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Transcriptomic analysis reveals differential gene expression in response to aluminium in common bean (*Phaseolus vulgaris*) genotypes

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• *Background and Aims* Aluminium (Al) resistance in common bean is known to be due to exudation of citrate from the root after a lag phase, indicating the induction of gene transcription and protein synthesis. The aims of this study were to identify Al-induced differentially expressed genes and to analyse the expression of candidate genes conferring Al resistance in bean.
• *Methods* The suppression subtractive hybridization (SSH) method was used to identify differentially expressed genes in an Al-resistant bean genotype ('Quimbaya') during the induction period. Using quantitative real-time PCR the expression patterns of selected genes were compared between an Al-resistant and an Al-sensitive genotype ('VAX 1') treated with Al for up to 24 h.
• *Key Results* Short-term Al treatment resulted in up-regulation of stress-induced genes and down-regulation of genes involved in metabolism. However, the expressions of genes encoding enzymes involved in citrate metabolism were not significantly affected by Al. Al treatment dramatically increased the expression of common bean expressed sequence tags belonging to the citrate transporter gene family *MATE* (multidrug and toxin extrusion family protein) in both the Al-resistant and -sensitive genotype in close agreement with Al-induced citrate exudation.
• *Conclusions* The expression of a citrate transporter *MATE* gene is crucial for citrate exudation in common bean. However, although the expression of the citrate transporter is a prerequisite for citrate exudation, genotypic Al resistance in common bean particularly depends on the capacity to sustain the synthesis of citrate for maintaining the cytosolic citrate pool that enables exudation.

Key words: Aluminium resistance, aluminum, citrate exudation, common bean, *MATE*, *Phaseolus vulgaris*, transcriptomic analysis, differential gene expression.

INTRODUCTION

Common bean (*Phaseolus vulgaris*) is produced in the tropics on small-scale farms where unfavourable edaphic factors limit the yield potential. Among others, soil acidity which affects about 40 % of the world arable land (Von Uexküll and Mutert, 1995) accounts for 30–40 % yield reduction in Africa and Latin America (CIAT, 1992). The crop yield on acid soils is mainly limited by aluminium (Al) toxicity. In addition, other soil acidity-related stresses, such as proton toxicity, Mn toxicity, and nutrient deficiencies particularly of P, Mg, Ca and Mo are also important constraints (Marschner, 1995). Al toxicity causes inhibition of root growth by injuring primarily the root apex of the growing plant (Ryan *et al.*, 1993; Sivaguru and Horst, 1998; Rangel *et al.*, 2007).

Common bean is generally less adapted to acid soil environments and improving Al resistance of common bean to reduce the dependence of small-scale farmers on lime and nutrient inputs is a major challenge (Rao, 2001). However, efforts to develop adapted genotypes indicate that there are genotypic differences in Al resistance in the bean germplasm (Rao, 2001; Rangel *et al.*, 2005; Manrique *et al.*, 2006).

Comparing two contrasting bean cultivars 'Quimbaya' (Al-resistant) and 'VAX 1' (Al-sensitive), Rangel *et al.* (2010) found that Al resistance in common bean is attributed to the release of citrate by the root apex. Organic acid anions such as citrate, malate and oxalate detoxify Al through forming a non-phytotoxic organic acid–Al complex. Ma *et al.* (2001) described two patterns of organic acid secretion: pattern I plants release organic anions immediately after the onset of Al treatment while in pattern II plants, organic anion release starts after a lag phase of several hours. This suggests that in pattern I the organic anion release mechanism is constitutively expressed, whereas in pattern II plants the induction of the resistance mechanism involves gene expression, and new protein synthesis. Common bean proved to be a typical pattern II plant (Rangel *et al.*, 2007, 2010). The delay in citrate exudation was not due to the limitation of internal citrate reserve but to the absence of citrate permeases. The role of organic anion permeases in Al resistance was recently reviewed by Delhaize *et al.* (2007). Aluminium resistance genes of several plant species have been identified and found to

encode membrane proteins which mediate the exudation of organic acid anions from the root. These proteins belong to two families, ALMT (Al-activated malate transporter) and MATE (multidrug and toxic compound extrusion). The ALMT facilitates malate efflux in plant species that depend on malate exudation as the Al resistance mechanism (Sasaki *et al.*, 2004; Hoekenga *et al.*, 2006; Ligaba *et al.*, 2006). On the other hand, the MATE proteins are citrate transporters which play a decisive role in Al-induced citrate exudation (Furukawa *et al.*, 2007; Magalhaes *et al.*, 2007; Ryan *et al.*, 2009).

On the basis of the results published by Rangel *et al.* (2010), it was hypothesized that the expression of a citrate transporter and the enhanced synthesis of citrate are crucial for sustained Al resistance in common bean. Thus, the objectives of this work were: (*a*) to study the transcriptional changes occurring between the onset of Al treatment and the beginning of citrate release in the Al-resistant common bean genotype 'Quimbaya' using suppression subtractive hybridization (SSH); and (*b*) to analyse the expression of selected candidate genes which may have significant roles in Al resistance using quantitative real-time PCR (qRT-PCR).

MATERIALS AND METHODS

Plant material and growth condition

Two common bean (*Phaseolus vulgaris* L.) genotypes with known differential Al resistance (Rangel *et al.*, 2005, 2010) were used in this study. The seeds of Al-resistant genotypes 'Quimbaya' and Al-sensitive genotype 'VAX 1' were germinated in filter papers sandwiched between sponges soaked with tap water. After 4 d, uniform seedlings were transferred to 18-L pots with a continuously aerated nutrient solution (containing 5 mM $CaCl_2$, 1 mM KCl and 8 μM H_3BO_3) in a controlled climate chamber, with a 16/8 h light/dark regime, 27/25 °C day/night temperatures, 70 % relative air humidity and a photon flux density of 230 μmol m^{-2} s^{-1} (photosynthetic active radiation) at the plant canopy. The pH of the nutrient solution was gradually lowered to 4·5 within 2 d. Then the plants were treated with or without 20 μM $AlCl_3$ for various durations of time up to 24 h. Root growth was measured at 4, 8 and 24 h of Al treatment.

