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Genome-wide identification and expression analysis of beta-galactosidase family members during fruit softening of peach [*Prunus persica* (L.) Batsch]

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ABSTRACT

Plant β-galactosidase (β-Gal) is an important glycosyl hydrolase, which is associated with cell wall biogenesis and modification during fruit softening. However, the roles of *β-Gal* family members in peach fruit softening remain unclear. In the present study, we identified 17 *PpBGAL* genes in peach genome, and these were investigated using bioinformatics including chromosomal locations, phylogenetic relationships, gene structure and domain and promoter analyses. Furthermore, quantitative real-time PCR analysis during fruit storage of four peach varieties with different softening characteristics suggested that *PpBGAL* genes, especially *PpBGAL2* and *PpBGAL16*, may be required for peach fruit softening. These results will be useful for further functional analyses of the *β-Gal* gene family in plants.

1. Introduction

Peach [*Prunus persica* (L.) Batsch] is a typical climacteric fruit, and its short shelf life and rapid softening after harvest unfavorably affect its market value (Karabulut et al., 2002; Yoshioka et al., 2010). Therefore, understanding the physiological and molecular processes regulating peach softening will be very beneficial in extending the fruit shelf life and increasing its market value.

Modification of cell walls is considered to be the foundation of changes in fruit firmness and texture (Brummell et al., 2004). Generally, the plant cell wall is composed of pectin, cellulose, hemicellulose and structural proteins (Carpita and Gibeaut, 1993; Yin et al., 2010). Several enzymes including β-galactosidase [β-Gal (EC 3.2.1.23)], α-L-arabinofuranosidase and polygalacturonase that metabolize cell walls contribute to modifying of cell wall structures, and may result in fruit softening (Fischer and Bennett, 1991; Goulao and Oliveira, 2007; Jongpil and Huber, 2000; Smith et al., 2002). Polygalacturonase has been suggested to play central roles in pectin degradation (Atkinson et al., 2002; Hadfield and Bennett, 1998). However, it has been reported that polygalacturonase is not the sole determinant of fruit softening (Giovannoni et al., 1989; Smith et al., 1998; Tateishi, 2008; Tateishi et al., 2001a). β-Gal may be important because it is correlated with cell wall modification and biogenesis (Liu et al., 2013), and characterized by the ability to hydrolyze terminal, non-reducing β-D-galactosyl residues from numerous substrates including pectin and hemicellulose (Smith and Gross, 2000; Tateishi, 2008). Furthermore, β-Gal is reportedly involved in softening in different fruit such as Japanese pear (β-Gal III) and tomato (β-Gal II) (Carrington and Pressey, 1996; Kitagawa et al., 1995; Pressey, 1983), apple (Ross et al., 1994) and avocado (Tateishi et al., 2001b). In addition, cDNAs encoding several β-Gal isoforms have been isolated from different plants (Smith et al., 1998; Tateishi et al., 2001a). For example, in tomato, silencing of the *TBG4* gene resulted in decreased fruit softening (Smith et al., 2002). In strawberry, antisense down-regulation of *Fa0205Gal4* reduced fruit firmness during ripening (Paniagua et al., 2016). Thus, β-Gal could greatly contribute to modification of fruit cell walls and play roles in fruit softening (Smith et al., 2002).

All plant β-Gals belong to the glycosyl hydrolase 35 family (GH35) (Chandrasekar and Ra, 2016), which catalyze the hydrolysis of terminal β-galactosyl residues from galactolipids, carbohydrates, and glycoproteins (Ahn et al., 2007). In higher plants, they are believed to play central roles in modification of cell wall ingredients during fruit ripening (Carey et al., 1995; Smith and Gross, 2000; Smith et al., 1998). Additionally, many *β-Gals* possessing the active site consensus sequences G-G-P-[LIVM](2)-x(2)-Q-x-E-N-E-[FY] are thought belong to the GH 35 family members (Henrissat, 1998), such as tomato (17 members), *Arabidopsis* (17 members), Japanese pear (8 members), rice (15 members) and *Brassica campestris* ssp. *chinensis* (27 members) (Chandrasekar and Ra, 2016; Liu et al., 2013; Smith and Gross, 2000; Tanthanuch et al., 2008; Tateishi et al., 2005). *β-Gals* have also been

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identified in other species including grape (Nunan et al., 2001), strawberry (Trainotti et al., 2001), pear (Mwaniki et al., 2005) and avocado (Tateishi et al., 2007).

Despite the role of β-Gal in peach softening was confirmed in several studies (Brummell et al., 2004; Strong, 2003) the roles of *β-Gal* family members in softening of peach fruit remains unclear. To clarify which members may be involved in peach fruit softening, we identified and characterized *PpBGAL* gene members from sequences of the peach genome and analyzed the expression profiles of *PpBGAL* genes during fruit softening in four peach cultivars with distinct softening characteristics: stony-hard varieties which maintain fruit firmness and hardly synthesize ethylene during storage, melting varieties which rapidly soften and exhibit an ethylene production peak during storage (at room temperature). The results will provide a foundation for researching the evolution and biological functions of *β-Gal* gene families in plants.

