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ISOLATION AND CHARACTERIZATION OF Δ^1-PYRROLINE-5-CARBOXYLATE SYNTHETASE GENE FROM SUGARCANE (*SACCHARUM* SPP. L.)

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ABSTRACT

Sugarcane variety ROC22 was used as the experimental material and water stress treatment was performed for the 4 or 5-leafed plants with 25% polyethylene glycol (PEG) 6000 solutions. The cDNA sequence of ScP5CS gene in sugarcane with 2151 bp in length was isolated by homologous cloning, which contained an open reading frame encoding a protein of 716 amino acids, with GenBank accession number EU005373. Comparing the sequence of ScP5CS with that of sugarcane P5CS reported in GenBank, the nucleotide acid showed high identity (98%), but the deduced amino acid was only 92%. The deduced protein contains putative ATP-binding site, putative leucine domains, Glu-5-kinase domain, putative NADPH-binding domain, conserved GSA-DH domain and feedback inhibition site. Besides, there were more differences in Glu-5-kinase domain from the deduced amino acid of sugarcane P5CS reported (ABM30223), but less for the P5CSs from rice (Oryza sativa) and wheat (Triticum aestivum). So it is confirmed this gene is a new gene of sugarcane P5CS.

Key words: sugarcane; Δ^1-pyrroline-5- carboxylate synthetase (P5CS) gene; isolation and characterization; proline

Proline is accumulated in plants under drought and salinity stress in a number of species and is thought to play an important role in plant cells for adaptation to water stress (Delauney and Verma, 1993). The role of proline biosynthesis in osmoregulation in bacteria is well established. In *Escherichia coli*, the reaction starts with the phosphorylation of glutamate, catalyzed by γ-glutamyl kinase (γ-GK; encoded by the *proB* gene), to form γ-glutamyl

phosphate, which is reduced to glutamic-5-semialdehyde (GSA) by GSA dehydrogensae (encoded by the *proA* gene). GSA spontaneously cyclizes to Δ^1-pyrroline-5-carboxylate (P5C), which is reduced by P5C reductase (P5CR; encoded by the *proC* gene) to proline. The *E. coli proB*, *proA*, *proC* loci have been cloned and sequenced.

The proline biosynthesis route in plants is thought to resemble the pathway in bacteria. Whereas proline can be synthesized from either glutamate (Glu) or ornithine (Orn) in plants and animals, stable isotope and radioisotope labeling experiments indicate that Glu, rather than Orn, is the primary precursor for proline biosynthesis in osmotically stressed plant cells. High level expression of Δ^1-pyrroline-5-carboxylate synthetase (P5CS), a bifunctional enzyme that catalyzes the first and second reactions of proline biosynthesis, which exhibited both γ-GK and GSA dehydrogenase activities, has been reported to result in increased salinity stress tolerance in transgenic tobacco plants (Kishor *et al*., 1995). In *Vigna aconitifolia* and *Arabidopsis*, the first two steps of proline biosynthesis from Glu are catalyzed by P5CS (Hu *et al*., 1992; Savouré *et al*., 1995; Yoshiba *et al*., 1995).

Several genes encoding the enzymes in the route of proline biosynthesis have been identified in several plant species and all have been reported to be upregulated in response to water deprivation and/or salinization (Hare and Cress, 1997; Hare *et al*., 1998; Yamada *et al*., 2005; Ma, 2005; Igarashi *et al*., 1997). In *Arabidopsis thaliana*, P5CS is encoded by two differentially regulated genes, named *AtP5CS1* and *AtP5CS2* (Strizhov *et al*., 1997; Zhang *et al*., 1997). Divergence of the physiological functions of P5CS isolforms has been observed in alfalfa (*Medicago sativa*) and tomato (*Lycopersicon esculentum*). It was found in differentiated tissues, but could not be detected in dividing cell cultures in the absence of stress stimuli, *AtP5CS2*, which is solely responsible for the synthesis of transcript encoding P5CS in rapidly dividing *Arabidopsis thaliana* cell cultures (Strizhov *et al*., 1997). The genes encoding P5CS have been cloned from *Arabidopsis thaliana*, rice (*Oryza sativa*), wheat (*Triticum aestivum*), alfalfa (*Medicago sativa*), tomato (*Lycopersicon esculentum*), lettuce (*Lactuca sativa*), soybean, radish (*Raphanus sativus*), grape (*Vitis vinifera*), and moth bean (*Vigna aconitifolia*).

