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ORIGINAL ARTICLE

A single QTL harboring multiple genetic variations leads to complicated phenotypic segregation in apple flesh firmness and crispness

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Abstract

Key Message **Within a QTL, the genetic recombination and interactions among five and two functional variations at *MdbHLH25* and *MdWDR5A* caused much complicated phenotype segregation in apple FFR and FCR.**

Abstract The storability of climacteric fruit like apple is a quantitative trait. We previously identified 62 quantitative trait loci (QTLs) associating flesh firmness retainability (FFR) and flesh crispness retainability (FCR), but only a few functional genetic variations were identified and validated. The genetic variation network controlling fruit storability is far to be understood and diagnostic markers are needed for molecular breeding. We previously identified overlapped QTLs F16.1/H16.2 for FFR and FCR using an F1 population derived from 'Zisai Pearl' × 'Red Fuji'. In this study, five and two single-nucleotide polymorphisms (SNPs) were identified on the candidate genes *MdbHLH25* and *MdWDR5A* within the QTL region. The SNP1 A allele at *MdbHLH25* promoter reduced the expression and SNP2 T allele and/or SNP4/5 GT alleles at the exons attenuated the function of *MdbHLH25* by downregulating the expression of the target genes *MdACS1*, which in turn led to a reduction in ethylene production and maintenance of higher flesh crispness. The SNPs did not alter the protein–protein interaction between *MdbHLH25* and *MdWDR5A*. The joint effect of SNP genotype combinations by the SNPs on *MdbHLH25* (SNP1, SNP2, and SNP4) and *MdWDR5A* (SNPi and SNPii) led to a much broad spectrum of phenotypic segregation in FFR and FCR. Together, the dissection of these genetic variations contributes to understanding the complicated effects of a QTL and provides good potential for marker development in molecular breeding.

Keywords Apple · Flesh firmness and crispness · Genetic variation · QTL

Introduction

Apple (*Malus domestica* Borkh.), as one of the most grown and commercialized fruit, is a typical climacteric fruit. Ethylene plays an important role in controlling the downstream activation of ethylene-related genes involved in several fruit quality aspects (Brummell and Harpster 2001; Shi et al. 2021). Therefore, quantitative trait loci (QTLs) or genetic variations in genes involved in ethylene synthesis, receptor, signaling, and cell wall metabolic genes are often associated with postharvest storability of climacteric fruit. QTLs for fruit firmness or flesh texture have been mapped on at least 11 chromosomes in apple (King et al. 2001; Longhi et al. 2012; Bink et al. 2014). We previously identified 62 QTLs for apple flesh firmness retainability (FFR) and the flesh crispness retainability (FCR) (Wu et al. 2021b).

Generally, one functional genetic variation can be identified within a QTL. From the QTL region on chromosome 15 of apple, variation in 1-aminocyclopropane-1-carboxylic acid synthase gene (*MdACS1*) causes low flesh ethylene production (Costa et al. 2005; Harada et al. 2000). A QTL-derived functional single-nucleotide polymorphism

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(SNP) marker in *MdPG1* was identified on apple chromosome 10 contributing to apple flesh softening rate (Costa et al. 2010). To date, a number of variations have been reported to regulate ethylene-related genes, such as *MdACS* and 1-aminocyclopropane-1-carboxylic acid oxidase (*MdACO*), while the allelic variations in *MdACS1* and *MdACO1* have been used in marker-assisted breeding in apple (Zhu and Barritt 2008). The 8-bp deletion on *MdERF3* promoter resulted in reduced expression of *MdERF3*, while MdERF3 binds directly to the promoter of *MdACO4* and represses *MdACO4* expression (Wu et al. 2021b). A 3-bp deletion in the *MdERF118* promoter decreased its expression by disrupting the binding of MdRAVL1, which increased *MdPGLR3* and *MdACO4* expression and reduced apple flesh FFR and FCR (Wu et al. 2021b). An SNP in the EAR motif in the coding region of *MdERF4* caused a reduction in the protein–protein interaction between MdERF4 and MdTPL4, which resulted in reduced repression of *ERF3* expression and subsequently reduction in *MdACO1* expression, and ethylene production, but increase in apple fruit firmness (Hu et al. 2020). Genetic variations in cell wall metabolism genes and the regulation factors are also frequently reported to control flesh softening or fruit storability. A functional SNP in *MdPG1* exon is associated with apple fruit softening (Longhi et al. 2013). Genetic variations in the *MdPAE10* gene leading to longer postharvest shelf life were identified using map-based cloning strategy (Wu et al. 2021a). Similarly, a functional allelic variation in *MdExp7* also affects apple fruit softening process (Costa et al. 2008).

Recently, dozens of SNPs and insertion/deletions (InDels) in both the coding and promoter sequences were identified in *MdNAC18.1* (Larsen et al. 2019; Migicovski et al. 2021b). A genetic marker within *MdNAC18.1* exhibited better predictability than the markers currently used by breeders (ACS1, ACO1, and PG1) for both firmness at harvest and firmness after 3 months of cold storage (Migicovski et al. 2021b). The results from Migicovski et al. (2021a) imply a possibility that more than one genetic variation within a gene could be putatively causal variants, and a single allelic variation often has weak contribution to the phenotypic segregation.

The bHLH transcription factor plays also important roles in participating in fruit flesh softening or postharvest storability. In apple, several fruit softening related genes, such as *MdPGs*, *MdPL*, *MdXET*, and *MdbGAL*, have been reported to be indirectly regulated by *MdbHLH3,* intermediated by ethylene production (Hu et al. 2019). Likewise in banana (*Musa acuminata* Colla), *MabHLH7* binds directly to the promoters of several cell wall metabolism genes to accelerate fruit ripening, such as *MaXTH12*, *MaEXP2/21*, *MaPME4/5*, *MaPG4*, and *MaPL1/2* (Song et al. 2020). Genetic variations in bHLH gene family members have not been reported to affect apple FFR and FCR.

In this study, within a single QTL region associated with apple FFR and FCR, five and two variants were identified within the region of *MdbHLH25* and *MdWDR5A*, respectively. The combinations of these variations led to a broad spectrum of phenotypic variations in apple flesh firmness (FF) and crispness (FC) at harvest, as well as FFR and FCR. The findings reported here suggest that a single QTL can explain a large phenotypic variability by harboring different genetic variations.

Materials and methods

Plant materials and phenotyping

All plant materials were obtained from China Agricultural University. The experimental research on plants, including field investigation and sample collection, was performed under institutional guidelines in accordance with local legislation.

The phenotype data of FF, FC, FFR, and FCR have been obtained previously using 2664 hybrid plants derived from three F1 populations, as described by Wu and colleagues (Wu et al. 2021b). In this study, the plant materials used for SNP genotyping were 478 hybrid plants randomly chosen from the three F1 populations of crosses between 'Zisai Pearl' (*M. asiatica* Nakai.) × 'Red Fuji' (*M. domestica* Borkh.) (278); 'Zisai Pearl' × 'Golden Delicious' (*M. domestica* Borkh.) (146); and 'Jonathan' (*M. domestica* Borkh.) × 'G' (54). For fruit ethylene production assay, 27 hybrid plants were selected from the above-mentioned populations based on the *MdbHLH25* SNP1 genotypes. The hybrid plants (on the own roots) were grown since 2009 in the Fruit Experimental Station, China Agricultural University (Changping District, Beijing, China) at a density of 2.5 × 0.5 m under conventional management and pest control. Fruit of 'Golden Delicious' was used in transient transformation at 140 days after full bloom (DAFB). The 3-week-old leaves of *Nicotiana benthamiana* were used for transient co-transformation, subcellular localization, and bimolecular fluorescent complimentary (BiFC) assay.

