times E^{-147}$	495	Milla *et al.* (2002)
Transporter					
GW316815	Protein of unknown function UPF0005; Rho GTPase activation protein; putative Al-activated malate transporter 1 (ALMT1)	0·95†	$4{\cdot}00\times E^{-73}$	484	Sasaki *et al.* (2004)
GW316816	Amino acid transmembrane transporter (CAT2)	0·75*	$4{\cdot}00\times E^{-65}$	470	Milla *et al.* (2002)
GW316820	Glucose-6-phosphate/phosphate translocator-related	0·78*	$3{\cdot}00\times E^{-104}$	492	Milla *et al.* (2002)
GW316822	Putative aquaporin	0·84*	$3{\cdot}00\times E^{-144}$	579	Eticha *et al.* (2010)
GW316824	Vacuolar H^+-ATPase subunit A	0·91*	$9{\cdot}00\times E^{-89}$	260	Milla *et al.* (2002)
Cell structure and cell growth					
GW316842	Chitinase class 3	0·87*	$1{\cdot}00\times E^{-101}$	341	Chandran *et al.* (2008*a*)
GW316835	Expansin-like protein	0·52†	$3{\cdot}00\times E^{-33}$	447	Milla *et al.* (2002)
GW316837	Putative annexin	0·81*	$3{\cdot}00\times E^{-72}$	412	Milla *et al.* (2002)
GW316838	S-adenosyl-L-methionine: caffeic acid 3-0-methyltransferase	0·72*	$1{\cdot}00\times E^{-46}$	429	Eticha *et al.* (2010)
GW316840	Xyloglucan endotransglucosylase	0·90*	$3{\cdot}00\times E^{-82}$	241	Chandran *et al.* (2008*a*)

* The sequence blast with the NCBI non-redundant nucleotide database using BLASTN.
† The sequence blast with the NCBI non-redundant protein database using BLASTX.

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Table 3. *Selected novel genes potentially associated with Al stress*

Accession no.	Putative function	Identity*†	*E*-value	Organism	Size (bp)
Metabolism and energy					
GW342425	Acyl desaturase (ACPD)	0·98*	0·00	*M. truncatula*	591
GW342442	Putative serine decarboxylase	0·78†	$3{\cdot}00\times E^{-40}$		457
GW342443	Serine acetyltransferase	0·84*	$5{\cdot}00\times E^{-127}$	*Pisum sativum*	448
GW342446	Triosephosphate isomerase	0·86*	0·00	*Glycine max*	837
GW342447	Triosephosphate isomerase	0·84*	$1{\cdot}00\times E^{-102}$	*Glycine max*	359
GW342448	Type IIB calcium ATPase MCA2 gene	0·95*	$7{\cdot}00\times E^{-175}$	*M. truncatula*	407
Defence, stress and cell death					
GW342451	Abscisic acid 8′-hydroxylase	0·82*	$2{\cdot}00\times E^{-111}$	*Glycine max*	437
GW342452	Abscisic stress ripening-like protein	0·72*	$5{\cdot}00\times E^{-50}$	*Glycine max*	376
GW342457	Epoxide hydrolase	0·64†	$7{\cdot}00\times E^{-36}$	*Nicotiana benthamiana*	500
GW342464	Peroxisomal short-chain dehydrogenase/reductase family protein	0·87*	$1{\cdot}00\times E^{-174}$	*Glycine max*	570
Signal transduction and transcription					
GW342491	Salt- and drought-induced ring finger 1	0·76*	$4{\cdot}00\times E^{-106}$	*Arabidopsis thaliana*	880
GW342494	WD40-like protein	0·60†	$8{\cdot}00\times E^{-05}$	*M. truncatula*	274
GW342495	WD-40 repeat family protein	0·92*	$5{\cdot}00\times E^{-72}$	*Trifolium pratense*	684
Transporter					
GW342528	Inositol transporter 2	0·73*	$9{\cdot}00\times E^{-22}$	*Arabidopsis thaliana*	451
GW342529	Organic anion transmembrane transporter (ANTR2)	0·80*	$4{\cdot}00\times E^{-124}$	*Arabidopsis thaliana*	724
GW342533	Substrate-specific transmembrane transporter	0·99†	$1{\cdot}00\times E^{-50}$	*M. truncatula*	474

* and † are described as in Table 2.

the Genetyx program (Software Development, Tokyo, Japan). Phylogenetic relationships between MsALMT1 and ALMT1 family proteins were analysed using the Dnaman program (Version 6.0.40, Lynnon Biosoft, USA). The dense alignment surface (DAS) Program (Cserzo *et al.* 1997) was used for predicting the location and transmembrane domains of MsALMT1.

Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content analysis

The roots of YM1 were treated with 5 μM $AlCl_3$ for 2, 4, 6, 10 and 24 h under constant light as described above at 25 °C. For the determination of H_2O_2 content, the roots (100 mg) were homogenized in 1 ml of tris(hydroxymethyl)aminomethane (Tris-HCl; 50 mM, pH 7·0). The homogenate was centrifuged at 13 000 ***g*** (4 °C) for 20 min. The H_2O_2 content was measured as described by Gay & Gebicki (2003). The MDA content was determined with the 2-thiobarbituric acid (TBA) method (Baccouch *et al.* 1998). The roots (100 mg) were homogenized in 1 ml of 0·10 mg/ml (w/v) trichloroacetic acid (TCA) and centrifuged at 13 000 ***g*** (4 °C) for 20 min. Then 0·5 ml aliquots of the supernatants were added to 0·4 ml of 0·01 mg/ml (w/v) TBA in 0·10 mg/ml (w/v) TCA and the tubes were incubated in a water bath at 100 °C for 15 min with occasional shaking. The reaction was stopped by placing the reaction tubes into an ice bath. After centrifugation at 13 000 ***g*** (4 °C) for 20 min, the absorbance of the supernatant was measured at 532 and 450 nm.

Determination of malate content

The roots of YM1 were treated as described for the H_2O_2 and MDA analyses. Roots and exudates were harvested and collected after Al treatments of 0 (control), 2, 4, 6, 10 and 24 h. The estimation of malate contents in the YM1 roots and exudates was performed using the enzymatic method according to Wang *et al.* (2010).

Measurement of Al content

The roots of YM1 were treated as described for the H_2O_2 and MDA analyses. The root apices (six tips for each sample) were ground with liquid nitrogen. The powder was suspended in 1·5 ml of 2 M hydrochloric acid (HCl) at room temperature for 24 h with

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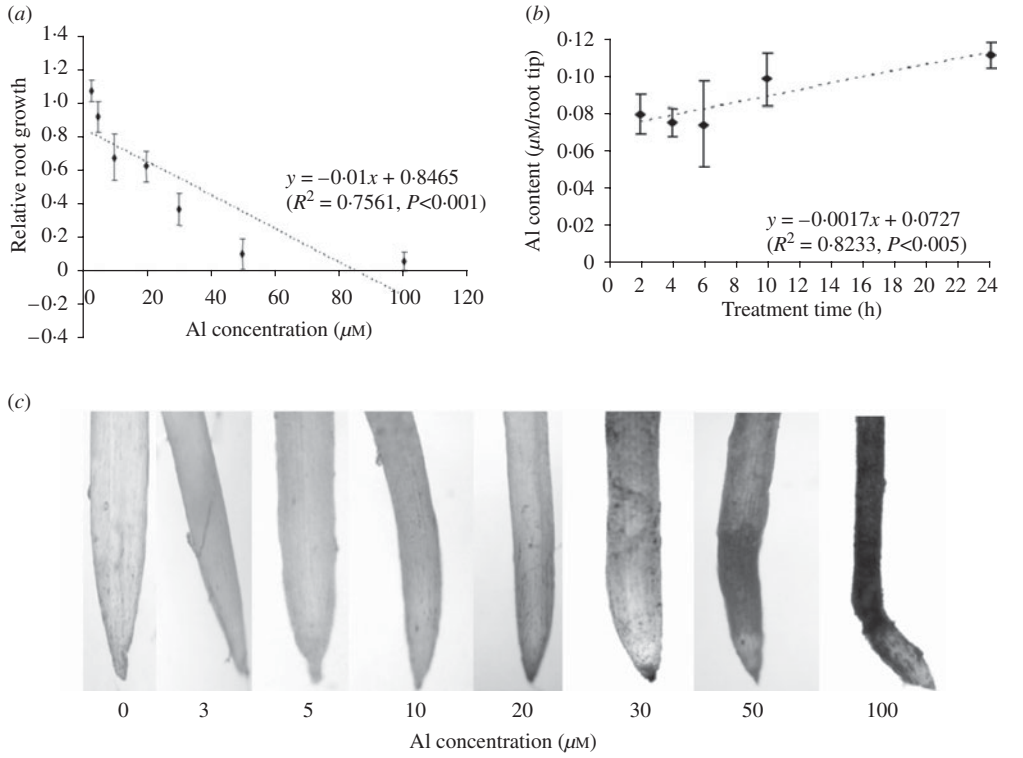

Fig. 1. The effect of Al on YM1 root growth and Al content in YM1 roots after Al treatment. (*a*) RRG of 5-day-old YM1 seedlings. Values are means ± S.D. ($n = 12$). (*b*) The Al content in YM1 roots after treatment with 5 μM Al (pH 4·2) for 2, 4, 6, 10 and 24 h, respectively. Values are means ± S.D. ($n = 3$). (*c*) Chrome azurol S staining of YM1 roots after exposure to 0, 3, 5, 10, 20, 30, 50 and 100 μM $AlCl_3$ (pH 4·2) for 24 h.

occasional shaking (100 rpm). The Al contents in the YM1 root tips were measured spectrophotometrically with pyrocatechol violet (PCV) as described by Zheng *et al.* (2004) with some modification. Assays were performed by mixing 0·05 ml of the sample solution with 1·35 ml of deionized water and then 0·1 ml of the PCV (37·5 mg/ml, w/v) reagent and 0·5 ml of Tris-HCl (50 mM, pH 6·0) buffer were added to start the reaction. The absorbance was read at 590 nm after 15 min.

