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Note

Expression of genes encoding transporters and enzyme proteins in response to low-pH and high-aluminum treatments in *Acacia mangium*, a stress-tolerant leguminous tree

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Abstract Several genes of model and crop species that function in plant adaptation to acid soils have recently been characterized, but little is known about the molecular basis of the stress tolerance of woody plants. In the present study, using cell suspension cultures of *Acacia mangium*, a leguminous tree habituated to tropical acid soils, genes up-regulated in response to lowering the medium pH and addition of $AlCl_3$ were screened by successive differential display and semi-quantitative RT-PCRs, followed by full-length determination by RACE (rapid amplification of cDNA ends)-PCR. A total of 57 genes were shown to be induced by low-pH and/or aluminum stresses, and 44 full-length sequences were identified and cloned. They included genes of a multidrug and toxic compound extrusion transporter that secretes citrate ions to chelate aluminum, ATP-binding cassette transporters, a plasma membrane H^+-ATPase, and a CYP94A, known in other plants to be responsive to low-pH and/or aluminum stresses. Genes that have not been recognized to be stress-responsive were also up-regulated by low-pH/aluminum treatments. Many of these genes were induced in the stressed *A. mangium* seedling roots. The cloned genes should provide the resources for the identification of the factors that play roles in the adaptation of *A. mangium* to acid soils.

Key words: Acid soil, multidrug and toxic compound extrusion (MATE), stress resistance, suspension culture, woody plant.

Soil acidity is a worldwide problem in agriculture. In low-pH soils, solubilized aluminum ions (Al^{3+}, referred to as Al hereafter) and protons (H^+) particularly limit plant growth. Plant factors that resist or tolerate these stresses have long been examined, and remarkable progress at the molecular level has been made during this decade (Reviews: Delhaize et al. 2012; Ryan et al. 2011). Secretion of organic anions such as malate and citrate from roots into the rhizosphere to chelate and detoxify Al is a major mechanism of Al resistance, and genes encoding transporters for organic anion exudation such as *ALMT* (*aluminum-activated malate transporter*) and *MATE* (*multidrug and toxic compound extrusion)* family genes from wheat and several other plants have been characterized. Genes of an Al transporter called *Nrat1* (*Nramp aluminum transporter 1*) and half-size ATP-binding cassette (ABC) transporters (*STAR* (*sensitive to aluminum rhizotoxicity*) and *ALS* (*aluminum sensitive*)) that sequester Al from essential metabolism were also identified. The regulatory genes, *STOP1* (*sensitive to proton rhizotoxicity 1*) involved in proton and Al resistances in *Arabidopsis thaliana* and *ART1* (*aluminum resistance transcription factor 1*) in Al resistance in rice, both encoding Cys2/His2 zinc finger transcription factors, were shown to coordinate the expression of resistance/tolerance genes of their respective plant species. Comprehensive gene expression analyses using microarray approaches have further yielded increasing numbers of candidate genes involved in plant stress adaptation (Tsutsui et al. 2012).

The plant species so far investigated for low pH and Al adaptation are mainly herbaceous plants such as *A. thaliana* and agriculturally important crops, and subtractive hybridization of cDNAs and map-based cloning utilizing genotypic variants and induced mutants have been employed for identification of the responsible genes (Ryan et al. 2011). In contrast, some woody plants adapt themselves to acid soils, but only a few reports are available regarding the molecular mechanisms of stress adaptation of tree species; e.g., Al-responsive induction

Abbreviations: ABC, ATP-binding cassette; ALS, aluminum sensitive; β'-COP, coatomer subunit β'; DD, differential display; EST, expression sequence tag; MATE, multidrug and toxic compound extrusion; MS, Murashige and Skoog; P450, cytochrome P450; PDR, pleiotropic drug resistance.

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of genes encoding mitochondrial citrate synthase in *Paraserianthus falcataria* (Osawa and Kojima 2006), and two transporters, *ALS* and *MATE* homologs in aspen (*Populus tremula*) and *Eucalyptus camaldulensis*, respectively (Grisel et al. 2010; Sawaki et al. 2013). Because stress-sensitive variants and mutants of tree species are usually unavailable, these studies used the plant materials under stress (so far Al) treatments.

