

A 5.2-kb insertion in the coding sequence of PavSCPL, a serine carboxypeptidase-like enhances fruit firmness in *Prunus avium*

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Summary

Fruit firmness is an important trait in sweet cherry breeding because it directly positively influences fruit transportability, storage and shelf life. However, the underlying genes responsible and the molecular mechanisms that control fruit firmness remain unknown. In this study, we identified a candidate gene, *PavSCPL*, encoding a serine carboxypeptidase-like protein with natural allelic variation, that controls fruit firmness in sweet cherry using map-based cloning and functionally characterized *PavSCPL* during sweet cherry fruit softening. Genetic analysis revealed that fruit firmness in the 'Rainier' × 'Summit' F₁ population was controlled by a single dominant gene. Bulk segregant analysis combined with fine mapping narrowed the candidate gene to a 473-kb region (7418778–7 891 914 bp) on chromosome 6 which included 72 genes. The candidate gene *PavSCPL*, and a null allele harbouring a 5244-bp insertion in the second exon that completely inactivated *PavSCPL* expression and resulted in the extra-hard-flesh phenotype, were identified by RNA-sequencing analysis and gene cloning. Quantitative RT-PCR analysis revealed that the *PavSCPL* expression level was increased with fruit softening. Virus-induced gene silencing of *PavSCPL* enhanced fruit firmness and suppressed the activities of certain pectin-degrading enzymes in the fruit. In addition, we developed functional molecular markers for *PavSCPL* and the *Pavscpl*^{5.2-k} allele that co-segregated with the fruit firmness trait. Overall, this research identified a crucial functional gene for fruit firmness. The results provide insights into the genetic control and molecular mechanism of the fruit firmness trait and present useful molecular markers for molecular-assisted breeding for fruit firmness in sweet cherry.

Keywords: sweet cherry, fruit firmness, BSA-seq, *PavSCPL*, molecular marker.

Introduction

Sweet cherry (*Prunus avium* L.) is an important horticultural and commercial crop globally. The fruit are highly valued by consumers for their attractive colour, the depth and concentration of flavours, and as a rich source of nutrients beneficial for human health, including vitamins, minerals, antioxidants, anthocyanins and carotenoids (Liu *et al.*, 2011; Quero-García *et al.*, 2017). However, the fruit softens rapidly during ripening, resulting in reduced postharvest transportability, storability and shelf life, which causes severe economic losses to growers and traders. Annual postharvest losses may be up to 30%, which greatly affects the market value of sweet cherry fruit and restricts the development of the sweet cherry industry (Quero-García *et al.*, 2017; Wang *et al.*, 2015a,b; Zhao *et al.*, 2019). Therefore, the selection and breeding of new cultivars of sweet cherry with improved storability is vital for the growth of the sweet cherry industry.

Fruit firmness is an important fruit-quality trait that involves changes in a variety of physical properties, including cell wall structure (Seymour, Østergaard, *et al.*, 2013) and cuticle attributes (Li *et al.*, 2020). Degradation of cell wall structural components, including cellulose, hemicellulose and pectin and

reduction in intercellular cell wall adhesion result in fruit softening (Bashline *et al.*, 2014; Brummell and Harpster, 2001; García-Gómez *et al.*, 2021; Wang *et al.*, 2015a,b; Xin *et al.*, 2020). This process is accompanied by the elevated expression of a suite of genes that encode cell-wall-degrading enzymes, including endopolygalacturonase (PG), pectate lyase (PL), pectin methylesterase (PME), endo-1,4-β-glucanase, β-galactosidase (β-Gal), α-L arabinofuranosidase (α-AF), xyloglucan endotransglycosylase/hydrolase (XTH) and expansin, which leads to fruit softening (García-Gómez *et al.*, 2020; Uluisik *et al.*, 2016; Wang *et al.*, 2018). Thus, fruit softening is a complex physiological process and is genetically controlled by polygenes and influenced by diverse environmental factors (García-Gómez *et al.*, 2021). Deciphering the genetic mechanism that regulates fruit firmness is, therefore, essential for the efficient modification of this trait in breeding and will accelerate the improvement of fruit storability in sweet cherry.

Considerable research in recent decades has focused on the mining of fruit firmness-related genes in sweet cherry by means of quantitative trait locus (QTL) analysis to elucidate the genetic control of fruit firmness. Several QTLs implicated in fruit firmness have been identified to date, but no candidate genes that control sweet cherry fruit firmness have been identified on chromosomes

(Calle and Wünsch, 2020). Campoy *et al.* (2015) identified firmness QTLs on linkage groups (LGs) 1–6 and 8 using 'Regina' × 'Lapins' and 'Regina' × 'Garnet' mapping populations. A major QTL was detected on LG2 that explained 20% of the total phenotypic variation in fruit firmness (Campoy *et al.*, 2015). Cai *et al.* (2019) identified a fruit firmness QTL located on LG4 (*qP-FF4.1*) using three sweet cherry populations, namely, a 'Fercer' × 'X' (an unknown parent) F₁ population, the INRA sweet cherry germplasm collection and the RosBREED pedigreed population (Cai *et al.*, 2019). A subsequent study also identified fruit firmness QTLs on LG4 (*qP-FF4.1*), which explained as much as 64.1% of the phenotypic variation (Calle and Wünsch, 2020). In addition, Calle *et al.* (2020) detected three QTLs for fruit firmness in an 'Ambrunés' × 'Sweetheart' F₁ population, comprising two on LG1 (*qP-FF1.1* and *qP-FF1.2*) identified as alleles of breeding interest, and one on LG6 (*qP-FF6.1*), with explained phenotypic variation of 12.7%–18.8%, 12.9%–22.5% and 14.3%, respectively (Calle *et al.*, 2020). In a genome-wide association study (GWAS), Crump *et al.* (2022) detected fruit firmness QTLs on LGs 1 (*qFirm-LG1.2*), 3 (*qFirm-LG3.2*) and 6 (*qFirm-LG6.2*), which explained 8.8%–21.8%, 1.7%–10.1% and 4.2% of the phenotypic variance, respectively (Crump *et al.*, 2022). In addition, several SNP-trait associations with fruit firmness were identified in other GWASs (Donkpegan *et al.*, 2023; Holušová *et al.*, 2022). Holušová *et al.* (2022) identified 18 SNP markers associated with the fruit firmness-related traits, located on chromosomes 1, 2, 3, 4, 6, 7 and 8 in GWASs with 235 sweet cherry accessions (Holušová *et al.*, 2022). The most significant SNP marker (chr4_16000421) for fruit firmness was colocalized with QTLs *qP-FF4.1* identified by Cai *et al.* (2019) and Calle and Wünsch (2020). Another SNP marker (chr1_31855038) associated with fruit firmness was colocalized with *qP-FF1.2* and *qP-FF1.1* identified by Calle *et al.* (2020). Donkpegan *et al.* (2023) identified seven SNPs associated with fruit firmness in GWASs using 116 sweet cherry accessions, located on chromosomes 3, 4, 5, 6 and 8 (Donkpegan *et al.*, 2023). And one SNP marker Chr4-15 932 813 bp using 'Satonishiki' was identified on chromosome 4 at 15.9 Mbp explaining 20.6% of the variance (Donkpegan *et al.*, 2023), which was also identified on chromosome 4 at 16.0 Mbp using 'Tieton' by Holušová *et al.* (2022). The identification of different QTLs and SNPs suggests that sweet cherry fruit firmness is a quantitative trait; therefore, precise identification of the corresponding genes associated with fruit firmness phenotypes remains a daunting task. Accordingly, the genes and underlying molecular mechanisms that control sweet cherry fruit firmness remain unknown.

The aim of the current study was to fine-map QTL associated with the fruit firmness trait, identify the key gene and develop practicable and functional molecular markers to facilitate breeding for firm sweet cherry fruit. Here, we observed that the fruit firmness of a proportion of individuals in the F₁ population derived from the cross 'Rainier' (hard-flesh fruit) × 'Summit' (hard-flesh fruit) was significantly higher than that of both parents. Genetic analysis of the progeny determined that fruit firmness may be controlled by a single gene in this F₁ population. Bulk segregant analysis sequencing (BSA-seq), a comparative transcriptome analysis and gene cloning were implemented to identify the candidate gene and develop the functional molecular markers. The function of candidate gene in sweet cherry fruit was investigated using virus-induced gene silencing (VIGS). The objective of this study, therefore, identify a key functional gene

for fruit firmness and provide novel insights into the genetic mechanism of fruit firmness in sweet cherry. In addition, the functional molecular markers developed in this study can be used for the genetic improvement of the fruit firmness trait in sweet cherry breeding programmes through marker-assisted selection (MAS).

Results

Phenotypic evaluation and genetic analysis of fruit firmness in the sweet cherry F₁ population

To evaluate the variation in fruit firmness among 204 F₁ individuals derived from the cross 'Rainier' (hard-flesh fruit) × 'Summit' (hard-flesh fruit) (Figure 1a), the fruit firmness of the F₁ individuals and the two parents was measured in 2016, 2017, 2018 and 2019. The phenotypic analyses revealed distinct differences in fruit firmness among the F₁ individuals, which ranged from 0.5 to 12.5 kg/cm², whereas the fruit firmness of the 'Rainier' and 'Summit' parents was 4.65 and 4.55 kg/cm², respectively (Figure 1b,c). These results indicated that the fruit firmness of some F₁ individuals was significantly higher or lower than that of the two parents. The frequency distribution of fruit firmness in the F₁ population exhibited a continuous and bimodal distribution with a distinct separation of the two groups of individuals in 2016–2019 (Figure 1d). Based on the fruit firmness of the two parents and the frequency distribution in the F₁ population, the F₁ population was artificially divided into soft-flesh fruit (fruit firmness ≤3.5 kg/cm²), hard-flesh fruit (fruit firmness 3.5–7.0 kg/cm²) and extra-hard-flesh fruit (fruit firmness ≥7.0 kg/cm²). Based on these criteria, the F₁ population comprised 151 individuals with non-soft-flesh fruit (103 individuals with hard-flesh fruit and 48 individuals with extra-hard-flesh fruit) and 53 individuals with soft-flesh fruit, corresponding to a 3 : 1 segregation ratio ($\chi^2 = 0.1045$, $P = 0.8196$). This segregation ratio indicated that a single dominant gene may control fruit firmness in the 'Rainier' × 'Summit' F₁ population.

Identification of a major QTL locus on chromosome 6 contributing to fruit firmness

To rapidly identify the candidate chromosomal region for fruit firmness in the F₁ population, the two parents and two DNA bulks, Soft-pool and Extra-hard-pool, constructed from the F₁ population were sequenced using an Illumina high-throughput platform. In total, 58 082 598 and 60 676 954 clean reads were obtained for the extra-hard-pool and Soft-pool, respectively, and the percentage coverage of the *Prunus avium* 'Satonishiki' reference genome was 94.65% and 94.62%, respectively (Table S1). Overall, 573 702 single-nucleotide polymorphisms (SNPs) and 91 145 small (≤50 bp) insertions/deletions (InDels) were detected between the parents and the two bulks. The indices $\Delta(\text{SNP index})$ and $\Delta(\text{InDel index})$ were calculated to analyse the SNPs and the small InDels between the two bulks (Figures S1 and S2). A significant signal region associated with fruit firmness was observed on chromosome 6, comprising approximately 6.5 Mb from 5 582 631 to 12 091 212 bp, and was identified as the candidate region for fruit firmness based on the $\Delta(\text{SNP index})$ and $\Delta(\text{InDel index})$ values (Table S2; Figure 2a).

