Isolation of Genes Involved in Soybean Response to Al Toxicity under Low pH Condition

Vladimir Ermolayev

Institute of Plant Genetics and Crop Plant Research, Germany

ABSTRACT

Al toxicity in plants is one of the major limitation of crop production on acid soils. Because of complex interaction between Al and a plant, it is very likely that there are a number of different mechanisms used by plants to confer Al resistance. Genetic studies of Al resistance have shown it to be a dominant, multigenic traits controlled by one or a few major genes and several minor genes. The aim of our investigations was to study such gene activities involved in molecular mechanisms of plant stress resistance. Therefore our research started with application of Differential Display RT PCR (DD) approach to find some differences in mRNA population between roots and suspensions cultured cells of sensitive (Malabar and Lumut) and tolerant (Tambora and Wilis) soybean (*Glycine max* L. Merril) lines under stress and non-stress conditions. After set of DD experiments 12 clones specific to mRNA of stress treated tolerant lines were obtained and sequenced. Marathon cDNA amplification was used to prolong the DD fragments. Eight prolonged PCR fragments were obtained and enhanced expression of corresponding mRNA under stress conditions was verified by Reverse Northern Blotting. In order to isolate complete protein coding cDNA sequence, selected fragments were used to screen λ-ZAP cDNA library produced from root tip and suspension cultured cell cDNA from tolerant soybean lines. After putative search, clones were obtained. Their differential expression under conditions of aluminum stress was confirmed by Reverse Northern or Northern Blotting experiment. The clone II-58 1b2 is coding for protein of 168 amino acids. It has 80% homology to translationally controlled tumor protein from plants. The clone II-63 2a3 is coding for protein of 319 amino acids having 77% identity with inosine-5'-monophosphate dehydrogenase from *Arabidopsis thaliana*. The clone I-31 bb is coding for protein of 539 amino acids having 35-42% identity with ATP-dependent transporter family. In addition, several genes of probable interest in Al resistance mechanisms were tested for their Al-responsiveness by Reverse Northern Blotting. Eight genes, for example G-protein, α-subunit, glutathione-S-transferase, calreticulin, malate dehydrogenase, metallothionein MT1, either gave equal signal or did not give any signal at all. For soybean malate synthase, Wali1 and Wali4 (wheat aluminum induced) genes as well as for PEP carboxylase turned out to be possible to prove that their expression was enhanced under conditions of Al stress. In order to study influence of obtained genes on plant resistance to Al stress it is planned to over express selected genes in soybean protoplas and *Nicotiana plumbaginifolia* as a model organism.

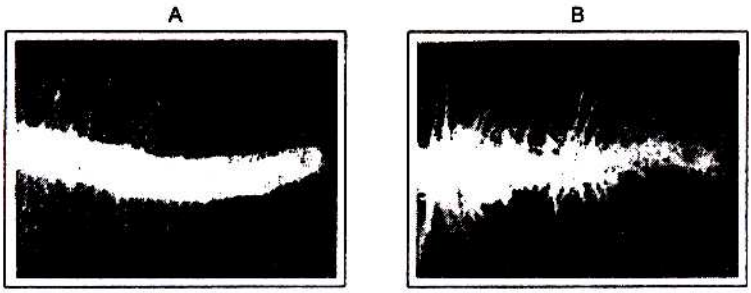
Key words: Al-toxicity, low pH, *Glycine max*, genes

INTRODUCTION

Al is the most abundant metal in the Earth's crust and occurs in different forms. In neutral and basic soils, Al is mostly found as oxide or silicate precipitates that are not toxic to plants. However, in acidic soil (pH<5.0), Al speciates to a soluble form, octahedral hexahydrate which belongs to phytotoxic Al species (Kindraide, 1991).

Al toxicity is the primary factor limiting agronomic production on acidic soils. Such soils constitute 30% of the arable land on the Earth causing very extensive research on the problems of Al-plant interaction in the world.

The primary zone of Al attack is meristem of the root tip, not the cap or other root regions. Initial symptom of Al toxicity is inhibition of root growth (Figure 1) which results in damaged root system and mineral deficiencies (Haug, 1984).



Notes: A = non-treated control root tips, B = root tips of seedlings grown in the presence of 60 μM aluminum in the nutrient solution. Root growth is severely inhibited as it is seen in alteration of root shape in comparison with non-treated control.

