Title

Transcriptomic analysis reveals differential gene expression in common bean (Phaseoulus vulgaris) for aluminum resistance

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Introduction

Common bean (*Phaseoulus vulgaris*) is produced in the tropics by small scale farms where unfavorable edaphic factors limit the yield potential. Among others, soil acidity which covers 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 aluminum toxicity. In addition, other 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 (Ryan et al., 1993; Sivaguru and Horst, 1998). Genotypic differences in Al resistance have been observed among bean genotypes (Rangel et al., 2005). Comparing two contrasting bean cultivars Quimbaya (Al-resistant) and VAX1 (Al-sensitive), Rangel et al. (2009) found out 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, activation, and protein synthesis. In bean, citrate release started four hours after Al treatment followed by recovery from Al stress in an Al-resistant genotype (Rangel et al., 2007; Rangel et al., 2009), confirming that common bean is typical pattern II plant. Thus, the objectives of this work were to study 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). Gene-expression analysis after full recovery from Al stress (24 h) is in progress.

Materials and methods

Plant growth and root tip harvest

Seeds of Al-resistant common bean genotype Quimbaya were germinated in filter papers sandwiched between sponges. After 4 days, uniform seedlings were transferred to a continuously aerated simplified 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 two days. Then the plants were treated without or with 20 µM Al for 0, 4, 8, and 24 hours. At the end of the treatment time, roots were rinsed with distilled water and 10 root tips (1 cm long) per plant were harvested and shock frozen in liquid N_2.

RNA isolation and construction of SSH libraries

Root tips of 15 plants per treatment were bulked and ground to powder in liquid nitrogen. Total RNA was isolated using NucleoSpin RNA Plant kit (MACHEREY-NAGEL GmbH and Co., KG, Düren, Germany) following the manufacturer's protocol. Testers and driver cDNAs were prepared from the total RNA of each treatment (0 and 4 h Al) using Super SMART PCR cDNA Synthesis Kit (Clontech Laboratories, Inc.). Suppression-subtractive hybridization (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 subcloned into pCR2.1-TOPO Vector (Invitrogen, US) by T-A cloning. The vectors were used to transform *E. coli* TOP10 competent cells. Transformed clones were grown on LB plate containing X-gal and ampicillin for blue/white screening. Positive clones were checked for the presence of gene inserts after plasmid isolation and EcoR1 digestion. A total of 144 clones having differentially expressed genes were further analyzed and the gene inserts were sequenced.

Sequence homology search

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

Results

Root growth of common bean genotype Quimbaya used for constructing subtractive libraries was monitored during Al treatment. Maximum inhibition of root elongation was observed within 4 h of Al treatment but it gradually recovered and reached a growth rate which is comparable to the control after 24 h Al treatment period (Fig. 1). This indicates that the maximum Al injury is experienced during the early hours while complete recovery is attained within 24 h period.

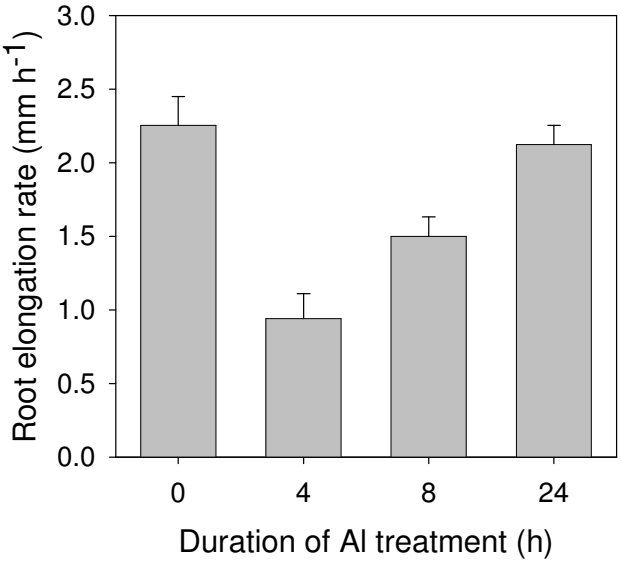

Figure 1. Root growth rate of bean genotype Quimbaya grown in nutrient solution treated without or with 20µM Al treatment.

