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# CHARACTERIZATION OF ENDO-β-MANNANASE ACTIVITY AND GENE EXPRESSION DURING 'YANHONG' PEACH STORAGE

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## ABSTRACT

Fruit softening is mainly caused by the cell wall modifications. Mannan is one of cell wall components, and its complete breakdown requires endo-β-mannanase (EC 3.2.1.78, β-Man). In the present study, the expression pattern of β-Man activities and genes in fruits, as well as the changes of fruit firmness and ethylene production were monitored during peach fruit storage. The results showed that the β-Man activity in both skin and mesocarp increased concomitantly with the loss of firmness and ethylene release during peach fruit storage. In addition, β-Man activity in skin was significantly higher than that in mesocarp. Two partial cDNAs of *PpMAN*1and *PpMAN*2 were obtained from mature peach fruit. The accumulation of *PpMAN*1 and *PpMAN*2 in both tissues exhibited a steady decline with the peach fruit softening during storage, which positively correlated with the loss of fruit firmness, but negatively correlated with the increase of β-Man activity and ethylene production.

**Key words:** Endo-β-mannanase, peach fruit, activity, gene expression, softening.

## INTRODUCTION

Fruit softening is a typical character of ripening fruit particularly those in the climacteric category that directly affects the fruit quality and shelf life, causing great economic loss. Because of the complexity of fruit softening process, the mechanism of fruit softening is still poor understand. But up to now, the changes in cell wall structures and compositions catalyzed by the action of several cell-wall modifying enzymes and proteins are considered as the key factors for fruit softening (Choudhury *et al*., 2009).

Mannans are one of the components of cell wall hemicelluloses, which have been reported to play a role in determining tissue firmness and flexibility (Schröer *et al*., 2006; Ordaz-Ortiz *et al*., 2009; Prakash *et al*., 2012). During fruit softening process, mannans were modified by the action of a number of hydrolytic enzymes, with endo-β-mannanase (EC 3.2.1.78, β-Man) being the most important. β-Man can catalyze the hydrolysis of the internal β-1,4-mannopyranosyl linkage in the backbone of mannans (Lin *et al*., 2011). The β-Man activity has been found in many fruits (Bourgault *et al*., 2001), and β-Man has also been well studied for its roles in tomato fruit ripening.

It has been reported that β-Man involves in tomato fruit ripening and softening (Carrington *et al*., 2002). The activity of β-Man increases with the decline of fruit firmness during tomato fruit ripening. However, the activity of β-Man in non-ripening mutants at maturity was lower than that in wild-type cultivars at the same maturity stage (Lin *et al*., 2011). In addition, β-Man belonged to a large gene family encoding multiple proteins and isoforms, and different genes showed different expression patterns during plant growth and development. It has been identified that there are eight, nine and eleven β-Man genes in the genomes of *Arabidopsis*, rice and poplar, respectively (Yuan *et al*., 2007).

Peach (*Prunus persica* L. Batsch) is a typical climacteric fruit, and it is difficult to preserve fruits for a long period because of fruit softening rapidly after harvest. Different softening-related cell wall enzymes/proteins have been better characterized in peach fruit (Di Santo *et al*., 2009). A great increase in the activity of β-Man has also been observed in O'Henry peach fruits during ripening (Brummell *et al*., 2004). However, little information is available about the pattern of β-Man gene expression in peach fruits. In addition, expression patterns of β-Man activity and genes in the skin during peach fruit storage have not been investigated. Therefore, the goal of this study was to investigate the profiles of β-Man activity and gene expression in skin and mesocarp in associated with the fruit softening and ethylene production during peaches storage.

## MATERIALS AND METHODS

**Plant material and treatments:** Peach (*Prunus persica* (L.) Batsch *cv.* Yanhong) fruits were harvested at commercial maturity stage from a commercial orchard in Guiyang, Guizhou, in China and transferred to the laboratory immediately. Fruits with uniform size and free of mechanical injury and defects were selected, and then stored at room temperature. Fruits were sampled at 0, 4, 8 and 12 days after storage.

**Fruit firmness and ethylene production:** Fruit firmness was determined at four spots in the equatorial regions per fruit and five fruits were used per replicate (3 replications per sampling time) using a hand penetrometer (GY-3, Zhejiang Top Instrument Co., Ltd., Hangzhou, China).

