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RESEARCH ARTICLE

Promoter characterization of chickpea delta-1-pyrroline-5-carboxylate synthetase (P5CS) gene provides novel insights into its stress responsiveness

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Abstract

Proline is an osmolyte that accumulates in response to various environmental stresses and serves several protective functions in plants. The gene P5CS (delta-1-pyrroline-5-carboxylate synthetase) codes a key regulatory enzyme for proline biosynthesis. In the present study, the promoter region of the chickpea P5CS gene (CaP5CS) was isolated and in-silico characterized. The expression of the gene was examined under various abiotic stresses, such as cold, salinity and dehydration and also on the application of various phytohormones and chemicals to understand the changes in gene expression which are driven by the promoter. Structurally, the promoter sequence was enriched in many cis-regulatory elements (CREs) recognized by transcription factors (TFs) involved in both ABA (Abscisic acid)-dependent and independent signaling pathways for proline biosynthesis. The gene was observed to be both spatially and temporally regulated. It was observed that the gene was highly up-regulated under heat and dehydration stress at 3 hours of stress treatment. Under dehydration, for the same tissues, the proline content was also estimated to increase by more than 3-fold from 3 to 6 hrs. Notably, under cold and IAA (indole-3-acetic acid) treatment, the gene was down-regulated, which confirms the role of the gene primarily under osmotic stress. This study provides novel insights into the regulation of proline biosynthesis in chickpea. Also, the promoter isolated can be utilized to enable spatial and temporal control in transgene expression in genetically modified crops developed for enhanced stress tolerance.

Keywords: Chickpea, proline, P5CS, promoter, drought stress, cis-regulatory elements

Introduction

Chickpea (*Cicer arietinum* L.) is one of the most important pulse crops and is a source of nutrition for the burgeoning world population (Zhang et al. 2024). It is grown in more than 50 countries globally, with countries of the Indian subcontinent accounting for almost 70% of the world's production (Arriagada et al. 2022; Mehrotra et al. 2023). The production of chickpea is adversely affected by various abiotic stresses like drought, salinity, cold and high temperature (Yadav et al. 2022; Jain et al. 2023; Panigrahi et al. 2024), which can cause 40 to 50% reduction in yield worldwide (Yadav et al. 2024). To ensure sustainable crop production in chickpea, it is important to develop climate-resilient chickpea varieties. Identification of the candidate genes governing stress tolerance and the underlying mechanisms involved in their regulation holds the key to crop improvement and is, therefore a constant endeavour of the scientific community involved in chickpea improvement.

Various metabolites have been shown to impart stress tolerance in plants (Arbona et al. 2013; Panahirad et al. 2023). Amongst these, proline, an amino acid, is an osmolyte that plays a highly significant role in plants exposed to various stress conditions. Under stress, it accumulates not only in plants but also in bacteria, algae, fungi, protozoa and marine invertebrates (El Moukhtari et al. 2020; Hosseinifard et

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al. 2022). The reactive oxygen species, like ROS (e.g., OH·, O_2·, etc.) which are increasingly synthesized during stress, are highly damaging to sub-cellular structures (Huang et al. 2019). Proline acts as a scavenger of these free radicals and, in this way, it provides protection against oxidative damage in plants (Renzetti et al. 2024). The biosynthesis of proline is an evolutionarily conserved metabolic pathway involving a key enzyme, the Pyrroline 5-Carboxylate Synthetase (P5CS), which catalyses the rate-limiting step in the glutamate pathway, enabling the synthesis of proline from glutamate (Meena et al. 2019). P5CS is one of the extensively studied enzymes and its expression in different organisms under several abiotic stresses, such as drought, salt, cold, etc., has been analysed (Amini et al. 2015; Maghsoudi et al. 2018). Overexpression of the *Vigna aconitifolia P5CS* (*VaP5CS*) in pigeon pea (Surekha et al. 2014), wheat (de Lima et al. 2019), rice (Karthikeyan et al. 2011), and chickpea (Ghanti et al. 2011) has been shown to lead to increased biomass production under various abiotic stress conditions. This suggests that the *P5CS* gene is a candidate gene for genetic engineering to increase the yield and biomass of a plant under several abiotic stresses. Signaling mechanisms by which stress is known to induce proline biosynthesis includes several biomolecules like ABA (Abscisic acid), auxin, gibberellins (GA), cytokinin, ethylene, and salicylic acid (SA), however the mechanisms underlying the regulation of the gene via phytohormones is not known in chickpea (Per et al. 2017; Sharma et al. 2019; Hussain et al. 2024). To understand how the *P5CS* gene is regulated under various stress conditions, the promoter of the gene was isolated from the drought-tolerant chickpea variety, ICC 4958. The sequence was structurally characterized *in-silico*. Promoter isolation and characterization have many applications in plant breeding, notable in the development of transgenic plants with enhanced tolerance to adverse conditions, where the expression of the transgene is driven by a promoter that is highly inducible by environmental factors like stress (e.g., drought, salt) or hormones. The present investigation identifies one such promoter that can be deployed to develop transgenic chickpea varieties that are tolerant to heat and drought stress.