Fifteen apples per hybrid plant were harvested and sampled for the assay of ethylene release rate by the criteria of starch degradation degree as seven and fruit skin background color changed from green to light yellow or white (Blanpied and Silsby 1992). Flesh firmness and crispness were measured using a texture analyzer (TA.XT; Stable Micro Systems, UK). The instrument settings were pre-test speed 1.0 mm/s, test speed 1.0 mm/s, and post-test speed 10.00 mm/s. The probe (2 mm in diameter) was pressed into the apple flesh to a depth of 5 mm. Three apples were

measured in each treatment, while three punctures per apple were set as experimental replicates (Costa et al. 2012).

Measurement of ethylene release rate

Three freshly picked apples or after transient transformation were weighed and put into the gastight vessel, and the volume of the vessel and the retention time were recorded. One milliliter gas was sampled from the vessel using a microsyringe (No. 309602, BD, Franklin Lakes, NJ, USA) and was immediately injected into gas chromatography (GC-9A, Shimadzu, Japan). The injection temperature and column temperature were 100 ℃ and the flame ionization detector temperature was 380 ℃. Five biological replicates were designed for apples collected from hybrid plants, and the ethylene concentration was measured three times to get an average value.

DNA/RNA extraction and qRT-PCR

DNA was extracted from leaf samples of the 478 hybrid plants. Total RNA was extracted from transiently transformed tobacco leaves or the mesocarp of transiently transformed apples using the CTAB method and cDNA was generated using HiScript® II 1st Strand cDNA Synthesis Kit (+ gDNA WIper) (Vazyme, Nanjing). HamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing) was used for qRT-PCR test, and PCR primers were designed using Primer 5 software (Table S1).

Cloning and sequencing of candidate genes

Candidate genes were screened from the region of an overlapped region of QTL F16.1/H16.2 for FFR and FCR based on the apple genome GDDH13.1 (Wu et al. 2021b). Genes were selected based on SNPs and InDels using the parental re-sequencing data (Shen et al. 2019). Then, 2000 bp segment upstream of the ATG codon and the complete coding sequence (CDS) of the four parents were amplified, cloned, and Sanger sequenced to validate the genetic variations (Shen et al. 2019; Wu et al. 2021b).

Glucuronidase (GUS) staining and GUS gene expression analysis

The fragments of bHLH-PRO_C-GUS and bHLH-PRO_A-GUS were constructed into pCAMBIA1391 vector and transformed into GV3101 (*Agrobacterium tumefaciens*), respectively. These vectors were transiently transformed into 3-week-old tobacco leaves. The GUS staining and qRT-PCR were performed after incubation (Zhang et al. 2016).

The three SNP haplotypes of *MdbHLH25* CDS, bH T-T-GT, bH G-C-TC, and bH T-C-GT, were constructed on pRI101 vector, respectively. Upstream 2 kb sequences of *MdACS1* promoter were constructed on pCAMBIA1391 vector. The two vectors were combined, transformed into *Agrobacterium tumefaciens,* and co-injected into tobacco leaves. The samples were stained with GUS Dye and decolorized with alcohol (Wu et al. 2021b).

Subcellular localization

The constructs 35S:*MdbHLH25*-eGFP with the three SNP haplotypes were interlinked into pRI101 vector and were transferred to GV3101, respectively. These vectors were transiently transformed into mcherry-tobacco with good growth. After incubation, the transformants were sampled and the fluorescence was observed with confocal microscopy (LSM 510-Meta, Zeiss, Germany) (Hu et al. 2017).

Transient transformation of apple fruit

The three SNP haplotypes of *MdbHLH25* CDS, bH T-T-GT, bH G-C-TC, and bH T-C-GT, were constructed on pRI101-AN (35S) vector, respectively. These vectors were transiently transformed via GV3101 into 'Golden Delicious' apple fruit (140 DAFB) by a vacuum pump. The transient transformation was performed with at least three apples for each vector, and the experiments were repeated at least three times. Changes in phenotypes were determined after 8 days of incubation (Li et al. 2016).

Yeast-one-hybrid (Y1H) assay

The three SNP haplotypes of MdbHLH25 CDS, bH T-T-GT, bH G-C-TC, and bH T-C-GT, were constructed on pJG4-5 vector, respectively. Upstream 2 kb sequences of *MdACS1* and *MdACO1* promoters were constructed on pLaczi vector. After the yeast competent cells were transformed, the yeast was screened by SD/-TRP/-URA screening plate. Finally, x-Gal staining was used for visualization (Zheng et al. 2020a).

Yeast-two-hybrid (Y2H) assay

The three SNP haplotypes of *MdbHLH25* CDS, bH T-T-GT, bH G-C-TC, and bH T-C-GT, were constructed into pGADT7. The three SNP haplotypes of *MdWDR5A* CDS, WD C-G, WD G-C, and WD C-C, were constructed into pGBKT7 vector, respectively. The 16 vector pairs were transformed into the competent cell y2hGold of yeast to perform Y2H (Zheng et al. 2020a).

BiFC assay

The three SNP haplotypes of *MdbHLH25* CDS, bH T-T-GT, bH G-C-TC, and bH T-C-GT, were constructed into SPYCE (M)-35S. The three SNP haplotypes of *MdWDR5A* CDS, WD C-G, WD G-C, and WD C-C, were constructed into SPYNE173-35S vector, respectively. The 16 vector combinations were transformed into GV3101 and were injected into 3-week-old tobacco leaves to perform BiFC assay (Walter et al. 2004).

Pull-down assay

The three SNP haplotypes of *MdbHLH25* CDS, bH T-T-GT, bH G-C-TC, and bH T-C-GT, were constructed into pET32a. Similarly, the three SNP haplotypes of *MdWDR5A* CDS, WD C-G, WD G-C, and WD C-C, were constructed into pGEX-4T vector, respectively. The sequenced plasmid was transformed into BL21 competent cell to perform pull down assay. His-protein purification kit was used for purification and the effluent was collected for Western blot (Hu et al. 2016).

Kompetitive allele-specific PCR (KASP) assay

KASP primers were designed based on the 100 bp flanking sequences of a specific SNP, SNP2 (bH113), SNP4 (bH723), SNPi (WD103), or SNPii (WD292). The genotypes of SNP1 (bH-827), i.e., SNP SNP38573599, have been already known (Wu et al. 2021b). KASP assay was performed on 478 hybrid plants randomly chosen from the three hybrid populations. Fluorescence detection was performed using an Omega PHERAstar (BMG PHERAstar, BMG LabTech, Ortenberg, Germany). SNP VIEWER software (LGC) was used to output genotype data (Chen et al. 2011). Genomics-assisted prediction (GAP) models for FF, FR, FFR, and FCR were developed using the 478 hybrid plants as a training population (Wu et al. 2021b; Shen et al. 2022).

