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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].

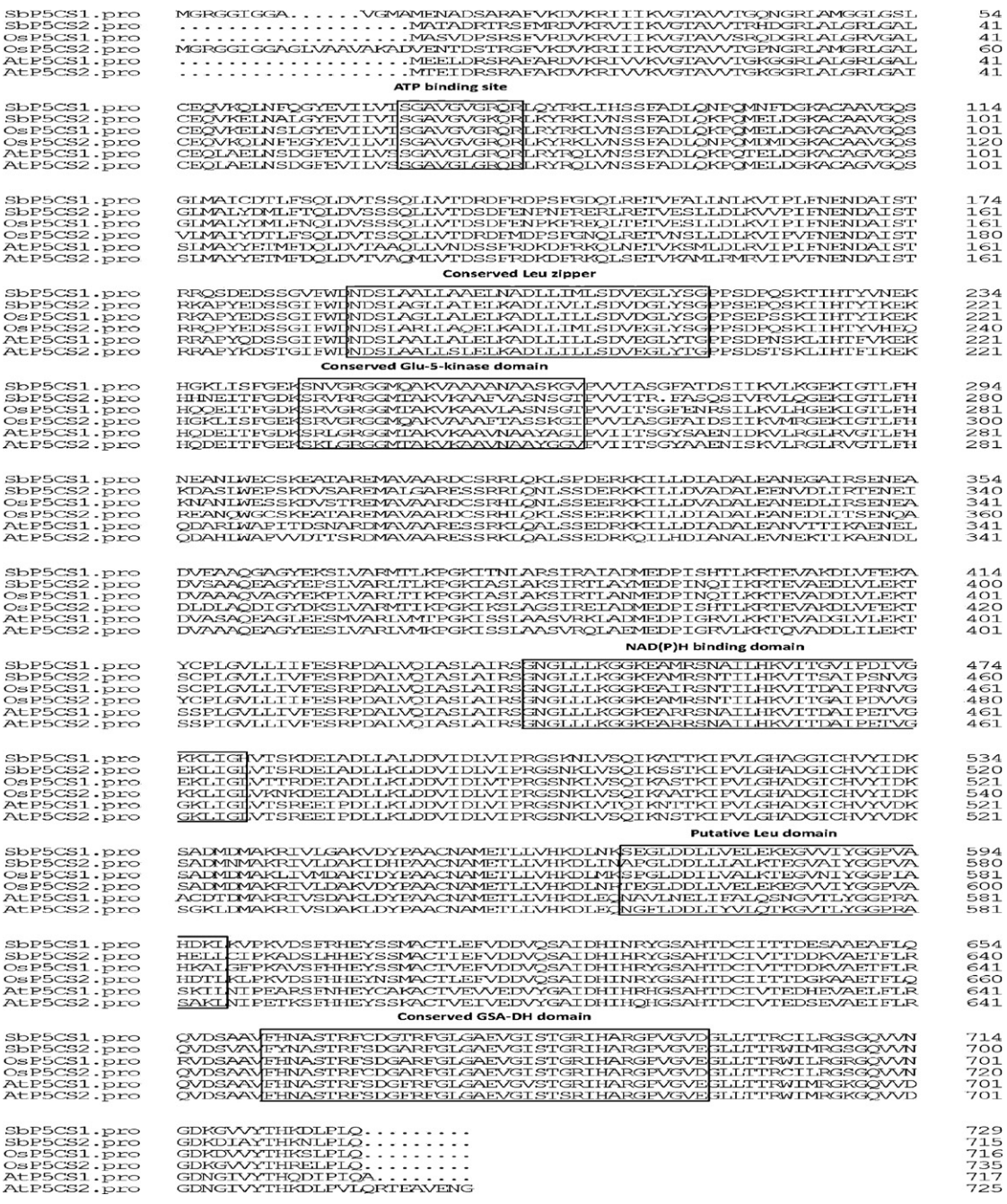

Fig. 1. Alignment of SbP5CS1 (accession no. ACU65226) and SbP5CS2 (accession no. ACU65227) with AtP5CS1 (accession no. NP181510), AtP5CS2 (accession no. NP191120), OsP5CS1 (accession no. BAA19916) and OsP5CS2 (accession no. NP001044802). Boxed sequences show conserved putative ATP and NAD(P)H-binding sites, Gk and GSA-DH domains, and putative Leu-rich regions.