Materials and methods

Isolation and in-silico analysis of promoter sequence

The promoter region of 1322 bps, located at 1,000 bp upstream of the translation start site of the chickpea *P5CS* gene (*CaP5CS*), was isolated by utilizing sequence-specific markers designed based on the flanking sequences of the gene (Supplementary Table S1). The amplified PCR product was eluted, sequenced and aligned with the known sequence and submitted to NCBI GenBank (KC464465). The PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) database was used for identifying the *cis*-regulatory elements (CREs) in the promoter sequence. These elements were depicted along the length of the sequence through the tool TBtools v2.142 (Chen et al. 2020).

Plant material and treatments

Hormone and chemical treatment

Chickpea seeds of the variety ICC 4958 were sown in soil-filled pots (8-inch plastic pots) in a glass house for 10 days with ambient temperature ranging between 25 to 28°C (night/day). Three seeds were sown in each pot and in total of 25 pots were used. At 10 days after sowing (DAS), the plantlets (a few of which were uniform in growth and healthy) were transferred to tubes containing Hoagland's solution (hydroponic condition) for another 11 days. During this period, the Hoagland solution was changed every 3 days to ensure proper availability of the nutrients to the plantlets. On the 12th day, the growth medium was supplemented with various phytohormones and chemicals and the plantlets were allowed to grow for varying durations, namely 1, 3, 6, 12 and 24 hours. The various chemicals used were 20 µM abscisic acid (ABA), 20 µM jasmonic acid (JA), 100 µM salicylic acid (SA), 10 µM BAP (6-benzylaminopurine), 200 µM ethephon (Et), 100 µM gibberellic acid (GA3), 20 µM indole acetic acid (IAA), 50 µM H_2O_2 and 150 mM sodium chloride (NaCl). All the experiments were conducted in triplicate (3 plants for each treatment). At the end of the stipulated durations, the samples were immediately frozen in liquid nitrogen and stored at -80°C till further analysis. The samples frozen immediately without being grown on any supplemented medium are considered as the 0 hr or control samples for each treatment.

Stress treatment

For heat and cold stress, the plantlets (10-day-old) transferred to Hoagland's solution were grown for another 11 days and on the 12th day, these were transferred to a growth chamber at 37 and 4°C, respectively. Likewise, for wound stress, on the 12th day, leaves of seedlings were scratched by a needle. For dehydration stress, the plantlets were placed on 1MM Whatman paper on the 12th day at room temperature. On the 12th day, samples were collected at different time points. The samples frozen immediately, without being given any heat, cold, or dehydration stress, are considered to be the 0 hr or control samples for each treatment.

Field samples

To study the *CaP5CS* expression in different tissues, namely leaves, roots, shoots, flowers (both unopened and opened) and pods, respective tissues were collected from the chickpea plants (ICC 4958) grown in the field, at the reproductive stage. Samples were collected in triplicate, snap frozen in liquid nitrogen and stored at -80°C till further analysis.

Proline estimation

About 21-day-old seedlings of hydroponically grown plants, which were used for the dehydration experiment, were harvested in triplicate at 0, 1, 3, 6, 8 and 12 hours time points. Leaf tissue from these samples was used for proline estimation as described by Bates et al. 1973.

