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# ANALYSIS AND EXPRESSION OF AL-TOLERANT GENES FROM SOYBEAN [*Glycine max* (L.) Merryl] ON FORAGE CROPS AND *Escherichia coli*

S. Anwar, Sumarsono, Karno and F. Kusmiyati
*Faculty of Animal Agriculture, Diponegoro University*
*Email: syaifulanwar06@yahoo.com, syaifulanwar2011@gmail.com*

## ABSTRACT

In order to analyze and to study expressions of the Al-tolerant genes, we have examined five clone genes that were isolated from soybean cv. Lumut. Soybean cv. Lumut and Slamet, *Centrocema pubescens, Pennisetum purpureum* and *Escherichia coli* were selected for futher analysis. Based on the DNA sequencing, searching enzyme restriction sites and searching DNA homology with the genebank database; the clones encoding: (1) Catalase (*gmali*12, that function as an antioxidant), (2) Proliferating cell nuclear antigen like protein/PCNALP (*gmali*15, that involved as one of transcriptional regulator in the eucaryotic cell cycle), (3) Growth hormone (*gmali*22, this gene may play a role on stimulation of cell growth/development), (4) Amine oxidase (*gm*AO, genebank accession number AF313622, a gene that function as amine oxidation and/or antioxidant), and (5) Aminoacyl peptidase (*gm*AP, genebank accession number AF091304, a serine protease gene). Expressions of the clone genes either on forage crops or *Escherichia coli* indicated that all of the clones are basic genes, but its expression increased with aluminium induction (Al-induced genes) and involved in detoxification to Al stress. From this research, we also found similar responses between oxidative stress and Al stress to gene responses.

*Keywords: Analysis, Expression, Al-Tolerant Genes, Soybean, Forage, E. coli*

## INTRODUCTION

Aluminum (Al) is regarded as one of the main toxic factors which exist in most acidic soil in Indonesia (Notohadiprawiro, 1983), even of the world, comprising $1x10^9$ hectares in the tropical and cool temperature regions (Van Wambeke, 1976) or approximately 8% by Weight (Moller *et al*., 1984). Most Al in soil is insoluble, associated with complex aluminosilicates and oxides. However, under acidic soil condition (pH < 5) Al is converted from insoluble forms into soluble $Al^{+3}$ (Marschner, 1991; Driscoll and Schecher, 1990; and Kinraide, 1991), which block growth of plant roots (Rajaram and Villegus, 1990; Kinraide and Ryan, 1991; Foy *et al*., 1978; Wagatsuma *et al*., 1987; and Taylor, 1991). Thus, Al toxicity is one of the most important soil problems that limits plant growth, particularly in the tropical regions.

Approximately 40% of the world's arable soils are too acid, and Indonesia has over 47,6 millions hectares. A problem that is becoming increasingly severe, because of the use of nitrogenous fertilizer, industrial pollution and acid rain (Van breeman, 1985). Eventhough, normal rainfall can also cause acidification of soils by promoting the leaching of basic cations such as $Ca^{+2}$, $Mg^{+2}$, $K^+$ and $Na^+$ (Foy, 1984). Thus, Al toxicity is one of the most important soil problems that limits plant growth, particularly in the tropical regions (Kochian, 1995; Taylor, 1995; Matsumoto, 2000).

Identifying genetic resistance to aluminium toxicity would be a valuable contribution toward the development of tolerant crops in the tropical areas, especially in Indonesia. In these low pH aluminosilicate soils, the susceptibility of field crops to aluminium toxicity leads to the inhibition of root growth into the lower soil horison. Aluminium saturates the charged sites of the soil particle and, along with the restriction of root growth, acts to impede cation exchange with subsoil elements ($Ca^{+2}$, $K^+$, and $Mg^{+2}$), which are critical for normal plant development. Determining the molecular basis of tolerance to increase levels of aluminium in certain crops (such as soybean) poses a significant challenge.