Statistical analysis

Experiments were conducted from 3 to 12 replicates and data were expressed as means ± S.D. For statistical analysis, one-way ANOVA (SPSS 13.0 program; Norusis 2005) and the *t*-test were used to determine the significance at $P < 0·05$.

RESULTS

Phenotypic performance of YM1 roots under Al stress and Al content in the root tips

The Al sensitivity and phenotypic performance of YM1 were tested using the RRG method. Increasing Al concentration led to lower RRG (linear regression $R^2 = 0·7651$, $P < 0·001$, Fig. 1*a*). The inhibitions at the Al concentrations of 10, 20 and 30 μM were *c.* 0·30, 0·40 and complete inhibition was observed at 50 and 100 μM $AlCl_3$. The results were similar to that of Al-sensitive *M. truncatula* A17 (Chandran *et al.* 2008*b*). CAS staining is an indicator of the toxic effects of Al on the apices of the roots, and the staining degree of the root tips provides a semi-quantitative measurement of the Al content in the root tips. The YM1 roots were stained with CAS after Al treatments (Fig. 1*c*). No visible staining was observed in YM1 roots treated with 3, 5 and 10 μM Al, respectively. Weak staining appeared in the root tips after treatment with 20 μM Al, and visible staining was observed in the root tips after treatment with 30 μM Al. The roots were stained deeply after treatment with 50 μM Al. When the concentration was increased up to 100 μM, the root tips of YM1 were fully stained and exhibited severe damage. These results confirm that YM1 is Al sensitive.

The Al content in the YM1 roots was measured after different periods of treatment with 5 μM Al. There was a significant increase in Al content with increasing treatment time (linear regression $R^2 = 0·8233$,

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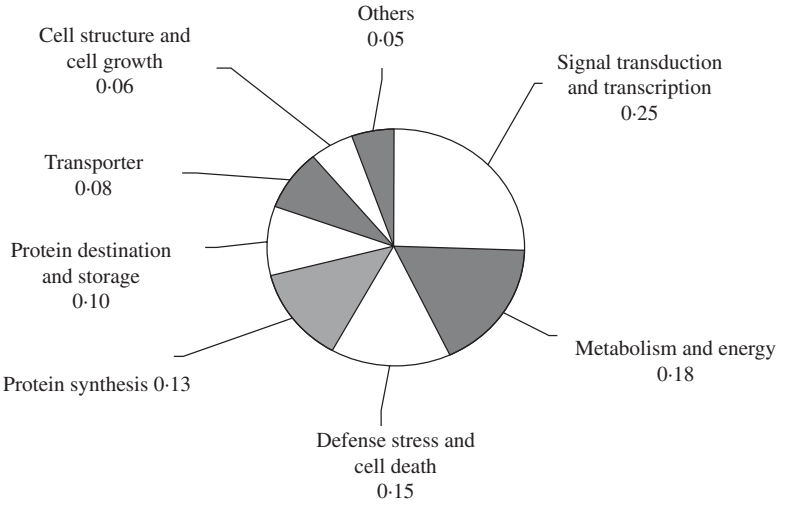

Fig. 2. The distribution of differentially expressed genes in YM1 roots under Al stress. Two hundred and twenty-nine known ESTs were grouped into eight functional categories according to their putative functions. The proportions of the ESTs representing each category are shown.

$P < 0{\cdot}005$, Fig. 1*b*). The Al content in the roots was significantly higher after Al treatment as compared to the control (without Al treatment). Longer periods of exposure led to greater Al content in the root tip. This suggests that Al was efficiently absorbed by the YM1 root tips during the treatment time.

Construction of a forward SSH cDNA library for YM1 under Al stress

Based on the above results, YM1 roots were treated with 5 μM $AlCl_3$ for a period of 10 min to 24 h in order to construct the SSH cDNA library. The library constituted 1209 clones. Colony PCR with the nested primers was used to determine the length of cDNA inserts. The results show that 0·90 were positive clones, and the lengths of the cDNA inserts ranged from 200 to 1200 bp, with an average length of *c.* 500 bp (data not shown). A total of 326 clones with inserts $\geqslant$500 bp were picked for sequencing, and 291 high-quality ESTs were obtained (Tables 2 and 3). Also, see supplementary Tables S1–S3 available at http://journals.cambridge.org/AGS).