Gene expression analysis

Total RNA was isolated using Trizol reagent according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). Purity of RNA was checked by determining the absorbance ratio at 260/280 (1.8–2.0) and 260/230 (2.0–2.2) by Perkin Elmer lambda 35 UV/V Spectrophotometer and the RNA integrity was confirmed by visualization of intact bands of 25S rRNA and 18S rRNA in formaldehyde agarose (FA) gel.

Semi-quantitative RT- PCR analysis

RNA was quantified and diluted to 25 ng/µl for reverse transcriptase PCR reaction using one-step RT-PCR (reverse transcriptase) kit (Qiagen, USA). All experiments were performed in three biological replicates and three technical replicates for each of the 13 different treatments. PCR amplicons were taken at 21, 24 and 27 cycles, resolved on 1.2% agarose gel and only the 27-cycle amplicons, which were found to be best among the experimental replications, are shown in the picture. *Elongation Factor-1 alpha* (AJ004960) gene was used as an internal control in all the cases (Primer sequences listed in Supplementary Table S1).

qRT-PCR analysis

For quantitative RT-PCR, primers targeting the transcript of *CaP5CS* were designed with the default parameters (GC content 50%, primer Tm 60 ± 2°C, primer length 24± 6 nucleotides, and a product size in the range of 80–200 bp) by using the IDT primer quest online tool. The RNA isolated from different tissues (root, shoot, leaf, unopened and opened flower, pod) was used for cDNA synthesis by using the Superscript III first strand synthesis kit (Life Technologies, Carlsbad, CA, USA) and the cDNAs were diluted to a final concentration of 5 ng/µl for qRT-PCR reaction. SYBR green chemistry was used for quantification and the reaction was performed using SYBR FAST qPCR Master Mix (2x) Universal (KAPA Biosystems) according to the manufacturer's instructions. Reaction was performed with three biological replicates along with the negative control (no template) in 96-well plates using the Stratagene Mx3005P system. PCR was performed under the following conditions: 3 min at 95°C (enzyme activation and initial denaturation), 40 cycles each of 3 seconds at 95°C (denaturation) and 30 seconds at 60°C (anneal/extend). Subsequently, a melting curve with increments of 0.5°C from 65 to 95°C for each cycle 5 sec. *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, AJ010224) and *Elongation Factor-1 alpha* were used as reference genes to normalize the expression and 2–ΔΔCt method was used for calculating relative fold expression (Livak and Schmittgen 2001) (Primer sequences listed in Supplementary Table S1).

Tissue-specific transcriptome dataset

The RNA-Seq data were obtained from the BioProject ID PRJNA413872 at NCBI and this was utilized to analyse the expression of *CaP5CS* across root, shoot, leaf, flower and pod at both vegetative and reproductive stages. The FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values were plotted in the form of a heatmap drawn through TBtools v2.142.

Results and discussion

Isolation and in-silico analysis of the CaP5CS promoter region

An upstream sequence of 1322 bp for the *CaP5CS* gene was isolated and sequenced. *In-silico* promoter analysis was done to identify the CREs (Supplementary Figure S1A and B). A total of 123 *cis*-regulatory elements were identified across the length of the promoter. Noteworthy amongst these were the ABRE (element involved in the abscisic acid responsiveness), ERE (Ethylene-responsive element), GRE (gibberellin-responsive element), MBS (MYB binding site involved in drought-inducibility), MRE (MYB binding site involved in light responsiveness), TATA-box, CAAT box, G-Box, F-Box, etc. The presence of these elements potentially explains the responsiveness of the gene to hormones and various biotic and abiotic stresses. The binding sites for various transcription factors (TF) like DREBs (Dehydration-responsive element binding), PIFs (Phytochrome-interacting factors), WRKY, bZIP (basic leucine zipper transcription factor), MYB, etc, were identified. The promoter sequence possessed the cis-elements recognized by TFs mediating both ABA-dependent and independent signaling pathways for proline biosynthesis (Zarattini and Forlani 2017). G boxes with ACGT core sequence were identified, which are recognized by the bHLH and bZIP (ABF- ABRE-BINDING FACTOR and GBF- G-BOX BINDING FACTOR) TFs, as part of the ABA-dependent pathway. *Cis*-elements for the binding of DREBs, notably DREB2A, were also identified, indicative of the gene as part of the ABA-independent pathway.

