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Expression of *sHSP* genes as affected by heat shock and cold acclimation in relation to chilling tolerance in plum fruit

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ABSTRACT

Three full-length cytosol small heat shock protein (sHSP) genes, including two class I sHSP (CI sHSP) and one class II sHSP (CII sHSP) cDNAs, termed *Ps-CI sHSP1*, *Ps-CI sHSP2* and *Ps-CII sHSP1* respectively, were isolated and characterized from plum fruit at harvest. Their expression in relation to heat shock and cold acclimation-induced chilling tolerance were investigated. Heat shock treatment by dipping the fruit in water at 55 °C hot for 2 min and cold acclimation by conditioning the fruit at 8 °C for 5 d prior to storage at 2 °C could effectively reduce malondialdehyde (MDA) content and alleviate chilling injury. Furthermore, accumulation of *Ps-CII sHSP1* mRNA transcripts in the fruit during the subsequent storage at 2 °C was remarkably enhanced by heat shock and cold acclimation treatments. These data suggest that heat shock and cold acclimation treatments induced the expression of *Ps-CII sHSP1*, which may be involved in chilling tolerance of the fruit caused by these treatments.

1. Introduction

Plums are highly perishable due to their rapid rate of ripening at room temperature and storage at low temperature is used widely to extend postharvest fruit life and maintain quality. However, plum fruit are particularly susceptible to chilling injury (CI) (Manganaris et al., 2008). Flesh browning and translucency are two of the main CI symptoms observed and the susceptibility of fruit to chilling mainly depends on the cultivar and storage temperature (Crisosto et al., 1999). Flesh browning (internal breakdown) appears as a brown discolouration of the flesh and flesh translucency (gel breakdown) manifests itself as a translucent gelatinous breakdown of the mesocarp tissue around the stone (Taylor et al., 1993; Candan et al., 2008; Manganaris et al., 2008). Chilling symptoms mainly develop during shelf-life after removal of fruit from low temperature storage (Crisosto et al., 1999). Therefore, it is important to develop ways to alleviate CI of fruit stored at low temperature and to understand the underlying CI molecular mechanisms. Ethylene contributes to the development of CI, and application of hot-water dips or 1-MCP in combination with modified atmosphere packaging can prevent CI in plum fruit (Abu-Kpawoh et al., 2002; Candan et al., 2008; Khan and Singh, 2008; Manganaris et al., 2008).

It has been suggested that exposure to high or intermediate low temperatures can be used as effective postharvest methods for alleviating CI at subsequent low temperatures. Heat pretreatments have been shown to be effective in controlling decay in citrus (Porat et al., 2000a), reducing chilling injury in tomato fruit (McDonald et al., 2000), mangoes (Pesis et al., 1997), grapefruit (Porat et al., 2000b), avocados (Woolf et al., 1995) and grape berries (Zhang et al., 2005), and maintaining cold storage quality of strawberries (Vicente et al., 2002), peaches (Zhou et al., 2002) and pears (Abreu et al., 2003).

Plant organisms respond to thermal stress with inducted synthesis of heat shock proteins (HSPs) (Brodl, 1989; Vierling, 1991). HSPs comprise a diverse group of proteins, ranging in molecular weight from 15 to 115 kDa, which are believed to play a major role in thermotolerance (Howarth and Ougham, 1993). Among these proteins, small HSPs (sHSPs) represent the major family of HSPs induced by heat stress in plants (Waters et al., 1996). The molecular mass of these sHSPs ranges from 15 to 30 kDa, and plant sHSPs can be divided into six classes based on amino acid sequence, immunological cross-reactivity, and intracellular localization (Waters et al., 1996). Three classes (classes I, II and III) are localized in the cytosol or nucleus, and the other three in the plastids (Vierling, 1991), endoplasmic reticulum (Helm et al., 1995) and mitochondria (Lenne et al., 1995; LaFayette et al., 1996).

