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DNA Polymorphism of the Drought Tolerance Gene *GmDREB2* of Indonesian local Varieties Soybean (*Glycine max* L. Merr).

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ABSTRACT

The drought-tolerant gene *Gm*DREB2 is categorized as a regulatory gene which has a role in the formation of *drought responsive element binding transcription factors*. This study analyzed *Gm*DREB2 sequence variability of some drought-tolerant and susceptible Indonesian local varieties using the Polymerase Chain Reaction (PCR)-sequencing method. PCR analysis using *Gm*DREB2 primers resulted in a 415 bp PCR product. The sequence of the PCR product showed 97-87% homology to the *Gm*DREB2 of soybean species in the NCBI database. Alignment analysis of the sequence of all varieties used in this experiment found 14 mutation sites, and each variety has a different number of mutation sites. Although the mutation caused alteration of amino acid, it did not change the level of drought tolerance. This indicates that *Gm*DREB2 is not the only gene that influences the drought tolerance.

Keyword: Soybean, drought stress, *Gm*DREB2, mutation site

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INTRODUCTION

Soybean (*Glycine max* L.) is one of the most important crops in the world [1], which serves as a source of food and feed protein. In Indonesia, the production of soybean has fluctuated. In 2008, soybean production reached 775,710 tons in a planting area of 590,956 ha, while in 2009 it reached 972,945 tons in a planting area of 721,499 ha. Soybean production in 2010 was estimated at 962,539 tons with a total planting area of 709,071 ha [2] One important problem that is usually faced by farmers is drought. Drought stress on the reproductive phase of soybean causes a reduction in productivity of up to 50% [3]. So, development of a drought-tolerant soybean variety is needed to overcome the problem.

To develop a drought-tolerant variety, the availability of genetic information related to the mechanism of responses toward drought stress is needed. One preliminary attempt is to identify the existence of drought-resistant genes in soybean plants. There are several genes associated with drought-resistant properties [4], among which are *DREB*1, *GmDREB2*, *PIP*1, *PIP*2 and *LEA* [5]. The *DREB*1 and *GmDREB2* genes are categorized as regulatory genes, which play a role in signal transduction and regulation of gene expression. The gene *DREB1* is responsible for the formation of a *drought-responsive element binding protein*, whereas *GmDREB2* has a role in the formation of *drought-responsive element binding transcription factor* [6]. Among other genes, *Mn-sod,* controls the formation of *manganese-superoxide dismutase* ([7], and *P5CS* is responsible for the formation of *delta 1-pyrroline-5-carboxylate synthetase*, which has a role in proline acumulation [8]. The genes *PIP1, PIP2* and *LEA8* produce some functional proteins related to resistance to drought stress [9]. The *PIP1* and *PIP2* genes are responsible for the formation of aquaporin, a protein related to the availability of water in plants [10], whereas *LEA8* codes the formation of dehydrin during a period of drought stress [11]. It has also been indicated that *HVA1* is accumulated at the time of seed desiccation [12]. Other gene responsible in drought tolerance *TPS1* has been found [13] codes trehalose-6-phosphate synthetase and is involved in the biosynthesis of trehalose [14, 15], whilst *SacB* produces the enzyme *SOD* (superoxide dismutase) [16]. Furthermore, *betA* codes choline dehydrogenase and *betB* codes betaine aldehyde dehydrogenase, which has a role in the biosynthesis of glycine betaine, initiating tolerance to drought [16, 17].

The tolerance of some soybean varieties toward drought stress in the fields has been identified [3]. Soybean lines which were categorized as drought-tolerant varieties were Dieng, Tidar and Wilis. Some studies using a physiological and genomic-fingerprint approach indicated that Burangrang and Anjasmoro are susceptible to drought stress ([18, 19]. However, information is not yet available about whether the tolerance toward drought stress is converted by genes and whether the alteration of tolerance showed by those varieties is caused by gene mutation. In this experiment, identification of drought-resistant genes *GmDREB2* in drought- tolerant and susceptible varieties by PCR using primers specific to drought-resistant genes was done. The alteration of the gene sequences between varieties was also investigated.

MATERIAL AND METHODS

Plant material and DNA isolation

Plant materials used in this experiment were drought-tolerant varieties Dieng, Tidar, and Wilis and drought-sensitive varieties Burangrang, Anjasmoro, and Grobogan. The plants were grown in polybags in a greenhouse, one plant per polybag. The DNA genome was isolated from young soybean leaves using the CTAB method of Doyle and Doyle [20].

Polymerase Chain Reaction (PCR) and Sequencing

PCR was conducted using forward and reverse primers, which were designed based on the sequence of *GmDREB2* obtained from the GenBank. The sequences of the primers were 5'-ATG GAA GAA GCG TTA GGT GGA GA-3 ' (forward) and 5'-TGG AGG ACG TCG AGT ATT GTG G-3 ' (reverse).

