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CROPS AND SOILS RESEARCH PAPER

Physiological and transcriptional responses of broad bean (*Vicia faba* L.) leaves to aluminium stress

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Summary

Aluminium (Al) toxicity is the major factor-limiting crop productivity in acid soils. In the present study, physiological and transcriptional responses of broad bean leaves to Al stress were investigated. Malondialdehyde (MDA) content, H_2O_2 content and protein carbonyls (PC) level in leaves were increased after 100 μM $AlCl_3$ stress treatment, whereas the total protein content was decreased, compared with the plants without Al treatment. Stomatal closure in leaves of broad bean was increased after Al stress, suggesting that the photosynthesis rate might be affected by Al stress. The relative citrate secretion in leaves was decreased after Al treatment for 24 h according to the ^{13}C-NMR analysis, indicating that citrate in leaves might be transported to the root to chelate Al^{3+}. To investigate the molecular mechanisms of Al toxicity in leaves of broad bean, a suppression subtractive hybridization (SSH) library was constructed to identify up-regulated genes: cDNA from leaves subjected to 12, 24, 48 and 72 h of 50 and 100 μM $AlCl_3$ stress were used as testers and cDNA from leaves subjected to 0 μM $AlCl_3$ treatment for the same lengths of time as above were used as a driver. The SSH analysis identified 156 non-redundant putative Al stress-responsive expressed sequence tags (ESTs) out of 960 clones. The ESTs were categorized into ten functional groups, which were involved in metabolism (0·21), protein synthesis and protein fate (0·10), photosynthesis and chloroplast structure (0·09), transporter (0·08), cell wall related (0·06), signal transduction (0·05), defence, stress and cell death (0·05), energy (0·03), transcription factor (0·03) and unknown proteins (0·30). The effect of Al treatment on expression of 15 selected genes was investigated by reverse transcription polymerase chain reaction (RT–PCR), confirming induction by Al stress. The results indicated that genes involved in organic acid metabolism, transport, photosynthesis and chloroplast structure, defence, stress and cell death might play important roles under Al stress.

INTRODUCTION

Acidic soils occupy up to 0·40 of world arable land (Kochian 1995) and the extent of acid soil is increasing worldwide. In China, *c*. 0·21 of arable lands are acidic (Liu *et al*. 2004). As soil pH falls below 5·0, aluminium (Al) will be released mainly in the phytotoxic form of Al^{3+}. The Food and Agricultural Organization (FAO) of the United Nations regards Al toxicity as the second largest soil constraint to agriculture globally (Hoekenga & Magalhaes 2011).

In recent years, much research has been directed towards elucidation of the mechanisms of Al toxicity and resistance in plants, but most of these papers have addressed mainly the mechanisms of Al toxicity and tolerance in the plant root system (Zhou *et al*. 2007; Chen *et al*. 2011, 2012), because inhibition of root growth is one of the earliest symptoms of Al injury and the most easily recognized symptom in solution culture (Chen 2006). Accumulating evidence shows that Al affects photosynthesis, organic acid metabolism, nitrogen (N) metabolism and photoprotective systems in plant shoots (Chen 2006). Aftab *et al*. (2010) noted that net photosynthetic rate, stomatal conductance and internal carbon dioxide (CO_2) concentration were reduced upon exposure to the toxic levels of Al. Furthermore, Al stress has been shown to induce changes in oxidative metabolism, caused by an increase in the

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concentration of reactive oxygen species (ROS) and an altered expression of antioxidant genes (Dong *et al*. 2002; Panda & Matsumoto 2010).

Different mechanisms of Al^{3+} resistance have been reported. Plants may resist Al toxicity by avoidance (Al exclusion) and/or tolerance mechanisms (detoxification of Al inside the cells). Aluminium exclusion involves the exudation of organic acid anions from the root apices, whereas tolerance mechanisms comprise internal Al detoxification by organic acid anions (Inostroza-Blancheteau *et al*. 2011). It is almost impossible for plants to avoid Al uptake into the root, so plants have mechanisms for internal tolerance to some levels of Al stress. Internal tolerance may result from intracellular chelation of Al, ROS scavenging, modifications to lipid or cell-wall synthesis or other unknown mechanisms (Hoekenga & Magalhaes 2011).

Broad bean (*Vicia faba* L.) is a legume that can be used as a source of protein in human diets or as animal forage (Duc *et al*. 2010; Rubiales 2010; Ma *et al*. 2011). Broad bean has been shown to have a strong ability to uptake immobilized phosphorous (P) through the release of organic acids and protons from their roots to adjust the rhizosphere pH in P-deficient soils (Li *et al*. 2007). Antioxidant enzyme activities were enhanced in an Al-resistant broad bean cultivar under Al stress (Zhang *et al*. 2009). Chen *et al*. (2012) found that broad bean (*V. faba* L. cvar 8363) from Yunnan province of China was more resistant to Al than a cultivar of broad bean from Anhui Province and investigated the physiological and molecular responses of the broad bean root to Al stress. The present study investigated the physiological and transcriptional responses of Al-tolerant broad bean (*V. faba* L. cvar 8363) to Al stress in order to understand the mechanisms of Al resistance in broad bean leaves.