Ethylene production was determined by incubating three fruits in a 2.5 L airtight crisper at room temperature for 2 h. A 1 mL gas sample was withdrawn from the headspace using a gastight syringe, and injected into a gas chromatograph (Agilent 7890, Agilent Technologies Co., Ltd, Santa Clara, CA, USA). After the measurement of fruit firmness and ethylene production, fruits were separated into skin and mesocarp using blade and then immediately frozen in liquid nitrogen and separately stored at -80 °C for enzyme and RNA extraction.

**β-Man activity:** Extracts for β-Man activity determination were prepared according to the method of Bourgault and Bewley (2002). 0.2 g of fruit sample was homogenized in 500 µL of 0.1 M $L^{-1}$ Hepes buffer (pH 8.0), then the homogenate was centrifuged at 13,000 g, 4 °C for 10 min, and the supernatant used to determine the activity of β-Man. β-Man activity was determined using a gel-diffusion assay (Bourgault and Bewley, 2002). The proteins were quantified by the method of Bradford (1976). β-Man activity was expressed as pKat $mg^{-1}$ protein.

**Tissue prints:** The region of β-Man present within peach fruit was determined according to the method of Ren *et al*. (2008). Freshly-cut surfaces of the fruit were put on the top of an agarose gel containing locust bean gum (Sigma-Aldrich Co., Ltd., St Louis, MO, USA) for 1 min at 25 °C. Then the fruit surfaces were removed from the gel and the gel was stained with Congo Red (Sigma-Aldrich). The clearing zones on the gel indicated where the enzyme activity was present.

**RT-PCR**: Total RNA was isolated from skin and mesocarp as described by Meisel *et al*. (2005). First-strand cDNA synthesis of all samples was generated by kit of Reverse Transcriptase M-MLV (RNase H-) using oligo-$dT_{12-18}$ as primer following the manufacturer's instruction (Takara Biotechnology Co., Ltd., Dalian, Liaoning, China). Two pairs of specific primers (*PpMAN*1-F: 5'-AATGGTTATCTGGGTCAA-3', *PpMAN*1-R: 5'-CCTTCTAATCTCCCTTGC-3'; *PpMAN*2-F: 5'-CGGCTTCAATGGCTACTGG-3', *PpMAN*2-R: 5'-CGACACCCACATCCAAGAT-3') were designed for cloning *PpMAN*1 and *PpMAN*2 cDNA based on the sequences available in NCBI GenBank. The thermal cycling conditions for *PpMAN*1 was an initial step of 94 °C for 5 min and then followed by 35 cycles of 94 °C for 30 s, 49 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The thermal cycling conditions for *PpMAN*2 was an initial step of 94 °C for 5 min, then 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C , with a final extension at 72 °C for 10 min. The PCR products were cloned using pMD18-T Vector (Takara) and transformed into the DH5α E. *Coli*. The nucleotide sequences were analyzed by AuGCT DNA-SYN Biotechnology Company in Beijing, China.

**3' RACE:** Gene specific primers (3'-RACE-*PpMAN*1: 5'-CCAAGACGGGTACGGCGTTG-3'; 3'-RACE-*PpMAN*2: 5'-CCTCAGAGGATGAGACTCAA-3') were designed in according to the sequence of the cloned DNA fragments of *PpMAN*1 and *PpMAN*2. 3' RACE was performed using the SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories, Inc., Mountain View, CA, USA). The RACE-ready cDNA was also generated from total RNA of mesocarp of peach. First-strand cDNA synthesis of all samples was generated by kit of Reverse Transcriptase M-MLV (RNase H-) following the manufacturer's instruction (Takara Biotechnology Co., Ltd., Dalian, Liaoning, China). RACE reactions were performed under the following program for *PpMAN*1: one cycle for 30 s at 94 °C and then subjected to 5 cycles of 94 °C 10 s and 72 °C 2 min; 5 cycles of 94 °C 10 s, 70 °C 2 min; 30 cycles of 94 °C 10 s, 68 °C 2 min and 58 °C 3 min. RACE reactions were performed under the following program for *PpMAN*2: one cycle for 30 s at 94 ◦C and then subjected to 5 cycles of 94 °C 10 s and 68 °C 2 min; 5 cycles of 94 °C 10 s, 66 °C 2 min; 30 cycles of 94 °C 10 s, 64 °C 2 min and 58 °C 3 min. The RACE products were also cloned and sequenced as above mentioned.