2. Materials and methods

2.1. Identification and analysis of PpBGAL family members

To identify *PpBGAL* family members, peach genome sequences, coding sequences and protein sequences were downloaded from the Phytozome v11.0 database (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Ppersica), and hidden Markov Model (HMM) searches (Finn et al., 2011) were performed locally in the peach protein database (version 2.1) using the HMM profile of the GH35 domain [PF01301 in Pfam (http://pfam.xfam.org/)]. The candidate *β-Gal* family members that contained the conserved GH35 domain (Chandrasekar and Ra, 2016) were considered to be *PpBGALs*. The online program SMART (http://smart.embl-heidelberg.de/) was used to ensure the presence of the conserved domain. Conserved domain and multiple sequence alignment were performed using DNAMAN software (Lynnon Corp, Quebec, Canada). To further investigate conserved motifs, 17 PpBGAL protein sequences were aligned with the online tool MEME set to output six motifs (Bailey and Elkan, 1994). The basic physical and chemical parameters of all predicted proteins were calculated using the online ProtParam tool (http://www.expasy.org/tools/protparam.html) (Gasteiger et al., 2005), including the length of sequences, molecular weight (*Mw*) and isoelectric point (p*I*). Signal peptides were analyzed using SignalP4.0 (Petersen et al., 2010).

2.2. Analysis of genome distribution and gene structures

The *PpBGAL* chromosomal locations were obtained using the Jbrowse tool in JGI (https://phytozome.jgi.doe.gov/jbrowse/index.html) and then the locations of *PpBGAL* genes were drafted using the MapInspect tool (Song et al., 2015). Gene Structure Display Server 2.0 software (http://gsds.cbi.pku.edu.cn/index.php) was used to analyze the exon and intron statuses (Bo et al., 2014).

2.3. Analysis of promoter sequences in PpBGALs

To investigate the *cis*-elements in promoter sequences of *PpBGAL* genes, genomic DNA sequences located 1500 bp upstream of the translation start site for each *PpBGAL* were obtained from the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html). The PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to identify the *cis*-elements in the promoter regions (Lescot et al., 2002).

2.4. Sequence alignment and phylogenetic analysis

Multiple sequence alignments of PpBGAL proteins were performed using the ClustalX 2.0.12 software with its default settings (Larkin et al., 2007). Phylogenetic analysis was carried out with MEGA 6.0 using the neighbor-joining (NJ) method and a bootstrap test was set at 1000 to test confidence for the tree (Tamura et al., 2011). The tree was drawn using the Fig Tree 1.4.2 software (Rambaut, 2015)

2.5. Fruit materials

Fruit from peach [*Prunus persica* L. Batsch] cvs. 'Xia Hui 8′ (XH8), 'Hu Jing Mi Lu' (HJML) and 'Yumyeoung' (YM) from the National Peach Germplasm Repository in Nanjing, Jiangsu, China and 'Xia Cui' (XC) from Nantong, Jiangsu, China were used in this study, and all the fruits were picked during 2016. All fruit samples reached commercial maturity, had uniform maturity with no diseases, had no mechanical damage and were randomly collected. Of each accession, 300 fruit were stored in an air-conditioned room at 25 ± 0.5 °C and a relative humidity of 75%–85% for up to 16 d. For XH8, fruit samples were taken at 0, 2, 4, 6, 9 and 12 d postharvest; for XC at 0, 2, 4, 6, 8, 12 and 16 d; and for HJML and YM at 0, 4, 8 and 12 d. Thirty fruit were used to measure ethylene production and fruit flesh firmness in each cultivar every time, in three independent biological replicates. After determination of ethylene production and fruit firmness, the pulp from the same samples was collected and frozen in liquid nitrogen and stored at − 80 °C until further analysis.

2.6. Fruit firmness and ethylene production

Fruit flesh firmness was measured using the TA-XT. Plus Texture Analyser (Stable Micro Systems, Texture Technologies Scarsdale, NY, USA) equipped with a probe (8.0 mm); 10 fruit were measured and the inserted location was the middle area beside the fruit suture line. Ethylene production was measured using a gas chromatograph (Agilent 7890A, CA, USA). Six peach fruits were stored in an air tight container equipped with a rubber stopper for 2 h at 25 ± 0.5 °C; a 1-mL gas sample was withdrawn from the headspace of the containers with a syringe (Agilent), and injected into the gas chromatograph fitted with an Hp-Plot q column (20 m × 0.53 mm × 20 μm) and a flame ionization detector, with injector temperature of 220 °C, column temperature of 40 °C and detector temperature of 220 °C. Helium was used as a carrier gas. Three independent biological replicates were conducted for these measurements.

2.7. RNA isolation and expression analysis

Total RNA was extracted from fruit samples using a MiniBEST Plant RNA Extraction Kit (TaKaRa, Dalian, China). An ultraviolet spectrophotometer (Eppendorf, Hamburg, Germany) and electrophoresis in 1% agarose gels were used to test the RNA integrity and quality, respectively. cDNA was synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa). The Beacin Designer 8.13 (Premier Biosoft International, Palo Alto, CA, USA) was used to design the specific primers for each *PpBGAL* gene, the primers of *PpPG21* and *endo-PG* were obtained from Qian et al. (2016) and Hayama et al. (2006), respectively. All primer sequences are listed in Supplemental Table 1. The *translation elongation factor 2* (*TEF2*) was used as the internal reference gene based on the study of Tong et al. (2009). Quantitative real-time (qRT)-PCR was performed using a 7500 Real Time PCR System (Applied Biosystems, NY, USA) with the SYBR®Premix Ex Taq™ (TaKaRa) and gene specific primers in a total volume of 20 μL. PCR conditions were as follows: an initial denaturation at 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 34 s. The specificity of the primer amplifications was tested by analysis of a melting curve. The comparative cycle threshold method (ΔΔCt) was used to analyze the relative expression level data (Livak and Schmittgen, 2001). Each sample was analyzed in triplicate.

2.8. Statistical analysis

Microsoft Excel 2010 was used to calculate standard errors (SEs). Graphs were produced using Origin 8.0 software. Significant differences