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Cloning, characterization and expression analysis of Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*) gene in harvested papaya (*Carica papaya*) fruit under temperature stress

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ABSTRACT

Δ^1-Pyrroline-5-carboxylate synthetase (P5CS) catalyzes the rate-limiting step in proline biosynthesis in plants. In the present study, a full-length cDNA, denominated as *CpP5CS2* for Δ^1-pyrroline-5-carboxylate synthetase (P5CS), was cloned from papaya using *in silico* cloning and 3′- or 5′-rapid amplification of cDNA ends (RACE). The full-length cDNA of *CpP5CS2* was 2583 bp, with a 2151 bp open reading frame (ORF) encoding a 717 amino acid polypeptide. Sequence analysis showed that CpP5CS2 contained several substrate-binding and catalytic domains and had high homology to other plant P5CSs. The expression pattern of *CpP5CS2* in papaya under low (7 °C) and high temperature (35 °C) stresses was examined using real-time quantitative PCR. The results showed that both stresses induced *CpP5CS2* expression during the storage period, and the increased expression of *CpP5CS2* preceded proline accumulation. In addition, the high temperature caused a more significant induction of *CpP5CS2* expression and a higher level of accumulated proline than low temperature.

1. Introduction

Extreme temperature, drought, and high salinity are common stresses that adversely affect plant growth, crop production and quality. Proline is an important osmoprotectant that is thought to be critical for the plant adaptation to environmental stresses (Delauney & Verma, 1993; Hsu, Hsu, & Kao, 2003; Kishor, Hong, Miao, Hu, & Verma, 1995; Öztürk & Demir, 2002). It not only acts as an osmolyte for osmotic adjustment but also stabilizes membrane and subcellular structures under stresses, and protects cells against oxidative damage (Ashraf & Foolad, 2007; Verslues, Agarwal, Katiyar Agarwal, Zhu, & Zhu, 2006). In higher plants, proline accumulation is a common physiological response in response to a wide range of biotic and abiotic stresses (Delauney & Verma, 1993; Verbruggen & Hermans, 2008). The positive effect of proline accumulation on osmotic stress tolerance was firstly proved by the fact that bacteria strains with over-production of proline show enhanced salt tolerance (Csonka, 1981). Furthermore, proline is also thought to be an indicator of osmotic tolerance to drought stress in plants (Ashraf & Foolad, 2007; Chiang & Dandekar, 1995).

In higher plants, proline is synthesized in the cytosol from glutamic acid (Glu) and ornithine, and free proline accumulation is controlled by increasing synthesis and reducing degradation (Delauney & Verma, 1993; Nanjo et al., 2003; Verbruggen & Hermans, 2008). Biochemical studies indicate that proline biosynthesis *via* Glu pathway dominates under stress conditions (Delauney & Verma, 1993; Kishor et al., 2005). In this pathway, the bifunctional enzyme Δ^1-pyrroline-5-carboxylate synthetase (P5CS) is a key enzyme which catalyzes the conversion of Glu into Δ^1-pyrroline-5-carboxylate (P5C) and then the reduction of P5C to proline (Wang et al., 2011). P5CS activity mediates the rate-limiting step in proline biosynthesis, which is controlled at the transcriptional level and through feedback inhibition by proline (Savouré et al., 1995; Strizhov et al., 1997; Yoshiba et al., 1995).

Papaya (*Carica papaya* L.) is the only species within the genus *Carica* and the most commercially important species in the family *Caricaceae* (Badillo, 2000). It has been widely cultivated in tropical and subtropical regions for its nutritional benefits and pharmacological effects (De Oliveira & Vitoria, 2011; Fernandes, Rodrigues, Gaspareto, & Oliveira, 2006). However, the fruit after harvest ripen rapidly, has physiological disorders and is susceptible to biotic or abiotic stresses. These problems usually result in a high percentage of product loss (Chen et al., 2003; De Oliveira & Vitoria, 2011; Shiga

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et al., 2009). Inappropriate storage temperature is the most common problem for papaya fruit during postharvest storage and transportation. Understanding the effects of different storage temperatures on the expression patterns of the key genes such as *P5CS* involved in proline biosynthesis will enable us to select the appropriate storage temperature for overcoming this problem.

