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Characteristics of the gene encoding pyrroline-5-carboxylate synthase (P5CS) in Vietnamese soybean cultivars (*Glycine max* L. Merrill)

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***Abstract*—This research evaluates the ability of six soybean local cultivars and a control cultivar (DT84) to tolerate severe drought. In this research, SL5 and SL6 have the most stress tolerance while DT84 and SL1 have the least stress tolerance. We have isolated P5CS gene as it relates to stress tolerance by using PCR with primers. The results show that the size of the P5CS gene of the studied cultivars is 2,148 bp, which encodes for 715 amino acids. When these proteins are compared with the P5CS proteins of the other species, the result shows that the isolated P5CS proteins have a higher identity than other bean species. They are located on the same branch of the phylogenetic tree. Proline (Pro) is one of the most accumulated osmolytes in salinity and water deficit conditions in plants. P5CS is a key regulatory enzyme involved in prolin biosynthesis in plants and is subject to feedback inhibition by prolin. The conserved aspartate residue at 126 and the phenylalanine at 129 are involved in the feedback inhibition by Pro binding. A gene encoding a feedback insensitive P5CS enzyme was obtained by mutating the P5CS gene. The mutation was due to a change of an aspartate at position 125 to alanine in the P5CS. This mutated gene was cloned in pBT vector and sequenced. This gene will be transfered to plants to increase the level of proline overproduction and to enhance the drought tolerance ability.**

Keywords—drought, Glycine max, point mutation, P5CS, proline.

I. INTRODUCTION

Soybeans are short-duration industrial crops which have high economic values and an important position in the structure of agricultural crops in Vietnam. They are also the ideal crops in the rotation system due to their potential capability for enriching the fertility of the soil. Soybean is a species very sensitive to adverse conditions of the environment such as heat, cold, drought, insect attack,… especially drought because soybeans are poor in drought-resistance. Drought makes plants mature about 8 days earlier than usual and prolonged drought limits the process of forming seeds (Hall & Twidwell, 2002) [13]. The previous studies show that the process of adjustment of osmotic pressure is one of the primary methods to help plants adapt to the condition of water shortages in the environment. The osmotic pressure in plant cells is adjusted through the synthesis and accumulation of substances such as sugar exhange, polyamine, Betaine glycine, proline... Among these substances, proline is an exchange factor which has been studied in detail. Proline is known as one of the substances playing an important role in the process of adjusting the osmotic pressure when plants live in adverse conditions such as drought and salinity (Delauney *et al*., 1993) [7]. Under adverse conditions of the environment, the phenomenon of increasing the proline accumulation was found in different organisms such as a bacteria, protozoa, species of marine molluscs and plants (Verbruggen & Hermans, 2008) [22]. In many plants, the proline content in the individual plant's ability to deal with drought can be increased more than 100 times in comparison with those in the control cultivar. Currently, it is believed that the accumulation of proline plays an important role in the resistance of plants to drought conditions.

In plants, proline is synthesized from two different precursors, glutamate and Ornithine, by two different synthesis cycles (Adams & Frank, 1980 [1]; Delauney *et al.*, 1993 [7]). In the first cycle, proline is produced through two reduction reactions of glutamate. These reactions are catalyzed by two enzymes, which are Δ-pyrroline-5-carboxylate synthase (P5CS) and pyrroline-5-carboxylate reductase (P5CR). P5CS is an enzyme activating glutamate through the phosphorylation process. This enzyme also reduces the product to form glutamate semialdehyde (GSA) (Hu *et al*., 1992) [11]. In the second cycle of proline synthesis, ornithine is aminised to form pyrroline-5-carboxylate with the catalysis of orn-δ-aminotransferase (OAT). This enzyme exists in mitochondria (Verbruggen & Hermans, 2008) [22]. However, when plants experience adverse environmental conditions, proline is synthesized mainly through the first cycle. This has been demonstrated through analyses of the expression of P5CS and P5CR in *Arabidopsis thaliana* and moth bean plants (Hu *et al*.,1992 [11]; Verbruggen *et al.,* 1993 [23]; Yoshiba *et al.,* 1995 [25]). It has been concluded from the previous studies that the

P5CS is an enzyme playing the primary role in proline synthesis process and its activity depends on the content of proline in plant cells (Zhang *et al.,* 1995) [24]. These proofs show that P5CS plays an important role in regulating proline synthesis in plants under adverse environmental conditions (Porcel & Ruiz-Losato, 2004) [17].

