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ORIGINAL PAPER

Cloning and genetic diversity analysis of a new *P5CS* gene from common bean (*Phaseolus vulgaris* L.)

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Abstract Δ^1-pyrroline-5-carboxylate synthetase (P5CS) is the rate-limiting enzyme involved in the biosynthesis of proline in plants. By the 3′ rapid amplification of cDNA ends (3′-RACE) approach, a 2,246-bp cDNA sequence was obtained from common bean (*Phaseolus vulgaris* L.), denominated *PvP5CS2* differing from another P5CS gene that we cloned previously from common bean (*PvP5CS*). The predicted amino acid sequence of *PvP5CS2* has an overall 93.2% identity GmP5CS (*Glycine max* L. P5CS). However, *PvP5CS2* shows only 83.7% identity in amino acid sequence to *PvP5CS*, suggesting *PvP5CS2* represents a homolog of the soybean P5CS gene. Abundant indel (insertion and deletion events) and SNP (single nucleotide polymorphisms) were found in the cloned *PvP5CS2* genome sequence when comparing 24 cultivated and 3 wild common bean accessions and these in turn reflected aspects of common bean evolution. Sequence alignment showed that genotypes from the same gene pool had similar nucleotide variation, while genotypes from different gene pools had distinctly different nucleotide variation for *PvP5CS2*. Furthermore, diversity along the gene sequence was not evenly distributed, being low in the glutamic-*g*-semialdehyde dehydrogenase catalyzing region, moderate in the Glu-5-kinase catalyzing region and high in the intervening region. Neutrality tests showed that *PvP5CS2* was a conserved gene undergoing negative selection. A new marker (Pv97) was developed for genetic mapping of *PvP5CS2* based on an indel between DOR364 and G19833 sequences and the gene was located between markers *Bng126* and *BMd045* on chromosome b01. The relationship of *PvP5CS2* and a previously cloned pyrroline-5-carboxylate synthetase gene as well as the implications of this work on selecting for drought tolerance in common bean are discussed.

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Introduction

In higher plants, Δ^1-pyrroline-5-carboxylate synthase (P5CS) is a rate-limiting enzyme involved in the biosynthesis of proline from glutamate (Hu et al. 1992; Yoshiba et al. 1995). Proline in turn is an important osmo-protectant that is thought to be critical for adaptation to several abiotic

stresses such as drought and salt by the increasing osmotic potential of cells and protecting proteins, membranes and cell structures against dehydration or oxidative stress (Delauney and Verma 1993; Bohnert and Jensen 1996; Verslues et al. 2006). P5CS is encoded by the *P5CS* gene, which has been cloned from many higher plants (Hu et al. 1992; Zhang et al. 1997; Fujita et al. 1998; Armengaud et al. 2004) including recently, common bean (Chen et al. 2009). Studies (Hu et al. 1992; Igarashi et al. 1997; Strizhov et al. 1997; Dombrowski et al. 2008; Chen et al. 2009) showed that the expression of *P5CS* was significantly induced by salt and drought.

Common bean (*Phaseolus vulgaris* L.) is one of the most ancient crops of the Americas; and is also the most important grain legume worldwide for direct human consumption (Broughton et al. 2003; McClean et al. 2004a). Evidence from phaseolin seed proteins and DNA markers have shown that Ecuador and northern Peru are a center of wild bean diversity from where the species diversified into South and Central America. Subsequently, human cultivation led to the formation of two distinct domesticated gene pools, the Andean and the Mesoamerican, which are predominant in their respective primary centers of diversity (Gepts 1998). Molecular marker studies have shown that these two gene pools spread throughout the world and sometimes hybridize in secondary centers of diversity (Duran et al. 2005; Zhang et al. 2008). Selection for different phenotypes of common bean led to additional genome variation between the two gene pools.

Single nucleotide polymorphisms (SNPs) are the most abundant sequence variations encountered in most genomes (Griffin and Smith 2000). The ubiquity and interspersed nature of SNPs make them ideal candidates as molecular markers for marker-assisted plant breeding, physical mapping, and association studies. Various large-scale SNP discovery projects have been conducted for a broad range of organisms (Karchin et al. 2005; Balasubramanian et al. 2002), including for EST collections from crop plants (Rafalski 2002; Soleimani et al. 2003; Barbazuk et al. 2007). An alternative to the analysis of SNPs in arbitrary sequences is the careful analysis of variability in genes of specific interest which can be useful for association of allelic variants with phenotypic differences and for subsequent marker-assisted selection of the associated trait. In the present study, we describe the cloning of a new full-length cDNA for *P5CS* from common bean and the mapping of the gene based on the development of a new PCR-based marker. We also analyze the allelic diversity for the gene among cultivated and wild common bean accessions discovering an abundance of single nucleotide polymorphisms and other variants.

Materials and methods

Genotypes and nucleic acid extraction

A drought-tolerant common bean accession from Shanxi Province in China, Huang Hua Dou, was used to clone the *PvP5CS2* gene. Seeds of this landrace were obtained from the Institute of Crop Sciences of the Chinese Academy of Agricultural Sciences (CAAS) and germinated in plates irrigated with sterile water for 3 days. Seedlings were transferred into pots filled with 25 g vermiculite for greenhouse culture 5 days under a 12 h light/12 h dark photoperiod at 25°C with ventilation. Several young trifoliolates from each common bean plant were harvested, lyophilized and ground for DNA and RNA extraction.

Subsequently, 27 common bean accessions (shown in the Supplementary Table), including wild and domesticated accessions (11 dry beans and 13 snap beans), were used to evaluate nucleotide variation. The wild beans included three accessions from the International Center for Tropical Agriculture (CIAT), two from Mexico (G2771 and G40199) and one from Colombia (G12822). Meanwhile the cultivated beans included two additional genotypes from CIAT: (1) G19833, a standard Andean landrace used for genomic analysis and (2) DOR364 which is a released variety from Central America that has been crossed with G19833 to create a mapping population (Blair et al. 2003), as well as a group of Chinese, Japanese and Rumanian snap beans from the CAAS collection (V07A0376, V07A0396, V07A1080, V07A1744, V07A1757, V07A2305, V07A3056, V07A3162, 7A373, 7A2125, 7A2303, 7A2902, 7A2968) and nine Chinese dry bean genotypes (F493, F531, F666, F1282, F1434, F2082, F2798, F3370 and F4524) also from CAAS.