Differential gene expression in response to Al stress was assessed by using suppression subtractive hybridization (SSH). The forward and reverse subtraction of cDNA samples (4 h Al treatment *vs.* control) showed that Al treatment affected the transcription of many genes involved in a wide range of functions. Some were up regulated while others were down regulated. These genes are grouped into several functional categories as indicated in Fig. 2. Transcripts which were up-regulated due to Al stress include genes involved in stress response/plant defense, signal transduction and translation. In contrast genes involved in metabolism were largely down-regulated. This indicates that Al toxicity induces manifold changes in the plant root, ranging from

perception of stress signal, gene transcription and translation to downstream physiological processes. The up-regulated stress responsive genes include those encoding peroxidases, heat shock proteins and dehydrins. These genes are transcribed as a general response to different stress factors and may not be specific to Al stress. Moreover, they probably have no significance for Al resistance. However, we selected a few other differentially expressed genes which are presumed to contribute to Al resistance in bean. These include genes encoding isocitrate dehydrogenase, anion-selective channel, mitochondrial ATP synthase and cytochrome P450 monooxygenase. Their possible contribution to Al resistance is briefly summarized in Table 1.

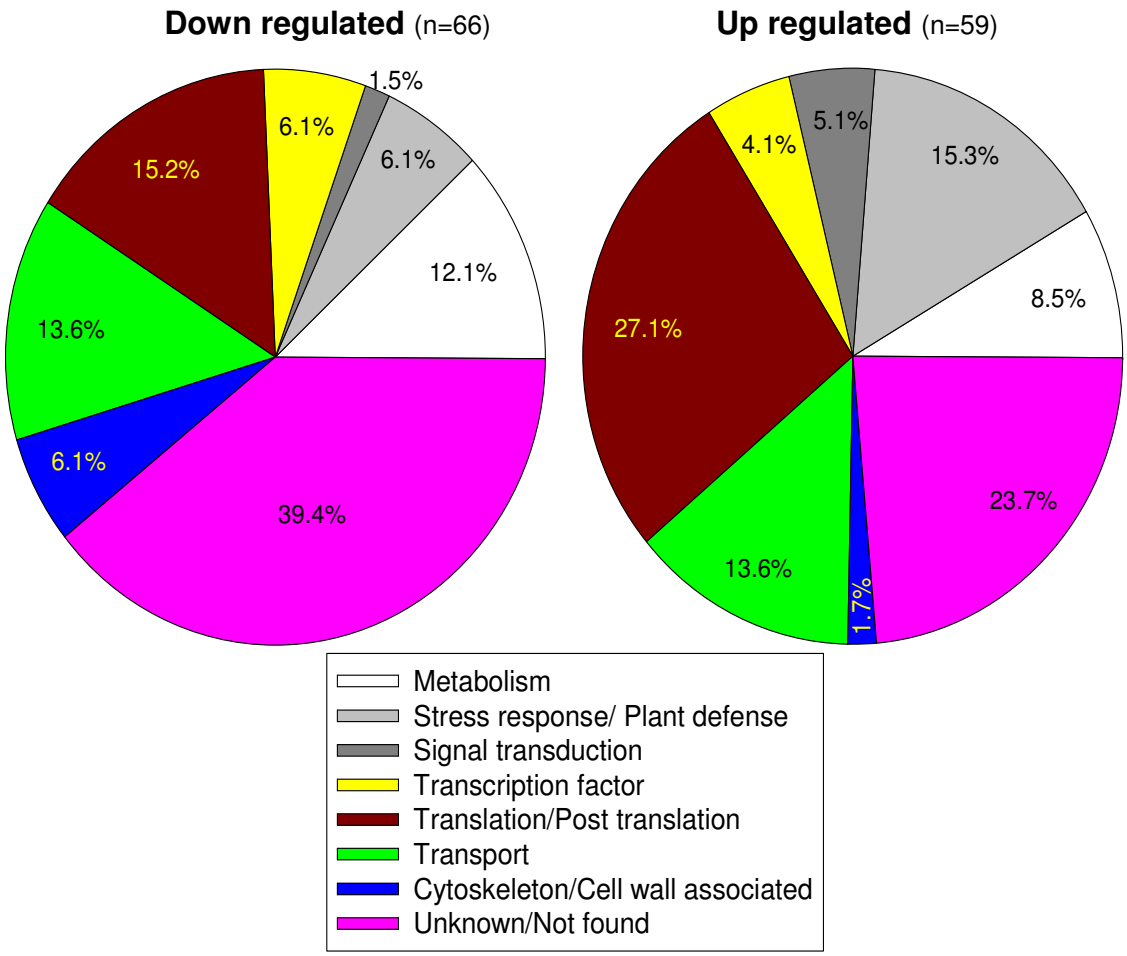

Figure 2. Functional categories of genes differentially expressed by 4 h Al treatment in root tips of Al-resistant bean genotype Quimbaya. Up-regulated genes are those whose transcripts are more abundant in Al-treated than in the control sample, and *vice versa* for down-regulated genes.