2.5. RT-PCR analysis

Total RNA isolation and first-strand cDNA synthesis was performed under the same conditions as described previously. PCR was performed as followed program: 5 min denaturation at 94 °C, followed by 28 cycles of PCR (94 °C 30 s; 55 °C, 30 s; 72 °C, 30 s). The *Actin* gene was amplified by the protocol: 5 min denaturation at 94 °C, followed by 26 cycles of PCR (94 °C 30 s; 55 °C, 30 s; 72 °C, 30 s). The *SbP5CS*-specific PCR primers were used as follows: (SP1-F: 5′-TAATGTTGGAAGAGGTGGC-3′; SP1-R: 5′-CAAGGCCCTCACTCTTGT-3′) for *SbP5CS1* and (SP2-F: 5′-GCTCTGGGTAGATTAGG-3′; SP2-R: 5′-GTGCTGCAGATACATCTAT-3′) for *SbP5CS2*; the *Actin* primers used were (ACT-F: 5′-TCACCATCGGGGCAGAG-3′; ACT-R: 5′-GGGAGGCAAGGA-TGGAC-3′).

2.6. Proline determination

Free proline was extracted using the method of Magne and Larher [26], 0.2–0.5 g tissue was homogenized by 3% sulfosalicylic acid followed by boiling in water for 10 min, briefly chilling in ice, and then centrifuged at 9000 × *g* for 10 min. After this, 2 ml of supernatant was added with 3 ml 2.5% ninhydrin and 3 ml HAc. The mixture was boiled for 1 h, kept on ice, and then the mixture was extracted using 5 ml toluene. Proline content was measured using a UVB 2450 UV spectrophotometer (SHIMADZU, Japan) at 520 nm.

Table 1
Prediction of stress response cis-acting element of (a) *SbP5CS1* and (b) *SbP5CS2* promoter.

Factor or site name	Site	Signal sequence	Function
(a) *SbP5CS1*			
MYB1AT	1193(+)	WAACCA	MYB recognition site found in the promoters of the dehydration-responsive gene rd22 and many other genes
MYBCORE	1508(−)	CNGTTR	Binding site for regulation of genes that are responsive to water stress
MYCCONSENSUSAT	436(+); 887(+); 923(+); 956(+); 1094(+); 1197(+); 1587(+); 1672(+); 1817(+); 436(−); 887(−); 923(−) 956(−); 1094(−); 1197(−); 1587(−); 1672(−); 1817(−)	CANNTG	Regulates the transcription of CBF/DREB1 genes in the cold
GARE	968(−); 1096(−);	AAACAGA	Gibberellin-responsive element
ABRE	1671(+)	RYACGTGGYR	ABA responsive element
CGTCA-motif	243(+);1741(−); 1749(+);	CGTCA	Cis-acting regulatory element involved in the
TGACG-motif	243(−);1741(+); 1749(−);	TGACG	MeJA-responsiveness
WBOXNTERF3	106(+);210(+);279(+);351(+);363(+);456(+); 1711(+); 399(−);534(−);1058(−);1513(−)	TGACY	May be involved in activation of ERF3 gene by wounding;
TC-rich repeats	229(+);972(+)	GTTTTCTTAC	Cis-acting element involved in defense and stress responsiveness
TCA-element	1372(+)	CCATCTTTTT	Cis-acting element involved in salicylic acid responsiveness
WRKY71OS	106(+); 210(+); 244(−); 279(+); 351(+); 400(−); 363(+); 1514(−); 456(+); 939(+); 1091(+); 1750(−); 1711(+);1741(+); 1123(−);1059(−); 535(−);	TGAC	Binding site of rice WRKY71, a transcriptional repressor of the gibberellin signaling pathway
(b) *SbP5CS2*			
MYBCORE	1091(+); 1132(+); 1685(+); 1379(−)	CNGTTR	Binding site for regulation of genes that are responsive to water stress
MYCCONSENSUSAT	289(+); 321(+); 371(+); 963(+); 1050(+); 1091(+); 1116(+); 1275(+); 1525(+); 1711(+); 289(−); 321(−); 371(−); 963(−); 1050(−); 1091(−); 1116(−); 1275(−); 1525(−); 1711(−)	CANNTG	Regulates the transcription of CBF/DREB1 genes in the cold
ABREOSRAB21	1276(+)	ACGTSSSC	ABA responsive element
ABRE	1275(+)	RYACGTGGYR	ABA responsive element
CGTCA-motif	759(−); 9251(−); 820(+); 1138(−); 1719(−)	CGTCA	Cis-acting regulatory element involved in the
TGACG-motif	759(+); 9251(+); 820(−); 1138(+); 1719(−)	TGACG	MeJA-responsiveness
WBOXNTERF3	613(+); 643(+); 1387(+); 1407(+); 1030(−); 1532(−); 1991(−)	TGACY	May be involved in activation of ERF3 gene by wounding;
TC-rich repeats	110(−)	GTTTTCTTAC	Cis-acting element involved in defense and stress responsiveness
TCA-element	351(+); 383(−)	CCATCTTTTT	Cis-acting element involved in salicylic acid responsiveness
WRKY71OS	613(+); 643(+); 759(+); 925(+); 1138(+); 1387(+); 1407(+); 1607(+); 1719(+); 1933(+); 330(−); 821(−); 1031(−); 1207(−); 1533(−); 1992(−)	TGAC	Binding site of rice WRKY71, a transcriptional repressor of the gibberellin signaling pathway

