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Cloning two *P5CS* genes from bioenergy sorghum and their expression profiles under abiotic stresses and MeJA treatment

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ABSTRACT

Sweet sorghum (*Sorghum bicolor* (Linn.) Moench) has promise as a bioenergy feedstock in China and other countries for its use in the production of ethanol as the result of its high fermentable sugar accumulation in stems. To boost biofuel production and extend its range, we seek to improve its stress tolerance. Proline acts as an osmolyte that accumulates when plants are subjected to abiotic stress. P5CS (Δ1-pyrroline-5-carboxylate synthetase) is a key regulatory enzyme that plays a crucial role in proline biosynthesis. We isolated two closely related *P5CS* genes from sweet sorghum, designated *SbP5CS1* (GenBank accession number: GQ377719) and *SbP5CS2* (GenBank accession number: GQ377720), which are located on chromosome 3 and 9 and encode 729 and 716 amino acid polypeptides, respectively. The homology between the two sweet sorghum *P5CS* genes was 76%. Promoter analysis of the two *P5CS* genes revealed that both sequences not only contained the expected cis regulatory regions such as TATA and CAAT boxes, but also had many stress response elements. Expression analysis revealed that *SbP5CS1* and *SbP5CS2* transcripts were up-regulated after treatment of 10-day-old seedlings of sweet sorghum with drought, salt (250 mM NaCl) and MeJA (10 μM). The expression levels of the both *SbP5CS* genes were significantly increased after 3-day drought stress. Under high salt treatment, peak *SbP5CS1* expression was detected at 4 h and 8 h for *SbP5CS2* in roots, while the trends of expression were nearly identical in leaves. In contrast, under drought and high salt stress, the up-regulated expression of *SbP5CS1* was higher than that of *SbP5CS2*. When the seedlings were exposed to MeJA, rapid transcript induction of *SbP5CS1* was detected at 2 h in leaves, and the *SbP5CS2* expression level increase was detected at 4 h post-treatment. *SbP5CS1* and *SbP5CS2* also show different temporal and spatial expression patterns. *SbP5CS2* gene was ubiquitously expressed whereas *SbP5CS1* was mainly expressed in mature vegetative and reproductive organs. Proline concentration increased after stress application and was correlated with *SbP5CS* expression. Our results suggest that the *SbP5CS1* and *SbP5CS2* are stress inducible genes but might play non-redundant roles in plant development. The two genes could have the potential to be used in improving stress tolerance of sweet sorghum and other bioenergy feedstocks.

1. Introduction

Drought and salinity are the most serious abiotic stresses to plants. These adverse factors restrict areas of potential cultivation and reduce productivity [1,2]. Therefore, research to develop plant resistance to these stress factors is a crucial undertaking needed to expand the range of crop growth to marginal or barren soils, especially saline-alkaline soils. Proline accumulation is a common metabolic response when higher plants are exposed to water deficits and high salt. Proline acts as an osmolyte that not only stabilizes protein structures, but also acts as the regulator of cellular redox potential [3,4]. The ability to accumulate proline under stress is often associated with stress tolerance in various plant species.

Proline is synthesized by either glutamate or arginine/ornithine pathways in higher plants; the glutamate pathway is the major route for proline synthesis during stress [5]. Under the glutamate pathway, proline is synthesized from glutamic acid via the intermediate γ-glutamic semialdehyde (GSA) and Δ1-pyrroline-5-carboxylate (P5C). Δ1-pyrroline-5-carboxylate synthetase (P5CS),

Abbreviations: ABA, abscisic acid; cDNA, DNA complementary to RNA; CDS, coding sequence; DNase, deoxyribonuclease; P5C, Δ1-pyrroline-5-carboxylate; P5CDH, Δ1-pyrroline-5-carboxylate dehydrogenase; P5CS, Δ1-pyrroline-5-carboxylate synthetase; MeJA, Methyl jasmonate; GSA, γ-glutamic semialdehyde; RT-PCR, reverse transcriptase PCR; ABRE, ABA-responsive element; ATP, adenosine-triphosphate; NAD(P)H, nicotinamide adenine dinucleotide phosphate; NJ, neighbor-joining; NCBI, The National Center for Biotechnology Information.