MATERIALS AND METHODS

Plant material and stress treatment

Broad bean (*V. faba* L. cvar 8363) seeds were supplied by the Yunnan Academy of Agricultural Science, Kunming 650205, China). Uniform and healthy seeds were selected and surface-sterilized with 75% alcohol for 5 min and rinsed 4–5 times with sterile water. After soaking in water for 24 h, seeds were germinated in the dark on moist filter paper at 25 °C. Germinated seeds were then cultured on a nylon net placed on a plastic pot containing tap water in the greenhouse of Kunming University of Science and Technology under natural conditions at 20–25 °C during the day and 13–18 °C at night. After 15 days growth, seedlings were initially grown in 0·5 mM $CaCl_2$ solution (pH 4·2) for 24 h, and then were transferred to 0·5 mM $CaCl_2$ solution containing 50 or 100 μM $AlCl_3$ for 12, 24, 48 and 72. The plants were maintained in a 16-h-light/8-h-dark regime and the control plants were grown without the addition of $AlCl_3$. After the treatment, the young leaves of broad bean were collected, frozen in liquid nitrogen immediately and stored at −80 °C.

Lipid peroxidation, H_2O_2 content, protein carbonyl levels and total protein content analysis

The lipid peroxidation of leaves was determined after 100 μM $AlCl_3$ treatment for 0, 12, 24, 48 and 72 h by estimating the formation of malondialdehyde (MDA), a product of lipid peroxidation, using the thiobarbituric acid reaction method described by Madhava & Sresty (2000). The concentration of MDA was calculated from the absorbance at 532 nm (correction was done by subtracting the absorbance at 600 nm for unspecific turbidity) by using extinction coefficient of 155 mM/cm. Hydrogen peroxide (H_2O_2) levels in the leaves of broad bean were examined according to Patterson *et al*. (1984). Measurement of protein carbonyl (PC) levels was performed as described by Gurel *et al*. (2005). Protein content was measured using bovine serum as a standard according to the method of Bradford (1976).

The analysis of leaf stomatal closure under aluminium stress treatment

Broad bean plants were pre-treated with $CaCl_2$ overnight and then treated with 100 μM $AlCl_3$ for 5 and 24 h. The first and second fully expanded leaves were taken and washed with double-distilled water (ddH_20) before peeling off the epidermis, washing it again with ddH_20 and cutting into strips 5 mm long. At least 20 cells per epidermal peel were measured under a microscope with a micrometre (Olympus). Measurements were performed on three to five plants per treatment.

^{13}C-nuclear magnetic resonance (NMR) analysis

Broad bean plants (2 weeks old) were pre-treated with $CaCl_2$ (0·5 mM, pH 4·2) overnight and then treated with 50 μM $AlCl_3$ for 24 h. Leaf samples (3 g each) were ground into powder with liquid N and then extracted with 4 ml 100 mM potassium phosphate buffer

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(KPB, pH 7·4, containing 100 mM maleic acid). The samples for ^{13}C-NMR analysis were prepared following the method of Chen *et al*. (2010). After thawing, the extract was boiled for 3 min and centrifuged at 12 000 rpm for 10 min to remove cellular debris. The supernatant was frozen, lyophilized and resuspended in 0·5 ml of 100 mM KPB. The methanamide was sealed in a capillary that was inserted into the 5-mm NMR tube containing the sample.

The ^{13}C-NMR analysis was performed on a Bruker DRX 500-MHZ instrument as described previously (Chen *et al*. 2010) and the data were collected using described parameters (Chen *et al*. 2010). Chemical shifts were obtained by reference to maleic acid at 130·66 ppm. The resonances were performed based on chemical shifts of reference samples. To calculate the relative contents of the ^{13}C relative contents of the metabolite, the target peaks (C4: 179 ppm, C1: 174 ppm, C2:73·5 ppm, C3: 42·7 ppm) of citrate were integrated relative to the maleic acid (reference) at 130·66 ppm.

Extraction of RNA and mRNA isolation

Leaves of broad bean were harvested at 0, 12, 24, 48 and 72 h after treatment with 0 (control), 50 and 100 μM $AlCl_3$ solution. Total RNA was extracted with TRIZOL reagent (Takara, Japan) according to the instructions of the manufacturer. The quantity and quality of the isolated total RNA were examined by spectrophotometry and gel electrophoresis, respectively. Genomic DNA was removed by DNAase I (Qiagen). For suppression subtractive hybridization (SSH), equal amounts of total RNA for each treatment or control were mixed and the mRNA was purified from the mixed total RNA using the Oligotex® mRNA Mini Kit (Qiagen) following the manufacturer's protocol.

Construction of the suppression subtractive hybridization library

A forward ($AlCl_3$ stress treatment as tester and control as driver) SSH library was constructed with the Clontech PCR-Selected™ cDNA Subtraction kit (BD Bioscience, USA) following the manufacturer's instructions, with some modifications. Briefly, driver and tester cDNAs were digested with *Rsa*I, phenol/chloroform-extracted, ethanol-precipitated and resuspended in water. Tester cDNA was split into two pools and then ligated with different adaptors (supplied in the cDNA subtraction kit). Two rounds of hybridization and PCR amplification were carried out to normalize and enrich the differentially expressed cDNAs according to the protocol: the primary PCR was performed as follows: 75 °C for 5 min, 94 °C for 25 s, and then 27 cycles of 94 °C for 10 s, 66 °C for 30 s and 72 °C for 1·5 min. The second PCR was performed for 12 cycles (94 °C for 10 s, 68 °C for 30 s, 72 °C for 90 s), and then secondary PCR products were purified and inserted into pMD18-T vector (Takara, Japan) and transformed into *Escherichia coli* DH5α-competent cells. The bacteria were plated on ampicillin-containing LB agar plates, which were overlaid with X-Gal and IPTG and incubated overnight at 37 °C.

Amplification of cDNA inserts

Nine hundred and sixty cDNA clones were selected randomly from the SSH library. The clones were freshly grown in 96-well plates overnight at 37 °C and used as templates. The cDNA inserts were amplified with nested PCR primers 1 and 2R provided in the PCR Select-cDNA Subtraction Kit, which were complementary to sequences flanking both ends of the cDNA insert.

