Natural variations in a pectin acetyl esterase gene, \textit{MdPAE10}, contribute to prolonged apple fruit shelf life

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Abstract
Room-temperature shelf life is a key factor in fresh market apple (\textit{Malus domestica} Borkh.) quality and commercial value. To investigate the genetic and molecular mechanism underlying apple shelf life, quantitative trait loci (QTL) were identified using bulked segregant analysis via sequencing (BSA-seq). Ethylene emission, flesh firmness, or crispness of apple fruit from 1,273 $F_1$ plants of \textit{M. asiatica} Nakai ‘Zisai Pearl’ × \textit{M. domestica} ‘Golden Delicious’ were phenotyped prior to and during 6 wk of room-temperature storage. Segregation of ethylene emission and the flesh firmness or crispness traits was detected in the population. Thirteen QTL, including three major ones, were identified on chromosome 03, 08, and 16. A candidate gene encoding pectin acetyl esterase, \textit{MdPAE10}, from the QTL Z16.1 negatively affected fruit shelf life. A 379-bp deletion in the coding sequence of \textit{MdPAE10} disrupted its function. A single nucleotide polymorphism (SNP) in the \textit{MdPAE10} promoter region reduced its transcription activity. These findings provided insight into the genetic control of fruit shelf life and can be potentially used in apple marker-assisted selection.

1 | INTRODUCTION

The softening of fleshy fruit is a critical trait that results in reduced shelf life. Cold chain facilities are used worldwide during packing, shipping, storage, and at the retail phase to maintain the quality of fleshy fruit, especially those such as apple (\textit{Malus domestica} Borkh.) that undergo climacteric ripening. Most apple cultivars gradually lose their firmness starting soon after harvest, but some, such as ‘Red Fuji’, retain flesh firmness for at least a month at room temperature after harvest (Gussman et al., 1993). Breeding for cultivars with better fruit shelf life at ambient temperatures has the potential to reduce production costs substantially by avoiding cold chain conditions and prolonging the time of market availability (Wakasa et al., 2006).

The two fruit texture attributes that determine shelf life—flesh firmness and crispness—are closely correlated but clearly distinct. Consumers prefer crisp but less firm apple cultivars such as ‘Honeycrisp’ (McKay et al., 2011). Both

Abbreviations: 1-MCP, 1-methylcyclopropene; BSA-seq, bulked segregant analysis via sequencing; cDNA, complementary DNA; CDS, coding sequence; CEP, chelator-extractable pectin; InDel, insertion–deletion; KASP, Kompetitive allele-specific PCR; NEP, Na$_2$CO$_3$–extractable pectin; PCR, polymerase chain reaction; QTL, quantitative trait loci; SNP, single nucleotide polymorphism; VIGS, virus induced gene silencing; WEP, water-extractable pectin.
flesh firmness and crispness are known to be quantitatively inherited and linked with multiple quantitative trait loci (QTL) (Costa et al., 2005; Matas et al., 2015). For example, QTL associated with apple fruit firmness or crispness have been mapped to chromosome 01, 08, 10, 15, and 16 (Baumgartner et al., 2015; Costa et al., 2010; Costa et al., 2005; Costa et al., 2008; Harada et al., 2000; Zhu & Barritt, 2008). A total of 22 QTL for mechanical and acoustic fruit texture components have been identified in apple, explaining variance from 10 to 49% (Longhi et al., 2012). Using a Bayesian multi-QTL pedigree-based approach, a large number of QTL for apple hypanthium firmness after 2 mo of cold storage were also mapped to chromosome 01, 03, 06, 10, 15, and 16, which was consistent with multigenic inheritance of fruit firmness (Bink et al., 2014). Based on six full-sib pedigreed families and a collection of 233 apple accessions, pedigree-based analysis and a genome-wide association study were employed to decipher the genetic control of fruit texture; chromosome 10 was found to be important in controlling the mechanical properties, and chromosome 02 and 14 were more associated with the acoustic response (Di Guardo et al., 2017). Natural mutations in several functional genes have been identified and validated via QTL-based gene mining. Molecular markers that mapped to the ethylene biosynthesis genes, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (MdACS1) and ACC oxidase (MdACO1), were shown to be independently linked to apple fruit shelf life. Apple cultivars with MdACS1-2 and MdACO1-1 homozygous genotypes showed superior shelf life (Costa et al., 2005).

Similarly, the expression of ethylene biosynthetic proteins was much lower in high-firmness phenotypes (Marondedze & Thomas, 2012). During fruit ripening, the expression of MdACS1, MdACS3, and MdACSSA increased rapidly in the fruit cortex of the short-shelf-life apple M. domestica Borkh. cultivar Golden Delicious but not in the long-shelf-life cultivar Fuji. MdACO1 transcript levels increased in the fruit cortex of both cultivars (Li et al., 2010). Increased expression of the ethylene receptor genes, MdETR1, MdETR2, MdERS1, and MdERS2 has also been observed in apple fruit cortex during ripening (Li et al., 2010). Mutations that affect apple fruit firmness have also been linked to a group of genes encoding cell-wall modifying proteins. For example, β-galactosidase, α-arabinofuranosidase (α-AFase), and pectin methylesterase (MdPME) play roles in modifying cell-wall structure and fruit firmness. The expression of these proteins is usually higher in the apple genotypes with softer fruit (Gwanpua et al., 2014; Marondedze & Thomas, 2012). Some highly significant fruit texture QTL have been reported to colocalize with genes involved in cell-wall metabolism, such as the pectinases pectinlyase 1 (MdPG1) and pectate lyase (MdPel), as well as the ripening regulators MdNOR and MdRIN (Longhi et al., 2012). Downregulation of MdPG1 by transgenesis in apple reduced pectin depolymerisation and fruit softening (Atkinson et al., 2012). MdPG1 expression increased rapidly during fruit ripening in the cortex of the short-shelf-life Golden Delicious (Li et al., 2010). The expression of MdPG1 was very weak in cultivars that retain their firmness after long-term cold storage irrespective of flesh ethylene production (Wakasa et al., 2006). Relatively low MdPG and MdPME activities during early fruit developmental stages were associated with increased cell adhesion and reduced fruit softening rate in the slow-softening ‘Scifresh’ cultivar (Ng et al., 2013). The expression of an α-L-arabinofuranosidase gene (MdAF3) was consistently lower in nonmealy genotypes than in those that are mealy (Ng et al., 2013). Moreover, α-AFase activity was significantly and negatively correlated with fruit firmness but positively correlated with mealliness during a 2-mo cold storage (Nobile et al., 2011). Another class of cell-wall modifying proteins, called expansins, has also been associated with fruit softening. Expansins are thought to be involved in the disruption of the noncovalent bonds between the hemicellulose matrix and cellulose microfibrils, thus exposing these polymers to the action of other cell-wall degrading enzymes (Cosgrove, 1997, 2000). The apple expansin gene MdExp7 is linked with fruit softening (Costa et al., 2008).