To further confirm and more precisely narrow down the candidate region for fruit firmness, fine-mapping was performed with the 'Rainier' × 'Summit' fruit firmness-segregating F₁ individuals based on observation of their fruit firmness

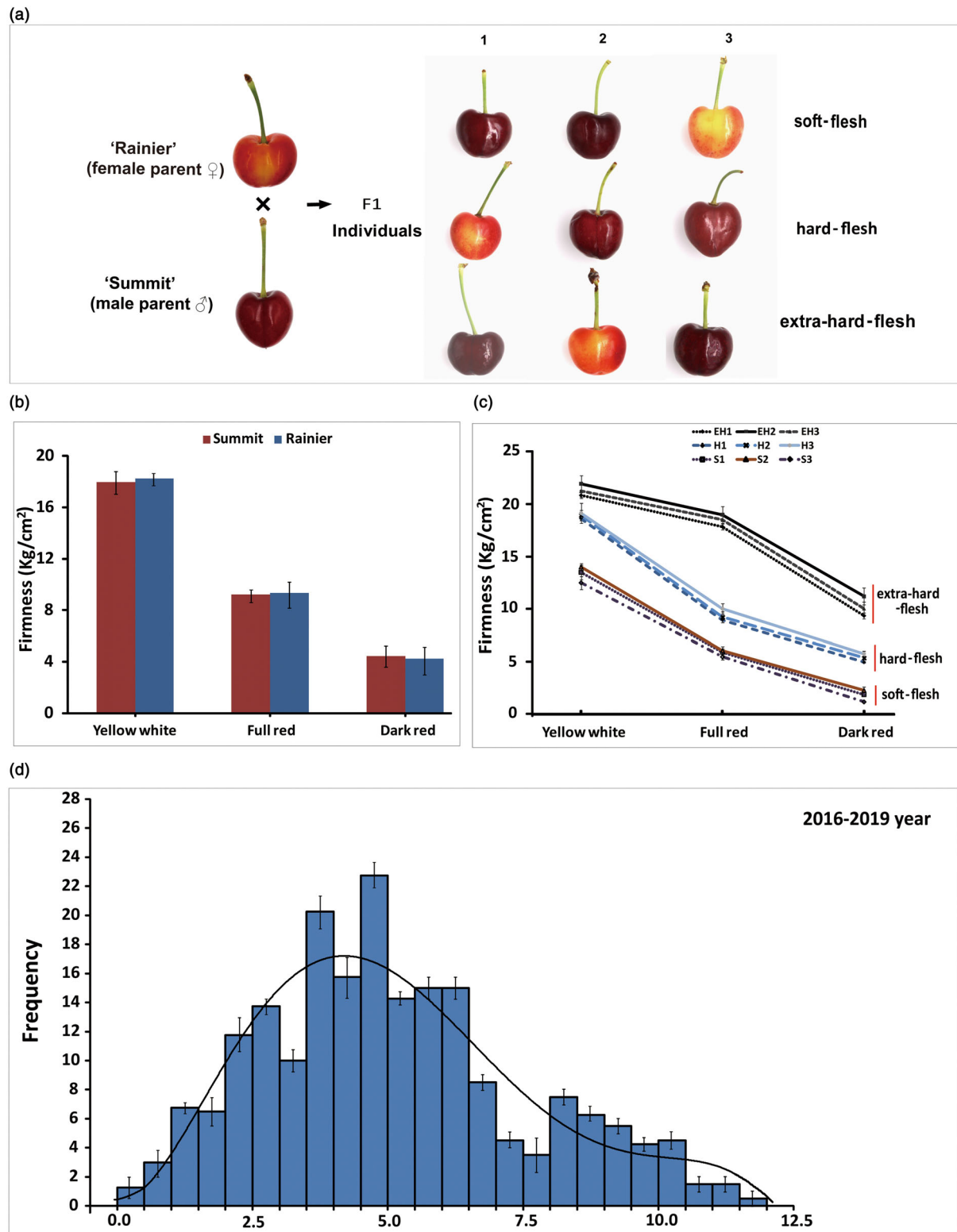


Figure 1 Phenotypic characterization and statistical analysis of sweet cherry fruit firmness in the 'Rainier' (hard-flesh fruit) × 'Summit' (hard-flesh fruit) F₁ population. (a) Fruit phenotypes of the parents 'Rainier' × 'Summit' and F₁ individuals derived from the 'Rainier' × 'Summit' cross at dark red (DR) or dark colouring stages (double-colour fruit). 1, 2 and 3 represent three different individuals of three fruit firmness types (extra-hard-flesh, hard-flesh and soft-flesh fruits, respectively) in the F₁ population. (b) Fruit firmness of two parents ('Rainier' and 'Summit') assessed at yellow-white (YW), full red (FR) and dark red (DR) stages. (c) Fruit firmness of three extra-hard-flesh fruit individuals (EH 1, EH 2 and EH 3), three hard-flesh fruit individuals (H 1, H 2 and H 3) and three soft-flesh fruit individuals (S 1, S 2 and S 3) from F₁ population assessed at YW, FR and DR stages. Data are mean ± SD of three independent experiments. (d) Frequency distribution of fruit firmness in the F₁ population exhibiting a continuous and bimodal distribution with a distinct separation of the two groups of individuals in 2016–2019.

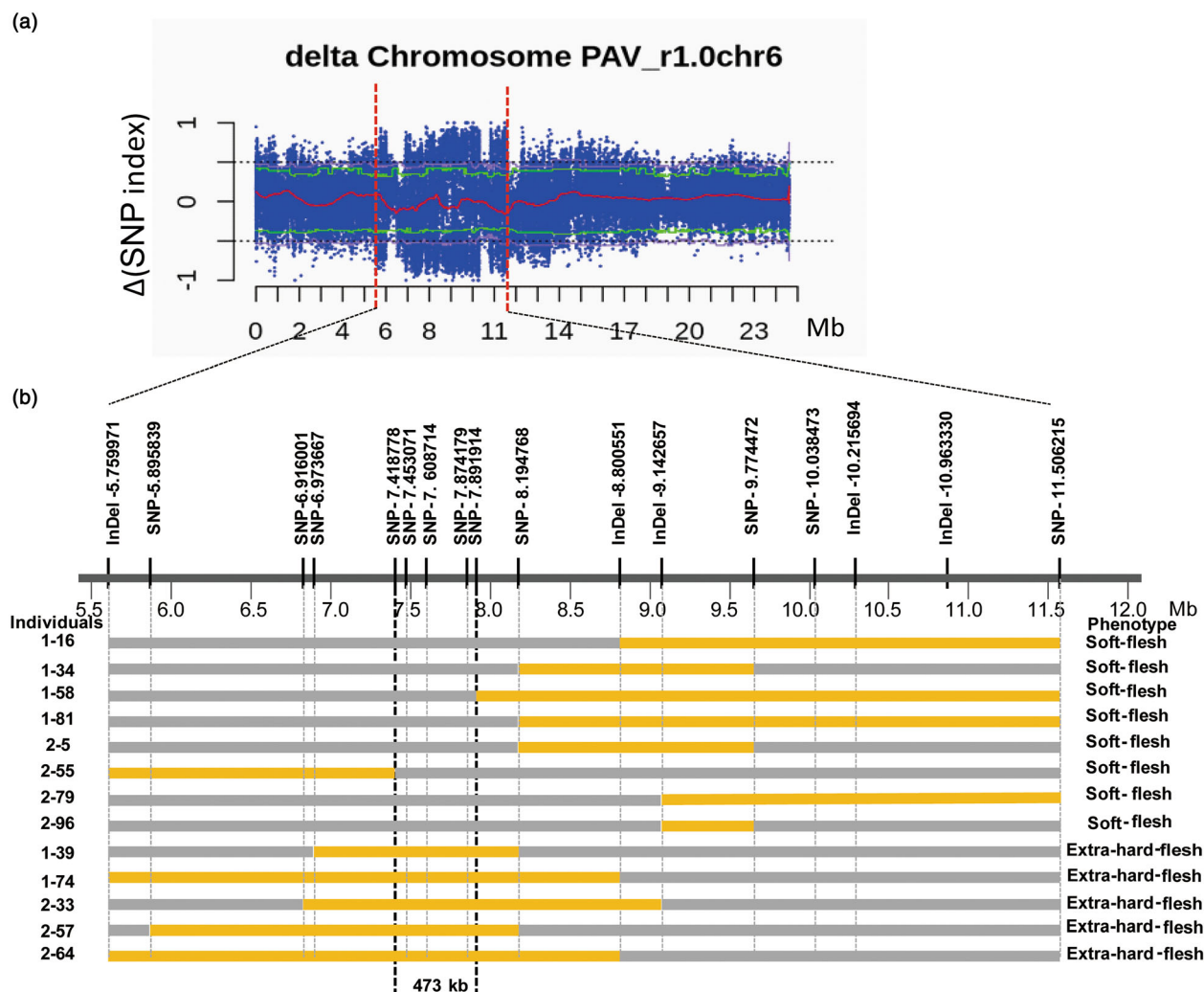


Figure 2 Identification of the candidate region for fruit firmness by BSA-seq analysis and fine-mapping in the 'Rainier' × 'Summit' F_1 population. (a) Initial mapping of the candidate region using BSA-seq by calculation of the $\Delta(\text{SNP index})$ value. (b) Fine-mapping of the fruit firmness locus. The recombinant individuals were screened using the 15 SNP and InDel markers listed above the figure. The F_1 individuals are listed to the left of the figure, and the fruit firmness phenotype is specified to the right of the figure. Yellow and grey shading represent chromosome fragments corresponding to the extra-hard-flesh fruit and soft-flesh fruit phenotypes, respectively. The candidate region was delimited to a 473-kb region on chromosome 6 between the markers SNP-7.418778 and SNP-7.891914. Mb, million bases.

phenotypes for 4 consecutive years. On the basis of BSA-seq results, 15 polymorphic InDel and SNP markers (Figure 2b; Table S3) located in the interval between the markers InDel-5.759971 and SNP-11.506215 were newly developed. These markers were located in the candidate region of chromosome 6 and differed between the Extra-hard-pool and Soft-pool bulks. The markers were subsequently used to genotype the 204 F_1 individuals. We identified 13 recombinant individuals using these polymorphic markers. Thus, the candidate locus was narrowed down to a 473-kb region (7 418 778–7 891 914 bp) on chromosome 6 between the markers SNP-7.418778 and SNP-7.891914; this region was designated q-FF6.1 (Figure 2b).

PavSCPL, encoding a serine carboxypeptidase-like protein, is the candidate gene for fruit firmness

To evaluate the possible molecular mechanisms for differences in sweet cherry fruit firmness, a comparative transcriptome

sequencing analysis was conducted between individuals with extra-hard-flesh fruit and those with soft-flesh fruit to identify candidate genes that control fruit firmness. Based on the sweet cherry genomic database (<http://cherry.kazusa.or.jp/map.html>; Shirasawa *et al.*, 2017), 72 genes were annotated or predicted within the q-FF6.1 genomic region (Table S4; Figure 3). Subsequently, the transcript abundance of these genes was assessed by performing RNA-sequencing (RNA-seq) and quantitative RT-PCR (qRT-PCR) analyses of extra-hard-flesh fruit and soft-flesh fruit individuals. The transcript levels of only two genes (Pav_sc0000480.1_g920.1.mk and Pav_sc0000480.1_g990.1.mk) differed significantly between extra-hard-flesh fruit and soft-flesh fruit individuals (Figure 3a). The gene Pav_sc0000480.1_g990.1.mk (designated as PavXTH14, Zhai *et al.*, 2021) showed more than 6.1-fold differences in its transcript levels between extra-hard-flesh fruit and soft-flesh fruit individuals (Table S4; Figure 3a). In particular, transcripts of Pav_sc0000480.1_g920.1.mk were not detected in the extra-hard-flesh fruit individuals, but its transcript levels

were high in the soft-flesh fruit individuals. The Pav_sc0000480.1_g920.1.mk gene was located at bp 7 611 383 to 7 613 903 on Chr 6 in the sweet cherry genome. Its 1521-bp coding sequence encoded a serine carboxypeptidase-like family protein consisting of 506 amino acid residues. The phylogenetic analysis revealed that 506 amino acid residues were most similar to peach PpSCPL, plum blossom PmSCPL, pear PbSCPL and apple MdSCPL and clustered with other SCPL proteins in the same clade, including FvSCPL, ZjSCPL, VvSCPL and DzSCPL48 (Figure S7A). Thus, Pav_sc0000480.1_g920.1.mk was identified as a likely candidate gene for the q-FF6.1 locus and was designated as *PavSCPL* (Figure S3).

