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Identification and characterization drought tolerance of gene *LEA-D*11 soybean (*glycine max* L. Merr) based on PCR-sequencing

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ABSTRACT

Drought is one of the most damaging abiotic stress. Different plants response differently to drought stress. Abiotic stresses such as drought induced diverse physicological and molecular responses in plants. These responses include changes in gene expression. One of drought tolerance gene is a gene encoding dehydrin which is belongs to the group II or *D*-11 *LEA* protein family. *LEA-D*11 gene produce dehydrin protein which has a role in stabilization of membrane structures and protection of macromolecules in the presence of drought. The aims of the study was to identify and to characterize the *LEA-D*11 gene in various soybean varieties. This research used seven varieties of soybean: Tanggamus, Nanti, Seulawah, Tidar (drought tolerant), Wilis and Burangrang (drought moderate) and Detam-1 (drought susceptible). DNA genome of those varieties was isolated using the methods from Doyle & Doyle [1]. DNA amplification was conducted using *Polymerase Chain Reaction* (PCR) with specific primers designed based on *GmLEA-D*11 gene sequence database from the NCBI. The DNA targets were sequenced using automatic sequencing machine, ABI 3130xl Genetic Analyzer, in Eijkman Institution. The result of this study showed that the sequences of *GmLEA-D*11 gene possessed by drought tolerance varieties (Tanggamus, Nanti, Seulawah and Tidar) and moderately tolerance (Wilis and Burangrang) were similar. However, the sequence of *GmLEA*-D11 gene detected in the drought susceptible variety Detam-1 was different from the two groups. Similarity between drought tolerance and moderately tolerance indicate that there is not only *LEA-D*11 gene responsible to drought tolerance but also others. The primer and sequences *GmLEA-D*11 gene can be used as molecular marker and capable of differentiating between drought susceptible and drought moderate to drought tolerant.

Keywords: Drought Tolerance; *LEA-D*11 Gene; Soybean

1. INTRODUCTION

Abiotic stress such as drought, salinity, and frozen cause greatly damage and decrease yield. Under severe condition, these adverse environmental stresses can result in death of plant. Plants must respond and adapt to these adverse environmental condition to avoid or decrease cell injury caused by water deficit. Among the diversity of reponses, plants can adapt to water deficit by the induction of specific gene [2,3], including the changing of gene expression related drought tolerance. One of the gene related drought tolerance is *LEA-D*11 gene encoding family dehydrin protein [4,5].

Dehydrin are part of these LEA proteins (group II) and are built up by many charged and polar amino acids without cystein and tryptophan ever occurring [6]. Dehydrin are expressed during the late stages of embryogenesis [7,8] and also accumulated in vegetative tissues in response to water deficit [9]. Dehydrin have been found to accumulate in the cytoplasm, nucleus, plasma membrane and mitochondria [8,10-12].

Protein produced by drought-inducible genes which are identified through the recent microarray analysis can be classified into two groups [13]. The first group include proteins that most probably function in abiotic stress tolerance, the second group is comprised of regulatory protein. One of the gene products may play a role in drought tolerance is late embryogenesis abundant (LEA) protein. LEA is a functional protein which plays a role in stabilization of membrane structures and protected macromolecules [8]. Transgenic plant carrying genes for drought tolerance has been developed by the introduction of LEA gene, prolin synthesis and betaine

[14-16]. Dehydrin like protein may also have role similar to compatible solute (such as proline, sucrose and glycine be taine) in osmotic adjustment. Another possible role of stress proteins is to bind with the ion accumulated (ion sequestering) under drought stress and to control solute concentration in the cytoplasma [17].

In addition, recently, it has been suggested that some dehydrin probably play role in antioxidative defence response directly by their radical scavenging activity [18] or indirectly by their capability of binding toxic metals and preventing production of ROS [19]. Dehydrin scavenged the hydroxyl radical and peroxyl radical, but did not superoxide anion and hydrogen peroxide [20]. Several residue such as Lys, His, Glyn d Ser, maybe related to the radical scavenging because the residue were modified when the dehydrin scavenging the hydroxyl radical. Dehydrin may protect cellular components from oxidative stress [21].

Identification and characterization of drought tolerance gene for developing molecular marker and selecting genetic variation in plants are very useful. The aims of this study is to identify and to characterize drought tolerance *LEA-D*11 gene in soybean varieties which tolerant, moderate and susceptible of drought.

2. MATERIAL AND METHOD

Growth Condition and Plant Material. Seven soybean varieties were utilized: Tanggamus, Nanti, Seulawah, Tidar (tolerant drought), Wilis, Burangrang (moderate drought), Detam-1 (susceptible drought). The experiment consisted of two treatments. Plants were grown in pots in a greenhouse. Control plants were well-watered throughout the experiment at about 100% field capacity; the drought stress treatment was conducted by maintaining soil water at about 25% field capacity throughout early vegetative growth until seed fulfill. After the last watering, soil water content was measured daily by weighing. The volume of water added afterward was calculated based on the weight difference between the soil before and after plant transpired in one day.