Soybean is one of important crops in Indonesia. Its specific material for Indonesian tradisional food such as tempe, tofu, sauce and soybean milk have brought the soybean to an important position in Indonesian nutrition. Demand for soybean is increasing with the increase on protein need due to improvement program on Indonesian nutrition. The development of animal husbandry in Indonesia have also increased the demand of this crop. Unfortunately, the increase in demand for soybean can not be responded by the sufficient increase in production of this crop. This research was conducted to support soybean breeding programs by molecular approach. Considering the importance of molecular information on soybean tolerance to Al, we proposed the research on molecular biology of soybean tolerance to al stress as follow-through from previous research, by two approach: (1) Analysis of the Al-tolerant and (2) Study of expression of the cloned genes. The genes also have been evaluated to forage crop by northern/slot blot hybridization (heterologous approach) and *E. coli*.

## RESEARCH METHOD

The research consist of two programs: Research I (Analysis of Al-tolerant genes) and Research II (Study expression of Al-tolerant genes).

**Research 1**. There are 3 steps in this program: (a) Analysis of clone genes by nucleotide sequencing, (b) Analysis of clone genes by searching restriction enzyme sites and (c) Analysis of clone genes by searching homology with GeneBank database.

DNA synthesis for chain-termination sequencing is carried out two steps. In the first, the primed strand of DNA is extended and at the same time labelled by the incorporation of dye-nucleotide. . In the second step, dideoxynucleotides are added to the population of labelled DNA molecules (ranging in length from a few to many hundreds of nucleotides) and synthesis continues until a ddNTP is incorporated, thus terminating the chains.

Analysis of clone genes by nucleotide sequencing was started with cDNAs cloned from our previous study that is not analyzed yet (Anwar, 1999). Plasmid cDNAs cloned are prepared using the alkaline lysis method (Sambrook *et al.,* 1989). The selected cDNA clones was sequenced by dideoxynucleotide chain-termination method (Sanger *et al.,* 1977).

Analysis of clone genes by searching restriction enzyme sites using the amino acid and restriction enzyme sites software that have been developed by DCRG-team database, which provided information about analysis of DNA especially for searching of restriction enzyme sites, start and stop codon, amino acid sequence, including number of ATGC and amino acid.

Analysis of clone genes by searching homology with GeneBank database. The resulted cDNA sequences are then compared to the existing genes sequences in Genebank. First, we access to the NCBI (National Center for Biotechnology Information) website (http://www.ncbi.nlm.nih.gov), and then select GeneBank database for searching similarity/homology sites for nucleotide sequence (BLAST program/BLAST web). Finally, follow instruction provided in the web electronic guide till resulted kinds of the genes.

**In Research II**, expression of the cloned genes have been studied by (a) using mRNA analysis by northern/slot blot hybridization method both on soybean and forage crop and (b) *Escherichia coli's* exposed to Al toxic level. There are 4 steps for analysis of transcript level/mRNA analysis i.e: (a) Planting material, (b) total RNA Isolation, (c) probe preparation and (d) northern/slot blot hybridization.

Planting material was planted described by Anwar (1999). Total RNA was isolated from the root tips (± 5 mm) and/or leaf of soybean and forage crop treated and untreated with $Al^{+3}$, using phenol/SDS method (Ausabel *et al*., 1987).

Northern/Slot Blot Hybridization. Total RNA (10-15 μg) samples was denatured with glyoxal and DMSO, and followed incubation in 65°C for 15 minutes. Then, the RNA was transferred to Hybond-N+ membranes (Amersham) by Slot-Hybridization (prior to use, the slot must be cleaned with 0.1 N NaOH and washed by steril water- DEPC treated). Probes was prepared from cDNA inserts isolated from agarose gels and labelled by non radioactive system (ECL-system). Hybridization was performed as described in Virca *et al.* (1990). The filter was washed twice with 2xSSC+0.4%SDS for 10 min at 42°C, and twice with 2xSSC for 5 min at room temperature. Filter was stripped by immersion in warm (60°C) 0.1% SDS and reprobed up to three times as described by Sambrook *et al.* (1989).

For Expression of clone genes by identifying tolerant-genes with its expression on *Escherichia coli* to Al toxic level was implemented by addition of Al toxic level on LB (Luria Bertani) culture (2% bactotryptone, 0.5% yeast extract, 10 mM NaCl and 1% bactoagar. First of all, to set up assay for Al stress, *E. coli* and *E. coli* containing vector was cultured in LB with various Al treatment (0-500 ppm). Assay for Al-toxic level based on the reduction of *E. coli's* growth on media at least 75% from control (without Al). Secondly, all of the clones was cultured at LB plate with addition of Al-toxic level based on previous study for 2 days. The clones that involved to Al tolerance was indicated by *E. coli* (contained the clones) growing well in the selected media.