The research on proline synthesis and lysis demonstrates that proline plays a role as a primary source of energy, nitrogen and carbon in plant cells under drought conditions (Kohl *et al*., 1988 [15]; Kavi kishor *et al*., 1995 [14]; Peng *et al*., 1995 [16]; Hua *et al*., 1997 [12]). In *Vigna aconitifolia* and *Arabidopsis* plants, pyrroline-5-carboxylase (P5CS) is one of the essential enzymes in the synthesis of proline from glutamate. This is a multi-functional enzyme with the activities of γ-glutamyl kinase (γ-GK) and glucose-5-semialdehyde (GSA) dehydrogenase (Hu *et al*., 1992 [11]; Savoure *et al*., 1995 [18]; Yoshiba *et al*., 1995) [25]. Two gene loci of P5CS were discovered in the nuclear genome of tomatoes: a locus encodes for enzymes P5CS and a locus is a polycistronic mRNA encoding for the enzymes γ-glutamyl kinase (γ-GK) and glucose-5-semialdehyde (GSA) dehydrogenase in two separate forms of peptide (Garcia-Rios *et al*., 1997) [8]. Two similar P5CS genes are also found in *Arabidopsis* and *Medicago sativa* (Strizhov *et al*., 1999 [21]; Ginzberg *et al*., 1998 [9]). The gene P5CS1 in *Arabidopsis* expresses in most organs and especially expresses intensely when plants live under adverse conditions (Strizhov *et al*., 1999 [21], Zhang *et al*., 1995 [24]; Yoshiba *et al*., 1995 [25]). In *Medicago sativa*, both P5CS1 and P5CS2 were expressed intensely in roots when plants were salt treated, but P5CS2 was expressed more clearly in the research conditions (Ginzberg *et al*., 1998) [9].

The previous studies demonstrated that when plants were experiencing adverse conditions, the accumulation of proline in plant cells was regulated through feedback inhibition mechanisms which modified the structure of the protein P5CS (Boggess *et al*., 1976a, 1976b) [2], [3]. In *Escherichia coli*, the synthesis of proline is adjusted through the inhibition of activity of the enzyme γ-GK by the end product of the cycle that is proline, by which the activity of this enzyme is reduced to 100 times in the laboratory strains (Smith *et al*., 1984) [20]. However, in *Samonella typhimurium* mutant strains which resist 3,4-dehydro-D, L-Pro (a homologous type of proline) shows that the accumulation of proline in cells is directly proportional to the tolerance of cells under adverse conditions of osmotic pressure (Csonka *et al*., 1981) [4]. When studied in E. coli, Dandekar and Uratsu (1988) found that, A single base pair change has been found in a site corresponding to a regulatory region of the first enzyme in the proline biosynthetic pathway [6]. When carrying out the molecular analysis of these mutant strains, the authors have discovered a mutation replacing aspartate (Asp) with asparagine (ASN) at position 107 in the amino acid sequence of the enzyme γ-GK. This replacement makes the enzyme γ-GK lose its sensitivity under the impact of proline (Csonka *et al*., 1988) [5]. Comparing the P5CS gene sequences of bacteria plants shows that the amino acid Asp at position 107 corresponds to Asp at position 128 in the amino acid sequence of the P5CS enzyme of higher plants. However, when applying the site directed mutation method to change Asp into Asn, this position is not related to feedback inhibition reactions to the P5CS by proline in higher plants. In contrast, when Asp at position 125 and 128 are changed, the activity of P5CS is more durable than the original enzyme when carrying out reactions at high concentration of proline (Zhang *et al*., 1995) [24]. The transgenic tobacco lines which increased the expression of genes encoding for genotypes of enzyme P5CS that have mutations that replace Asp at two positions 126 and 129 expressed salt-resistance better than transgenic tobacco plants with no P5CS in the environment containing NaCl 200mM (Hong *et al*., 2000) [10]. With the aim of making materials for transgenic in order to create soybean cultivars which are able to survive under drought conditions, we used the point mutation method to eliminate the feedback inhibition by proline for P5CS.

II. MATERIALS AND METHODS

Six Viet Nam local cultivars and a hybrid cultivar of soybean were provided by the Center for Research and Development of Soybeans – Vietnam Institute of Agricultural Sciences. They are Muong La (SL1), Quynh Nhai (SL2), Mai Son (SL3), Moc Chau (SL4), Song Ma (SL5), Yen Chau (SL6) and control cultivar (DT84) .

Total RNA was extracted with Trizol Reagent by the procedure of Invitrogen. cDNA is synthesized by the process RevertAidTMH Minus First Strand cDNA Synthesis Kit (Fermentas). The P5CS gene which was amplified by the PCR technique with specific pairs of primers designed based on gene sequences of P5CS of soybean (*Glycine max*) has been published in international gene bank and numbered AY492005. The P5CS gene size is approximately 2150 bp. We designed two pairs of specific primers P5CSfor/SacI-P5CS and BamHI-P5CS/P5CSrev. The sizes of PCR products with these pairs of primer size are approximately 1300 and 1100 bp.