Finally, a set of 87 recombinant inbred lines (RILs) for the DOR364 × G19833 population was used to genetically map the *PvP5CS2* gene. And 221 common bean accessions evaluated with SSR by Zhang et al. (2008) were used in this study to validate the Pv97 marker. All genotypes used for the diversity analysis and mapping work were germinated on paper towels to harvest young cotyledonary leaves for DNA extraction.

Total RNA was extracted with RNAprep Plant kits (TIANGEN Corporation, China), and then treated with deoxyribonuclease I before reverse transcription (RT). Genomic DNA for CAAS genotypes was extracted following a modified hexadecyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980), while tissue from the CIAT genotypes and the mapping population were extracted with a mini-prep method from Afanador et al. (1993).

Cloning of the *PvP5CS2* gene

For the amplification of the *P5CS* gene of common bean, a primer pair P0 (forward: 5′ ATGGAGAACACAGATCCT TGTAGAC 3′, reverse: 5′ GTGCCCCATCACTGAAT CTTG 3′) was designed from the soybean (*Glycine max* L.) homologue *GmP5CS* (Genebank AY492005). PCR was performed using the template of first-strand cDNA, which was synthesized from total RNA treated with deoxyribonuclease I using M-MLV reverse transcriptase (Promega, Carlsbad, USA) and Oligo dT_{16} primer.

A PCR technique called 3′ rapid amplification of cDNA ends (3′-RACE) was then used to obtain the 3′ end of the cDNA sequence of the *PvP5CS2* gene. To do this, total RNA was again treated with deoxyribonuclease I and then reverse transcribed using M-MLV reverse transcriptase and an oligo-dT adaptor primer (5′ GACTCGAGTCGACATCGATTTT TTTTTTTTTTTT 3′). Following this step, PCR amplification of the 3′ end of the cDNA was performed using two primers, GSP (gene-specific primer: 5′ CACTGGCTCGTA CATTACATCTC 3′) and AP (adaptor primer: 5′ GACTCG AGTCGACATCG 3′). The product of the 3′-RACE reaction was sequenced as described below and two primer pairs were designed from the full-length cDNA sequence: namely, primer pair 1 or P1 (forward: 5′ ATGGAGAACACAGATC CTTGTAGAC 3′, reverse: 5′ CGGTTCGTTTTAGCACT CG 3′) and primer pair 2 or P2 (forward: 5′ GATGTGAAA AATCCTTGGTGGC 3′, reverse: 5′ TCAAATAGCAAG-GTCTTTGTGGG 3′) which amplified genomic sequences at the 5′- and 3′ ends of the *PvP5CS2* gene, respectively.

Sequence analysis

Sequencing of the original cDNA, of the 3′RACE product and of the PCR amplification products of the P1 and P2 primer pairs was performed on an ABI3700 automated sequencer (Applied Biosystems, Foster City, USA). Editing of sequences was performed with the program DNAStar (Lasergene, USA). Alignments were made using Clustal W (Thompson et al. 1994) and a phylogenetic tree for the P5CS gene was built with the software programs Phylip and MEGA 4.0 based on protein sequences. Polymorphic sites (both SNPs and indels) as well as synonymous and nonsynonymous SNPs were identified using DnaSP4.0 (Rozas et al. 2003). The nucleotide diversity, including π (Nei 1987) and Θ (Watterson 1975), and the neutrality test statistics, including Tajima's *D* (1989) as well as Fu and Li's *D* and *F* (1993), were estimated using the same software.

Mapping of *PvP5CS2* gene

A specific primer pair (forward: 5′ AGGTTGGGACTGCT GTGGTTAC 3′ and reverse: 5′ TTTCACAGTTTCGCTG AGTTGC 3′) was designed around an indel found between the parents of the DOR364 × G19833 population and was named Pv97 (the indel is 97 bp). Genomic DNAs from both parents and each of the 87 RILs from the population were used for PCR amplification of this marker and separation of the resulting DNA bands on 1.5% agarose gels. The PCR fragments obtained for G19833 and DOR364 showed size differences (differential fragment length polymorphisms, DFLPs) and these were scored in the progeny. The data from the marker segregation were then analyzed for the presence or absence of G19833- or DOR364-specific alleles and segregation analysis was performed using the MAPMAKER 3.0b software to integrate the map positions for *PvP5CS2* into a combined AFLP/RFLP/SSR linkage map described in Blair et al. (2003).

Results

Isolation and sequence analysis of PvP5CS2 gene from common bean

Sequence for a 1,981-bp cDNA was determined using the primer pair P0 for the *PvP5CS2* gene based on reverse transcription (RT) from RNA isolated from common bean leaves. Sequence analysis showed that the cDNA had an initiation codon but not a termination codon. Subsequently, we designed a gene-specific primer based on the 1,981-bp cDNA sequence and used this with an adaptor primer for a 3′-RACE reaction. Using the two primers, a 578-bp cDNA sequence, including 21-bp long polyA tail, was cloned from the RT product of common bean leaves. A 219-bp overlap sequence between the 1,981-bp cDNA sequence and the 578-bp cDNA sequence shared 100% identity, suggesting that the 578-bp cDNA sequence was the 3′ end of the 1,981-bp cDNA sequence. Splicing the two cDNA sequences together resulted in a 2,340-bp sequence, which contained 2,148 bp of open reading frame, 171 bp of a 5′ untranslated region (UTR) and 21 bp of a 3′ untranslated polyA tail. The native molecular weight of the PvP5CS2 protein was estimated to be 77.785 kDa.