In this paper, we reported the isolation and structure of the ScP5CS gene in sugarcane as well as the features of the encoded polypeptide.

Materials and methods

Plant growth and salt-stress treatment

Sugarcane variety ROC22 was used as the plant material. The seedcanes were planted in the laboratory. Water stress treatment was performed when the plants grew with 4-5 leaves. The plants were treated with the liquids containing 25% polyethylene glycol (PEG) 6000, and the plants treated with deionized water were used as the control. Leaf samples were taken for RNA

extraction in 18-24 hours after treatment.

Cloning and sequencing of P5CS cDNA from leaves of sugarcane

The total RNA was extracted from the young leaves of sugarcane and the PCR amplification with procedures described by Wang and Fang (2002). The P5CS cDNA fragment was amplified from the total pool of cDNA using gene specific primers designed with reference to the known P5CS gene sequences (GenBank accession number: EF155655, AY574031, D49714, AY888045, DQ864376). The cDNA for PCR was synthesized in a standard first strand reaction using 0.5-5 µg of total RNA from leaves with 20 units of avian myeloblastosis virus reverse (AMV) transcriptase (Takara). The cDNA was subjected to 35 cycles of amplification in a 25µl reaction mixture containing 0.4 µM of each primer, 0.2 mM each of dATP, dTTP, dGTP, and dCTP, 10×PCR Buffer ($MgCl_2$ plus) , and 1.25 units of *Taq* DNA polymerase. Each amplification cycle consisted of 3 min at 94℃ firstly, then 1 min of denaturation at 94℃, 40 s of annealing at 53℃, and 90 s of extension at 72℃, with a final extension for 10 min at 72℃. The PCR products (10µl) were determined by electrophoresis through 1% agarose gels. The amplified cDNA was cloned and sequenced, and named ScP5CS.

Sequence analysis

The analyses of DNA and protein sequences were performed using DNAMAN software package and BLAST program of the NCBI. Homology alignment was performed using the Clustalx 1.83 and ClustalW program.

Results and analysis

Isolation, sequencing, and characterization of sugarcane ScP5CS cDNA

The complete nucleotide sequence of a P5CS cDNA clone, ScP5CS, was shown in Fig. 1. The sequence of 2151 base pairs contained a single major open reading frame encoding a polypeptide of 77762.0 Da for a putative protein of 716 amino acids with a calculated isoelectric point of 6.29 and GenBank accession number EU005373, and the translation would be initiated from the first ATG codon at 5' end of the coding strand. Comparing the sequence of ScP5CS with that of sugarcane P5CS reported in GenBank, the nucleotide acid showed higher identity (98%), but the deduced amino acid was only 92%. It showed 85% identity and 84% overall similarity to those of rice (*Oryza sativa*) and wheat (*Triticum aestivum*).

Interestingly, the putative ATP-binding site, putative leucine domains, Glu-5-kinase domain, putative NADPH-binding domain, conserved GSA-DH domain and feedback inhibition site were present in each of the enzymatic domains of sugarcane ScP5CS (Chiang *et al*., 1995; Ma, 2005; Hu *et al*., 1999). There were more differences in Glu-5-kinase domain from the deduced amino acid of the reported sugarcane P5CS (ABM30223), but less for the P5CSs from rice (*Oryza sativa*) and wheat (*Triticum aestivum*) (Fig. 2).

Sugarcane ScP5CS contains domains homologous to *E. coli proA* and *proBA* proteins

The ScP5CS clones efficiently complemented *E. coli*, *proA* and *proBA*, but not *proC*, the predicted two domains showed high homology to the *E. coli proBA* and *proA* proteins, and the recombinant ScP5CS protein showed GSA-dependent γ-GK enzyme activity. It was also found high homolog to bacterial and yeast γ-glutamyl kinase at the N-terminus and to bacterial γ-glutamyl phosphate reductase at the C-terminus of the ScP5CS protein. Searching in GenBank resulted in the conserved domain of the deduced amino acid sequences of sugarcane ScP5CS which was shown in Fig. 3.