Figure 1. Root tip morphology alteration of *Nicotiana tabacum* plants under conditions of Al stress

Source: De La Fuente-Martinez and Herrera-Estrela, 1999

Al stress causes quite many different breaches in the cell physiology even in the first hours of stress. Major molecular symptoms are:

1. Inhibition of nucleic acid and protein biosynthesis (Haug *et al.*, 1994),
2. Alteration of membrane potential and work of membrane channels (Huang *et al.*, 1992),
3. Rigor in cell microskeleton network (Kochian, 1995).

There is no consensus in scientific community about how plants react to toxicity despite very extensive research in this field. There are two basic strategies possibly in use to tolerate Al:

1. The ability to exclude or dramatically restrict Al entry into the cells of root apex and root hairs (apoplasmic mechanisms) and
2. Mechanisms that allow the plants to tolerate toxic Al concentrations within the cell (symplasmic mechanisms).

Organic acid exudation, increasing of rhizosphere pH, mucilago exudation, adsorbtion to charged cell wall substances, callose synthesis and lignification can be attributed to apoplasmic mechanisms (Kochian, 1995). Symplasmic mechanisms include transport of Al in the vacuolar or extracellular compartments of the cells presumably by special transporters or internal chelating inside the cells (Foy *et al.*, 1978).

Genetic studies of Al resistance have shown it to be a dominant, multigenic trait controlled by one or a few major genes and several minor genes (Aniol and Gustafson, 1994). There are several gene activities found to be involved in plant reaction on Al attack. They are represented in the Table 1.

As shown in Table 1, all genes identified to this moment represent general stress response genes or genes with unknown functions. No genes detected to be upregulated during Al stress could be directly attributed to molecular mechanisms of Al detoxification.

The aim of investigations was to study gene activities involved in molecular mechanisms of plant stress resistance.

RESULTS AND DISCUSSION

In order to study such gene activities, two general strategies were used. The first one included study of genes potentially important in the mechanisms of plant stress response and tolerance. Total RNA was isolated from root tips or suspension cultured cells from non-stressed and Al-stressed plants. Tolerant (Tambora and Wilis) and sensitive (Malabar and Lumut) soybean lines were used in this experiment. The RNA had been used in Northern Blotting experiments with available genes or gene fragments

Table 1. The genes known to be involved in Al stress response

No.	Gene name	Plant	Function or homology to
1.	Wali1[1]	Wheat	metallothionein-like proteins from several plants
2.	Wali2[1]	Wheat	no sufficient homology, unknown function
3.	Wali3[1] Wali5[1] Wali6[1]	Wheat	51 to 54% amino acid similarity to Bowman-Birk Ser proteinase inhibitors from several plant species
4.	Wali4[1]	Wheat	clone encoding part of phenylalanine ammonialyase
5.	Wali7[1]	Wheat	no sufficient homology, unknown function
6.	pAL139[2] pAL141[2] pAL142[2]	Tobacco	strong similarity with glutatione S-transferase gene family from many plant species
7.	pAL201[2]	Tobacco	codes moderately anionic peroxidase
8.	Zmal1[3]	Maize	gene coding for protein with expression restricted to root tip
9.	Mtn29[4]	*Medicago trincaluta*	gene induced during nodule development
10.	Sali5-4[5] Sali3-2[5]	Soybean, *Glycine max* cv Bedford (tolerant) and cv Peking (sensitive)	very high homology to auxin down regulated gene ARD6 from soybean (*Glycine max*)

Source: [1] Snowden and Gardner, 1993; [2] Ezaki *et al.*, 1995; [3] Menossi *et al.*, 1997; [4] Gamas, 1998; [5] Rhagland and Soliman, 1997

to test their Al-responsiveness. When certain fragments were unavailable, RT-PCR approach had been involved. In this case the literature data for the sequences of interest were used in EMBL gene bank search to find conservative regions in several similar proteins and corresponding genes. PCR primers were designed with use of sequences from these regions and applied for RT-PCR technique to obtain PCR fragments for further use in Northern Blotting experiments with RNA isolated from Al-stressed and non-stressed plants as it was mentioned above for directly used genes. As shown in Table 2, the 8 genes, for example G-protein. α-subunit, glutathione-S-transferase, calreticulin, malate dehydrogenase, metallothionein MTI either gave equal signal for control and Al-treated samples or did not give any signal at all. For soybean malate synthase, Wali1 and Wali4 (Table 1) genes as well as for PEP carboxylase it was possible to prove that their expression was enhanced under conditions of Al stress.