Genes encoding P5CS have been cloned and characterized from several types of plants including *Arabidopsis* (Strizhov et al., 1997), *Medicago truncatula* (Armengaud, Thiery, Buhot, Grenier De March, & Savouré, 2004), alfalfa (Ginzberg et al., 1998), tomato (Fujita, Maggio, Garcia-Rios, Bressan, & Csonka, 1998), sorghum (Su et al., 2011), and bean (Chen et al., 2010; Hu, Delauney, & Verma, 1992). Furthermore, there have been a few studies with focus on the regulation and function of these genes in plants by over-expressing them through transgenic approaches (Kishor et al., 1995; Nanjo et al., 1999). However, there have been fewer studies on postharvest horticultural crops and no report on the cloning of *P5CS* genes from papaya. Therefore, it is important to study the role of *P5CS* gene in mediating the stress response of papaya. In the present work, we first cloned a full-length cDNA of *CpP5CS2* from papaya using *in silico* cloning and 3′- or 5′-rapid amplification of cDNA ends (RACE), then characterized it and investigated its expression profile and proline accumulation in harvested papaya exposed to high and low temperature stresses. This study provided a foundation for further study on papaya fruit stress tolerance and fruit quality control.

2. Methods

2.1. Plant materials and experimental conditions

Papaya fruits (*C. papaya*) at color break stage (5%<peel color<15% yellow) (Blas et al., 2010) were harvested from a local commercial plantation near Guangzhou, South China, then transported to the laboratory and sorted by size, shape and maturity. Uniform fruits free from visual symptoms of any disease or blemishes were randomly selected. The selected fruits were first cleaned, dipped in a 1% hypochloride solution for 10 min to astringe cut and then soaked in 0.2% (w/v) Sporgon solution (Bayer, Leverkusen, Germany) for 1 min to eliminate potential microbes. The fruits were air dried at 25 °C for 3 h and then placed into unsealed plastic bags (0.02 mm thick) and subsequently transferred to 7 °C, 25 °C and 35 °C for storage, respectively. Samples stored at 25 °C were collected at 0, 2, 4, 6, 8, 10 and 12 d after treatment; samples stored at 35 °C were collected at 0, 2, 4, 6, 8 and 10 d after treatment; samples stored at 7 °C were collected at 0, 2, 6, 10, 14, 18 and 22 d after treatment. Three independent biological triplicates were performed for each temperature setting. For all samples collected, fruit core was removed and the flesh with peel was crushed in liquid nitrogen and stored at −80 °C until use.

2.2. Fruit evaluation under temperature stress

Fruit peel color was rated on a scale from 1 to 6 as described by Miller and McDonald (1999), where 1 = entirely green, 2 = <25% yellow, 3 = 25–50% yellow, 4 = 50–75% yellow, 5 = ≥75% yellow, and 6 = orange blush/yellow. Fruit coloring index was calculated on a daily basis as: coloring index = ∑(coloring grade × number of fruit)/total number of fruit. Subjective firmness was evaluated based on the whole fruit resistance to moderately applied finger pressure.