Acacia mangium Willd., a woody species of the Leguminosae (subfamily Mimosoideae) native to northeastern Australia and adjacent tropical areas, can grow rapidly and vigorously in acid soils, and therefore is an important tree for environmental restoration (Umezawa et al. 2008). A subtractive cDNA library of flower (Wang et al. 2005) and expression sequence tags (ESTs) of developing secondary xylem and shoots (Suzuki et al. 2011) from *A. mangium* as well as ESTs of the inner bark from *A. auriculiformis*×*A. mangium* hybrid (Yong et al. 2011) have been constructed, but there has been no report focused on genes involved in stress responses in *A. mangium*. As an initial step to clarify the molecular basis for the stress adaptation of *A. mangium*, we examined the genes induced by low-pH and Al treatments of cultured *A. mangium* cells. Several full-length cDNAs of candidate genes that may play roles in stress adaptation were then cloned and are reported here.

Cells of the callus culture derived from hypocotyls of *A. mangium* (B & T World Seeds, Aigues-Vives, France) were suspension-cultured in Murashige and Skoog (MS) medium (pH 5.8) containing 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 0.1 mg l^{-1} kinetin at 25°C in the dark at 140 rpm. The cells grew ca. 5-fold during 3-week culture periods. When the cells were transferred to media whose pH were adjusted to as low as 2.8, the growth was not affected significantly, and the cell cultures could even be maintained in the medium at pH 2.5 (data not shown). To examine the effect of Al toxicity on the cells, 3-week-old cultures were transferred to 1/2 strength MS medium (pH 4.3, the same hormone condition) containing $AlCl_3$ at different concentrations, which has been proposed for Al toxicity studies (Martínez-Estévez et al. 2001), and cultured for 15 days. As a result, the cell growth was not inhibited severely in the media containing $AlCl_3$ at concentrations below 0.5 mM, but it decreased by ca. 10% and 60% at concentrations of 1–2 mM and 5 mM, respectively (Supplementary Figure S1). This growth inhibition may not solely be the result of direct Al toxicity, and indirect effects should also be taken into account, but the influence of pH changes caused by Al would have been small. Also, an *A. mangium* cell suspension culture line habituated to the pH 4.3 medium containing 3 mM $AlCl_3$ was established. *A. mangium* cells thus appeared to be extremely resistant to Al compared to other plant cell cultures, e.g., coffee (*Coffea arabica*) in which the LD_{50} of Al is 0.025 mM (Martínez-Estévez et al. 2001). Therefore, in the following experiments to select stress-responsive genes, *A. mangium* cultures were subjected to rather severe stress conditions, and the early transcriptional responses were examined.

A. mangium genes whose expression levels are changed in response to low-pH and/or Al stress were first screened by differential display RT-PCR (DDRT-PCR) using RAPD (random amplified polymorphic DNA) primers (OPA01-OPE20, OPERON 10mer kits, Operon Technologies, Alameda, CA, USA; Yoshida et al. 1994). Total RNA was extracted from cultured *A. mangium* cells (13-day-old) harvested 1 h and 24 h after adding 0.5 M aq. H_2SO_4 to the medium to adjust the pH to 3.0 (indicated as low-pH treatment) and also the cells obtained by simultaneously adjusting the medium pH to 3.0 and addition of $AlCl_3$ to a final concentration of 0.1 mM (indicated as low-pH/Al treatment). Also, separately, the 13-day-old cells were transferred to 1/2 strength MS media (pH 4.3) containing high concentrations (2 and 5 mM) of $AlCl_3$, and cultured for 24 h (high-Al treatment). Among the PCR bands displayed from low-pH and low-pH/Al treated cells, 66 bands were estimated to be fragments of the up-regulated genes, and 108 nucleotide sequences were obtained from the excised bands cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA). Similarly, 87 up-regulated bands, which yielded 100 nucleotide sequences, were detected from the high-Al treated cells. Narrowing down the fragments of up-regulated genes was carried out by semi-quantitative RT-PCR with gene-specific primers designed for the sequences from DDRT-PCR fragments. Final characterization of full-length sequences was performed by 3′- and 5′-RACE (rapid amplification of cDNA ends) PCR employing redesigned gene-specific primers, and then full-length cDNAs were cloned. PCR conditions for each step of the screenings and the procedure for cDNA cloning are described in the Supplementary Methods.

These experimental steps eventually yielded 33 genes that were up-regulated by low-pH and low-pH/Al treatments, and full-length coding sequences of 27 cDNAs were determined. Among these 33 genes, 30 genes induced by low-pH were also up-regulated by low-pH/Al (marked LpA (23 genes) and LH (7 genes) for detection condition in Table 1 and Supplementary Table S1), while two genes were induced only by low-pH (PH) and one gene only by low-pH/Al (LAL), indicating that most of these genes are basically responsive to low pH. From the candidate fragments induced by high-Al treatment, 31 sequences (HAL for 24 genes and LH for 7 genes) were actually amplified by RT-PCR with gene-specific primers, and 24 full-length sequences were obtained. Among the genes identified in these procedures, seven genes were found to be up-regulated

by both low-pH/Al and high-Al treatments (LH as shown above), although these two analyses were performed independently.