## RESULTS AND DISCUSSIONS

### Analysis of Al-tolerant Genes

There are five clones that are already analyzed (cDNA isolation and sequencing, searching enzyme restriction sites and searching homology with GeneBank database) as shown on Table 1 and Figure 1-5. Based on the searching homology with the genebank database, the clones encode: (1) Catalase (*gmali*12, that function as an antioxidant), (2) Proliferating cell nuclear antigen like protein/PCNALP (*gmali*15, that involves as one of transcriptional regulator in the eucaryotic cell cycle), (3) Growth hormone (*gmali*22), (4) Amine oxidase (*gm*AO, genebank accession number AF313622), and (5) Aminoacyl peptidase (*gm*AP, genebank accession number AF091304).

Table 1. Characteristics of clones

| No. | Clones | Characteristics |
|---|---|---|
| 1. | *gmali*12 | Nucleotide length=252 bp; Amino acid=84 aa; Encode=Catalase (CAT) |
| 2. | *gmali*15 | Nucleotide length=254 bp; Amino acid=84 aa; Encode=PCNALP |
| 3. | *gmali*22 | Nucleotide length=247 bp; Amino acid=65 aa; Encode= Growth Hormon (GH) |
| 4. | *gm*AO | Nucleotide length=830 bp; Amino acid=250 aa; Encode= Amine Oxidase (AO) |
| 5. | *gm*AP | Nucleotide length=657 bp; Amino acid=202 aa; Encode=Aminoacyl Peptidase (AP) |

Notes :
*gmali*12 = *Glycine max* aluminium induced number 12; *gmali*15 = *Glycine max* aluminium induced number 15
*gmali*22 = *Glycine max* aluminium induced number 22; *gm*AO = *Glycine max* Amine Oxidase
*gm*AP = *Glycine max* Aminoacyl Peptidas

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1 tggataatgaatttccacatactgacactgagtattagggttaatatgtggaaattcatt 60
61 atccaaaaacaactcaaacttgattcccatgcaagttatatgtctttgtttgatagtttt 120
121 ctttttttcttatttttttttatgtgttatatttacagatgggtatatgttctttgtttcat 160
161 tttttaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaggcggccgctcgcgat 240
241 ctaaaactagtc 252

Figure 1. Nucleotide and the deduced amino acid sequence of
The *gmali*12 Clone. Amino acid residues are shown in the single-letter codes (coding : Catalase)

1tgattatccattgtgttgatgttcacatagtctcatacaagcataacagtttaatatggg 60
61 tgatcagttgttcccatagtctcatacaagcatatcagtttaatatgggtgatcactgtt 120
121 acgaacacaaagcaaacaatcctagatgtggacagatacacacaactgattatccattgt 180
181 gttgttgttccaacatatatttttatgacaaaaaaaaaaaaaaaaaagggcggccgctcgcg 240
241 atctagaactagtc 254

Figure 2. Nucleotide and the deduced amino acid sequence of The *gmali*15 Clone. Amino acid residues are shown
in the single-letter codes (coding : PCNALP)

1 tgagcagaaaaggcatttatatatattacataacttatataagtgatttctatgtcttga 60
61 ttatttggtaccttacatcatccccaaatgcaggcaaaattttgaatagcctaaaaaagc 120
121 gtgatgcctatgtatgtcttaatcacatgaataatcatcatcccaaaaatgagaatcaaa 180
181 agaaggaaattagatgaaaagaaaacatgtaatgctacaaaatgaagttgttttacttta 240
241 CTGCTTC 247

Figure 3. Nucleotide and the deduced amino acid sequence of
The *gmali*22 Clone. Amino acid residues are shown
in the single-letter codes (coding : Growth Hormone)