Sequence analysis have shown that the cDNA sequence exhibited highest DNA homology in nucleotide sequence to the legume homologues of the P5CS genes, especially the soybean sequence *GmP5CS* (93.7%) and the alfalfa (*Medicago sativa*) sequences *MsP5CS*1 (88.3%), *MsP5CS*2 (87.9%). The sequence of the cDNA was also similar to another P5CS gene that we cloned previously from common bean *PvP5CS* (83.6%) as well as the mothbean (*Vigna aconitifolia*) homologue *VaP5CS* (83.4%) on which that previous cloning was based (Hu et al. 1992; Chen et al. 2009). Similarly, putative amino acid sequences shared highest identity with GmP5CS (93.2%), MsP5CS1

(89.0%), MsP5CS2 (88.8%), PvP5CS (87.5%) and VaP5CS (82.6%). A comparison of the protein sequence of PvP5CS2 with sequences from *Arabidopsis thaliana* P5CS2 (CAA70527.1), *V. aconitifolia* VaP5CS, *M. sativa* P5CS2 and *P. vulgaris* P5CS showed overall conservation in the amino acid sequence and order of the Glu-5-kinase domain, the GSA-DH domain, the putative ATP and NAD(P)H-binding sites and the Leu-rich regions (Fig. 1). These results indicated that the cDNA sequence was a new *P. vulgaris P5CS* gene and that the putative protein is involved in proline synthesis in common bean. Since we have already cloned a P5CS gene from common bean, we denominated the cDNA sequence *PvP5CS2* (*P. vulgaris* P5CS gene 2) and deposited this sequence in GenBank under the accession number EU407263.

Phylogenetic analysis of the deduced amino acid sequences of the *PvP5CS2* gene compared to *P5CS* genes from other organisms using ClustalW and PHYLIP found that the genes could be separated into four groups (Fig. 2). *PvP5CS2* belonged to group IV, which included the five legume homologues mentioned earlier (*PvP5CS*, *VaP5CS*, *GmP5CS*, *MtP5CS1* and *MsP5CS1*), and was especially closely related to *GmP5CS*. This group was related to other dicotyledon P5CS genes (group III), and more distantly to P5CS genes from monocotyledonous plants such as rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.). Finally, the prokaryotic P5CS genes GK and GPR from *Escherichia coli* were the outgroup.

Allelic diversity in the *PvP5CS2* gene

After cloning of the cDNA, we isolated two genomic fragments of 2,911 and 3,055 bp from DNA of the same genotype (Huang Hua Dou) using the *PvP5CS2* primer pairs P1 and P2, respectively. Sequence alignment showed that the two DNA sequences had a 207 bp overlapping sequence, which shared 100% identity. Therefore, the two sequences were spliced together to form a 5,761 bp full-length sequence. Comparisons of the *PvP5CS2* cDNA sequence with this genomic sequence showed that the spliced sequence included all ORF sequences of the *PvP5CS2* gene, suggesting that the spliced sequence was the DNA sequence of the gene. Sequence analysis further showed that the *PvP5CS2* gene sequence embodied 20 exons and 19 introns.

The same primer pairs were used to analyze sequence diversity in 27 common bean accessions, including 3 wild common beans and 24 domesticated accessions (11 dry beans and 13 snap beans), and to identify the distribution of SNPs and indels in the *PvP5CS2* gene. The 27 DNA sequences ranged in length from 5,760 to 5,861 bp and a total of 244 variant sites were found among the common bean accessions, including 63 nucleotide substitution sites (SNPs, 25.8%) and 183 insertions/deletions (indels, 74.2%) (Table 1). The SNP and indel frequency were similar between dry bean and snap bean accessions, but were higher within wild beans and between wild beans and cultivated beans. All 63 nucleotide substitution sites were bi-allelic having only two alternative nucleotides with 55.5% of these being transitions and 44.4% being transversions. SNPs were 1.5-fold more frequent in intronic (1/83 bp) than in exonic (1/119 bp) sequences. Meanwhile all 183 indels were found exclusively in the intronic regions. Among the 183 indels, there was one each of a 97-nucleotide-long indel and a 52-nucleotide-long indel, as well as two 4-nucleotide-long indels, one 3-nucleotide-long indel, two double-nucleotide indels and 19 single nucleotide indels. Among the 63 SNPs, 12 were found in two genotypes when comparing with the reference sequence from Huang Hua Dou, 51 were found in more than 2 genotypes, and no SNP was found in all accessions when compared to the reference sequence. Low-frequency variants (singletons and doubletons) accounted for 19% of the gene variants.

Single nucleotide polymorphism of *PvP5CS2* showing evolutionary history

Using the software MEGA 4.0 and a bootstrap test with 500 replicates based on the single nucleotide polymorphisms of the *PvP5CS2* gene, we were able to estimate the phylogenetic relationships of the 27 common bean accessions for this gene with the *E. coli* sequences for *ProA* used as an outgroup sequence (Fig. 3). The accessions were grouped into two clusters: cluster I included 24 domesticated accessions and 1 wild common bean and cluster II contained 2 wild common beans.

Cluster I was subdivided two subgroups: cluster A and cluster B where cluster A included G19833, a standard control accession for the Andean gene pool of common bean, and three snap beans (V07A1744, V07A3056, V07A3162) also from the Andean gene pool. Cluster B included DOR364, a standard control accession for Mesoamerican gene pool of common bean, five snap beans of Mesoamerican origin (V07A0376, V07A0396, V07A1080, V07A1757, V07A2306) plus the wild bean G12822 from Mexico.

We conclude therefore that the single nucleotide polymorphisms of the *PvP5CS2* gene reflected common bean domestication history and that sequences of accessions from the same gene pool origin had similar nucleotide substitutions. Based on the phylogeny presented above, the haplotype structure of the Andean and Mesoamerican gene pool genotypes was obtained based on the consensus sequences from clusters A and M, denominated haplotypes A and M, respectively (Table 2).