The functions of 49 genes from the total of 57 up-regulated genes were estimated based on homology search using NCBI BLAST programs with satisfactory E-values (equal or smaller than 2.0e-10), and they were functionally categorized by reference to the KEGG BRITE database. Of these, 26 were supposed to encode transporters and metabolic enzymes, and nine genes comprising eight protein kinases and one GTP-binding protein were categorized to signal transduction. While a detailed list of detected genes appears in Supplementary Table S1, genes of interest selected from the viewpoint of plant stress adaptation or defense response are shown in Table 1 and discussed below.

The gene *MATE family transporter* (E12c-2 in Table 1) was the sole transporter gene up-regulated by low-pH/Al treatment but not by low-pH, demonstrating its responsiveness to Al. Induction was observed as early as 1 h after the Al treatment and continued for 24 h. The closest *A. thaliana* homolog of this gene was *AtMATE* (At1g51340), a member of genes encoding MATE families, that mediates Al-activated citrate efflux (Liu et al. 2009). A small amount of Al-activated citrate efflux from roots of *A. mangium* has been reported (Osawa and Kojima 2006), although the role of citrate in *A. mangium* is speculated to be more likely to detoxify Al inside the cells. The present finding, together with two recent reports (Grisel et al. 2010; Sawaki et al. 2013), indicates that the release of organic anions is, at least in part, an Al resistance mechanism of tree species. Our preliminary results also show that cultured *A. mangium* cells release organic anions, and their increase after Al treatment was verified (data not shown).

A gene encoding plasma membrane H^+-ATPase (Al09′) was up-regulated in *A. mangium* cell cultures in response to both low-pH and high-Al. This gene is a homolog of *A. thaliana AHA2*, which encodes a plasma membrane proton pump functioning in ion homeostasis (Gaxiola et al. 2007), and thus the present finding of up-regulation of this gene under acidic conditions may be reasonable. The high plasma membrane H^+-ATPase activity in the roots of maize that had been acclimated to low-pH indicated its essential role in adaptation to low-pH stress (Yan et al. 1998). A partial sequence of a gene showing homology with proton pump interactor (E10c-1), which interacts with and hyperactivates plasma membrane H^+-ATPases (Janicka-Russak 2011), was also obtained from low-pH-treated *A. mangium* cells, further supporting the idea that induced H^+ secretion can be a mechanism of low-pH adaptation in *A. mangium*. Regarding Al resistance, H^+ gradients across the plasma membrane generated by plasma membrane H^+-ATPase have been supposed to regulate the Al-induced organic anion secretion, and the correlation between Al-induced up-regulation of plasma membrane H^+-ATPase and secreted citrate amount in an Al-tolerant soybean cultivar was reported (Shen et al. 2005).

ABC transporters form a large superfamily and exist

Table 1. Selected genes up-regulated by low-pH and/or aluminium treatments detected in cultured cells of *Acacia mangium* in this study.

Gene	Detection condition[a)]	Expression[b)]				Functional category and homology	E-value
		h*		mM**			
		1	24	2	5		
						Transporter	
E12c-2	LAL	+	+			MATE family transporter	0.0
Al09′	LH	+	+	+	+	Plasma membrane H^+-ATPase	0.0
Al46	LH	+	+	−	+	Pleiotropic drug resistance protein	0.0
Al52	LH	+	−	+	+	Pleiotropic drug resistance protein	0.0
A08c-1	LpA	+	−			ABC transporter A subfamily	0.0
						Metabolic enzyme	
D03c-1	PH	−	+			β-D-Xylosidase	0.0
B16c-3	LH	+	+	+	+	Rhamnogalacturonate lyase	0.0
Al02′	HAL			+	+	Purple acid phosphatase	0.0
A07c-2	LH	+	−	+	+	Cytochrome P450 94A1	0.0
Al13′	HAL			+	+	Cytochrome P450 71D	5.0e-175
						Other	
E10c-1	LpA	+	−			Proton pump-interactor 1-like	2.0e-11
Al01-2	HAL			+	+	Extensin-like protein	0.0
C03c-1	LH	−	+	−	+	Coatomer subunit β'-2-like	2.0e-179
B06c-1	LpA	+	−			Putative E3 ubiquitin ligase	0.0
C14c-1	LH	+	+	+	+	MYND type Zinc finger protein	4.0e-149

a) PH: low-pH (pH 3.0) treatment. LAL: low-pH/Al (pH 3.0, 0.1 mM) treatment. HAL: high-Al (pH 4.3, 2 and/or 5 mM) treatment. LpA: low-pH and low-pH/Al treatments. LH: low-pH, low-pH/Al and high-Al treatments. b) *: time for low-pH and/or low-pH/Al treatments. **: Al concentration of high-Al treatment. + and −: up-regulated and similar level compared with control, respectively.