2.3. Total RNA isolation and first strand cDNA synthesis

All frozen tissues were ground in liquid nitrogen for RNA isolation. Total RNA was extracted using hot borate method described by Wan and Wilkins (1994) and then treated with DNAse I digestion using RNAse-free kit (TaKaRa, Japan) to eliminate the potential DNA contamination. The RNA concentration and purity were determined from the ratio of absorbance readings at 230, 260 and 280 nm using a BioPhotometer plus (Eppendorf, Germany). RNA integrity was assessed with 1.2% agarose/formaldehyde gel electrophoresis. Only RNA samples with absorbance ratio at 260/280 nm of 1.8–2.1, and the ratio at 260/230 nm ≥ 2.0, as well as a density ratio 28S/18S rRNA bands of 2:1 were used for further analyses. To prepare cDNA for RT-qPCR, 2 μg of total RNA was reverse-transcribed using the ReverTra Ace qPCR RT kit (TOYOBO, Japan). The final cDNA products were diluted 150-fold prior to use. The cDNA for RT-PCR was prepared by reverse transcribing 1 μg of total RNA using the SMARTer™ RACE cDNA Amplification Kit (Clontech, USA).

2.4. In silico cloning of P5CS and sequence analysis

To amplify the *P5CS* gene from papaya, a homologous *P5CS* gene from *Arabidopsis* (GenBank accession number: NM_115419.4) was used as bait and seed sequence for retrieval blast in papaya EST database. A papaya *P5CS* EST sequence (GenBank accession number: EX303129.1) was obtained, and found to be highly homologous to the *P5CS* obtained from other plant species in the NCBI Blast program. A set of primers (sense: 5′CAAGAGACGAGATACCTGAT3′, antisense: 5′CAATCCTTCAA CTCCAACTG3′) was designed based on this EST sequence. RT-PCR was conducted under the following conditions: one cycle of 94 °C for 3 min, 35 cycles (94 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min), and then one cycle of 72 °C for 10 min. The PCR products of the predicted size (about 650 bp in length) were purified and cloned into PMD20-T vector (TaKaRa, Japan) and then sequenced by Beijing Genomics Institute (BGI, China). The sequenced fragment information was then used to design primers for obtaining the full-length cDNA of *CpP5CS2* by RACE using SMARTer™ RACE cDNA Amplification Kit (Clontech, USA). Two specific primer pairs for *CpP5CS2* (3′-RACE: outer, TTCAGCATGGAACTAG CCCTTGGTCCAC, inner TGCATCCAGAACAATGCGCCTTGCCATA; 5′-RACE: outer, ATCCTGCTCGTACTTATCCCAACCTCTG, inner, ATGAATCCTGCTCG TACTTATCCCAACC) were used for 3′- and 5′-RACE, respectively. The PCR products were cloned and sequenced as described above. After aligning and assembling the sequences of the internal conserved fragment, 3′-RACE and 5′-RACE products, the full-length cDNA sequence of the *CpP5CS2* gene was deduced and subsequently obtained.

2.5. Bioinformatics analysis

Identification of nucleotide sequences from RT-PCR clones was established using the NCBI Blast program (http://www.ncbi.nlm.nih.gov/BLAST). Sequence alignment was conducted using the DNAMAN software and the phylogenetic tree was constructed by ClustalW program (http://www.ebi.ac.uk/clustalw). Open reading frame and protein prediction were made using NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The theoretical isoelectric point (pI) and mass values for mature peptides were calculated using the Peptide-Mass program (http://us.expasy.org/tools/peptide-mass.html). Protein subcellular localization was predicted using WoLF PSORT (http://wolfpsort.org/). The three-dimensional structure of the CpP5CS2 protein domain was predicted using the SWISS-MODEL workspace (http://swissmodel.expasy.org) (Bordoli et al., 2008).

2.6. Selection of reference genes

As the success of RT-qPCR depends on the stability of the reference gene(s) used for data normalization (Chen et al., 2011; Udvardi, Czechowski, & Scheible, 2008), we selected 21 candidate reference genes based on previous reports (Zhu et al., in press) and conducted systematic analysis of the stability of these genes for the selection of superior reference genes for accurate transcript normalization in papaya under different experimental conditions. The used selected five reference genes were described in our previous study (Zhu et al., in press) and their characteristics were also listed in Supplemental Table 1 and Supplemental Fig 1, so did *CpP5CS2*.