Characteristics and regulatory pathway of the *PrupeSEP1* *SEPALLATA* gene during ripening and softening in peach fruits

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Abstract: *SEPALLATA* genes are members of a subfamily of MADS-box transcription factors, and have essential roles in floral organ development and fruit ripening. In this study, the *PrupeSEP1* gene was cloned from peach flesh. Its deduced amino acid sequence was very similar to that of *MdMADS8* and *MdMADS9* in apple and *MADS-RIN-like* in strawberry. During storage of melting flesh (MF) peach, the pattern of *SEP1* expression was similar to that of ethylene biosynthesis and ethylene signal transduction-related gene expression (*EIN2* and *ETR2*). The *SEP1* expression level was correlated with that of *EIN2* and *ETR2*. Furthermore, in MF, cell wall modification-related genes (*Endo-PG3*, *EXP2* and *PME3*), *N*-glycan processing genes (β -*Hex2* and α -*Man*) and *Lox1* exhibited similar expression patterns to that of *SEP1*. However, in non-melting flesh (NMF) peach, the *SEP1* expression pattern was different from that of MF peach. Moreover, in NMF, only *EXP2*, *LOX1*, and α -*Man* expression patterns were similar to that of *SEP1*. After *SEP1* expression was inhibited by virus-induced gene silencing technique (VIGS) in MF peach, compared with the control, the fruit remained firm and fruit softening was delayed. While expression levels of the ripening and softening related genes, *ACS2*, *EIN2*, *PME1*, *Endo-PG3*, *ACO1*, *ETR2*, β -*Hex2* and *Lox1*, were significantly decreased in *SEP1*-silenced peach, the transcription of *EXP2* and *PME3* were significantly enhanced except at the harvest stage. Yeast one-hybrid verification showed *SEP1* can interact with promoter of target genes *PGs*. Our results indicate *SEP1* may regulate fruit ripening and softening of MF peaches, while this regulation may be lost in the NMF peaches.

Keywords: *PrupeSEP1*; peach; fruit; ripening and softening; Virus-induced gene silencing (VIGS)

1. Introduction

Peaches (*Prunus persica* L. Batsch) generally soften rapidly after harvest, which may result in considerable decreases in fruit quality during postharvest handling, storage, and transportation [1]. Peach cultivars are usually classified as melting flesh (MF) or non-melting flesh (NMF) according to fruit firmness and texture characteristics. Melting flesh peaches soften rapidly and develop a melting texture, while NMF peaches lack the final melting phase of the softening process, so fruits remain relatively firm when fully ripe [2]. Ethylene release is closely related to fruit softening [3-6]. However, Haji reported that both MF and NMF mature peaches showed increased endogenous ethylene production and softer flesh, although the degrees differed [7], and Brovelli et al. also indicated that NMF trait in peaches is not related to low ethylene production rates. Therefore, there is no clear link between ethylene production and softening in NMF fruits [8].

Fruit softening is usually attributed to the degradation of the cell wall, with the depolymerization of pectins catalyzed by endo-polygalacturonase (endo-PG) having a central role [2, 4, 5, 9]. In MF peaches, the abundance of endo-PG is maintained at a relatively high level, and increases during the softening process. In NMF fruits, endo-PG levels are low during storage [10]. However, little is known about how the expression of endo-PG genes is regulated to control peach flesh texture.

SEPALLATA (*SEP*) genes form a MADS-box subfamily, and have a major role in regulating floral organ development, flowering time, and fruit development and ripening [11-13]. *SIRIN*, which is a member of the *SEP* subfamily, is an essential regulator of tomato fruit ripening and softening because the fruits failed to ripen in the *rin* mutants [14], and *SIRIN* directly influences the expression of several ripening-related genes, including those associated with ethylene biosynthesis and signaling, cell wall modification, fragrance formation, carotenoid accumulation, and ripening-related transcription factors [15-20]. Dong et al. determined that the pepper

SEP gene, *CaMADS-RIN*, could increase the transcription of cell wall metabolism genes (i.e., *PG*, *EXPI*, and *TBG4*) and lipoxygenase genes (i.e., *TomloxB* and *TomloxC*) in the tomato *rin* (ripening inhibitor) mutant [21]. In apple, the *SEP*-like *MdMADS8* and *MdMADS9* genes control processes such as starch degradation and ethylene synthesis. The *MdMADS9* gene product can activate the *ACO1* promoter as well as the transcription of *ACSI* [22]. Furthermore, the *SEP*-like *FaMADS9* and *VviSEP4* genes in the non-climacteric fruits strawberry and grape, respectively, have functions similar to that of the *RIN* gene [23, 24]. Therefore, *SEP*-type MADS genes appear to be global regulators of ripening, with conserved functions in climacteric and non-climacteric fruits [13].

Considering the essential role of *SEP-like* genes in fruits development and ripening, it is interesting to investigate the *SEP-like* genes in ripening peach fruit. Xu et al. identified three *SEP*-like genes, *PrpMADS2* (AAZ16241), *PrpMADS5* (ABO27621), *PrpMADS7* (ABO27622) in *Prunus persica*, and indicated that *PrpMADS5* and *PrpMADS7* could control flowering time and floral organ formation in *Prunus* species [25]. Tani et al. had studied again these three *SEP* genes with different name, *PPERSEP1* (ABO27622), *PPERSEP3* (ABO27621) and *PPERFBP9* (AAZ16241), and indicated that *PrpMADS7/PPERSEP1* strongly expressed in flower and fruit organs [26]. Furthermore, *PrpMADS7/PPERSEP1* clustered with ripening-related *MdMADS8*, *MdMADS9* and *FaMADS9* genes [22]. Therefore, *PrpMADS7/PPERSEP1* may be involved in peach fruit development and ripening. Based on a standard nomenclature for gene designation in the Rosaceae [27], we have renamed *PrpMADS7/PPERSEP1* gene as *PrupeSEP1*.

To discover the expression characteristics and potential functions of the peach *SEP1* gene in the ripening, we used MF and NMF fruits with differences in firmness

and texture characteristics during ripening, and analyzed the patterns of *SEP1* expression, expression of ethylene biosynthesis and signal transduction, cell wall metabolism, and other ripening-related genes. Furthermore, we silenced *SEP1* via virus-induced gene silencing (VIGS) in melting flesh fruit to evaluate *SEP1* functions in peach fruit ripening and softening.

