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IDENTIFICATION AND ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES ASSOCIATED WITH ALUMINUM RESPONSE IN TWO SOYBEAN CULTIVARS BY cDNA-RAPD

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Abstract

Identification of aluminum (Al) responsive genes is of great importance in illuminating the molecular mechanism of plant Al response. In this present study, we preliminarily identified several genes that possibly involved in Al-response by cDNA based random amplified polymorphic DNA (cDNA-RAPD) method from Al tolerant/sensitive soybean cultivars exposed to 0 or 50μM Al^{3+} solutions for two days. Totally one hundred random primers were used to identify the differentially expressed genes; however, only two primers generated eight stable PCR products. The eight gene fragments were cloned and sequenced, then compared with NCBI gene bank. We subsequently verified the expression profiles of these eight genes by real time quantitative PCR (RT-qPCR) and found that two genes were significantly up-regulated after Al treatment for 24, 48 and 72h. One gene, encoding nucleotide-diphospho-sugar transferase which is essential for polysaccharide synthesis and another gene, encoding polygalacturonase inhibiting protein which exerts its role in terms of inhibiting polysaccharide hydrolysis, suggesting the possibility that they might cooperate in response to Al stress through the modification of cell wall components. These findings provided valuable candidate genes for further study on the molecular mechanisms in plant Al tolerance.

Key words: Soybean, Aluminum stress, Aluminum tolerance, cDNA-RAPD.

Introduction

In acid soils, Aluminum (Al) has become one of the most restricting factor reducing the quality and production of crops (Foy, 1988; Delhaize, 2004). Plant can respond to Al stress in different levels with multiple genes involved, which constitutes a perplexing network that still remains unclear (Huang *et al*., 2013). Identification of key genes that involve in Al tolerance is the first step to dissect the mechanism of plant Al resistance and enhance the plant's capacity to survive in the Al-toxic environment. Transcriptome (Chandran *et al*., 2008) and cDNA Microarray (Duressa *et al*., 2011) technologies have proved to be the efficient way to study the differentially expressed genes. In addition, high throughput sequencing has also become a promising strategy for genome-wide analysis of differentially expressed genes (Myles *et al*., 2010). Comparing with these methods, cDNA based randomly amplified polymorphic DNA (cDNA-RAPD) is much more economical and time-saving. It is employed by some researchers to study *Cucurbita pepo* L. (Guo *et al*., 2010), *Triticum aestivum* L. (Mizumoto *et al*., 2009) and *Gossypium hirsutum* L. (Jagadeesh *et al*., 2009). Our previous work also used this method to identify the differentially expressed genes in Al-tolerant soybean cultivar (Huang *et al*., 2013). However, comparative analysis by combining two soybean genotypes with contrasting Al-tolerance capacities will be much more effective for mining Al-responsive genes. Therefore, the present work also used cDNA-RAPD method to identify the potential genes that really conferred to Al-response by using Al-tolerant soybean cultivar and Al-sensitive soybean cultivar. The PCR products amplified from cDNA-RAPD were collected for sequencing and submitted to NCBI gene bank for blast analysis and subsequently applied real-time quantitative PCR to investigate the expression characteristics of the interesting genes.

Materials and Methods

Plant materials: Soybean [*Glycine max* (L.) Merr] was used for present study. BaXi10 (BX10), an Al-tolerant soybean cultivar, and BeDi2 (BD2), an Al-sensitive soybean cultivar, were prepared. Plump seeds were surface sterilized by 0.1% mercuric chloride for 15 min and rinsed in distilled water for at least three times. Seeds were placed in dark at 26°C for germination. Then the germinated seedlings were transplanted in perlite containing Hoagland nutrient solution for 4 days. After, the uniformly grown seedlings were moved to a special growth chamber with Hoagland nutrient solution for further 2 days. Finally, pre-incubated seedlings were exposed to 0 or 50 μM Al^{3+} (both containing 100 μM $CaCl_2$, pH=4.5), then root samples were harvested at 24, 48 and 72h for RNA extraction. All seedlings were grown at 26°C/22°C (day/night) for 16h/8h photoperiod with luminous intensity at 400μmol.m^{-2}.s^{-1} and relative humidity of about 70%.