Determination of citrate exudation and citrate contents of root apices

Citrate exudatation from root tips and citrate contents of root tips were determined as described by Rangel *et al.* (2010). Briefly, plants were pre-treated with or without 20 μM Al for 3, 7 and 23 h at pH 4·5 as described above. To collect root exudates from intact root apices, 12 pre-treated plants were bundled in filter paper soaked with nutrient solution. Approximately 1 cm of the main root apex of each plant was immersed into 18 mL of a constantly aerated collection solution containing 5 mM $CaCl_2$, 8 μM H_3BO_3 and 0 or 40 μM $AlCl_3$, pH 4·5, in 20-mL poly prep filtration columns (BioRad Laboratories, Richmond, CA, USA). The Al concentration in the collection medium was doubled in order to compensate for the small volume and thus low total Al supply.

After 2 h of incubation, the collection solution containing the root exudates was immediately frozen at −20 °C for later citrate determination. At the end of incubation period, the root tips (1 cm) were excised with a razor blade, rinsed with double deionized water, transferred to Eppendorf reaction vials and fixed immediately in liquid nitrogen to measure the citrate content in the root tissue. The citrate concentrations in the root exudates as well as in the root tissue extracts were measured by isocratic high-pressure liquid chromatography (Kroma System 3000; Kontron Instruments, Munich, Germany).

RNA isolation and construction of the SSH library

For construction of the SSH library, only the Al-resistant genotype 'Quimbaya' was used. The plants were treated with or without 20 μM $AlCl_3$ for 4 h as described above. At the end of the treatment time, roots were rinsed with distilled water and ten root tips (1 cm long) per plant were harvested and shock-frozen in liquid nitrogen. Root tips of 15 plants per treatment were bulked and ground to powder in liquid nitrogen. Total RNA was isolated using the NucleoSpin RNA plant kit (MACHEREY-NAGEL GmbH and Co., KG, Düren, Germany) following the manufacturer's protocol. Tester and driver cDNAs were prepared from the total RNA of each treatment (control and 4 h Al treatment) using the Super SMART PCR cDNA synthesis kit (Clontech Laboratories, Inc., Mountain View, CA, USA). SSH was performed using PCR-Select cDNA subtraction kit (Clontech Laboratories, Inc.). Forward and reverse subtraction libraries were constructed using cDNA samples of control (no Al) versus 4 h Al treatment. The subtracted cDNAs were subjected to two rounds of PCR to normalize and enrich cDNA populations. The PCR products were sub-cloned into the pCR2·1-TOPO Vector (Invitrogen, Carlsbad, CA, USA) by T-A cloning. The vector was used to transform *Escherichia coli* TOP10 (Invitrogen) competent cells. Transformed clones were grown on LB plates containing X-gal and ampicillin for blue/white screening. Positive clones were checked for the presence of gene inserts after plasmid isolation and *Eco*R1 digestion. A total of 144 clones containing putative differentially expressed genes were further analysed and the gene inserts were sequenced (ITT, Bielefeld, Germany).

Sequence homology search

In order to identify putative gene functions of the differentially expressed genes, the cDNA sequences were compared with the GenBank database using the online Basic Local Alignment Search Tool (BLAST) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Candidate-gene selection and primer design

Candidate genes were selected either from the authors' SSH library or from a public database based on previous results on the physiological characterization of the mechanisms of Al resistance in common bean (Rangel *et al.*, 2005, 2007, 2010). The sequences of candidate genes were initially obtained from the arabidopsis database (TAIR). Then,

similar sequences of known genes of legumes and expressed sequence tags (ESTs) of common bean (*P. vulgaris*) were searched and gathered for sequence alignment. Finally, primers were designed in such a way that they anneal to part of the sequence which is well conserved among the legume species. Primers were designed using Primer3 software (Rozen and Skaletsky, 2000). The list of candidate genes and their respective primer pairs are shown in Table 1.

The citrate transporter gene *MATE* is a member of a large gene family. Several ESTs of *P. vulgaris* which have similarity with known *MATE* genes were gathered and aligned to assess their homology. Based on the alignment result (see Fig. S1 in Supplementary data, available online), they were grouped into three classes (*MATE-a*, *MATE-b* and *MATE-c*) and appropriate primers were designed as described above. *MATE-a* and *MATE-c* have nucleotide-sequence similarities of 81 % and 75 %, respectively, with the arabidopsis *MATE* gene (locus: AT1G51340). Likewise, *MATE-b* has 72 % similarity with the arabidopsis *FRD3* (ferric reductase defective 3) gene (locus: AT3G08040).

Quantitative RT-PCR

Two common bean genotypes 'Quimbaya' (Al-resistant) and 'VAX 1' (Al-sensitive) were grown and treated with or without 20 μM AlCl$_3$ for 1, 2, 3, 4, 8 and 24 h as described above. At the end of each treatment time, the roots were rinsed with distilled water and ten root tips (1 cm) per plant were harvested and shock-frozen in liquid nitrogen. Root tips of 15 plants per treatment were bulked and ground to powder in liquid nitrogen. Total RNA was isolated from the root tips as described above. First-strand cDNA was synthesized using RevertAid H-Minus first strand cDNA synthesis kit (Fermentas, www.fermentas.com). Random hexamer primers were used for this purpose. The reaction was stopped by heating at 70 °C for 10 min followed by 20 min incubation at 37 °C after addition of 10 U RNase-H (EPICENTRE Biotechnologies, www.epibio.com). Quantitative RT-PCR was undertaken using the Applied Biosystems StepOne Plus thermocycler (www.appliedbiosystems.com). The SYBR Green detection system was used with self-prepared SYBR Green master mix including a passive reference dye, ROX. The constituents of the qRT-PCR reaction mix were 1× hot-start PCR buffer, 3·6 mM MgCl$_2$, 0·5 μM ROX, 0·1× SYBR Green-I, 200 μM each dNTP (dATP, dTTP dCTP dGTP), 252 nM each forward and reverse primer, 0·75 U hot-start Taq DNA polymerase, 2 ng μL^{-1} cDNA template and ultra-pure DNase/RNase-free distilled water in a final volume of 25 μL. The qRT-PCR cycling stages consist of initial denaturation at 95 °C (3 min), followed by 45 cycles of 95 °C (15 s), 60 °C (30 s), 72 °C (30 s), and a final melting curve stage of 95 °C (15 s), 60 °C (1 min) and 95 °C (15 s). The fluorescence signal was recorded during the strand elongation step at 72 °C and the melting curve stage at every 0·3 °C temperature ramp. Samples for qRT-PCR