Retention of apple flesh firmness is significantly linked to slower ripening and softening (Marondedze & Thomas, 2012). However, a firmer initial texture does not always correlate with longer shelf life or increased cold storability (Costa et al., 2010). Notably, the mechanisms responsible for fruit firmness or crispness retention are not well understood, both during long-term cold storage and at room temperature. In this study, bulked segregant analysis via sequencing (BSA-seq) was employed to identify QTL for apple fruit room-temperature shelf life. From a major QTL, a pectin acytyleresterase (MdPAE10) was identified and the functional variations were validated.

2 | MATERIALS AND METHODS

2.1 | Materials

Bulked segregant analysis via sequencing and QTL identification were performed on 1,273 8-yr-old F1 individuals between
*M. asiatica* Nakai ‘Zisai Pearl’ as the maternal parent and Golden Delicious as the paternal parent. Another segregating population resulting from a cross between Zisai Pearl and Red Fuji containing 1,306 F₁ individuals, was employed to validate the role of allelic variations in fruit shelf life. Plants were grown at a density of 2.5 by 0.5 m under conventional management and pest control in the Fruit Experimental Station, China Agricultural University (Changping District, Beijing, China).

In 2015–2017, fruit samples were harvested at commercial maturity stage based on fruit characteristics such as starch degradation (selected at 7 on a scale of 1–10) and skin background color (Blanpied & Silsby, 1992). After harvest, apple fruits were stored at room temperature (25–30 °C) with ~95% relative humidity in polyethylene bags. Flesh firmness and crispness of each F₁ individuals were measured at 0, 2, 4, and 6 wk after harvest with three biological replicates and three apple fruits per replicate.

To test whether *MdPAE10* expression responded to ethylene signaling, apple fruits (Golden Delicious), prior to commercial ripening (140 d after full bloom), were collected and immediately treated with ethephon or 1-methylcyclopropene (1-MCP) as described by Tan, Li, and Wang (2013). The fruit were then stored at room temperature for 20 d. Unripe apple of Golden Delicious were also used for transient transformation experiments.

### 2.2 Methods

#### 2.2.1 Measurement of fruit ethylene production and flesh firmness and crispness

Flesh firmness and crispness were measured using a computer-controlled texture analyzer TAXT (fruit tester, Stable Micro Systems) as described by Costa et al. (2011, 2012). The diameter of the penetrating probe was 0.2 cm, and the penetration depth was 0.5 cm. The firmness or crispness readings were expressed in N (m kg s⁻²). Three trigonal symmetric punctures were performed on the maximum equator of each apple.

Because of alternate bearing, year-repeated phenotype data of 403 and 404 F₁ plants for flesh firmness and flesh crispness, respectively, were obtained for broad-sense heritability estimation in 2015–2017. The broad-sense heritability was calculated using the formula, \( H^2 = (\sigma^2 - \sigma_e^2)/\sigma^2 \), where \( \sigma^2 \) is the overall phenotype variance, \( \sigma_e^2 \) is the environmental variance, that is, the variance among years (Hallauer et al., 1988).

Each fruit was weighed and then enclosed in a gas-tight container and kept for 2 h at room temperature. One milliliter of gas was sampled from the headspace in the container using a BD syringe (No. 309602, Becton, Dickinson and Company). The ethylene concentration of gas samples was measured with a gas chromatograph HP 5890 series II (Hewlett-Packard) equipped with a flame ionization detector. Before the gas samples were assayed, the gas chromatograph was calibrated with ethylene gas (NO. 34489, Restek Corporation) at a series of concentrations (0.01, 0.1, 0.5, 1, 5, 10, and 100 mg L⁻¹). The fruit ethylene production was calculated with the following formula: \( E = C \times (V_1 - V_2)/\omega/T \), where \( E \) is the fruit ethylene production rate (µl g⁻¹ fresh weight h⁻¹); \( C \) is ethylene concentration (mg L⁻¹); \( V_1 \) indicates the volume of the container (mL); \( V_2 \) is the volume of fruit (mL) equivalent to fresh weight (ω) in grams, and \( T \) represents the time (h) kept in the container (Dougherty et al., 2016).

#### 2.2.2 Validation of known *MdACS1* and *MdACO1* markers

The *MdACS1* marker was developed by Sunako et al. (1999). The polymerase chain reaction (PCR) products were *MdACS1-1/1* (489 bp) and *MdACS1-1/2* (655 bp). A polymorphism of a 62-bp insertion–deletion (InDel) in the third intron of *MdACO1* was used to generate the *MdACO1* marker. *MdACO1-1/1* and *MdACO1-2/2* represented fragment size of 52S and 587 bp, respectively (Costa et al., 2005). Fresh young leaves (100 mg) of the F₁ individuals or their parental cultivars were used to extract genomic DNA using the modified hexadecyltrimethylammonium bromide (CTAB) protocol described by Li et al. (2013). The PCR reactions were performed and the products were visualized using agarose gel electrophoresis (Jia et al., 2018).