The PCR reaction contained 16·1 μl sterile water, 2·5 μl 10 × buffer (including Mg^{2+}), 0·5 μl dNTP (2·5 mM each), 0·5 μl of each primer (10 μM each), 0·5 units of Taq DNA polymerase and 1 μl of bacterial culture. Thermocycling conditions were as follows: initial denaturation at 94 °C for 5 min, and then 30 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1·5 min and a final extension of 72 °C for 10 min. The PCR products were electrophoresed on a 0·8% agarose gel to confirm the amplification quality and quantity.

Sequence analysis

The insert cDNAs of the selected clones were sequenced using M13F and M13R primers. After removal of sequences of the vector and adapters (low-quality sequences), the sequences were submitted to the National Center for Biotechnology Information (NCBI) database, and BLASTX programs were used to search for known proteins homologous to cDNA sequences. After BLASTX, query coverage of >0·50 or an *E*-value <0 were identified as effective. Functional classification of the expressed sequence tags (ESTs) was carried out according to the MIPS Functional Catalogue (http://mips.helmholtz-muenchen.de/proj/funcatDB/, accessed 19 July 2013).

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Table 1. *Primers used for RT–PCR analysis in the leaves of broad bean under $AlCl_3$ stress (MDH: malate dehydrogenase; VP1, vacuolar-type H-pyrophosphatase; LOX, lipoxygenase; SBP, sedoheptulose-1,7-bisphosphatase, chloroplast; CS, citrate synthase; RBC, rbcS-3A gene for ribulose 1·5-bisphosphate carboxylase (RBC) small subunit; TK, transketolase; PAT, patatin homolog mRNA; HA, P-type H^+-ATPase; ABC, ABC1 family protein; HSP, heat shock protein 70; POD, peroxidase 2; Lhcb3 gene for chlorophyll a/b-binding protein; LHC, PSI light-harvesting antenna chlorophyll a/b-binding protein; PCA, phytochrome A*

Gene name	Forward (5′–3′)	Reverse (5′–3′)	Product (bp)
MDH	CAGTGCCCTGTTATGGTC	GCTGCTTTCCCTCTTCTT	315
VP1	ATGGAAGCGTCGGTGGAG	TTGGTGCCTTTGTGAGCC	250
LOX	GATGGTCTTGTTCTTTGGGATG	GTTGACCGCTGCGTGATG	249
SBP	ATGAGTTTCTCCTGCTTG	CACCTTCCTTGAGCCTTG	456
CS	CTAACATTGCCGCCTTCA	GGTCTGGGAACCAACCTA	231
RBC	TTTCGTTCGTATCATCGG	GTAACATTTGCAGCTTTG	229
TK	CTAAGGTTCTTCCTGGTCT	TCTATTGGCTGATGAGTTG	331
PAT	GCACGCTTCTGTTTCATC	CTGCACCAACTTATCTACCTC	130
HA	AATGATGGCAGCCCTCTA	ATTACACCAGCCCAACCC	215
ABC	ACACGCTGTGAATGAGGA	CGTAGAGCTGGATTTGGA	348
HSP	AGTAGGCGGGAACAGTAA	TCAGGGTGATATGAAGCA	203
POD	CGGCGTTAGATGCTGTTC	GGATTGCGTATTGTTTGT	345
Lhcb3	TTTACCAGCCACAATAGC	GTAGAAGGTTTCCGCATC	201
LHC	GCTTGAACAAACCACCTC	GATGGCTACCGTTACTACAC	282
PCA	ACTTACACTACCCACTTAG	CTTGTCCTGGAAATGAGC	210
16S	TCAAGGTCGGGGACAACAGT	ACGGACTCTTTACGCCCAAT	394

RT–PCR analysis

Total RNA of leaves isolated by TRIZOL reagent (Takara, Japan) from 0, 12, 24, 48 and 72 h treatments were used for RT–PCR analysis. Genomic DNA was removed by DNAase I. First strand cDNA was synthesized from 2 μg total RNA from each sample using Moloney Murine Leukaemia Virus (M-MLV) reverse transcriptase (Invitrogen, USA) according to the supplier's instructions. Broad bean 16S rRNA was used as inner control for RT–PCR analysis. The cDNAs were diluted to 20- or 40-fold. All primers for the candidate genes and 16S rRNA are listed in Table 1. General PCR was conducted according to the following programme: an initial denaturation at 94 °C for 3 min, followed by 23–30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 3 min. The RT–PCR experiments were repeated three times and the PCR products were detected by 1% agarose gel in 1 × TAE with EtBr.

Statistical analysis

Data were analysed with OriginPro8 (Version8E, OriginLab Corporation, Massachusetts, USA) and presented as means of three replicates ± standard errors.