DNA Isolation. Total DNA was extracted from young soybean leaf, using the method of Doyle dan Doyle [1]. Fresh leaf with the weight of 0.1 - 0.2 g was grinded with addition of liquid nitrogen, and then 700 µL CTAB buffer was added and incubated for 30 minute in waterbath 65˚C. The DNA then was extracted using the mixture of chloroform: isoamyl alcohol (24:1). DNA was precipitated using 0.1 volume ammonium acetat and 2.5 volume ethanol absolute. The concentration and purity of extracted DNA was determined used spectrofotometric at the wavelength of 260 and 280 nm.

Primer Design. Primers were designed based on the sequence of complete CDS (coding DNA sequence) of *GmLEA-D*11 (ID: AM421515) from NCBI (The National Center for Biotechnology Information) database using the Oligo Analyzer 1.0.2., Oligo 1.1. software. The sequences of the primer were: forward 5'-ATGATCAGGGTCGCAAGGTC-3', and reverse 5' CTTGTCACTGTGTCCTCCAG-3' with the amplification product of 700 bp.

Polymerase Chain Reaction. The total volume of PCR mixture was 20 µL per-tube, which were consist of 11.9 µL dH_2O, 2 µL buffer Taq PCR; 1.6 µL $MgCl_2$; 1.6 µL dNTPs 2.5 mM, (Qiagen-Taq PCR Master Mix), 0.3 µL primer forward-reverse (10 - 100 ng/µL), 0.3 µL Taq-Polymerase (5 U/µL) and 2 µL (1 µg/µL) DNA. The PCR program was set on 93˚C for 1 minute preheating, continued with 30 cycles consisting of 1 minute denaturation at a temperature of 93˚C, 1 minute annealing at a temperature of 57˚C, and 1 minute extension at a temperature of 72˚C. A final extension was conducted for 1 minute at a temperature of 72˚C. The PCR product was visualized on 1% agarose gel.

Sequences Analysis. Sequencing of the PCR products were performed with ABI automatic sequencer (ABI 3130xl Genetic Analyzer) using fluorescence-labelled nucleotides. The sequences were analyzed using multiple sequence alignment by *Sequence Scanner v*1.0, *ClustalW*, *Bioedit* and BLAST (Basic Local Alignment Search Tool) programme from NCBI.

3. RESULT AND DISCUSSION

3.1. Identification of *GmLEA D*-11 Gene on Various Soybean

Using the primer derived from the sequence of *GmLEA-D*11 gene, PCR products with the size of about 701 bp were produced. The results showed that both of the DNA genome of soybean varieties treated with drought stress treatment and the control can be amplified by the primer (**Figure 1**). These indicates that the tolerant, moderate

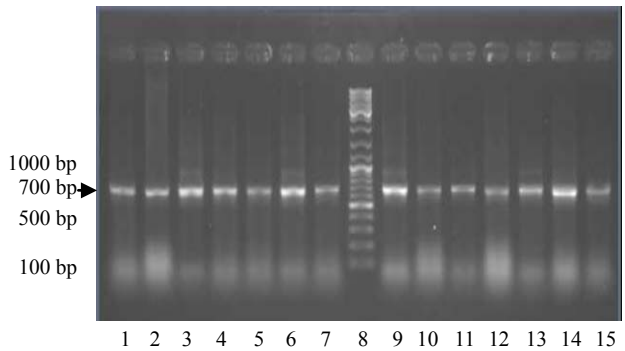

Figure 1. The PCR product in some varieties of soybean plants using primers *LEA-D*11 Lanes 1-7 (control); 1: Tanggamus; 2: Nanti; 3: Seulawah; 4: Tidar; 5: Wilis; 6: Burangrang l; 7: Detam; 8: Marker. Lane 9-15 (drought); 9: Tanggamus 10: Nanti; 11: Seulawah; 12 : Tidar; 13: Wilis; 14: Burangrang; 15: Detam 1.

and susceptible drought varieties both in control and drought stress treatment posses *LEA-D*11 gene.

Drought did not alter *LEA-D*11 gene, this is indicated by the appearance of bands at 700 bp in control and drought condition. Basically, a gene provides the instructions for making a protein and proteins influence the characteristics of plants. Gene is genetic material which more stable than protein. Environmental stresses do not change the gene but may change the expression of the gene such as protein alteration. However gene variation can be induced by mutagenic agents such as radiation and certain chemicals [22].