#### 2.2.3 BSA-seq and candidate gene prediction

Consumers generally accept apple fruit as firm when the flesh firmness >2.2 N; likewise the apple fruits with >0.22 N crispness are referred to as crisp (Costa et al., 2010, 2011; McKay et al., 2011; Nybom et al., 2012). We therefore defined firmness retainability as the maximum number of weeks an apple retains a firmness >2.2 N during room-temperature storage. By the same way, the number of weeks an apple retains >0.22 N flesh crispness was defined as flesh crispness retainability. The least retainability of flesh firmness or crispness was considered as the room-temperature shelf life.

To constitute the long shelf life bulk, 30 F₁ plants from Zisai Pearl × Golden Delicious, which exhibited 6-wk fruit shelf life, were selected. Conversely, the short shelf life bulk was comprised of 30 F₁ individuals with 0-wk shelf life, that is, the flesh firmness was <2.2 N and crispness <0.22 N just after harvest. To reduce the potential contribution of *MdACS1* genotype to the phenotype of fruit shelf life, these 60 F₁ plants for BSA-seq analysis were confirmed to have the same *MdACS1-1/2* genotype because apple cultivars with
MdACS1-1/2 genotype produced relatively lower levels of ethylene and had longer fruit shelf life than those with MdACS1-1/1 genotype. Genomic DNA from each F1 individual was extracted from young leaves and pooled to construct segregant bulks. Each bulked DNA sample and DNA of one parent, Zisai Pearl, were sequenced to give 30× genome coverage using a paired-end 150-bp read strategy (Illumina X10). The pollen parent, Golden Delicious, has been resequenced previously (Shen et al., 2019).

The resequencing data were processed, and QTL were identified using the BSATOS software package (Shen et al., 2019). Burrows–Wheeler Aligner was used to map clean reads to the apple genome (Li & Durbin, 2009), whereas the GDDH13 genome was used as reference (http://www.rosaceae.org/) (Daccord et al., 2017). Default parameters were used for reads mapping, genotyping, and filtering, and only uniquely mapped reads with a minimum phred-scaled map quality score of 20 were kept for further analysis. SAMtools was used to identify SNPs and InDels between the aligned sequencing reads and the reference genome. (Li & Durbin, 2009; Li., 2011). To predict the possible function of the variations, genes were annotated. The annotation was performed with ANNOVAR based on the functional domain at the coding sequence (CDS) or the cis-elements at the promoter (Rombauts et al., 1999; Wang & Hakonarson, 2010). A modified G value method was used for the statistical analysis of allelic variations between the two bulks (Magwene et al., 2011). By combining the BSA-seq data and the parental resequencing data, biallele SNPs were categorized into three subsets: G-type, Z-type, and H-type. The Z-type SNPs meant that the SNP genotype of the maternal parent, Zisai Pearl, was homozygous, while the SNP genotype of the pollen parent, Golden Delicious, was homozygous. Alternatively, G-type SNPs indicated that the SNP genotype was homozygous in Zisai Pearl but was heterozygous in Golden Delicious. The H-type SNPs were those SNP genotypes that were heterozygous in both parents. Quantitative trait loci mapping was run three times with G-type, Z-type, and H-type SNP subsets, and the slide window was set as 1 Mb (Shen et al., 2019). The allele combinations of markers for G-type, Z-type, and H-type QTL were heterozygous in only paternal, maternal, or both parents, respectively. Thus the names of the QTL were initiated with G, Z, or H, respectively.

The genes from QTL interval were downloaded from the apple genome GDDH13 (https://www.rosaceae.org/) (Daccord et al., 2017). By comparing parental resequencing data, genes with identified genetic variations on the parental cultivar on which the QTL was not mapped were removed from the list (Shen et al., 2019). Of these genes, those with SNPs or InDels that did not affect the cis-element on the upstream sequence or the functional domain on the coding region were excluded. For the remaining genes, the UniProt database (http://www.UniProt.uniprot.org/) was used to assess the function of the corresponding proteins. Genes that were clearly not related to the target trait were excluded. The pipeline for candidate prediction has been published previously (Liu et al., 2020; Shen et al., 2019).

2.2.4 Relative gene expression assay using quantitative reverse transcription PCR

The complete DNA and complementary DNA (cDNA) sequences of coding region of MdPAE10 were cloned, Sanger sequenced, and sequence compared between Zisai Pearl and Golden Delicious. The three-dimensional structure of MdPAE10 peptide was analyzed by using online tools (https://swissmodel.expasy.org). The primer pairs of MdPAE10 for quantitative reverse transcription PCR were designed using Primer 5.0 (Premier, Canada). The primer sequences were listed in Supplemental Table S1.

Total RNA was extracted from apple flesh tissue as previously described (Hu et al., 2016). Complementary DNA was synthesized from total RNA using a cDNA Synthesis Kit. Quantitative reverse transcription PCR was carried out as previously reported (Jia et al., 2018). EFla (Loc103447856) was used for internal reference (Jia et al., 2018) (Supplemental Table S1).

2.2.5 KASP assay

To validate the identified variations in MdPAE10, 100-bp upstream and downstream sequences flanking the SNPs were used for the development of Kompetitive allele-specific PCR (KASP) markers (Chen et al., 2011). The KASP primers are listed in Supplemental Table S1. The KASP assay was performed using the LGC SNPline (LGC Genomics, Laboratory of the Government Chemist) and the data were analyzed using SNPviewer software (LGC).

2.2.6 Vector construction and transient transformation of apple

To determine the impact of InDel2 (a 379-bp deletion) in the MdPAE10 CDS on fruit shelf life, the complete MdPAE10 cDNA sequence containing InDel2 (MdPAE10-D) was amplified from Zisai Pearl. The intact MdPAE10 CDS without InDel2 (MdPAE10) were amplified from Golden Delicious. These segments were cloned into the KpnI and BamHI sites, respectively, of the pRI101 vector. The empty pRI101 was used as a control. The pRI101 vector already contains a 35S promoter and a green fluorescent protein reporter gene. The primer pairs used are listed in Supplemental Table S1. The three vectors were transiently transformed into
immature Golden Delicious fruit (140 d after full bloom) by using the method described previously (Li et al., 2017). The inoculation sites were recognized by the slightly watery lesions. Flesh texture was measured by penetration at 3–5 mm away from the inoculation sites. The assays were performed with three biological replicates and six apple fruits per replicate per vector.