Statistical analysis

Differences between the control and experimental treatments were analyzed by one-way analysis of variance (ANOVA) through Dunnett's multiple comparison at a significance level of $\alpha = 0.05$.

Results

Candidate gene screening and variation validation

Using the hybrid population of 'Zisai Pearl' × 'Red Fuji' and BSATOS software (Shen et al. 2022), we previously identified six overlapped QTLs for apple FFR and FCR on chromosome 16, and the G' values were as high as 42.43, 81.44, 8.86, 14.93, 9.83, and 9.85 (Wu et al. 2021b). However, the marker effect of SNP38573599, which was developed within the overlapped QTL region, was estimated as low as 1.47 and 1.43 months on FFR and FCR, respectively (Wu et al. 2021b). To address this issue, the genetic variation was screened and validated. The overlapped QTL region was located on 38,426,419–39,485,064 bp of the chromosome 16 (Fig. 1A). Five genes were annotated in this region according to the GDDH 13 v1.1 apple genome (Table S2). Using the re-sequencing data of the parental cultivars, 'Zisai Pearl' and 'Red Fuji' (Shen et al. 2019), genetic variations at upstream core element region or CDS domain were found in all the five genes (Table S2). Since several bHLH family members were reported to affect flesh softening and storability in apple and banana (Hu et al. 2019; Song et al. 2020), WD proteins have been reported to interact with bHLH (Dubos et al. 2008). Therefore, *MdbHLH25* (*MD16G1282500*) and *MdWDR5A* (*MD16G1282400*) were selected as candidate genes for further experiments.

The sequences of the two genes were cloned from leaf samples of the four parental cultivars and Sanger's sequenced. Five non-synonymous SNPs on *MdbHLH25* were found between the two parents 'Red Fuji' and 'Zisai Pearl', one of them, SNP1 C/A (i.e., SNP38573599), was located at − 827 bp upstream of the ATG codon and the other four were located in the CDS region: SNP2 G/T (+ 113 bp Ala/Ser), SNP3 C/T (+ 158 bp Thr/Ile), SNP4 G/T (+ 723 bp Lys/Asn), and SNP5 T/C (+ 724 bp Ser/Pro) (Fig. 1B). Two SNPs were identified in the CDS region of *MdWDR5A,* SNPi C/G (+ 103 bp Arg/Gly) and SNPii C/G (+ 292 bp Leu/Val) (Fig. 1C). The SNPs in *MdbHLH25* exhibited three haplotypes: *pro*A-*bH* T-T-GT, *pro*C-*bH* G-C-TC, and *pro*A-*bH* T-C-GT (Fig. 1D). Three haplotypes in *MdWDR5A,* WD C-G, WD G-C, and WD C-C were identified (Fig. 1C). One SNP was found within the 2 kb region upstream of the ATG codon of *MdWDR5A*, but the expression of *MdWDR5A* did not vary among apples of different storability (Wu et al. 2021b). All the SNPs at the CDS of the two genes did not alter the three-dimensional structure of the coding proteins predicted using Swiss-model software.

The SNP1 allele A at MdbHLH25 promoter causes decrease in gene expression

The transcriptome data by Wu et al (2021b) showed that the expression of *MdbHLH25* was significantly lower during 0–6 weeks of postharvest cold storage in fruit with SNP38573599 AA genotype with regards to CA genotype (Fig. 2A). To validate whether SNP1 C/A affects *MdbHLH25* promoter activity, the 2 kb promoter sequence