2. Materials and methods

2.1. Plant materials

Peach (*P. persica* L. Batsch) fruits [MF cultivar: ‘Qian Jian Bai’ (QJB) and ‘Sha Hong’ (SH); NMF cultivar: ‘Ge Gu’ (GG)] were grown at the Experimental Station of the College of Horticulture at Northwest A & F University in Yangling, Shaanxi, China. Fruits were harvested when they were commercially mature (i.e., fruits with fading peel color with light green, partially red, and slightly hard flesh). At least 200 fruits without visible defects were randomly collected and stored at 25 ± 1 °C. During storage, fruit samples were taken every other day. Three biological repetitions were designed, and each repetition was carried out by mixing flesh samples from 3 fruits. All samples were frozen with liquid nitrogen and stored at -80 °C.

2.2. Flesh firmness and ethylene production

Flesh firmness of 12 randomly selected fruits was measured daily using the GY-4 penetrometer equipped with a 7.9-mm probe. After the skin was removed, fruits were measured on two places. Ethylene production was determined following the method described by Liguori et al. [28]. Briefly, 8–10 fruits were weighed and sealed in a jar for 1 h. An air sample (1 ml) from the jar was analyzed using a gas chromatograph (Trace GC Ultra, Thermo Fisher, Italy).

2.3. Gene cloning and bioinformatics analysis

Peach total RNA was isolated using cetyltrimethylammonium bromide according to the method of Xing et al. [29]. First-strand cDNA was synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara). The primers used for cloning were designed with Primer Premier 6.0 software (Premier Biosoft International) based on the *SEPI* coding sequence, which is available in the GenBank database (Supplementary Table S1). The *SEPI* fragment was amplified by a polymerase chain reaction (PCR) using the Phanta HS Super-Fidelity DNA Polymerase (Vazyme) and the GeneAmp PCR System 9700. The amplified product was cloned into the pMD18-T vector (Takara) for sequencing. Sequence alignments involving the cloned gene were completed using the blastx tool available on the National Center for Biotechnology Information website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and DNAMAN software (Lynnon Biosoft, USA). A phylogenetic tree was generated using the MEGA 5.1 software, and sequence data can now be aligned using the MUSCLE program, and statistical method of molecular evolution was MP [30]. Analyses of the promoters of ripening-related genes were completed using online tools (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

2.4. Gene expression according to quantitative reverse transcription PCR

Quantitative reverse transcription (qRT)-PCR was completed using the CFX Connect Real-Time System (Bio-Rad, USA). Gene-specific primers for *ACS2*, *ACO1*, *EIN2*, *ETR2*, *Endo-PG3*, *PME3*, *EXP2*, β -*Hex2*, *LOX1*, and α -*Man* were designed using the Beacon Designer 7.92 software (Premier Biosoft International) (Supplementary Table S1) according to published methods [31-34]. The PCR was completed in a 10 μ l volume containing 5 μ l SYBR Premix Ex Taq II (Tli RnaseH Plus) (2 $\times$) (Takara), 1 μ l 50 nM cDNA, 1 μ l of each primer, and 2 μ l sterilized

distilled water. The PCR program was as follows: 95 °C for 1 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s. The mixed sample was heated to 95 °C for 10 s and cooled to 65 °C for 15 s. The sample was then heated up to 95 °C at a rate of 0.1 °C/s for melting curve analyses [32]. Peach 18S ribosomal RNA (18S rRNA) was used as a reference. Transcript accumulation was calculated using the $2^{-\Delta\Delta C_t}$ method [35]. Each sample was analyzed in triplicate.

2.5. Virus-induced gene silencing (VIGS)

For the construction of VIGS vector (Tobacco rattle virus TRV2), a 295-bp cDNA fragment of the *SEP1* gene (ppa010548m) was amplified using primers that introduced *EcoR* I and *BamH* I restriction sites (Table 3). The PCR product was first cloned into T-vector pMD19-T (TaKaRa), and then this recombinant vector was digested with *EcoR* I and *BamH* I, and the fragment was ligated into the virus vector pTRV2 cut with *EcoR* I and *BamH* I.

pTRV1, pTRV2 (empty plasmid as control), or the recombinant plasmid pTRV2-SEP1 were transformed into *Agrobacterium* strain GV3101 as described by Jia et al. [36]. *Agrobacterium tumefaciens* GV3101 containing pTRV1 and pTRV2 or pTRV2-SEP1 were injected into QJB fruits according to the protocols reported by Jia et al. [36]. For virus injection, 400 intact fruits were selected randomly, and evenly divided into two groups. One group of fruits were injected with pTRV1 and pTRV2-SEP1 for gene silencing, while the other group were injected with pTRV1 and pTRV2 as the control. Fruits were selected for infiltration at the end of the second exponential growth phase, and one week after infiltration, the infiltrated peach fruits were hand-picked and stored at 25 °C. For *SEP*-silenced and control fruits, the ethylene production was determined with whole fruits. The positions on fruits for firmness measurement and for sampling with gene expression analysis were shown in Supplementary Figure S1. Three biological repetitions were designed, and each repetition was carried out by mixing flesh samples from 5 fruits.

2.6 Y1H Assay

Y1H assays were performed using the Matchmaker Gold Yeast One-Hybrid System Kit (Clontech) according to the manufacturer's protocols. The coding sequence of *SEP1* was amplified with the primer pairs of 5'-GGAGGCCAGTG AATTCATGGGGAGGGGAAGAGTGGAAC -3' (F) and 5'-CGAGCTCGATGGA TCCTCACAGCATCCATCCAGG -3' (R). The PCR product was ligated to pGADT7 to generate the construct pGADT7-SEP1 (AD- SEP1).