TABLE 1. *List of genes and specific primer pairs used for quantitative gene expression analysis*

Candidate gene	Primer pairs (5′ → 3′)*	Amplicon size (bp)	GenBank accession no.
PFK (phosphofructokinase)	(+) ACCCTTGCAAGTCGAGATGT (−) CTGCACACTCTCGGAAACAA	171	FE688067
PEPC (phosphoenolpyruvate carboxylase)	(+) TGGCTCCTTCCAAAGTGAGT (−) TCCTCCCCGTGTAAATTCTG	100	AF288382
CS (citrate synthase)	(+) CGACGGATATTCAAGGATGG (−)CGTGATCACTGTGGATGGAA	142	FE693132
Aco (aconitase)	(+) TCCAGTGTGTTGCCTGACAT (−) GACTTGGGGTCCCATGAGTA	116	CV536664
ICDH (isocitrate dehydrogenase)	(+) GCTCATTTTGCCTTTCCTG (−) TTCACACGAGCTTCATCTGG	165	SSH library
MDH (malate dehydrogenase)	(+) CCAACTGCAAGGTTCTGGTT (−) GCCCTGTTGTGATCCAATCT	124	FE677903
STOP1 (transcription factor)	(+) GCTCTAACTGCCGATGAGAA (−) TCTCTCCAGCTCCTCCTGAA	159	SSH library
MATE-a (citrate transporter family)	(+) GCTGGATGCAGTTTCAAGAGAG (−) ACTCCAGCAGCTGCAAGTTC	138	CV535133
MATE-b (citrate transporter family)	(+) TGCTGTTCAAGCCATTCTAGC (−) TCCAACAGCAAGAGAGAGTCC	124	CV534527
MATE-c (citrate transporter family)	(+) GTGACACTGGCTGCATCATT (−) GAGAAACTGCCAACCAAACC	91	FD792891
ALS3 (Al-sensitive3)	(+) ACAAGCTTGGCCTCCAGATA (−) GCGTTGTCCTGGTTGAAGAT	106	CV532021
ALMT1 (Al-activated malate transporter)	(+) TTCGCCCCATCTGGGCTGGT (−) TCCGGGGTTTCACTGCCATCA	118	CV543751
VDAC (voltage dependent anion channel)	(+) TGCCTCGTTGACTCTGAATG (−) CCGAGGTACCAAGGATGTGA	146	SSH library
ACC-oxidase[†]	(+) GAAGATGGCGCAAGAAGAAG (−) TGGAGCAAAGGTTCAAGGAG	105	SSH library
β-Tubulin	(+) CCGTTGTGGAGCCTTACAAT (−) GCTTGAGGGTCCTGAAACAA	117	CV530631

* (+) and (−) indicate forward and reverse primers, respectively.
[†] ACC-oxidase = aminocyclopropane-1-carboxylic acid oxidase.

were run in three biological replicates and two technical replicates. Relative gene expression was calculated using the comparative $\Delta\Delta C_T$ method according to Livak and Schmittgen (2001). For the normalization of gene expression, three housekeeping genes, namely 18S rRNA, actin and β-tubulin, were tested; β-tubulin was found to be most stable. Accordingly, β-tubulin was used as an internal standard and the control plants of the Al-resistant genotype 'Quimbaya' were used as reference sample. The PCR efficiencies of the β-tubulin and the target genes were comparable and thus relative gene expression was calculated without efficiency correction.

Statistical analysis

A completely randomized experimental design with three to six replicates was used for each experiment. The general linear model procedure of the statistical program SAS 9·1 (SAS Institute, Cary, NC, USA) was used for analysis of variance as well as multiple mean comparison using the Tukey test. For qRT-PCR data, relative gene expression and standard deviation were calculated using StepOne Plus software (Applied Biosystems). Expression levels were considered to be significantly different only if the fold change is at least three times higher/lower than the control and if the standard deviations are not overlapping.