cDNA-RAPD and sequence analyses: RAPD procedures were performed according to the previous work (Huang *et al*., 2013). Totally 100 random primers (S1~S100) were synthesized by Sangon Biotech Co., Ltd (Shanghai). For validating the repeatability of the primer, we selected only the one that could generate stable and visible products in three rounds PCR in four

samples. PCR products were separated on 1.5% agarose gel electrophoresis and the DNA pellets were collected and then cloned into pMD-18 T vector (Takara) for DH5-α-transformation. PCR verified positive transformants were prepared for sequencing. Then the sequences were submitted into NCBI gene bank (http://www.ncbi.nlm.nih.gov/blast.cgi) for homology analysis and subsequently ran blast against Soybase database (http://soybase.org) for the annotations of the genes.

RT-qPCR analysis: For real-time quantitative PCR (RT-qPCR) procedure, total RNA from the roots of four samples were extracted using the Trizol reagent (Takara). First-strand cDNA was synthesized using 1 μg total RNA, following the manufacturer's instructions. 4μl of 12-fold dilution cDNA from each sample, 3μl of 10-fold dilution of each primer from 20μM stock-solution was used for the quantitative analysis of gene expression with 10μl SYBR Green Real-time PCR Master Mix (Toyobo) in the final 20μl reaction system and subsequently proceeded in 7300 Real Time PCR System (Applied Biosystems). GAPDH was used as an internal control to normalize the expression of the genes. The PCR program was 95 1min, followed by 40 cycles of 95 15s, 55 15s and 72 45s. We used the $2^{-\Delta\Delta Ct}$ method to calculate the relative expression of the each gene. All the RT-qPCR primers are listed in Table 2.

Results and Discussion

We were interested in the Al-responsive genes that showed differential expression in both BX10 and BD2 after Al^{3+} treatments. Because of the limitation of RAPD procedure, not all the primers could generate positive amplification in the samples. To make the results more persuasive, we performed three rounds of PCR using 100 random primers (S1~S100) and finally found two of them (S86 and S97) showed stable amplification in the two soybean genotypes (Fig. 1). The two primers generated dozens of PCR products and we selected eight fragments for sequencing (a~h). The results indicated that the length of these eight gene fragments ranged from 400 to 2000bp. We subsequently submitted these sequences into NCBI gene bank database for blast analysis and found that each of the fragments could find its homologue with highly identity in soybean genome (Table 1).

Table 1. Summary of eight gene fragments and their blast information.

Sequence ID	Length (bp)	Blast information				
		Max Score	E value	Identity	Accession	Annotation
a	1983	3483	0.0	98%	XR_137649.1	carbamoyl-phosphate synthase large chain-like
b	867	737	0.0	99%	FJ225394.1	dehydration responsive protein
c	794	1467	0.0	100%	XM_003550858.1	Nucleotide-diphospho-sugar transferases
d	911	1664	0.0	100%	XM_003531017.1	polygalacturonase inhibiting protein 1
e	790	1454	0.0	99%	XM_003531873.1	chitinase-like protein 1-like
f	562	374	6e-100	96%	XM_003545940.1	PHYLLO, chloroplastic-like
g	506	931	0.0	100%	XM_003534996.1	vesicle-associated membrane protein 726-like
h	403	468	2e-128	99%	NM_001253004.2	chaperone protein dnaJ 11

Table 2 RT-qPCR primers used in the present study.