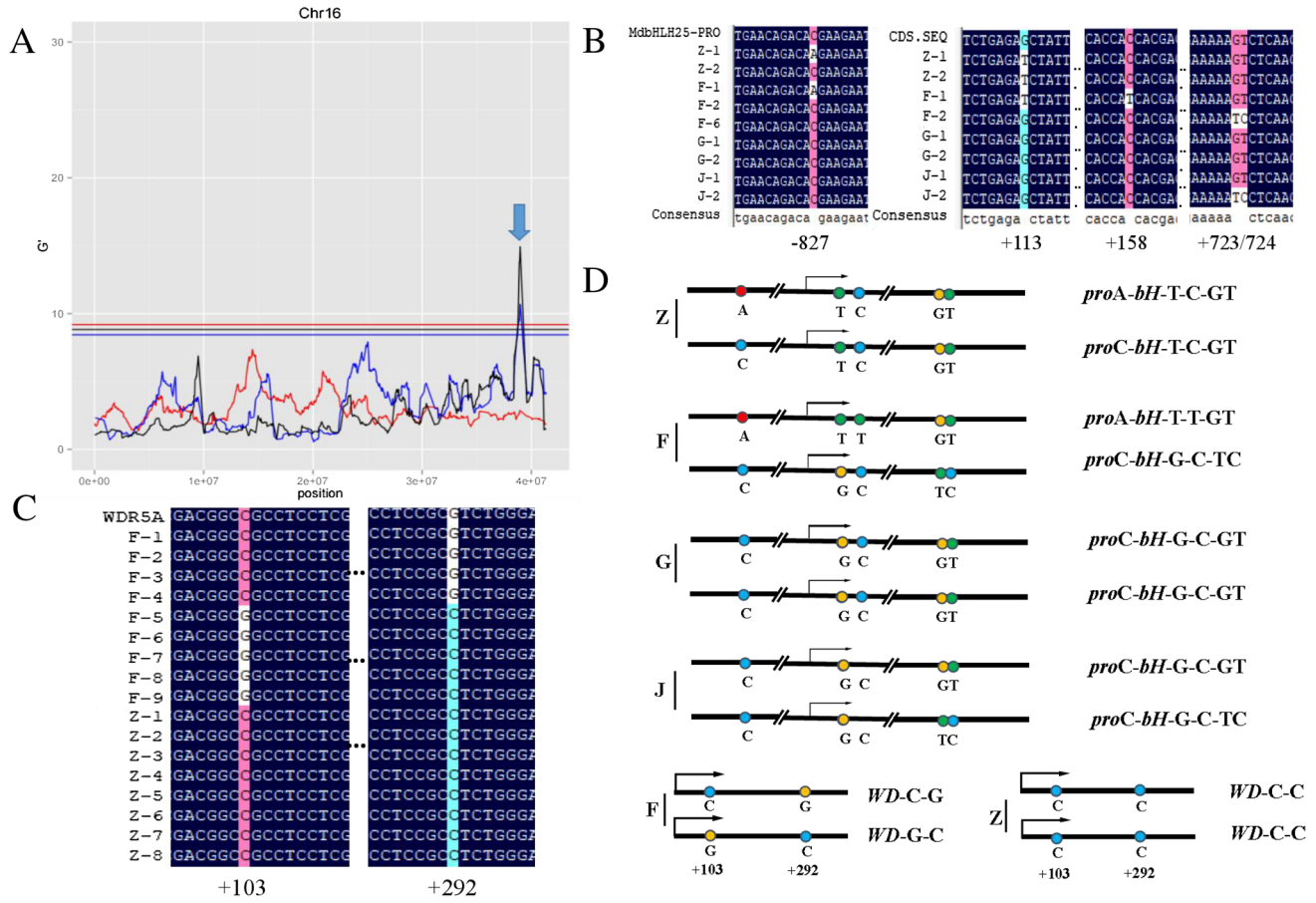

Fig. 1 A quantitative trait locus (QTL) and the harboring genetic variations associated with flesh firmness and crispness retainability in a hybrid population [*Malus asiatica* Nakai 'Zisai Pearl' (Z)×*M. domestica* Borkh. 'Red Fuji' (F)]. **A** The QTL F16.1/H16.2. The solid horizontal lines represent significant thresholds for QTLs. The line coloration indicates the parentage of the QTLs which were mapped on the maternal (red), paternal (blue), and both (black) parents. **B**, **C** Sanger sequencing indicated genetic variations in *MdbHLH25* (**B**) and *MdWDR5A* (**C**). **D** Schematic diagram showing the SNP haplotypes of *MdbHLH25* and *MdWDR5A* of the parental cultivars, the SNP haplotype of *MdWDR5A* in 'Golden Delicious' (G) and 'Jonathan' (J) was the same as 'Red Fuji' and 'Zisai Pearl', respectively. *proA* or *proC* indicates the SNP1 C/A genotype at the promoter region of *MdbHLH25* (color figure online)

of *MdbHLH25* with SNP1 C or SNP1 A allele was transiently transformed into tobacco leaves. GUS staining and GUS gene expression indicated that the promoter activity of *MdbHLH25* with SNP1 A allele was significantly reduced than that with SNP1 C allele (Fig. 2B, C). The ethylene production rate was significantly higher in apples from hybrid plants with *MdbHLH25* SNP1 CC genotypes than that with SNP1 CA and AA genotypes (Fig. 2D). To determine whether the SNPs at *MdbHLH25* CDS affect its subcellular localization, subcellular localization assay was performed using the three SNP haplotypes, bH T-T-GT, bH G-C-TC, and bH T-C-GT. Fluorescence clearly appeared in the nucleus (Fig. S1), which implied that the nuclear localization of MdbHLH25 protein was not altered by any of the SNPs at the CDS.

SNPs at MdbHLH25 CDS impair its function

Apple fruit transient transformation was performed to explore whether SNPs at *MdbHLH25* CDS affect ethylene release and flesh softening. Apparent fruit skin de-greening phenotype was observed in 'Golden Delicious' apples overexpressing *MdbHLH25* with bH G-C-TC genotype in comparison to those transformed with the other genotypes or the empty vector (Fig. 3A). Consistent to fruit skin de-greening, ethylene production rate was significantly higher in apples overexpressing *MdbHLH25* with bH G-C-TC genotype than the others (Fig. 3B). The high ethylene emission was confirmed by the expression of ethylene synthetic genes, such as *MdACS1* and *MdACO1,* the expression of which was significantly higher in apples

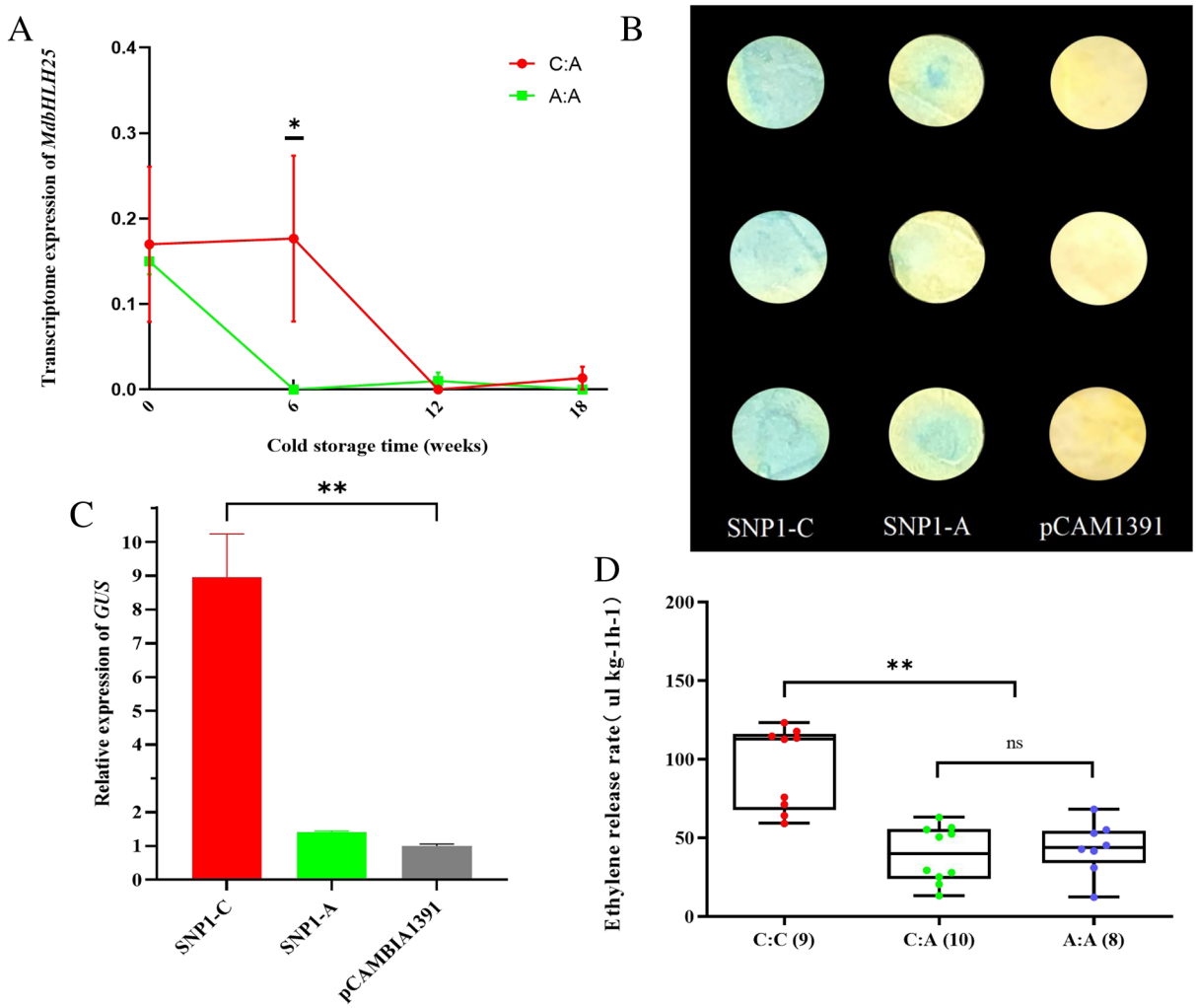

Fig. 2 The SNP1 allele A at *MdbHLH25* leads to a decreased promoter activity. **A** Transcriptome data (Wu et al. 2021a, b) showed that *MdbHLH25* expression (in FPKM) was lower in apples with SNP1 AA genotype than that with SNP1 CA genotype during 0–6 weeks of cold storage. **B**, **C** A 2 kb promoter of *MdbHLH25* with SNP1 A allele caused weaker GUS staining (**B**) and lower GUS gene expression (**C**) than that with SNP1 C allele using transient transgenic tobacco leaves. **D** Ethylene production rate of apples after 6 weeks of cold storage from hybrid plants 'Zisai Pearl' (Z) × 'Red Fuji' (F), Z × 'Golden Delicious' (G), and 'Jonathan' × G with different *MdbHLH25* SNP1 genotypes, and the number of hybrid plant was given in the parentheses following the genotype. Statistically significant differences were determined by t tests: $*P < 0.05$, $**P < 0.01$