To summarise the results, assumtions were made such as follows:

1. Negative results can be explained by genetic differences between soybean and the plant species from which Northern Blotting probes were originated,
2. No differences in gene expression were found for calreticulin and malate dehydrogenase,
3. Wali1 and Wali4 (Table 1) were also induced by Al-stress. However, their function in plant response is not yet clear,
4. Malate synthase and PEP carboxylase are involved in organic acid biosynthesis, increasing of their activity can lead to enhanced synthesis and exudation of organic acids.

Second approach included direct evaluation of differences between mRNA population in root or suspension cultured cells of Al-sensitive and Al-tolerant soybean lines under stress and non-stress conditions. The most direct and quick technique for this strategy is Differential Display RT-PCR (DD) (Liang *et al.*, 1993). This method starts with isolation of total RNA from appropriate samples with further cDNA synthesis and special PCR. For this PCR the short random primers and the ones binding to the part of cDNA corresponding to mRNA poly-A tail are used.

Table 2. Differences of gene expression in non-treated and Al-treated root tip cells of tolerant soybean line Tambora

No.	Gene of interest	Northern signal	Plant sources of the genes probed	Mode of use
1.	G-protein, a-subunit	-	Tobacco	Direct
2.	GST	-	Tobacco	Direct
3.	Calreticulin	+	Tobacco	Direct
4.	Malate dehydrogenase	+	Tomato	Direct
5.	Metallothionein, MT1	-	Tomato	Direct
6.	Metallothionein, MT4	-	Tomato	Direct
7.	Wali1	+ (diff. expression)	Wheat	RT-PCR
8.	Wali4	+ (diff. expression)	Wheat	RT-PCR
9.	Phenylalanine ammonialyase	-	Potato	Direct
10.	Pea metallothionein	-	Pea	Direct
11.	PEP carboxylase	+ (diff. expression)	Tobacco	Direct
12.	Malate synthase	+ (diff. expression)	Soybean	RT-PCR

After PCR reaction, a pool of short (50-500 bp) random cDNA fragments was obtained representing appropriate mRNA transcripts of the sample taken for the experiment. To obtain cDNA fragments probably belonging to the specific genes of plant response to Al stress and not general stress gene expression, only the fragments existent in Al-stressed cells of tolerant soybean lines was selected but not in sensitive lines or in non-treated controls. After DD experiments, 12 clones specific to mRNA of stress treated tolerant lines were isolated. Evaluation of Al-responsiveness for corresponding mRNAs by Reverse Northern Blotting allowed us to find 2 cDNA fragments (TA5-2 of size 75 bp and TA2-2-202 bp) showing enhanced expression in comparison with non-treated controls. For the other DD fragments the results of hybridizations were not clear.

Amplified DD fragments were located in the region corresponding to the terminal 3'part of mRNA and were very short as it was mentioned above. Such disadvantages of the method did not allow them to be used in further studies without prolongation. In order to obtain longer fragments, we used Marathon cDNA amplification method. This method is based on double strand cDNA synthesis and ligation with specific adapters. After cDNA synthesis and ligation PCR with use of gene-specific primers is subsequently performed to obtain prolonged cDNA fragments usable in cDNA bank screening in order to obtain the full sequence for the genes interest.

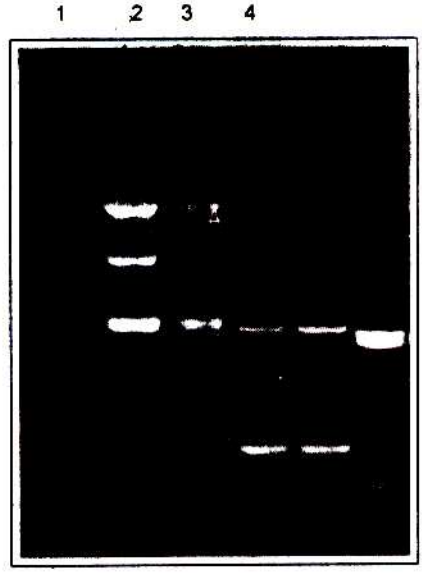
Marathon amplification experiments with primers designed using DD fragments TA5-2, TA2-2, and HT3-1 (Figure 2) gave 8 PCR fragments existing only in cDNA from Al-treated tolerant cells isolated either from suspension culture or root tips, but not in non-treated controls or in sensitive lines.

The prolonged PCR fragments were isolated and sequenced by dideoxynucleotide chain termination method (Sanger *et al.*, 1977) with an automated DNA sequencer (A.L.F. DNA sequencer, Pharmacia). Enhanced expression of corresponding mRNA under stress conditions was verified by Reverse Northern Blotting as shown in Figure 3 and 4.

