Identification of Fruit Firmness QTL *ff2.1* by SLAF-BSA and QTL Mapping in Melon

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Abstract

Fruit firmness is an important target of melon breeding, as it is associated with shelf life and economic value; however, the precise mechanism determining fruit firmness during fruit ripening remains elusive. In the present study, one hundred forty-four F_2 plants and $F_{2\text{-}3}$ families derived from the high-firmness melon line M2-10 and the low-firmness melon line ZT091 were used to identify major quantitative trait loci (QTLs) by specific-locus amplified fragment (SLAF) sequencing with bulked segregant analysis (BSA). Simple sequence repeat (SSR) and cleaved amplified polymorphic sequence (Caps) markers based on the resequencing of parental lines were also used to narrow the associated region to identify candidate genes. Two regions associated with fruit firmness were investigated, including a 4.87 Mb region on chr. 2 and a 28.7 Mb region on chr. 5 of the melon genome. SSR and Caps markers were used to construct a genetic map of the associated regions: QTL *ff5.1* was located between CmSSR13509 and CmSSR13423 and explained 38.44% of the observed variation, with an LOD threshold of 17.44; *ff2.1* was located between CmSSR07709 and SNP22228 and explained 28.14% of the variation, with an LOD threshold of 3.8, and this region included 106 Kb and 10 candidate genes. Quantitative Real-time PCR (qRT-PCR) was used to investigated the investigate candidate gene expression at 15, 20 and 25 days after pollination (DAP) in the parental lines, and significant expression levels were detected for most of the genes, including four genes of unknown function and MELO3C017519, MELO3C017520, MELO3C017522, MELO3C029506, and MELO3C029520. These results revealed a new QTL, *ff2.1*, for melon fruit firmness-related gene identification.

Introduction

Melon (*Cucumis melo*. L) is an important *Cucurbitacea* species not only due to the diversity of its fruit but also because it produces both climacteric and nonclimacteric fruit types. Melon exhibits extensive variation in fruit traits, including soluble sugar content, aroma volatile content, flesh colour, and fruit firmness, shape and size (Galpaz et al. 2018). This makes melon an important model crop for studying the genetics of fruit-related traits. QTLs and genes affecting fruit-related traits, including ethylene synthesis (Ríos et al, 2017), aroma (Ayub et al. 1996; Gonda et al. 2016) and fruit acidity (Cohen et al. 2014), were recently identified. In contrast to other agronomic traits of melon, research aimed at identifying QTLs for fruit weight and fruit size has identified more than 200 QTLs arranged on 12 melon chromosomes. Fruit firmness is a complex trait that is important for melon breeding, especially for transportation and storage, but the understanding of melon fruit firmness is poor. Fruit firmness is a typical quantitative trait affected by germplasm inheritance and cultivation management, and it represents an important fruit harvest and maturity index. This complex trait involves numerous physical components, including cell wall, cell turgor, and cuticle characteristics. Previous research has indicated that the disintegration and degradation of the cell wall are intimately involved in fruit softening (Shim et al. 2014). In horticultural plants, the *LeExp1* (Solyc06g051800) (Brummell et al. 1999), *SlCOBRA-like* (Solyc02g065770) (Cao et al. 2012), *SlPL* (Solyc03g111690) (Uluisik et al. 2016), *GDSL1* (Solyc02g071620) (Girard et al. 2012) and *AP2* transfoator *SlSHN3* (Solyc06g053230) genes were all shown to be related to fruit development and firmness (Shi et al. 2013). More recently, the map-based cloning of a fruit firmness regulator gene in tomato (*Solanum lycopersicum* L.) indicated that an *FIS1* mutation increased the bioactive gibberellin content, enhanced cuticle and wax biosynthesis, and increased fruit firmness.