The thermal cycles of PCR were: 94^0C/5 mins; 30 cycle: 94^0C/30 seconds, 58^0C/45 seconds, 72^0C/2 minutes 30 seconds; 72^0C/10 min. PCR products were detected by electrophoresis on 0.8% agarose gel, cleaned by using to the QIAquick Gel Extraction Kit (Bioneer), then integrated to the cloning vector PBT and transformed into competent cells *E. coli* DH5α by the heat shock method. Plasmids were extracted following the procedure described by Sambrook *et al*., (2001) [19] and purified by using Plasmid Miniprep Kit (Qiagen). The nucleotid sequence of the P5CS gene was identified by the nucleotide sequence automatic reading machine ABI PRISM @ 3100 Advant Genetic Analyzer (Applied Biosystem). The result of gene sequencing was analyzed using the software DNAstar and BioEdit.

III. RESULTS AND DISCUSSION

A. Drought-resistant ability of local soybeans cultivars

We evaluated quickly the drought-resistant ability of soybean cultivars at the juvenile stage (when the plants have three leaves) by determining the relative drought-resistant index base on analysing morphological phyological and

biochemical characteristics. The results show that the two cultivars SL5 and SL6 were the most drought-resistant, and the two cultivars SL1 and DT84 were the least drought-resistant (Table I).

TABLE I. INDEX OF DROUGHT-RESISTANCE AND RATE INCREASES OF PROLINE CONTENT IN SOYBEAN CULTIVARS

Cultivars	Index of drought-resistance	Rate increase proline content after 5 days (%)	Rate increase proline content after 7 days (%)	Rate increase proline content after 9 days (%)
SL1	11108.24	109.29	133.88	156.83
SL2	9509.91	202.94	227.94	272.79
SL3	9582.35	158.16	209.18	229.59
SL4	9565.23	152.87	159.24	184.71
SL5	12701.30	200.75	233.08	377.44
SL6	12486.58	144.83	268.97	351.72
DT84	6788.75	101.06	129.26	146.81

B. Cloning and comparison of the sequence of P5CS gene from local soybean cultivars

The total RNA from leaves of four soybean cultivars SL5, SL1, SL6 and DT84 was separated, extracted and synthesized cDNA. The P5CS gene of four soybean cultivars DT84 and SL5, SL1, SL6 has been amplified by PCR and detected by electrophoresis on 0.8% agarose gel. The results in Figure 1 shows that the size of the fragment corresponds to the size calculated theoretically.

To obtain the complete P5CS gene, PCR products were mixed and used as the template for PCR reactions with primer pairs P5CSfor/P5CSrev. The obtained PCR products size of about 2100 bp (Figure 1).

P5CS gene was ligated to the cloning vector PBT and transformed into E.coli DH5α cells. Transformants was cultured on the environment LB supplemented with carbenicillin and X-gal/IPTG. The white cell lines carrying P5CS gene was selected by colony-PCR with specific primer pairs P5CSfor/P5CSrev, choose positive results to culture and extract plasmids. To identify the inserted gene, plasmids were cut with BamHI restriction enzyme (the results are shown in Figure 2). Electrophoresis test results showed a band size corresponding with gene P5CS (≈ 2100bp). The rest band was the cut pBT (≈ 2.7 bp). Thus, the recombinant vector was cut completely and it can be certainly concluded that the P5CS gene was inserted into vector PBT.

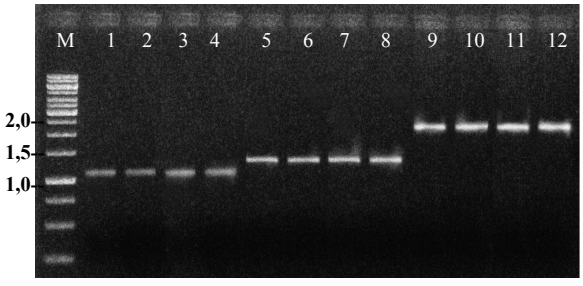

Figure 1. Electrophoresis products P5CS gene cloning. PCR products with primer pairs P5Csfor/SacI-P5CS (5,6,7,8); BamHI-P5CS/P5Csrev (1, 2,3,4); P5CSfor/P5Csrev (9,10,11,12). DT84 (1, 5, 9); SL5 (2, 6, 10), SL1 (3, 7, 11), SL6 (4, 8, 12). M: 1 kb ladder DNA standard.