1 gcaagaatggcacttcgttgaatggcagaagtggaattttcgtattggattcactcctag 60
61 ggagggtttggtaattcattcagtagcctatattgatggaagtcggggacgaagacctgt 120
121 ggcccatagattgagctttgttgagatggtagtcccgtatggagatcctaatgatcctca 180
181 ctataggaaaaatgcttttgacgctggggaagatggcctgggtaaaaatgctcattctct 240
241 caagaagggctgcgattgtttaggctatatcaaatactttgatgcgcacttcacaaactt 300
301 ctatggaggtgttgaaacaattgagaactgtgtttgtttgcatggagaagatcatggtat 360
361 tttatggaagcatcaagattggagaacaggattggctgaagttcgaaggtctagaaggct 420
421 gacagtgtctttatatgcactgtggctaactatgagtatggatttttctggcacttttat 480
481 caggatggaaaaatagaagcagagatcaagctcacaggaattctcagcttaggatcactt 540
541 caaccaggtgaactcgaaaatatggcacaaccattgcacctggactatatgcgcctgtcc 600
601 accaacatttttttgttgctcgtatggacatggcagtaaattgcaagcctggtgaaacatt 660
661 taatcaggttgttgaaggtgaatgtcaaaattgaaaaaccagaaacaataatgttcctaa 720
721 caatgcattttatgctgaaaaaaaaactgcttaaatcaaaaatggaagcaatgccttgat 780
781 tgtgacctttatctgcccctccctgggattgtttggaaccctaggacttt 830

Figure 4. Nucleotide sequence of the *gm*AO (*Glycine max Amine Oxidase*) Clone.

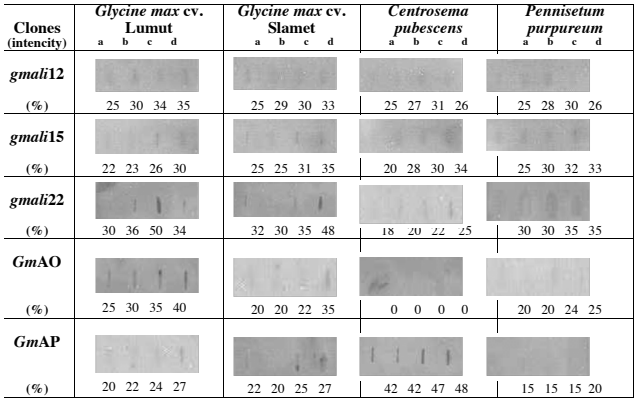
1 atggcagctactcaggaagatgtgtactctgatcccggttctcctatgatgcggagaact 60
61 caagctgggacatacattattgccaggataaagaaggaaagtgatgaaggaagatatatt 120
121 tatactgaatggaaatggtgctacaccagaaggaaacattccattccttgatctgtttga 180
181 cataaatacaggtaaaaaaatggaacgaatctgggagagcgataaggagaagtattatga 240
241 gactgttgttgctctaatgtctgatcaagaagaaggggatttgtatttagataaactgaa 300
301 gaagatactgacttctaaagagtcaaaaactgaaaacacccaatactactttgttagctg 360
361 ggccagataaaaacatagttcaggttacaaatttccctcatccataccctcagcttgcat 420
421 ccattgcagaaagagatgatcagatatgaaagaaaagacggggttcaacttactgctaca 480
481 ttatacctaccaccaggttacaatccatcaacagatggccctttgccatgcctggtttgg 540
541 tcttacccaggagaatttaagaacaaagatgctgctggacaagttcgtggtctccaaatg 600
601 aatttgtaggctccacatcttcctgagtagctgccatcgcccgaaacttcattcgtt 657

Figure 5. Nucleotide sequence of the *gm*AP (*Glycine max Aminoacyl Peptidase*) Clone.

## Expression of Al-tolerant Genes

Plants show spesific responses to many kinds of stress (biotic and abiotic) including aluminium stress. Genes response to Al stress will be reflected by increasing transcription (production mRNA) level of one or more genes. The molecular basis of these responses has not been completely worked out but there are clear examples of the expressions of many induced genes by aluminium stress.

Based on slot blot analysis (Figure 6), all of the genes are basic genes (appear at all of control media/media pH 6.0 without Al) , but its expression increased with Al stress (media pH 4.0 with Al stress). Clone g*mali*12 is coding catalase which involved as antioxidant. This result indicated that genes response to Al stress is similar with oxidation stress responses. This novel information is useful for genetic engineering. Similar result from the genes are expressed on *Escherichia coli*.