Genomic DNA from peach cv. SH was isolated using a DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. The promoter of *PG2* was amplified with the primer pairs of *p PG2* (F) (5'-GAAAAGCTTGAATTCGAGCTCAAATAGGAGTG GAAACAGGAT -3') and *p PG2* (R) (5'- AGCACATGCCTCGAGGTCGACCGTG ATTGCTTCCATGTTGAA -3'). Due to far distance between two CArG-boxes, the promoter *PG3* were divided two segments of *PG3-1* and *PG3-2*, and they were amplified respectively with primer pairs: *p PG3-1* (F) (5'- GAAAAGCTTGAATTCGAGCT CTTCCAGCACTTCCTATTACC -3') and *p PG3-1* (R) (5'- AGCACATGCCTCGAG GTCGACGTGTGGCTATGGCTATGG -3'); *p PG3-2* (F) (5'- GAAAAGCTTGAATT CGAGCTCCGTGTTATCCTAGTCCAAGT -3') and *p PG3-2* (R) (5'- AGCACATGC CTCGAGGTCGACCGCATCCGATTA ACTCAATT -3'). The fragments of the promoter of *PG2*, *PG3-1* and *PG3-2* were ligated to pAbAi vector to generate pAbAi-bait plasmids. The bait plasmids were linearized and transformed into yeast strain Y1HGold, which were cultured on a selective synthetic dextrose medium lacking uracil plate. The constructs of AD-SEP1 were transformed into strain Y1HGold holding pAbAi-bait and screened on an SD/-Leu/AbA plate. All transformations and screenings were performed three times.

2.7. Statistical analysis

Microsoft Excel 2010 and DPS v7.05 was used for data processing and correlation analysis, and Origin 7.0 was used to prepare figures.

3. Results

3.1. Cloning and phylogenetic analysis of *PrupeSEP1*

We isolated *SEP1* from SH fruit (MF variety) using RT-PCR with specific primers designed based on sequence of NCBI. Sequencing results revealed that the cDNA sequence of *PrupeSEP1* was consistent with the corresponding *PrpMADS7* sequence in Genbank [25]. The *N*-motif is a conserved region in the MADS-box family (i.e., MADS domain), and the *C*-motif is characteristic of *SEP1* genes [26]. Phylogenetic analysis of SEP proteins in selected fruit species was carried out. The results showed that *PrupeSEP1* was highly similar to genes encoding SEPALLATA-like MADS-box proteins from apple (*MdMADS8*, CAA04919; *MdMADS9*, CAA04920), pear (*SEP1-like*, XP-009333662), grape (*VvMADS2*, AAM21342), papaya (*CpMADS1*, ACD39982), and strawberry (*Fragaria × ananassa MADS-RIN-like*, AAO49380; *Fragaria vesca SEP1*, XP_004304204) (Fig. 1). According to blastx and DNAMAN analyses, the amino acid sequence of *PrupeSEP1* was more than 93.00% similar to that of apple *MdMADS8* and *MdMADS9* and strawberry *MADS-RIN-like* and *SEP1*. It was also 87.09% similar to *Arabidopsis thaliana* *SEP1* and *SEP2*, and 59.18% similar to tomato *MADS-RIN*.

3. 2. Changes to fruit firmness, ethylene production and *PrupeSEP1* expression during storage

Fruit firmness of the SH MF decreased significantly during the whole storage. The firmness of GG fruit (NMF variety) also dropped significantly in the first 4 days of storage, and then decreased slowly, eventually dropping to 67.3% of the fruit firmness at harvest (Fig. 2a).

During storage, the rate of endogenous ethylene release increased significantly in SH fruits, peaking on day 4, and then decreased rapidly. In contrast, in GG fruits,

ethylene production increased slowly during the early storage period, and then decreased slowly until reaching a stable level by the end of the storage period (Fig. 2b).

Quantitative RT-PCR results indicated that in SH fruits, *SEP1* expression levels increased obviously during the first 4 days of storage, and then rapidly decreased and maintained stable. However, in GG fruits, *SEP1* expression levels decreased during the first 4 days of storage, and also remained relatively stable (Fig. 2c).

3.3. Relative expression of ripening- and softening-related genes during storage

The expression of ethylene biosynthesis genes (i.e., *ACS2* and *ACO1*) and signal transduction-related genes (i.e., *EIN2* and *ETR2*) were analyzed. During the storage of SH peach, the expression patterns of *ACO1*, *EIN2*, and *ETR2* were similar to that of *SEP1*. The highest expression levels were observed on day 4. In contrast, the expression levels of *ACS2* were highest on days 4 and 8. In GG fruits, *ACS2* expression initially decreased from the relatively high levels at harvest. This was followed by an increase in expression on day 4, and then a rapid decrease. The expression levels of *ACO1*, *EIN2*, and *ETR2* initially increased from the low levels at harvest, and then tended to decrease until the end of the storage period (Fig. 3).

In SH, the expression patterns of cell wall modification-related genes (i.e., *Endo-PG3*, *PME*, *EXP2*) and *Lox1* were similar to that of *SEP1*. Their expression levels increased during the first 4 days of storage, and then decreased rapidly. In GG, the pattern of expression for *EXP2* was similar to that of *SEP1*, with a rapid decrease in expression during the early stages of storage. In contrast, *PME3* expression levels initially increased, peaking on day 4 and 8. *Endo-PG3*, *PME3*, and *Lox1* had relative low expression levels in GG, especially *Endo-PG3* and *Lox1*, whose transcripts were almost undetectable throughout the storage period (Fig. 3).

Our qRT-PCR results also revealed that the expression patterns of the *N*-glycan processing genes (i.e., *Hex2* and α -*Man*) were highly similar to that of *SEP1* in SH. In GG, *Hex2* expression increased during the initial storage period, and then decreased starting on day 2. The expression levels of α -*Man* exhibited a decreasing trend, and stabilized at a low level for the duration of the storage period (Fig. 3).

3.4. Correlation between *PrupeSEP1* and the ripening- and softening-related genes during storage

Because the expression patterns of ripening- and softening-related genes were similar to that of *SEP1* during storage, we assessed the correlations between them. Correlation analysis results indicated that in SH MF, the expression of *SEP1* was positively correlated with the expression of the other ripening- and softening-related genes ($P < 0.05$), including *EIN2*, *ETR2*, *Endo-PG3*, *PME3*, *Lox1*, β -*Hex2*, and α -*Man*. Genes lacking a positive correlation were *EXP2*, *ACS2*, and *ACO1*. In contrast, in GG, *SEP1* expression was positively correlated only with *EXP2* expression, and negatively correlated with *PME3* expression ($P < 0.01$) (Table 1).