Given that *PavSCPL* was not expressed in extra-hard-flesh fruit individuals and *PavXTH14* was highly expressed in soft-flesh fruit individuals, the genomic DNA sequences for *PavSCPL* and *PavXTH14*, including the promoter and coding region sequences, were amplified using gene-specific primers (Table S3). The sequences were compared between the extra-hard-flesh fruit and soft-flesh fruit individuals. The *PavSCPL* and *PavXTH14* promoter sequences and the *PavXTH14* coding region sequences were not different between extra-hard-flesh fruit individuals and soft-flesh fruit individuals (Figure S4). However, a 5244-bp (5.2-kb) insertion in the second exon of *PavSCPL* (where the full *PavSCPL* sequence included nine exons and eight introns) was identified in the extra-hard-flesh fruit individuals (Figure 3b). Therefore, we selected *PavSCPL* as the possible candidate gene for the q-FF6.1 locus and hypothesized that increased fruit firmness may be associated with the 5.2-kb sequence insertion in *PavSCPL* in the extra-hard-fleshed sweet cherry individuals.

Co-segregation analysis of *PavSCPL* and allelic variation with a 5.2-kb insertion in the coding region associated with fruit firmness in the F₁ population and different sweet cherry accessions

Based on the 5.2-kb sequence (Table S5) insertion in *PavSCPL* in extra-hard-flesh fruit individuals, we developed four co-dominant gene-specific primers, PavSCPL-1-F/R and PavSCPL-2-F/R (Table S3), to further ascertain that the 5.2-kb sequence insertion influences the fruit firmness phenotype in the F₁ population. Three genotypes were designated, namely the homozygous insertion genotype [*Pavscpl*^{5.2-kb}/*Pavscpl*^{5.2-kb}(AA)], heterozygous insertion genotype [*Pavscpl*^{5.2-kb}/*PavSCPL*(Aa)] and no-insertion genotype [*PavSCPL*/*PavSCPL*(aa)]. Forty-eight individuals were classified as having extra-hard-flesh fruit, with the *Pavscpl*^{5.2-kb}/*Pavscpl*^{5.2-kb}(AA) genotype; 103 individuals were classified as having hard-flesh fruit, with the *Pavscpl*^{5.2-kb}/*PavSCPL*(Aa) genotype; and 53 individuals were classified as having soft-flesh fruit, with the *PavSCPL*/*PavSCPL*(aa) genotype. This was entirely consistent with the fruit firmness phenotype in the F₁ population, with an accuracy of 100% (Table S6). This result suggested that *PavSCPL* genotypes co-segregated with the fruit firmness phenotype (Figures 4a and S5). Furthermore, the PavSCPL-1- and PavSCPL-2-specific primer pairs were used to screen the *PavSCPL* genotype in 118 accessions of sweet cherry. We detected two *Pavscpl*^{5.2-kb}/*Pavscpl*^{5.2-kb}(AA) genotypes; 20 *Pavscpl*^{5.2-kb}/*PavSCPL*(Aa) genotypes, which co-segregated with the fruit firmness phenotype; and 96 *PavSCPL*/*PavSCPL*(aa) genotypes, which included nine accessions with a *PavSCPL* genotype inconsistent with the fruit firmness phenotype, i.e., accuracy of 70.97% (Figures 4b and S6; Table S7). These results confirmed that the PavSCPL-1- and PavSCPL-2-specific primer pairs could be used as diagnostic genotyping markers to predict the fruit firmness phenotype through MAS.

Expression pattern of *PavSCPL* in relation to fruit softening

To characterize the function of *PavSCPL* gene, a subcellular localization analysis was conducted to visualize the transient expression of *PavSCPL* fused with the green fluorescent protein gene (*PavSCPL-GFP*) in *Nicotiana benthamiana* leaves. The *PavSCPL-GFP* fluorescence signal was observed in the apoplast (Figure S7B; Conley et al., 2009), while that of free GFP was uniformly distributed throughout the whole cell (Figure S7B). To further determine whether *PavSCPL* is associated with fruit softening during fruit development and ripening in sweet cherry, the expression patterns of *PavSCPL* were investigated in the leaf, flower bud, blossom and fruit at different developmental stages [small green, big green, initial degreening, yellow-white, initial red or initial colouring (blush type), full red or full colouring (blush type) and dark red or orange-red (blush type)] for three accessions that differed in *PavSCPL* genotype, namely 'Chunlu' [*PavSCPL*/*PavSCPL*(aa)], 'Summit' [*Pavscpl*^{5.2-kb}/*PavSCPL*(Aa)] and 'Ruby' [*Pavscpl*^{5.2-kb}/*Pavscpl*^{5.2-kb}(AA)]. At the same time, the fruit firmness of 'Chunlu', 'Summit' and 'Ruby' during different fruit developmental stages (initial degreening, yellow-white, initial red, full red and dark red) was measured (Figure 5a). *PavSCPL* was ubiquitously expressed in all analysed tissues in 'Chunlu' and 'Summit', and was most highly expressed in the fruit, followed by the flower bud and blossom. However, *PavSCPL* expression was not detected in various organs of 'Ruby' (Figure 5b), consistent with 'Ruby' belonging to the *Pavscpl*^{5.2-kb}/*Pavscpl*^{5.2-kb}(AA) genotype, in which the *PavSCPL* gene was completely inactivated by the 5.2-kb sequence insertion (Figures 4b and S6; Table S7). The transcript level of *PavSCPL* remarkably and gradually increased during fruit development and ripening in 'Chunlu' and 'Summit', with transcript levels reaching the maximum at the fruit full red stage, and remaining high thereafter. The expression levels of *PavSCPL* in the leaf, flower and fruit of hard-flesh fruit and soft-flesh fruit individuals were further evaluated, and similar *PavSCPL* expression patterns were observed (Figure 5c).

Silencing of *PavSCPL* improved fruit firmness by influencing pectin degradation in sweet cherry

To further examine the physiological function of *PavSCPL* in sweet cherry, Tobacco rattle virus (TRV)-mediated VIGS technology was used to downregulate the expression of *PavSCPL* in individuals with a soft-flesh fruit genotype. Compared with the control fruit transfected with the empty TRV vector, the expression of *PavSCPL* was significantly reduced by 74.52% in TRV-*PavSCPL*-infected fruit at 15 days post-inoculation (dpi) (Figure 6a). Phenotypic investigation and fruit firmness measurement revealed a significant increase in fruit firmness in *PavSCPL*-silenced fruit compared with that of the control fruit at 21 and 28 dpi (Figure 6b,c). Collectively, these results show that the knockdown of *PavSCPL* significantly enhanced fruit firmness, consistent with the fruit firmness characteristics of the extra-hard-flesh fruit individuals of the F₁ population.

To investigate how *PavSCPL* affected fruit softening, the activities of cell wall modification enzymes, pectin methylesterase (PME), endo-polygalacturonase (PG), β -galactosidase (β -Gal), pectate lyase (PL) and α -L arabinofuranosidase (α -AF), as well as the transcript abundance of softening-related genes were estimated in *PavSCPL*-silenced and control fruit. Interestingly, the activities of total PME and PG were significantly reduced in *PavSCPL*-silenced fruit at 21 and 28 dpi compared with those of

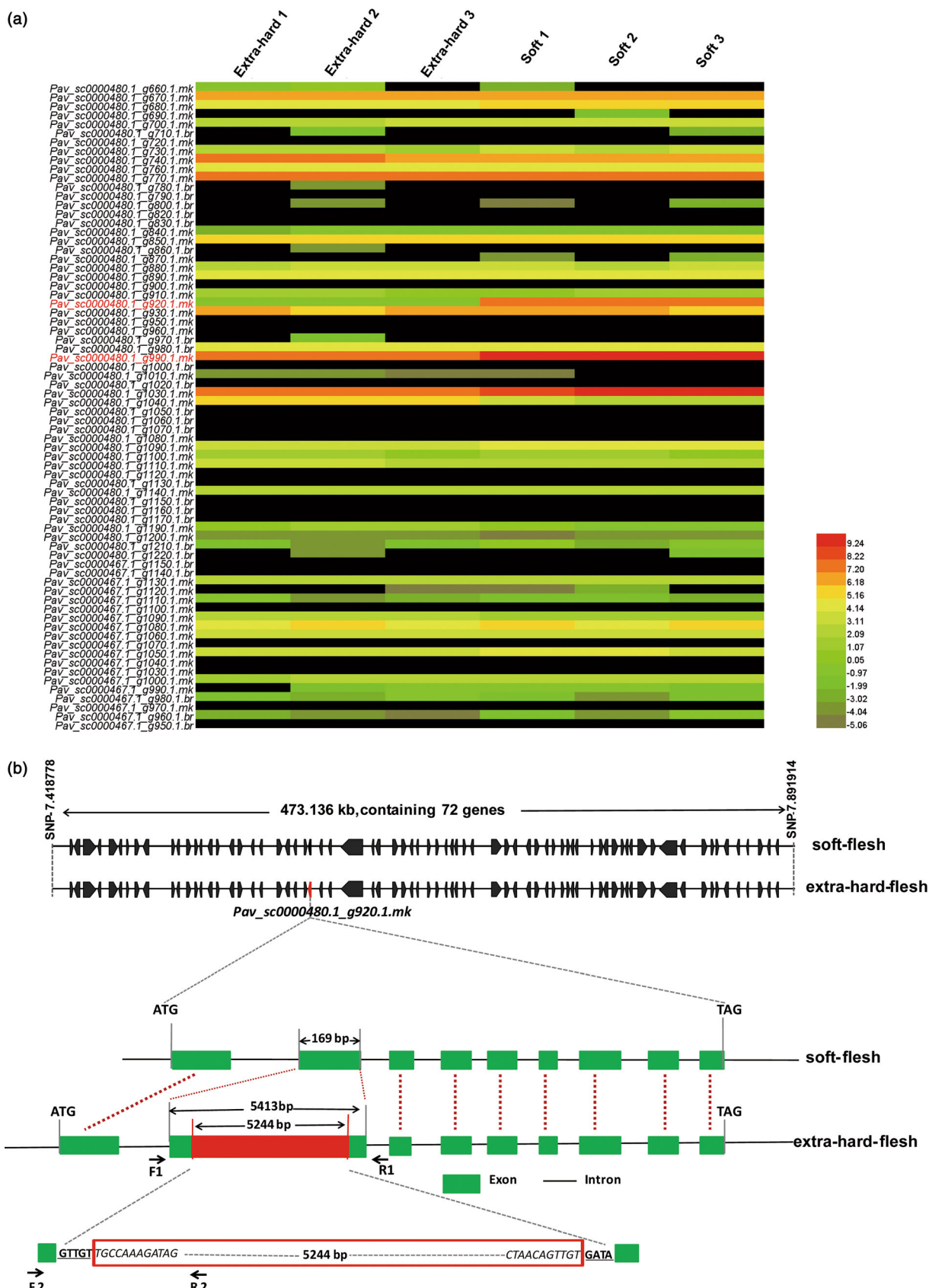


Figure 3 Identification and validation of the candidate gene controlling fruit firmness. (a) Expression analysis of 72 genes located in the 473-kb genomic DNA region in the interval between SNP-7.418778 and SNP-7.891914 (upper element in b), based on the *Prunus avium* reference genome, in the Hard-pool and Soft-pool groups determined by RNA-sequencing and quantitative RT-PCR analyses, respectively. (b) Gene structure of *PavSCPL* and its allele. The exon–intron structure of *PavSCPL* and its allele in the extra-hard-flesh fruit and soft-flesh fruit phenotypic groups is shown in the middle element. Green boxes represent exons and a black line between exons indicates an intron. ‘F1/R1’ and ‘F2/R2’ indicate the developed AFLP markers *PavSCPL*-1-F/R and *PavSCPL*-2-F/R based on the two *PavSCPL* alleles. The structure and sequence of the 5.2-kb transposable element insertion are listed in Table S5.