overexpressing *MdbHLH25* with bH G-C-TC genotype (Fig. 3C). However, *MdACO1* expression was significantly lower in apples overexpressing *MdbHLH25* with bH T-T-GT genotype (Fig. 3C). The expression of *MdbHLH25* varied between transformants but without statistical significance (Fig. 3C). As expected, significantly higher flesh crispness was retained in apples overexpressing *MdbHLH25* with bH T-T-GT genotype, but transformants with bH G-C-TC haplotype without showing any significant changes in flesh firmness among transformants (Fig. 3D). These data indicated that SNP2 T allele and/or SNP4/5 GT alleles extensively attenuated MdbHLH25 function in addition to a slight suppression by SNP3 T allele.

Interaction between MdbHLH25 and MdACS1

Y1H indicated that MdbHLH25 protein bound directly to the promoter of *MdACS1* but did not directly interact with *MdACO1* promoter (Fig. 4A). The SNPs at the *MdbHLH25* CDS did not affect the qualitative interaction between MdbHLH25 and the promoter of *MdACS1* (Fig. 4A). Transient transformation assay confirmed the interaction between MdbHLH25 and *MdACS1*, and the GUS staining and GUS gene expression further revealed that the SNP haplotype bH G-C-TC of *MdbHLH25* exhibited a significantly higher activity to promote *MdACS1* expression than the other SNP haplotypes (Fig. 4B, C).

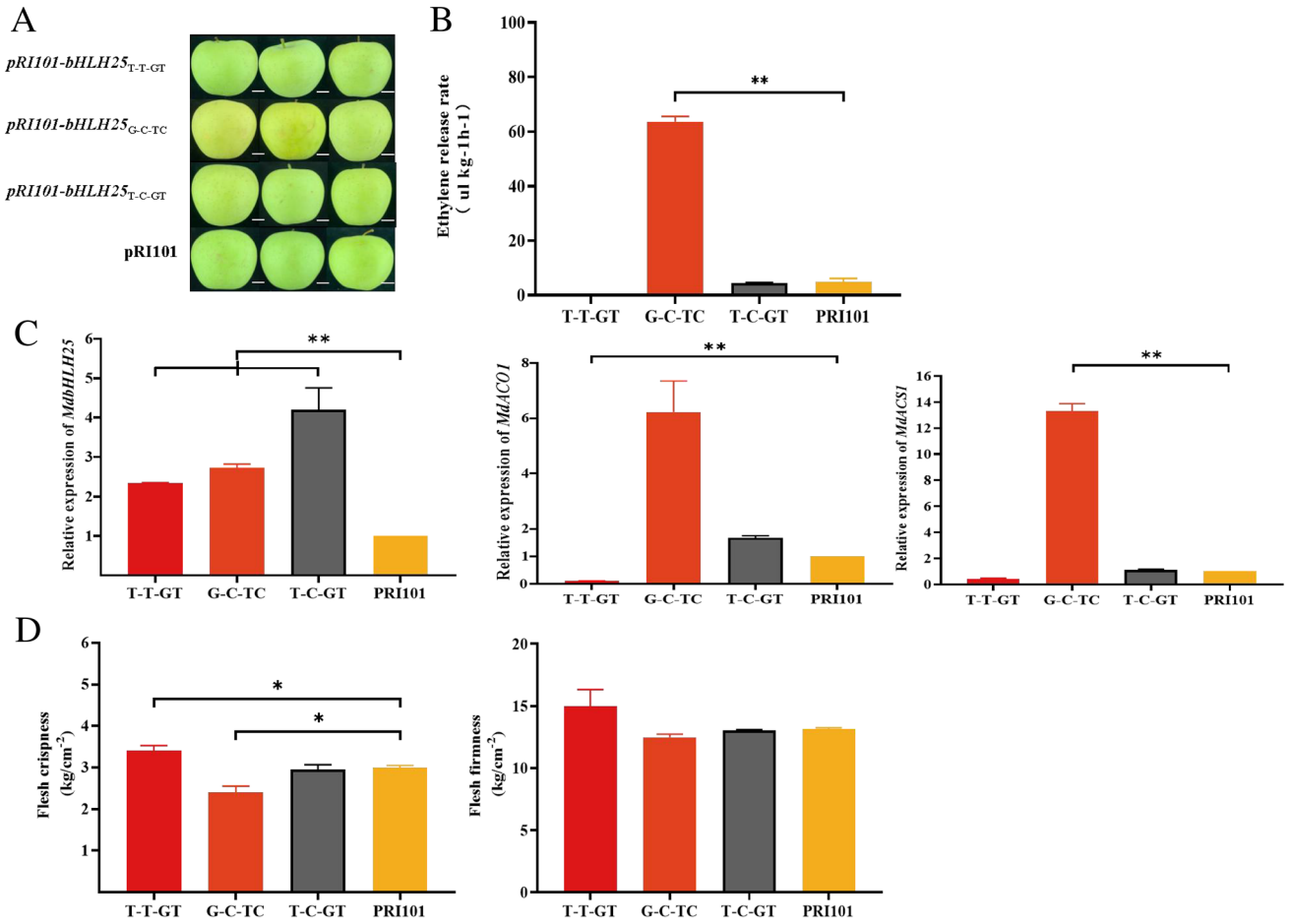

Fig. 3 Functional validation of SNPs at *MdbHLH25* coding sequence via 'Golden Delicious' apple fruit transient transformation. **A** Fruit skin de-greening phenotype of transformed fruit. **B** Ethylene release rate of transformed fruit. **C** Relative expression of *MdbHLH25*, *MdACO1*, and *MdACS1* of transformed fruit. **D** Changes in flesh crispness and firmness of transient transformed fruit. All the data were collected on day 8 after transformation. Three replicates were set for each experiment. Statistically significant differences were determined by *t* tests: $^{*}P < 0.05$, $^{**}P < 0.01$

Effect of SNP genotype combinations of MdbHLH25 and MdWDR5A

Interaction between bHLH and WD proteins has been reported years ago (Dubos et al. 2008). The protein–protein interaction between *MdbHLH25* and *MdWDR5A* was confirmed by Y2H, BiFC, and pulldown assay; however, none of the SNPs at *MdbHLH25* or *MdWDR5A* CDS qualitatively affected the interactions at molecular level (Fig. 5A–C).