**β-Man genes expression:** Total RNA samples from skin and mesocarp of peach fruit were treated with DNase I (Ambion, Life Technologies Corporation, NY, USA) for 30 min at 37 °C to remove any minor genomic DNA contamination. Quantitative real-time PCR (qRT-PCR) of *PpMAN*1 and *PpMAN*2 was performed with real-time PCR using a CFX96 real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using 1 µL 6 µM $L^{-1}$ of each gene-specific primers (*PpMAN*1-F: 5'-TTCACCACAGTCTACTCGGC-3', *PpMAN*1-R: 5'-AGCCTGGCATACATCT TCCG-3'; *PpMAN*2 -F: 5'-GAGACTTGCTCTTCAACACT-3', *PpMAN*2-R: 5'-TGATAGAGC TTGTGAGATTG-3'), 1 µL cDNA, 7 µL sterile water and 10 µL THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd., Osaka, Japan). Cycling conditions included an initial hot start at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 15 s and 72 °C for 45 s, plate read at 80 °C and a final extension step of 5 min at 72 °C. All the reactions were repeated three times. Mean threshold cycle values normalized with the TEF2 and UBQ10 as reference genes (Tong *et al*., 2009).

**Statistical analysis:** The data were analyzed with SPSS 16.0 statistical software and presented as means ± SE of three individual experiments.

## RESULTS AND DISCUSSION

**Firmness and ethylene production:** As shown in Fig. 1, 'Yanhong' peaches were harvested at firmer stage. With the process of storage, the fruit firmness gradually declined. The large decrease in firmness occurred between 4 to 8 days after storage, firmness decreased by 51.4 %. After 12 days of storage, fruit firmness decreased by nearly 85.0 %. With the process of storage, ethylene production increased and exhibited climacteric peak at 8 days of storage, which consistent with the fast-softening stage. These results are agree with the previous report on peach fruits (Tonutti *et al*., 1997; Trainotti *et al*., 2006; Murayamaa *et al*., 2009).

**Tissue print:** As shown in Fig. 2, the peach slices exhibited higher β-Man activity, which was localized in both skin and mesocarp at the beginning of storage. With the progress of storage, the β-Man activities in both tissues gradually increased. This indicated that both skin and mesocarp are the major sites of hydrolase production during peach storage.

**Expression pattern of β-Man activity:** As shown in Fig. 3, β-Man activity in peach mesocarp firstly increased then decreased, and the highest value of β-Man activity appeared at day 8 of storage. A similar trend of β-Man activity in the skin was also observed during storage. In addition, β-Man activity in skin tissue was significantly higher than that in mesocarp during storage, and the average β-Man activity in skin is nearly 2.1 fold higher than that in mesocarp. Bewley *et al*. (2000) explained that a high β-Man activity in the skin is due to more cell wall material present in the skin than that in the mesocarp. Corresponding to the rise in β-Man activity in skin and mesocarp, firmness of peach fruit declined (Fig. 1), which showed a temporal correlation between fruit softening and β-Man activity during peach storage. A similar trend of β-Man activity was also reported in ripening tomato (Bewley *et al*., 2000) and pawpaw fruits (Koslanund *et al*., 2005). However, in O'Henry peaches, β-Man activity shows a continuous increasing trend during ripening stage (Brummell *et al*., 2004). The discrepancies might result from differences in the cultivars.

**Expression pattern of *PpMAN*1 and *PpMAN*2:** By aligning and assembling the RT-PCR products and 3' RACE products, the length of 593 bp and 1440 bp cDNA fragments were obtained with 99.8 % and 99.6 % identity to the published sequences *PpMAN*1 (GI:157313307) and *PpMAN*2 (GI:157313310) at the nucleotide level indicated that they are the partial cDNA fragments of *PpMAN*1 and *PpMAN*2.