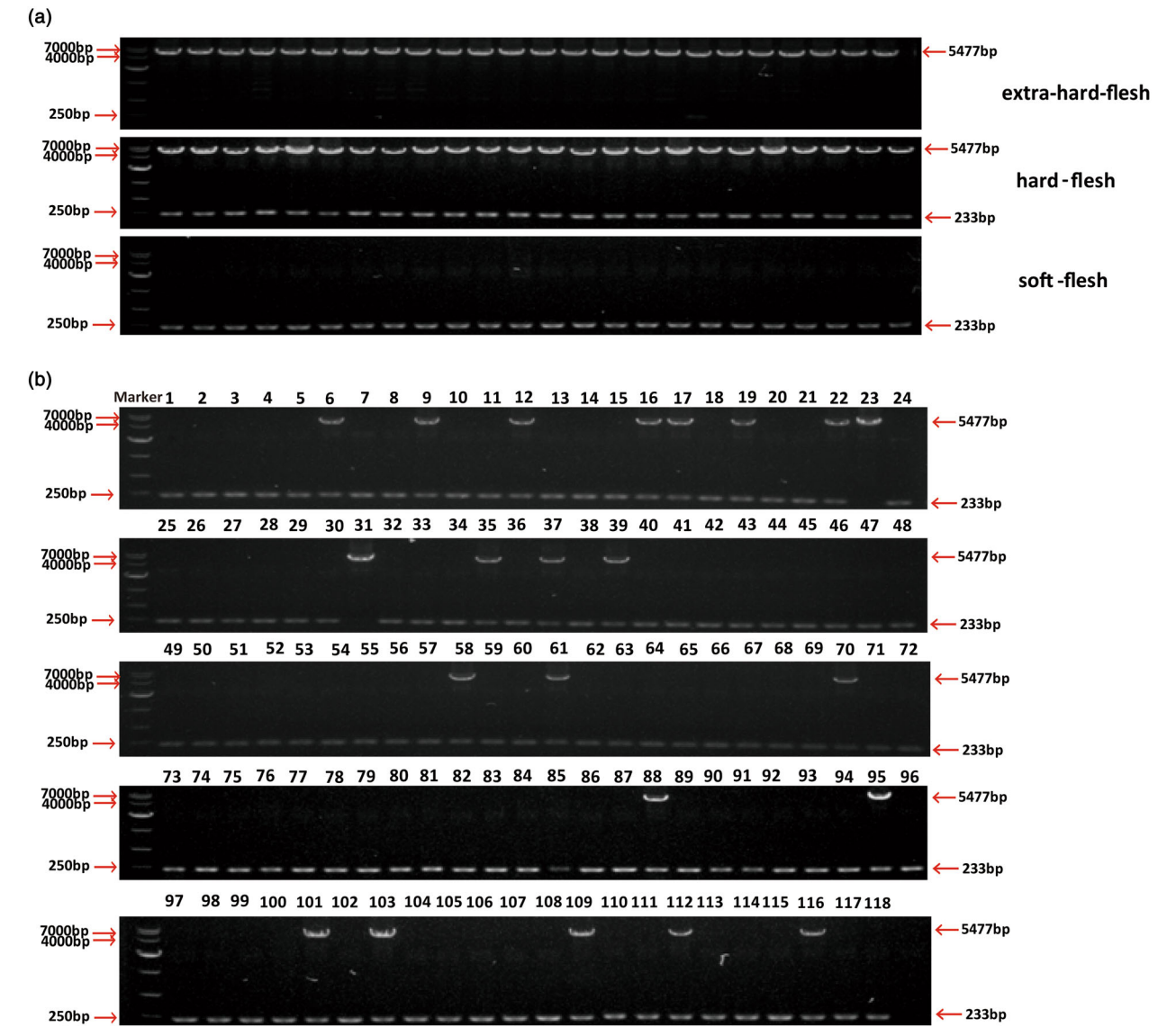


Figure 4 Co-segregation analysis for genotyping of the *PavSCPL* alleles with fruit firmness using the AFLP marker *PavSCPL*-1-F/R in the ‘Rainier’ × ‘Summit’ F₁ population and 118 sweet cherry accessions. (a) PCR analysis of three fruit firmness phenotypes (extra-hard-flesh, hard-flesh and soft-flesh fruits) in the F₁ population corresponding to three genotypes: Homozygous insertion genotype, AA (*Pavscpl*^{5.2-kb}/*Pavscpl*^{5.2-kb}, only a 5477-bp long fragment); heterozygous insertion genotype, Aa (*Pavscpl*^{5.2-kb}/*PavSCPL*, two fragments, a 5477-bp long fragment and a 233-bp short fragment); and no-insertion genotype, aa (*PavSCPL*/*PavSCPL*, only a 233-bp short fragment) based on *PavSCPL* and *Pavscpl*^{5.2-kb} alleles using the AFLP marker *PavSCPL*-1-F/R. (b) *PavSCPL* genotype analysis in sweet cherry 118 accessions by PCR using the AFLP marker *PavSCPL*-1-F/R. Number corresponding to each individual sweet cherry accession is listed in Table S7.

expression levels of *PavPME* (encoding pectin methylesterase), *PavPG* (encoding polygalacturonase), *PavXTH14* (encoding a xyloglucan endotransglycosylase/hydrolase) and *PavEXPA2* (encoding an expansin) in *PavSCPL*-silenced fruit were significantly lower than those in the control fruit at both 21 and 28 dpi (Figure 6e). Compared with the control fruit, a noteworthy increase in expression of *PavPGI* (encoding a polygalacturonase inhibitor) was observed in the *PavSCPL*-silenced fruit at 21 and 28 dpi. No distinct difference was detected in the expression level of *PavCEL8* (encoding endo-

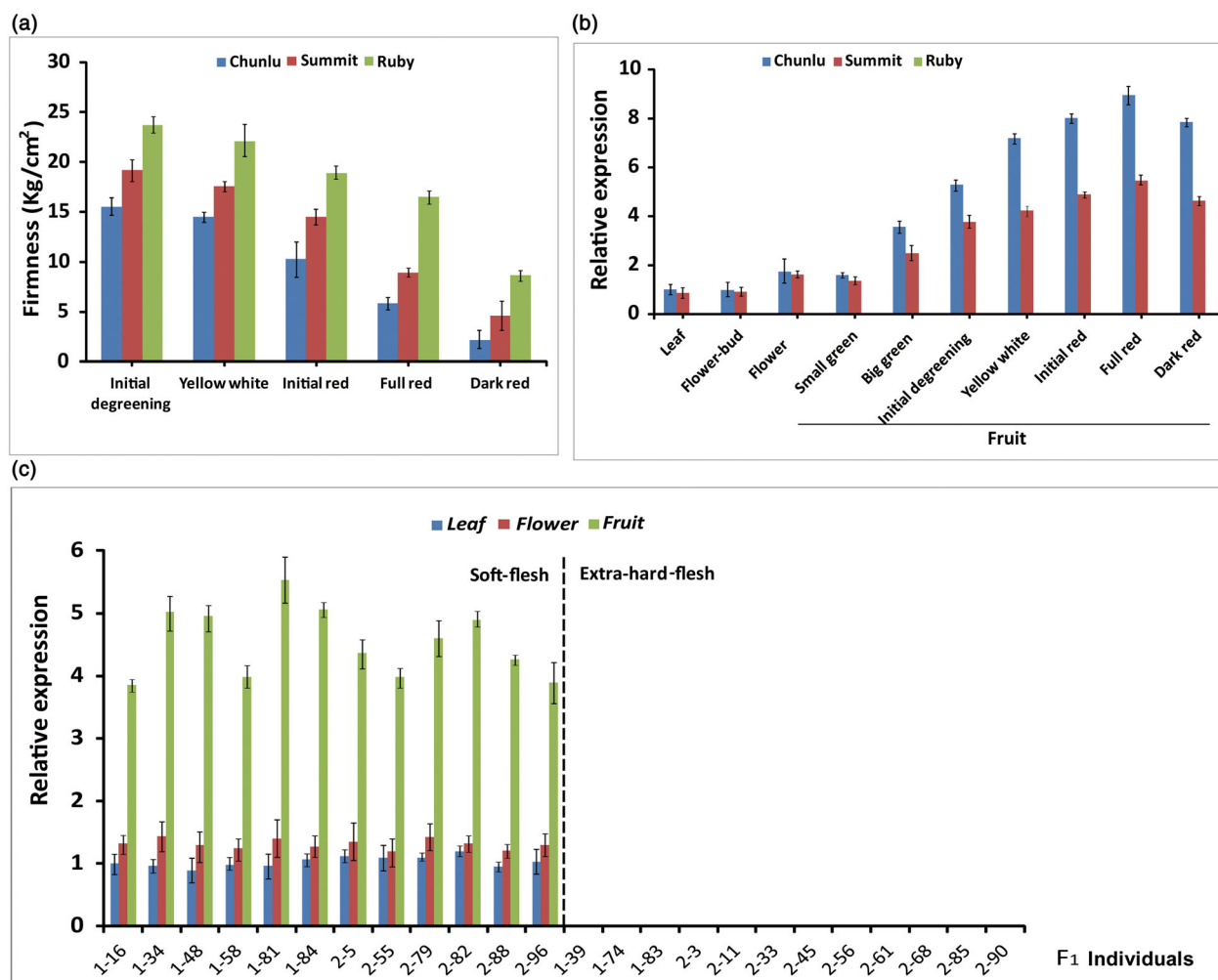


Figure 5 Characterization of the *PavSCPL* gene. (a) The fruit firmness of ‘Chunlu’, ‘Summit’ and ‘Ruby’ during different fruit developmental stages (initial degreening, yellow-white, initial red, full red and dark red). Data are the mean $\pm$ SD from three biological replicates. (b) Expression patterns of *PavSCPL* in the leaf, flower and fruit at different developmental stages of three sweet cherry accessions differing in fruit firmness phenotype: ‘Chunlu’ (soft-flesh fruit), ‘Summit’ (hard-flesh fruit) and ‘Ruby’ (extra-hard-flesh fruit). Data are the mean $\pm$ SD from three independent replicates. (c) Quantitative real-time PCR analysis of *PavSCPL* expression in F₁ individuals with soft-flesh fruit or extra-hard-flesh fruit at the mature fruit stage. *Pavactin* (*Pav_sc0002247.1_g030.1.mk*) was used as the internal control. Data are the mean $\pm$ SD of three independent experiments.

glucanase 8) between *PavSCPL*-silenced fruit and the control fruit at either 21 or 28 dpi (Figure 6e).