RESULTS

Root growth of both genotypes 'Quimbaya' and 'VAX 1' was reduced by >50 % within 4 h of Al treatment (Fig. 1A). However, 'Quimbaya' gradually recovered from the initial Al stress and recovered to nearly full growth within 24 h. In contrast, 'VAX 1' showed a transient recovery after 8 h, but later the growth was again severely inhibited. Citrate exudation from root tips was determined after 4, 8 and 24 h Al treatment (Fig. 1B). Citrate exudation was induced by Al treatment after a lag phase of 4 h in genotype 'VAX 1', but in genotype 'Quimbaya' the lag phase lasted >4 h. After 8 h the exudation rate remained constant in genotype VAX-1, but in genotype 'Quimbaya' the citrate exudation-rate steeply increased up to 24 h of Al treatment. The Al-induced exudation of citrate might be related to the citrate contents of the root-tip tissue. Therefore, the citrate contents of the 1-cm root tips were determined after the collection of root exudates in order to study the effect of Al-treatment duration on the dynamics of the citrate content in the root tissue (Fig. 1C). Genotype 'Quimbaya' had constitutively higher citrate contents than 'VAX 1'. After 8 h Al treatment the citrate content of both genotypes were significantly reduced compared with their respective controls. This was related to the enhanced citrate exudation (Fig. 1B). Striking differences between the two genotypes were observed in the ability to restore the tissue citrate content. While the Al-resistant genotype 'Quimbaya' was able to restore its tissue citrate level, in the Al-sensitive genotype 'VAX 1' the citrate pool was depleted after 24 h. A close relationship existed between the dynamics of citrate exudation after the lag phase (Fig. 1B) and the recovery of root growth after 8–24 h Al treatment (Fig. 1A).
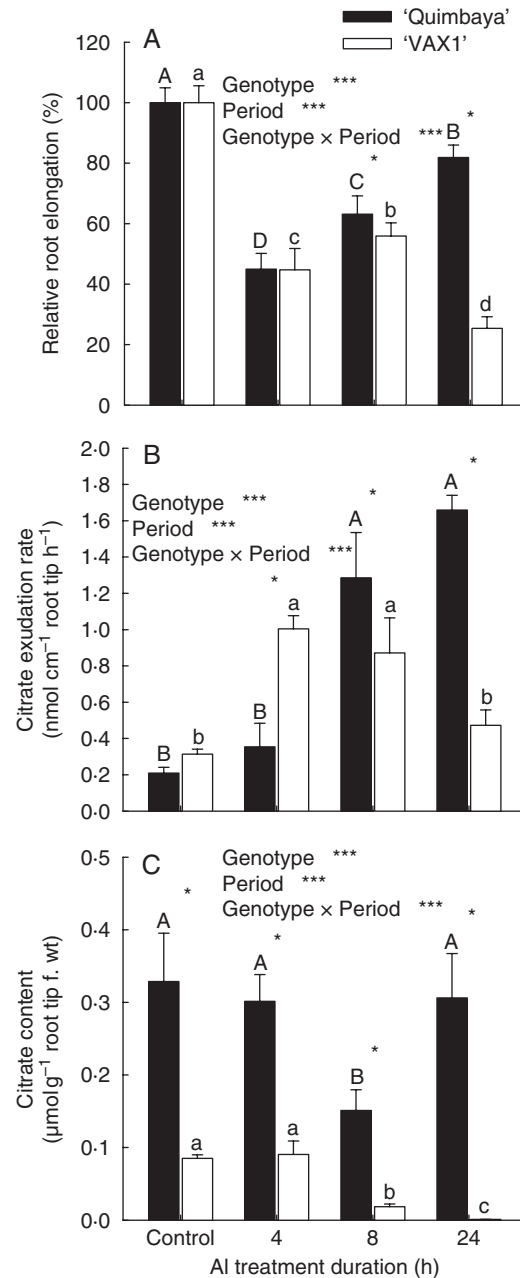

FIG. 1. (A) Root growth, (B) citrate exudation rate and (C) citrate content of 1-cm root tips of two common bean genotypes, 'Quimbaya' (Al-resistant) and 'VAX 1' (Al-sensitive), grown in simplified nutrient solution treated with or without 20 μM Al, pH 4·5, for up to 24 h. For the determination of citrate exudation and root-tissue citrate-content, plants were pre-cultured in nutrient solution with or without 20 μM Al, pH 4·5 for 3, 7, or 23 h. Root exudates were collected for a period of 2 h in simplified collection solution with or without 40 μM Al, pH 4·5. Citrate content of the root tissue was determined at the end of collection period. Bars are means + standard deviation of four to six replicates. For the analysis of variance, three asterisks denote significance at $P < 0.001$. Means with the same letter are not significantly different between exudation periods for 'Quimbaya' (upper case) and VAX-1 (lower case); asterisks over data points show significant differences between genotypes within each treatment time (Tukey test, $P < 0.05$). Control plants were not treated with Al.

Differential gene expression in Al-resistant genotype 'Quimbaya', in response to Al stress was assessed by using SSH. Through forward and reverse subtraction of cDNA
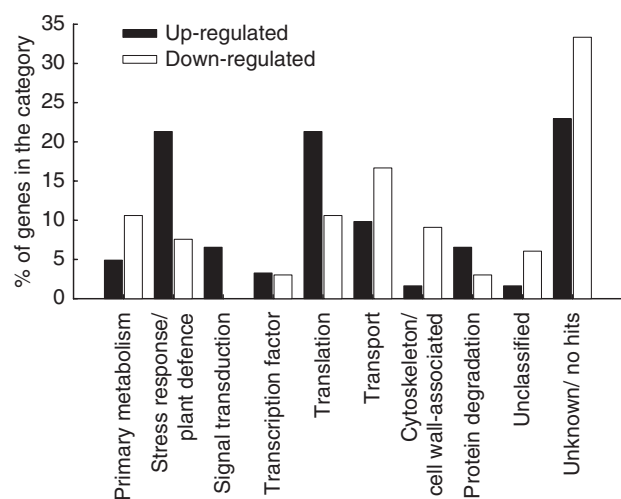
FIG. 2. Functional categories of genes differentially expressed (SSH) by 4 h Al treatment in root tips of Al-resistant bean genotype 'Quimbaya'. Up-regulated genes are those whose transcripts were more abundant in Al-treated than in the control sample and vice versa for down-regulated genes.

samples (4 h Al treatment vs. control) 127 Al-induced differentially expressed transcripts, 61 up-regulated and 66 down-regulated genes were obtained (see Tables S1 and S2 in Supplementary data, available online). These genes were grouped into several functional categories as shown in Fig. 2. Al treatment affected the transcription of genes involved in a wide range of functions. Transcripts which were up-regulated due to Al stress include genes involved in stress response, plant defence, signal transduction and translation. In contrast, genes involved in primary metabolism, transport processes, cytoskeleton and cell wall organization were largely down-regulated. This indicates that Al toxicity induces manifold changes in the plant root, ranging from perception of stress signals, gene transcription and translation to downstream physiological processes. The up-regulated stress-responsive genes include those encoding peroxidases, heat shock proteins and dehydrins. Peroxidases may contribute to the plant Al resistance by detoxifying reactive oxygen species which are produced as a result of oxidative stress induced by Al. Down-regulated genes such as auxin influx-carrier and cytoskeleton-associated proteins confirm the physiological characterization of the mechanism of Al toxicity. However, this study was restricted to the genes which could be involved in citrate exudation and metabolism which was the focus of the physiological studies (Fig. 1). These include genes encoding isocitrate dehydrogenase, an anion-selective channel, a mitochondrial ATP synthase and cyto-chrome P450 monooxygenase. Their possible contribution to Al resistance is briefly summarized in Table 2.