To test whether the allelic variations in the upstream MdPAE10 region may affect its promoter activity, the promoter fragments of MdPAE10.1 (containing InDel1 and SNP1) and MdPAE10-D (containing SNP2) were amplified from genomic DNA of Zisai Pearl. The intact MdPAE10 promoter segment was amplified from Golden Delicious. All these fragments were sequence compared and digested with BamHI/NcoI. The fragments were then cloned into the pCAMBIA1391-GUS (β-glucuronidase) plant transformation vector as previously reported (Jia et al., 2018). Thus, the three constructs were pro-MdPAE10::GUS (intact); pro-MdPAE10.1::GUS (containing InDel1 and SNP1), and pro-MdPAE10-D::GUS (containing SNP2). Both 35S::GUS and the empty pCAMBIA1391-GUS vector were used as controls. The resulting constructs were transformed into leaf epidermal cells of 4-wk-old tobacco (Nicotiana benthamiana Domin) plants by vacuum infiltration with Agrobacterium tumefaciens strain GV3101.

To test whether variants of the MdPAE10 promoter respond to ethylene, a 1,000 mg L⁻¹ aqueous ethephon solution was sprayed onto the surface of the transformed tobacco leaves 48 h after infiltration. After 2–3 d of transfection, the transgenic leaves were stained using the GUS reporter gene staining kit (Solarbio) in three biological replicates as described by Zhang et al. (2016). Four technical replicates were performed. All primer sequences for vector construction are listed in Supplemental Table S1.

2.2.7 Virus induced gene silencing

A 622-bp MdPAE10 CDS fragment was cloned from Golden Delicious into the XbaI/KpnI site of the pTRV2 virus vector as previously described (Li et al., 2017). Agrobacterium tumefaciens (GV3101) cells harboring the resultant plasmids were suspended in infiltration buffer supplemented with 150 mM acetosyringone. The inoculum preparations were adjusted to OD 600 = 1.0. The mixed vectors and A. tumefaciens solution were transformed into Golden Delicious apple fruit by infiltration with a vacuum air pump (Hu et al., 2016; Li et al., 2016). The inoculation sites were recognized by the slightly watery lesions. Flesh texture was measured by penetration at 3–5 mm away from the inoculation sites. The assays were performed with three biological replicates and at least six apple fruits per replicate per vector.

2.2.8 Observation of flesh cellular structure

Flesh tissue was fixed with 10% formalin for 24 h at room temperature and subjected to paraffin embedding. Flesh-tissue-embedded paraffin blocks were sectioned (25 μm thick), dewaxed, and the cellular structure was observed using confocal microscopy (Jia et al., 2018).

2.2.9 Pectin extraction and fraction assay

Flesh materials were excised on the lesion from transiently transformed apple cortex tissue (~10 g). An alcohol-insoluble cell-wall preparation was made before the extractions were performed. Then, pectins were extracted sequentially with water, trans-1,2-diaminocyclohexane-N,N,N,N-tetraacetic acid (CDTA), and Na₂CO₃ to obtain water-extractable pectin (WEP), chelator-extractable pectin (CEP), and Na₂CO₃-extractable pectin (NEP) fractions, respectively (Fan et al., 2018). The absorbance was read at 530 nm by a UV–visible spectrophotometer (UV-1800, SHIMADZU). The pectin content was calculated by a standard curve of D-(+)-galacturonic acid.

2.2.10 Statistical analysis

Significant differences were analyzed by pairwise comparisons between corresponding genotypes or treatments using the independent samples Student’s t test (SPSS Statistics 21; IBM).

3 RESULTS

3.1 Segregation of flesh firmness and crispness and retainability

To investigate the segregation of apple flesh firmness or crispness, a collection of 1,273 F₁ plants derived from the Zisai Pearl × Golden Delicious cultivars were phenotyped. Both initial flesh firmness and crispness immediately after harvest were found to segregate considerably and exhibited Gaussian distribution (P < .0001) (Figure 1a, 1b). The broad-sense heritability of flesh firmness and crispness was 88.68 and 86.12%, respectively. Of the 1,273 F₁ individuals, the flesh firmness ranged from 1.06 to 6.20 N, while the flesh crispness ranged from 0.07 to 0.87 N. Among them, 1,185 and 1,171 showed high flesh firmness (>2.2 N) and high crispness (≥0.22 N) at harvest, respectively, while 1,138 exhibited both high flesh firmness and high crispness. The flesh firmness of Zisai Pearl and Golden Delicious were 4.23 and 2.41 N, respectively. The
Phenotype segregation for room-temperature shelf life in an interspecific hybrid population (*Malus asiatica* Nakai ‘Zisai Pearl’ × *M. domestica* Borkh. ‘Golden Delicious’). Frequency distribution of (a) flesh firmness or (b) crispness at harvest. The phenotype values of the parental cultivars were indicated with red arrows. Segregation in (c) flesh firmness retainability or (d) crispness retainability of *F*$_1$ plants with high initial values of flesh firmness and crispness at harvest during room-temperature storage. One dot in the box plot represents one *F*$_1$ plant.

To measure fruit flesh firmness and crispness retainability, 119 *F*$_1$ plants with both high initial flesh firmness and crispness at harvest were randomly selected for room-temperature storage. The retainability of flesh firmness and crispness segregated from 2 to 6 wk (Figure 1c, 1d). A significant correlation coefficient ($r = .2439$, $P < .01$) was obtained between flesh firmness and flesh crispness. Finally, 30 and 35 of the 119 *F*$_1$ plants showed high values of flesh firmness (>2.2 N) or flesh crispness (>0.22 N) after 6 wk of room-temperature storage, respectively (Figure 1c, 1d). The intersection of 30 *F*$_1$ plants with 6 wk of flesh firmness and crispness retainability were referred to as long-shelf-life phenotype. Whereas, the *F*$_1$ plants that exhibited both low flesh firmness and low flesh crispness at harvest (0-wk flesh firmness and crispness retainability) were referred as short-shelf-life phenotype. These *F*$_1$ plants were used for subsequent experiments.