Functional classification of the Al-responsive genes in the SSH cDNA library

All 291 ESTs obtained from the SSH cDNA library were screened against the NCBI nucleotide and protein databases. The results indicated that 229 ESTs (0·79) showed similarities to proteins of known function (Tables 2 and 3). Also, see supplementary Tables S1 and S2 available at http://journals.cambridge.org/AGS), 53 ESTs (0·18) showed similarities to genes or genomic sequences of unknown function, and nine ESTs (0·3) were new ESTs (see supplementary Table S3 available at http://journals.cambridge.org/AGS). All 229 ESTs with known functions were classified into eight functional categories (Fig. 2). The genes related to signal transduction and transcription accounted for the largest group, with a corresponding ratio of 0·22. The second categories were genes related to defence, stress and cell death (0·19), and metabolism and energy (0·17), respectively. Protein synthesis and protein destination/storage represented the third categories, with the corresponding ratios of 0·14 and 0·09, respectively. Genes related to transport, cell structure and cell growth and others represented the smallest categories, with the corresponding ratios of 0·80, 0·60 and 0·50, respectively. The genes with known function were carefully compared with the Al-responsive genes that have been reported in other plants. The results showed that 137 ESTs were previously reported to be Al-responsive genes (Table 2). Also, see supplementary Table S2 available at http://journals.cambridge.org/AGS). The other 92 ESTs were not reported and thus were referred to as novel Al-responsive genes (Table 3). Also, see supplementary Table S2 available at http://journals.cambridge.org/AGS).

Up-regulation of known Al-responsive genes

The SSH data (Table 2). Also, see supplementary Table S2 available at http://journals.cambridge.org/AGS) showed that 42 known Al-responsive genes were involved in signal transduction and transcription. The Al and H^+ tolerance-related C2H2-type zinc finger

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protein (sensitive to proton rhizotoxicity 1 (STOP1)), phosphatase 2C, calcineurin B-like-interacting protein kinase, GTP-binding protein, calcium and calcium/calmodulin-dependent serine/threonine-protein kinase DMI-3 and mitogen-activated protein kinase were up-regulated. However, receptor family proteins and cell wall-associated protein kinases, which have been shown to be related to Al resistance, did not show up-regulation in the YM1 roots. The stress, defence and cell death category contains 30 known Al responsive genes. Typical Al-induced marker genes, such as genes related to pathogen stress (class 10 PR protein), disease-resistance proteins, PR10-1 protein and thaumatin-like protein PR-5b, antioxidants (glutathione S-transferase (GST), peroxidase (POD), superoxide dismutase (SOD), germin-like proteins (GEP) and thioredoxin h), cell death (putative senescence-associated protein, cysteine protease and cysteine proteinase precursor) and metal resistance were present in the library. However, blue copper-binding protein and GDP dissociation inhibitor, which have been determined to be Al resistance-associated genes, were absent. Al up-regulated genes related to metabolism and energy included 20 known Al-responsive genes. Several genes encoding important enzymes involved in the metabolism of flavonoids, such as chalcone synthase, chalcone reductase and chalcone isomerase, were up-regulated. Genes related to lipid metabolism such as fatty acid desaturase 1-like and lipase were also up-regulated. However, genes associated with organic acid metabolism, such as citrate synthase (CS), phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH), were absent from the library. The up-regulation of several known Al-responsive transporter genes, such as Zn transporter, cationic amino acid transporter (AAT) and vacuolar H^+-ATPase subunit A, was observed. It is worth noting that a putative Al-activated malate transporter (ALMT1) gene, which had been confirmed to be an Al-resistance gene, was also up-regulated. However, genes including multi-drug and toxin efflux protein (MATE, Al-activated citrate transporter), ATP-binding cassette transporter (ABC transporter), Mg transporter, K transporter and mitochondrial ATPase did not show up-regulation. Known Al-responsive genes associated with cell structure modification and cell growth, including caffeic acid O-methyltransferse, xyloglucan endortransglycosylase, expansion-like protein, annexin-like protein (ANN) and chitinase, were up-regulated. However, genes encoding the cytoskeleton, coumarated: CoA ligase, cellulose microfibril and glycosylphosphatilylinositol-anchored protein were not induced in YM1 roots.

Up-regulation of novel genes associated with Al stress

Of the genes with known functions, 92 novel genes (Table 3). Also. see supplementary Table S2 available at http://journals.cambridge.org/AGS) potentially associated with Al stress were up-regulated. There were 18 genes related to metabolism and energy. Gene encoding enzymes involved in lipid, carbohydrate and serine metabolism, such as acyl desaturase, putative serine decarboxylase, serine acetyltransferase and triosephosphate isomerase were up-regulated. In the category of genes related to defence, stress and cell death, genes encoding ABA 8′-hydroxylase, ABA stress ripening protein, epoxide hydrolase and the peroxisomal short-chain dehydrogenase/reductase family protein were induced. Of the signal transduction and transcription category, genes related to salt- and drought-induced ring finger 1, the WD40-like protein and WD-40 repeat family of proteins were induced. The up-regulation of several novel transporter genes, including inositol transporter 2, organic anion transmembrane transporter and substrate-specific transmembrane transporter, were observed.