3. Results

3.1. Sequence analysis and phylogenetic tree construction

Two full-length cDNAs of *P5CS* were isolated from sweet sorghum and registered in NCBI GenBank (GenBank accession No. GQ377719 and GenBank accession No. GQ377720), which encode 729 and 716 amino acid polypeptides, respectively. Sequence analysis showed that *SbP5CS1* was 76% homologous to the *SbP5CS2* in nucleotide sequence. *SbP5CS1* share 84% homology with *OsP5CS2*, and *SbP5CS2* had high homology with *SaP5CS* (94%), *SoP5CS* (94%), and *ZmP5CS* (91%).

The two SbP5CS protein sequences were compared with AtP5CS1, AtP5CS2 and the duplicate of *O. sativa* P5CS showed conserved regions including putative ATP, NAD(P)H-binding sites, Glu-5-kinase, GSA-DH domains and Leu-rich regions were well conserved in sweet sorghum (Fig. 1). A conserved residue was also found in the sequences of SbP5CS (Phe, at position 141 in SbP5CS1, at position 128 in SbP5CS2) (Fig. 1), which functions as proline feedback inhibition.

In order to analyze the evolutionary relationship of SbP5CS proteins and other species' P5CS, the phylogenetic tree was deduced from amino acid sequence. It was constructed by ClustalW and MEGA 4.1 (Fig. 2) using NJ method. Except for tomPRO1 and MsP5CS1, the tree could be divided into two major groups, separating P5CS proteins of monocots from dicots. The two SbP5CSs clustered within the monocot group. SbP5CS2 was most closely with SoP5CS and SaP5CS, whereas SbP5CS1 shared significant homology to OsP5CS2.

3.2. Promoter analysis

In order to identify putative cis-acting regulatory elements, about 2000 bp upstream sequences from the start codon of *SbP5CS* genes were isolated and analyzed using PLACE and PlantCARE databases. Many known stress response elements were identified in the *SbP5CS* promoter sequences (Table 1).

3.3. Expression levels

RT-PCR was performed to determine the expression patterns of the two *SbP5CS* genes response to drought, salt and MeJA stresses. Both genes were activated by water deficit (Fig. 3). The transcript levels of *SbP5CS* genes began to increase at 3 d then continued to increase, peaking at 6 d. The same expression trends of the two *P5CS* genes were observed in roots (Fig. 3A) and leaves (Fig. 3B), but the expression level of *SbP5CS1* was stronger than that of *SbP5CS2*. In addition, the up-regulation in roots was higher than in leaves. High salt (250 mM NaCl) also induced transcription of both genes (Fig. 3C and D). The *SbP5CS1* transcript showed significant increase at 4 h in roots and leaves and declined at 24 h and 12 h in roots