To estimate the genotype effect on FF, FC, FFR, and FCR, a training population was used consists of 478 hybrid plants from three F1 populations. The phenotype data have been collected previously (Wu et al. 2021b). SNP2, and SNP4 of *MdbHLH25*, as well SNPi and SNPii of *MdWDR5A* were developed as KASP markers (Table S3). SNP3 and SNP5 of *MdbHLH25* were failed to be developed into KASP markers, because they were too close to SNP2 and SNP4 in the genome, respectively. The markers differed in the effects on FF (0.10–2.55 kg/cm^2), FC (0.01–0.28 kg/cm^2), FFR (0.22–1.45 months), and FCR (0.32–1.03 months) (Table S4). The SNP1 (SNP38573599) of *MdbHLH25* exhibited over-dominance allelic effect, because the genotype effects of CA on FF, FC, FFR, and FCR were less than that of AA (Table S4). The effects of *MdbHLH25* SNP2 GT genotypes on the four traits were partial-dominant, because the effects of GT genotype deviated far from the median of the effects of GG and TT genotypes (Table S4). The joint effects of genotype combinations of SNP1, SNP2, and SNP4 of MdbHLH25, and SNPi and SNPii of MdWDR5A were 4.51 kg/cm^2, 0.40 kg/cm^2, 2.80 months, and 3.10 months on FF, FC, FFR, and FCR, respectively (Fig. 6A) (Table S5). GAP models for FF, FC, FFR, and FCR were developed using the joint effects of SNP genotype combinations (Fig. 6B). The prediction accuracy was 0.4025, 0.3692, 0.4267, and 0.3915 for FF, FC, FFR, and FCR, respectively ($n = 451$, $P < 0.01$) (Fig. 6B). These data indicated that the

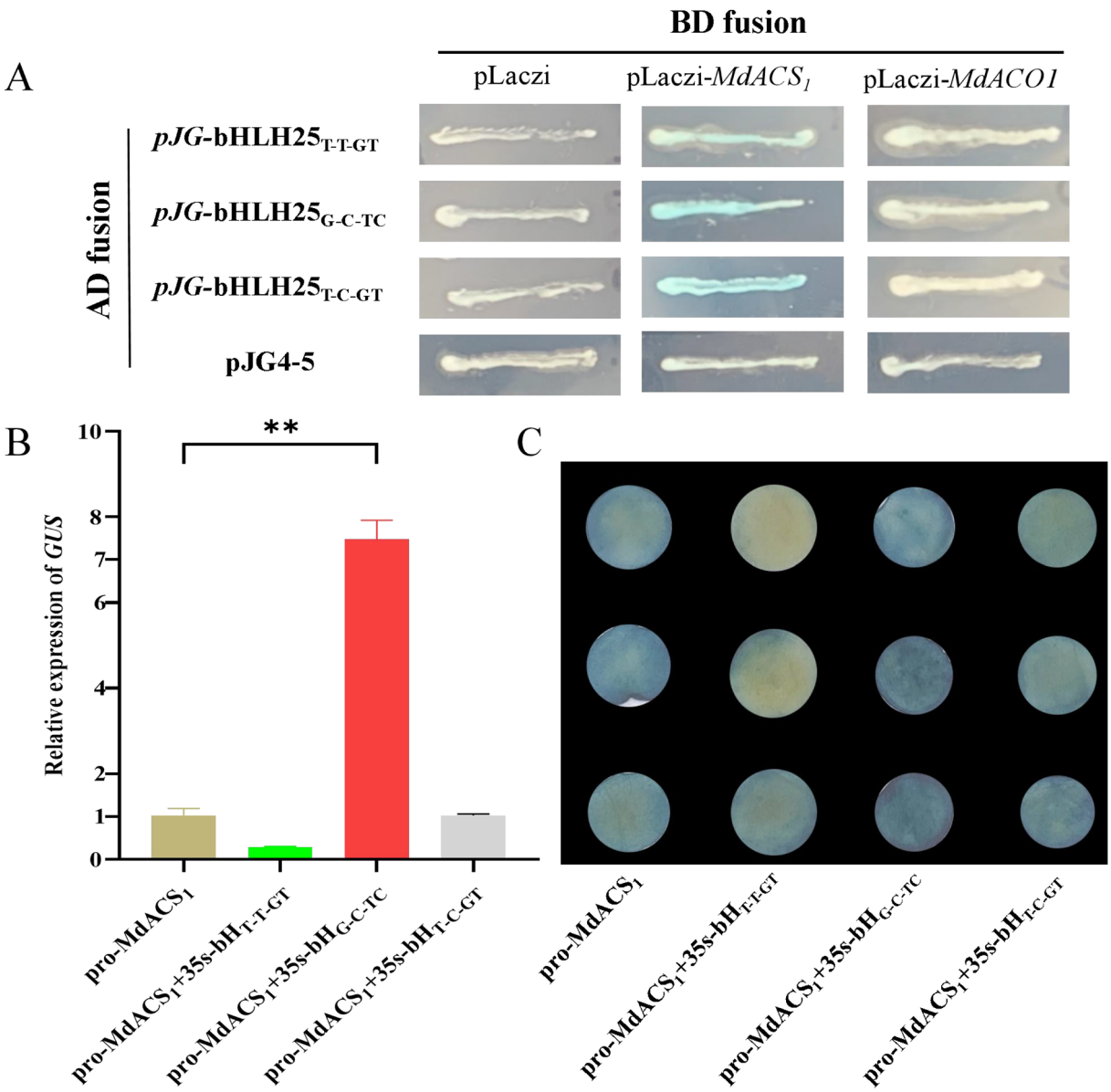

Fig. 4 Yeast-one-hybrid assay and GUS reporter assay indicate the interaction between MdbHLH25 protein and the promoter of *MdACS1* but not *MdACO1*. **A** Yeast-one-hybrid images. Full-length coding sequence of *MdbHLH25* with different SNP haplotypes of SNP2, SNP3, and SNP4/5 was constructed on pJG4-5 vector as AD, while upstream 2 kb sequences of *MdACS1* and *MdACO1* promoters were, respectively, constructed on pLaczi vector as BD. **B**, **C** Images of GUS reporter assay showing that the GUS gene expression (**B**) was higher and that the GUS staining (**C**) was more intensive when 35S:MdbHLH25G-C-TC was co-injected with pro-MdACS1 than the other treatments

non-allelic epistasis of the five SNPs at *MdbHLH25* and *MdWDR5A* contributed to broaden the phenotype segregation of FF, FC, FFR, and FCR in apple.

Discussion

The storability is primarily characterized by the FFR and FCR, which are impaired by the FF, FC, and the rate of flesh softening during storage (Costa 2015; Johnston et al. 2001; Nybom et al. 2013). At the molecular level, the storability was controlled by a large quantity of genetic variations on a wide spectrum of genes which have been mapped on at least 11 chromosomes (Longhi et al. 2012; Costa 2015; Hu et al. 2020; Liang et al. 2020; Wu et al. 2021b). Therefore, a great challenge must be faced to implement molecular breeding for fruit storability, depending upon the accurate dissection of the genetic variations of these complex traits.