In 2008, an introgression line (IL) population founded from PI 161375 and PS, both of which are nonclimacteric, led to the identification of QTLs for flesh firmness (*ff2.2*, *ff3.5*, *ff8.2*, *ff8.4* and *ff10.2*) in melon. Pe´reira et al. (2020) used recombinant inbred lines (RILs) to study traits related to climacteric maturation and indicated that flesh firmness in F_1 fruits was lower than that in either parent, and the analysis of a flesh firmness QTL (FIRQV) further suggested that *FIRQV2.1*/*FIRQV2.2* is incapable of triggering autocatalytic ethylene production by itself. Studies have been conducted on the molecular mechanism of fruit ripening and softening, especially on the relationship among fruit ripening, fruit softening and cell wall enzyme activity (Peter et al. 2007; Luis et al. 2007). The ripening and softening of melon are closely related to ethylene. Concerning the molecular mechanism of this process, the *CmACS1* and *CmACO1* genes have been successfully cloned and shown to be related to fruit softening (Gao et al., 2021). Additionally, genes encoding β-D-xylosidase, glyoxysomal malate synthase, chloroplastic anthranilate phosphoribosyltransferase (MELO3C011963) and histidine kinase (MELO3C020055) have been demonstrated to be associated with flesh firmness in melon (Nimmakayala et al. 2016).

Fruit QTL mapping is a critical method for selecting candidate regions and genes for important quantitative traits, and tightly linked markers can be used for marker-assisted selection (MAS) in plant breeding. Traditional QTL analysis based on genetic map construction requires a large population size and many markers arranged on each chromosome. Bulked segregant analysis (BSA) is a simplified strategy for rapidly identifying markers linked to target genes or QTLs that affect a trait of interest; this approach is based on genotyping only one pair of pooled DNA samples from two sets of individuals with distinct or opposite extreme phenotypes (Michelmore et al. 1991; Du et al. 2019), and it can the time and labour costs. To date, BSA has been useful for the primary mapping and development of target putative QTLs/genes in numerous horticultural crops, including watermelon (Dong et al. 2018; Sun et al. 2020; Yang et al. 2021), melon (Li et al. 2017a, b; Liu et al. 2019; Zhang et al. 2019), cucumber (Zhang et al. 2015), and tomato (Zhao et al. 2016). Specific-locus amplified fragment sequencing (SLAF-seq), developed with next-generation sequencing (NGS) technologies, is a strategy for identifying single-nucleotide polymorphisms (SNPs) to investigate target regions/genes or to construct genetic maps (Geng et al. 2016). The combination of SLAF-seq and BSA technologies (SLAF-BSA) has been successfully used to identify major QTLs in pepper (Xu et al. 2015;Xu et al. 2018; Guo et al. 2017), cucumber (Zhang et al. 2018;) and melon (Qiu 2019; Gur et al. 2017).

In the present study, to determine the firmness of mature fruit, high-firmness and low-firmness parental lines were used to perform crosses, and F_2 high-/low-firmness bulks were constructed based on F_3 phenotype data. The SLAF-BSA strategy was further used to rapidly identify regions associated with mature fruit firmness in melon, and QTL mapping was performed to verify the regions associated with fruit firmness and identify linked markers.

Materials And Methods

Plant materials and inheritance analysis

F_2 plants and F_{2-3} families were derived from a cross of M2-10 × ZT091 melon for the evaluation of fruit firmness and the identification of related genes in the greenhouse of Heilongjiang Bayi Agricultural University. M2-10 is a thin-skinned melon line with high fruit firmness when the melon ripens (reaching maturity ~28 days after pollination (DAP), fruit firmness of ~10.8). ZT091 was obtained from the Zhengzhou Fruit Research Institute and is a thin-skinned melon with low fruit firmness when the melon fruit ripens (~25 DAP, fruit firmness of ~3.2). To detect the inheritance character of fruit firmness, one hundred forty-four F_2 and $F_{2:3}$ families(each family contained ~15 plants) were planted in the greenhouse field in 2019 and 2020, respectively.

Phenotype collection and bulked construction

Fruit firmness of fifteen plants of parental lines and F_1 plants, each F_2 individual, and the average of F_3 families were collected when fruit ripened. Fruit firmness was measured by three replications in the top, middle and bottom of a single fruit.

For bulk construction, thirty F_2 plants with fruit firmness >9.0 or <3.0 were employed as hard and soft bulks, respectively.

Whole-genome sequencing of parental lines and bulk SLAF-seq

The two bulked pools and the parental lines M2-10 and ZT091 were subjected to sequencing on an Illumina GAIIx system according to Sun et al. (2013). The preparation of genomic DNA from the parental lines and two bulked pools and the PCR settings followed the Illumina sample preparation guide. The samples were gel-purified, and products with appropriate sizes (260~420 bp) were excised and diluted for sequencing on the Illumina GAIIx platform (Illumina, San Diego, CA, USA). The selected reads were compared to the reference genome (http://cucurbitgenomics.org/).