RESULTS

The lipid peroxidation, H_2O_2 content, protein carbonyl levels and total protein content under aluminium stress in broad bean leaves

To understand the toxic effects of Al on broad bean leaves at the physiological level, changes in physiological characteristics of broad bean plants exposed to 100 μM Al stress were investigated. As shown in Fig. 1, MDA content, H_2O_2 content and PC levels were increased after 100 μM Al stress treatment compared with the control plants. The MDA content increased after 100 μM Al treatment for 12 h and then the content decreased in 48 and 72 h, but the MDA content was still higher than the control without Al treatment in leaves of broad bean (Fig. 1*a*). The results indicated that there was lipid peroxidation after Al stress in broad bean leaves. After Al treatment for 12, 24, 48 and 72 h, the H_2O_2 content increased at 12 h and then decreased at 24 h (Fig. 1*b*), which meant that Al stress-induced oxidative stress in broad bean leaves. The PC content after Al stress treatment showed a trend similar to that of the H_2O_2 content (Fig. 1*c*). The PC contents in broad bean leaves of 100 and 0 μM Al stress treatment for 12 h were 1·53 and 0·972 mmol/kg, respectively. After 12 h treatment, the total protein content increased, and then the content decreased gradually,

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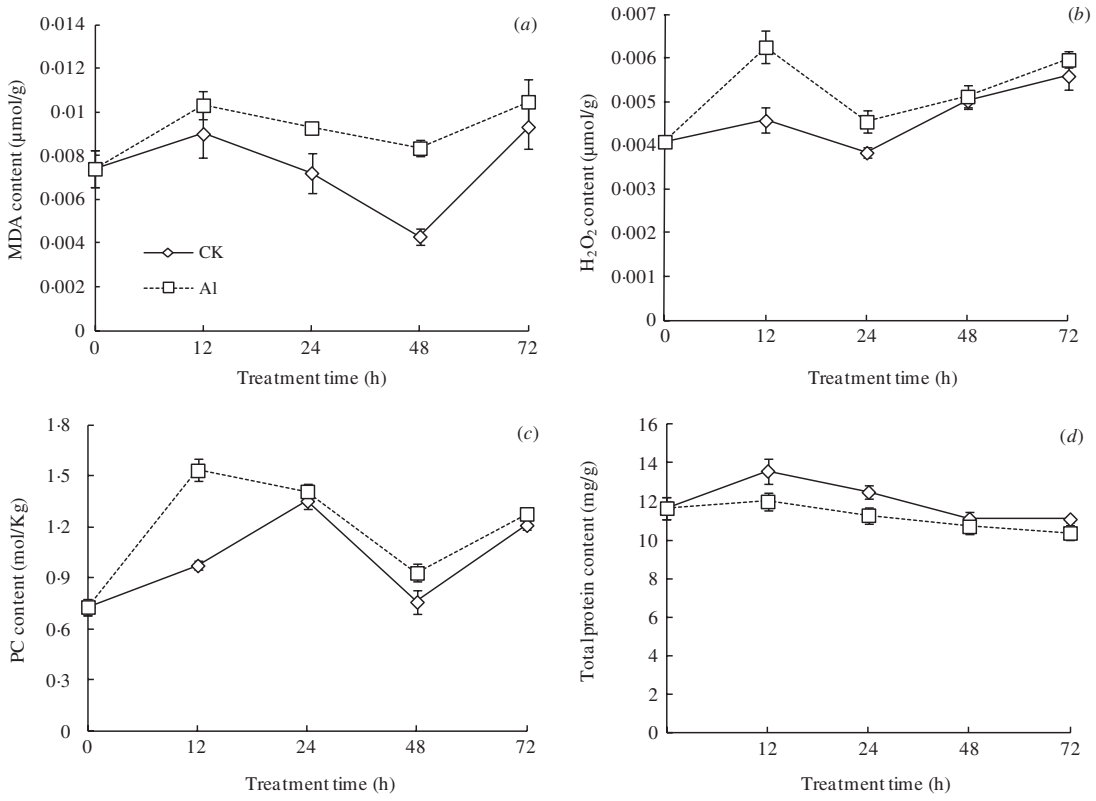


Fig. 1. Effect of Al stress on (*a*) MDA content, (*b*) H_2O_2 content, (*c*) PC levels and (*d*) total protein content in leaves of broad bean after 100 μM Al treatment for 0, 12, 24, 48 and 72 h. Values presented were means of three replicates.

but the content was lower than the control plants (Fig. 1*d*).

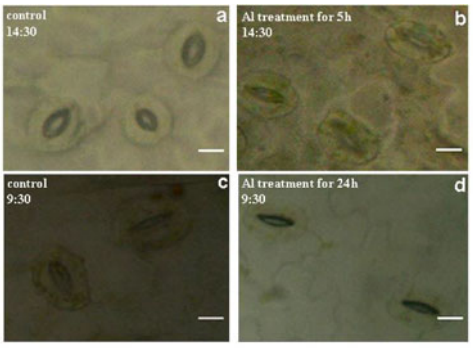
Stomatal conductance of broad bean leaves

To evaluate the effect of Al stress on the leaf stomatal conductance, the stomata were observed using a microscope and the lengths and widths of the stomata after Al treatment for different lengths of time were measured. As shown in Fig. 2, the stoma after Al treatment for 5 h were more fully closed than in the control plants without Al treatment. The ratio of length to width of the stoma was increased after Al stress treatment, especially after 24 h treatment (24·0 ± 2·0, compared with the control of 6·33 ± 0·83) (Fig. 2*b*). These results indicated that the photosynthesis rate of broad bean leaves might be affected by Al stress treatment.

^{13}C-nuclear magnetic resonance analysis of the relative citrate content in leaves of broad bean

The synthesis of citrate took place mainly within mitochondria and was catalysed by citrate synthase (CS).

(*a*)



(*b*)

Treatment	Ratio of length to width of stomata	
Time (h)	Control	Al treatment
5	2·8 ± 0·07	4·9 ± 0·17
24	6·3 ± 0·83	24 ± 2·0

Fig. 2. (*a*) Observations of stomatal conductance in broad bean leaves after 100 μM Al treatment for different times. (*b*) The ratio of length to width of stomata of broad bean after 100 μM Al stress treatment. Colour version available online.