Validation of SSH data by RT-PCR analysis

RT-PCR is an economic and a simple method of gene expression profile analysis. Nine genes (Table 1), including two stress-related genes (*GEP* and *PR10*), three genes for metabolism and energy (*SQS, PPCK* and *MCA*), one gene for signal transduction and transcription (*STOP1*), one gene for cell structure and cell growth (*ANN*) and two genes for transporters (*AAT* and *MsALMT1*), were selected for RT-PCR analysis. RT-PCR analysis was performed as described in the Materials and Methods. The RT-PCR data (Fig. 3) suggested that the transcription profiles of the selected nine genes were all up-regulated. The results indicated that the data of the SSH library are reproducible and reliable.

Cloning and expression analysis of full-length MsALMT1 cDNA

ALMT1s are proteins that contain the UPF0005 motif and possess five to seven transmembrane domains. An *ALMT*-like gene (*MtALMT1*, ABD32183) was found in the *M. truncatula* genomic sequence (Delhaize *et al*. 2007). Using the BLASTX program, it was found that MtALMT1 also contains the UPF0005 motif. Based on the coding region sequence of *MtALMT1*, the full-length (1347 bp) *MsALMT1* ORF (GU550122) was cloned from the YM1 roots by PCR amplification. Sequence analysis indicates that the *MsALMT1* ORF encodes a deduced protein with 448 amino acid residues. Based on the sequences of the ALMT1 protein family published by the NCBI, a phylogenetic tree was constructed (Fig. 4). As shown in Fig. 4, all 10 sequences can be classified into three main groups, namely A, B and C at a genetic identity score of 0·40. At a genetic identity score of 0·60, group A was further divided into four subgroups (subgroups A1–A4). The deduced amino

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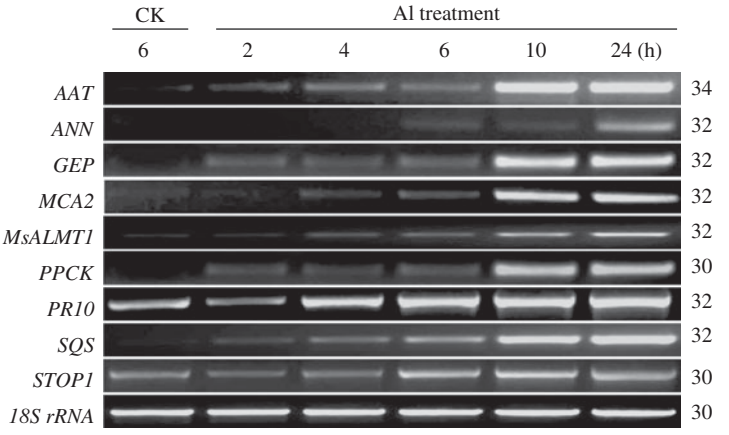

Fig. 3. Expression profile analysis of genes selected from the SSH library by RT-PCR. RT-PCR analysis was performed as described in the Materials and Methods. Number of PCR cycles was shown on the right side. CK (control) indicates the sample without Al treatment. Al treatment indicates samples treated with 5 μM Al (pH 4·2) for 2, 4, 6, 10 and 24 h. 18S rRNA was used as an internal control. AAT, cationic amionic acid transporter; ANN, annexin-like protein; GEP, germin-like protein; MCA, type IIB calcium ATPase (MCA2) gene; MsALMT1, Al-activated malate transporter; PPCK, phosphoenolpyruvate carboxykinase; PR10, class 10 PR protein; SQS, farnesyl-diphosphate farnesyltransferase; STOP1, sensitive to proton rhizotoxicity 1.

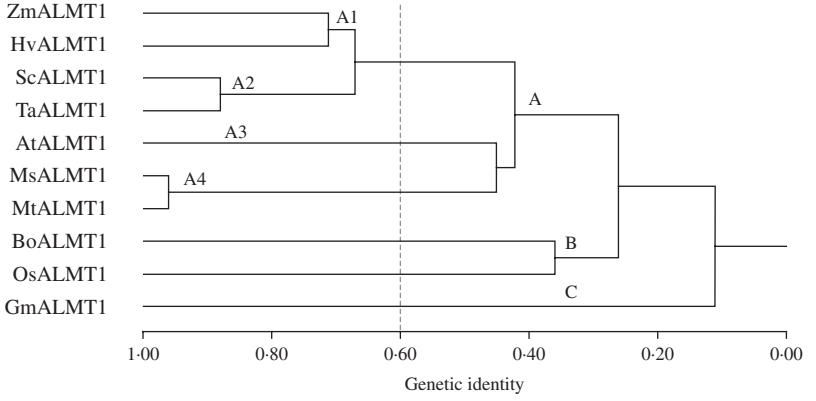

Fig. 4. The phylogenetic relationship between MsALMT1 and its homologues in other species. AtALMT1, *A. thaliana* AAF22890; MtALMT1, *M. truncatula* ABD32183; GmALMT1, *Glycine max* EU586179; BnALMT1, *Brassica napus* BAE97280; BoALMT1, *Brassica oleracea* AAW81734; OsALMT1, *O. sativa* NP-001042433; ScALMT1, *Secale cereale* ABA62397; TaALMT1, *Triticum aestivum* BAD10882; ZmALMT1, *Zea mays* ABC86748; HvALMT1, *Hordeum vulgare* EF424084.