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which catalyzes the first two reactions of proline biosynthesis, is a bifunctional enzyme containing both the γ-glutamyl kinase and the glutamic-γ-semialdehyde dehydrogenase activities. The γ-glutamyl kinase activity of P5CS represents the rate-limiting step in this pathway and also features feedback inhibition by proline [5].

P5CS genes have been isolated from several plants species. A *P5CS* gene was first cloned from *Vigna aconitifolia* [6]. In some species two closely related *P5CS* genes have been identified, but they apparently have no unified functions. For example, in *Arabidopsis thaliana*, *AtP5CS1* is inducible by drought, salt and ABA, whereas *AtP5CS2* is apparently a housekeeping gene active in dividing tissues [7–9]. The expression patterns of two *P5CS* orthologues were also different under NaCl stress in tomato. The transcript of *tomPRO2* increased more than 3-fold, whereas the transcript of *tomPRO1* was undetectable [10]. In *Brassica napus*, however, both *BnP5CS1* and *BnP5CS2* were inducible by ABA, NaCl and PEG [11]. In *Oryza sativa* and *Phaseolus vulgaris*, *OsP5CS1* and *OsP5CS2*, *PvP5CS1* and *PvP5CS2* also were up-regulated by various stresses [12–15]. *OsP5CS1* is ubiquitously expressed contrasting with *OsP5CS2*, which is mainly expressed in mature organs. Prior studies demonstrated the up-regulation of the expression levels of *P5CS* and the accumulation of proline had a cause-and-effect relationship, except in tomato. Kishor et al. showed that overexpression of *VaP5CS* in transgenic tobacco resulted in significant accumulation of proline and an increase of biomass production compared with the non-transgenic parents under drought-stress conditions [16]. Overexpression of *P5CS* also increased stress tolerance of transgenic potato, rice and wheat as a result of the increased proline content [17–20].

Sweet sorghum (*S. bicolor* (Linn.) Moench) has been recognized as a promising energy plant owing to its high sugar content in stems, which could yield as much sugar as sugarcane. Vegetative biomass of sweet sorghum could be also used as livestock fodder [21]. Moreover, sweet sorghum is a highly productive species and can produce biomass ranging from 58.3 t to 80.5 t of fresh stems per hectare in semi-arid zones [22]. However, the development of bioenergy feedstocks is limited by the high cost of arable land. If bioenergy plants could be grown on lands that are currently marginal for many other crops, the two-fold problem of feedstock production and rural poverty could be addressed. Stress tolerance seems to be key trait to target in this regard.

Because conventional breeding is time-consuming and laborious, genetic engineering is an alternative approach to generate stress-tolerant plants. The functions of *P5CS*s make them candidates for genetic engineering. As an aid to target one or both of these genes for overexpression, we need to know the endogenous expression patterns of the *P5CS* genes. In this study, two full-length cDNAs of *P5CS* were cloned from sweet sorghum. The promoters of the two genes were also isolated and analyzed using bioinformatic methods. Expression levels of *SbP5CS* were analyzed in leaves and roots of sweet sorghum seedlings when exposed to drought, salt stresses and MeJA. The relationship between accumulation of proline and up-regulation of the expression levels was also demonstrated. The results of this study are promising in that both *SbP5CS* genes could be used as targets for genetic engineering of sweet sorghum to improve the stress tolerance. In particular, we envisage the overexpression of *SbP5CS1* as a means to endow broad stress tolerance in sorghum.