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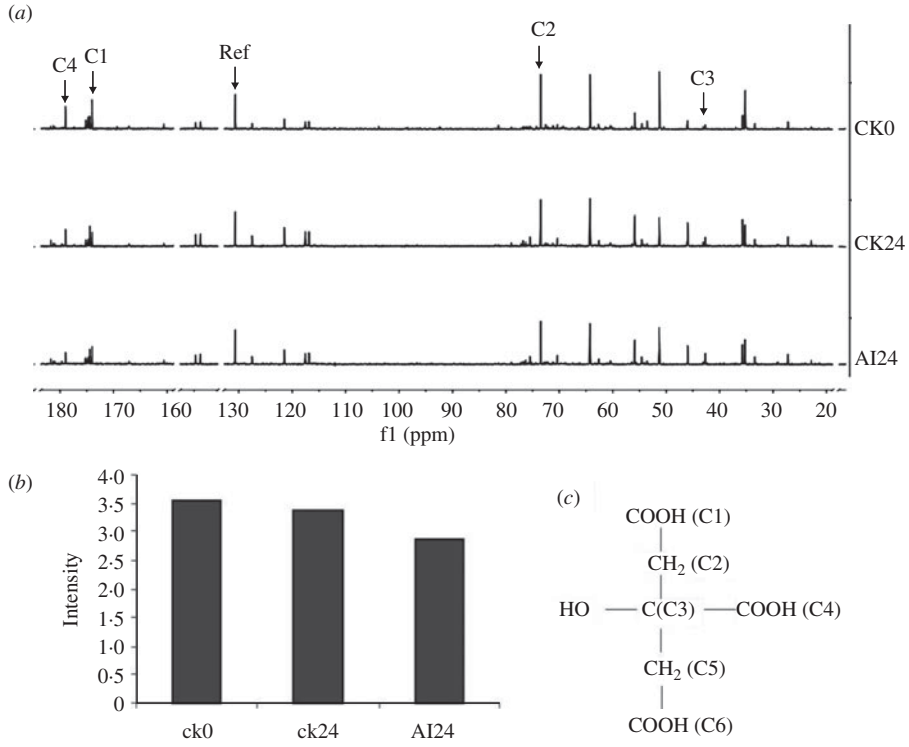

Fig. 3. ^{13}C-NMR analysis of relative citrate content in broad bean leaves with or without Al stress treatment. (*a*) Complete ^{13}C-NMR spectra of broad bean leaves without Al treatment for 0 and 24 h (CK0 and CK24) and 50 μM Al treatment for 24 h (Al24). Peak assignments of C1, C2, C3, C4 indicate different C atoms of citrate as shown in (*c*). Ref indicates the resonance peak of malate acid in 130·66 ppm. (*b*) Relative intensity of CK0, CK24 and Al24 was estimated by adding the sum of C1, C2, C3 and C4.

The relative citrate content was analysed using ^{13}C-NMR. The relative citrate content of broad bean leaves after Al treatment for 24 h (Al 24) decreased to 80% of that in the control plants (Fig. 3*b*).

Construction of the suppression subtractive hybridization library

In order to identify up-regulated genes during Al stress, a forward SSH library was constructed with 50 and 100 μM $AlCl_3$ treated leaves for 0, 12, 24, 48 and 72 h as tester and treated with 0 mM Al for the same as the driver set. A set of 960 bacterial colonies were picked randomly from the library and assayed for the presence of a cDNA insert with nested primers. Electrophoresis of the PCR product showed that the insert fragments ranged from *c*. 200 to 1200 base pairs (bp) (data not shown).

Sequence analysis of the cDNA clones

The size distribution and the sequence of cDNAs were determined for 301 clones. All of the clones from these 301 that showed a single PCR product were sequenced. The insert length varied from 210 to 1172 bp. Using BLASTX, a total of 208 putative ESTs (including 156 non-redundant ESTs) were identified, of which 47 failed to match any EST in the public database and were therefore considered as new.

The 156 non-redundant differentially expressed genes were functionally annotated by blasting against the GenBank protein database, and subsequently classified into ten functional categories according to their putative functions as follows: cell-wall-related, metabolism, signal transduction, transcription factor, transporter, defence stress and cell death, energy, protein synthesis and protein fate, photosynthesis and chloroplast structure and unknown (Fig. 4). The category of cell wall-related contained ten distinct proteins deduced from 16 ESTs, which accounted for 0·06 of the classified ESTs. Other categories were as follows: metabolism (33 distinct proteins; 43 ESTs, 0·21), signal transduction (eight distinct proteins; eight ESTs, 0·05), transcription factor (four distinct proteins; four ESTs, 0·03), defence, stress and cell death

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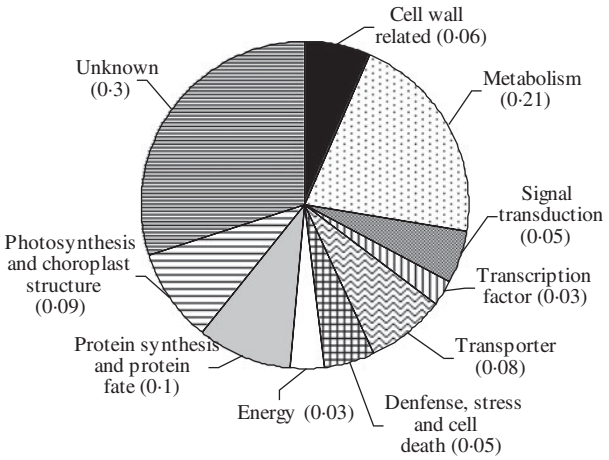

Fig. 4. Distribution of differentially expressed genes in broad bean under Al stress. A total of 156 non-redundant ESTs were grouped into ten categories based on MIPS functional categories. The proportion of gene transcripts in each group is listed.