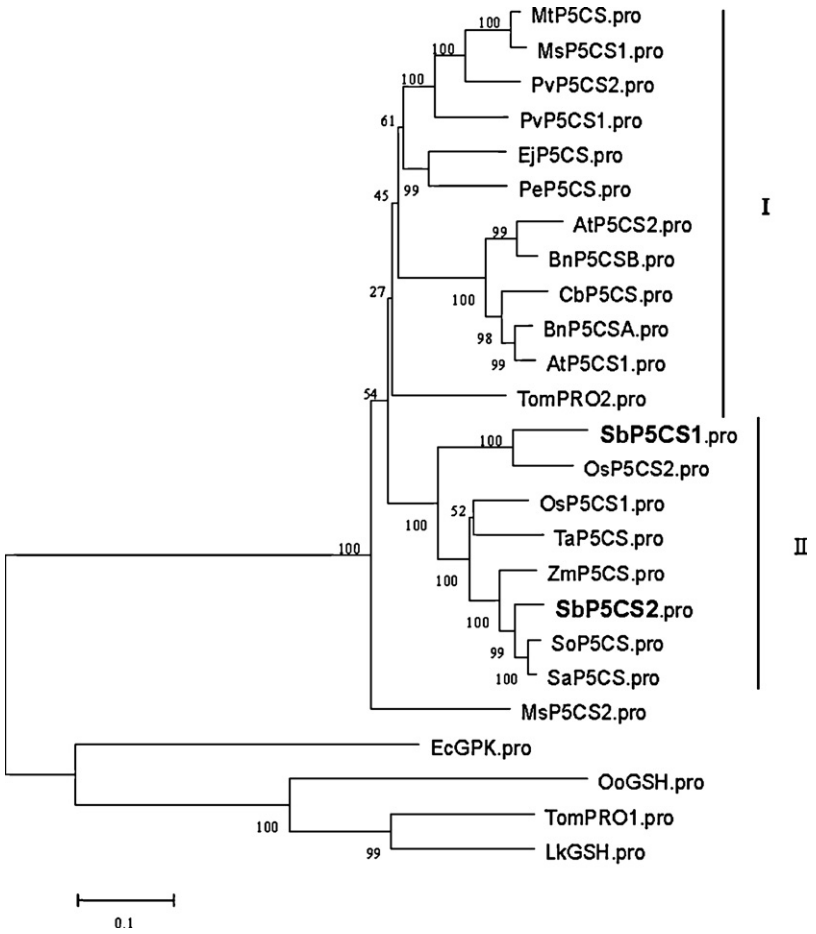

Fig. 2. Phylogenetic relationships of SbP5CS1 and SbP5CS2 (bold) with VaP5CS (*V. aconitifolia* P5CS), MsP5CS1 (*M. sativa* P5CS1), MsP5CS2 (*M. sativa* P5CS2), MtP5CS (*M. truncatula* P5CS), AtP5CS1 (*A. thaliana* P5CS1), AtP5CS2 (*A. thaliana* P5CS2), TomPRO1 (*L. esculentum* PRO1), TomPRO2 (*L. esculentum* PRO2), TaP5CS (*T. aestivum* P5CS), OsP5CS1 (*O. sativa* P5CS1), OsP5CS2 (*O. sativa* P5CS2), BnP5CSA (*B. napus* P5CSA), BnP5CSB (*B. napus* P5CSA), CbP5CS (*C. bungeana* P5CS), EjP5CS (*E. japonicus* P5CS), PeP5CS (*P. euphratica* P5CS), PvP5CS1 (*P. vulgaris* P5CS1), PvP5CS2 (*P. vulgaris* P5CS2) and EcGPR (*E. coil* GPK), OoGSH (*O. oeni* GSH), LkGSH (*L. kimchii* GSH). The Phylogenetic tree was divide in two major groups, (I) for dicots group, (II) for monocots group.

and leaves, respectively. Transcript levels of *SbP5CS2* reached a maximum at 8 h in roots and leaves. Because the MeJA responsible element was present in the putative *SbP5CS* promoter region, we treated the seedlings with 10 μM MeJA. Interestingly, under the treatment of MeJA, the expression level of *SbP5CS2* was more highly up-regulated than the levels of *SbP5CS1* in roots, but the response in leaves was opposite. The plateau of *SbP5CS2* expression was detected at 8 h in leaves whereas in roots the transcript level appeared to undergo periodic expression. Meanwhile, the expression of the *SbP5CS1* was slightly increased and diminished quickly (Fig. 3E and F). Tissue special expression patterns for the *SbP5CS* genes were also studied by RT-PCR, using samples from seedlings and inflorescences (Fig. 4). *SbP5CS2* were transcribed in most organs, in contrast, *SbP5CS1* was mainly expressed in mature vegetative tissue and reproductive organs, preferentially in panicles.