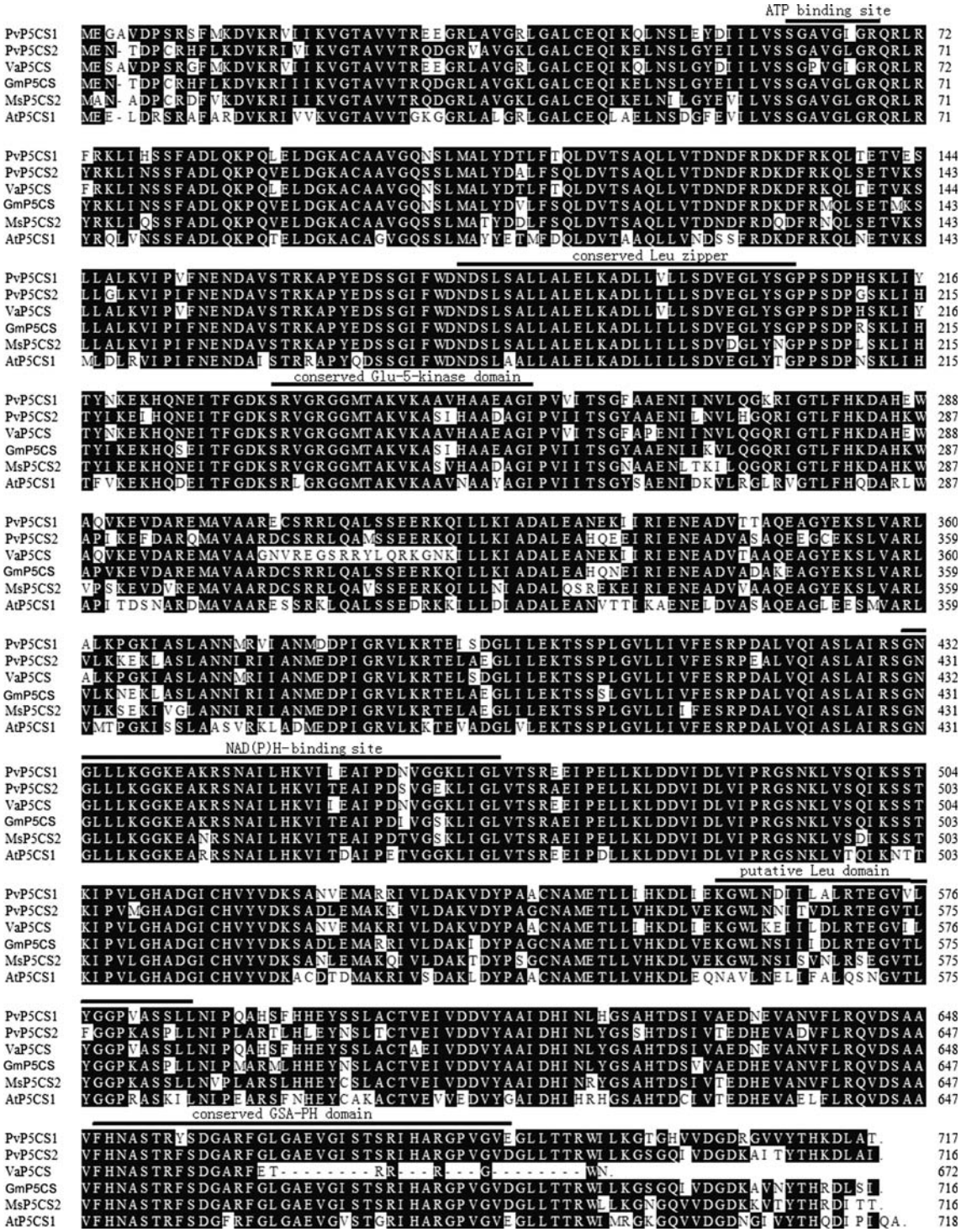

Fig. 1 Alignment of putative amino acid sequence of the common bean gene, *PvP5CS2* (Accession No. EU407263) with common bean *PvP5CS1* (Accession No. EU340347), mothbean *VaP5CS* (Accession No. M92276), soybean *GmP5CS* (Accession No. AY492005), alfalfa *MsP5CS2* (Accession No. X98422) and Arabidopsis *AtP5CS1* (Accession No. X87330) as described in text. Sequences under *dark lines* show conserved Gk and GSA-DH domains, putative ATP and NAD(P)H-binding sites and Leu-rich regions, respectively

Development of a PCR-based marker for *PvP5CS2*

A PCR-based, genetic marker for *PvP5CS2* was developed based on the conserved consensus sequences of both haplotypes discussed above (A and M). The primer pair (forward: 5′ AGGTTGGGACTGCTGTGGTTAC 3′, reverse: 5′ TTTCACAGTTTCGCTGAGTTGC 3′) was designed to target a 97-bp insertion/deletion event (In1 of Table 2) and

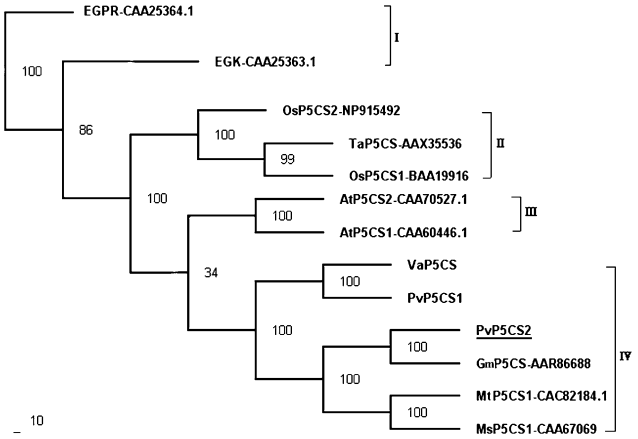

Fig. 2 Phylogenetic relationships of the new gene PvP5CS2 (*underlined*) with *PvP5CS1* (*Phaseolus vulgaris* P5CS), VaP5CS (*V. aconitifolia* P5CS), GmP5CS (*G. max* P5CS), MsP5CS1 (*M. sativa* P5CS1), MtP5CS1 (*M. truncatula* P5CS1), AtP5CS1 (*A. thaliana* P5CS1), AtP5CS2 (*A. thaliana* P5CS2), TaP5CS (*T. aestivum* P5CS), OsP5CS1 (*O. sativa* P5CS1), OsP5CS2 (*O. sativa* P5CS2), EGK (*E. coil* GK) and EGPR (*E. coli* GPR)

hence was named marker Pv97. The expected PCR product of Pv97 had an amplicon length difference between haplotypes A and M which included this 97 bp indel and therefore the G19833 A haplotype was shorter (785 bp) than the DOR364 M haplotype as seen in Fig. 4. This result showed that the DOR364 and G19833 as parents could be distinguished by PCR amplification using Pv97 and that their progeny would be segregated for this marker.