2. Materials and methods

2.1. Plant materials and stress treatments

Sweet sorghum (*S. bicolor* cv. Rio) seeds were dehusked and germinated on plates for 2 d, after which the seedlings were transferred into 8 cm diameter pots filled with vermiculite, with each pot containing 6 seedlings. Seedlings were grown in a greenhouse with 16 h light/8 h dark photoperiod, 25–18 °C (day and night, respectively) thermoperiod, and fertilized with Hoagland solution [23] every 3 d. Experiments were performed after 10 d when the nutrients of seeds were exhausted. A total of 21 pots with 126 plants were used for drought treatment and separate sets of 24 pots with 144 plants were subjected to salt (250 mM NaCl) and MeJA (10 μM) treatments. For drought stress, sweet sorghum seedlings were withheld water for 3 d. Leaf and root samples were collected at 0, 1, 2, 3, 4, 5, 6 d after the initiation of the treatment. Salt stress was imposed by application of Hoagland nutrient solution containing 250 mM NaCl. Leaf and root samples were collected at 0, 1, 2, 4, 8, 12, 24, 48 h after the initiation of treatment. For the MeJA stress, suspensions of 10 mM MeJA (Sigma–Aldrich) in deionized water were applied to seedlings with a hand sprayer until the solution began to drip off leaves. Leaf and root samples were collected at 0, 1, 2, 4, 8, 12, 24, 48 h after the initiation of treatment. A total of 3 pots with 18 plants were collected each time point under the above stresses, and then snap frozen in liquid nitrogen. The snap frozen material was then used for total mRNA extraction and determination of proline content.

2.2. Isolation of total RNA and synthesizing first strand cDNA

Total RNA was extracted with Trizol reagent (TIANGEN Corporation, China). A sample of 1 μg of total RNA was used for the first strand cDNA synthesis with PrimeScript RT-PCR kit (Takara Corporation, Japan) according to the instructions of the manufacturer.

2.3. Cloning P5CS cDNA and sequence analysis

For amplification the *P5CS* gene from sweet sorghum, two pairs of primers (P51-F: 5′-CACCGAGCCGAGCCACTTA-3′; P51-R: 5′-GGATCTCATCACACATGCA-3′; P52-F: 5′-AGACGGAGGACCAGGAG-3′; P52-R: 5′-CAGAATGAACCACCAGAAT-3′) were designed based on the monocot *P5CS* conserved region sequence then screened using the JGI (http://www.jgi.doe.gov/) sorghum genome database. The 25 μl PCR mixture contained 1 μl cDNA template, 200 μM dNTP, 0.5 units LATaq polymerase (Takara Corporation, Japan), 2.5 μl 10× LATaq buffer, and 0.4 μM primers. The PCR program was set up as follows: denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2.5 min, followed by extension of 72 °C for 10 min. The fragment obtained was subcloned in the PMD-18T vector (Takara Corporation, Japan), then transformed into DH5α competent cells (TransGen Biotech Corporation, China). The positive clones were subjected to M13 primer-based Sanger sequencing. Alignments were made using DNAMAN. ClustalW and MEGA 4.1 were used to construct a phylogenetic tree of P5CS proteins.

2.4. Promoter isolation

Genomic DNA was prepared from seedlings of sweet sorghum using the Plant Genomic DNA Rapid Extraction kit (Bioteke Corporation, China). Approximately 0.1 g of tissue was ground to a fine powder in liquid nitrogen. Primers used to isolate the promoter of the *SbP5CS1* were (P1-F: 5′-TTGCTGATCCCTTGCTGC-3′; P1-R: 5′-ATCCCTCCTCTCCCCATT-3′) and of the *SbP5CS2* were (P2-F: 5′-TTTTTCTCGCGAGTCCGG-3′; P2-R: 5′-CGCGTTTAACATCCCTCA-3′). The following PCR protocol was used for amplification the fragment: 5 min denaturation at 94 °C followed by 35 cycles of PCR (94 °C, 30 s; 60 °C, 30 s; 72 °C, 2.5 min), and a final extension at 72 °C for 10 min. Promoter regions were identified by the online promoter prediction database PLACE [24] and PlantCARE [25].