3.4. Proline content

In order to test whether proline accumulation correlates with the expression level of *SbP5CS* genes, proline content was determined using the same samples as taken for RT-PCR assays (Fig. 5). Under drought treatment, the content of proline gradually increased from the first day and reached the highest level of 2338 μg/g and 2585 μg/g at 6 d in leaves and roots, respectively. It had a 60-fold increase compared with the control (Fig. 5A and B). Under the salt stress, the peak of proline content was detected at 48 h both in roots and in leaves. It was about 8-fold and 5.5-fold higher, respectively, than the control (Fig. 5C and D). During the MeJA treatment, proline content was increased to 1.5-fold in roots and 1.6-fold in leaves compared with the control at 8 h and 4 h, respectively (Fig. 5E and F).

4. Discussion

As observed with other plant species, the P5CS enzymes were encoded by two closely related *P5CS* genes in sweet sorghum. A BLAST search using the completed sorghum genome database (http://www.jgi.doe.gov/) greatly aided identification of *P5CS* gene variant copy number in the species [27]. The two *SbP5CS* genes are located on chromosomes 3 and 9 and each query gave a single BLAST hit to the sorghum genome (Fig. 6). The sequence of the *SbP5CS1*, located on chromosome 3 also hit chromosome 9, the result of the conserved sequence between the two genes; the reverse case was found with query of *SbP5CS2*.

The P5CS gene tree clearly classified groups of genes into monocot and dicot clades with the exception of TomPRO1 and MsP5CS1. The tomato TomPRO1 is similar to prokaryotic GSH or GPK, whereas TomPRO2 was similar to other dicotyledon P5CS genes. Our two sweet sorghum P5CS proteins were clustered with those of other monocots. Gene duplication could be one source of evolution for the variety of P5CS genes [28]. From the phylogenetic tree we could

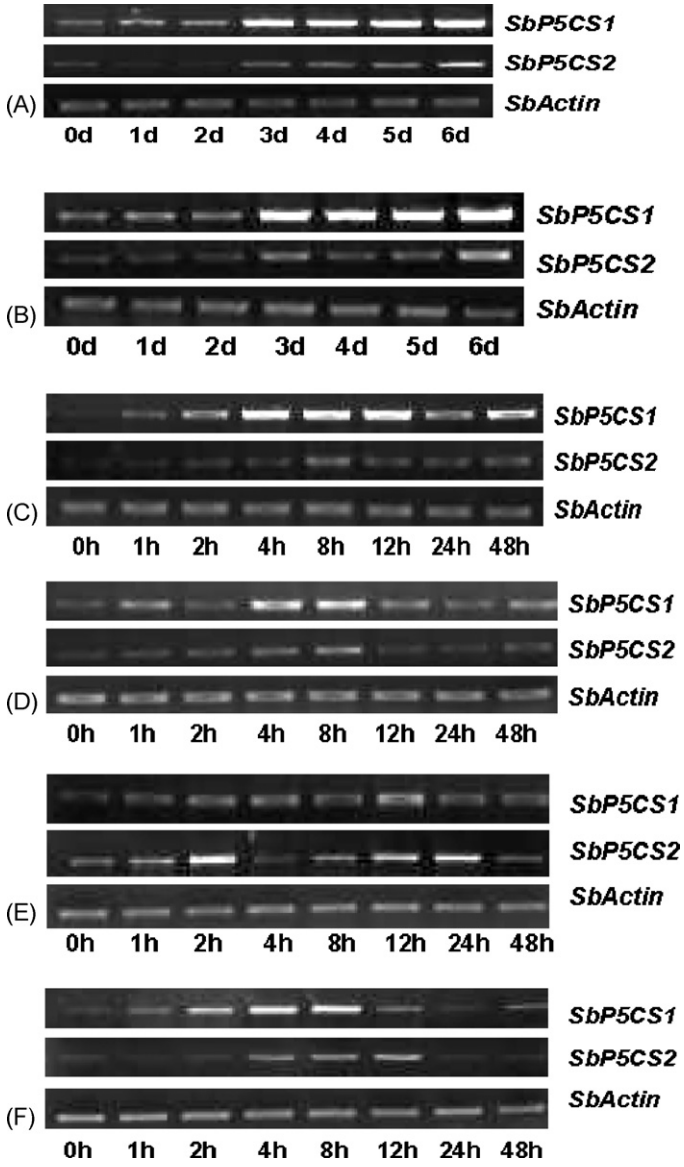

Fig. 3. Expression patterns of *SbP5CS* genes under abiotic stresses. The treatments were: Drought stress (A, roots and B, leaves) for 0 d, 1 d, 2 d, 3 d, 4 d, 5 d, 6 d. 250 mM NaCl (C, roots and D, leaves) and 10 μM MeJA (E, roots and F, leaves) for 0, 1, 2, 4, 8, 12, 24, 48 h, respectively.