Table 1. Differentially expressed genes related to Al resistance in common bean genotype Quimbaya

Clone ID	GenBank accession #	Annotation	Identity (%)	E-value	Up/down regulated	Putative function
0_153	DQ072165.1	VD[1] anion-selective channel	97	1E-154	up	Anion transport
0_156	M64246.1	ATP synthase (F1 alpha)	99	0E+00	up	ATP production through proton gradient
0_178	DQ340249.1	Cytochrome P450 monooxygenase	92	0E+00	up	Detoxification of toxic compounds
4_86	L12157.1	NADP-specific isocitrate dehydrogenase	88	2E-138	down	Conversion of isocitrate to 2-oxoglutarate in the TCA cycle.

[1]VD = voltage-dependent

Discussion

One of the well known mechanisms of plant Al resistance 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. (2009) observed that Al-activated exudation of citrate plays a major role in Al resistance of common bean. Citrate exudation started after about four hours of Al treatment despite the abundant citrate content in the root tissue. Moreover, Al-resistant and Al-sensitive bean genotypes did not differ in root growth within this lag period indicating that Al resistance in common bean is not constitutively expressed. The lag phase between the beginning of Al treatment and the onset of citrate exudation show that the induction process involves gene transcription and de novo synthesis of proteins which are necessary for citrate transport.

Using the SSH method we identified a range of genes which are differentially expressed in response to Al stress in common bean. Al triggered the expression of genes related to plant stress response, plant defense and signal transduction. The expression of many of these genes is also triggered by other stresses such as heat, drought and disease infection, and as such may not be specific for Al. Genes that may be related to the citrate-mediated Al resistance of bean include those encoding a voltage-dependent anion-selective channel (VDAC) and NADP-specific isocitrate dehydrogenase (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 and references therein). 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 peroxisomes (Arai et al., 2008). Thus, VDAC may mediate citrate exudation of bean root tips. The gene encoding the Al-activated malate transporter (TaALMT1) is responsible for malate release in Al resistant wheat. TaALMT1 functions as a ligand-activated and voltage-dependent anion channel to facilitate malate efflux across the plasma membrane of root cells. Similarly, Al-activated citrate efflux from barley

(Furukawa et al., 2007), sorghum (Magalhaes et al., 2007) and wheat (Ryan et al., 2009) is controlled by members of the multidrug and toxin extrusion (MATE) family of genes.

Rangel et al. (2009) observed that citrate exudation in bean genotypes was followed by a reduction in citrate content of the root tissue. While the resistant genotype was able to replenish its tissue citrate content, the Al-sensitive genotype shortly ran out of tissue reserve and eventually stopped exudation. Citrate content in the root tissue is a function of synthesis, degradation, and exudation. 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 the cell (Kihara, et al., 2003). Similarly, Rangel et al. (2009) reported that Al treatment rapidly decreased the activity of NADP-isocitrate dehydrogenase yet without any significant increase in citrate accumulation. Al induced down regulation of NADP-ICDH gene thus indicates that the activity of this enzyme is regulated at the transcript level.

Similar to our current observation, Cytochrome P450 monooxygenase expression was up regulated in Al-resistant near isogenic wheat lines under Al stress (Guo at al., 2007; Houde and Diallo. 2008) and were implicated to play a role in Al resistance. Cytochrome P450s may serve as monooxygenases in the biosynthetic pathways for lignin, defense compounds, hormones, pigments, fatty acids, and signaling molecules, and in the detoxification pathway to catalyze 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 condition.

Mitochondrial F1-ATPase (alpha-subunit) was up regulated in bean root tips upon Al treatment. F1-ATPase is involved in mitochondrial oxidative phosphorylation by which ATP is produced through proton gradient. The energy stored in ATP could fuel the metabolic processes involved in Al resistance. Hamilton et al. (2001) hypothesized that induction of the 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-resistance phenotype (Taylor et al., 1997). This indeed suggests that ATPase plays a role in Al resistance.

In short our transcriptomic analysis indicates that Al-induced activation of an anion-selective channel and down regulation of NADP-specific ICDH may play a role in the continuous release of citrate from the root tip to ensure Al resistance of common bean. Verification of the expression of these genes using qPCR is underway.

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