ubiquitously in living organisms (Verrier et al. 2008). Two genes of the PDR (pleiotropic drug resistance) transporters in the G subfamily of ABC transporters (Al46 and Al52) were shown to be up-regulated in low-pH- and Al-treated *A. mangium* cells. This type of the ABC transporters has been reported to be induced by a variety of stimuli in several plants (Rea 2007), and the closest *A. thaliana* homologs of Al46 and Al52 were *AtPDR12* (At1g15520), implicated in Pb resistance (Lee et al. 2005), and *AtPDR8* (At1g59870) in Cd resistance (Kim et al. 2007), respectively. Al46 is also a homolog of a gene up-regulated in an Al-tolerant maize root (Maron et al. 2008). The present study showed for the first time that these PDR-type ABC transporters are also induced by low-pH in *A. mangium*. Another ABC transporter of the A subfamily (A08c-1) was also responsive to low-pH/Al, implicating possible involvement of multiple ABC transporters during the stress adaptation in *A. mangium*.

The genes encoding a broad variety of enzymes up-regulated in low-pH- and Al-treated *A. mangium* cells included defense-related pathways such as lipoxygenase of jasmonate biosynthesis, 1-aminocyclopropane-1-carboxylate oxidase of ethylene biosynthesis, a class I chitinase, and serine acetyltransferase of the cysteine pathway eventually leading to glutathione and phytochelatins (see Table S1). Several genes related to cell wall modification and/or maintenance, such as β-D-xylosidase (D03c-1), rhamnogalacturonate lyase (B16c-3) and extensin-like protein (Al01-2), may be involved in repair of damaged cell walls, which is considered a tolerance mechanism under low-pH and Al stresses. Another metabolism-related gene that encodes purple acid phosphatase (Al02′) was also up-regulated on high-Al stress. This observation agrees with the suggestion that secretion of acid phosphatase from plant roots is one of the phosphorus acquisition strategies in an Al-rich environment where phosphorus forms low-soluble Al compounds (Hiradate et al. 2007).

Characteristically, two cytochrome P450 (P450) genes homologous to *CYP94A1* (A07c-2) and *CYP71D* (Al13′) were induced in response to both low-pH and high-Al treatments, and high-Al treatment, respectively. *A. thaliana* CYP94A1 catalyzes ω-hydroxylation of fatty acid, and ω-hydroxy fatty acids have been supposed to be involved in defense signaling and cutin/suberin metabolism (Pinot and Beission 2011). Up-regulation of the *CYP94A1* gene during Al treatment (48 h) has also been reported in soybean (Duressa et al. 2010). In the present study, the response of *A. mangium CYP94A-like* gene to low-pH was demonstrated for the first time. However, identification of the real function of this P450 in *A. mangium* awaits future investigation, and the situation is the same for CYP71D, because the P450s of this subfamily constitute a large number of enzymes including hydroxylases of an alkaloid, a monoterpene, and a flavanone.

Coatomer subunit β' (β'-COP, C03c-1) and MYND-type zinc finger protein (C14c-1) were shown to be induced by both low-pH and high-Al treatments in *A. mangium* cultures. β'-COP is one component of a COPI coat protein complex and is involved in intra-Golgi trafficking of proteins (Hwang and Robinson 2009). A soybean homolog of this gene has been reported to be induced by 48 h Al treatment (Duressa et al. 2010). Up-regulation of E3 ubiquitin ligase (B06c-1), which is involved in protein turnover in the ubiquitin-proteasome system, was also observed 1 h after low-pH treatment. These observations may indicate rapid protein degradation and turnover during stress adaptation in *A. mangium*. Also, the function of the MYND-type zinc finger protein in plants is currently unknown, whereas the MYND domain is known to be involved in specific protein-protein interactions (Gamsjaeger et al. 2007), which could indicate that it functions in the plant stress adaptation networks. Induction of MYND-type zinc finger protein gene under both low-pH and Al stresses is a unique finding in *A. mangium*, because no preceding report regarding the induction of this gene in other plants is available.