Association analysis

The analysis of SLAF-seq data was conducted according to Abe et al. (2012). The SNP index indicates the proportion of reads harbouring a SNP that differs from the reference sequence. The allelic frequency was calculated by Euclidean distance followed by Loess regression analysis, which identifies the region in which an MA trait is located and generates a list of

candidate regions in the linked genomic segment. The variations in the samples and the results of BSA association analysis were plotted with Circos software, http://circos.ca/.

DNA preparation and SSR phenotyping

Genomic DNA was extracted from the tender leaves of each individual using the CTAB method according to Luan et al. (2010). Each bulk was produced by mixing equal amounts of DNA from 30 plants with high fruit firmness or low fruit firmness. DNA quality and concentrations were measured by 1% agarose gel electrophoresis, and the final DNA concentration was adjusted to 75~100 ng/μL.

Each PCR mixture contained 30 ng template DNA, each of forward and reverse primer at 1.0 μM, 0.2 mM dNTPmix, 0.1 unit of Taq DNA polymerase and 1× PCR buffer (Takara, China) in a total volume of 10 μL. A restriction enzyme was added to the PCR mixture. After performing the specific primer-based PCR programme, incubation was performed for 2 h at the appropriate temperature according to the manufacturer's instructions. Then, 6% polyacrylamide gel electrophoresis with silver staining was used to separate the digestion products.

Development of SSR markers and mapping of associated regions

Ninety molecular markers were used in the present study, most of which were obtained from the published literature (Zhu et al. 2016). SSR markers between candidate regions were used for linkage analysis of the bulked pools to narrow the candidate region for fruit firmness. All plants were genotyped with morphological markers between preliminary candidate regions and internal regions (LG VIII and LG IX), and a genetic map was constructed with JoinMap 4.0. The Kosambi map function was used to calculate the distance between markers in the genetic map.

Candidate locus sequencing assembly and DNA analysis

The DNA sequences of candidate regions were retrieved from the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi/) and the database of *Cucurbitaceae* of the Weng Lab at the University of Wisconsin, Madison, USA (http://cuke.vcru.wisc.edu/wenglab/). Softberry was employed to predict candidate gene structure with the Open Reading Frame Finder and FGENESH tools (http://linux1.softberry.com).

qRT-PCR

Total RNA was isolated from the fruit sarcocarp of hard-FF M2-10 and soft-FF ZT091, and RNA was extracted for candidate gene expression analysis. Flesh sarcocarp samples from three developmental stages (15, 20 and 25 DAP) were selected for RNA isolation using RNA Plus (Takara, China). qRT-PCR was performed using the IQ5 system (Bio-Rad, United States) in a volume of 20 μL. The PCR primers were designed by using Primer 5.0 software (Supplementary Table S1). Each reaction contained 10 μL of 2× TransStart Top Green qPCR Supermix (Transgene, China), 10 pmol of each primer, 2 μL of the cDNA template, and distilled water added to a volume of 20 μL. The two-step method of thermal cycling was employed. qRT-PCR was performed under the following conditions: 95 °C for 15 s and then 55 °C for 15 s, followed by a slow increase in temperature of 0.5 °C per cycle to 95 °C, with continuous measurement of fluorescence. Three replicates were performed for the qRT-PCR experiment, and the ΔCt method was used to analyse mRNA expression.

Results

Performance of fruit firmness

As observed in the field, M2-10 ripened at ~28 DAP, with a fruit firmness of 10.8 ± 1.24, and ZT091 ripened at ~25 DAP, with a fruit firmness of 3.2 ± 0.32. There was a significant difference between the parental lines (Fig. 1). F_1 fruit ripened ~30 DAP, with a fruit firmness of 8.7 ± 1.15, and the average fruit firmness values of the F_2 population and F_3 families were 5.3 ± 1.12 and

5.93 ± 0.89, respectively. The fruit firmness of each population was continuously distributed, reflecting the fact that it is a quantitative trait controlled by multiple genes (Fig. 2).