Discussion

Among the major fruit-quality traits in commercial sweet cherry, fruit firmness is important for the grower, distributor and consumer and influences the fruit transportability and shelf life. Recent studies on sweet cherry have mapped several QTLs associated with fruit firmness on LG1 (*qP-FF1.1* and *qP-FF1.2*), LG2 (*qP-FF2.1*), LG4 (*qP-FF4.1*) and LG6 (*qP-FF6.1*) in the different F₁ sweet cherry populations. However, the QTLs for the sweet cherry fruit firmness trait on various LGs showed diversity among different F₁ populations. For example, using the ‘Ambrunés’ $\times$ ‘Sweetheart’ F₁ population, *qP-FF1.1*, *qP-FF1.2* and *qP-FF6.1* were identified, while only *qP-FF4.1* was identified in the ‘Fercer’ $\times$ ‘X’ (an unknown parent) F₁ population (Cai *et al.*, 2019; Calle *et al.*, 2020; Calle and Wünsch, 2020; Campoy *et al.*, 2015). Meanwhile, several significant SNPs associated with

the fruit firmness trait have been identified and localized with QTLs *qP-FF1.1*, *qP-FF1.2*, *qP-FF4.1* and *qP-FF6.1* in GWASs with 116 or 235 sweet cherry accessions (Donkpegan *et al.*, 2023; Holušová *et al.*, 2022), especially *qP-FF4.1* (approximately 1.8 Mbp, 12 928 603–14 860 789 bp). Despite the identification of numerous SNP markers for the sweet cherry fruit firmness trait, few have been successfully applied to breeding for fruit firmness (Calle *et al.*, 2020; Calle and Wünsch, 2020). To date, the candidate genes that influence fruit firmness have not been identified and cloned from QTLs (Calle and Wünsch, 2020).

In the present study, a major QTL for fruit firmness, *qP-FF6.1*, was fine-mapped to a 470-kb region on Chr 6, which co-located with the QTLs *qP-FF6.1* identified by Calle *et al.* (2020) and *qFirm-LG6.2* identified by Crump *et al.* (2022). Both Holušová *et al.* (2022) and Donkpegan *et al.* (2023) identified SNPs associated with fruit firmness-related traits at this locus on chromosome 6. Furthermore, the *PavSCPL* gene at the *qP-FF6.1* locus, which encodes a serine carboxypeptidase-like protein, was

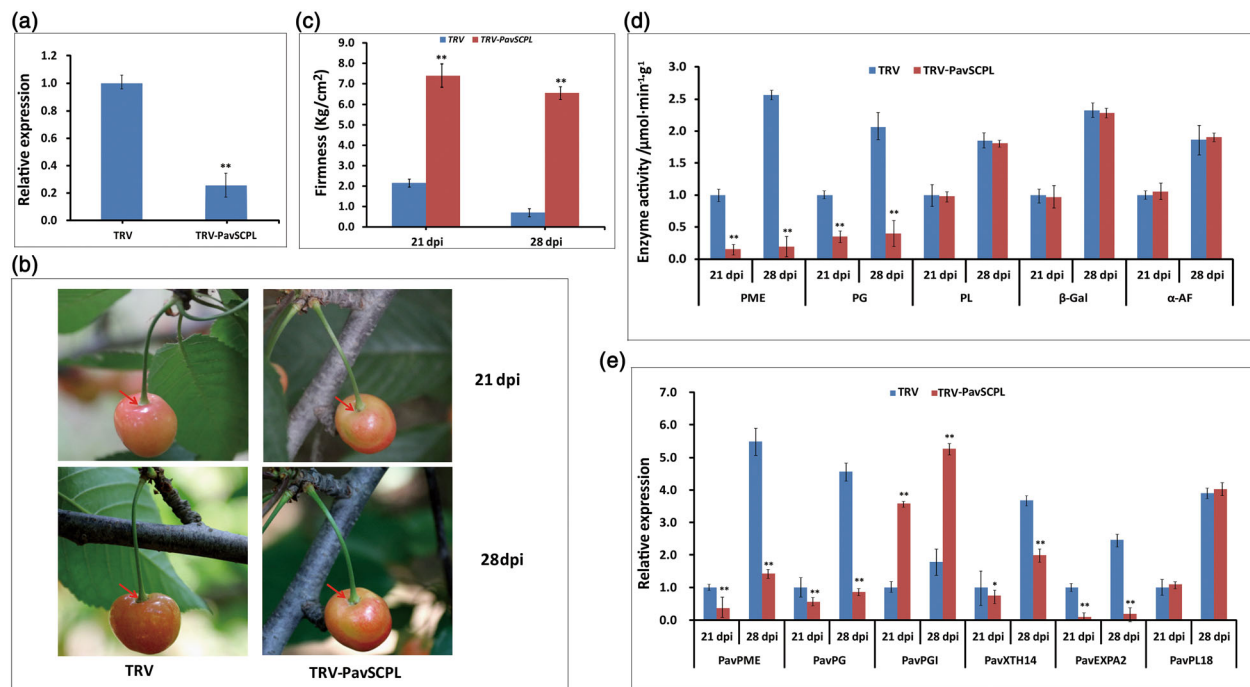


Figure 6 Virus-induced silencing of *PavSCPL* improved sweet cherry fruit firmness and suppressed pectin degradation. (a) *PavSCPL* transcript abundance relative to that of *Pavactin* in *PavSCPL*-silenced fruit and control fruit at 15 days post-infiltration (dpi), as detected by qRT-PCR. (b) Phenotypes of *PavSCPL*-silenced fruit and control fruit at 21 and 28 dpi. The red arrow indicates the location of vector injection. (c) Fruit firmness of *PavSCPL*-silenced and control fruit at 21 and 28 dpi. (d) Change in activities of the cell wall modification enzymes pectin methyl esterase (PME), polygalacturonase (PG), β-galactosidase (β-GAL), pectate lyase (PL) and α-L arabinofuranosidase (α-AF) in *PavSCPL*-silenced fruit at 21 and 28 dpi. (e) Expression analysis of the cell wall metabolism-related genes *PavPME* (Pav_sc0000130.1_g870.1.mk), *PavPG* (Pav_sc0000254.1_g1300.1.mk), *PavPGI* (Pav_sc0005028.1_g040.1.mk), *PavXTH14* (Pav_sc0000480.1_g990.1.mk), *PavEXPA2* (Pav_co4016743.1_g010.1.br) and *PavPL18* (Pav_sc0000229.1_g150.1.mk) in *PavSCPL*-silenced fruit at 21 and 28 dpi. Data are the mean ± SD of six biological replicates. Statistical significance was determined using Student's t-test. ** *P*-value < 0.01.

identified as having a novel regulatory role in fruit firmness in sweet cherry. *PavSCPL* and the *Pavscpl*^{5.2-k} allele, which harbours a 5.2-kb insertion in the *PavSCPL* coding region, co-segregated with the fruit firmness phenotype among 118 sweet cherry accessions, with an accuracy of 70.97%. We speculated that the *Pavscpl*^{5.2-k} allele, which is specifically present in sweet cherry accessions, is strongly associated with firmer fruit in sweet cherry germplasm (Figures 4b and S6; Table S7).

Serine carboxypeptidase-like proteins are members of the peptidase S10 family, which share a highly conserved α/β hydrolase tertiary structure with PF00450 as the representative domain in the InterPro online database (<https://www.ebi.ac.uk/interpro/>), and are widespread among higher plants. The SCPL proteins contain a conserved catalytic triplet composed of three amino acid residues—a serine, an aspartate and a histidine (Ser-Asp-His) (Mugford and Milkowski, 2012)—that participates in transacylation reactions in plant secondary metabolism pathways (Weier et al., 2008). Members of the SCPL class have been identified and characterized in wheat (*Triticum aestivum*) (Xu et al., 2021), rapeseed (*Brassica napus*) (Liu et al., 2022), grape (*Vitis vinifera*) (Wang et al., 2021), cotton (*Gossypium hirsutum*) (Wang et al., 2022) and tea (*Camellia sinensis*) (Ahmad et al., 2020). Previous studies have ascertained that SCPLs play essential functions in determining seed weight and size (Dong et al., 2023; Li et al., 2011), signal transduction (Xu et al., 2015), development and senescence of organs (Cercós et al., 2003; Domínguez et al., 2002), plant abiotic stress responses (Xu et al., 2021) and plant disease resistance (Mugford et al., 2009;

Wang et al., 2022). For instance, a SCPL protein GS5 positively regulates grain size by promoting mitosis in *Oryza sativa* (Li et al., 2011). In rapeseed, SCPL19 influences seed weight through abscisic acid signalling (Dong et al., 2023). In cotton, GhSCPL42 plays a crucial role in resistance to *Verticillium* wilt through its involvement in the salicylic acid and jasmonic acid signalling pathways (Wang et al., 2022). The present results revealed that *PavSCPL* played a critical role in controlling fruit firmness during fruit ripening and softening in sweet cherry, which enriches the functions of SCPL proteins in fruit growth and development. Given the rapid development of genomics, transcriptomics and metabolomics, we strongly believe that the molecular functions of more SCPLs will be characterized and unravelled in future studies on account of the acyltransferase activity of SCPLs.

Fruit ripening and softening is a coordinated complex developmental and process, particularly in climacteric fruits, such as those of *Prunus* species, which have a short ripening period and a fast softening period (García-Gómez et al., 2020, 2021). Numerous studies on fruit firmness or softening have focused on the roles of cell wall-modifying enzymes (Atkinson et al., 2012; García-Gómez et al., 2020, 2021; Phan et al., 2007; Shi et al., 2022; Zhang et al., 2022); softening-related transcription factors, such as members of the MADS-box and NAC (NAM, ATAF and CUC) families, which directly regulate cell-wall-modifying genes (Fujisawa et al., 2011; Gao et al., 2020; García-Gómez et al., 2021; Qi et al., 2022; Shi et al., 2022); and ethylene/abscisic acid biosynthetic genes (Jia et al., 2011; Shi

et al., 2022; Seymour, Poole, et al., 2013). Interestingly, in the present study, a serine carboxypeptidase-like protein, PavSCPL, was shown to play a vital function in controlling fruit firmness by BSA-seq and fine-mapping analysis, and this is the first report of a SCPL-encoding candidate gene involved in the control of fruit firmness among perennial Rosaceae crops. Furthermore, silencing of PavSCPL increased sweet cherry fruit firmness, simultaneously reduced the activities of total PME and PG enzymes, and downregulated the expression of the corresponding genes (Figure 6). Pectin modification enzymes, such as PMEs, PGs and PLs, are responsible for fruit softening in tomato and in Rosaceae crops, such as apple, plum, cherry, peach and strawberry (Atkinson et al., 2012; García-Gago et al., 2009; Zhang et al., 2022). We speculated that the alteration of pectin degradation in the fruit might be responsible for the increase in fruit firmness caused by the knockdown of PavSCPL. Therefore, further studies are necessary to determine the mechanism through which PavSCPL influences pectin-modifying enzymes at the molecular level. Another gene identified in this study within the q-FF6.1 genomic region, PavXTH14, was differentially expressed between extra-hard-flesh fruit individuals and soft-flesh fruit individuals. However, the genomic DNA sequences for PavXTH14, including promoter sequences and coding region sequences, were not different between extra-hard-flesh fruit individuals and soft-flesh fruit individuals (Figure S4). We found no functional genetic molecular markers for sweet cherry fruit firmness associated with PavXTH14. When PavSCPL was silenced in sweet cherry fruit, the expression of PavXTH14 was significantly reduced, indicating that PavXTH14 may be associated with sweet cherry fruit softening. Recently, Zhai et al. (2021) confirmed that PavXTH14 promotes fruit softening in sweet cherry (Zhai et al., 2021). Similarly, Witasari et al. (2019) found that enhancing expression of FvXTH9 and FvXTH6 accelerated strawberry fruit softening; however, they only annotated the gene functions and did not apply these genes for breeding improvement of the fruit firmness trait.