The patterns of *PpMAN*1 and *PpMAN*2 transcript level in peach tissues were analyzed by using qRT-PCR. As shown in Fig. 4A and Fig. 4B, the accumulation of both *PpMAN*1 and *PpMAN*2 transcripts in peach fruit were shown to be highly expressed in skin and mesocarp at the beginning of storage. Thereafter, although *PpMAN*1 transcript fluctuated to some extent at 8 days of storage, the expression levels of *PpMAN*1 and *PpMAN*2 in skin and mesocarp continued to decline until the end of storage. Compared with level of *PpMAN*1 transcript, the level of *PpMAN*2 transcript in both tissues decreased more rapidly (Fig. 4B). The similar accumulation pattern of *PpMAN*2 mRNA was also found in 'Chiripá' peach fruits during cold storage (Pegoraro *et al*., 2010). The expression patterns of *PpMAN*1 and *PpMAN*2 in both tissues were positively correlated with the decrease of fruit firmness (Fig. 1), but negatively correlated with the increase of β-Man activity (Fig. 3).

The inconsistent relationships among gene expression, activity of β-Man enzymes and fruit firmness observed here was also found in the other ripening-related cell wall enzymes. For example, Mwaniki *et al*. (2005) reported that the β-Gal activity exhibited increase trend with the decrease of 'La France' pear fruit firmness, however, the abundance of *PpGAL*6 and *PpGAL*7 transcripts decreased gradually. In 'Fuji' apple fruit, the change of PG activity was also not consistent with the pattern of gene expression during fruit storage (Wei *et al*., 2010). In sapodilla fruit, *MzEG* was expressed in unripe but mature fruit, however, no expression was observed during fruit softening after harvest (Kunyamee *et al*., 2010).

As we known, β-Man is a multigene family and different β-Man gene shows different expression pattern with the fruit ripening and softening. In tomato and banana fruits, *LeMAN*4 and *MaMAN* showed increased expression during fruit ripening and softening (Carrington *et al*., 2002; Zhuang *et al*., 2006). It was inferred that the increased expression of a β-Man gene was the cause of at least part of the loss of tomato and banana fruit firmness. In this study, the absence of a relationship between the expressions of *PpMAN*1 and *PpMAN*2 and β-Man activity in peach fruits might indicate that the expressions of the other different β-Man genes are responsible for the increase in β-Man activity.

It has been found that β-Man has a duel role: mannan hydrolases and mannan transglycosylase. Each of the different β-Man could potentially have endotransglicosylase, hydrolase or both activities (Schröer *et al*., 2006). Like β-Man, XTHs are bifunctional enzymes-xyloglucan endotrans glycosylase/ hydrolase which could modified xyloglucans, one of main hemicelluloses component (Marcus *et al*., 2010). A similar expression pattern has also been found in the

XTH genes from tomato (Miedes and Lorences, 2009) and apple fruits (Atkinson *et al*., 2009). From the results in the study, it could be speculated that *PpMAN*1 and *PpMAN*2 might have a strong preference for transglycosylase, which could act to incorporate the newly synthesized low molecular weight GGM into the cell wall. Since the expressions of *PpMAN*1 and *PpMAN*2 decreased with the fruit softening, the potential rearrangement and remodeling roles of cell wall were also effected by the β-Man and the softening process was fastened.

It has been reported that the expression of some cell wall-associated genes, including PG (Hiwasa *et al*., 2003), expansin (Rose *et al*., 1997), EGase (Lashbrook *et al*., 1994) and Gal (Mwaniki *et al*., 2005; Nishiyama *et al*., 2007) could be regulated by ethylene. However, no information is available about how β-Man is regulated by ethylene. The results presented here (Fig. 1 and Fig.4) indicated that the expressions of *PpMAN*1 and *PpMAN*2 genes might be down-regulated by ethylene during peach storage, as the decrease of *PpMAN*1 and *PpMAN*2 transcripts was negatively correlated with the increase in ethylene production. This ethylene-regulated expression was also found in the other ripening-related cell wall enzymes, like *PpGAL*6 and *PpGAL*7 in 'La France' pear fruits (Mwaniki *et al*., 2005) and *FcXTH*2 in the *F. chiloensis* fruits (Opazo *et al*., 2013). This research has laid a foundation for further study on the contribution of other β-Man genes to fruit softening and the mechanisms by which their expression and hormone regulation are coregulated as part of the ripening process.