**3.2 Effects of known markers, *MdACS1* and *MdACO1*, on apple fruit shelf life**

Many references reported that alleles of *MdACS1*, *MdACS1-1/1*, *MdACS1-1/2*, and *MdACS1-2/2* can result in high, intermediate, and small ethylene production rates in apple flesh (Harada et al., 2000; Oraguzie et al., 2004; Sunako et al., 1999). To determine the effect of *MdACS1* and *MdACO1* genotypes on apple fruit shelf life, the parental cultivars of the hybrid populations were genotyped. The genotype of Zisai Pearl, Red Fuji, and Golden Delicious cultivars was *MdACS1-1/1:*MdACO1-1/1, *MdACS1-2/2:*MdACO1-2/2, and *MdACS1-1/2:*MdACO1-1/1, respectively (Supplemental Figure S1). According to the parental genotypes, *MdACO1* genotype would not segregate in the two *F*$_1$ populations, Zisai Pearl × Red Fuji and Zisai Pearl × Golden Delicious. The *MdACS1* genotype in Zisai Pearl × Golden Delicious population segregated in a 7:9 ratio (*MdACS1-1/2:*MdACS1-1/1), which is consistent with 1:1 ($\chi^2 = 0.25$, $\chi^2_{0.05} = 7.88$) (Supplemental Figure S1; Supplemental Table S2).

The ethylene production rate at harvest, as well as during room-temperature storage, in fruit with the *MdACS1-1/2* genotype was significantly lower than that with the *MdACS1-1/1* genotype (Figure 2a, 2b, 2g). However, the flesh firmness or crispness in the *F*$_1$ plants with the same *MdACS1-1/1* genotype (28-037, 25-031, 31-126, 23-059, 25-129, and 29-172) segregated during storage as higher in 28-037, 25-031, and 31-126 or as lower in 23-059, 25-129, and 29-172 (Figure 2c, 2d). Completely similar dynamic change in flesh firmness or flesh crispness during storage was observed in *F*$_1$ plants with *MdACS1-1/2* genotypes (20-002, 29-123, 30-115, 33-101, 31-161, and 28-162) (Figure 2e, 2f). These data indicated that there should be other factors determining fruit shelf life than *MdACS1* genotype.
FIGURE 2  Ethylene production and dynamic changes in flesh firmness or crispness during room-temperature storage in apple F₁ plants of *Malus asiatica* Nakai ‘Zisai Pearl’ × *M. domestica* Borkh. ‘Golden Delicious’. Dynamic changes in ethylene production in F₁ individuals with the (a) *MdACS1-1/1* and (b) *MdACS1-2* genotypes during 6 wk of room-temperature storage. Changes in (c) flesh firmness or (d) flesh crispness in F₁ individuals with the *MdACS1-1/1* genotype. Changes in (e) flesh firmness or (f) flesh crispness in hybrids with the *MdACS1-1/2* genotype. In panels (a) through (f), the error bars represent standard deviation of three biological replicates. (g) Box plot shows the ethylene production rate in individuals with *MdACS1-1/1* and *MdACS1-1/2* genotypes. Statistically significant differences were determined by *t* tests (** **P < .0001)
3.3 Identification of QTL for fruit shelf life

Thirty F₁ individuals with long shelf life (6 wk) and 30 F₁ individuals with short shelf life (0 wk) were used to constitute long and short shelf life extremity bulks, respectively (Figure 3a, 3b). The MdACS1 genotype of all these 60 F₁ individuals to be used for bulked segregant analysis bulking was MdACS1-1/2. By comparing the resequencing data of Zisai Pearl (unpublished data in our lab) and Golden Delicious (Shen et al., 2019), we identified 10,844,493 SNP markers. These SNPs included 1,823,942 G-type, 7,271,424 Z-type, and 1,749,127 H-type markers between the two parents. Resequencing of the two bulks generated 243,212,629 clean reads (114,767,700 for short shelf life and 128,444,929 for long shelf life). In total, 227,712,155 (93.6%) reads were mapped to the GDDH13 genome. After mapping, genotyping, and filtering, 4,850,112 SNP markers were obtained and the marker densities ranged from 1,324 to 3,661 Mb⁻¹. All BSA-seq raw data have been deposited in the NCBI Sequence Read Archive under the accession number PRJNA546037.

We identified 13 significant QTL for apple fruit shelf life on three chromosomes (Figure 3c; Supplemental Table S3). Seven QTL were mapped to chromosome 03 of Golden Delicious, one QTL was identified on chromosome 16 of Zisai Pearl, and five on chromosome 08 and 16 of both parents (Figure 3c; Supplemental Table S3). Three QTL (H08.2, Z16.1, and H16.2) with G′ values >30% of the corresponding thresholds were referred to as major QTL (Supplemental Table S3). The allele effects of nine QTL were positive (δ-AF > 0) on fruit shelf life, whereas that of four QTL were negative (δ-AF < 0) (Supplemental Table S4). The contribution of the parents to longer fruit shelf life varied with QTL (Supplemental Table S4).