The results from gene sequencing shows that the products of cloning gene from four studied samples are 2148 nucleotides long, coding for 715 amino acids. The nucleotide sequences of four soybean cultivars SL5, SL6, SL1, DT84 were compared with each other and with the sequence having the accession number AY492005 in NCBI GenBank. Results showed the high homology (99.2 to 99.5%), the differences were in 70 positions. Comparing amino acid sequences of these five soybean cultivars showed there was high homology (96.8% - 99.2%), the differences were in 38 positions. P5CS of all these five cultivars have amino acid Asp and Phe in the position 125 and 128, respectively. They are two positions that cause inhibition of activity of enzyme P5CS due to the increase of proline content in cells (Zhang *et al*., 1995 [24]; Hong *et al*., 2000 [10]).

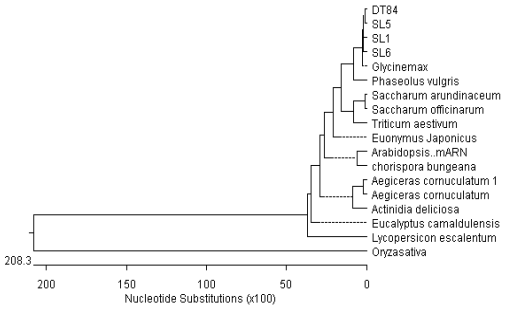

Figure 2. Genetic Relationship of soybean and other crops based on comparison of sequence P5CS gene

The results from comparing the sequence of P5CS protein of four studied species with those published in the NCBI gene bank showed that P5CS sequence of four soybean cultivars DT84 and SL5, SL1, SL6 has the high homology with legumes (96 to 99.2%). These sequences had been registered on the NCBI Genebank with accession numbers: FM999729.1; FM999730.1; FN564571.1; FN564570.1.

C. Create point mutation of the gene encoding pyrroline-5-carboxylate synthase (P5CS)

P5CS is a key regulatory enzyme involved in prolin biosynthesis in plants and can be inhibited by prolin. A gene encoding a feedback insensitive P5CS enzyme was obtained by create point mutation of P5CS gene. Basing on the sequence of P5CS of SL5 published in NCBI GenBank (FM999729.1), size of it is 2148bp. To create point mutation at position 125 of protein P5CS, we have designed three primer pairs: M125for2/SacI-P5CS and BamHI-P5CS/M125rev1 and P5Csfor/P5Csrev for PCR to make C at position 374 of the GCC triplet is replaced by A and triplet became GAC. It means that in amino acid sequence of protein, Asp were replaced with Ala at position 125. PCR

products with primer pairs with expectant size is 400 bp, respectively, 700 bp and 1770 bp.

PCR products were detected by electrophoresis on 0.8% agarose gel, the results showed that the obtained DNA fragments have the match sizes with the theoretical calculated. Product of PCR include three DNA fragments with size is 400 bp, 1800 bp, 700 bp. Use of the OE-PCR method (Overlay Extension PCR) to amplify the gene and OE-PCR product obtained have molecular size of about 2100bp. Products of the OE-PCR reaction (the mutant gene in the nucleotide at position 374) continue to be cloning, checked and read the sequence of the gene to determine the exact point mutation.

The result showed that C at position 374 of triplet GCC was replaced by A and GAC is new triplet and in amino acid sequence of protein Asp were replaced with Ala at position 125. When changing Asp at position 125 and 128, the activity of enzyme P5CS is more durable than the original enzyme when performing reactions at high concentrations of proline (Zhang et al., 1995) [24]. The transgenic tobacco lines which increased the expression of genes encoding for types of enzyme P5CS have mutations that replace Asp at two positions 126 and 129 expressed the salt-resistance ability better than transgenic tobacco plants with no mutation P5CS in the environment containing NaCl 200mM (Hong et al., 2000) [10]. The mutation of gene encoding pyrroline-5-carboxylate synthase is material to construct the recombinant vector used in transformation of P5CS gene to enhance the ability of drought-resistance of soybean cultivars.

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CONCLUSIONS

We have identified from six soybean cultivars that two cultivars SL5 and SL6 are the most drought-resistant; two cultivars SL1 and DT84 are the worst drought-resistant. P5CS gene of the four soybean cultivars SL5, SL6, SL1, DT84 was cloned successfully. The molecular size of gene isolated is 2148 nucleotides, coding for 715 amino acids. Nucleotide sequences of SL5, SL6, SL1, DT84 and of the sequence with accession number AY492005 show the differences in the 70 position, and the amino acid sequences show differences in 38 positions. We have successfully created point mutation at position 374 of the gene encoding pyrroline-5-carboxylate synthase (P5CS) in order to eliminate the feedback inhibition by proline against enzymes P5CS in soybean cultivars. This is the first successful in Vietnam. Mutated gene will be materials for transgenic in order to create soybean cultivars which are able to live in the drought conditions.

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