In order to isolate complete protein coding cDNA sequence, λ-ZAP cDNA bank from both root tip and suspension cultured cell cDNA from tolerant soybean lines was created. For screening of the cDNA library, two selected Marathon PCR fragments HT3-1F3 and TA5-2F3 were labeled with ^{32}P and used for hybridization. Positive cDNA clones were isolated and used for sequence analysis.

After cDNA bank screening with two selected Marathon PCR fragments, 3 clones were obtained II-58 1b2, II-63 2a3 (TA5-2F3 fragment), and I-3 1 bb (HT3-1F3 fragment). Their differential expression under conditions of aluminum stress was confirmed by Reverse Northern Blotting experiments as shown in Figure 5.

BLAST search analysis was used for identification of our cDNA clones. The clone II-58 1b2 is coding for protein of 168 amino acids. It has 80% homology to Translationally Controlled Tumor Protein (TCTP) from plants. These proteins have tubulin binding activity (Thiele *et al.*, 1998) and up-regulated by heavy metals in earthworms (Stürzenbaum *et al.*, 1998).



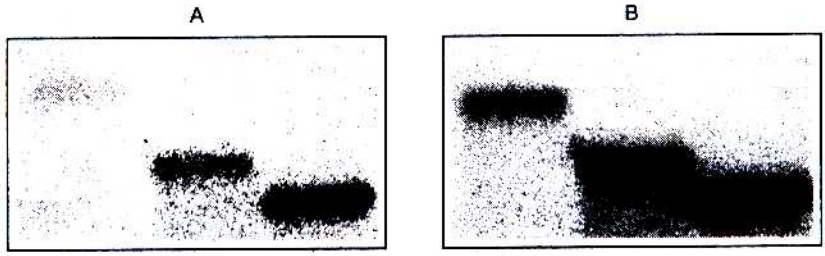
Notes: 1 = marker, 2 = PCR with non-treated control cDNA from tolerant soybean line Tambora, 3 = PCR with cDNA from tolerant soybean line Tambora treated with $AlCl_3$, 4 = PCR with cDNA from sensitive soybean line Lumut treated with $AlCl_3$, A = the band corresponding to HT3-1F1 cDNA fragment, B = the band corresponding to HT3-1F2 cDNA fragment, C = the band corresponding to HT3-cDNA fragment

Figure 2. Marathon PCR amplification with use of HT3-1 DD fragment primers

The clone II-63 2a3 is coding for protein of 319 amino acids having 77% identity with inosine-5'-monophosphate dehydrogenase from *Arabidopsis thaliana* known as functional enzyme in purine biosynthesis in animals and plants (Huberman *et al.*, 1995).

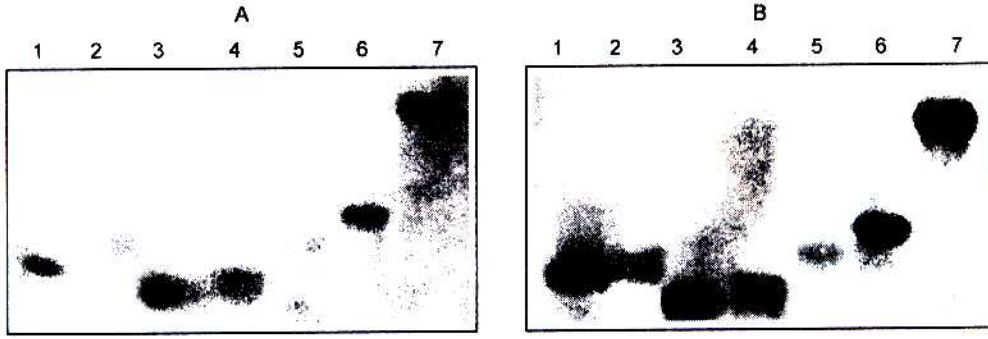
The clone I-31 bb is coding for protein of 539 amino acids having 35-42% identity with ABC transporter family. This enzyme family consists of multisubunit ATP-dependent transporters locating in tonoplast (Tommasini *et al.*, 1998) or plasmalemma of bacteria (Goldman *et al.*, 1997) and plants (Sidler *et al.*, 1998).

At present the physiological function of the obtained genes differentially expressed in Al-resistant soybean lines under stress conditions is not clear. However, it is well known that molecular symptom of Al stress is the binding of Al to microtubules of the cytoskleleton (Kochian, 1995). It means that TCTP may interact with the microskeleton by its tubulin binding activity and could be important to diminish binding of Al to microtubules resulting in enhanced Al resistance to plants.