3.4 Candidate gene screening and variation verification

Quantitative trait locus Z16.1 was chosen for candidate gene prediction because Z16.1 exhibited the largest G′ value (11.3) (Supplemental Table S3). One hundred thirty-one genes were annotated in the Z16.1 interval of GDDH13 genome. Of the 131 genes, 17 were removed from the list because the genetic variations occurred in Golden Delicious but not in Zisai Pearl. Of the remaining 114 genes, 82 were selected because the genetic variations of these genes affected the cis-element on the upstream sequence or the functional domain on
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FIGURE 4 Allelic variations in MdPAE10 between Malus asiatica Nakai ‘Zisai Pearl’ and M. domestica Borkh. ‘Golden Delicious’. (a) Diagram showing haplotypes of intact MdPAE10 in Golden Delicious and MdPAE10.1 containing SNP1 and InDel1 or MdPAE10-D containing SNP2, SNP3, and InDel2 in Zisai Pearl. The grey rectangle indicates coding sequence and the thick black line shows the upstream region. (b) DNA sequence comparison of the MdPAE10 coding region and upstream segment of Zisai Pearl (Z) to GDDH13 genome sequence. The variations are located at −737, −729, and −490 bp upstream and +113 and +728 downstream of the ATG codon. (c, d) Bioinformatics predicted three dimensional structures of the intact MdPAE10 protein (c) without the variations and (d) MdPAE10-D protein containing SNP3 and InDel2 the CDS (Supplemental Table S5). For the remaining genes, by using the UniProt database, the biological functions of a pectin acetyl esterase gene (MdPAE10, MD16G1132100) and a pectin lyase-like superfamily protein gene (MdPel, MD16G1140500) are apparently well associated with flesh shelf life (Supplemental Tables S6 and S7). The variations in MdPAE10, but not MdPel, were confirmed by PCR product Sanger sequencing (Supplemental Tables S6 and S7). MdPAE10 was used for further variation validation.

Sanger clone sequencing of MdPAE10 identified five variations between Golden Delicious and Zisai Pearl (Supplemental Table S8). The variations included three variations at the promoter region, InDel1, T to − at −737 bp; SNP1, A to G at −729 bp; and SNP2, C to T at −490 bp prior to the ATG codon. There were two variations in the CDS. One was SNP3, A to G at +113, which alters a glutamine (Gln) to an arginine (Arg). The other was InDel2, a 379-bp nucleotide deletion at +728 downstream of the ATG codon (Figure 4a, 4b; Supplemental Table S8). The three-dimensional structure of the MdPAE10 peptide was predicted to be altered as a consequence of the SNP3 and the InDel2 (Figure 4c, 4d). The SNP2, SNP3, and the InDel2 of MdPAE10 exhibited complete linkage in the parental cultivars and the F1 individuals (Supplemental Table S9).

3.5 Validation of the relationship between allelic variation in MdPAE10 and fruit shelf life

To determine the association of the allelic variations in MdPAE10 with fruit shelf life, MdPAE10 SNP3 of 537 (344 from Zisai Pearl × Red Fuji and 193 from Zisai Pearl × Golden Delicious) randomly chosen F1 plants were genotyped (Figure 5a, 5b). Chi-square test revealed a 1:1 segregation between MdPAE10 SNP3 A:A and G:A genotypes in both of the two F1 populations (χ² = 0.19; 0.75, χ² 0.05 = 7.88) (Figure 5c–f). After 6 wk of room-temperature storage, flesh firmness or crispness of the apple fruits with MdPAE10 SNP3 A:A genotype were significantly lower than that of the G:A genotype from both populations (Figure 5c–f). These data
confirmed the association between allelic variation SNP3 in *MdPAE10* and the phenotype of fruit shelf life. Owing to the tight linkage among SNP2, SNP3, and InDel2, all of the three allelic variations exhibited close association with room-temperature shelf life (Supplemental Figure S2; Supplemental Table S9).

### 3.6 *MdPAE10* expression pattern and promoter activity

To investigate whether *MdPAE10* expression differs between the long and short shelf life F1 individuals, *MdPAE10* expression were analyzed using three F1 plants with long shelf life (19-174, 27-003, and 23-212 with SNP2 C:T/SNP3 A:G/InDel2 Ins:Del genotype) and three F1 plants with short shelf life (24-127, 19-213, and 20-002 with SNP2 C:C/SNP3 A:A/InDel2 Ins:Ins genotype) (Supplemental Table S9). The changes in flesh firmness and crispness during room-temperature storage are shown in Figures 6a and 6b. The *MdPAE10* expression was significantly and consistently lower in the long-shelf-life F1 individuals and Zisai Pearl than that in the short-shelf-life F1 individuals and Golden Delicious during 2–6 wk of storage (Figure 6d). The data indicated that the high *MdPAE10* expression was associated with short fruit shelf life, which was possibly attributed to the promoter variations in *MdPAE10*.

Then, to test the effects of variations in *MdPAE10* promoter on the transcription activity, tobacco leaves were transiently transformed.Transformants of pro-*MdPAE10::GUS* (intact)
FIGURE 6  MdPAE10 expression pattern and promoter activity analysis in F1 plants with long (19-174, 27-003, and 23-212) and short shelf life (24-127, 19-213, and 20-002) derived from a cross between Malus asiatica Nakai ‘Zisai Pearl’ (Z) × M. domestica Borkh. ‘Golden Delicious’ (G). Changes in (a) flesh firmness or (b) crispness of F1 plants during room-temperature storage. (c) Promoter activity assay of MdPAE10 variants using transiently transformed tobacco leaves and treatment with ethephon before staining. The leaf discs were stained using β-Glucuronidase (GUS) Reporter Gene Staining Kit. The three horizontally placed leaf discs represent three replicates. (d) MdPAE10 expression profiles in F1 plants with different flesh firmness and crispness retainability and their parents during room-temperature storage. Error bars indicate standard deviation of three biological replicates, each containing at least three technical replicates. EF1a (Loc103447856) was used as the internal reference. Statistical significance was compared between individuals with long and short shelf life by Student’s t tests (***P < .01)

and pro-MdPAE10.1::GUS (containing InDel1 and SNP1) exhibited the same GUS staining intensity, indicating that InDel1 and SNP1 did not affect promoter activity. The GUS staining intensity was considerably lower in pro-MdPAE10-D::GUS (containing SNP2) transfectants than that of pro-MdPAE10.1::GUS (containing InDel1 and SNP1) or pro-MdPAE10::GUS (intact) (Figure 6c). This suggested that the SNP2-T allele reduced the MdPAE10-D promoter activity (Figure 6c). When the transfectants were treated with 1,000 mg L−1 ethephon, an ethylene precursor, the GUS staining was more intensive, indicating that the MdPAE10 promoter from any of the genotypes responded to ethylene (Figure 6c).