Comparing the sequence of Tanggamus varieties (drought tolerant) to the sequence of *LEA-D*11 of soybean in the NCBI datase resulting in the high homology of those sequences (**Table 1**).

The gene sequences of Tanggamus varieties had 100% similarity with *Glycine max LEA-D*11 gene for dehydrin. This means that the gene is amplified genes *LEA-D*11.

3.2. Comparison of *LEA-D*11 Sequence of Several Varieties of Soybeans

Sequence alignment between *GmLEA-D*11 Tanggamus varieties (drought tolerant) with other varieties used in this experiment (Nanti, Seulawah, Tidar, Wilis, Burangrang and Detam 1) treated with drought stress and the control without drought stress (**Figure 2**). The results showed that both in control and drought stress condition the sequence of *LEA-D*11 possessed by drought tolerant soybean varieties Tanggamus, Nanti, Seulawah and Tidar are not different from the sequence of *GmLEA-D*11 possessed by moderately tolerant varieties Burangrang and Wilis, however some sequence differences were detected in the drought-susceptible varieties, Detam-1.

Comparing the sequence of *GmLEA-D*11 gene possessed by Tanggamus with other soybean varieties, Nanti, Seulawah, Tidar, Wilis, Burangrang and Detam-1 under conditions without stress (control = K) with a variety Tanggamus, Nanti, Seulawah, Tidar, Wilis, Burangrang and Detam 1 in stress conditions (treatment = C) shows 6 mutation site. These mutation site were only found in Detam 1 but were not detected in other varieties. The changes of DNA sequence occur in Detam alter the amino acid in mutation site number 2 and 4. There is no changing the amino acids in mutation site number 1, 3, 5 and 6.

Mutation site 2 and 4 shows the nitrogen base changes. Mutation site number 2 shows the changing of amino acid from proline to serine, and mutation site 4 shows the changing of amino acid valine to Ileusine. This suggests that the difference in some nitrogen bases in DNA sequences have changed expression in response to drought stress become drought susceptible. However the sequences of *GmLEA-D*11 identified in this experiment were similar to the gene sequences possessed by drought tolerant varieties Tanggamus, Nanti, Seulawah, Tidar and moderately drought varieties Wilis and Burangrang. That similarity indicate that there is not only *LEA-D*11 gene which is responsible to drought tolerance but also other gene. There are hundreds of genes induced by drought stress has been identified [13].

Examined the drought resistance genes in soybean, and found that the sequence of *GmDREB*2 gene on different varieties of soybean are different, but the difference did not affect expression of the nature of drought tolerance [23]. It was suggested that not only *GmDREB*2 genes responsible for drought tolerant. There could be many genes that influence resistance to drought stress. [24] examined drought resistant gene *DREB*1 in several genotype of soybean, and discovered that the tolerance level of several soybean genotypes was not affected by variations in the sequences of *DREB*1 gene.

*LEA-D*11 gene is a gene that produces a functional protein dehydrin which is regulated by several genes. *LEA* genes work is influenced by other member of drought resistance gene family that can be expressed in certain circumstances, either simultaneously or alternately expressed depending on environmental conditions [6,25].

Some stress-responsive genes regulated by ABA [26-29] shows two regulatory pathway of dehydrin accumulation in sunflower, which is ABA-dependent and ABA-independent. Transcription factors for *LEA* are *DREB*2 and *DREB* 1 which act to initiate the transcription of the gene [30].

Table 1. Homology sequence Tanggamus varieties comparison with soybean NCBI database.

Gene database soybean from NCBI	Accession number	Length of sequence (bp)	Similarity (%)
*Glycine max LEA-D*11 gene for dehydrin	AM421515.1	751	100
*Glycine max LEA*2-*D*11 for dehydrin	AM420412.1	729	99
*Glycine max LEA-D*11 gene for dehydrin Cultivar M103	AJ583802.1	729	99
*Glycine max LEA-D*11 gene for dehydrin Cultivar V74	AJ583800.1	729	98
*Glycine max LEA-D*11 gene for dehydrin Cultivar Cuc Vang	AJ583799.1	681	88
*Glycine max LEA-D*11 gene for dehydrin Cultivar MV1C	AJ583801.1	681	87