We were interested in confirming the association of this marker with the gene pools of common bean and to do this we analyzed the Pv97 genotype for 221 common bean accessions from Zhang et al. (2008). When PCR was performed using the marker on these genotypes, we found that Pv97 status agreed with genepool status 91.8% of the time, with 76 accessions having the Andean haplotype and 145 accessions having the Mesoamerican haplotype. Introgression of the Andean haplotype into the Mesoamerican gene pool occurred in eight genotypes and the opposite situation occurred for ten genotypes, showing the value of this marker for gene pool evaluations.

Genetic mapping of *PvP5CS2* gene

Following the determination of the Andean and Mesoamerican haplotypes, the *PvP5CS2* gene was mapped as the Pv97 marker in the recombinant inbred line (RIL) population DOR364 × G19833. Among the 87 progeny RILs the genotypes of 44 lines were the same as that of G19833, while 36 lines showed the PCR product expected for DOR364, and 7 were heterozygous showing both bands for this co-dominant marker. Genetic mapping was used to place the Pv97 marker for the *PvP5CS2* gene into linkage group (chromosome) b01 flanked at distances of 2.4 and 27.5 cM, respectively, by markers *Bng126* and *BMd045* as shown in Fig. 5.

Nucleotide diversity and neutral test of *PvP5CS2* gene

Two estimates of nucleotide variation were calculated (Table 3). The nucleotide diversity (π), i.e., the average pairwise sequence difference between two random sequences in a sample, was 0.0483 per site. The average estimate of Θ (from S), which is based on the observed number of polymorphic sites in a sample, was 0.00287 per site. Nucleotide diversity (π) was 1.6-fold greater in non-coding than in coding regions, 1.2-fold greater for snap beans than for dry beans, and 1.3-fold greater for Mesoamerican gene pool accessions (accessions in cluster B) than Andean gene pool accessions (accessions in cluster A).

Tajima's test and Fu and Li's test were conducted to examine whether variation in the sequenced region deviated from the mutation-drift model (Kimura 1983). Significant positive values were observed for each statistic indicating an excess of intermediate frequency polymorphism that can be the result of balancing selection, diversifying selection or population subdivision (Hartl and Clark 1997). To consider these possibilities, the data was subdivided relative to breeding history (dry bean or snap bean) and gene pool (Mesoamerican or Andean), and the population genetic statistics were recalculated (Table 3). Nucleotide diversity values for both dry beans and snap beans were lower than that observed for the

Table 1 Comparison of SNP and indel polymorphisms found in 27 accessions of common bean for the *PvP5CS2* gene

Status	Number of accessions	Sites	SNP		Indel	
			Number	Frequency	Number	Frequency
Dry bean	11[a]	5,872	50	1/117	164	1/36
Snap bean	13	5,872	51	1/115	164	1/36
Wild	3	5,872	19	1/309	76	1/77
Cultivated	24	5,872	51	1/115	164	1/36
Total	27	5,872	63	1/93	183	1/32

[a] Excludes three wild dry beans

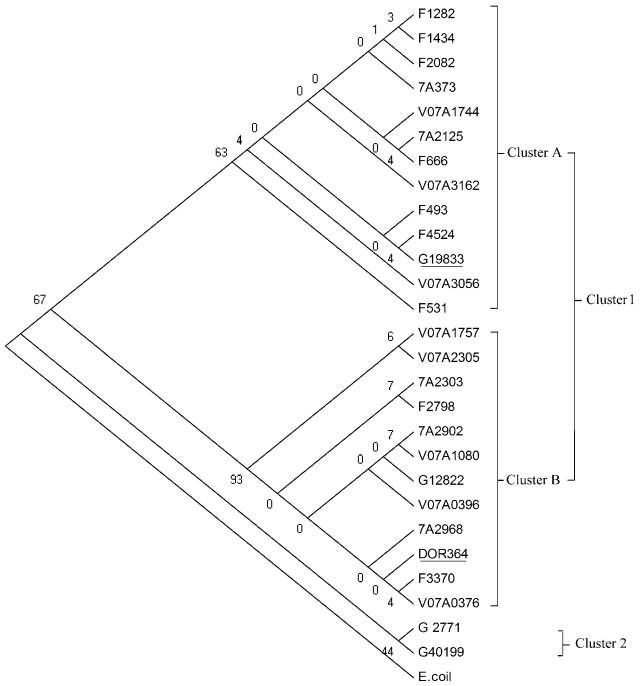

Fig. 3 The neighbor-joining tree of 27 *PvP5CS2* gene sequences from common bean

entire population. This pattern was also observed when the genotypes were analyzed by gene pool where the Mesoamerican gene pool was much more diverse than the Andean gene pool. However, significant negative values were observed for both gene pools indicating an excess of low-frequency polymorphism that can be the result of negative selection or background selection. These results suggested that *PvP5CS2* has undergone selection during common bean evolution and domestication. The rates of nonsynonymous to synonymous substitutions (Ka/Ks) were all smaller than 0.5 in the five subdivision of Table 3 implying that negative selection has occurred for this gene in common bean.