gene ID	sequence (5'to 3')
a	CTGGCTGACTCACAAGGTAA
	ACAGTTCTGGTGGGAATAGG
b	CATCAGTAGCGACTATTTGG
	ATGCTATGGATGGCAGGTTA
c	AGCATTCTTGCGGCTTACAC
	GCAGCAGAAAGGCATTGACC
d	CGACTCCTTCGGCTCCTTCT
	TATCTGCACCGTGTCTTTCT
e	TGAGCAGAGGGCTGTGACTT
	TGACTACTATGGACGTGGAG
f	GAGGCTGGATGGACCTGTGC
	TCCCTCCTCCAATGTCAACG
g	CAGGCACAAGATTTCAGGAC
	TTCCACAGTTGAACCCACGA
h	ATGGCTTCCCTCTATGACGT
	ATTGGTTCGCTGAGCTTTCC
GAPDH	TGGACACTGGAAGCATCACG
	AACAGTCTTCTGGGTAGCGG

We observed that above eight gene fragments showed different abundances on the agarose gel in the four samples, implying that they possibly had different expression in the root. However, it is not sufficient to draw a conclusion that which gene exhibits differential expression in response to Al stress because RAPD cannot be used for quantitative analysis. To verify the expression levels of these genes, we applied RT-qPCR to discover the differential expression patterns of these eight genes in the four samples. As shown in Fig. 2, six genes (a, b, e, f, g and h) did not show differential expression, which could be considered as consecutive genes and were not responsive to Al stress. However, two genes (c and d), were found to be dramatically altered in Al-treated samples comparing with the controls, suggesting that these two genes probably conferred to Al response. In order to investigate the dynamic expression characteristics of these two genes under Al stress, we further verified the expression profiles at different time points (0, 24, 48 and 72h) after

Al^{3+} treatments (Fig. 3). Obviously, both of them exhibited strong elevation over time (except 0h) comparing with the controls. At 24h, gene c and d were up-regulated by 27.2-fold and 72.6-fold in BX10 while increased by 42.3-fold and 101.8-fold in BD2, respectively. However, at 48h, gene c and d were up-regulated by 8.3-and 62.1-fold in BX10 while increased by 12.7-fold and 14.5-fold in BD2, respectively (Fig. 4). The possible explanation for this fluctuation is that different soybean genotypes might lead to the expression differences and gene d might play much more important role in response to Al stress. In addition, gene d reached its expression peak at 24h while gene c exhibited highest expression at 48h after Al treatment, implying that gene d probably underwent earlier response than gene c.

To predict the possible functions of these differentially expressed genes, we used the Soybase database for their functional annotations. We found that gene c encodes a nucleotide-diphospho-sugar transferase (NDPST) and gene d encodes a polygalacturonase inhibiting protein (PGIP). It is known that NDPST catalyzes the glycosyl transfer from donor to specific acceptor (Charnock & Davies, 1999a) and is one of the most important group of enzymes on earth (Charnock & Davies, 1999b). NDPST is responsible for the formation of glycosylic bond which is very essential for polysaccharide synthesis. Meanwhile, polysaccharides such as cellulose, hemicellulose and pectin are well characterized to be involved in Al tolerance by absorbing Al^{3+} in the cell wall (Liu *et al.*, 2008; Yang *et al*., 2011). Therefore, we presumed that NDPST might indirectly associate with Al response through catalyzing the formation of polysaccharide, by which chelating Al^{3+} in the cell wall. To the authors' knowledge, this is the first time reported that NDPST is connected with abiotic stress (Fig. 5).

PGIP is a cell wall-associated protein that belongs to the leucine-rich repeat (LRR) protein family which plays crucial roles in the development of plants (Di Matteo *et al.*, 2003). Kalunke *et al.* (2014) revealed that there were two PGIP loci and each locus was composed of three clustered genes which were highly conserved in legume species. PIGPs inhibit the activity of polygalacturonase (PG), which is essential for fungal infection in plants (HuangFu *et al.*, 2014). It is reported that PGIPs involved in *Phaseolus vulgaris* defense against wounding, elicitors and fungal infection (Bergmann *et al.*, 1994; De Lorenzo *et al.*, 2001). Faize *et al.* (2004) found that PGIP could resist scab in *Japanese pea*. HuangFu *et al.* (2014) reported the transformation of PGIP2 into *Brassica napus* and observed that overexpression of PGIP2 could enhance *Sclerotinia rot* resistance.