Notes : a = media pH 6.00 without Al; b = media pH 4.0 without Al
c = media pH 4.0 with 0.8 mM Al d = media pH 4.0 with 1.6 mM Al

Figure 6. Slot blot hybridization of clone genes on *Glycine max, Centrocema pubescens* and *Pennisetum purpureum*

Assay for Al-toxic level on *Escherichia coli*, based on the reduction of *E. coli's* growth on media at least 75% from control (without Al). We found that 300 ppm Al is a critical assay for *E. coli*, and used it for study of expression of Al-tolerant genes on *Escherichia coli*. The result of research is listed on Table 2-3 and Figure 7.

## CONCLUSIONS

From this research, we concluded that nucleotide and sequencing of Al-tolerant genes are coding: (1) Catalase (*gmali*12, that function as an antioxidant), (2) Proliferating cell nuclear antigen like protein (*gmali*15, that involved as one of tanscriptional regulator in the eucaryotic cell cycle), (3) Growth hormone (*gmali*22), (4) Amine oxidase (*gm*AO, genebank accession number AF313622), and (5) Aminoacyl peptidase (*gm*AP, genebank accession number AF091304)

Expression of Al-tolerant genes (*gmali*12, *gmali*15, *gmali*22, *gm*AO, and *gm*AP) either on plant or *Escherichia coli*, indicated that all of the clones are genes response to Al-induction and involved in detoxification to Al-stress.

Table 2. Optical density value ($OD_{550}$) of growth of *E. coli* in Luria

| Clone | Time of Stress (h) | | | | |
|---|---|---|---|---|---|
| | 0 | 2 | 8 | 24 | 48 |
| ***gmali*12: B+none** | 0 | 0 | 0 | 0 | 0 |
| **B+VnR** | 0 | 0 | 0.115 | 0.247 | 0.225 |
| **B+VR** | 0 | 0.157 | 0.388 | 0.748 | 1.166 |
| ***gmali*15: B+none** | 0 | 0 | 0 | 0 | 0 |
| **B+VnR** | 0 | 0 | 0.115 | 0.247 | 0.225 |
| **B+VR** | 0 | 0.125 | 0.315 | 0.685 | 1.225 |
| ***gmali*22: B+none** | 0 | 0 | 0 | 0 | 0 |
| **B+VnR** | 0 | 0 | 0.115 | 0.247 | 0.225 |
| **B+VR** | 0 | 0.095 | 0.250 | 0.595 | 1.247 |
| ***gm*AO: B+none** | 0 | 0 | 0 | 0 | 0 |
| **B+VnR** | 0 | 0 | 0.088 | 0.282 | 0.200 |
| **B+VR** | 0 | 0.210 | 0.517 | 1.025 | 1.825 |
| ***gm*AP B+none** | 0 | 0 | 0 | 0 | 0 |
| **B+VnR** | 0 | 0 | 0.088 | 0.282 | 0.200 |
| **B+VR** | 0 | 0.144 | 0.414 | 0.661 | 0.934 |

Notes :
B+none = DH10B *E.coli*; B+VnR = DH10B *E.coli* withVector/plasmid insite;
B+VR = DH10B *E.coli* with Vector Recombiant

Expression of Al-tolerant genes (*gmali*12, *gmali*15, *gmali*22, *gm*AO, and *gm*AP) either on plant or *Escherichia coli*, indicated that all of the clones are genes response to Al-induction and involved in detoxification to Al-stress.

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Table 3. Growth of *E. coli* in Luria Bertani agar media with 300 ppm Al for 24 h

| Stress | Clones | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *gmali*12 | | | *gmali*15 | | | *gmali*22 | | | *gm*AO | | | gmAP | | |
| | a | b | c | a | b | c | a | b | c | a | b | c | a | b | c |
| -Al:S | 125 | 130 | 120 | 125 | 130 | 135 | 125 | 130 | 120 | 87 | 110 | 126 | 87 | 110 | 106 |
| % | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| +Al:S | 0 | 20 | 91 | 0 | 20 | 110 | 0 | 20 | 104 | 0 | 21 | 110 | 0 | 21 | 83 |
| % | 0 | 15 | 76 | 0 | 15 | 80 | 0 | 15 | 87 | 0 | 19 | 87 | 0 | 19 | 78 |

Notes : a = B+none = DH10B *E.coli;* b = B+VnR = DH10B *E.coli* withVector/plasmid insite;
c = B+VR = DH10B *E.coli* with Vector Recombiant

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