In addition candidate genes were selected based on the physiological mechanisms of Al resistance in bean, which is a sustained release of citrate from the root tips (Fig. 1). The expression of two groups of genes was closely investigated using qRT-PCR. The first group included genes encoding the enzymes involved in citrate metabolism, while the second group consisted of genes encoding ion transporters. Aluminium treatment did not significantly alter the expression of genes coding for enzymes which play a role in citrate synthesis and degradation (Fig. 3). Besides, there were few or no genotypic differences in Al-induced changes in gene expression. However, constitutively lower expression of genes coding for phosphoenolpyruvate carboxylase (PEPC) and isocitrate dehydrogenase (ICDH) was observed in 'VAX 1' than in 'Quimbaya'. The expression pattern did not change when the plants were treated for 8 or 24 h (data not shown).

The release of organic acid anions is mediated by membrane-localized anion transporters, whose expressions were known to be regulated by the *STOP1* transcription factor. In beans, short-term Al treatment (4 h) slightly increased the expression of *STOP1* (Fig. 4) but the expression levels of *MATE-a*, *MATE-b* and *ALS3* were greatly enhanced by Al treatment in both bean genotypes. Yet, Al did not affect the transcript abundance of *MATE-c*, *ALMT1* and *VDAC* (voltage-dependent anion-selective channel; Fig. 4).

Upon extended Al treatment duration, the expression of *MATE-a* and *MATE-b* continued to increase in the Al-sensitive genotype 'VAX 1' but not in the Al-resistant genotype 'Quimbaya' (Fig. 5). In order to find out the exact timing of *MATE*-gene induction, root samples were collected at 1-h intervals during the first 4 h of Al treatment. Enhanced expression of the *MATE-a* gene was observed as early as 2 h Al treatment in 'VAX 1', but in 'Quimbaya' the expression was delayed until 3 h (Fig. 6). The expression levels continued to increase with time in both genotypes. Similar to Fig. 5, the expression level was much higher in 'VAX 1' than in 'Quimbaya'. The earlier induction of *MATE* genes in this genotype is in agreement with the earlier induction of citrate exudation in 'VAX 1' (Fig. 1B).

Finally, the expression of a typically stress-induced gene coding the ACC-oxidase (1-aminocyclopropane-1-carboxylic acid oxidase) which was differentially expressed according

TABLE 2. *Differentially expressed genes (SSH) related to Al resistance in common bean genotype 'Quimbaya'*

| Clone ID	GenBank acc. no.	Annotation	Identity (%)	E-value	Up-/down-regulated	Putative function
0_153	DQ072165	VD anion-selective channel*	97	1E-154	up	Anion transport
0_156	M64246	ATP synthase (F1 alpha)	99	0E + 00	up	ATP production through proton gradient
0_178	DQ340249	Cytochrome P450 monooxygenase	92	0E + 00	up	Detoxification of toxic compounds
4_86	L12157	NADP-Isocitrate dehydrogenase	88	2E-138	down	Conversion of isocitrate to 2-oxoglutarate in the TCA cycle.

* VD = voltage-dependent.
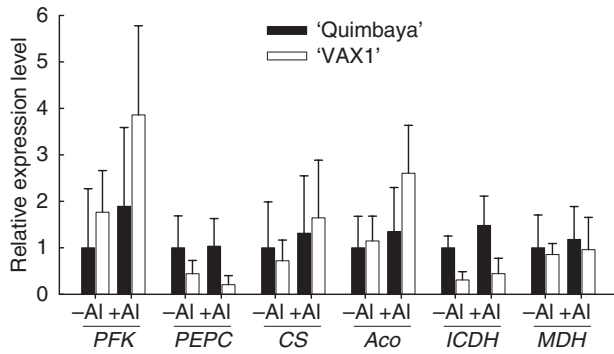
FIG. 3. Expression of genes encoding enzymes involved in citrate metabolism in common bean genotypes 'Quimbaya' (Al-resistant) and 'VAX 1' (Al-sensitive) grown in nutrient solution treated with or without 20 μM Al for 4 h. Total RNA was extracted from root tips. Quantitative RT-PCR was performed using the β-tubulin gene as internal standard and untreated plants of the Al-resistant genotype 'Quimbaya' as calibrator. Relative gene expression was calculated from three biological and two technical replicates; standard errors are shown.
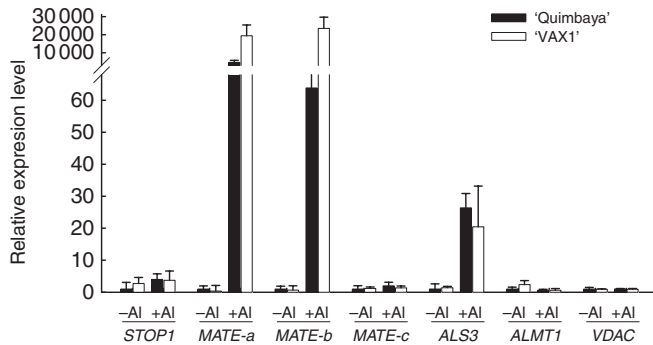

FIG. 4. Expression of genes regulating/encoding ion transporters in the common bean genotypes 'Quimbaya' (Al-resistant) and 'VAX 1' (Al-sensitive) grown in nutrient solution treated with or without 20 μM Al for 4 h. Total RNA was extracted from root tips. Quantitative RT-PCR was performed using the β-tubulin gene as internal standard and untreated plants of the Al-resistant genotype 'Quimbaya' as calibrator. Relative gene expression was calculated from three biological and two technical replicates; standard errors are shown.

to the SSH study (Table S1 in Supplementary data) was determined (Fig. 7). Early Al stress (4 h) resulted in a >10-fold increase in the expression of ACC-oxidase gene in both bean genotypes. In a subsequent analysis, enhanced gene expression could be found in both genotypes as early as 1 h after Al treatment (data not shown). But with Al treatment duration, it decreased to the original expression level in 'Quimbaya' while it continued to increase in 'VAX 1'. This stress response is similar to the response in Al-induced inhibition of root growth (Fig. 1A) and thus might be used as an internal marker of Al stress.