3.5. Virus-induced *PrupeSEP1* gene silencing (VIGS) in MF fruit led to delay of ripening and softening

At the 7th day after agrobacterium infection, QJB fruits (NMF variety) were harvested at commercially mature. The surfaces of control fruits infiltrated with empty *TRV2* vector turned red normally (Fig. 4a), while the infected portions on the surfaces of *TRV2-SEP1* infiltrated fruits remained light green (Fig. 4b). Real-time PCR analyses showed that the mRNA level of the *SEP1* gene was reduced in RNAi fruits compared with that in control fruits during storage (Fig. 5c).

During the first 6 days of storage, the firmness of control fruits decreased significantly (Fig. 5a) and the rate of endogenous ethylene release increased

significantly, peaking on day 6, and then decreased rapidly (Fig. 5b). The firmness of *SEP1* silenced fruits was higher than that of the control fruits in the first 4 DAH (Fig. 5a), and endogenous ethylene release of gene silenced fruits was significantly lower than that of control fruits on 2nd, 4th and 10th DAH during storage (Fig. 5b).

To elucidate the mechanism of *SEP1*-silencing on delay of peach fruits ripening and softening, the expression of several ripening and softening related genes were monitored in control and *TRV2-SEP1* infiltrated fruits by real-time quantitative PCR. In control fruits infiltrated with empty *TRV2* vector, the expression patterns of *ACS2*, *ACO1*, *EIN2*, *Endo-PG3*, *PME1*, *Lox1* and *Hex2* were similar to that of *SEP1*. However, the expression of *ETR2*, *EXP2* and *PME3* initially decreased from the high levels at harvest, and increased from the 2nd DAH to 6th DAH, and then tended to decreased (Fig. 6). In *TRV2-SEP1* infiltrated fruits, the relative expression of *ACS2*, *EIN2* and *endo-PG3* were lower than in the control fruits during storage except at the day of harvest (Fig. 6). The expression levels of *PME1* and *Lox1* in *SEP*-silenced fruits were also decreased in the first 6 or 8 DAH (Fig. 6). The transcripts abundance of *ACO1*, *ETR2* and β -*hex2* in *SEP*-silenced fruits were significant lower than that of control fruits on 6th DAH ($P<0.01$) and higher than that of control fruits ($P<0.05$) on 2nd or 4th DAH (Fig. 6). However, the relative expression levels of *EXP2* and *PME3* in *TRV2-SEP1* infiltrated fruits were higher than in the control fruits during storage except at the day of harvest (Fig. 6).

3.6. CArG-boxes in the promoters of ripening-induced genes

To detect potential *SEP1*-binding sequences, the promoters (i.e., 3 kb upstream of the transcription start site) of the ripening-induced genes were examined for a possible CArG-box motif [C(C/T)(A/T)₆(A/G)G]. The motif includes three groups of CArG-box sequences: SRF-like [canonical CArG-box, CC(A/T)₆GG], MEF2-like [CTA(A/T)₄TAG], and intermediate [CC(A/T)₆AG] (Fujisawa et al., 2011). A motif

search revealed that all of the examined genes had at least one typical CArG-box sequence in their promoters (Table 2, Fig. S2). Some genes (i.e., *EIN2*, *PME3*, *EXP2*, *Lox1* and β -*Hex2*) enriched with 5 or more typical motifs, and even appeared the clusters of CArG-boxes. CarG-box motifs focused on promoter within 2 kb except *PME3* and β -*Hex2*. And it first appeared within 500bp upstream of the most of those genes transcription start site (i.e., *ACS2*, and *ACO1*, *EIN2*, *ETR2*, *EXP2*, *Lox1*) (Table 2, Fig.S2).

3.7. Interaction of *PrupeSEP1* gene with promoters of *PG2* and *PG3*

A yeast one-hybrid (Y1H) assay was performed to test the association of *SEP1* with promoters of *PG2* and *PG3*. We cloned coding sequence of *SEP1* from MF cultivars SH and QJB, and isolated the promoter sequences of *PG2* (ppa006839m, 22647220-22650220), *PG3-1* and *PG3-2* (ppa006857m, 22679500-22684600). We tested the binding of SEP1 to the promoters of PG2, pPG3-1 and pPG3-2, respectively. The results showed that SEP1 can interact with CArG-box in pPG2 and pPG3-1, whereas SEP1 does not interact with pPG3-2 (Figure 7a, b).

4. Discussion

4.1. *PrupeSEP1* involved in regulation of ripening and softening in melting flesh peach

The *SEPALLATA* subfamily genes is involved in the regulation of floral organ development and flowering time, and also has important roles during the regulation of fruit development and ripening [11-13]. Members of the *SEP* subfamily that regulate fruit ripening have been identified in many plants, such as tomato [14], pepper [21], apple [22], strawberry [23], and grape [24]. Ireland et al. reported that *MdMADS8*- and *MdMADS9*-suppressed apples did not ripen properly in terms of developmentally controlled ripening processes, such as starch degradation, and ethylene-modulated

ripening traits [22]. Silencing of the strawberry *FaMADS9* gene led to abnormal development and ripening of petals, achenes, and receptacle tissues [23]. Mellway and Lund observed that *VviSEP4* can partially complement the non-ripening phenotype of the tomato *rin* mutant [24]. In this study, a phylogenetic analysis confirmed that there was a close genetic relationship among *PrupeSEP1*, *MdMADS8*, *MdMADS9*, *VvMADS2*, and *FaMADS9* (Fig. 1), which suggests that the function of *PrupeSEP1* may be similar to that of *MdMADS8*, *MdMADS9*, and *FaMADS9* during fruit ripening and softening. Furthermore, Xu et al. and Tani et al. reported that there are three *SEP*-like MADS genes in peach fruits, but only *PrupeSEP1* (*PPERSEP1*) exhibits elevated expression in the mesocarp [25, 26]. However, according to our best knowledge, no more information regarding the possible role of *PrupeSEP1* in peach fruit ripening and softening was reported so far.