Temporal regulation of CaP5CS under stress and chemical treatment

To study the expression of *CaP5CS* under 13 different treatments, semi quantitative RT PCR was performed. Treatments employed in this present study can be categorized as abiotic (dehydration, heat, cold and wound), exogenous hormonal (ABA, JA, SA, BAP, ethephon, GA3, IAA) and chemical (H_2O_2 and NaCl) supplementation. Noteworthy increase in the expression of *CaP5CS* was observed in treatments after 1-hour in Et., 3 hours in heat

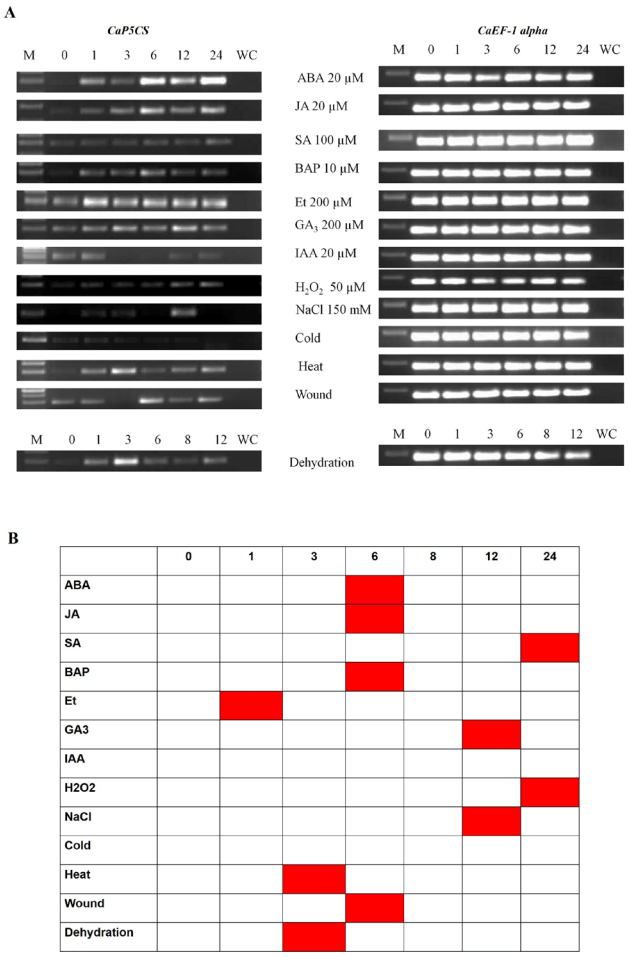

Fig. 1. Temporal variation in *CaP5CS* expression: (A) Semi-quantitative RT-PCR analysis of the *CaP5CS* expression under various abiotic, hormonal and chemical treatments. *CaEF-1* alpha was used as an internal control. M- 1Kbp ladder; 0, 1, 3, 6, 8, 12 and 24 represent time points in hrs; WC-Water Control. (B) A representation (in the form of a heatmap) depicting the pattern of gene expression under various treatments, with red color depicting the time point with the highest expression

and dehydration, 6 hr in ABA, JA, BAP and wound, 12 hours in GA3 and NaCl, 24 hours in H_2O_2 and SA (Fig. 1A and B). In comparison to all the other phytohormones studied, the gene exhibited consistent up-regulation across all the time periods for Et. treatment. However, the presence of significant expression even in the 0 hr control for this treatment demonstrates the lack of responsiveness of the promoter for ethephon relative to the other phytohormones. No increase in the expression of *CaP5CS* was observed in response to cold and IAA treatment. Exogenous application of IAA resulted in reduced accumulation of proline in wheat under salt and drought stress (Sadiqov et al. 2002), validating the inverse relation observed in the present study. The lack of significant up-regulation of the gene under cold stress has been previously reported in grapevine, where only one of the two copies of the gene, *P5CS2,* was up-regulated under cold stress and the other copy, *P5CS1,* was down-regulated (Wei et al. 2022). The gene *CaP5CS* could therefore be an ortholog to the grapevine *P5CS1* and might potentially play a more important role in countering osmotic stress like salt, dehydration and drought stresses. The lack of any cis-acting elements, which are primarily recognized by the DREB1-type genes involved in cold-responsive pathways (Zarattini and Forlani 2017), could also be a possible reason for this.