acid sequence of MsALMT1 displayed 0·96, 0·46, 0·43 and 0·42 homology with MtALMT1, AtALMT1, GmALMT1 and TaALMT1, respectively. The DAS program analysis predicted that the MsALMT1 protein contains seven transmembrane domains (see supplementary Fig. S1 available at http://journals.cambridge.org/AGS). RT-PCR analysis confirmed that the transcript level of *MsALMT1* in the YM1 roots was induced under Al stress (Fig. 3), and no expression of *MsALMT1* was detected in the shoots of the plants treated with or without Al (data not shown). The expression pattern of *MsALMT1* is similar to that of *ALMT1s* in other plants.

Malate content in the YM roots and its exudation rate from the YM1 roots under 5 μM stress conditions

To validate the function of *MsALMT1* in the YM1 roots, malate exudation was determined after 0, 2, 4, 6, 10 and 24 h of Al treatment. The malate exudation-rate steeply increased during the period of 2–6 h of Al treatment (Fig. 5*a*). The data confirm the function of

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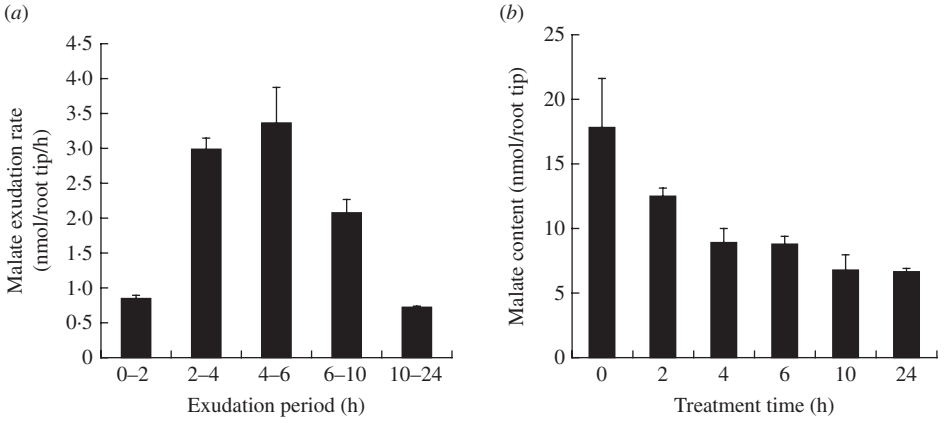

Fig. 5. The malate exudation rate (*a*) and content (*b*) of YM1 roots under 5 μM Al stress conditions. YM1 roots were treated with 5 μM Al (pH 4·2) for 0, 2, 4, 6, 10 and 24 h, respectively, as described in the Materials and Methods. Values are means ± S.D. ($n = 3$).

MsALMT1 in the YM1 roots. According to the criteria described by Ma *et al.* (2001), the malate exudation pattern in YM1 was characterized as Pattern II with a 6-h lag phase before the maximum period of malate exudation. However, YM1 could not maintain such a high level of Al-induced malate exudation beyond the 6–10 h and 10–24 h periods of Al treatment (Fig. 5*a*). Moreover, the SSH data showed that the genes involved in organic acid synthesis, including PEPC, CS and MDH were not up-regulated in YM1 roots. To verify whether the decrease in malate exudation was associated with the internal malate synthesis, the malate content in YM1 roots was determined. As shown in Fig. 5*b*, YM 1 root-tip tissue had higher malate content (17·73 nmol/root tip) before Al treatment (control). However, the malate content was gradually reduced after Al treatment. At 6, 10 and 24 h, malate contents in the YM1 root tips decreased by approximately 0·50, 0·38 and 0·37, respectively, compared to that in the control. The results suggested that the decrease in Al-induced malate exudation might be due to a reduction of malate synthesis in YM1 roots under Al stress conditions.