We then performed the analysis of organ-specific expression of selected genes. Eight-day-old *A. mangium* seedlings germinated on 0.3% agar were cultured hydroponically for 11 days using the nutrient solution described by Osaki et al. (1997) under 12 h light/12 h dark at 30°C, and transferred to the low-pH (pH 3.0) medium or the media (pH 4.3) containing 0.1 or 2 mM $AlCl_3$. RT-PCR of the roots and shoots of the seedlings 1 and 24 h after the treatments were then performed (Figure 1). In the roots, genes of the PDR-type ABC transporters (A146 and A152), MATE family transporter (E12c-2), CYP94A (A07c-2), acid phosphatase (Al02′), and β'-COP (C03c-1) were clearly shown to be induced by both low-pH and 0.1 mM and/or 2 mM Al treatments for 1 and 24 h, and the induction of the plasma membrane H^+-ATPase (Al09′), PDR-type (A146) and A subfamily (A08c-1) ABC transporter genes by low-pH was also verified. In shoots, no conspicuous alteration in the expression of the genes tested was displayed, except for the up-regulation of a PDR-type ABC transporter (A152), MATE family transporter (E12c-2), and β'-COP (C03c-1) genes at 24 h in response to high-Al. Therefore, screening stress–responsive genes with *A. mangium* cell cultures is a reliable method to select candidate genes that function in the adaptation of the intact roots to the chemical environments.

Summarizing the results, several genes homologous or similar to those so far reported to function in low-pH and Al stresses in other plants were detected in *A. mangium* cells in the present study. MATE and other transporter families were suggested to function in this

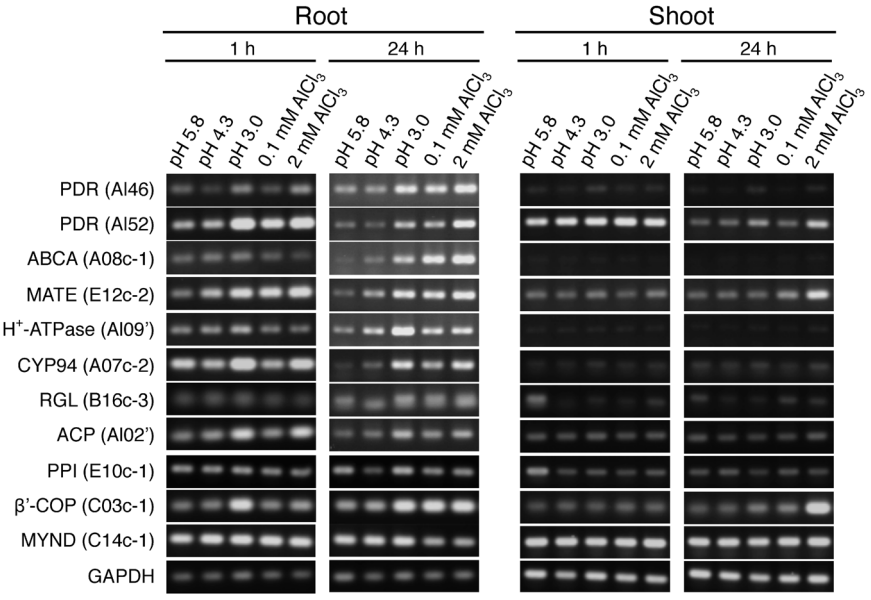

Figure 1. Organ-specific gene expression of selected genes in *Acacia mangium* seedlings. Details of PCR conditions and primers are described in Supplementary Methods and Table S2, respectively. Abbreviations used in Figure 1: ABCA, A subfamily ABC transporter; ACP, acid phosphatase; PPI, proton pump interactor 1-like; RGL, rhamnogalacturonate lyase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

tree species as in herbaceous plants. Mechanisms of plant protection by enzymes for cell wall and membrane modification and of phosphorus uptake utilizing acid phosphatase appeared to be operative. Genes that have not been known so far as low-pH and Al responsive were also shown to be up-regulated and encode enzymes of primary and secondary metabolism, a component of intracellular protein trafficking and a zinc finger protein. When comparing information on the ESTs from the aerial parts of *A. mangium*, only nine genes detected in this study were found in the database (Table S1). This suggests that the expression patterns are very different between the cells and tissues. Also, some of the constitutively expressed organ-specific genes listed in the EST database may be inducible and stress responsive in the roots. In conclusion, the present study represents the first, to our knowledge, examination of genes responsive to low-pH and Al stresses using a cell culture system of woody plant species. Further exploration of the functions of the gene products in the adaptation to low-pH and Al toxicity will be facilitated by taking advantage of the full-length cDNAs that are now available.