During the past decade, MAS for important agronomic traits has been practically applied in breeding programmes. This selection method can accelerate the traditional breeding process through the use of molecular markers (Laurens et al., 2018; Liu et al., 2012). In *Prunus*, MAS has been used to select for several strong candidate genes linked to fruit quality traits, such as the R2R3 MYB transcription factor gene linked to fruit colour in peach and sweet cherry, and the NAC transcription factor gene linked to ripening date in peach (García-Gómez et al., 2021). In the present study, we developed an amplified fragment length polymorphism (AFLP) marker to distinguish the two alleles of PavSCPL associated with fruit firmness for MAS in sweet cherry breeding. Similarly, the allelic variation at the PpYUC11-like locus has been ascertained to control the 'stony hard' trait, a recessive monogenic trait, in peach (Cirilli et al., 2018; Pan et al., 2015). In tomato, a GA2-oxidase FIS1 allele was selected during tomato domestication for the improvement of fruit firmness (Li et al., 2020). We suspected that the extra-hard-flesh fruit trait in sweet cherry may be controlled by a single gene that mutated during domestication—such a gene would provide a valuable resource for the genetic improvement of cultivars. In addition, the PavSCPL and Pavscpl alleles co-segregated with the fruit firmness phenotype, which enabled prediction, with 70.97% accuracy, of the fruit firmness variation among 118 sweet cherry accessions. In nine sweet cherry accessions were the fruit firmness phenotypes inconsistent with the PavSCPL genotype, implying that other genes may contribute to the fruit firmness in sweet cherry. In

apple, two functional markers, MdERF4 located on chromosome 3 and MdPG1 located on chromosome 10, explained 10%–49% of the phenotypic variation in fruit firmness; an additional two functional markers, MdERF3 and MdERF118 located on different chromosomes, for fruit postharvest firmness and crispness have been validated and used in breeding programmes to improve fruit firmness (Costa et al., 2010; Hu et al., 2020; Wu et al., 2021). These results indicate that the fruit firmness-related trait is a highly complex trait that is controlled by multiple factors, such as genetic factors, and environmental factors. Moreover, as sweet cherry accessions are becoming more abundant, the percentage of PavSCPL and Pavscpl alleles co-segregated with the fruit firmness phenotype will be more accurate among the wider sweet cherry germplasm. However, our findings demonstrate that a single gene, PavSCPL, with a 5.2-kb insertion, has led to firmer fruit in the F₁ population derived from the cross 'Rainier' × 'Summit'. The AFLP markers of PavSCPL associated with fruit firmness developed in this study, as well as several SNPs markers associated with fruit firmness-related traits identified by Holušová et al. (2022) and Donkpegan et al. (2023) will be used for MAS in breeding improvement of the fruit firmness trait.

In summary, we fine-mapped a major QTL for fruit firmness to a 473-kb region on chromosome 6 in sweet cherry using BSA-seq and fine-mapping. The candidate gene PavSCPL encoded a serine carboxypeptidase-like protein, which was confirmed to play a critical role in the regulation of fruit firmness. The functional alleles of PavSCPL could be implemented in MAS to improve the efficiency of sweet cherry breeding programmes by the early selection of new genotypes with very firm fruit. To the best of our knowledge, this is the first report of fine-mapping and functional confirmation of a candidate gene controlling fruit firmness in sweet cherry. The identification of PavSCPL provides new insights into the genetic regulation of fruit firmness in sweet cherry, and is a novel and invaluable genetic resource with the potential for improvement of fruit firmness in other fruit trees.

Experimental procedures

Plant materials

An F₁ sweet cherry population ($N = 213$) was derived from the cross 'Rainier' (Origin: Washington State University, USA). Parentage: 'Bing' × 'Van'. Fruit characteristics: slightly obovate, large, firm, blush type. Quero-García et al. (2017) × 'Summit' (Origin: Summerland, Canada). Parentage: 'Van' × 'Sam'. Fruit characteristics: heart-shaped, very large, medium firm, red. Quero-García et al. (2017) in 2009 and grown at the Fruit Experimental Station of the Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Sciences (ZFRI, CAAS), located in Xinxiang, Henan, China. The trees were planted at a density of 2.5 m × 1.0 m. In total, 204 surviving F₁ individuals were selected for investigation of fruit firmness at the mature fruit stage from 2016 to 2019. The fruit firmness of two parents ('Rainier' and 'Summit'), three extra-hard-flesh fruit individuals (EH 1, EH 2 and EH 3), three hard-flesh fruit individuals (H 1, H 2 and H 3) and three soft-flesh fruit individuals (S 1, S 2 and S 3) from the F₁ population was assessed at the yellow-white, full red or full colouring (blush type) and dark red or orange-red (blush type) stage from 2016 to 2019 (García-Gómez et al., 2020, 2021; Zhai et al., 2022). In addition, 118 sweet cherry accessions were used as a natural segregating population for marker validation (Table S7), which were maintained and grown in the cherry germplasm resource platform of the National Horticultural

Germplasm Repository, ZFRI, CAAS, located in Zhengzhou, Henan, China. The fruit firmness of them was assessed at the yellow-white, full red or full colouring (blush type) and dark red or orange-red (blush type) stage from 2016 to 2018 and 2020 to 2022. Accessions comprised commercial and older historic cultivars released in Canada, the USA, Japan, Ukraine and other countries in Europe, and cultivars bred and released by Chinese breeding programmes more or less recently. The Chinese accessions represented about 40% of the 118 sweet cherry accessions. For VIGS, fruit from the same 10-year-old tree of 'Heishatangjin' [*PavSCPL/PavSCPL(aa)*] were used. The leaves, blossom buds and flowers were collected from 'Chunlu', 'Summit' and 'Ruby' for RNA extraction, and fruits at seven different developmental stages [small green, big green, initial degreening, yellow-white, initial red or initial colouring (blush type), full red or full colouring (blush type) and dark red or orange-red (blush type)], corresponding to 7, 17, 21, 25, 31, 36 and 42 days after full blooming (DAFB), respectively, for 'Chunlu'; 7, 21, 27, 32, 38, 49 and 56 DAFB, respectively, for 'Summit'; and 7, 21, 30, 35, 40, 47 and 56 DAFB, respectively, for 'Ruby' were collected for fruit firmness analysis and RNA extraction (Zhai et al., 2022). All plants were cultivated under natural conditions with standard management practices.

Phenotyping of flesh firmness

The fruit firmness of individuals in the F_1 population ($N = 204$, from 2016 to 2019) and 118 sweet cherry accessions (from 2016 to 2018 and 2020 to 2022) was measured or at the mature fruit stage using a fruit hardness tester (KM-model, Fujiyama Co., Tokyo, Japan). For each individual in the F_1 population, 60 randomly selected fruits were collected and measured with three biological and three technical replicates. For each fruit, firmness was measured at three positions and the maximum force (kg/cm^2) was recorded with three technical replicates.

Bulked segregant analysis (BSA) by resequencing and fine-mapping of the candidate fruit firmness gene

For BSA-seq, based on the phenotypic characterization of fruit firmness for the F_1 population, 21 F_1 individuals with the extreme maximum firmness phenotype (extra-hard-flesh fruit, fruit firmness $\geq 7.0 \text{ kg}/\text{cm}^2$) and an additional 21 individuals with the extreme minimum firmness phenotype (soft-flesh fruit, fruit firmness $\leq 3.5 \text{ kg}/\text{cm}^2$) were selected to generate the respective DNA bulks. The two DNA pools, Extra-hard-pool and Soft-pool, were constructed by mixing equal amounts of DNA from the F_1 individual fruits. The two DNA pools and DNA from the two parents were resequenced on an Illumina HiSeqTM PE150 platform using an approximately 350-bp library (Novogene, Beijing, China). The clean sequencing reads were aligned to the *Prunus avium* 'Satonishiki' reference genome using the Burrows–Wheeler Alignment (BWA) software. We used the Genome Analysis Toolkit (GATK 3.8; McKenna et al., 2010) to identify SNPs (filtering parameters: $-\text{clusterWindowSize } 4$, $-\text{filterExpression 'QD < 4.0 || FS > 60.0 || MQ < 40.0'}$, $-\text{G_filter 'GQ < 5'}$) and InDels (filtering parameters: $-\text{filterExpression 'QD < 4.0 || FS > 200.0'}$) that differed between the two parents and the two pools. By comparison with the reference genome sequence, the SNP-index and InDel-index of the two pools were calculated in 1-Mb intervals with a 10-kb sliding window, then $\Delta(\text{SNP index})$ and $\Delta(\text{InDel index})$ values between the Extra-hard-pool and the Soft-pool were calculated to derive a preliminary candidate interval associated with the fruit firmness trait.

For fine-mapping, 15 polymorphic SNP and InDel markers located within the preliminary major QTL interval were identified by BSA-seq and verified through polyacrylamide gel electrophoresis (PAGE) between the two bulks to screen extreme recombinant individuals from the 'Rainier' $\times$ 'Summit' F_1 population. Seventeen primer pairs, including InDel-5.759971 and SNP-11.506215, were designed with Primer Premier 5.0 software (Singh et al., 1998; Table S3). PCR amplifications were conducted using 2 $\times$ Taq Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China) in a 50- μL reaction mixture containing 1 μL of 0.5 μM forward and reverse primers and 50–100 ng DNA, according to the following programme: 10 min 95 $^{\circ}\text{C}$; 35 $\times$ 30 s 95 $^{\circ}\text{C}$, 30 s 56 $^{\circ}\text{C}$, 30 s 72 $^{\circ}\text{C}$; 10 min 72 $^{\circ}\text{C}$. The PCR products were analysed by PAGE or sequenced by Sangon Biotech Co., Ltd. (Shanghai, China). The individuals with multiple polymorphic markers were used as the recombinants and their association with fruit firmness was analysed to narrow down the candidate region.

Transcriptome analysis

Fruit from three F_1 individuals (as three biological replicates) from each of the two bulks (Extra-hard-pool and Soft-pool) were collected at the mature fruit stage for RNA-seq analysis. Sixty fruits were randomly collected from each individual and then randomly divided equally into three groups of 20 as three biological replicates. The 20 fruit in each biological replicate were mixed to generate one sample, and three technical replicates were analysed for each sample. The RNA-seq analysis was performed by LC-Bio Technology Co., Ltd. (Hangzhou, China) on an Illumina NovaseqTM 6000 platform using 150-bp paired-end reads. Total RNA was isolated and purified using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. High-quality clean data were obtained by removing reads containing adaptors, low-quality bases and undetermined bases using Cutadapt software. The clean reads were aligned to the *Prunus avium* 'Satonishiki' reference genome using HISAT2 software (Kim et al., 2015). The mapped reads from each sample were assembled using StringTie software with default parameters. Then, all transcriptomes from all samples were merged to reconstruct a comprehensive transcriptome. The final transcriptome was generated using GFFcompare software. The DEGs were selected based on the criteria of fold change > 2 or < 0.5 and P -value < 0.05 using the edgeR or DESeq2 R packages. Gene Ontology annotation and enrichment for all DEGs were performed with the Blast2GO software. Kyoto Encyclopedia of Genes and Genomes pathway annotation and enrichment analysis was performed using the KOBAS2.0 software.

Cloning and sequence analysis of the candidate gene and development of PCR-based markers

DNA from the two bulks (21 individuals) was extracted for sequence analysis. The primer pairs PavSCPL-F/PavSCPL-R and PavSCPL-Pro-F/PavSCPL-Pro-R (Table S3) were designed using the Primer Premier 5.0 software, according to the *Prunus avium* reference genome sequence, to amplify the *PavSCPL* candidate gene coding sequence and promoter regions, respectively. The PCR amplification reaction mixture included Ex Taq enzyme (TaKaRa, Dalian, China) and 50–100 ng gDNA, and the thermal cycling conditions were as follows: 95 $^{\circ}\text{C}$ for 3 min, followed by 35 cycles of denaturation at 95 $^{\circ}\text{C}$ for 30 s, annealing at 56 $^{\circ}\text{C}$ for 30 s, and extension at 72 $^{\circ}\text{C}$ for 2 min, then extension at 72 $^{\circ}\text{C}$ for 10 min. The PCR products were sequenced by Sangon Biotech Co., Ltd. to identify DNA polymorphisms.