To investigate whether nucleotide diversity was evenly distributed along the sequence, the π values were estimated within a 500-bp interval sliding window analysis which is graphically represented in Fig. 6. The distribution of nucleotide diversity along the sequence was found to be uneven and *PvP5CS2* DNA sequence diversity level could be subdivided according to three approximate regions. Among these three regions, the Glu-5-kinase catalyzing region (from bp 1 to 2,000) had moderate diversity ($\pi = 0.00554$), the glutamic-*g*-semialdehyde dehydrogenase catalyzing region (3,000–5,761 bp) had low diversity ($\pi = 0.00231$), and the intervening region (2,000–3,000 bp) had the highest level of diversity ($\pi = 0.010586$). The transition between each domain was punctuated by higher levels of diversity and the impact of selection on polymorphism was narrowly focused on these intervening regions of the *PvP5CS2* gene. Notably, the glutamic-*g*-semialdehyde dehydrogenase domain was more conserved than the glutamyl kinase domain in *PvP5CS2*.

Table 2 Polymorphic sites among the Andean (A) and Mesoamerican (M) haplotypes

Position	0	0	0	0	0789	0	0	1	1	1	1	1	1	1	1	1	1	1	2
	5	5	6	6		9	9	1	1	2	2	2	3	3	4	4	5	7	0
	5	5	6	7		0	7	1	6	2	6	6	0	6	2	2	9	1	9
	0	2	5	1	0885	0	4	5	5	9	4	7	7	6	1	2	7	3	1
Haplotype A	C	C	T	A	*	C	C	T	G	T	A	C	A	A	T	G	G	T	G
Haplotype M	*	T	A	G	In1	A	T	C	A	G	T	A	G	G	C	C	A	C	A

Position	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	1	1	1	2	2	2	2	2	3	3	3	3	3	3	4	4	4	5	5
	2	7	9	1	1	2	6	9	1	2	8	8	8	8	1	3	9	3	6
	3	8	0	1	9	3	0	2	3	0	2	3	4	5	8	0	7	4	1
Haplotype A	G	T	A	C	*	*	T	A	T	G	*	*	*	*	G	C	T	C	G
Haplotype M	A	G	G	G	G	T	A	G	*	T	T	A	T	G	A	T	C	T	A

Position	2	2	2	2	3	3055	3	3	4	4	4	4	4	4	5	5	5
	6	7	7	8	0		5	9	1	2	5	5	7	7	2	4	7
	5	4	9	3	1		5	0	0	6	1	9	3	6	3	9	7
	8	8	3	8	3	3106	5	9	5	7	5	6	2	3	2	7	6
Haplotype A	T	G	T	G	C	In2	G	G	C	T	C	C	A	C	T	A	C
Haplotype M	G	T	A	T	T	*	*	A	A	C	T	G	*	A	C	T	T

Numbers the position of each nucleotide, * deleted nucleotides of the following insertions, *In1* GTTGTTTT GTTTAGTCAG GTAATTGTTGTTAATTGAT GTTCATGTCTGAACTAT CATCTTATGCCTGCTTTCA TTCCAATTGTGTTGTGTAG ATTTT, *In2* ATGGGTCATGG TAGTTTTACATTGT GCTAA GTTAAATTTTGTTCCTTCT ATT

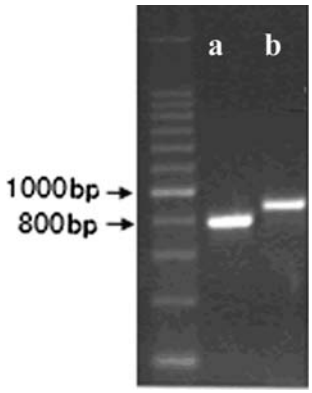

Fig. 4 Amplification length difference of a primer pair Pv97 between genotypes G19833 (*a*) and DOR364 (*b*) on a 1.5% agarose gel

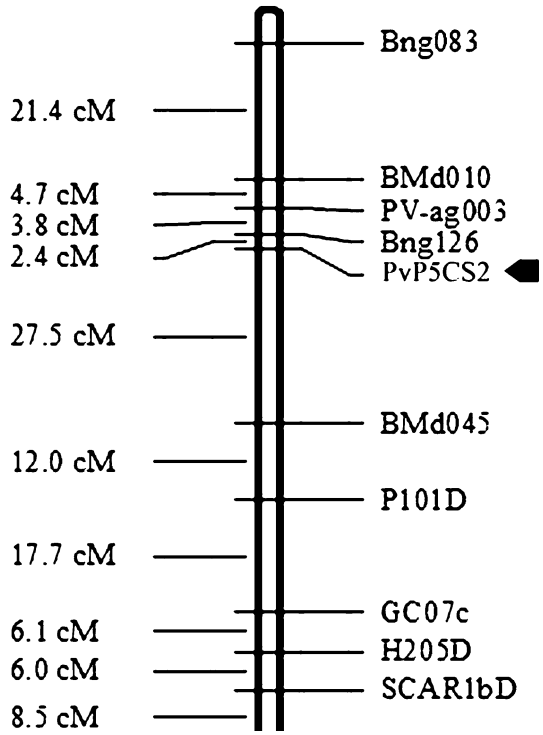

Fig. 5 Genetic mapping of *PvP5CS2* on linkage group (chromosome) b01

Recombination and linkage disequilibrium within *PvP5CS2*

The minimum number of recombination events (RM) in the *PvP5CS2* gene was calculated using the four-gamete test (Hudson and Kaplan 1985). When the entire population (27 accessions) was analyzed the value was RM = 3. This was reduced to RM = 1 when only the cultivars (24 accessions) were analyzed. Further analysis of Andean versus Mesoamerican genotypes revealed that no recombination events were likely.

Linkage disequilibrium (LD) was calculated for all informative sites (Fig. 7). The linkage disequilibrium statistic of Hill and Robertson (1968), R^2, was plotted relative to distance. In the present context, each nucleotide was considered to be a locus. Despite the presence of recombination events in the full dataset of 27 accessions, 66% of LD comparisons were significant after the Bonferroni correction was applied, including 16% linkage disequilibrium and 49% close linkage pairwise comparisons. LD decay over nucleotide distance was found to be a steady decline as shown by the significant linkage disequilibrium ($R^2 \geq 0.2$) which was maintained over a 5.1 kb distance within the 5.872 nucleotides sequences for the *PvP5CS2* gene. These values for the decay in LD are long when compared to maize but shorter than for soybean and *Arabidopsis* (Rafalski and Morgante 2004).