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Supplementary methods and data

Supplementary methods

Total RNA isolation and first strand cDNA synthesis

Total RNA was isolated using the CTAB method and purified using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Contaminated genomic DNA was digested using an RNase-Free DNase Set (Qiagen) following the manufacturer's instructions. First strand cDNA synthesis was performed using SuperScript III First-Strand Synthesis System (Life Technologies, Carlsbad, CA, USA).

Differential display RT-PCR (DDRT-PCR)

DDRT-PCR was done with GoTaq Green Master Mix (Promega, Madison, WI, USA) with 100 pmol of single RAPD primer and 12.5 ng of the first strand cDNA as the template. The PCR condition was: 94ºC for 5 min, 40–45 cycles of [94ºC for 60 s, 35ºC for 60 s, and 72ºC for 120 s] and 72ºC for 5 min. Amplified fragments were visualized by ethidium bromide staining after 1.5% agarose gel electrophoresis. Fragments showing specific amplification patterns were excised from the gel by Wizard SV Gel and PCR Clean-Up System (Promega) and cloned into pGEM-T Easy Vector (Promega) and sequenced.

Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR was done with GoTaq Green Master Mix (Promega) with 4 pmol of forward and reverse primers and 25 ng of the first strand cDNA as the template. The PCR condition was: 94ºC for 5 min, 22–40 cycles of [94ºC for 30 s, 55ºC for 30 s, and 72ºC for 30 s] and 72ºC for 5 min. As an internal control, the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene of *A. mangium* was identified (AB839225), and then specific primers were designed. Primers used in semi-quantitative RT-PCR analysis are shown in Table S2.

5'- and 3'- RACE PCR

To obtain the 5'- and 3'-regions of stress-responsive genes, RACE (rapid amplification of cDNA ends) PCR was performed with a GeneRacer Kit (Life Technologies) and Ex Taq DNA polymerase (Takara Bio, Otsu, Japan) with 30 pmol of the RACE primer, 10 pmol of gene-specific primer, and 50 ng of the cDNA as the template. The first strand cDNA was prepared following the manufacturer's instructions. The first PCR condition was: 94ºC for 5 min, 35 cycles of [94ºC for 30 s, 65ºC for 30 s, and 72ºC for 60 s/1 kbp] and 72ºC for 5 min. The second nested PCR was performed with 10 pmol each of the nested RACE and gene specific nested primers, and 1 µl of the first PCR product as the template. The second nested PCR condition was: 94ºC for 5 min, 25 cycles of [94ºC for 30 s, 65ºC for 30 s, and 72ºC for 60 s/1 kbp] and 72ºC for 5 min. Amplified fragments were cloned into pGEM-T Easy Vector (Promega) and sequenced.

Cloning of cDNAs encoding stress-responsive genes

Full-length cDNAs encoding stress responsive genes were amplified using PrimeStar Max DNA Polymerase (Takara Bio) with 10 pmol of forward and reverse primers and 25 ng of the first strand cDNA as the template. The PCR condition was: 98ºC for 5 min, 35 cycles of [98ºC for 10 s, 65ºC for 15 s, and 72ºC for 60 s/1 kbp] and 72ºC for 5 min. Amplified cDNAs were cloned into either pENTR/D-TOPO or pCR8/GW/TOPO vector (Life Technologies).

Table S1. Genes up-regulated by low-pH and/or aluminium treatments detected in cultured cells of *Acacia mangium* in this study