infer that the duplication of the SbP5CS event occurred after divergence of monocots and dicots and then later, after the divergence of cereals. Gene duplication is considered to play a major role in evolution. Gene duplication creating one or more copies of a gene in a genome could result in the evolution of new gene functions or gene redundancy [29]. Gene redundancy is the sets of paralogous genes that perform the same role to some extent [30].

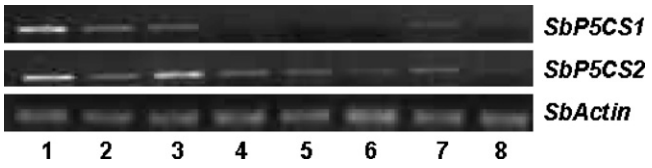

Fig. 4. Expression patterns of *SbP5CS* transcript levels from various organs. 1: young panicle; 2: mature leave; 3: mature root; 4: phloem; 5: stem node; 6: stem pith; 7: seedling root; 8: seedling leave.

In *Arabidopsis* and rice, the distinct functions of two closely related *P5CS* genes were studied. *P5CS* genes in *Arabidopsis* showed vast differences in transcription levels. In normal growth conditions, *AtP5CS1* transcript levels are highly expressed in most organs but silent in dividing cells, in contrast, *AtP5CS2* is mainly expressed in dividing cells. When exposed to stress conditions, the *AtP5CS1* is induced by salt, drought, glucose and sucrose treatments. Analysis of *Arabidopsis AtP5CS1* and *AtP5CS2* T-DNA insertion mutant showed that knockout mutations of *AtP5CS1* resulted in reduction of stress-induced proline synthesis and hypersensitivity to salt stress. On the other hand, *AtP5CS2* knockout mutant had embryo abortion. Cellular localization studies with *P5CS-GFP* gene fusions showed *P5CS2-GFP* also had a distinct cell-type-specific and subcellular localization pattern when compared to *P5CS1-GFP* [9]. These data indicate that the *Arabidopsis P5CS* genes show non-redundant functions. In rice, both rice genes are inducible by salt and cold stress; *OsP5CS1* has high expression levels in both vegetative and reproductive organs. In contrast, *OsP5CS2* is preferentially expressed in mature plants, especially in the stamens. Knockout mutant seedlings of *OsP5CS2* are more sensitive to salt and cold stress, under normal growth conditions, *OsP5CS2* knockout plants can grow normally and produced seeds without any significant loss of yield. Because the *OsP5CS1* gene is ubiquitously expressed, the researchers supposed that a mutation in *OsP5CS1* gene may be lethal [12]. These results demonstrate that *OsP5CS2* is essential for the stress response of rice and the function of two *P5CS* genes in rice are also non-redundant. Our sweet sorghum *P5CS* genes have mostly the same expression patterns with rice *P5CS* gene, the results of expression patterns of the two *P5CS* gene under abiotic stress showed that both the *SbP5CS* genes were stress inducible, but the *SbP5CS1* was drastically up-regulated under abiotic stresses. The two genes also have different tissue-specific expression patterns. In normal growth condition, expression levels of *SbP5CS1* was very low in most organs, it was mainly expressed in mature organs, on the contrary, the *SbP5CS2* was ubiquitously expressed. From the expression patterns we could infer that the *SbP5CS2* is a housekeeping gene that mainly function in basic proline metabolism, on the other hand, the *SbP5CS1* gene could play major roles in stress responses. Base on our research and the results of prior study, we believed the two *SbP5CS* gene have non-redundant functions. Additional research is needed to study mutations in each *SbP5CS* gene and double mutations in both genes to further address the question of function.

Transcriptional regulation is a central component in the control of gene expression. Identification of functional cis-elements in promoter regions is a key method to understanding gene regulation and the results could serve as a guide for functional experiments. The silico analysis of the two *P5CS* promoters revealed that many stress induced transcription factors binding sites like MYCCONSENSUSAT, WRKY, and MYBCORE were located in the *SbP5CS* promoter region. Transcription factors can bind to the cis-elements of the promoter regions to regulate gene expression. These stress response elements represent binding sites of transcription factors which could be involved in cold, wound and dehydration responses. We also found that ABRE (abscisic acid responsive element) existed within the both promoters, which indicated that synthesis of proline by the *SbP5CS* genes might be ABA dependent. Moreover, more than one MeJA-responsive motifs were identified in the both promoter sequences. MeJA has been known as a phytohormone induced by wounding and insect herbivory.