It has also been reported that mRNA levels of various sHSPs may increase following exposure to low temperatures; thus sHSPs may have chaperone activity under low-temperature stress (Sung et al., 2003; Wang et al., 2004; Mamedov and Shono, 2008). In tomato fruit, grape berries and grapefruit, there is increasing evidence suggesting that sHSPs could be involved in the ability of heat shock to increase cross-resistance in harvested fruit to chilling tolerance

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(Sabehat et al., 1998; Zhang et al., 2005; Sapitnitskaya et al., 2006). However, information is needed on the mechanisms underlying this alleviation of chilling injury and of chilling tolerance in relation to sHSP expression.

In the present study, three full-length cytosol sHSP genes, including two class I sHSP (CI sHSP) and one class II sHSP (CII sHSP) cDNAs, termed *Ps-CI sHSP1*, *Ps-CI sHSP2* and *Ps-CII sHSP1* respectively, were isolated and characterized from plum fruit at harvest. Their expression as affected by heat shock and cold acclimation treatments, and their relation to chilling tolerance were also investigated. Our results suggest that expression of *Ps-CII sHSP1* gene enhanced by heat shock and cold acclimation treatments may be involved in chilling tolerance in harvested plum fruit.

2. Materials and methods

2.1. Plant materials

Preclimacteric fruit of plum (*Prunus salicina* L. cv. Sanhua) were harvested from a commercial orchard in Weng-yuan County, Guangdong province, China. Fruit were transported to the laboratory within 6 h, selected for freedom from visual defects and for uniformity of weight, shape and maturity, washed in 500 mg L^{-1} Sportak (a.i. prochloraz) fungicide solution to control disease for 1 min and then air-dried at 25 °C for 2 h.

2.2. Treatments

The fruit were divided at random into three groups of 800 fruit per group for the following treatments: group 1 (control, non-conditioned group) fruit were placed into unsealed plastic bags (0.04 mm thick) and then stored at 2 °C for 50 d. Fruit for group 2 (heat shock pretreatment) were dipped into hot water at 55 °C for 2 min, then placed into unsealed plastic bags and stored at 2 °C for 50 d, while fruit for group 3 (cold acclimation pretreatment) were firstly stored at 8 °C for 5 d in unsealed plastic bags for cold acclimation, and then transferred to 2 °C for 50 d. During low temperature storage, sub-samples of these three groups were taken every 10 d for analyses. For chilling injury evaluation, fruit from the three different treatment groups were transferred to 22 °C after 50 d of storage at 2 °C, and stored at 22 °C for 4 d. Chilling injury was evaluated every day.

2.3. Chilling injury evaluation

The incidence of chilling injury was assessed on three fruit replicates (with each replicate contained 20 individual fruit) after 50 d of storage at 2 °C, immediately after removal from storage, and after 1, 2, 3 and 4 d of shelf-life at 22 °C, by the method of Candan et al. (2008). Symptoms of chilling injury were visually assessed by cutting each fruit in half along its equatorial axis and determining the percentage of fruit affected by chilling.

2.4. Determinations of membrane oxidation

Membrane oxidation was assessed by determining the malondialdehyde (MDA) concentration according to Chen et al. (2008). Flesh tissue samples (1.0 g) from three individual fruit were homogenized in 4 mL of 10% trichloroacetic acid and then centrifuged for 15 min at 10,000 × *g*. The supernatant phase was collected and 1 mL was mixed with 3 mL of 0.6% thiobarbituric acid. The mixture was heated to 100 °C for 20 min, quickly cooled and centrifuged at 10,000 × *g* for 10 min. The supernatant was collected and absorbances at 532, 600 and 450 nm were then measured in a Shimadzu UV spectrophotometer (Shimadzu UV-2450). The MDA concentration was calculated according to the formula: $6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$. Each assessment was repeated three times.