Our results indicated that in MF SH peach, the expression patterns of *ACO1*, *EIN2* and *ETR2*, were similar and to that of *SEP1* expression (Fig. 2c, 3), and the expression level of *SEP1* was significantly correlated ($P < 0.05$) with that of *EIN2* and *ETR2* (Table1). In QJB control fruits infiltrated with empty *TRV2* vector, the expression patterns of *ACS2*, *ACO1* and *EIN2* were also similar to that of *SEP1* (Fig. 5c, 6), and a significant correlation was found between the expression of *ACS2* and *SEP1*. Furthermore, the expression levels of *ACS2* and *EIN2* were obviously inhibited in *TRV2-SEP1* infiltrated MF fruits during storage (Fig. 6). Recent studies reported that tomato *RIN* participates in ethylene production by inducing the expression of ethylene synthesis- or signaling-related genes [37, 16, 17, 15, 19], and apple *MdMADS9* gene activates *ACS1* transcription [22]. These results suggest *PrupeSEP1*

probably regulates the ripening and softening of MF peach through the ethylene synthesis and signal transduction pathway. Additionally, although both SH and QJB are MF type fruits, there are some difference in softening properties and expression characteristics of genes related to ethylene synthesis or signaling (Fig. 3, 6). For example, the SH had quite a different *ACS2* expression from QJB, which suggests that peach softening is a complex process, there may be different ACS gene family members involved in softening in different varieties.

Cell wall modifications are believed to contribute to fruit softening. This is supported by the fact some cell wall modification-related genes undergo expression level changes during fruit ripening [38, 39]. *N*-glycan processing also plays an important role in peach fruit ripening and softening [33]. Our findings revealed that in MF SH fruits, the expression patterns of *Endo-PG3*, *PME3*, *EXP2*, β -*Hex2*, α -*Man* and *Lox1* were similar to that of *SEP1* (Fig. 2c, 3). There were significant correlations among the expression levels of these genes and *SEP* except for *EXP2* ($P < 0.01$ or $P < 0.05$) (Table1). In MF QJB control fruits infiltrated with empty *TRV2* vector, the expression patterns of *Endo-PG3*, *PME1*, *Lox1* and *Hex2* were also similar to that of *SEP1* (Fig. 5c, 6). At the same time, after *SEP1*-silencing in QJB fruits, the expression levels of *PME1*, *Endo-PG3* and *Lox1* were markedly suppressed, while *PME3* and *EXP2* were promoted during storage except for the day of harvest (Fig. 6). In tomato, the SIRIN proteins can directly or indirectly regulate the transcription of ripening-induced genes (*PG*, *TBG4*, *MAN4*, *EXPI* and β -*Hex*) [16, 20]. Overexpressing *SEP* gene of pepper, CaMADS-RIN, in ripening inhibitor (*rin*) mutant tomato plants, ripening phenotype was showed and the transcripts of cell wall metabolism genes (*PG*, *TBG4* and *EXPI*) and lipoxygenase genes (*loxB* and *loxC*) in fruits were up-regulated [21]. Silencing of the *FaMADS9* gene in strawberry inhibits

the expression of genes associated with ripening [23]. These results indicate that in MF peach fruit, *PrupeSEP1* may regulate fruit ripening and softening by directly or indirectly influencing processes such as cell wall metabolism, *N*-glycan metabolism and flavor volatiles metabolism. As for the expression of *PME3* and *EXP2* were promoted in *SEP1*-silencing fruits from day 2 to day 10 of storage, which suggesting that *PME3* and *EXP2* may be affected by other developmental factors in addition to *SEP1*.

The SEP-like protein can regulate downstream gene transcription by binding to the CArG-box sequences in promoters [16, 40, 41, 42, 43]. Therefore, we analyzed the promoter regions of the genes included in this study, and determined that some genes (i.e., *EIN2*, *PME3*, *EXP2*, *Lox1* and β -*Hex2*) enriched with 5 or more typical motifs, and even appeared the clusters of CArG-boxes. The binding sites can lie up to 4kb upstream of the transcription start sites (TSSs), and the occurrence frequency of binding sites is higher in the vicinity of the TSS (-500 to +500) [16, 43]. Our results showed that CarG-box motifs first appeared within 500bp upstream of the most of those genes transcription start site (i.e., *ACS2*, and *ACO1*, *EIN2*, *ETR2*, *EXP2*, *Lox1*) (Table 2). Furthermore, our study demonstrated the direct interaction of SEP1 with promoter sequences of *PG2* and *PG3* (Fig. 7). These results indicated that in MF peach fruit, transcription factor SEP1 may regulate the expression of ripening- and softening- related genes containing CarG-box motifs in their promoter sequences.

4.2 Possible differences occurring between melting and non-melting peach fruit during ripening and softening

Fruit softening is considered to be mainly the result of modifications to cell wall architecture, including disassembly of polysaccharide networks in the primary cell wall and middle lamella, and turgor loss [44-47]. The MF phenotype is associated with a large increase in the amount of soluble pectin and pectin depolymerization. In

contrast, NMF peaches lack the final melting phase of softening so the fruits remain relatively firm when fully ripe, with little solubilization or depolymerization of pectin [9, 39]. Endo-PG activity has a very important role in the metabolism of cell wall pectin during fruit ripening, and is needed for the development of melting flesh texture [2, 10]. As expected, we observed that *endo-PG* gene expression was almost undetectable in NMF GG fruits (Fig. 3). At the same time, *PME3* also had relative low expression levels in NMF GG (Fig. 3), which agrees with the report that PME activity in NMF peaches was generally lower than that of the MF peaches after storage [48]. However, *EXP2* expression showed a higher level in GG fruit at the harvest day (Fig. 3), and expression levels of *EXP2* were significantly enhanced in *SEPI*-silenced MF peach fruit (Fig. 6). Expansin (EXP) contributes to cell wall disassembly through a non-enzymatic mechanism by disrupting non-covalent linkages at the cellulose/ hemicelluloses interface [49]. Hayama et al indicated that *EXP2* is more likely to play a role in the expansion process in ripe fruit, and not related to the loss of firmness [50]. Therefore, *EXP2* may be involved in NMF fruit ripening process.