Gene	Length[a] (bp)	Accession number	Detection condition[b]	Expression[c] h* 1	h* 24	mM** 2	mM** 5	Functional category and homology	Organism	E-value	Identity to EST[d]
Transporter											
E12c-2	1518	AB839199	LAL	+	+			MATE family transporter	*Eucalyptus camaldulensis*	0.0	
A09c-1	1713	AB839182	PH	−	+			Cation/calcium exchanger	*Medicago truncatula*	0.0	
A08c-1	2838	AB839181	LpA	+	−			ABC transporter A subfamily	*Vitis vinifera*	0.0	
E25c-2	1461	AB839203	LpA	+	+			Amino acid permease	*Populus trichocarpa*	0.0	FS584846
E24c-4	1362	AB839202	LpA	+	+			Nitrate and chloride transporter	*M. truncatula*	0.0	
Al09'	2865	AB839220	LH	+	+	+	+	Plasma membrane H^+-ATPase	*Sesbania rostrata*	0.0	
Al46	4302	AB839212	LH	+	+	−	+	Pleiotropic drug resistance protein	*Glycine max*	0.0	
Al52	4446	AB839214	LH	+	−	+	+	Pleiotropic drug resistance protein	*M. truncatula*	0.0	
Al08'	1575	AB839219	HAL			−	+	Hexose transporter	*M. truncatula*	0.0	
Al06'	2328	AB839218	HAL			+	+	Potassium transporter	*Gossypium hirsutum*	0.0	
Metabolic enzyme											
D03c-1	2292	AB839196	PH	−	+			β-D-Xylosidase	*G. max*	0.0	FS590492 GR480913
B04c-1	1767	AB839184	LpA	−	+			Asparagine synthetase	*G. max*	0.0	
E16c-1	2778	AB839200	LpA	+	−			Lipoxygenase	*S. rostrata*	0.0	GR480838
C02c-1	732	AB839189	LpA	+	+			Methyltransferase-like	*G. max*	3.0e-117	
D04c-1	918	AB839197	LpA	+	+			Neomenthol dehydrogenase	*G. max*	9.0e-133	
C14c-2	858	AB839194	LpA	+	−			Phosphoglycerate mutase-like	*G. max*	2.0e-162	
A07c-2	1524	AB839180	LH	+	−	+	+	Cytochrome P450 94A1	*V. vinifera*	0.0	FS585227
B16c-3	2079	AB839187	LH	+	+	+	+	Rhamnogalacturonate lyase	*M. truncatula*	0.0	
Al03-2	964*		HAL			+	+	1-Aminocyclopropane-1-carboxylate oxidase-like	*G. max*	9.0e-170	
Al24-2	1542	AB839209	HAL			+	+	4-Hydroxycinnamoyl-CoA ligase	*Coffea arabica*	0.0	
Al35	972	AB839211	HAL			+	+	Class I chitinase	*Acacia koa*	0.0	
Al13'	1512	AB839221	HAL			+	+	Cytochrome P450 71D	*G. max*	5.0e-175	
Al33-2	1620	AB839210	HAL			+	+	Dihydropyrimidinase	*M. truncatula*	0.0	FS588531
Al47	1410	AB839213	HAL			+	+	Glycosyltransferase	*Pueraria montana*	0.0	
Al05-1	1158	AB839205	HAL			+	+	NADH dehydrogenase subunit 4	*Silene latifolia*	0.0	FS587598
Al02'	1869	AB839216	HAL			+	+	Purple acid phosphatase	*G. max*	0.0	
Al21	975	AB839208	HAL			+	+	Serine acetyltransferase	*Nicotiana tabacum*	2.0e-125	
Al24'	1407	AB839223	HAL			+	+	UDP-glycosyltransferase	*G. max*	0.0	
Transcription factor											
C01c-4	2562	AB839188	LpA	+	−			Auxin response factor-like	*G. max*	0.0	FS592678
E24c-1	1362	AB839201	LpA	+	−			SHOOT2 protein	*G. max*	7.0e-158	
Al05'-1	1353	AB839217	HAL			+	+	Basic leucine zipper protein	*Phaseolus vulgaris*	0.0	FS586684

Table S1. continued

						Signal transduction			
B03c-1	1221*		LpA	+	−	Calcium-dependent protein kinase-like	*G. max*	2.0e-11	
C07c-1	1935	AB839191	LpA	+	−	Cysteine-rich receptor-like protein kinase	*G. max*	0.0	
C11c-3	624	AB839192	LpA	+	−	Protein MKS1-like	*G. max*	8.0e-71	
E08c-1	1926	AB839198	LpA	+	−	Receptor-like protein kinase	*G. max*	0.0	
B01c-1	2400	AB839183	LpA	+	−	Serine/threonine protein kinase-like	*G. max*	0.0	
Al22	538*		HAL			− + GTP-binding protein	*V. vinifera*	2.0e-30	
Al23'	558	AB839222	HAL			+ + Protein kinase	*G. max*	1.0e-115	
Al16-2	1423*		HAL			+ + Serine/threonine protein kinase	*G. max*	1.0e-145	
Al19	1149	AB839207	HAL			+ + Serine/threonine protein kinase	*G. max*	0.0	
						Other			
D02c-1	1986	AB839195	LpA	+	−	Armadillo repeat-containing protein	*M. truncatula*	0.0	
B05c-2	682*		LpA	+	+	DNA-directed RNA polymerase	*M. truncatula*	2.0e-10	
B07c-1	363	AB839186	LpA	+	−	Heavy metal associated domain containing protein	*G. max*	2.0e-29	
E10c-1	297*		LpA	+	−	Proton pump-interactor 1-like	*G. max*	2.0e-11	
B06c-1	3048	AB839185	LpA	+	−	Putative E3 ubiquitin ligase	*P. trichocarpa*	0.0	
C03c-1	939	AB839190	LH	−	+	− + Coatomer subunit β'-2-like	*G. max*	2.0e-179	
C14c-1	1098	AB839193	LH	+	+	+ + MYND type Zinc finger protein	*Arabidopsis thaliana*	4.0e-149	FS587256
Al01-2	1257	AB839204	HAL			+ + Extensin-like protein	*G. max*	0.0	
Al32	788**		HAL			+ + 60S Ribosomal protein-like	*G. max*	1.0e-46	
						Unknown			
Al11	1230	AB839206	HAL			+ + Uncharacterized protein	*G. max*	3.0e-155	
Al63	366	AB839215	HAL			+ + Uncharacterized protein	*G. max*	2.0e-42	
A01c-1	1290*		LpA	+	−	No hits			
E09c-4	811*		LpA	+	−	No hits			
C19c-1	1131**		LpA	+	−	No hits			
Al17'	932*		HAL			+ + No hits			
Al21'	1377*		HAL			− + No hits			
Al24-1	1241**		HAL			+ + No hits			