The PGIP-PG interaction leads to the accumulation of oligogalacturonides (OGs) that are perceived in *Arabidopsis* by the receptor Wall Associated Kinase 1(WAK1) to activate the plant defense responses (Bellincampi *et al.*, 2014). Similarly, another elicitor, Al^{3+}, could also induce the expression of WAK1 in *Arabidopsis* and overexpression of WAK1 in transgenic *Arabidopsis* resulted in elevated Al tolerance (Sivaguru, 2003). Cell wall is the major site of Al accumulation (Taylor *et al.*, 2000), and Al treatment altered the cell wall components (Van *et al.*, 1994). It is reported that cell wall polysaccharides (pectin and hemicellulose) played an important role in excluding Al specifically from the rice root apex (Yang *et al.*, 2007). According to these literatures, we speculated that PGIP might involve in the pathway of cross adaptation that inhibits the activity of PG, and subsequently hinders the degradation of cell wall component (such as pectin), which in turn increases the resistance of soybean to Al toxicity (Fig. 5). Our results indicated that gene d (PGIP) experienced sharp increase in both BX10 and BD2 soybean genotypes at 24h Al treatment, suggesting the stress response to Al toxicity.

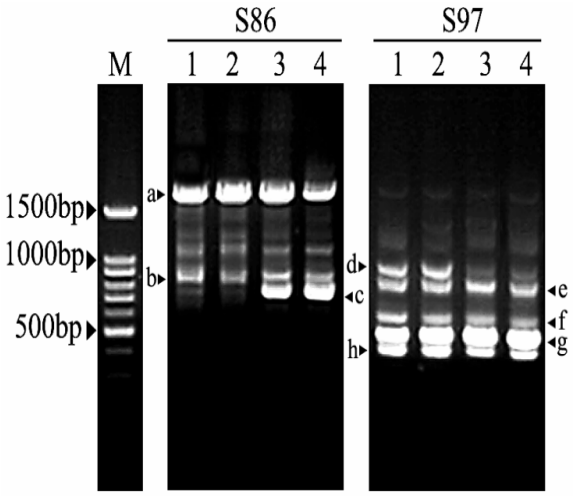

Fig. 1. RAPD products of two random primers (S86 and S97) in the four soybean samples.
Lanes M, 1, 2, 3, 4 indicate DNA ladder, BX10-Al, BX10+Al, BD2-Al and BD2+Al, respectively.

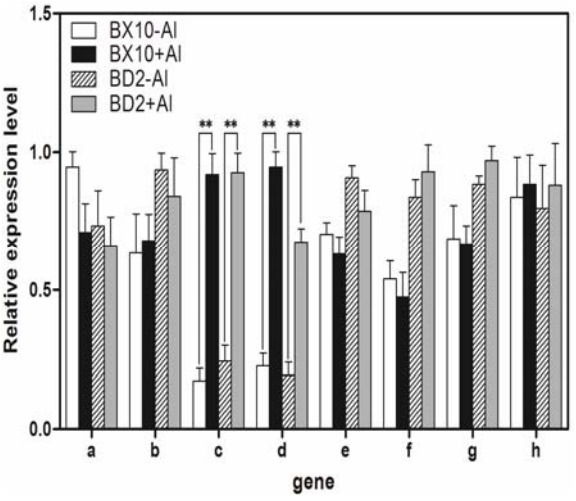

Fig. 2. Expression patterns of 8 genes in the four samples verified by RT-qPCR.
Data was given as mean ± SD (n=3). Double stars (**) on the graph indicate significant differences ($p<0.01$) between Al treated and Al free samples of each soybean genotype.