Using BSA-seq strategy and 2664 hybrids from three cross populations as plant materials, 62 significant QTLs

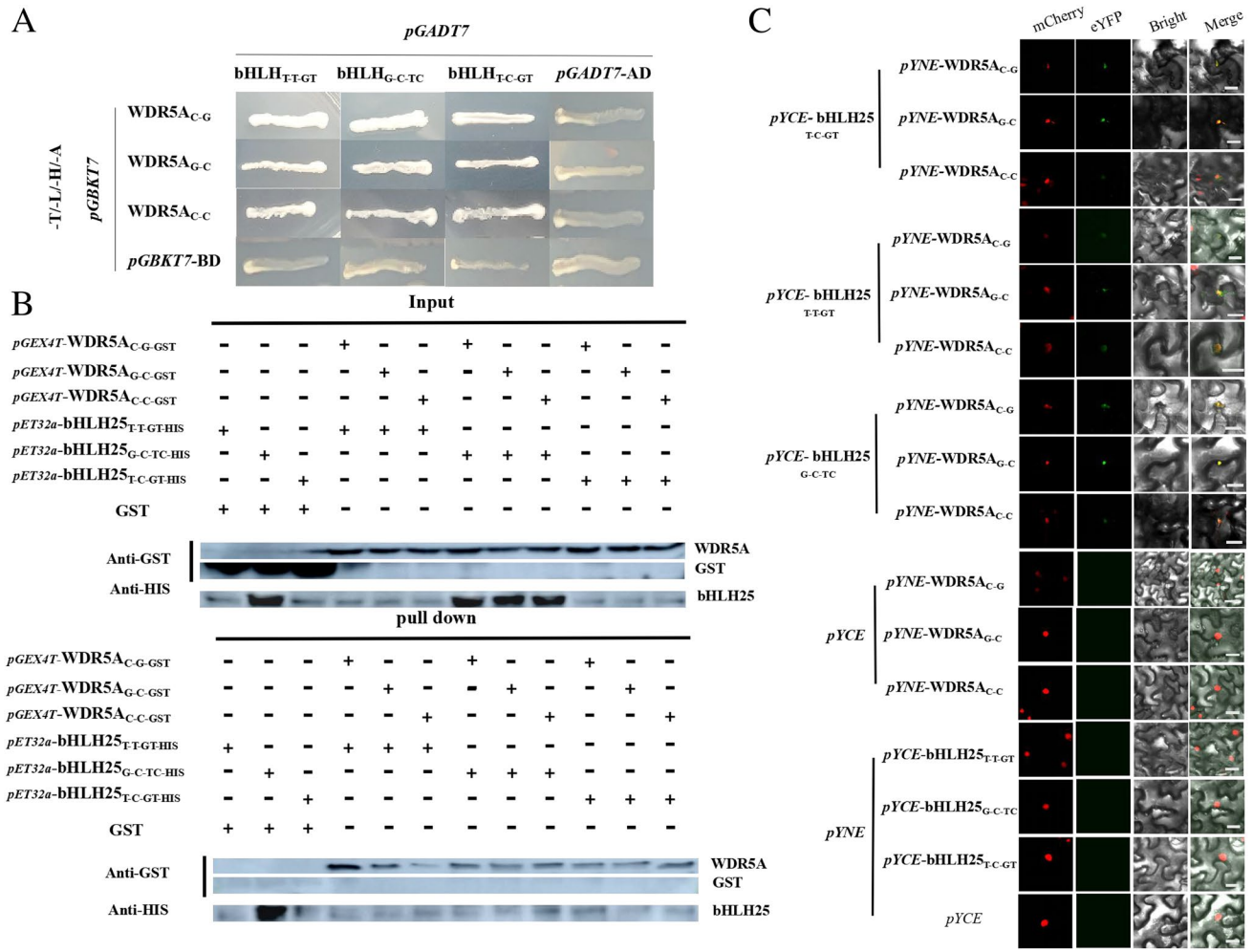

Fig. 5 None of the SNPs affected the interaction between MdbHLH25 and MdWDR5A proteins. **A** Yeast-two-hybrid assay, *MdbHLH25* CDS with different haplotypes of SNP2, SNP3, and SNP4/5 was constructed into pGADT7 as AD, while *MdWDR5A* CDS with different haplotypes of SNPi and SNPii was constructed into pGBKT7 vector as BD, respectively. No differences were identified among AD and BD combinations. **B** Pull-down assay, pET32a vector was used for *MdbHLH25* CDS with different haplotypes of SNP2, SNP3, and SNP4/5, and pGEX-4T vector was used for *MdWDR5A* haplotypes of SNPi and SNPii. No differences were found among treatments. **C** Bimolecular fluorescent complimentary assays, *MdbHLH25* and *MdWDR5A* CDS with different SNP haplotypes, were constructed into SPYCE(M)-35S and SPYNE173-35S vectors, respectively. No differences were found among treatments. Bars 50 μm

were previously mapped on nine chromosomes for apple FFR and FCR (Wu et al. 2021b). The FF and FC phenotype values of the parental cultivar 'Zisai Pearl' were higher than that of the other parents, but the FFR and FCR phenotype values of 'Red Fuji' were longer than the other cultivars (Table S6). Chromosome 16 was reported as a 'QTL hotspot' for apple flesh texture traits (Bink et al. 2014; Di Guardo et al. 2017; Wu et al. 2021b). In this study, five SNPs were detected and at least three of them were functional variations in *MdbHLH25* from the QTL F16.1/H16.2. In addition, two SNPs exhibiting genotype effect on the FFR and FCR phenotypes were found at a nearby gene *MdWDR5A*. The genetic recombination and the epistasis between the non-allelic variations of these SNPs acted both to alter the genetic effect on the target trait and to broaden the phenotypic segregation spectrum. Similarly in apple rootstock, at the promoter of *MdLAZY1*, two functional SNPs caused wider segregation in root growth angle (Zheng et al. 2020a). These data indicated that a single marker designed on a certain QTL region may sometimes not be sufficient to represent the genetic variation when GAP models were developed (Zheng et al. 2020b; Liu et al. 2020; Wu et al. 2021b). Multiple markers developed from a QTL may have advantages to improve the accuracy of the prediction; to the utmost, genome-wide random markers were explored in the genomic selection (GS) strategy (Kumar et al. 2012; Muranty et al. 2015). The prediction accuracy of the GAP models for FF, FC,