Although ethylene plays an important role in the softening of peach fruits, the NMF trait in peaches is not related to ethylene production [8]. In our study, the ethylene climateric peak was reached faster in NMF than MF fruits, while the peak of ethylene production is higher in MF SH than that of NMF GG, which is in agreement with the results of Haji [7]. However, Kao et al. and Onelli et al. reported that the NMF cultivars generally had higher ethylene production at harvest and throughout ripening than the MF cultivars [48, 51]. Therefore, the difference in ripening and softening between NMF and MF may be independent to ethylene production. In addition to, ACS is usually thought to be the rate limiting step in ethylene production, while our results also showed that the expression of *ACS2* cannot match ethylene evolution sometimes. This may be due to other ACS family members are also involved in the ethylene production during fruit ripening.

A SEP-like protein was proposed to function early in the transcriptional activation cascade regulating ripening related processes [52]. Our results showed that similar to the SIRIN in tomato, PrupeSEP1 may regulate the expression of most ripening- and softening- related genes in MF peach fruit. However, *PrupeSEP1* expression patterns were different between MF and NMF peaches cultivars (Fig. 2c). Furthermore, in NMF fruits, only *EXP2*, *LOX1*, and *α-Man* expression patterns were similar to that of *SEP1*, with a positive correlation ($P < 0.05$) only between *SEP1* and *EXP2* expression levels (Table 1). Therefore, it is speculated that the mechanism of SEP1 regulating the ripening and softening in MF peach may be lost in NMF peach, but the exact mechanisms need to be clarified.

5. Conclusions

During storage of melting flesh (MF) peach, the expression pattern of *SEP1* was similar to that of most ripening and softening- related genes. However, in non-melting flesh (NMF) peach, the *SEP1* expression pattern was different with that in MF peach, and only a few genes expression patterns were similar to that of *SEP1*. After *SEP1* expression was inhibited by virus-induced gene silencing technique (VIGS) in MF peach, the fruit firmness remained firm and fruit softening was delayed. Moreover, expression levels of the ripening and softening- related genes, *ACS2*, *EIN2*, *PME1*, *Endo-PG3*, *ACO1*, *ETR2*, *β-Hex2* and *Lox1*, were significantly decreased in *SEP1*-silenced peach. Furthermore, SEP1 can direct interact with promoter of *PG2* and *PG3*. Our results show *SEP1* may regulate fruit ripening and softening of MF peaches by controlling the transcription of related genes.

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Figure legends:

Fig. 1. Phylogenetic analysis involving SEPALLATA proteins from selected fruit species. A phylogenetic tree of full-length sequences from peach (*Prunus persica*), apple (*Malus domestica*), *Arabidopsis thaliana*, *Prunus mume*, grape (*Vitis vinifera*), papaya (*Carica papaya*), strawberry (*Fragaria × ananassa* and *Fragaria vesca*), pepper (*Capsicum annuum*), persimmon (*Diospyros kaki*), citrus (*Citrus sinensis*),

pear (*Pyrus × bretschneideri*), kiwi (*Actinidia chinensis*), and tomato (*Solanum lycopersicum*) was constructed using MEGA 5.1 software.

Fig. 2. Fruit firmness (a), ethylene production (b) and Relative expression of *PrupeSEP1* (c) in ‘Sha Hong’ (SH) melting flesh (MF) and ‘Ge Gu’ (GG) non-melting flesh (NMF) during storage at 25 °C. Expression levels determined by qRT-PCR are relative to the expression in SH fruits at the start of the storage period (i.e., day 0). Each qRT-PCR was normalized using the cycle threshold value of the peach 18S rRNA gene. Bars correspond to the mean $\pm$ standard error (n = 3). The overall least significant difference ($p < 0.05$) was calculated and used to separate means.

Fig. 3. Relative expression of ripening- and softening-related genes (*ACS2*, *ACO1*, *ETR2*, *EIN2*, *Endo-PG3*, *PME3*, *EXP2*, *Lox1*, β -*Hex2* and α -*Man*) in ‘Sha Hong’ (SH) melting flesh (MF) and ‘Ge Gu’ (GG) non-melting flesh (NMF) during storage. Expression levels determined by qRT-PCR are relative to the expression in SH fruits at the start of the storage period (i.e., day 0). Each qRT-PCR was normalized using the cycle threshold value of the peach 18S rRNA gene. Bars correspond to the mean $\pm$ standard error (n = 3). The overall least significant difference ($p < 0.05$) was calculated and used to separate means.

Fig. 4. Phenotypes of the *TRV2* control (a) and *TRV2-SEP1* (b) infiltrated fruits. The black arrows indicate the injection site.

Fig.5. Fruit firmness (a), ethylene production (b) and Relative expression of *PrupeSEP1* (c) during control (*TRV2*) and RNAi (*TRV2-SEP1*) fruits storage.

Expression levels determined by qRT-PCR are relative to the expression in control (*TRV2*) fruits at the start of the storage period (i.e., day 0). Each qRT-PCR was normalized using the cycle threshold value of the peach 18S rRNA gene. Bars correspond to the mean $\pm$ standard error (n = 3). The ‘*’ indicates a significant difference (P<0.01) and the ‘**’ indicate significant difference (P<0.05) from control, using the two-tailed Student’s *t* test.

Fig.6. Relative expression of ripening and softening-related genes during control (*TRV2*) and RNAi (*TRV2-SEP1*) fruits storage. Expression levels determined by qRT-PCR are relative to the expression in control (*TRV2*) fruits at the start of the storage period (i.e., day 0). Each qRT-PCR was normalized using the cycle threshold value of the peach 18S rRNA gene. Bars correspond to the mean $\pm$ standard error (n = 3). The ‘*’ indicates a significant difference (P<0.01) and the ‘**’ indicate significant difference (P<0.05) from control, using the two-tailed Student’s *t* test.

Fig. 7 Interaction between PrupeSEP1 and promoter of *PrupePG2* (a), *PrupePG3* (b) are determined by Y1H assay. CARG-boxs are indicated by blue triangles. Numbers under dotted line indicate locations of CARGs, and numbers in brackets indicate fragments of *PG2*, *PG3* promoter used for Y1H assays. Each fragment of the *PG* promoter was ligated to pAbAi vector to generate pAbAi-bait plasmids. AD-PrupeSEP1 was transformed into the Y1H gold strain harbouring pAbAi-bait and then transformants were screened on selective synthetic dextrose medium lacking Leu/AbA. An empty pGADT7 vector (AD) was transformed as a negative control.