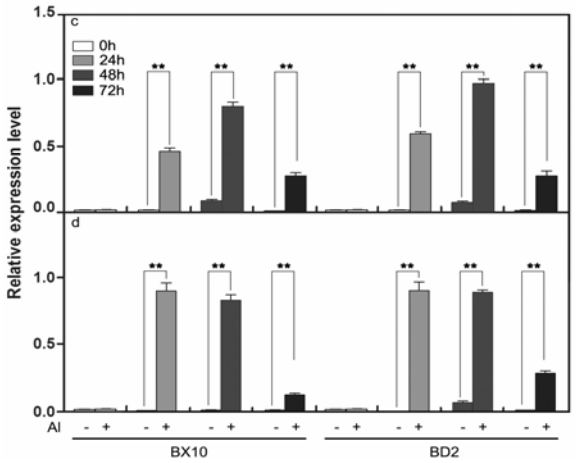

Fig. 3 Expression patterns of two genes (c and d) in four samples at different times after Al treatments
Data was given as mean ± SD (n=3). Double stars (**) on the graph indicate significant differences ($p<0.01$) between Al treated and Al free samples of each soybean genotype.

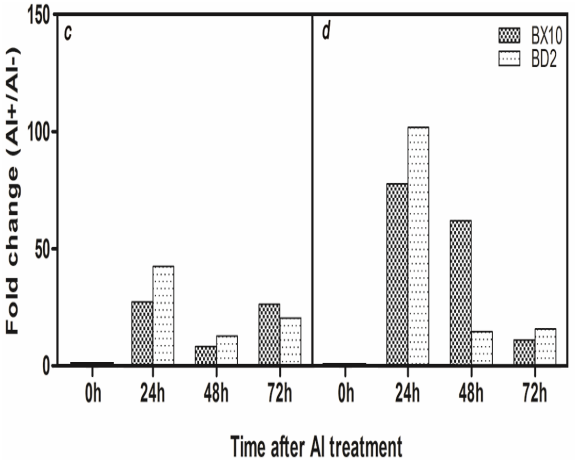

Fig. 4 Expression fold change of two genes (c and d) at different times after Al treatments in Al-tolerant and Al-sensitive soybean.

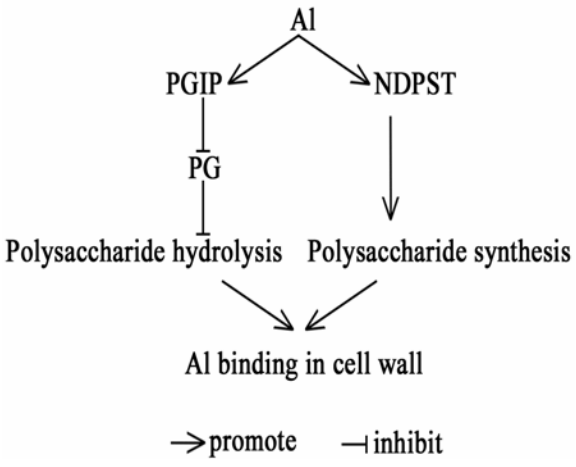

Fig. 5 Supposed pathway of PGIG and NDPST cooperated in the regulation of Al response.

Conclusions

cDNA-RAPD is a simple method to identify differentially expressed genes in the organisms. In the present study, we identified two genes that differentially expressed in Al-treated BX10 and BD2 samples comparing with the controls. These two genes could be considered as potential Al-tolerant genes because they were significantly up-regulated in both genotypes under Al stress. They are encoding two important proteins, NDPST and PGIP, respectively, and are first time reported to be involved in Al response. However, the definite functions and molecular mechanisms of these two genes are not clear and need further investigation.

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