a) *: partial sequence and not cloned. **: estimated pseudogene and not cloned.

b) PH: low-pH (pH 3.0) treatment. LAL: low-pH/Al (pH 3.0, 0.1 mM) treatment. HAL: high-Al (pH 4.3, 2 and/or 5 mM) treatment. LpA: low-pH and low-pH/Al treatments. LH: low-pH, low-pH/Al and high-Al treatments.

c) *: time for low-pH and/or low-pH/Al treatments. **: Al concentration of high-Al treatment. + and -: up-regulated and similar level compared with control, respectively.

d) >98% identity to EST databases (FS: Suzuki et al., 2012; GR: Yong et al., 2011).

Table S2. Primers used for organ-spcific gene expression analysis

Gene	Forward primer	Reverse primer
PDR (Al46)	CCACTCGCCACATGTTACTGTC	AACCTCTTGCGTTGTTCGGTTG
PDR (Al52)	CTCCATAGCAGTCGAAGTTCGT	ATTGTCCCCACCATTAGACCAG
ABCA (A08c-1)	ATCGGGCAATCATACTGACCAC	TGCCTTGGTAGCATTTGGAGAC
MATE (E12c-2)	TGTTGCAGTGACTCAGCCCATC	CCCTGTTTCAGACCCTAGCCTC
H^+-ATPase (Al09')	CTGGCTCTACAGCATTGTCTTC	CTTTCAGCTTCACCACTGACTC
CYP94A (A07c-2)	CGTCGAACAGGTTGTGGACAACGAG	CTCTGCCCCAGTGATGGTGACAAG
RGL (B16c-3)	CTCACGACGAGACCTCAGTGAGAAG	GGACAGGGTGATCTGAACAATGCC
ACP (Al02')	CACCTTATCCTGGACAGAACTCTC	GATAAGCTGGTCAGTGGTGTTG
PPI (E10c-1)	CGAATGTTGCAGTGGAGGCTACAG	GCCCATACCCAGTAACTCTTGGAC
β'-COP (C03c-1)	CCGACGATCAATTTATCCGCGTCTAC	CAAGGTGGGATGTATGACCAAACTCC
MYND (C14c-1)	CGACGCATGCCAGATGGAATGGTC	CACCCAATGTGCGCGCATAGTCTC
GAPDH	CGAGATGGGTTACAGCACAC	GGAGGGTAGACTGCAACCAG

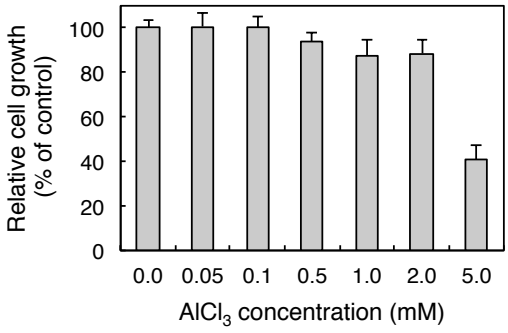

Figure S1. Effect of aluminum on cell growth in suspension cultures of *Acacia mangium*. The fresh weight of 15-day-old cultured cells grown in 1/2 strength MS medium (pH 4.3) containing $AlCl_3$ at different concentrations was measured, and the relative growth rate compared to control (0.0 mM Al; 33.67 ± 1.28 g) was determined. Note that free Al concentrations in the media were not necessarily the same as that of added $AlCl_3$ due to the formation of insoluble Al derivatives during pH adjustment and the existence of other media components. Error bars represent standard deviation (n = 16).