2.5. RNA extraction, isolation of plum sHSP full-length cDNAs

Total RNA from plum flesh tissue was extracted using the hot borate method of Wan and Wilkins (1994). Frozen tissues (10 g) were ground to a fine powder in a mortar using a pestle in the presence of liquid nitrogen. The extracted total RNA was used as templates for RT-PCR. The product (the first-strand cDNA) was subjected to PCR amplification. Degenerate primers of *CI sHSPs* (i.e. sense: 5′-AGCAACRTNTTCGAYCCNTTYTC-3′ and antisense: 5′-CTNCK YTCCAYRCGRTGCCA-3′) and *CII sHSPs* (i.e. sense: 5′-CCACGGTCACCTTCAGC ACNCCRTCYTG-3′ and antisense: 5′-GCGATCTTCACGTCGATGGTYTTNGGYTT-3′) were designed with reference to the conserved amino acid sequences of sHSPs. Reactions for the RT-PCR were subjected to one cycle of 94 °C for 3 min, 35 cycles each at 94 °C for 1 min, 45 °C for 2 min and 72 °C for 2 min, and then one cycle of 72 °C for 10 min. PCR products of the predicted size (about 400 bp in length for both CI sHSP and CII sHSP) were purified and cloned into pGEM-T easy vector (Promega, USA). The nucleotide sequences of the cDNA inserts were determined using the thermo sequenase dye terminator cycle sequencing kit and a 3730 DNA sequencer (PerkinElmer Applied Biosystems).

Consequently, 3′- or 5′-rapid amplification of cDNA ends (3′- or 5′-RACE-PCR) was performed using cDNA amplification kits (Takara, Shiga, Japan) according to the manufacturer's protocol. In order to amplify 3′-end and 5′-end fragments, the specific primers for *Ps-CI sHSP1* (3′-RACE: outer, AGAAGAGGTGAAGGTGGAGG and inner, GCGGAGAGAGGAAAATAGAG; and 5′-RACE: outer, TGACACTCAAAACCCCATTCTCC, middle, TCTGTGCCACTGGTCGTTCTTGT, and inner, TCAACCTCCACCTTCACCTCTTC), *Ps-CI sHSP2* (3′-RACE: outer, GACATCTGGGACCCCTTTGA and inner, GAGGAGGTGAAAGTTGAGGT; and 5′-RACE: outer, CCTCCCGTCATCAACCTCAACTT, middle, ATGTGGGCTTCTGGGGTCTCTTT, and inner, AGTCAATGCGAGTGTTGGCGATG), and *Ps-CII sHSP1* (3′-RACE: outer, GAGACATCAAGGTCCAGGTG and inner, AGGAAGAGGGAGGAGGAGAA; and 5′-RACE: outer, AGTCACAGTCAGGACCCCATCTT, middle, TTCTCAGGCAGCACAAACTTCCA, and inner, AGAAGCACATTGTCGTCCTCCAC) were designed based on the nucleotide sequences of the cDNA fragments already cloned by RT-PCR. The 3′- and 5′-RACE-PCR products were cloned and sequenced as described above.

2.6. DNA sequence analysis, alignment, and comparisons

Identification of nucleotide sequences from RT-PCR clones was established using the NCBI Blast program [http://www.ncbi.nlm.nih.gov/BLAST]. Alignment and comparison of sequence were made using the 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 (p*I*) and mass values for mature peptides were calculated using the PeptideMass program [http://us.expasy.org/tools/peptide-mass.html].

2.7. Northern blot analysis

Total RNA (10 μg) was separated on a 1.2% agarose-formadehyde gel and capillary blotted onto positively charged nylon membrane (Biodyne® B, 0.45 μm, PALL Co. Sarasota, FL). The RNA was fixed to the membrane by baking for 2 h at 80 °C and then cross-linked to the membranes using an ultraviolet cross-linker (Amersham Biosciences, Piscataway, NJ). The membranes were prehybridized for more than 3 h in SDS buffer [50% deionized