Estimation of proline

The level of proline (µg/g wt of leaf tissue) was estimated from the plants kept under dehydration stress and harvested at different time points of 0, 1, 3, 6, 8 and 12 hours was estimated to be 10, 23.8, 29.76, 89.28, 110 and 178.57 µg/g, respectively (Fig. 2).

Previously, in wheat seedlings as well, under short-term osmotic stress, the content of proline was estimated to be significantly enhanced within a 2 to 4-hour interval post osmotic stress treatment (Koenigshofer and Loeppert 2019). This increase in proline content, with a more than 3-fold increase from 3 to 6 hours, is in corroboration with the enhanced transcript accumulation observed for the same samples, particularly at 3 hrs of dehydration stress, which is possibly due to transcript abundance of the enzyme prior to the actual enzymatic conversion of the intermediate metabolite (glutamate semialdehyde) into proline. This confirms that the gene *CaP5CS* is involved in proline biosynthesis in chickpea.

Spatial regulation of CaP5CS

Transcript level of *CaP5CS* in the tissues was estimated in unopened flowers (1.0 fold), roots (0.88 fold), pods (0.66 fold), opened flowers (0.36 fold), leaves (0.27 fold) and shoots (0.2 fold) (Fig. 3A). Highest expression of the gene in unopened flowers is in agreement with previous reports also. The expression pattern of the gene was highly conserved as

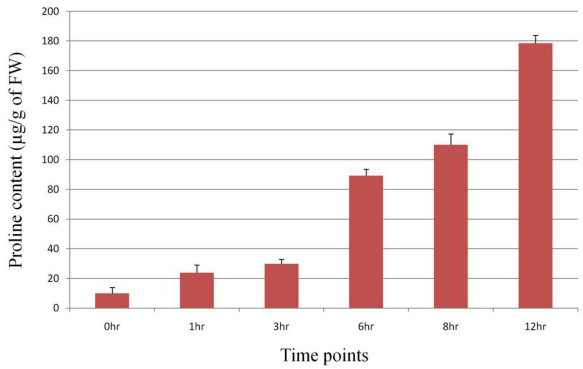

Fig. 2. Increase in proline content during dehydration stress. Proline was estimated at different time points, i.e., 0, 1, 3, 6, 8 and 12 hours in plants subjected to dehydration stress.

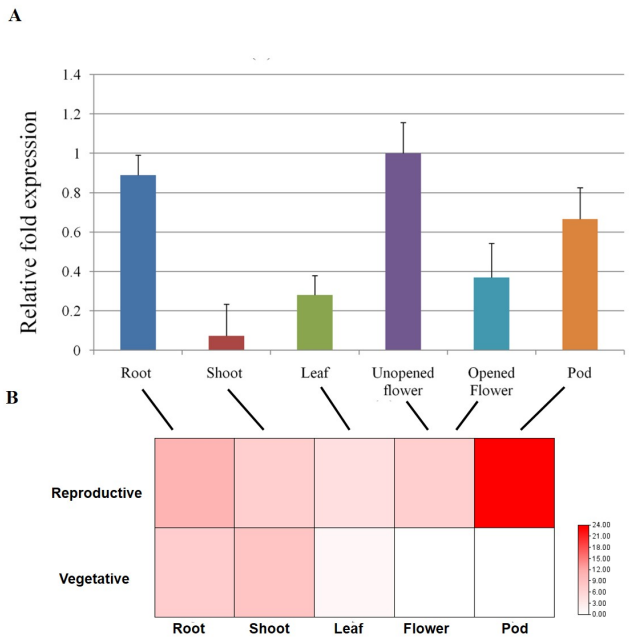

Fig. 3. Spatial variation in CaP5CS expression: (A) Expression analysis of the gene in different plant tissues through qRT-PCR. (B) Heatmap depicting the RNA-Seq derived FPKM values of the gene across different tissues at vegetative and reproductive stages

was validated through the RNA-Seq-based expression of the gene across various tissues, collected at both vegetative and reproductive stages (Fig. 3B).