Discussion

Similarity and differences between *PvP5CS2* and other plant *P5CS* genes

In plants, proline biosynthesis from glutamate is catalyzed by pyrroline 5-carboxylate synthetase (P5CS), a bifunctional enzyme exhibiting both *g*-glutamyl kinase (γ-GK) and glutamic-*g*-semialdehyde dehydrogenase (GSA-DH) activities which correspond to the ProB and ProA proteins of *E. coli*, respectively. In higher plants, the *P5CS* (Va*P5CS*) gene was first isolated from mothbean (*V. aconitifolia*) by a functional complementation technique (Hu et al. 1992). Here, we cloned a cDNA named *PvP5CS2* from 10-day-old seedlings of common bean by a candidate gene approach and found it to have a complete ORF that encoded a full-length P5CS protein and to be similar in DNA sequence to a previously cloned PvP5CS from common bean (Chen et al. 2009). Many eukaryotic P5CS genes encode a full-length P5CS protein, whereas a tomato *P5CS* sequence, *tomPRO1*, was similar to prokaryotic P5CS genes, resembling a polycistronic operon encoding the γ-GK and GSA-DH proteins separately (Garcia-Rios et al. 1997). The peculiar internal structure of the *tomPRO1* sequence (Garcia-Rios et al. 1997) was not found in either *PvP5CS or PvP5CS2*.

The deduced amino acid sequence of *PvP5CS2* was similar to that of VaP5CS and other legume homologues, and comprised five regions typical of P5CS protein functional domains in higher plants (ATP-binding site, conserved Leu zipper and Glu-5-kinase domain, NAD(P)H-binding site, Leu-rich regions and GSA-DH) showing that the putative protein is involved in common bean's synthesis of proline. Hong et al. (2000) showed that the replacement of Phe at position 129 by Ala eliminated VaP5CS enzyme feedback

Table 3 Nucleotide diversity and neutrality tests for *PvP5CS2*

Item	π	Θ	Ka/Ks	Tajima's *D*	Fu–Li's *D*	Fu–Li's *F*
Total accessions	0.00483	0.00287	0.3883	2.6049**	1.828**	2.449**
Dry bean[a]	0.00378	0.00299	0.4114	1.242	1.544**	1.665*
Snap bean	0.00467	0.00288	0.3862	2.699**	1.646**	2.208**
A	0.00107	0.00224	0.4088	−2.322***	−3.036**	−3.255**
M	0.00144	0.00261	0.3750	−2.0439**	−2.682**	−2.866**
Exon	0.00356	0.00217	–	2.2383**	1.2666	1.8337**
Intron	0.00567	0.00344	–	2.3187**	1.5149**	2.0870**

π nucleotide diversity (Nei 1987), *Ka* the average nonsynonymous substitution per nonsynonymous site, *Ks* the average synonymous substitution per synonymous site, *A* Andean gene pool, *M* Mesoamerican gene pool

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

[a] Excludes three wild dry bean

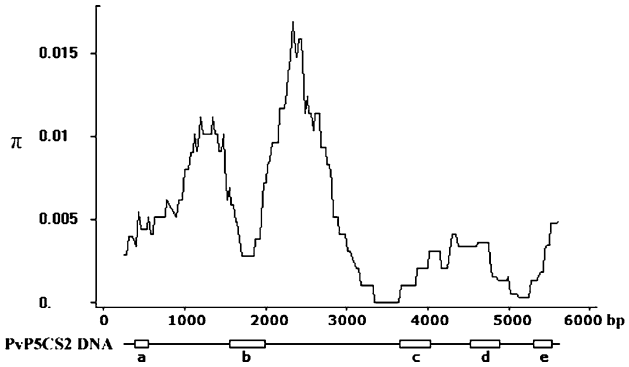

Fig. 6 Nucleotide diversity distribution along *PvP5CS2* genome sequence where *a*, *b*, *c*, *d* and *e* represent putative ATP-binding site, conserved Leu zipper and Glu-5-kinase domain, NAD(P)H-binding site, Leu-rich regions and GSA-DH domain of *PvP5CS2* gene, respectively

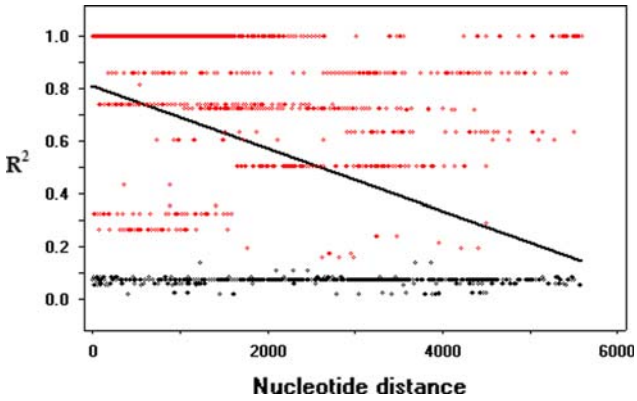

Fig. 7 Plot of R^2 (the linkage disequilibrium statistic of Hill and Robertson (1968)) versus nucleotide distance for *PvP5CS2* gene among 27 common bean accessions

inhibition by proline. A comparison of PvP5CS2 with VaP5CS showed that the feedback inhibition amino acid residue of VaP5CS (at position 129) was conserved in the PvP5CS2 sequence (at position 128) (Fig. 1), suggesting that this activity may be inhibited by the accumulation of proline.

Although the amino acid length was similar between PvP5CS and PvP5CS2, there were 106 variant amino acid sites between their putative proteins. Eighteen sites were found in six functional domains: one in ATP-binding site and putative Leu zipper, three in conserved Glu-5-kinase domain and NAD(P)H-binding site, eight in Leu-rich region and two in GSA-DH domain (Fig. 1). Of the 18 differences, 10 amino acid variants (1 in conserved Glu-5-kinase domain 1, 2 in NAD(P)H-binding site and 7 in Leu-rich region) resulted in changes in protein properties and characteristics. Among the five functional domains, the amino acid differences were greatest in the Leu-rich region which has been postulated to facilitate inter- or intra-molecular interaction of the P5CS protein with other proteins. The tertiary structure of the Glu-5-kinase and GSA-DH domains of the P5CS enzyme may be maintained by the Leu-rich domain (Kavi Kishor et al. 2005), so we presume that the variants between the PvP5CS and PvP5CS2 sequences for this domain may affect the spatial structure of the different resulting proteins.