Notes: Obtained PCR fragments were transferred to the membrane and hybridized with ^{32}P-labeled cDNA from suspension cultured cells of tolerant line Wilis, non-treated (A) and treated (B) with 100 μM $AlCl_3$

Figure 3. Verification of Marathon PCR fragments obtained with use of HT3-1 DD sequence by Reverse Northern Blotting



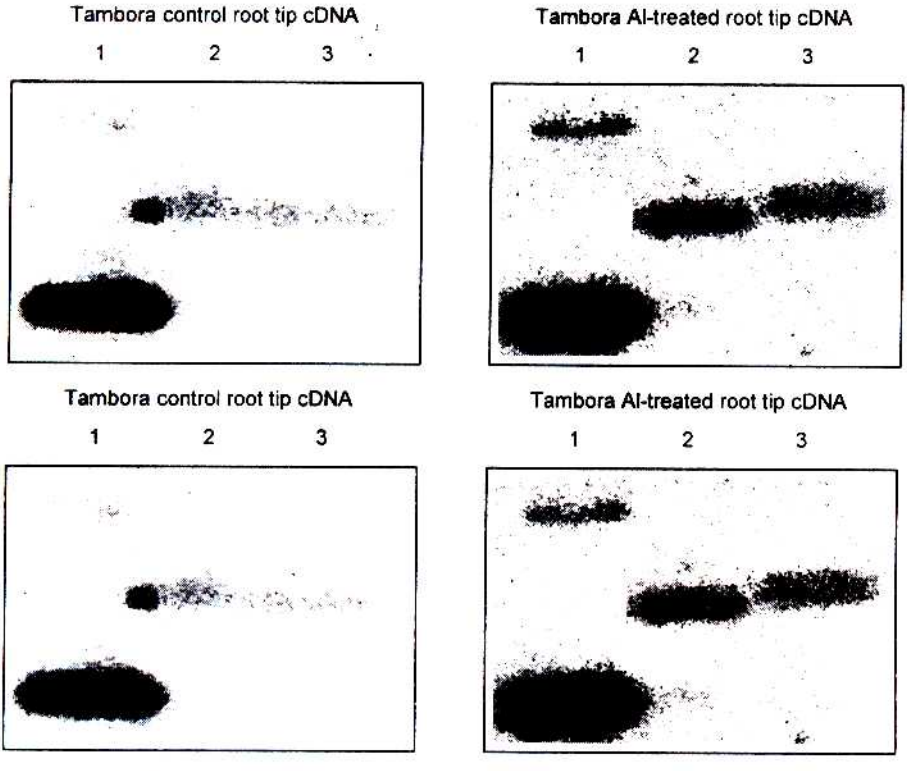
Notes: Obtain PCR fragments were transferred to the membrane and hybridized with ^{32}P-labeled cDNA from root tip cells of tolerant line Tambora, non-treated (A) and treated (B) with 300 μM $AlCl_3$

Figure 4. Verification of Marathon PCR fragments obtained with use of TA2-2 (lanes 1, 2) and TA 5-2 (lanes 3-7) DD sequences by Reverse Northern Blotting

Inosine-5'-monophosphate dehydrogenase may play key role in attemps to enhance nucleic acid biosynthesis severely affected by Al during stress.

ABC transporter family possess important physiological function in plant detoxification actively transporting different toxic substances across the tonoplast membrane. However, Al-resistant species (for example wheat and soybean) have lower Al uptake and accumulation in the cell. Therefore ABC transporters could not be directly involved in detoxification of Al itself. It is known that Al stress cause peroxidation of membrane lipids (Ezaki *et al.*, 1995). That is why ATP-dependent transporters could be involved in detoxification of lipid peroxidation products on membrane surface.

In order to study influence of the isolated genes on plant resistance to Al stress we plan to express transiently the genes *in vitro* cultured protoplasts of soybean as a homologous system. On the other hand stable over expression of the genes of interest will be studied in *Nicotiana tabacum* as a model organism. Analysis of transgenic plants



Notes: DNA was isolated from positive clones and hybridized with the same quantity of appropriate ^{32}P-labeled cDNA: 1 = II-58 1 b2 cDNA full fragment, 2 = II-63 2a3 cDNA full fragment, 3 = I-31 bb cDNA full fragment

Figure 5. Differential expression of the genes corresponding to the selected clones detected by Reverse Northern Blotting

along with protoplasts under conditions of Al stress in comparison to non-treated controls can bring some light in the problem of plant response to Al stress.

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