Based on the DNA polymorphisms of the *PavSCPL* gene (5.2-kb transposable element insertion), we developed primers for two AFLP markers, *PavSCPL*-1-F/R and *PavSCPL*-2-F/R (Table S3), associated with the fruit firmness phenotype in sweet cherry. The markers were used to genotype the F_1 population and 118 sweet cherry accessions.

Quantitative real-time PCR analysis

Using the EASYspin RNA Plant RNA Rapid Extraction Kit (Yuanpinghao Bio, Beijing, China), total RNA was extracted and purified for all samples. Reverse transcription was performed with the purified RNA samples in a 20 μ L volume using the FastQuant RT (with gDNase) Kit (Tiangen Bio, Beijing, China). The real-time PCR reactions were performed on the ABI7500 PCR thermocycler (Applied Biosystems, Foster City, CA) using the TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China) containing the SYBR green fluorescent intercalating dye, according to the following programme: 30 s 94 °C; 40 $\times$ 5 s 94 °C, 15 s 56 °C, 40 s 72 °C and the melting-curve analysis was applied. The sweet cherry *actin* gene (*Pav_sc0002247.1_g030.1.mk*) was used as the internal control. Each target gene (*PavSCPL*, *PavPME*, *PavPG*, *PavPGI*, *PavXTH14*, *PavEXP1* or *PavPL18*) was analysed with three biological and three technical replicates, and the relative expression level of the target genes was calculated using the $2^{-\Delta\Delta C_t}$ method. The gene-specific primers used for the qRT-PCR analysis are listed in Table S3. Six biological replicates and three technical replicates were performed for the qRT-PCR analyses.

Phylogenetic tree construction and subcellular localization analysis

A phylogenetic tree was constructed using the amino acid sequences of sweet cherry *PavSCPL* and the homologues from different species, which were downloaded from the GenBank database (Table S8). MEGA 6.0 software was used to align the sequences and construct the dendrogram using the neighbour-joining method with 500 bootstrap replicates (Tamura *et al.*, 2013).

For *PavSCPL* protein subcellular localization, the full-length *PavSCPL* coding sequence was cloned into the pCambia1302-GFP vector to generate the fusion protein pCambia1302-*PavSCPL*-GFP. The fusion plasmid and the control vector were transformed separately into *Agrobacterium tumefaciens* strain GV3101. The epidermal cells of 5-week-old *N. benthamiana* leaves were infiltrated with the transformed *A. tumefaciens* strains to visualize the subcellular localization of the fusion proteins. The tobacco leaves were observed 2 days after infiltration and images were captured with a fluorescence microscope (Zeiss LSM 710, Wetzlar, Germany). The transient expression assays were repeated at least three times.

Plasmid construction and VIGS of *PavSCPL* in sweet cherry fruit

An approximately 300-bp coding region fragment of *PavSCPL* was cloned into the pTRV2 vector to generate pTRV2-*PavSCPL*, which was transformed into *A. tumefaciens* strain GV3101. The TRV-mediated silencing of *PavSCPL* in sweet cherry fruit was implemented as described previously (Li *et al.*, 2015; Qi *et al.*, 2017, 2022) with the following modifications. (i) *Agrobacterium tumefaciens* strain GV3101 was cultured overnight at 28 °C to an OD₆₀₀ of 0.7–0.8 and then resuspended in *A. tumefaciens* infiltration buffer (10 mM MgCl₂, 10 mM MES at pH 5.6 and 100 μ M acetosyringone) to a final OD₆₀₀ of 0.8–1.0.

(ii) *Agrobacterium tumefaciens* strain GV3101 cells containing the pTRV1 and pTRV2 or pTRV2-*PavSCPL* vectors were mixed at a 1 : 1 ratio and infiltrated using needle syringes into the basal pedicel of cherry fruit at 20 DAFB until the whole fruit was permeated. (iii) The inoculated fruit was bagged for 2 days. At 21 and 28 dpi, 10 randomly selected fruit per strain were collected and photographed. The TRV-mediated gene-silencing assays, which each involved 50 transformed fruit per strain from the same tree, were performed with six biological replicates using six 10-year-old trees.

Enzymatic activity assays

The activities of pectin methylesterase (PME), polygalacturonase (PG), β -galactosidase (β -GAL), pectate lyase (PL) and α -L arabinofuranosidase (α -AF) were determined using PME, PG, β -GAL, PL and α -AF activity assay ELISA kits (Shanghai COIBO Biotech Co., Ltd., Shanghai, China) in accordance with the manufacturer's instructions. Six biological replicates and at least three technical replicates were used for each measurement.

Statistical analysis

The statistical significance of differences between means was evaluated using Student's *t*-test at the $P < 0.05$ significance level. All data were analysed using SPSS version 17.0 (SPSS, Inc., Chicago, IL). Graphs were constructed using Origin 9.1 (Microcal Software, Inc., Northampton, MA).

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Conflict of interest

The authors declare that they have no conflicts of interest.

Author contributions

ML and XQ conceived the project. XQ designed the experiments. XQ, YD, CL, LS and LC performed the experiments. XQ and YD analysed the results and wrote the manuscript. ML provided scientific suggestions and revised the manuscript. All authors reviewed and approved the final manuscript.

Data availability statement

All the data supporting the study are available in the manuscript and supplementary data.

References

- Ahmad, M.Z., Li, P., She, G., Xia, E., Benedito, V.A., Wan, X.C. and Zhao, J. (2020) Genome-wide analysis of serine carboxypeptidase-like acyltransferase gene family for evolution and characterization of enzymes involved in the biosynthesis of galloylated catechins in the tea plant (*Camellia sinensis*). *Front. Plant Sci.* **11**, 848.
- Atkinson, R.G., Sutherland, P.W., Johnston, S.L., Gunaseelan, K., Hallett, I.C., Mitra, D., Brummell, D.A. *et al.* (2012) Down-regulation of POLY-GALACTURONASE1 alters firmness, tensile strength and water loss in apple (*Malus $\times$ domestica*) fruit. *BMC Plant Biol.* **12**, 129.
- Bashline, L., Lei, L., Li, S. and Gu, Y. (2014) Cell wall, cytoskeleton, and cell expansion in higher plants. *Mol. Plant*, **7**, 586–600.