(eight distinct proteins; 15 ESTs, 0·05), transporter (12 distinct proteins; 17 ESTs, 0·08), protein synthesis and protein fate (15 distinct proteins; 21 ESTs, 0·10), energy (five distinct proteins; six ESTs, 0·03), photosynthesis and chloroplast structure (14 distinct proteins; 31 ESTs, 0·09) and unknown proteins (47 distinct proteins, 47 ESTs, 0·30) (Fig. 4).

Genes involved in organic acid metabolism, transporter, defence, stress and cell death, photosynthesis and chloroplast structure

In the EST database, genes encoding enzymes involved in organic acid metabolism, transport, defence, stress and cell death, photosynthesis and chloroplast structure were identified and summarized (Table 2). There were genes encoding for organic acid metabolism in the SSH library created in the present work, such as cytosolic malate dehydrogenase and CS, which might play important roles under Al stress. In the present study, two clones encoding protein ABC were found. There were also some genes involved in defence, stress and cell death, such as heat shock protein (HSP), peroxidase, type 1 metallothionein to release Al stress. Fourteen distinct genes encoding photosynthetic and chloroplastic proteins were identified, such as ribulose bisphosphate carboxylase/oxygenase activase1, ribulose 1,5 bisphosphate carboxylase, chloroplast chlorophyll a/b binding protein, photosynthesis II protein.

Validation of the suppression subtractive hybridization results

To validate the reliability of the SSH library data, 15 genes were investigated for response to Al stress by RT–PCR, including organic acid metabolism [malate dehydrogenase (MDH), CS], photosynthesis and chloroplast structure (*Lhcb3, SBP, LHC, PCA, RBC*), transporter (*VP1, ABC*), defence, stress and cell death (*HSP, POD, PAT*) and other genes involved in Al stress. Total RNA was isolated from the leaves of additional plants under 50 and 100 μM Al stress for 0, 12, 24, 48 and 72 h. The expression analysis of the selected genes increased in a time-dependent manner (Fig. 5). The result confirmed that the expression of all 15 ESTs tested was regulated by Al treatment in broad bean and demonstrated that the SSH analysis was reliable.

DISCUSSION

Oxidative stress is an important part of Al toxicity (Richards *et al*. 1998). It has been reported that Al^{3+} can alter cell redox homeostasis as a consequence of enhanced production of ROS (Tamás *et al*. 2003). The MDA content, H_2O_2 content and PC levels increased after Al stress, which meant that Al stress produced oxidative stress in broad bean (Fig. 1). Several genes encoding antioxidative enzymes such as glutathione S-transferase, peroxidase and superoxide dismutase (SOD) were induced under Al stress. Peroxidase has previously been shown to be induced upon Al exposure (Richards *et al*. 1998; Milla *et al*. 2002). In the SSH library created through the present work, one gene encoding peroxidase (*HS514038*) was isolated and RT–PCR confirmed that the expression of peroxidase was up-regulated after Al treatment. Heat shock proteins are a key component of defence systems in the stress responses of higher plants. Induction of HSPs under stress helps to maintain normal protein structure and function in all organisms (Basha *et al*. 2004). The present study identified an HSP and RT–PCR analysis found that the HSP expression was increased dramatically, and expression maintained, 48 and 72 h after Al treatment.

Organic acids, such as malate and citrate are able to form strong complexes with Al by chelating Al ions present in the apoplast or by the internal detoxification of this metal by reversing its toxic effects inside the cell (Ma *et al*. 2011). In the SSH library of the present work, Al stress induced the expression of three transcripts involved in the organic acid pathway: CS (HS513973),

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Table 2. *Selected known Al-responsive genes in broad bean leaves*