Discussion

In the present study, it was successful in isolation and characterization of the cDNA and the corresponding gene of ScP5CS encoding the Δ^1-pyrroline-5-carboxylate synthetase from *Saccharum officinarum* L. as well as the features of the encoded polypeptide. Comparing the sequence of ScP5CS with that of sugarcane P5CS reported in GenBank (ABM30223), their nucleotide acid sequences showed high identity (98%), but the deduced amino acid homology was only 92%. The alignment of ScP5CS with all the presently available similar proteins in GenBank revealed two enzymatic domains corresponding to bacterial and yeast γ -glutamyl kinase and to bacterial γ-glutamyl phosphate reductase. The two residues in the positions 125 and 128 were conserved in sugarcane ScP5CS and other plants, which were aspartate (Asp, D) and phenylalanine (Phe, F), respectively. Site-directed mutagenesis might indicate whether they are implicated in the feedback inhibition by proline.

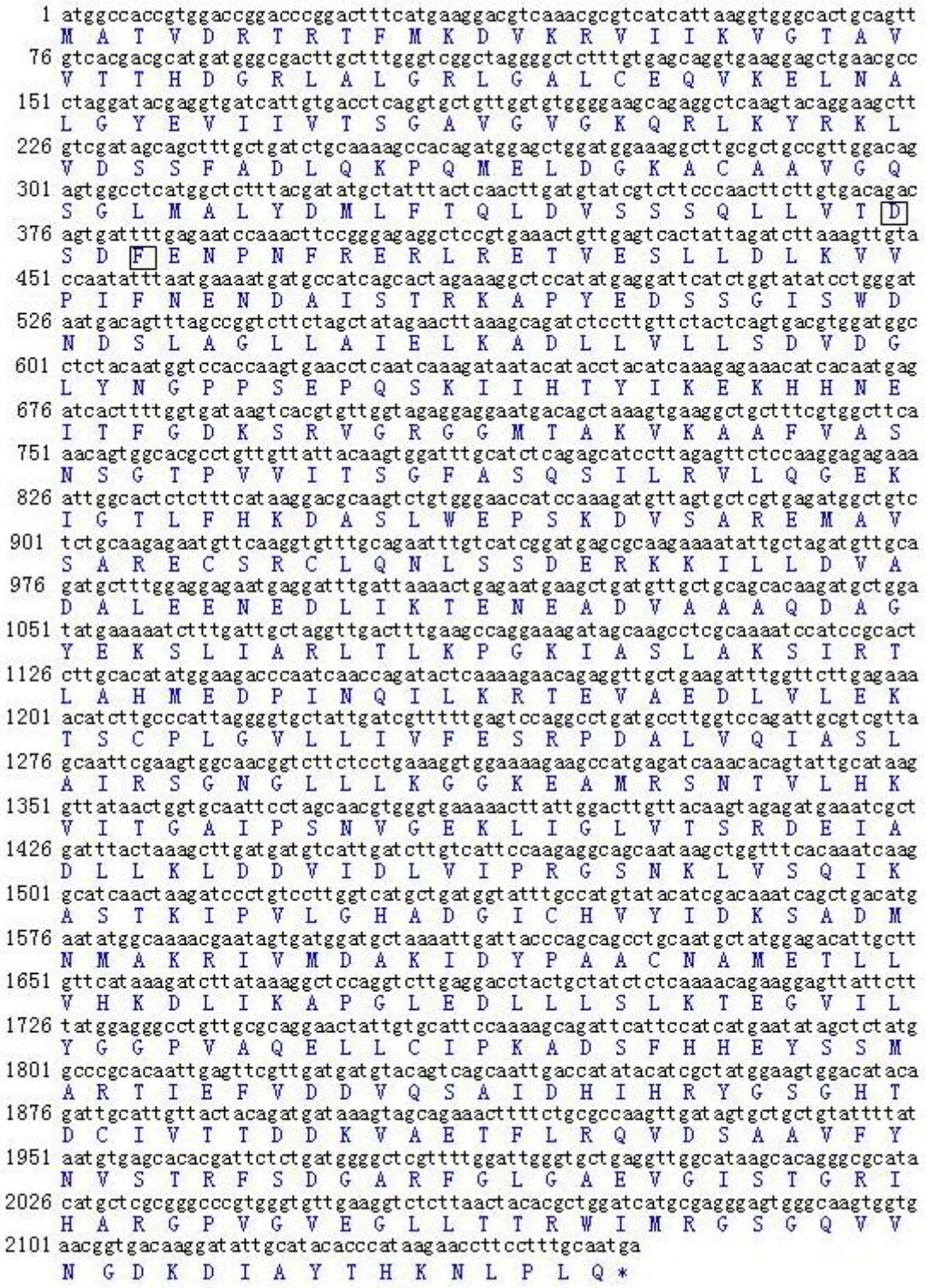

Fig.1 Nucleotide sequence of sugarcane ScP5CS cDNA and primary sequence encoding the protein of sugarcane ScP5CS