Oxidative stress levels in YM1 roots under 5 μM Al stress

A number of genes related to antioxidants were shown to be up-regulated in YM1 roots according to the SSH data (see Table 2). To better understand the molecular mechanisms within YM1 in response to Al stress, the oxidative stress level in the YM1 roots under stress of 5 μM Al was investigated. MDA and H_2O_2 contents are routinely used as the indicators of oxidative stress level. The contents of MDA and H_2O_2 in the YM1 roots were determined after treatment with 5 μM Al and both of them showed significant linear relationships with Al treatment time (Fig. 6). As shown in Fig. 6*a*, an H_2O_2 burst was triggered within 2 h after Al treatment. The H_2O_2 content was maintained at a higher level than that in the control throughout the entire treatment period. Similar changes were also observed with regard to the MDA content. The MDA content in YM1 roots was also markedly increased after exposure to 5 μM Al (Fig. 5*b*). The increase in H_2O_2 and MDA contents suggested that Al might induce ROS formation and lead to lipid peroxidation. Therefore, oxidation stress was severe in YM1 root cells under the 5 μM Al stress conditions. The up-regulation of antioxidant-related genes might be favoured of protecting root cells from damage by oxidative stress induced by Al toxicity.

DISCUSSION

In the present study, the SSH strategy was used to analyse gene expression changes in response to 5 μM Al stress in the roots of an Al-sensitive *M. sativa* L. cv YM1, which is an important alfalfa cultivar in China. The data obtained from the SSH cDNA library showed the up-regulation of 137 known Al-associated genes that had been found in other Al-tolerant species and 92 novel Al response genes under 5 μM Al stress conditions. The results suggest that the Al-sensitive YM1 shares some Al response mechanisms with Al-tolerant species but still has some mechanisms that are different from those in Al-resistant plants.

It is well known that antioxidant genes are up-regulated in response to Al stress in *Arabidopsis* (Richards *et al.* 1998), barley (Guo *et al.* 2007), sugarcane (Watt 2003) and wheat (Darkó *et al.* 2004) roots. The excessive production of ROS induced by Al causes oxidative damage to cell membrane lipids and

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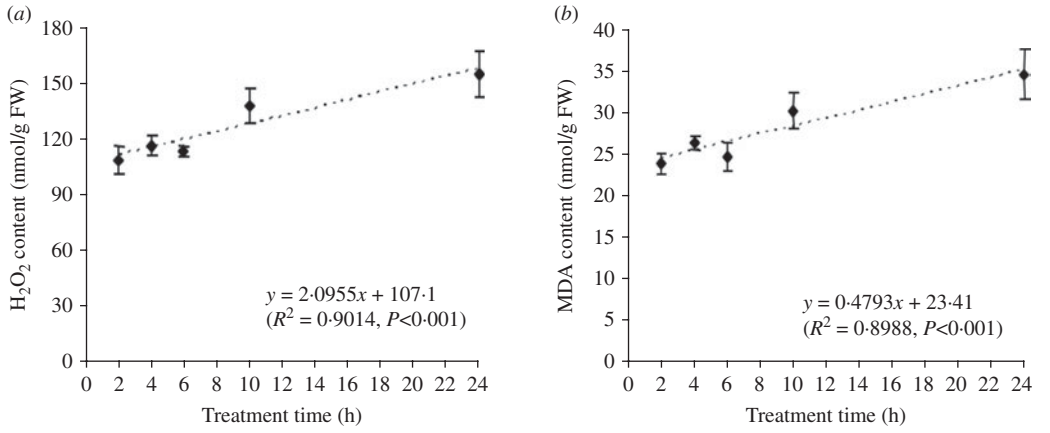

Fig. 6. Effects of 5 μM Al stress on H_2O_2 (*a*) and MDA (*b*) contents in YM1 roots. Values are means ± S.D. (n = 3). YM1 roots were treated as described in Fig. 5.

DNA strands (Guo *et al.* 2007). The cellular damages can be protected by antioxidant enzymes, such as GST, POD, catalase (CAT) and SOD. Darkó *et al.* (2004) showed that the activities of CAT and GST enzymes in Al-tolerant plants were greater than those in Al-sensitive plants, resulting in less accumulation of ROS in the roots of Al-tolerant plants. Furthermore, the over-expression of Al response genes, such as tobacco GST gene (*parB*) and POD gene from *Arabidopsis* (*AtPox*), conferred transgenic *Arabidopsis* with not only oxidative resistance but also Al tolerance (Ezaki *et al.* 2000). These results suggest that antioxidant enzymes may play a role in the detoxification of Al-induced ROS in Al-resistant plants. GEPs are ubiquitous plant proteins, and most of them have antioxidant enzyme activities by binding with Mn/Zn/Cu. The expression of GEPs is considered to be associated with biotic and abiotic stresses (Membre *et al.* 2000; Gucciardo *et al.* 2007). The analysis of H_2O_2 and MDA contents confirmed that 5 μM Al stress resulted in pronounced elevation of H_2O_2 and MDA levels in YM1 roots. MDA formation is used as a general indicator of lipid peroxidation. This suggests that 5 μM Al stress did cause a severe oxidative stress in YM1 roots. The SSH data revealed the up-regulation of a number of genes encoding antioxidant enzymes (GST, POD, SOD and GEP) in YM1 roots by Al stress. This might improve its resistance to the oxidative stress caused by Al toxicity.