DISCUSSION

Using the SSH method, a range of genes which are differentially expressed in response to Al stress were identified in common bean. Aluminium triggered the expression of genes related to plant-stress response, plant defence and signal
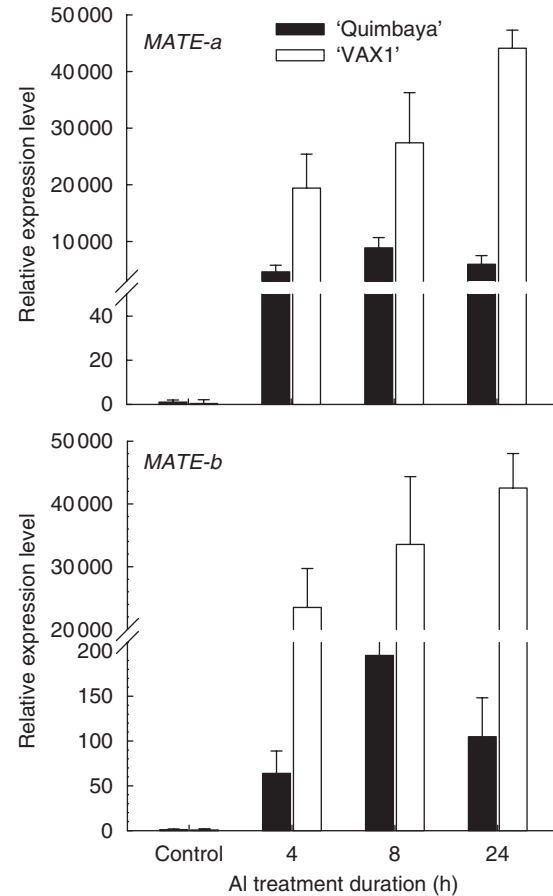

FIG. 5. Expression of two *MATE* genes under extended duration of Al treatment in the common bean genotypes 'Quimbaya' (Al-resistant) and 'VAX 1' (Al-sensitive) grown in nutrient solution treated with or without 20 μM Al for up to 24 h. Total RNA was extracted from root tips. Quantitative RT-PCR was performed using the β-tubulin gene as internal standard and untreated plants of the Al-resistant genotype 'Quimbaya' as calibrator. Relative gene expression was calculated from three biological and two technical replicates; standard errors are shown. Control plants were not treated with Al.
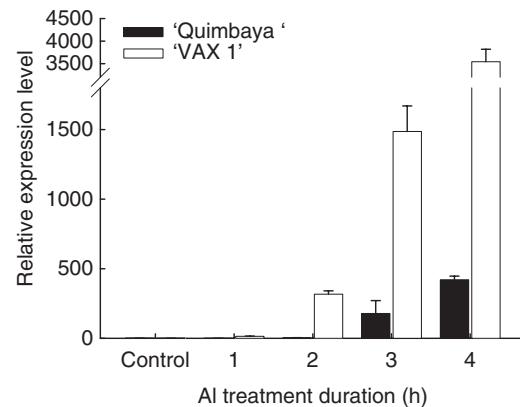

FIG. 6. Expression of *MATE-a* genes under short-term Al treatment in the common bean genotypes 'Quimbaya' (Al-resistant) and 'VAX 1' (Al-sensitive) grown in nutrient solution treated with or without 20 μM Al for up to 4 h. Total RNA was extracted from root tips. Quantitative RT-PCR was performed using the β-tubulin gene as internal standard and untreated plants of the Al-resistant genotype 'Quimbaya' as calibrator. Relative gene expression was calculated from three biological and two technical replicates; standard errors are shown. Control plants were not treated with Al.
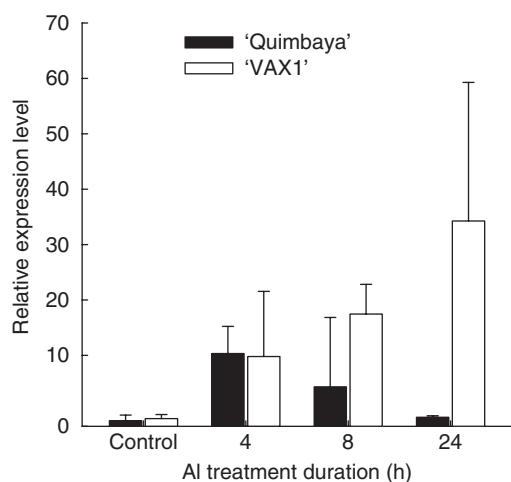
FIG. 7. Gene expression of ACC-oxidase in the common bean genotypes 'Quimbaya' (Al-resistant) and 'VAX 1' (Al-sensitive) grown in nutrient solution treated with or without 20 μM Al for up to 24 h. Total RNA was extracted from root tips. Quantitative RT-PCR was performed using the β-tubulin gene as internal standard and untreated plants of the Al-resistant genotype 'Quimbaya' as calibrator. Relative gene expression was calculated from three biological and two technical replicates; standard errors are shown. Control plants were not treated with Al.

transduction. For example, Al enhanced the expression of ACC-oxidase (Fig. 7). Similarly, Sun *et al.* (2007) reported increased gene expression and enzyme activity of ACC-oxidase leading to increased ethylene production in *Lotus japonicus* and *Medicago truncatula* under Al stress and suggested that ethylene is involved in the Al-induced inhibition of root elongation. However, the expression of ACC-oxidase and other differentially expressed stress-responsive genes observed in the present study are also triggered by other stress factors such as heat, cold, drought, waterlogging, or disease infection (Nie *et al.*, 2002; Fekete *et al.*, 2009; Zeller *et al.*, 2009) and as such may not be specific for Al.