Clone no.	Putative function	Identity (%)	*E*-value	Organism	Length (bp)	Accession no.
Metabolism						
1-B8	2-C-methyl-D-erythritol,2, 4-cyclodiphosphate synthase	98	3e-60	*Medicago truncatula*	342	HS513834
1-C4	Fatty acid 2-hydroxylase-like	81	2e-21	*Glycine max*	404	HS513961
1-C7	Cytosolic malate dehydrogenase	96	1e-90	*Malus x domestica*	428	HS513835
4-D6	Citrate synthase	93	3e-69	*Sesbania rostrata*	416	HS513973
2-A7	Lipoxygenase	76	7e-101	*Solanum lycopersicum*	580	HS513963
2-A8	Glucose-6-phosphate1-dehydrogenase	91	2e-89	*Vitis vinifera*	439	HS513837
2-F10	Glycine dehydrogenase P protein	94	5e-97	*Medicago truncatula*	354	HS513901
2-H8	Allantoate amidohydrolase	95	7e-83	*Medicago truncatula*	401	HS513966
3-C3	Sedoheptulose-1 7-bisphosphatase	95	3e-98	*Medicago truncatula*	461	HS513969
3-E12	1-aminocyclopropane-1-carboxylate oxidase	63	1e-59	*Medicago truncatula*	504	HS513970
4-B8	Phosphomannomutase/ phosphoglucomutase-like	87	2e-146	*Glycine max*	511	HS514052
4-C7	Steroid 5-alpha reductase	97	1e-48	*Cicer arietinum*	459	HS513972
5-B3	Dihydroflavonol-4-reductase	83	3e-145	*Medicago sativa*	524	HS513975
5-F10	Acyl-(acyl carrier protein) thioesterase	89	2e-34	*Medicago truncatula*	388	HS513978
5-H1	Methylmalonate-semialdehyde dehydrogenase	88	6e-78	*Medicago truncatula*	697	HS513979
8-H12	Beta amylase	91	4e-114	*Vigna unguiculata*	1132	HS513841
10-H4	Granule-bound starch synthase	97	9e-170	*Pisum sativum*	757	HS513994
5-G9	Menaquinone biosynthesis methyltransferase ubiE	89	9e-151	*Medicago truncatula*	440	HS514011
9-H5	ATP sulfurylase	98	2e-100	*Glycine max*	441	HS513989
9-H12	Transketolase	94	2e-100	*Platanus x acerifolia*	485	HS513990
10-D2	Ferredoxin-thioredoxin reductase	48	4e-11	*Vitis vinifera*	439	HS513991
10-E12	6-phosphogluconate dehydrogenase	98	6e-92	*Medicago sativa*	473	HS513992
10-G8	Violaxanthin de-epoxidase	81	3e-61	*Medicago truncatula*	418	HS513993
1-G8	Myo-inositol-1-phosphate synthase	93	3e-07	*Glycine max*	210	HS513842
1-E9	Light-dependent NADH: protochlorophyllide oxidoreductase 3	91	2e-35	*Solanum lycopersicum*	416	HS513995
3-B12	Alanine aminotransferase	96	3e-109	*Medicago truncatula*	875	HS513844
3-B3	Alanine glyoxylate aminotransferase	96	7e-73	*Medicago truncatula*	455	HS513968
8-H11	GDSL esterase/lipase APG	90	3e-80	*Medicago truncatula*	411	HS514014
2-B3	Amino acid aminotransferase	77	3e-84	*Glycine max*	728	HS513937
8-C6	Bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase	94	0	*Glycine max*	349	HS513958
9-B1	*N*-carbamoyl-L-amino acid hydrolase	79	1e-11	*Medicago truncatula*	1172	HS513951
6-G8	Chloroplast magnesium chelatase H subunit	97	3e-105	*Pisum sativum*	587	HS514029
H11	2-oxoisovalerate dehydrogenase	95	1e-88	*Medicago truncatula*	734	HS514035
Transporter						
2-G11	Vacuolar proton-inorganic pyrophosphatase	99	2e-117	*Medicago truncatula*	557	HS513965
3-C6	H+-pyrophosphatase	98	1e-51	*Vitis hybrid cultivar*	685	HS513996
1-B5	Aquaporin	98	7e-96	*Vicia broad*	824	HS514023
4-E12	Inner membrane protein PPF-1	92	5e-130	*Pisum sativum*	523	HS513904
1-F2	P-type H^+-ATPase	99	8e-94	*Vicia broad*	421	HS514024
1-F6	Rho guanine nucleotide exchange factor	94	7e-127	*Medicago truncatula*	1099	HS514025
4-A1	Triose phosphate/phosphate translocator	100	6e-109	*Pisum sativum*	513	HS514027

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Table 2. (*Cont*.)

Clone no.	Putative function	Identity (%)	*E*-value	Organism	Length (bp)	Accession no.
4-D7	Protein ABC1	88	9e-152	*Ricinus communis*	1129	HS513849
10-H5	ABC transporter G family member 21-like	86	4e-37	*Glycine max*	538	HS514017
10-D11	ATP synthase CF0 subunit IV	100	1e-116	*Medicago truncatula*	531	HS514031
4-H5	Mitochondrial 2-oxoglutarate/malate carrier protein	93	2e-42	*Medicago truncatula*	412	HS514032
9-H2	Ferritin-3	93	3e-73	*Medicago truncatula*	731	HS514050
Defence, stress and cell death						
1-H8	Heat shock protein	97	2e-105	*Medicago truncatula*	510	HS514033
7-F5	Heat shock protein, Hsp70	97	1e-122	*Medicago truncatula*	599	HS514042
5-E11	Peroxidase	92	7e-144	*Trifolium repens*	725	HS514038
3-F9	Type 1 metallothionein	79	3e-25	*Pisum sativum*	406	HS514036
4-H7	S-adenosylmethionine decarboxylase proenzyme	96	2e-40	*Vicia broad*	835	HS513974
2-B11	Dormancy-associated protein	82	1e-131	*Pisum sativum*	644	HS514034
6-F3	Seed maturation-like protein	82	2e-64	*Medicago truncatula*	711	HS513946
3-A6	Patatin group A-3-like	64	4e-56	*Glycine max*	553	HS514018
Photosynthesis and chloroplast structure						
2-D11	Phosphoribulokinase	81	2e-180	*Arabidopsis thaliana*	1117	HS513852
4-F5	Ribulose bisphosphate carboxylase/ oxygenase activase1	77	6e-52	*Glycine max*	579	HS514054
1-B6	Putative rubisco activase	94	5e-72	*Rhododendron kawakamii*	387	HS513833
7-D3	Ribulose 1,5 bisphosphate carboxylase	98	1e-23	*Pisum sativum*	341	HS513911
3-A9	Chloroplast chlorophyll a/b binding protein	99	4e-150	*Pisum sativum*	751	HS513919
4-H3	Photosystem I reaction centre subunit II	87	5e-69	*Medicago truncatula*	526	HS513923
8-E11	Photosystem II 5 kDa protein	70	5e-26	*Medicago truncatula*	587	HS513927
6-H2	Photosystem II protein D1	99	9e-117	*Phaseolus vulgaris*	627	HS513930
10-F1	Photosystem II 32 kDa protein	97	0	*Gackstroemia magellanica*	554	HS513899
4-H9	Photosystem I reaction centre subunit III	89	1e-25	*Vigna radiata*	390	HS513931
5-B6	Putative PSII-P protein	96	7e-61	*Trifolium pratense*	504	HS513932
6-B11	Photosystem II protein D2	88	0	*Parthenium argentatum*	1125	HS513881
2-G10	Photosystem II 22 kDa protein	96	2e-84	*Medicago truncatula*	485	HS513917
6-F5	Phytochrome A	96	1e-76	*Callerya megasperma*	769	HS513935