A mixture of 20 µL solution consisting of 10x Ex Taq polymerase buffer, 2 mM MgCl2, 200 uM dNTPs, 25 pmol primers, 1U Taq polymerase and distilled water was used for each PCR reaction. The PCR program was set on 93^{o} C for 2 minutes preheating, continued with 35 cycles consisting of 1 minute denaturation at a temperature of 93 ^{o} C, 1 minutes annealing at a temperature of 58 ^{o} C, and 90 seconds extension at a temperature of 72 ^{o} C. A final extension was conducted for 10 minutes at a temperature of 72 ^{o} C. The PCR

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product was visualized on 1,5% agarose gel. Sequencing for the *GmDREB2* PCR product was conducted at 1st BASE Pte Ltd in Singapore.

Data Analysis

Data were analysed using the Basic Local Alignment Search Tool (BLAST) program of the NCBI.

RESULTS AND DISCUSSION

Identification of *GmDREB2* gene

The gene *GmDREB2* has been identified to have homology to *DREB*2 which is expressed in response to osmotic stress, high salinity, and cold. The response is possibly ABA-independent or ABA-dependent [1]. Primers which were designed based on the sequence of *GmDREB2* in this experiment were capable of amplifying a band with the size of about 415 bp both in drought-sensitive and drought-tolerant varieties (Figure 1).

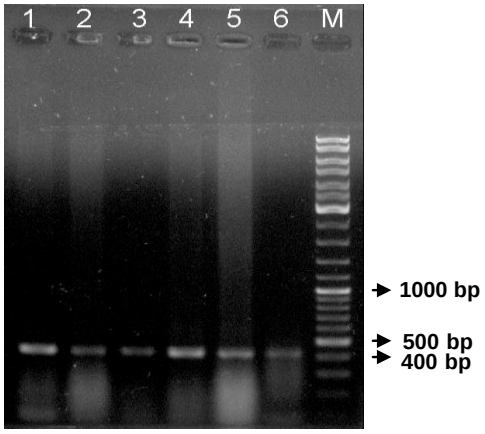

Figure 1: Amplification result of primer *GmDREB2* in sensitive varieties: Lane 1: Anjasmoro; 2: Burangrang; 3:Grobogan; and tolerance varieties: Lane 4: Dieng; 5: Tidar; 6: Wilis; M: Marker 1 Kb

The dehydration responsive element (DRE) is a cis-acting element which is important in regulating the expression of genes' response to drought, high salinity and cold stress. Unlike the *DREB*1 role in the cold response, the protein synthesised by the *DREB*2 gene is classified into a regulatory transcription factor which has a role in increasing plant tolerance to drought stress through an osmotic/dehydration response pathway [21] and tolerance toward high-salinity stress [9]. The *DREB*2 gene may also activate other genes involved in drought stress resistance [21].

Analysis sequence *GmDREB2* gene

An alignment analysis of the *GmDREB2* sequence of the Dieng variety (Ref. No. JF946769) showed a high similarity with the Glycine max recorded in the database, with reference numbers of DQ054363.1 (97%), AK244651.1 (96%), FJ965341.1 (96%), and BT091877.1 (87%). These indicate that the sequenced gene which was identified in this experiment indeed is the *GmDREB2* gene. Alignment analysis of *GmDREB2* sequence between the drought-tolerant variety, Dieng (Ref. No. JF946769), with other varieties, Tidar (Ref. No. JF946770), Willis (Ref. No. JF946771), Anjasmoro (Ref. No. JF946766), Burangrang (Ref. No. JF946767), and Grobogan (Ref. No. JF946768), showed 14 mutation sites with each variety having a different number of mutation sites. Most of the bases changing did not alter the resulted amino acid (silent mutation). The drought-tolerant variety, Tidar, has 1 mutation site, the Willis variety (drought tolerant) has 10 mutation sites, the Anjasmoro variety (drought susceptible) has 1 mutation site, the Burangrang variety (drought sensitive) has 11 mutation sites, and the Grobogan variety (drought sensitive) has 9 mutation sites. The changes of the nitrogenous bases caused some alteration in encoded amino acid (missense mutation) (Table 1).

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Table 1: Changes in nitrogenous bases on the gene mutation site sequences GmDREB2 in some varieties of soybean varieties compared to Dieng.