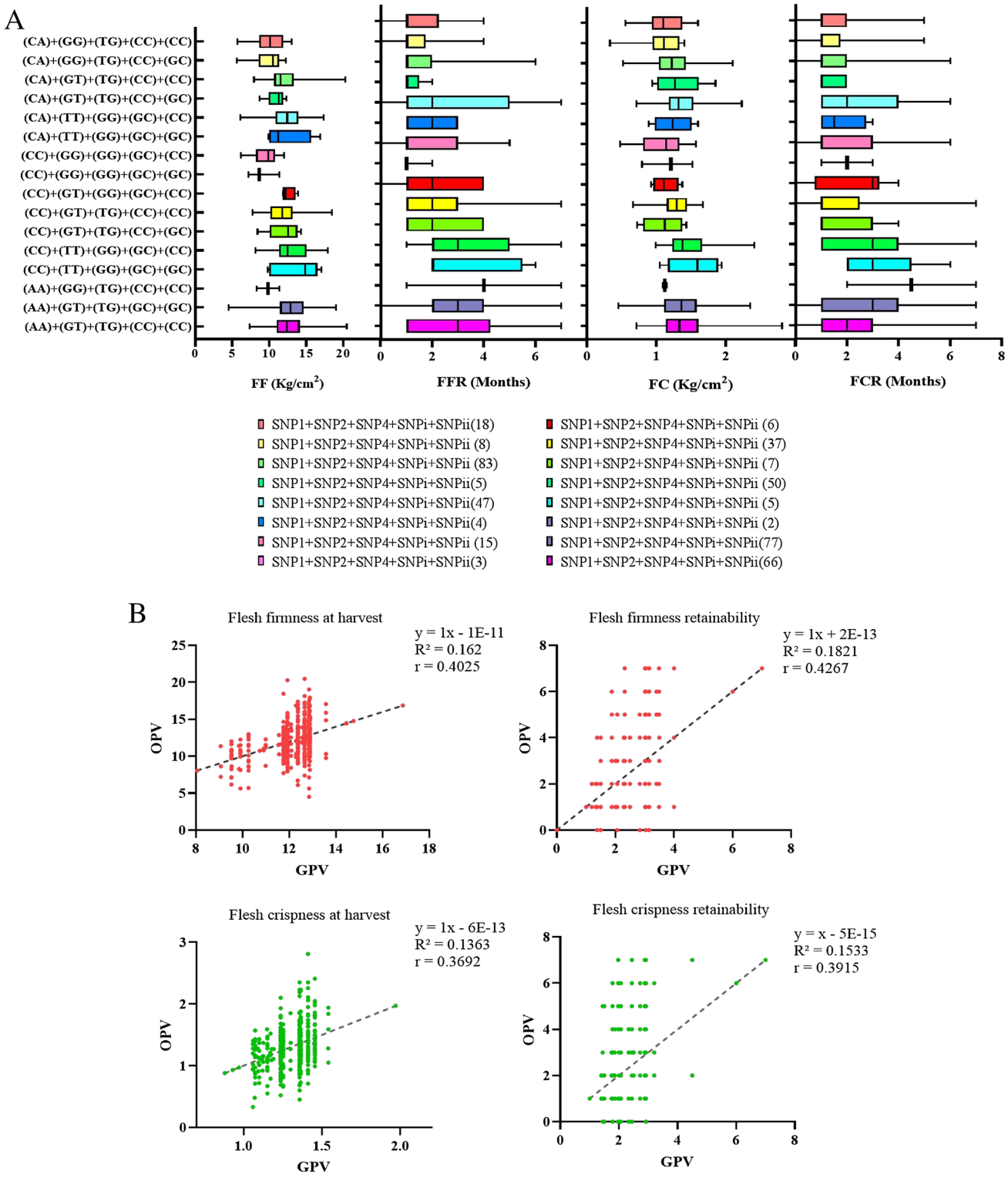

Fig. 6 Joint effect estimation and validation of SNP genotype combinations of *MdbHLH25* and *MdWDR5A* on apple flesh firmness at harvest (FF), flesh crispness at harvest (FC), flesh firmness retainability (FFR), and flesh crispness retainability (FCR). **A** Boxplot illustrating the joint effects of SNP genotype combinations. **B** The genomics-assisted prediction (GAP) models for FF, FC, FFR, and FCR using the joint effects of SNP genotype combinations. The prediction accuracy was represented by the linear regression between genotype predicted values (GPVs) and the observed phenotype value (OPV)

FFR, and FCR in this study was statistically significant, but was relatively low compared with GAP models using genome-wide QTL markers or pedigreed families (Bink et al. 2014; Wu et al. 2021b).

Some bHLH transcription factors have been reported to regulate ethylene biosynthesis and fruit ripening (Li et al. 2017; Hu et al. 2019; Song et al. 2020). We found that MdbHLH25 participated in controlling ethylene synthesis and thus apple FFR and FCR by directly binding to the promoter of *MdACS1*. SNP1 A allele at the *MdbHLH25* promoter caused a decrease in the expression, while SNP2 and/or SNP4/5 quantitatively attenuated the function to activate the target genes like *MdACS1*. *MdbHLH25* transient overexpression led to an increased *MdACO1* expression, but YIH and GUS reporter assay did not reveal the direct interaction between MdbHLH25 and *MdACO1* promoter, which indicated that MdbHLH25 might act indirectly on *MdACO1* expression. It has been reported that the key genes in ethylene synthesis pathway can be indirectly regulated by several transcription factors like *MdERF2* (Li et al. 2016).

In plants, bHLH interacts with WD40 protein, which is an important component of the WD40/MYB/bHLH ternary protein complex (Grotewold et al. 2000; An et al. 2012). In this experiment, the protein–protein qualitative interaction between *MdbHLH25* and *MdWDR5A* was not interfered by any of the SNPs at their CDS, but the joint genetic effects varied among SNP genotype combinations (Fig. 6A), implying that SNPi and/or SNPii of *MdWDR5A* could also be functional variation(s).

The dominant and additive effects among alleles of a genetic variation, as well the epistasis of non-allelic interactions have interpreted at the molecular level (Jia et al. 2018). Epistatic effect was identified between *MdbHLH25* and *MdWDR5A* in this study (Table S5), but the molecular mechanism was not explored. Genetic variation in downstream genes of a regulatory pathway, such as *MdALMTII* controlling apple fruit acidity, often exhibits epistasis on the genetic variation in the upstream genes, like *MdPP2CH* and *MdSAUR37* (Jia et al. 2018). Epistatic effect of *MdACS1* on *MdbHLH25* was implied by the direct regulatory interaction in this study, but the epistasis was not analyzed, because the genotype of the marker on *MdACS1* did not segregate in the F1 population of 'Zisai Pearl' × 'Red Fuji' (Zhu and Barritt 2008; Wu et al. 2021a). Over-dominant allelic effect has been reported in our previous work (Shen et al. 2022). As in the present case, over-dominant effects were observed for the CA genotype of *MdbHLH25* SNP1 on FF, FC, FFR, and FCR phenotypes (Table S4). Unfortunately, the molecular mechanism of the over-dominance, which was usually described in genetics, was not fully understood to date. Whether the over-dominance is attributed to the complicated interactions among multiple genetic variations' demands for further insight investigation.

Conclusion

From an overlapped QTL region for apple fruit FFR and FCR, five and two SNPs were identified on *MdbHLH25* and *MdWDR5A*, respectively. SNP1 A allele at the promoter of *MdbHLH25* reduced the promoter activity, while SNP2, SNP3, and SNP4/5 at the CDS of *MbHLH25* attenuated the function to activate the target gene *MdACS1*. Any of the SNPs at the CDS of *MdbHLH25* and *MdWDR5A* did not interfere the qualitative protein–protein interaction, but the joint effect of genotype combinations by SNP1, SNP2, SNP4 of *MdbHLH25* and SNPi, SNPii of *MdWDR5A* led to a much broad spectrum of phenotypic segregation.

Author contribution statement XY, BW, and XinZ conceived and designed the experiments. XW, HZ, YW, TW, XX, XiZ, XinZ, and ZH contributed to the plant materials. XY, BW, JL, ZZ, and XR performed the experiments. XY and XinZ wrote the paper. All authors have read and approved the manuscript.

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Data availability The apple genome used was a version of the *Malus* × *domestica* genome GDDH13_v1.1 (GDDH13, https://iris.angers.inra.fr/gddh13/).

Declarations

Conflict of interest The authors declare that they have no competing interests.

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