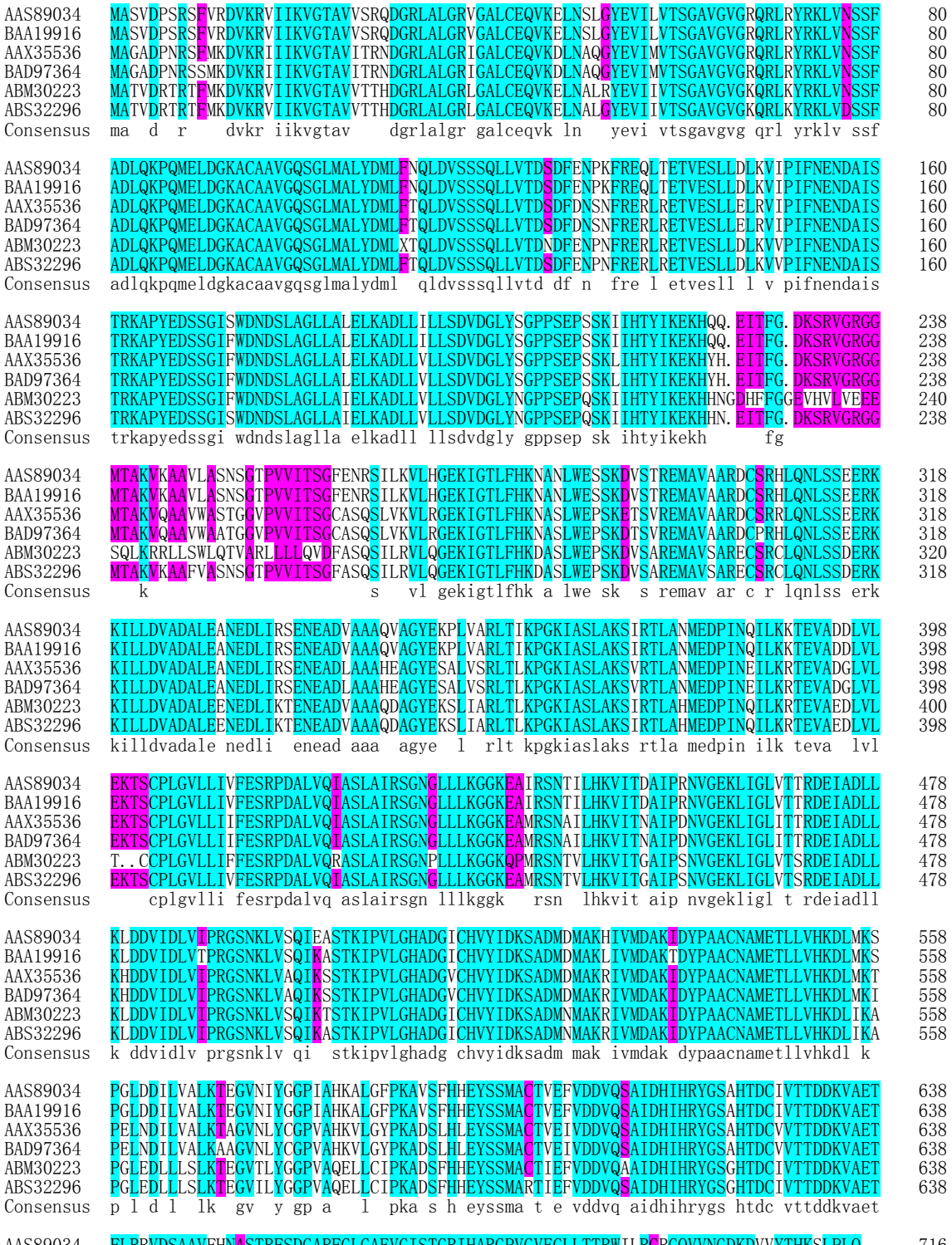

Fig. 2 Sequence alignment of the predicted ScP5CS amino acid sequence of sugarcane with the

P5CS protein sequences of gramineous plants, AAS89034, BAA19916 (*Oryza sativa*); AAX35536, BAD97364 (*Triticum aestivum*); and ABM30223, ABS32296 (*Saccharum officinarum*).

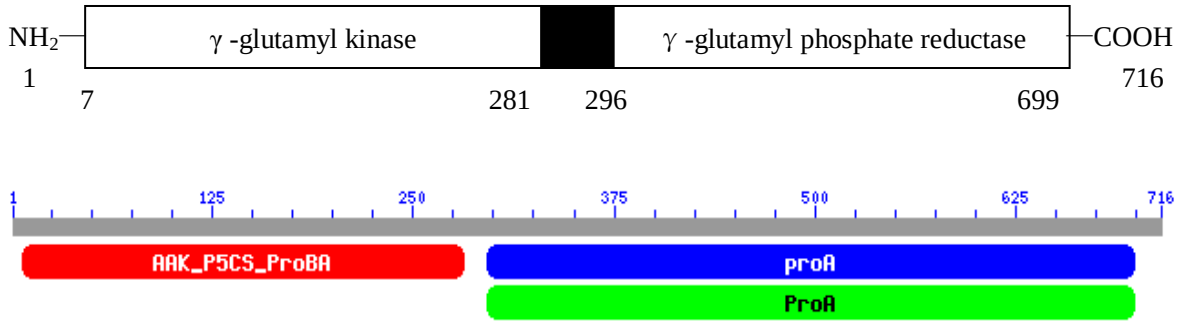

Fig.3 Conserved domain of the deduced amino acid sequences of sugarcane ScP5CS

Interestingly, putative ATP- and NAD(P)H-binding sites should also be included in the ScP5CS protein accord to other plants (Savouré *et al*., 1995). Amino acid sequence analysis revealed the identical sequences between the ScP5CS and gramineous plants. There were more differences in Glu-5-kinase domain from the deduced amino acid of the reported sugarcane P5CS (ABM30223), but less for the P5CSs from rice (*Oryza sativa*) and wheat (*Triticum aestivum*). Different molecular structures would lead to different functions for the ScP5CSs. Ahmed et al. (2015) reported that overexpression of a novel feedback-desensitized Δ^1-pyrroline-5-carboxylate synthetase increases proline accumulation and confers salt tolerance in transgenic *Nicotiana plumbaginifolia*. Proline content, expression level of P5CS (Δ1-pyrroline-5-carboxylate synthetase), and drought tolerance were closely related in *Tibetan hulless* barley (*Hordeum vulgare* var. nudum) (Deng et al., 2013). In Arabidopsis a *Lycium chinense*-derived P5CS-like gene is regulated by water deficit-induced endogenous abscisic acid and overexpression of this gene enhances tolerance to water deficit stress (Guan et al., 2014). Recently, Guerzoni et al. (2014) found that Stress-induced Δ^1-pyrroline-5-carboxylate synthetase (P5CS) gene confers tolerance to salt stress in transgenic sugarcane. In our experiment, the transformed antisence ScP5CS in tobacco inhibited the expression of tobacco P5CS. The transgenic plants grew slowly, the leaves were easy to get etiolational, the root system development was stunted, and the roots were shorter under the NaCl and PEG stress as compared with the control (data not shown). Further characterization of the events that underlie P5CS gene expression may also reveal proline insight into the stress-related changes in gene expression at post-transcriptional level.

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