Based on the results of promoter analysis we selected high salt and drought treatment to identify the expression patterns of sweet sorghum under stress condition. Gene expression patterns under drought and high salt treatments were used to guide in silico analysis of promoter regions. Because the two promoters had MeJA responsive elements, we treated the seedlings with 10 μM MeJA.

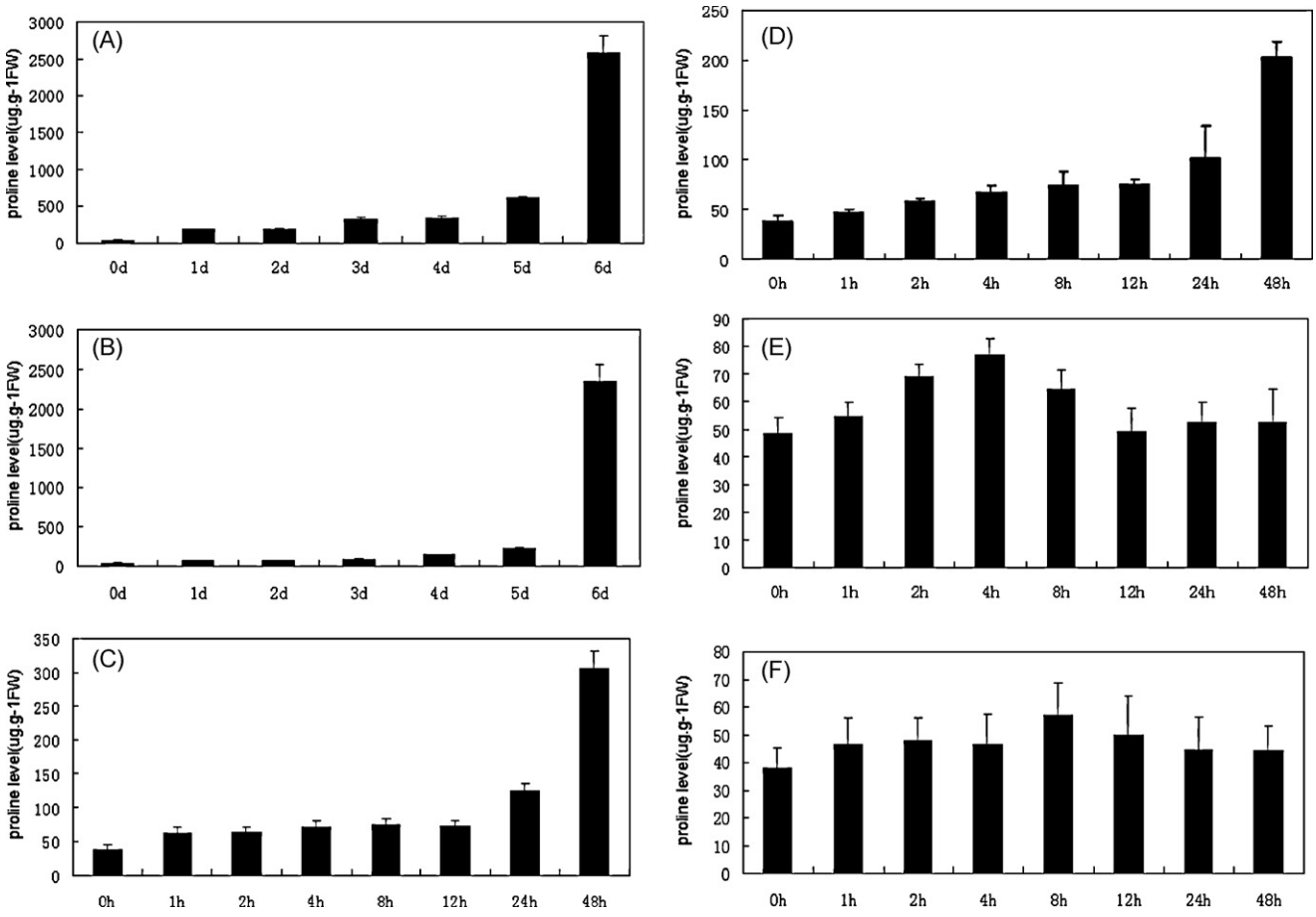

Fig. 5. Proline content in roots and leaves sampled from sweet sorghum seedlings over time courses of drought (A, roots and B, leaves), salt (C, roots and D, leaves) and MeJA stress (E, roots and F, leaves) treatments. All of the experiments were carried out repeated three times with plant materials from three different pots and error bars indicated the standard deviation.