- Brummell, D.A. and Harpster, M.H. (2001) Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. *Plant Mol. Biol.* **47**, 311–340.
- Cai, L., Quero-García, J., Barreneche, T., Dirlwanger, E., Saski, C. and Iezzoni, A. (2019) A fruit firmness QTL identified on linkage group 4 in sweet cherry (*Prunus avium* L.) is associated with domesticated and bred germplasm. *Sci. Rep.* **9**, 5008.
- Calle, A. and Wünsch, A. (2020) Multiple-population QTL mapping of maturity and fruit-quality traits reveals LG4 region as a breeding target in sweet cherry (*Prunus avium* L.). *Hort. Res.* **7**, 127.
- Calle, A., Balas, F., Cai, L., Iezzoni, A., López-Corrales, M., Serradilla, M.J. and Wünsch, A. (2020) Fruit size and firmness QTL alleles of breeding interest identified in a sweet cherry 'Ambrunés' × 'Sweetheart' population. *Mol. Breed.* **40**, 1–17.
- Campoy, J.A., Le Dantec, L., Barreneche, T., Dirlwanger, E. and Quero-García, J. (2015) New insights into fruit firmness and weight control in sweet cherry. *Plant Mol. Biol. Report.* **33**, 783–796.
- Cercós, M., Urbez, C. and Carbonell, J. (2003) A serine carboxypeptidase gene (PSCP), expressed in early steps of reproductive and vegetative development in *Pisum sativum*, is induced by gibberellins. *Plant Mol. Biol.* **51**, 165–174.
- Cirilli, M., Giovannini, D., Ciacciulli, A., Chiozzotto, R., Gattolin, S., Rossini, L., Liverani, A. et al. (2018) Integrative genomics approaches validate PpYUC11-like as candidate gene for the stony hard trait in peach (*P. persica* L. Batsch). *BMC Plant Biol.* **18**, 1–12.
- Conley, A.J., Joensuu, J.J. and Menassa, B.J.E. (2009) Induction of protein body formation in plant leaves by elastin-like polypeptide fusions. *BMC Biol.* **7**, 48.
- Costa, F., Peace, C.P., Stella, S., Serra, S., Musacchi, S., Bazzani, M., Sansavini, S. et al. (2010) QTL dynamics for fruit firmness and softening around an ethylene-dependent polygalacturonase gene in apple (*Malus domestica* Borkh.). *J. Exp. Bot.* **61**, 3029–3039.
- Crump, W.W., Peace, C., Zhang, Z. and McCord, P. (2022) Detection of breeding-relevant fruit cracking and fruit firmness quantitative trait loci in sweet cherry via pedigree-based and genome-wide association approaches. *Front. Plant Sci.* **13**, 823250.
- Domínguez, F., González, M. and Cejudo, F.J. (2002) A germination-related gene encoding a serine carboxypeptidase is expressed during the differentiation of the vascular tissue in wheat grains and seedlings. *Planta*, **215**, 727–734.
- Dong, H., Yang, L., Liu, Y., Tian, G., Tang, H., Xin, S., Cui, Y. et al. (2023) Detection of new candidate genes controlling seed weight by integrating gene coexpression analysis and QTL mapping in *Brassica napus* L. *Crop J.* **11**, 842–851.
- Donkpegan, A.S., Bernard, A., Barreneche, T., Quero-García, J., Bonnet, H., Fouché, M., Dantec, L.L. et al. (2023) Genome-wide association mapping in a sweet cherry germplasm collection (*Prunus avium* L.) reveals candidate genes for fruit quality traits. *Hort. Res.* **10**, 191.
- Fujiisawa, M., Nakano, T. and Ito, Y. (2011) Identification of potential target genes for the tomato fruit-ripening regulator RIN by chromatin immunoprecipitation. *BMC Plant Biol.* **1**, 26.
- Gao, Y., Wei, W., Fan, Z.Q., Zhao, X.D., Zhang, Y.P., Jing, Y., Zhu, B.Z. et al. (2020) Re-evaluation of the nor mutation and the role of the NAC—NOR transcription factor in tomato fruit ripening. *J. Exp. Bot.* **71**, 3560–3574.
- García-Gago, J.A., Posé, S., Muñoz-Blanco, J., Quesada, M.A. and Mercado, J.A. (2009) The polygalacturonase FaPG1 gene plays a key role in strawberry fruit softening. *Plant Signal. Behav.* **4**, 766–768.
- García-Gómez, B.E., Ruiz, D., Salazar, J.A., Rubio, M., Martínez-García, P.J. and Martínez-Gómez, P. (2020) Analysis of metabolites and gene expression changes relative to apicort (*Prunus armeniaca* L.) fruit quality during development and ripening. *Front. Plant Sci.* **11**, 1269.
- García-Gómez, B., Salazar, J.A., Nicolás-Almansa, M., Razi, M., Rubio, M., Ruiz, D. and Martínez-Gómez, P. (2021) Molecular bases of fruit quality in prunus species: an integrated genomic, transcriptomic, and metabolic review with a breeding perspective. *Int. J. Mol. Sci.* **22**, 333.
- Holušová, K., Čmejlová, J., Suran, P., Čmejla, R., Sedlák, J., Zelený, L. and Bartoš, J. (2022) High-resolution genome-wide association study of a large Czech collection of sweet cherry (*Prunus avium* L.) on fruit maturity and quality traits. *Hort. Res.* **1**, 233.
- Hu, Y., Han, Z., Sun, Y., Wang, S., Wang, T., Wang, Y., Xu, K. et al. (2020) ERF4 affects fruit firmness through TPL4 by reducing ethylene production. *Plant J.* **103**, 937–950.
- Jia, H., Chai, Y., Li, C., Lu, D., Luo, J., Qin, L. and Shen, Y. (2011) Abscisic acid plays an important role in the regulation of strawberry fruit ripening. *Plant Physiol.* **157**, 188–199.
- Kim, D., Langmead, B. and Salzberg, S.L. (2015) HISAT: a fast spliced aligner with low memory requirements. *Nat. Methods* **12**, 357–360.
- Laurens, F., Aranzana, M.J., Arus, P., Bassi, D., Bink, M., Bonany, J., Caprera, A. et al. (2018) An integrated approach for increasing breeding efficiency in apple and peach in Europe. *Hort. Res.* **5**, 11.
- Li, Y., Fan, C., Xing, Y., Jiang, Y., Luo, L., Sun, L., Shao, D. et al. (2011) Natural variation in GS5 plays an important role in regulating grain size and yield in rice. *Nat. Genet.* **43**, 1266–1269.
- Li, Q., Chen, P., Dai, S., Sun, Y., Yuan, B., Kai, W., Kai, W. et al. (2015) PacCYP707A2 negatively regulates cherry fruit ripening while PacCYP707A1 mediates drought tolerance. *J. Exp. Bot.* **66**, 3765–3774.
- Li, R., Sun, S., Wang, H., Wang, K., Yu, H., Zhou, Z., Xin, P. et al. (2020) FIS1 encodes a GA2-oxidase that regulates fruit firmness in tomato. *Nat. Commun.* **11**, 5844.
- Liu, Y., Liu, X., Zhong, F., Tian, R., Zhang, K., Zhang, X. and Li, T. (2011) Comparative study of phenolic compounds and antioxidant activity in different species of cherries. *J. Food Sci.* **76**, C633–C638.
- Liu, Y., He, Z., Appels, R. and Xia, X. (2012) Functional markers in wheat: current status and future prospects. *Theor. Appl. Genet.* **125**, 1–10.
- Liu, Y., Ce, F., Tang, H., Tian, G., Yang, L., Qian, W. and Dong, H. (2022) Genome-wide analysis of the serine carboxypeptidase-like (SCPL) proteins in *Brassica napus* L. *Plant Physiol. Biochem.* **186**, 310–321.
- Mugford, S.T. and Milkowski, C. (2012) Serine carboxypeptidase-like acyltransferases from plants. In *Natural Product Biosynthesis by Microorganisms and Plants, Part B. Methods in Enzymology*, Vol. **516** (Hopwood, D. A., ed), pp. 279–297. San Diego, CA: Academic Press.
- Mugford, S.T., Qi, X., Bakht, S., Hill, L., Wegel, E., Hughes, R.K., Papadopolou, K. et al. (2009) A serine carboxypeptidase-like acyltransferase is required for synthesis of antimicrobial compounds and disease resistance in oats. *Plant Cell*, **21**, 2473–2484.
- Pan, L., Zeng, W., Niu, L., Lu, Z., Liu, H., Cui, G., Zhu, Y. et al. (2015) PpYUC11, a strong candidate gene for the stony hard phenotype in peach (*Prunus persica* L. Batsch), participates in IAA biosynthesis during fruit ripening. *J. Exp. Bot.* **66**, 7031–7044.
- Phan, T.D., Bo, W., West, G., Lycett, G.W. and Tucker, G.A. (2007) Silencing of the major salt-dependent isoform of pectinesterase in tomato alters fruit softening. *Plant Physiol.* **144**, 1960–1967.
- Qi, X., Liu, C., Song, L., Li, Y. and Li, M. (2017) PaCYP78A9, a cytochrome P450, regulates fruit size in sweet cherry (*Prunus avium* L.). *Front. Plant Sci.* **8**, 2076.
- Qi, X., Dong, Y., Liu, C., Song, L., Chen, L. and Li, M. (2022) The PavNAC56 transcription factor positively regulates fruit ripening and softening in sweet cherry (*Prunus avium*). *Physiol. Plant.* **174**, e13834.
- Quero-García, J., Iezzoni, A., Pulawska, J. and Lang, G. (2017) *Cherries: Botany, Production and Uses*. Wallingford, UK: CAB International.
- Seymour, G.B., Østergaard, L., Chapman, N.H., Knapp, S. and Martin, C. (2013) Fruit development and ripening. *Annu. Rev. Plant Biol.* **64**, 219–241.
- Seymour, G.B., Poole, M., Giovannoni, J.J. and Tucker, G.A. (2013) *The molecular biology and biochemistry of fruit ripening*. Oxford, UK: John Wiley & Sons, Inc.
- Shi, Y., Li, B.J., Su, G., Zhang, M., Grierson, D. and Chen, K.S. (2022) Transcriptional regulation of fleshy fruit texture. *J. Integr. Plant Biol.* **64**, 1649–1672.
- Shirasawa, K., Isuzugawa, K., Ikenaga, M., Saito, Y., Yamamoto, T., Hirakawa, H. and Isobe, S. (2017) The genome sequence of sweet cherry (*Prunus avium*) for use in genomics-assisted breeding. *DNA Res.* **24**, 499–508.
- Singh, V.K., Mangalam, A.K., Dwivedi, S. and Naik, S. (1998) Primer premier: program for design of degenerate primers from a protein sequence. *Biotechniques*, **24**, 318–319.
- Tamura, K., Stecher, G., Peterson, D., Filipowski, A. and Kumar, S. (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **30**, 2725–2729.
- Ulluisik, S., Chapman, N.H., Smith, R., Poole, M., Adams, G., Gillis, R.B., Besong, T.M. et al. (2016) Genetic improvement of tomato by targeted control of fruit softening. *Nat. Biotechnol.* **34**, 950–952.

- Wang, L., Jin, P., Wang, J., Jiang, L., Shan, T. and Zheng, Y. (2015a) Effect of β -aminobutyric acid on cell wall modification and senescence in sweet cherry during storage at 20 °C. *Food Chem.* **175**, 471–477.
- Wang, Y., Bai, J. and Long, L.E. (2015b) Quality and physiological responses of two late-season sweet cherry cultivars 'Lapins' and 'Skeena' to modified atmosphere packaging (MAP) during simulated long distance ocean shipping. *Postharvest Biol. Technol.* **110**, 1–8.
- Wang, D., Yeats, T.H., Uluisik, S., Rose, J.K. and Seymour, G.B. (2018) Fruit softening: revisiting the role of pectin. *Trends Plant Sci.* **23**, 302–310.
- Wang, X., Wu, W., Zhou, B., Wang, Z., Qian, Y., Wang, B. and Yan, L. (2021) Genome-wide analysis of the SCPL gene family in grape (*Vitis vinifera* L.). *J. Integr. Agric.* **20**, 2666–2679.
- Wang, Y., Zhao, J., Deng, X., Wang, P., Geng, S., Gao, W., Guo, P. *et al.* (2022) Genome-wide analysis of serine carboxypeptidase-like protein (SCPL) family and functional validation of Gh_SCPL42 unichromosome conferring cotton Verticillium der Verticillium wilt stress in *Gossypium hirsutum*. *BMC Plant Biol.* **22**, 1–13.
- Weier, D., Mittasch, J., Strack, D. and Milkowski, C. (2008) The genes BnSCT1 and BnSCT2 from Brassica napus encoding the final enzyme of sinapine biosynthesis: molecular characterization and suppression. *Planta*, **227**, 375–385.
- Witasari, L.D., Huang, F.C., Hoffmann, T., Rozhon, W., Fry, S.C. and Schwab, W. (2019) Higher expression of the strawberry xyloglucan endotransglucosylase/hydrolase genes FvXTH9 and FvXTH6 accelerates fruit ripening. *Plant J.* **100**, 1237–1253.
- Wu, B., Shen, F., Wang, X., Zheng, W.Y., Xiao, C., Deng, Y., Wang, T. *et al.* (2021) Role of MdERF3 and MdERF118 natural variations in apple flesh firmness/crispness retainability and development of QTL-based genomics-assisted prediction. *Plant Biotechnol. J.* **19**, 1022–1037.
- Xin, Y., Jin, Z., Chen, F., Lai, S. and Yang, H. (2020) Effect of chitosan coatings on the evolution of sodium carbonate-soluble pectin during sweet cherry softening under non-isothermal conditions. *Int. J. Biol. Macromol.* **154**, 267–275.
- Xu, C., Liu, Y., Li, Y., Xu, X., Xu, C., Li, X., Xiao, J. *et al.* (2015) Differential expression of GS5 regulates grain size in rice. *J. Exp. Bot.* **66**, 2611–2623.
- Xu, X., Zhang, L., Zhao, W., Fu, L., Han, Y., Wang, K., Yan, L. *et al.* (2021) Genome-wide analysis of the serine carboxypeptidase-like protein family in *Triticum aestivum* reveals TaSCPL184-6D is involved in abiotic stress response. *BMC Genomics*, **22**, 1–19.
- Zhai, Z., Feng, C., Wang, Y., Sun, Y., Peng, X., Xiao, Y., Zhang, X. *et al.* (2021) Genome-wide identification of the xyloglucan endotransglucosylase/Hydrolase (XTH) and Polygalacturonase (PG) genes and characterization of their role in fruit softening of sweet cherry. *Int. J. Mol. Sci.* **22**, 12331.
- Zhai, Z., Xiao, Y., Wang, Y., Sun, Y., Peng, X., Feng, C., Zhang, X. *et al.* (2022) Absciscic acid-responsive transcription factors PavDof2/6/15 mediate fruit softening in sweet cherry. *Plant Physiol.* **190**, 2501–2518.
- Zhang, W.W., Zhao, S.Q., Gu, S., Cao, X.Y., Zhang, Y. and Niu, J.F. (2022) FvVRKY48 binds to the pectate lyase FvPLA promoter to control fruit softening in *Fragaria vesca*. *Plant Physiol.* **189**, 1037–1049.
- Zhao, H., Liu, B., Zhang, W., Cao, J. and Jiang, W. (2019) Enhancement of quality and antioxidant metabolism of sweet cherry fruit by near-freezing temperature storage. *Postharvest Biol. Technol.* **147**, 113–122.

Supporting information

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

Figure S1 Δ (SNP index) values from BSA-seq analysis.

Figure S2 Δ (InDel index) values from BSA-seq analysis.

Figure S3 Sequence alignment analysis of PavSCPL protein with its homologues showing conserved structural domains of SCPL.

Figure S4 Genomic DNA sequences of PavSCPL and PavXTH14, including promoter and coding region sequences, amplified from and compared between extra-hard-flesh fruit and soft-flesh fruit individuals.

Figure S5 Co-segregation analysis of 204 F₁ individuals from the 'Rainier' $\times$ 'Summit' for genotyping of PavSCPL alleles with fruit firmness phenotype using the AFLP markers PavSCPL-1-F/R (left) and PavSCPL-2-F/R (right).

Figure S6 Genotyping of PavSCPL alleles in 118 sweet cherry accessions using the AFLP marker PavSCPL-2-F/R.

Figure S7 Phylogenetic analysis and Subcellular localization of PavSCPL.

Table S1 Summary of sequence data from the bulk segregant analysis

Table S2 Bulk segregant analysis sequencing results for the fruit firmness locus

Table S3 Primer sequences used in this study

Table S4 Expression analysis of 72 genes located in the fruit firmness fine-mapping region on chromosome 6 based on RNA-seq data

Table S5 The 5.2-kb insertion sequence

Table S6 Genotypes and fruit firmness phenotypes in the F₁ population based on PavSCPL and the allele Pavscpl^{F5.2kb}

Table S7 Identification of genotypes and fruit firmness phenotypes of 118 sweet cherry accessions based on PavSCPL and the allele Pavscpl^{F5.2kb}

Table S8 GenBank accession numbers for protein sequences used in this study