malate dehydrogenase (HS513835) and alanine aminotransferase2 (HS513844). The synthesis of citrate takes place mainly in mitochondria and is catalysed by CS, which converts oxaloacetate to citrate and is the 'pace-maker' of the tricarboxylic acid cycle, modulating synthesis of other organic acids. Of the organic acids that participate in normal respiratory metabolism, citrate has the highest binding activity for Al (Samac & Tesfaye 2003). In the present study, it was found that the relative citrate content in broad bean leaves decreased after Al stress for 24 h compared with the control plants, indicating that the citrate synthesized in leaves might be transported to the root to alleviate Al stress. Tesfaye *et al*. (2001) found that overexpression of a malate dehydrogenase in transgenic alfalfa produced a 7·1-fold increase in root exudation of organic acids. However, Zhou *et al*. (2009) found that in tomato root, the MDH protein was suppressed.

Previous studies have shown that Al toxicity affects photosynthesis in many plant species (Jiang *et al*. 2008; He *et al*. 2011). Photosynthesis is principally conducted by leaves that can supply sufficient precursors for the synthesis of organic acids. In Al-tolerant rye plants, the decrease in net photosynthetic rate was found to be accompanied by stomatal closure after Al treatment (Silva *et al*. 2012). Stomata provide the major pathway of gas exchange. In the present experiment,

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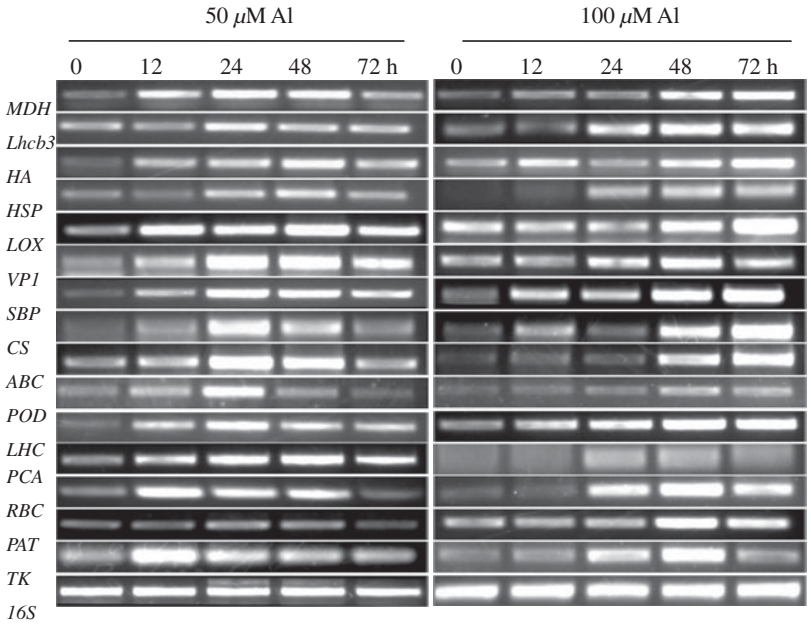

Fig. 5. RT–PCR analysis of ESTs of broad bean treated with 50 and 100 μM Al treatment for different times (*MDH*: malate dehydrogenase; *Lhcb3*:*Lhcb3* gene for chlorophyll a/b-binding protein; *HA*, P-type H^+-ATPase; *HSP*, heat shock protein 70; *LOX*, lipoxygenase; *VP1*, vacuolar-type H-pyrophosphatase; *SBP*, sedoheptulose-1,7-bisphosphatase, chloroplast; *CS*, citrate synthase; *ABC*, ABC1 family protein; *POD*, peroxidase 2; *LHC*, PSI light-harvesting antenna chlorophyll a/b -binding protein; *PCA*, phytochrome A; *RBC, rbcS-3A* gene for ribulose 1·5-bisphosphate carboxylase (RBC) small subunit; *PAT*, patatin homolog mRNA; *TK*, transketolase).

the stomata of Al-treated plants closed more fully than in control plants, which might affect the photosynthesis rate under Al stress. Light activates proton (H^+)-ATPases in guard cells, to drive hyperpolarization of the plasma membrane to initiate stomatal opening, allowing diffusion of ambient CO_2 to photosynthetic tissues (Merlot *et al*. 2007). The RT–PCR analysis showed that the expression of H^+-ATPases decreased after 24 h of 100 μM Al stress treatment, which meant that the H^+-ATPase might function to enhance the closure of stomata under Al stress.

The impact of Al toxicity in photosynthesis is indirect: it leads to a disturbance in the chloroplast architecture. Moreover, the reduction of electron transport in photosystem II (PSII) resulted in a decrease of photosynthesis (Panda *et al*. 2009). Ribulose 1, 5 bisphosphate carboxylase/oxygenase (Rubisco) has been found to be deactivated after heat stress (Law *et al*. 2001). However, in the forward SSH library of the present work under Al stress, the gene encoding ribulose 1, 5 bisphosphate carboxylase/oxygenase (Rubisco) was isolated. One gene with high homology to Rubisco activase was also found in the present study. It is generally believed that Rubisco activase plays a vital role in releasing sugar phosphate from the active site of Rubisco to facilitate CO_2 fixation. Enhanced expression of Rubisco activase might be important to maintain some protein functions under Al stress conditions.

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