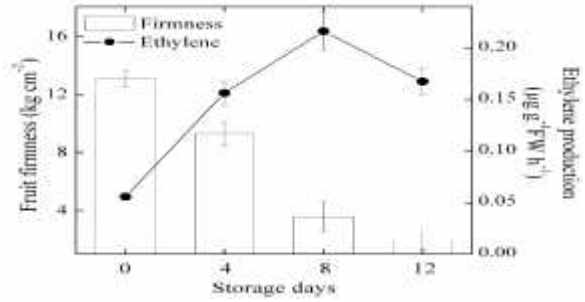


**Fig.1. Time-course of firmness and ethylene production of 'Yanhong' peach fruit during storage at room temperature.**

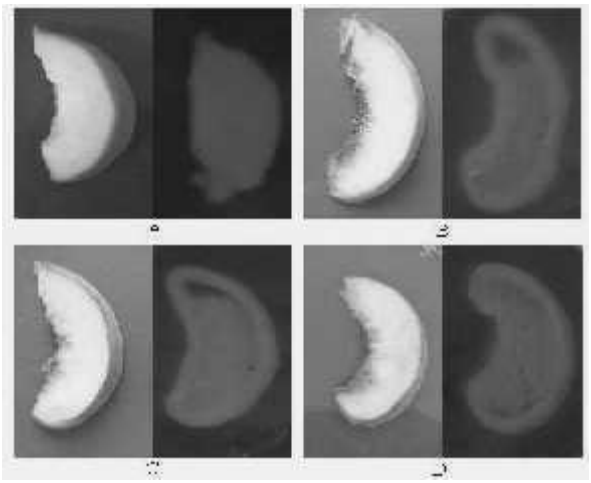

**Fig.2. The region of β-Man activity present in 'Yanhong' peach fruits determined by tissue prints. A, B, C and D denoted respectively 0, 4, 8 and 12 days after storage. Lighter areas indicate high enzyme activity**

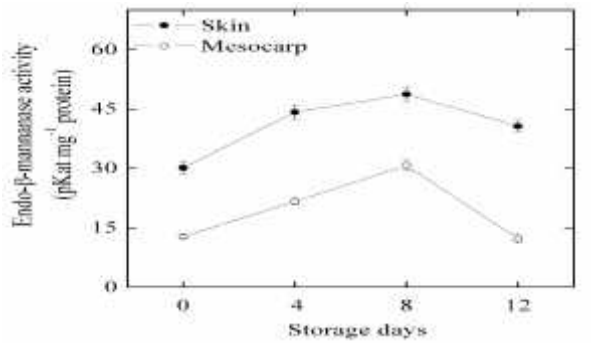


**Fig.3. Changes of endo-β-mannanase activities in the skin and mesocarp of 'Yanhong' peach fruits during storage at room temperature**

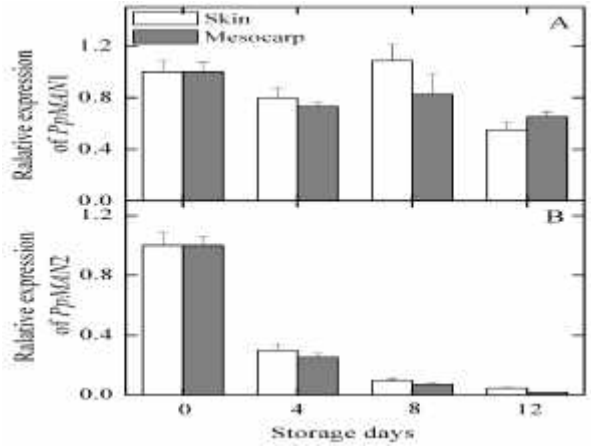


**Fig. 4. Patterns of *PpMAN*1 (A) and *PpMAN*2 (B) gene expression in skin and mesocarp of peach fruit measured by quantitative real-time PCR**

**Conclusions:** The present study indicated that β-Man is present in the skin and mesocarp of peach fruits during storage. The increase in β-Man activity is positively correlated with the increase of fruit softening and ethylene production, illustrating that β-Man likely participates in peaches softening process. However, there are no consistent relationships between β-Man activity and genes expression. The accumulation of *PpMAN*1 and *PpMAN*2 transcripts decreased with the progress of fruits softening and ethylene release. Since β-Man belongs to a multi-genic family and could have a dual role, the discrepancies between β-Man activity and gene expression maybe reflect the presence of the other β-Man gene contributing to its hydrolytic activity measured. The β-Man encoded by *PpMAN*1 and *PpMAN*2 maybe act as a mannan transglycosylase, which is helpful to keeping the cell wall intact. Therefore, it is necessary to further study the contribution of different β-Man genes to fruit softening.

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