Genes that may be related to the citrate exudation-mediated Al resistance of bean include those encoding a VDAC and an NADP-specific ICDH. Several studies indicate that the release of organic acid anions is mediated by anion channels located in the plasma membrane (Kochian *et al.*, 2004; Delhaize *et al.*, 2007; and references therein). The VDAC is a family of eukaryotic pore-forming proteins, originally discovered in the outer membrane of mitochondria where it allows free permeability of low molecular-weight solutes (Colombini, 1979). It is found to be not only expressed in the mitochondria, but also in the plasma membrane (Lawen *et al.*, 2005) and in peroxisomes (Arai *et al.*, 2008). Thus, VDAC may mediate organic anion exudation in bean. However, its differential expression in response to Al treatment could not be verified by qRT-PCR.

Similar to the current observation, cytochrome P450 monooxygenase expression was found to be up-regulated in Al-resistant near isogenic wheat lines under Al stress by Guo *et al.* (2007) and Houde and Diallo (2008) and was implicated to play a role in Al resistance. Cytochrome P450s may serve as monooxygenases in the biosynthethis of lignin, defence compounds, hormones, pigments, fatty acids and signalling

molecules and in the detoxification catalysing numerous endogenous and exogenous toxic compounds encountered in the environment (Schuler and Werck-Reichhart, 2003). Thus it may contribute to sustained root growth under Al stress conditions. In addition, mitochondrial F1-ATPase (alpha-subunit) was also up-regulated in bean root tips upon Al treatment. F1-ATPase is involved in the mitochondrial oxidative phosphorylation by which ATP is produced through a proton gradient. The energy stored in ATP could fuel the metabolic processes involved in Al resistance. Hamilton *et al.* (2001) hypothesized that the induction of V-ATPase and the F1F0-ATPase plays a role in Al resistance of wheat. The subunits of these enzymes were newly synthesized upon Al treatment and the proteins accumulate in an Al dose-dependent manner (Basu *et al.*, 1994). In addition, accumulation of V-ATPase and F1F0-ATPase subunits segregated with the Al-resistant phenotype (Taylor *et al.*, 1997). This indeed suggests that the up-regulated ATPase may play a yet unspecified role in Al resistance.

The best understood mechanism of Al resistance in plants is the release of organic acid anions such as citrate, malate and oxalate, which chelate Al and form non-toxic complexes (Ryan *et al.*, 2001; Ma *et al.*, 2001). Rangel *et al.* (2010) observed that Al-activated exudation of citrate plays a major role in Al resistance of common bean. Citrate exudation started after about 4 h of Al treatment despite the abundant citrate content in the root tissue (Fig. 1B and C). Moreover, the root growth of both Al-resistant and Al-sensitive bean genotypes was equally inhibited during this lag period, indicating that Al resistance in common bean is not constitutively expressed (Fig. 1A). This is in agreement with an earlier suggestion that Al resistance in bean is an inducible trait (Cumming *et al.*, 1992). The lag phase between the beginning of Al treatment and the onset of citrate exudation shows that the induction process involves gene transcription and *de novo* synthesis of proteins which are necessary for citrate transport.

In the present study, the expression of genes encoding organic anion transporters was examined. Among the candidate genes tested, *MATE-a* (GenBank accession no. CV535133) and *MATE-b* (GenBank accession no. CV534527) were strongly expressed upon Al treatment in bean. Both ESTs (denoted as *MATE-a* and *MATE-b*) of common bean have high sequence similarity to previously characterized *MATE* genes of *Lupinus albus* (GenBank accession no. AY631874) and *Glycine max* (GenBank accession nos EU591739 and EU591741). Nucleotide sequences of *MATE-a* and *MATE-b* have no significant similarity and also they do not belong to the same contig assembly of ESTs in the TIGR database. Whether they are two different genes or just different sequence regions of the same gene will be clarified in the ongoing study through full-length cDNA sequencing.

The MATE proteins are a large family of membrane transport-proteins which have 58 members (paralogues) known just in the arabidopsis genome (Hvorup *et al.*, 2003). The arabidopsis *FRD3* gene which is important for iron transport in the xylem as ferric citrate is also a MATE protein (Durrett *et al.*, 2007; Rogers *et al.*, 2009). The role of a MATE protein for Al resistance was first observed in sorghum (Magalhaes *et al.*, 2007) and barley (Furukawa

et al., 2007) almost simultaneously, later in wheat (Ryan *et al.*, 2009) and further characterized in arabidopsis (Liu *et al.*, 2009). The MATE protein was described as an Al-activated citrate transporter which is responsible for Al resistance of both sorghum and barley (Furukawa *et al.*, 2007; Magalhaes *et al.*, 2007). In sorghum *SbMATE* was expressed only in the root tips of the Al-resistant genotype in an Al-inducible way. Similarly, barley *HvMATE* was constitutively expressed mainly in the root apices and correlated with Al-activated citrate exudation and Al resistance in a set of barley cultivars. In contrast, the *MATE* gene of bean is highly expressed in both resistant and sensitive genotypes used in the present study (Figs 4–6). This result corroborates the observation that citrate exudation was induced by Al in both Al-resistant and -sensitive genotypes (Fig. 1B). Regardless of the ample amount of citrate in the root tissue (Fig. 1B), exudation started only after about 4 h of Al treatment, the time lag which is required for activation of *MATE*-gene transcription, translation and formation of the functioning protein (Fig. 6). Similarly, Al enhanced the expression of a citrate transporter gene in soybean after 4 h (Z. M. Yang, Jilin University, China, pers. comm.). After the MATE protein is in place, citrate exudation progressed and resulted in a reduction in the citrate content of the root tissue (Fig. 1B and C). As a result of citrate exudation, both bean genotypes transiently recovered from the stress which equally affected both of them in the early hours of Al treatment (Fig. 1A). The remarkable difference between the two bean genotypes was observed in their capacity to replenish the tissue citrate reserve and to sustain citrate exudation in order to protect the growing root tip. The Al-resistant genotype 'Quimbaya' was able to restore the citrate pool in the root tissue and to continue to release citrate, whereas the Al-sensitive genotype 'VAX 1' was unable to restore the internal citrate pool and failed to further release citrate after the short recovery period (Fig. 1B and C). These observations underline that sustained synthesis of citrate as well as constant expression and activity of a citrate transporter are vital for Al resistance in common bean.