Ma *et al.* (2001) described two patterns for Al-stimulated secretion of organic acids from plant roots. For pattern I, Al directly activates an anion channel on the plasma membrane and plants release organic acids immediately after Al treatment, whereas in pattern II, Al interacts with the cell wall and, perhaps via an unknown signal pathway, activates the transcription of genes involved in the metabolism of organic acids or their transports across the plasma membrane. The exudation of organic acids is mediated by membrane-localized organic acids transporters (Delhaize *et al.* 2007), and these proteins belong to two families, ALMT1 and MATE. Recently, genes encoding the ALMT1 and MATE protein families were identified from wheat (*TaALMT1*), *Arabidopsis* (*AtALMT1* and *AtMATE*), rye (*ScALMT1*), rape (*BnALMT1* and *BnALMT2*) and sorghum (*SbMATE*) (Delhaize *et al.* 2007). The full-length cDNA of the putative ALMT1 gene, *MsALMT1*, was also cloned from the YM1 roots, and the sequence analysis confirms that it belongs to the ALMT1 transporter family. In *Arabidopsis* and rice, the organic acid transporter genes are regulated by the STOP1 and ART1 transcription factors, respectively (Liu *et al.* 2009; Yamaji *et al.* 2009). In YM1 roots, *MsALMT1* and *STOP1* were up-regulated and their expression levels were increased in a time-dependent manner after Al treatment (see Fig. 3). The induction of malate exudation from YM1 roots was triggered efficiently by Al and occurred within 0–2 h following Al exposure. The malate exudation pattern in YM1 roots was characterized as pattern II. This might suggest that Al-induced malate exudation was achieved through the function of *MsALMT1*. It is possible that *STOP1* is involved in the regulation of *MsALMT1* expression in YM1 roots.

The malate exudation pattern of YM1 roots is similar to that of Al-sensitive pattern-II species (Delhaize *et al.* 1993; Li *et al.* 2000; Rangel *et al.* 2010) but different from that of Al-resistant species in which organic acid exudation is maintained at a constant level or an ascending trend after the induction period (Delhaize *et al.* 1993; Yang *et al.* 2001; Zheng *et al.* 2005; Ryan *et al.* 2009; Rangel *et al.* 2010). YM1 could not maintain high levels of malate exudation after the induction period. The high contents of Al in the YM1 roots during these periods (see Fig. 1*b*)

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indicated that the released malate failed to chelate the external Al effectively. Several studies have indicated that the Al-induced secretion of organic acids is poorly associated with the internal organic acid concentration and the activity of organic acid metabolism enzymes (Ryan *et al.* 2001; Hayes & Ma 2003). However, the studies performed by Yang *et al.* (2001) and Rangel *et al.* (2010) suggested that an internal accumulation of organic acids conferred the enhanced organic acid exudation in some typical Pattern-II Al-resistant species of soybean and common bean under Al stress. Moreover, the Al-resistant common bean genotype Quimbaya had higher specific activities of CS (4-fold) and PEPC (1·6-fold) than that in Al-sensitive genotype VAX-1 under Al stress (Rangel *et al.* 2010). In agreement with these results, over-expression of organic acid metabolism enzymes such as CS and MDH in transgenic plants was shown to be able to enhance organic acid exudation and Al-tolerance (Koyama *et al.* 2000; Tesfaye *et al.* 2001). The malate content of YM1 roots decreased gradually over the whole period of Al treatment. Genes encoding enzymes associated with organic acid metabolism, such as CS, PEPC and MDH, were absent in the SSH library. As a result, the up-regulation of *MsALMT1* did not confer YM1 Al resistance under Al stress conditions. This finding is also supported by the evidence that over-expressing neMDH enhanced the malate exudation and Al resistance of transgenic *M. sativa* (Tesfaye *et al.* 2001).

The conclusion from the present study is that very low levels (5 μM) of Al stress induce a severe oxidative stress in the Al-sensitive YM1 roots. This severe oxidative stress induced the expressions of a series of antioxidant genes in YM1 roots. This suggests that YM1 responds to oxidative stress caused by Al toxicity in the same way as that found in Al-tolerant species. The up-regulation of *MsALMT1* and *STOP1* by 5 μM Al stress might be associated with a steep increase in the malate exudation rate for a short period of Al treatment. However, the gradual reduction of malate synthesis in YM1 roots under Al stress might not allow the malate exudation to sustain a plateau period or an increasing trend as is the case in Al-resistant species. These results suggest that YM1 does not contain a perfect system to cope with Al toxicity. This may be the main reason for the sensitivity of YM1 to Al. Thus, over-expressing genes associated with the enhancement of organic acid synthesis under Al stress might be one of the effective strategies to improve the Al resistance of YM1.

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