The gene was observed to be relatively up-regulated at the reproductive stage and in reproductive tissues, compared to the vegetative stage and tissues. Previously, the expression of the gene was observed to be highest in the floral shoot apical meristems contributing to bloom improvement (Meena et al. 2019; Wei et al. 2022). In grapevine, the two copies of the *P5CS* genes were significantly up-regulated in the tendril and flower tissues compared to the other tissues (Wei et al. 2022). The higher expression of the gene in reproductive tissues was also demonstrated in switchgrass (*Panicum virgatum*), where the two *PvP5CS* genes were significantly up-regulated in panicle compared to the leaf, stem and root tissues (Guan et al. 2020).

Our analysis provides novel insights into the regulation of the *CaP5CS* gene. The *in-silico* promoter sequence analysis revealed the presence of several cis-regulatory elements that are recognized by a diverse array of TFs. The gene was also identified to be up-regulated under salinity, cold and dehydration stress and also on exposure to various phytohormones and chemicals. This proves the cross-linking of the stress signaling pathways leading to enhanced proline biosynthesis as a tolerance mechanism in chickpea. The promoter isolated in the present study could be deployed to drive the expression of a transgene in response to various stresses like heat and drought. Identification of such promoters that allow a calibrated response of a transgene under external environmental conditions remains an area of interest in molecular plant breeding and its application in crop improvement.

Supplementary materials

Supplementary Table S1 and Supplementary Fig. 1 are provided and can be accessed at www.isgpb.org.

Authors' contribution

Conceptualization of research (RS, PKJ); Designing of the experiments (VK, AAD); Execution of field/lab experiments and data collection (VK, AAD); Analysis of data and interpretation (DK, SY); Preparation of the manuscript (VK, AAD, DK, SY).

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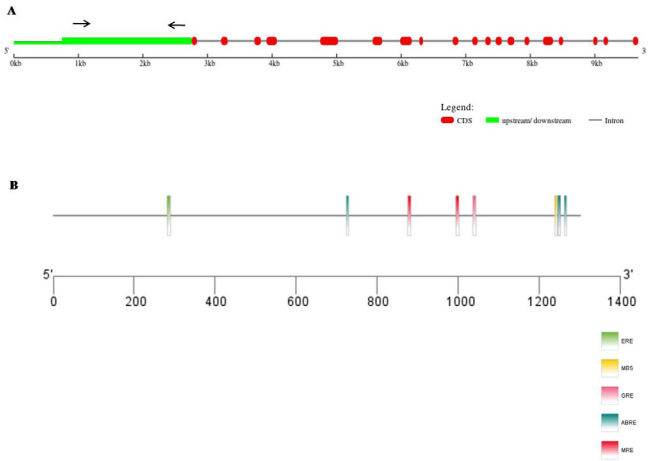

Supplementary Figure S1. *CaP5CS* promoter isolation and characterization. (A) The gene structure and the upstream promoter sequence, with sequence isolated flanked by the arrows depicting the position of PCR primers utilized. (B) The characterization of the promoter sequence isolated for the presence of cis-acting regulatory elements. ABRE (element involved in the abscisic acid responsiveness), ERE (Ethylene-responsive element), GRE (gibberellin-responsive element), MBS (MYB binding site involved in drought-inducibility), MRE (MYB binding site involved in light responsiveness)

Supplementary Table S1. Details of primers used in this study for PCR, qRT- PCR and semi-quantitative RT-PCR

Primer	Primer sequence
Promoter_F PCR	CAAGAGTTATTTGGGTGGAG
Promoter_R PCR	AAATGCGGAAATTGTTGGGA
GAPDH F	ATGACCACCGTCCATTCCATCACT
GAPDH R	AGACATCAACAGTTGGGACACGGA
EF-1alpha F	AGTCATCAAGCCTGGTGTGATCGT
EF -1alpha R	AGCTTCCTTAGCAGGGTCATCCTT
P5CS qRT-PCR F	AGTCACAAGCCCAATGAGTTTGCC
P5CS qRT-PCR R	AAAGGAGGAAAGGAAGCCCGAAGA
P5CS Semi-qRT-PCR F	CAGGCGGCAGGATAATCAGTCT
P5CS Semi-qRT-PCR R	AGGAACCAGTGGGTCGGATTTT