Although the role of organic acid anion exudation for Al resistance and the importance of organic acid anion transporters are currently well defined, the significance of organic acid metabolism and accumulation in the root tissue are still not well understood (Ryan *et al.*, 2001; Horst *et al.*, 2007). In plant species, where organic acid anion release started directly after Al treatment, no correlations were observed between internal organic acid concentrations and efflux. For example, Al-sensitive and Al-resistant wheat genotypes did not differ in root concentrations of malate, although the Al-resistant genotypes released up to 10-fold more malate than the Al-sensitive genotypes (Delhaize *et al.*, 1993). Similarly, contrasting maize genotypes did not differ in tissue citrate content and Al equally increased citrate accumulation in the root tissue of both genotypes but significant citrate exudation was only observed in the Al-resistant genotype (Pellet *et al.*, 1995). In contrast, in soybean (pattern II plant) the Al-enhanced internal accumulation of citrate contributed to the enhanced citrate exudation (Silva *et al.*, 2001). Reports on the role of enzymes involved in the organic acid metabolism for Al-induced organic acid anion efflux are also diverse. In wheat, Al-induced malate exudation occurred

without significant changes to the activities of PEPC or malate dehydrogenase (NAD-MDH). Moreover, the activities of these enzymes were not significantly different between genotypes (Ryan *et al.*, 1995). In contrast, an increased citrate synthase (CS) activity was reported in *P. vulgaris* (Mugai *et al.*, 2000) and *Cassia tara* (Yang *et al.*, 2004) after Al treatment. Similarly, Al treatment enhanced the gene expression as well as enzyme activity of mitochondrial CS in soybean (Z. M. Yang, Jilin University, China, pers. comm.). Furthermore, over-expression of enzymes involved in organic acid metabolism has been proven to be effective in enhancing exudation of organic acid anions leading to Al resistance in transgenic plants of arabidopsis (Koyama *et al.*, 2000), alfalfa (Tesfaye *et al.*, 2001) and canola (Anoop *et al.*, 2003).

Rangel *et al.* (2010) studied the changes in activities of enzymes involved in citrate metabolism. Al treatment reduced the activity of ICDH leading to reduced internal citrate consumption and enhanced exudation. The citrate content in the root tissue is a function of citrate synthesis, exudation, degradation or consumption for other metabolic functions. Accordingly, continuous release of citrate while maintaining normal citrate concentration in root tissue requires enhanced synthesis and/or reduced degradation of citrate. Reduction in cytosolic NADP-isocitrate dehydrogenase activity resulted in citrate accumulation and subsequent release from mutant carrot cells which were able to grow on insoluble phosphate sources (Kihara *et al.*, 2003). But according to Rangel *et al.* (2010), not only the reduction of NADP–ICDH but also maintaining the activities of CS and PEPC are important for sustained exudation of citrate in common bean. Failure of continuous citrate exudation in the Al-sensitive bean genotype 'VAX 1' was mainly attributed to the constitutively lower CS activity which was further inhibited by extended duration of Al treatment. In the current study, no significant change was observed in the expression of genes encoding enzymes involved in citrate metabolism (Fig. 3). Similarly, Kumari *et al.* (2008) who made a large-scale, transcriptomic analysis of root responses to Al, using a microarray representing about 93 % of the predicted genes in the genome of arabidopsis did not detect a significant increase in transcript abundance for any of the 52 genes of the TCA cycle present in the microarray, except for MDH. However this does not mean that there is no change in the activity of enzymes involved in the TCA cycle. Since Rangel *et al.* (2010) clearly demonstrate the changes in the enzyme activity of the above bean genotypes it can be concluded that the activities of these enzymes are regulated at the post-translational level.

The role of ATP-binding cassette transporter family proteins, ALS1 and ALS3 for Al resistance, was observed in arabidopsis (Larsen *et al.*, 2005, 2007). Plant ATP-binding cassette transporters that have been functionally characterized so far were known to detoxify organic and inorganic compounds by sequestering in the vacuole (Schulz and Kolukisaoglu, 2006). Arabidopsis *als1-1* and *als3-1* mutants were hypersensitive to Al but the exact functions and substrates of ALS1 and ALS3 are not known. Whereas ALS1 is located in the tonoplast and the gene is expressed in root apices and the vascular system (Larsen *et al.*, 2007), ALS3

is primarily located in the plasma membrane of leaf hydathode cells, the phloem and the root cortex (Larsen *et al.*, 2005). The expression of *ALS3* is induced by Al and was suggested to function in channelling accumulated Al away from Al-sensitive tissues in order to protect the growing root from Al toxicity. In agreement with Larsen *et al.* (2005) it was observed in the present study that Al treatment induced the expression of the *ALS3* gene in both in Al-resistant and sensitive bean genotypes (Fig. 4). However, the suggested function of *ALS3* could not be confirmed since the sensitive cultivar continued to accumulate Al in the root tissue (Rangel *et al.*, 2010) regardless of *ALS3* expression.

In conclusion, this study strongly suggests that in common bean a *MATE* gene is responsible for Al-induced citrate exudation. The expression of this gene is a prerequisite for Al resistance. However, sustained citrate release and genotypic Al resistance requires, in addition, the continuous synthesis and maintenance of a cytosolic citrate pool in the root apex.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxford-journals.org and consist of the following figure and tables. Figure S1: sequence alignment tree of *MATE* genes along with *Phaseolus vulgaris* ESTs having high similarity to the known *MATE* genes. Table S1: Up-regulated genes induced by 4 h of Al treatment in the Al-resistant common bean genotype 'Quimbaya'. Table S2: Down-regulated genes induced by 4 h of Al treatment in the Al-resistant common bean genotype 'Quimbaya'.

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