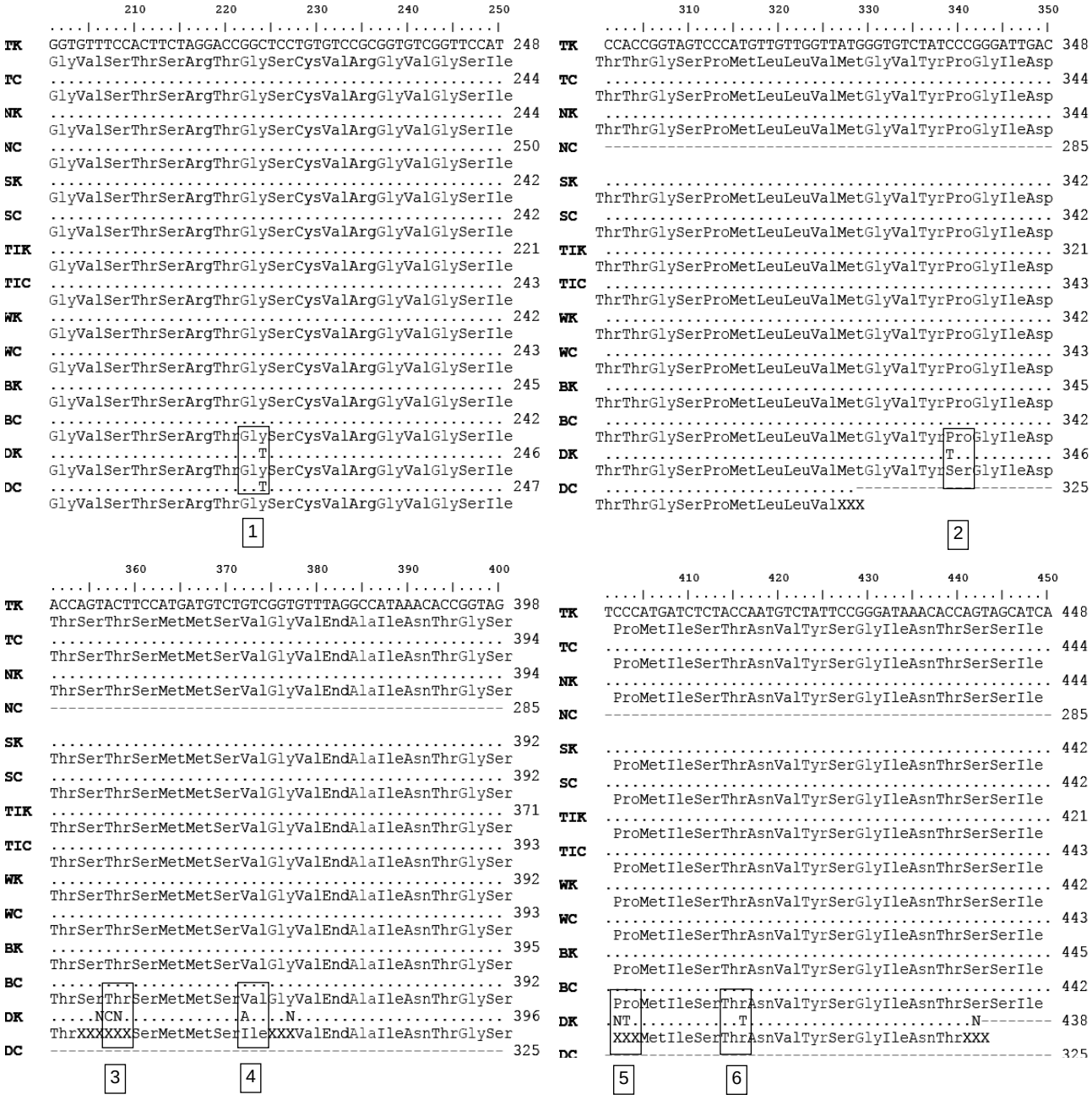

Figure 2. The results of amino acids alignments *GmLEA-D*11 Tanggamus varieties with some varieties of soybean under conditions without stress and drought stress conditions. TK = Tanggamus control, NK = Nanti control, SK = Seulawah control, TC = Tidar control, WK = Wilis control, BK = Burangrang control, DK = Detam control, TC = Tanggamus drought, NC = Nanti drought, SC = Seulawah drought, TIC = Tidar drought, WC = Wilis drought, BC = Burangrang drought, DC = Detam drought.

The expression of certain gene is influenced by a number genes that can be active (on) or inactive (off) as depend on time and environment. *DREB* transcription factors and *DRE* element serves as a signal transduction under conditions of drought, salinity and cold stress. *DREB* transcription factors can control the expression of several target functional genes involved in plant tolerance to drought conditions, salinity and cold temperatures [31].

Evaluate the role of genes coding for dehydrin proteins (*LEA-D*11) during drought stress in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* [32]. The results show that *GmLEA* gene generally expressed only in drought stress treatment. This supports that the dehydrin is essential for plants to adapt in drought stress [25,29, 33,34]. Significantly, the introduction of many stress-inducible genes transfer resulted in improved plant stress tolerance [35,36]. *LEA-D*11 gene specific primers de-

signed can be used as molecular marker and capable of differentiating between drought susceptible and drought moderate or drought tolerant.

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