3.7 Response of MdPAE10 expression to ethephon or 1-MCP treatment

The skin color of Golden Delicious apple fruits typically changes to yellow during ripening and senescence. Here, we observed no obvious changes in fruit skin color in Golden Delicious apple fruits treated with the ethylene inhibitor 1-MCP during room-temperature storage. However, the skin color of apple fruits treated with ethephon was more yellow than that of untreated control (Figure 7a). Either flesh firmness or crispness of ethephon treated apple fruits decreased more rapidly during storage than the untreated control, while this decrease was delayed by 1-MCP treatment (Figure 7b, 7c). MdPAE10 expression in apple flesh was significantly induced by ethephon treatment at 5–20 d; however, 1-MCP treatment resulted in significantly lower expression of MdPAE10 than that in untreated control at 15–20 d (Figure 7d). High fluorescence intensity was observed in apple fruits during 5–10 d storage, demonstrating the tissue structure remained intact (Figure 7e). During 15–20 d of room-temperature storage in ethephon treatment or nontreated controls, the apple cells became swollen or had irregular morphology (Figure 7e). Also, there was a low fluorescent signal, indicating that pectin fractions in the cell wall were degraded (Figure 7e). In contrast, the cells of 1-MCP-treated apple fruits were swollen but were regular in shape and richly fluorescent (Figure 7e). Taken together, both MdPAE10 expression and flesh firmness or crispness retainability was sensitive to ethylene.
FIGURE 7 Changes in apple fruit (a) skin color, (b) flesh firmness, (c) crispness, (d) expression of MdPAE10, and (e) structure of the parenchyma cells of ‘Golden Delicious’ apple fruits after ethephon or 1-methylcyclopropene (1-MCP) treatments. EF1a (Loc103447856) was used as the internal reference for (d). Statistical significance in panels (b), (c), and (d) was determined by the Student’s t tests between treatments at equivalent time points (*P < .05; **P < .01; ***P < .001). Scale bars = 50 μm. The cell wall is pointed out with arrows in (e).

3.8 Functional validation of InDel2 in MdPAE10

The function of InDel2 at MdPAE10 CDS was validated by using transient transgenesis. A significant increase in MdPAE10 expression, but no distinct changes in visual fruit skin color, was observed throughout the experiment in apple transiently transformed with pRI-MdPAE10-D and pRI-MdPAE10 than pRI101 empty vector control (Figure 8a, 8g). No robust differences in flesh firmness or flesh crispness were detected between pRI-MdPAE10-D and empty pRI101-transformed apple (Figure 8b, 8c). In contrast, both flesh firmness and crispness of pRI-MdPAE10-transformed apple fruits were significantly lower than that of empty pRI101 control.
and pRI-MdPAE10-D transformants 5–20 d after transformation (Figure 8b, 8c). Percentage of NEP fraction was relatively lower in pRI-MdPAE10-transformed apple than that in pRI-MdPAE10-D and empty pRI101 transformants (Figure 8d). Significantly higher percentages of WEP and CEP fractions were detected in pRI-MdPAE10-transformed apple than that in empty pRI101 transformants 5–20 d after transformation (Figure 8e, 8f). In pRI-MdPAE10-D-transformed apple, however, percentage of WEP and CEP was identical with empty pRI101 transformants throughout (Figure 8e, 8f). These data indicated that intact MdPAE10 functions to accelerate apple flesh softening but that the InDel2 disrupts the function of MdPAE10.

The effect of MdPAE10 on fruit firmness or crispness retention was further validated by using VIGS. Compared with Golden Delicious apple transiently transformed with an empty pTRV vector, apple transformed with pTRV-MdPAE10 exhibited slightly less visual yellowing of the skin (Figure 9a). The MdPAE10 expression was significantly reduced in pTRV-MdPAE10-transformed apple fruits 5–20 d after transformation (Figure 9g). However, significantly higher flesh firmness or crispness was detected in pTRV-MdPAE10-transformed apple 5–20 d after infiltration, except the flesh firmness at day 10 (Figure 9b, 9c). Relatively higher percentage of NEP was observed in pTRV-MdPAE10-transformed apple than that in the empty TRV control 5–20 d after treatment (Figure 9d). As a consequence, the percentages of WEP and CEP were significantly lower in pTRV-MdPAE10 apple than that in empty TRV-transformed control (Figure 9e, 9f). These data further confirmed that MdPAE10 negatively contribute to flesh firmness or crispness retention by pectin solubilization, hence, silencing MdPAE10 prolongs apple fruit shelf life.
4 | DISCUSSION

Room-temperature shelf life is dominantly determined by the retention of flesh firmness and crispness (Wakasa et al., 2006). The retainability of flesh firmness and crispness is a composite trait dependent on both the initial values of flesh firmness or crispness at harvest and the rate of softening. Most commercial cultivars have high initial flesh firmness and crispness at harvest but the softening rate varied extensively (Nybom et al., 2013). In fact, firmer initial texture does not always correlate with longer shelf life or better firmness or crispness retainability (King et al., 2000, 2001). The larger correlated coefficients between fruit firmness after storage and firmness loss were detected, but that between initial firmness at harvest and firmness loss were often not significant (Chagné et al., 2014). We observed that 93.1 and 91.9% of the F₁ individuals showed acceptable initial flesh firmness and crispness at harvest, respectively. After 6 wk of room-temperature storage, however, only 25.2 and 29.3% of the high-initial-values F₁ individuals retained acceptable flesh firmness and crispness. Therefore, on one hand, apple fruit shelf life is correlated with flesh firmness or crispness at harvest, which is consistent with the previous study (Marondedze & Thomas, 2012). On the other hand, low flesh softening rate is more critical for longer shelf life.