Table legends

Table 1. Correlations between the expression levels of *PrupeSEP1* and those of other genes during fruit ripening and softening in ‘Ge Gu’(GG) non-melting flesh and ‘Sha Hong’ (SH) melting flesh peach.

Notes: $r_{0.05} = 0.878339$; $r_{0.01} = 0.958735$; P: correlation coefficient; $|P| > r_{0.01}$: ** highly significant; $r_{0.01} > |P| > r_{0.05}$: * significant.

Table 2. CArG-box sequences in the promoters of ripening-induced genes.

- 1) CArG-box sequences are underlined.
- 2) The motif includes three groups of CArG-box sequences: SRF-like [canonical CArG-box, CC(A/T)₆GG], MEF2-like [CTA(A/T)₄TAG], and intermediate [CC(A/T)₆AG][16](Fujisawa et al., 2011).
- 3) Scaffolds indicated with numbers correspond to sequences that originated from *Prunus_persica_v1.0_genes* data. The (+) and (–) indicate that the CArG-boxes are presented in either the sense or antisense strands, respectively.

Notes: F and R correspond to forward and reverse primers, respectively.

PrupePG2 promoter 2000bp

pPG2(--1301_-490)

+1

PrupePG2

SD/-Leu 250 ng/ml AbA

PrupeSEP1

AD

-1257/-1201

-652/-638

a

PrupePG3 promoter 3500bp

pPG3-1(-3426_-2999)

PrupePG3

pPG3-1 SD/-Leu 900 ng/ml AbA

pPG3-2 SD/-Leu 650 ng/ml AbA

PrupeSEP1

AD

-3210

-633

pPG3-2(-1069_-618)

b

Figure 8

Figure 9

Table 1. Correlations between the expression levels of *PrupeSEP1* and those of other genes during fruit ripening and softening in ‘Ge Gu’(GG) non-melting flesh and ‘Sha Hong’ (SH) melting flesh peach.

<i>P</i>	SH(<i>PrupeSEP1</i>)	GG(<i>PrupeSEP1</i>)
<i>PrupeACS2</i>	0.239835	0.642472
<i>PrupeACO1</i>	0.698702	0.043483
<i>PrupeEIN2</i>	0.934065 *	-0.26008
<i>PrupeETR2</i>	0.943961*	-0.61596
<i>PrupeEndo-PG3</i>	0.908194*	-0.05919
<i>PrupePME3</i>	0.99812**	-0.97018**
<i>PrupeLox1</i>	0.91204*	0.850528
<i>PrupeEXP2</i>	0.819897	0.96553**
<i>Prupeβ-Hex2</i>	0.988131**	0.248395
<i>Prupeα-Man</i>	0.921773*	0.853992

Notes: $r_{0.05} = 0.878339$; $r_{0.01} = 0.958735$; P: correlation coefficient; $|P| > r_{0.01}$: ** highly significant; $r_{0.01} > |P| > r_{0.05}$: * significant.

Table 2. CArG-box sequences in the promoters of ripening-related genes.