However, our results showed that the up-regulation of two genes was not significant and the accumulation of proline was not evident too. It is probably a consequence of the MeJA involves in multiple signal pathways and the up-regulation of *P5CS* may eliminate by other pathway or the expression of *P5CS* gene is MeJA independent. Although the two promoters shared nearly the same response elements, the expression intensity of the two genes were different under stresses, *SbP5CS1* expression levels were stronger than *SbP5CS2*, which may have been the result of the different spatial and temporal regulations of the cis-acting regulatory elements within *SbP5CS* promoter regions. Testing the core promoter regions which contribute to efficient induction might be useful to explain this effect.

P5CS is a rate-limiting enzyme in the proline synthesis via the glutamate pathway. In most plant species such as *P. vulgaris* [6], *B. napus* [11], cactus pear [31] and *O. sativa* [12], proline accumulation was accompanied by the increase of *P5CS* transcripts. In sweet sorghum, the induction of *SbP5CS* transcript levels were preceded the accumulation of proline content during the stresses. We presumed that *SbP5CS* plays a key role in the biosynthesis of proline under stress and accumulation of the proline was correlated with increased expression of *SbP5CS* in sweet sorghum. To clarify the role of proline in various stress responses, proline biosynthetic pathways have been modified in transgenic plants. In the previous reports, an increased level of free proline was found to correlate with improved osmototolerance [32]. It has been reported that overexpression of the *P5CS* gene in potato [17], tobacco [33], *O. sativa*. [18,19] and wheat [20] could increase proline content and confer stress tolerance of the plant. Our results revealed differences in transcriptional control of the closely related genes in sweet sorghum under stress. The expression level of *SbP5CS1* was strongly induced whereas the *SbP5CS2* was more subtly induced.

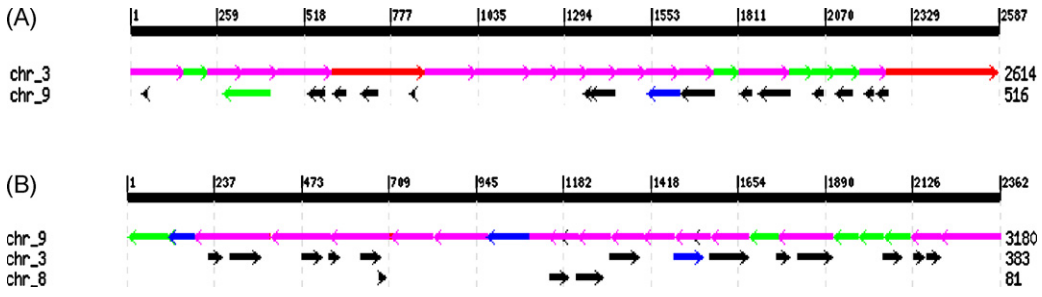

Fig. 6. The location of *SbP5CS* genes in JGI sorghum genome (A, *SbP5CS1* and B, *SbP5CS2*).

Therefore, we conclude that the *SbP5CS1* might be a good candidate gene for genetic engineering to enhance stress tolerance. Furthermore, site-directed mutagenesis could also enhance tolerance. Hong et al. replaced Phe of VaP5CS by Ala at position 129. The mutated enzyme (P5CSF129A) was no longer subject to feedback inhibition. When exposed to salt stress, transformed P5CSF129A plants produced more than twice the amount of proline compared with non-mutated P5CS transgenic plants [32]. In sweet sorghum, feedback inhibition might be associated with SbP5CS1 (at position 141) and SbP5CS2 (at position 128), which indicated that the SbP5CS may be inhibited by proline.

Considering the previous reports and our results, an effective way to enhance the stress tolerance of sweet sorghum is by the overexpression of *SbP5CS* genes, in particular, overexpression of *SbP5CS1* or its site-directed mutant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2011.03.002.

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