Flesh firmness and crispness are important texture attributes for fruit shelf life. From the QTL Z16.1 for fruit shelf life in this study, variations in the candidate gene MdPAE10 were associated with both flesh firmness and crispness. Another flesh texture attribute, flesh mealiness, was also considered as an index of fruit shelf life. The occurrence of flesh mealiness during storage does not always correspond with the firmness loss or softening (Iwanami, Moriya,
A QTL associating apple flesh mealinness has been mapped on chromosome 01 (Kuni-hisa et al., 2016). However, the interval of the QTL was very near the MdEXP7 locus, which affects fruit firmness or softening (Costa et al., 2008). We did not measure the degree of flesh mealinness, and no QTL were identified on chromosome 01, which, most likely, is due to the differences in mapping population.

Quantitative trait loci related to fruit shelf life have previously been identified, and chromosome 10 and 15 have been proposed as hot linkage groups (Costa, 2015; Kenis et al., 2008; King et al., 2000; Longhi et al., 2012). In this study, no significant QTL were mapped on chromosome 10 or 15. Instead, we identified 13 confident QTL associating with fruit shelf life on chromosome 03, 08, and 16. Of these QTL, the confident intervals of H16.1 and Z16.1 overlapped with the previously reported QTL at 4.05–4.94 and 9.97–11.27 Mb, respectively (Longhi et al., 2012).

Allelotypes of the functional markers MdACS1, MdACS1-1/1, MdACS1-1/2, and MdACS1-2/2 have been reported to be associated with high, intermediate, and low ethylene production during fruit ripening (Costa et al., 2005; Harada et al., 2000; Oraguizie et al., 2004; Sunako et al., 1999). Consistently, we observed that fruit of F1 plants with MdACS1-1/1 genotype produced higher levels of ethylene than that with MdACS1-1/2 genotype. However, it was not always reliable to predict fruit firmness at harvest or softening rate during cold storage by markers of MdACS1, MdACO1, MdEXP7, or MdPG1. These genes accounted for, at maximum, 15% of the observed variation in initial firmness and 18% for softening rate (Longhi et al., 2013; Nybom et al., 2013). For late-ripening cultivars, MdACS1-1/1 genotypes are often firmer at harvest, while the MdACS1-2/2 allele exhibits a slower rate of flesh softening during cold storage, suggesting that factors other than MdACS1 contribute substantially to fruit storability (Oraguizie et al., 2007). In this study, the ethylene release rate varied in the MdACS1-1/1 and MdACS1-1/2 genotypes, while the retainability of flesh firmness or crispness segregated independently of either MdACS1 genotype or the ethylene emission rate (Figure 2a). By using phenotypetype-segregating F1 plants with the same MdACS1-1/2 genotype, 13 QTL were detected in this study.

A number of cell-wall metabolism-related genes, MdPG1, MdPME, MdPel, and MdExp7, promote the degradation of fruit cell walls and therefore accelerate flesh softening (Costa et al., 2008; Longhi et al., 2013; Zhang et al., 2018). We observed that overexpressing intact MdPAE10, but not MdPAE10-D, caused a significant decrease in flesh firmness and crispness, whereas VIGS MdPAE10 led to better flesh firmness and crispness retention. Therefore, MdPAE10 acts to promote apple flesh softening. The 379-bp deletion InDel2 disrupts the function of MdPAE10 and prolongs the fruit shelf life. The less color change in MdPAE10 VIGS apple was unexpected and the cause was unknown. Besides the InDel2, the two SNPs, SNP2 and SNP3, showed complete genetic linkage with InDel2. The SNP2 in MdPAE10 promoter leads to a significant reduction in the promoter activity, thus a decrease in MdPAE10 expression, which also potentially contributes to longer shelf life. The SNP3 at MdPAE10 CDS altered an amino acid residue, whether or not the SNP3 might contribute to MdPAE10 function loss is now unclear.

Fruit softening commonly attributes to the loosening of the cell wall, which is related to the degradation of pectin (Bennett & Labavitch, 2008). The NEP is comprised mainly of highly branched rhamnogalacturonan 1 pectin molecules tightly bound to primary cell wall (Brummell, 2006; Renard, 2005). During the process of cell-wall loosening, there is generally an increase in soluble pectin and a decrease in insoluble pectin (Song et al., 2016). The action of debranching enzymes, PME, PAE, and PG produces shorter-chain molecules that are solubilized into CEP and WEP fractions (Gwanpua et al., 2017). The enzyme PAE specifically deacetylates acetylated carbohydrate polymer pectin (Philippe et al., 2017). In this study, transgenesis of pRI-MdPAE10 or pTRV-MdPAE10 caused only slight changes in the percentage of NEP. However, the percentage of soluble WEP and CEP was increased by pRI-MdPAE10 transformation and was significantly decreased by MdPAE10 VIGS. Such changes of pectin fractions lead to the loss of cohesion of pectin gel matrix, cell-wall dissolution and cell separation (Xie et al., 2017). As a consequence, the flesh firmness and crispness loss occurred more extensively in pRI-MdPAE10-transformed apple, but higher flesh firmness and crispness were retained in pTRV-MdPAE10 transformants. Hence the function of MdPAE10 on pectin degradation and cell-wall loosening was further confirmed by these data.

In summary, 13 confident QTL for apple fruit shelf life were identified including three major ones (H08.2, Z16.1, and H16.2). The candidate gene predicted in the QTL Z16.1, MdPAE10, was found to be associated with apple fruit shelf life. A 379-bp deletion in the CDS and a C-to-T SNP in the promoter region of MdPAE10 both perturbed the gene function and prolonged fruit shelf life. These results may contribute to further understanding of apple fruit shelf life, and the functional markers may potentially assist apple breeding.

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DATA AVAILABILITY STATEMENT
The data that support the findings of this study have been deposited in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA546037.

AUTHOR CONTRIBUTIONS
BW, CJC, and LL performed the lab experiments. FS analyzed the BSA-seq data and QTL mapping. XW, WYZ, YD, TW, ZYH, CX, QZ, YW, and TW contributed to the phenotyping. XZZ, ZHH, and XFX designed and supervised the experiments. BW and XZZ wrote the draft and revised the manuscript.

CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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