Site	CArG-box and its flanking sequences (5' to 3') ¹⁾	Motif ²⁾	CArG-box position (bps) ³⁾
<i>PrupeACS2</i>	CAGACA- <u>CTAATAATAG</u> -TGTTTC	MEF2-like	ppa004774m scaffold_2 (18629231-18629240) (-)
	ATACTT- <u>CTAATATTGG</u> -TTGTTG	Possible	ppa004774m scaffold_2 (18629589-18629598) (-)
	TTTCTA- <u>CTAAAAAAGG</u> -CCTTAA	Possible	ppa004774m scaffold_2 (18630426-18630435) (-)
<i>PrupeACO1</i>	TTCAGA- <u>CCTTAAAAAG</u> -GAGTTA	Intermediate	ppa008791m scaffold_3 (16206363-16206372) (-)
	TATTAA- <u>CCTATATACG</u> -TAAAGG	Possible	ppa008791m scaffold_3 (16206363-16206372) (-)
	CTGTTT- <u>CCAAATTTGG</u> -TGTCCT	SRF-like	ppa008791m scaffold_3 (16207139-16207148) (-)
<i>PrupeEIN2</i>	ACTCCC- <u>CAAAATATTG</u> -CGAGAG	Possible	ppa000305m scaffold_6 (21796557-21796566) (-)
	CATAAT- <u>CAATATTAAG</u> -TATTCT	Possible	ppa000305m scaffold_6 (21797334-21797343) (-)
	GCTAAA- <u>CATAAAAAAG</u> -AAAGAG	Possible	ppa000305m scaffold_6 (21797424-21797433) (-)
	CTTGCT- <u>CAAATTTTGG</u> -GAAAAG	Possible	ppa000305m scaffold_6 (21798372-21798381) (-)
	CGTTTA- <u>CCTATTTATG</u> -TTCTCT	Intermediate	ppa000305m scaffold_6 (21798947-21798956) (-)
<i>PrupeETR2</i>	AATGTA- <u>CATAAAAAATG</u> -GTGGTT	Possible	ppa001786m scaffold_1 (2383476-2383485) (+)
	TCACTC- <u>CCTTTTAATG</u> -GTATTT	Intermediate	ppa001786m scaffold_1 (2383592-2383601) (+)
	AACTTC- <u>CGTTATTTTG</u> -TTGAGG	Atypical	ppa001786m scaffold_1 (2383932-2383941) (+)
	GTCTTT- <u>CTTATTTTGG</u> -GACCGA	Possible	ppa001786m scaffold_1 (2384422-2384431) (+)
	TTATTA- <u>CTATAAATGG</u> -GCAGCG	Possible	ppa001786m scaffold_1 (2385338-2385347) (+)
<i>PrupeEndo-P G3</i>	CACTCT- <u>CTATAAATTG</u> -GCACTC	Possible	ppa006857m scaffold_4 (22682016-22682025) (+)
	TCTAGA- <u>CTTTTAATTG</u> -AGTTAA	Possible	ppa006857m scaffold_4 (22683958-22683967) (+)
<i>PrupeEndo-P G2</i>	GGGTAC- <u>CTTAATATTG</u> -CGCTTT	Possible	ppa006839m scaffold_4 (22647942-22647951) (+)
	ACAAC- <u>CAATTTTGG</u> -AATAAT	Possible	ppa006839m scaffold_4 (22648963-22648972) (+)
	TACCAT- <u>CCAATAAACG</u> -AAAGAT	SRF-like	ppa006839m scaffold_4 (22649019-22649028) (+)
	GAGATT- <u>CCATTTTGG</u> -GGATCC	SRF-like	ppa006839m scaffold_4 (22649568-22649577) (+)
	GGGGAT- <u>CCAATAATAG</u> -AGATTT	Intermediate	ppa006839m scaffold_4 (22649582-22649591) (+)
<i>PrupeEXP2</i>	CAAATT- <u>CTTATAAAGG</u> -CTATAG	Possible	ppa010379m scaffold_1 (27211281-27211290) (-)
	GTAATG- <u>CCTTTATTTG</u> -TTTAGT	Intermediate	ppa010379m scaffold_1 (27211356-27211365) (-)
	TAAGCT- <u>CATTAATTAG</u> -GTTTTG	Possible	ppa010379m scaffold_1 (27212109-27212118) (-)
	AATTCT- <u>CTATTATAGG</u> -AAAAAT	Possible	ppa010379m scaffold_1 (27212199-27212208) (-)
	TTTCTA- <u>CTTATATTG</u> -GCCATT	Possible	ppa010379m scaffold_1 (27212470-27212479) (-)
	CAAGTC- <u>CTTTTAAACG</u> -GCTGTA	Atypical	ppa010379m scaffold_1 (27213299-27213308) (-)
<i>PrupePME3</i>	AAACTG- <u>CTTAATTAAG</u> -TCAAAA	Possible	ppa003578m scaffold_1 (46510197-46510206) (-)
	AATTTA- <u>CATTAATTTG</u> -GTCCAA	Possible	ppa003578m scaffold_1 (46510613-46510622) (-)
	CGAGCA- <u>CATATATATG</u> -TACATA	Possible	ppa003578m scaffold_1 (46511069-46511078) (-)
	CGACCA- <u>CAAATAAAAG</u> -AATATT	Possible	ppa003578m scaffold_1 (46511625-46511634) (-)
	TCGTGA- <u>CAAATATATG</u> -ATCTAA	Possible	ppa003578m scaffold_1 (46511961-46511970) (-)
	CACTTT- <u>CTATATATAG</u> -ATACGA	MEF2-like	ppa003578m scaffold_1 (46512351-46512360) (-)
	CTTCTC- <u>CATTAATTAG</u> -AGCAA	Possible	ppa003578m scaffold_1 (46512411-46512420) (-)
<i>PrupeLox1</i>	CAATAC- <u>CCAAATTAGG</u> -AAGCTT	SRF-like	ppa001082m scaffold_4 (2278399-2278408) (+)
	TCTCAT- <u>CTTAATTAAG</u> -AAACCA	Possible	ppa001082m scaffold_4 (2278500-2278509) (+)
	GAAGAA- <u>CATTTAAAAG</u> -GCCATC	Possible	ppa001082m scaffold_4 (2278737-2278746) (+)
	CTCAAC- <u>CAAATAAATG</u> -CTCTGG	Possible	ppa001082m scaffold_4 (2279001-2279010) (+)
	TTATCT- <u>CATAATTTAG</u> -GTAGCT	Possible	ppa001082m scaffold_4 (2279711-2279720) (+)
	TCACTA- <u>CCAAAAAAGG</u> -GGGCAA	SRF-like	ppa001082m scaffold_4 (2279828-2279837) (+)
	ATTTAA- <u>CTAATTAAG</u> -ATAAAA	Possible	ppa001082m scaffold_4 (2280595-2280604) (+)
	GTCACC- <u>CAAAAAAATG</u> -TGGCTA	Possible	ppa001082m scaffold_4 (2280784-2280793) (+)
	GGCATC- <u>CCTAAAAACG</u> -TGTACA	Atypical	ppa001082m scaffold_4 (2281417-2281426) (+)
<i>Prupeβ-Hex2</i>	CAAAGA- <u>CAATAATTTG</u> -GATGTT	Possible	ppa002753m scaffold_2 (10410744-10410753) (+)
	TCGCAT- <u>CAAATATATG</u> -TCCACA	Possible	ppa002753m scaffold_2 (10410790-10410799) (+)
	CGTCGT- <u>CTATATTAAG</u> -TTAAGA	Possible	ppa002753m scaffold_2 (10411259-10411268) (+)
	ATTGTA- <u>CGATTTAAAG</u> -ATACCG	Atypical	ppa002753m scaffold_2 (10411781-10411790) (+)
	AAACTC- <u>CATTTTTTAG</u> -TTGTCT	Possible	ppa002753m scaffold_2 (10412119-10412128) (+)

	CAAAAG- <u>CAATAAAATG</u> -GCAATA	Possible	ppa002753m scaffold_2 (10412571-10412580) (+)
	ATTACA- <u>CAAATATTAG</u> -AAACAT	Possible	ppa002753m scaffold_2 (10412768-10412777) (+)
<i>Prupea-Man</i>	TTGTTA- <u>CCTATTATTG</u> -TTTGGT	Intermediate	ppa000717m scaffold_3 (21776119-21776128) (-)
	TTGCCA- <u>CAAATAATGC</u> -TTAACT	Atypical	ppa000717m scaffold_3 (21776208-21776217) (-)

- 1) CArG-box sequences are underlined.
- 2) The motif includes three groups of CArG-box sequences: SRF-like [canonical CArG-box, CC(A/T)₆GG], MEF2-like [CTA(A/T)₄TAG], and intermediate [CC(A/T)₆AG][15](Fujisawa et al., 2011)
- 3) Scaffolds indicated with numbers correspond to sequences that originated from *Prunus_persica_v1.0_genes* data. The (+) and (-) indicate that the CArG-boxes are presented in either the sense or antisense strands, respectively.