Genetic diversity for the *PvP5CS2* gene in common bean gene pools

In this study, 63 SNP sites were identified in 5,872 bp of DNA sequence for the *PvP5CS2* gene when comparing the initially sequenced variety with 27 other wild and cultivated common bean genotypes, giving one SNP every 93 bp, which was similar to the rate found in maize (Tenaillon et al. 2001) but higher than that found in soybean (Zhu et al. 2003), and less frequent than that found in barley (Bundock and Henry 2004). Single nucleotide polymorphisms were slightly less abundant in the comparison of ESTs from two common bean genotypes, G19833

(Andean) and a contrasting Mesoamerican variety (Ramírez et al. 2005) occurring one SNP every 387 bp. In contrast, SNP frequency when comparing 92 common bean genotypes for gene sequences of dihydroflavonol 4-reductase (*DFR*) intron 1 was 1 SNP every 8 bp (McClean et al. 2004b), which was ten times higher than within the *PvP5CS2* gene. However, the proportion of indels to total sequence variants in *PvP5CS2* (74.2%) was quite similar to that reported in the *DFR* intron 1 sequences (71%) for common bean (McClean et al. 2004b). By comparison, Schmid et al. (2003) determined that 14% of sequence polymorphisms they detected via the resequencing of 12 genotypes were indels. In cultivated barley, the proportion is 8% on the basis of an analysis of sequence polymorphisms in five diverse barley cultivars (Kanazin et al. 2002), while in soybean, about 15% of sequence variants were indels (Choi et al. 2007). Therefore, it appears that that the indel frequency is substantially higher in common bean genomic sequence than in these other inbred crops, perhaps because of the two gene pool origin of the domesticates.

The mean nucleotide diversity for common bean *PvP5CS2* ($\Theta = 0.00287$) was similar with values found for *Arabidopsis* ($\Theta = 0.0033$) (Olsen et al. 2002), but lower than in cultivated maize ($\Theta = 0.0181$) (Eyre-Walker et al. 1998) and barley ($\Theta = 0.0081$) (Morrell et al. 2005). The level of sequence diversity in an inbreeding species is expected to be lower than that of an outcrossing species because of smaller effective population size (Pollak 1987) and as a result of additional effects of background selection (Nordborg et al. 1996). Common bean, like *Arabidopsis* and barley, has lower mean nucleotide diversity than maize, suggesting the pressure of relatively high level of slightly deleterious mutations. The nucleotide diversity of intronic regions of *PvP5CS2* in this study ($\Theta = 0.00344$) was lower than that of the DFR intron 1 ($\Theta = 0.0256$) analyzed by McClean et al. (2004b), implying stronger negative selection pressure in general on *PvP5CS2* compared to *DFR*.

Another discovery we made was that variation at *PvP5CS2* among the Andean genotypes ($\pi = 0.00107$) was greatly reduced relative to the Mesoamerican genotypes ($\pi = 0.00144$). Using AFLP markers, Beebe et al. (2001) also observed reduced variation among Andean genotype; however, Blair et al. (2006) found similar levels of SSR-based diversity. On the other hand, variation in *DFR* intron 1 among Andean genotypes was also decreased compared with variation among Mesoamerican genotypes (McClean et al. 2004b). These results suggest that the Andean gene pool might have a narrower genetic basis than the Middle American gene pool although this depends on the genotype selection used in any of these studies. In this study, nucleotide diversity values were higher with dry beans than with snap beans of either the Andean or Mesoamerican gene pools perhaps reflecting the narrower germplasm within snap beans.

Mapping of the *PvP5CS2* gene based on an indel polymorphism

A concrete result of this study was the development of the Pv97 marker based on an indel, which could reliably distinguish Andean and Mesoamerican genotypes and which was useful in genetic mapping of *PvP5CS2* in an inter-gene pool population. indel-based markers have been useful in several crops (Bhattramakki et al. 2002; Rickert et al. 2003) and have been developed in common bean but mostly from anonymous genomic sequences (e.g., Blair et al. 2007). In contrast, here we have developed an indel marker for a gene encoding an important common bean protein (Δ^1-pyrroline-5-carboxylate synthase) that is involved in osmoprotection of drought stressed cells (Delauney and Verma 1993).

Genetic mapping showed the *PvP5CS2* gene to be located on linkage group b01 which is the same linkage group where determinacy and photoperiod adaptation genes are located (McClean et al. 2004a). As a co-dominant marker with a large amplicon size difference, the Pv97 was an easy-to-use marker for future mapping work. Other gene-based markers have also been used in common bean for genetic mapping and have been proposed for selection of various traits, as for example, the duplicate DFR genes that potentially function in seed coloration (McClean et al. 2004b) or the Terminal Flower 1 homologues (*PvTFL1x*, *PvTFL1y*, and *PvTFL1z*) that may play a role in plant architecture traits (Kwak et al. 2008). Future studies will determine if the *PvP5CS2* gene underlies proline accumulation or quantitative trait loci (QTL) for drought or other abiotic stress tolerances, in which case the marker may be useful for selection of adaptation of common bean genotypes to adverse environments. Earlier, we found that gene expression of the other P5CS gene in common bean, *PvP5CS*, is temporally associated with peak proline accumulation after drought, cold and salt stress treatments (Chen et al. 2009). Therefore, the cloning and characterization of this second P5CS gene in common bean is important for evaluating the gene expression of *PvP5CS2* as compared to *PvP5CS* under these abiotic stresses. Finally, the candidate gene approach to dissecting drought tolerance has taken on added importance with the increasing frequency and severity of severe drought events and appears promising for linking drought tolerance QTL with underlying factors (Fu et al. 2007).

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