Variety	Mutation site	Nitrogen bases changing	Codon changing	Amino acid changing	Mutation Type
Tidar	10	T→C (Transition)	TAT→ TAC	Tyr → Tyr	Silent
Wilis	**4**	**T→G (Transversion)**	**GAT→GAG**	**Asp→ Glu**	**Missense**
	5	A→G (Transition)	CAA→CAG	Gln→ Gln	Silent
	6	T→C (Transition)	AAT→AAC	Asn→ Asn	Silent
	7	C→A (Transversion)	CGT→AGG	Arg→ Arg	Silent
	8	T→G (Transversion)	CGT→AGG	Arg→ Arg	Silent
	9	C→T (Transition)	CTG→TTG	Leu→ Leu	Silent
	10	T→C (Transition)	TAT→TAC	Tyr→ Tyr	Silent
	11	C→T (Transition)	CTC→CTT	Leu→ Leu	Silent
	12	**C→G (Transvertion)**	**AAC→AAG**	**Asn→ Lys**	Missense
	14	C→G (Transversion)	GCC→GCG	Ala→ Ala	Silent
Anjasmoro	**13**	**C→A (Transversion)**	**CAA→ AAA**	**Gln→ Lys**	**Missense**
Burangrang	3	G→T (Transversion)	ACG→ACT	Thr→ Thr	Silent
	4	**T→G (Transversion)**	**GAT→GAG**	**Asp→ Glu**	**Missense**
	5	A→G (Transition)	CAA→CAG	Gln→ Gln	Silent
	6	T→C (Transition)	AAT→AAC	Asn→ Asn	Silent
	7	C→A (Transversion)	CGT→AGG	Arg→ Arg	Silent
	8	T→G (Transversion)	CGT→AGG	Arg→ Arg	Silent
	9	C→T (Transition)	CTG→TTG	Leu→ Leu	Silent
	10	T→C (Transition)	TAT→TAC	Tyr→ Tyr	Silent
	11	C→T (Transition)	CTC→CTT	Leu→ Leu	Silent
	12	**C→G (Transversion)**	**AAC→AAG**	**Asn→ Lys**	**Missense**
	14	C→G (Transversion)	GCC→GCG	Ala→ Ala	Silent
Grobogan	1	G→C (Transversion)	TCG-->TCC	Ser-->Ser	Silent
	2	**T→A (Transversion)**	**AAT-->AAA**	**Asn--> Lys**	**Missense**
	4	**T→G (Transversion)**	**GAT-->GAG**	**Asp-->Glu**	**Missense**
	5	A→G (Transition)	CAA-->CAG	Gln-->Gln	Silent
	7	**C→A (Transversion)**	**CGT-->AGT**	**Arg--> Ser**	**Missense**
	9	C→T (Transition)	CTG-->TTG	Leu-->Leu	Silent
	11	C→T (Transition)	CTC-->CTT	Leu--> Leu	Silent
	13	**C→A (Transversion)**	**CAA-->AAA**	**Gln--> Lys**	**Missense**
	14	C→G (Transversion)	GCC-->GCG	Ala--> Ala	Silent

The modification of bases which occurred in Tidar did not affect the resulting amino acids; on the other hand, the changing of bases in Wilis, Anjasmoro, and Grobogan Burangrang caused amino acid alteration. Wilis demonstrated two amino acid variations resulting from bases mutation at the sites 4 and 12, Anjasmoro showed an amino acid change at the mutation site 13, whereas Burangrang had two amino acid subtitutions at mutation sites 4 and 12, and Grobogan had an amino acid variation at mutation site 13. Codon mutation, which occurred in gene *GmDREB2* of the drought-tolerant variety Wilis and the drought-sensitive variety Burangrang, produced similar amino acids, but the ability of those varieties to respond to drought stress was different. This shows that the change of some nitrogen base, which causes alteration into a similar amino acid, does not necessarily modify the response toward drought stress. This emphasizes the previous finding that the drought stress response was encoded by several different codons [22], and the changes in amino acids within a gene do not necessarily alter its expression in response to drought stress.

Mutation that affects the less vital parts of the protein will provide a minor effect and still retain its substantial activity, and even in rare cases amino acid changes in proteins may result in a better functioning protein [22]. Study of the role of the *DREB*1 gene in somaclone soybean has indicated that the missense mutation in somaclones does not alter its expression in the resistance to drought stress.

Identification of *GmDREB2* on soybean local varieties indicated that the gene sequences were different. However, these differences did not affect the tolerance toward drought stress. So, this indicated that the nature of drought tolerance is not only influenced by the gene *GmDREB2* alone, but is also influenced by multiple genes in a family of drought-resistant genes through a complex mechanism. This finding is in accordance other researcher finding which reported that there are 16 candidate genes potentially involved in

drought stress response in rice [23]. Furthermore, drought stress induces several genes to produce proteins that can be classified into groups of functional proteins and regulatory proteins [9,24]. Group functional proteins include chaperones, *LEA* proteins (dehidrin), osmotin, antifreeze proteins, mRNA-binding proteins, aquaporin, sugar and proline transport proteins, key enzymes for osmolyte biosynthesis, detoxification enzymes, and various proteases, while groups of regulatory proteins include transcription factors, protein kinases, protein phosphatase, the enzymes involved in phospholipid metabolism and signals, and also other molecules such as a calmodulin-binding protein.

The members of the drought-resistant gene family can be expressed in a certain condition, either simultaneously or alternately, depending on environmental conditions. Tolerance to abiotic stress is a complex reaction because of the complicated interaction between stress factors and the various phenomena of molecular, biochemical and physiological factors that affect plant growth and development [25].

CONCLUSION

Different varieties of soybean were identified in this experiment to have differences in the sequence of GmDREB2 genes, but such differences do not affect the expression of tolerance toward drought stress. This indicates that drought tolerance is not only influenced by GmDREB2 genes.

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