SLAF-base sequence

In total, 18.53 million reads were obtained from the high-throughput sequencing of the constructed SLAF library. After filtering, 18.39 Gb of clean bases were acquired. The average Q30 values of the parental lines were 93.03% and 92.88%. The GC contents of the sequenced parental lines were 36.94% and 38.03, respectively. The numbers of total raw reads were 42,916,190 and 45,759,537 for M2-10 and ZT091, respectively, and the numbers of clean reads for the male and female parents were 42,680,203 and 45,492,988, respectively (Table 2). In total, 31,658,000 clean reads were obtained for the high-firmness bulk and 29,713,764 for the low-firmness bulk. The average Q30 was 92.45%, and the GC contents of the high-firmness and low-firmness bulks were 37.32% and 37.85%, respectively. After the tags were aligned with the reference genome, more than 93% of the sequences were mapped to the reference genome. The average sequencing depths were 26.5 X and 18.5 X for parental lines and bulks, respectively (Table 2).

Association analyses

SNP-based association analysis

In total, 12,365 high-quality SNP loci were identified. Based on the Euclidean distance method and the use of the median + 3 SD to select the correlation threshold of all loci, 5.66 was finally selected as the threshold, and five candidate regions were shown to be associated with fruit firmness. These regions covered a total of 88.73 Mb in the associated region and included 5,804 genes, within which 321 nonsynonymous SNP loci were detected. However, since the theoretical threshold was not reached (7.06), this region needs further verification.

SNP index analysis is an association analysis method based on the difference in genotypic frequency between bulks. In the present study, the DISTANCE method was used to analyse the ΔSNP index to select an association threshold. When the threshold was 0.99, eighty-nine associated regions containing 8,892 genes, covering 127.62 Mb of the genome and including 469 nonsynonymous SNP loci, were identified. Based on these SNPs, the results obtained for the associated regions via the two association analysis methods overlapped on chr. 2 chr. 5 and chr. 10 (Table 3 and Fig. 3).

InDel-based association analysis

Before the association analysis, 2,741 high-quality InDel loci were identified. Based on the Euclidean distance method, 5.66 was selected as the threshold, and six associated regions, covering 88.73 Mb and including 5,804 genes, were identified. In this region, 107 genes contained mutant InDel loci. Based on the InDel loci, the Δindel-index method was applied with a selected a threshold of 0.99; within fifteen associated regions containing 8,892 genes and covering 143.08 Mb, 117 genes containing mutant InDel loci were identified.

According to the results of the two index calculation methods, two associated regions (*ff2.1* and *ff5.1*) were identified. One of these regions was located on chr. 2 from 18996333 to 23870331, covering 4.87 Mb, and the other was located on chr. 5 from 49380 to 28751254, covering 28.7 Mb.

Narrowing the mapped locations of QTLs *ff2.1* and *ff5.1*

To narrow the associated regions, SSR markers were used to identify polymorphic loci between the parental lines. Eight and nine SSR polymorphic markers were obtained from 144 $F_{2:3}$ families (each family contained 15 plants) to narrow the associated regions on chr. 2 and chr. 5, respectively. QTL *ff2.1* was located on chr. 2 between CmSSR07709 and SNP22228 and explained 28.14% of the phenotypic variation; the LOD value of *ff2.1* was 3.8. The candidate region spanned genomic positions 22122424 to 22228642, covering 106 Kb, and it contained 10 candidate genes (Fig. 4).

QTL *ff5.1* was located on chr. 5 between CmSSR13509 and CmSSR13911 and explained 38.44% of the phenotypic variation; the LOD value of *ff5.1* was 17.44, which was relatively high and indicated that this QTL was a major QTL for fruit firmness. The candidate region spanned genomic positions 10503296 to 18014796, covering 7.5 Mb. Positive additive effects of *ff2.1* and *ff5.1* were contributed by the alleles of M2-10 (1.82 and 0.85, respectively; Table 5)

Gene annotation in the candidate region of chr. 2

A candidate region spanning 138 Kb with 10 genes was screened out, but only six genes were annotated in the region. These genes encoded an auxin response factor (ARF) (MELO3C029720), movement protein (MELO3C029506), cell division cycle 5-like protein (MELO3C017522), L-allo-threonine aldolase (MELO3C017520), adenyl sulphate kinase (MELO3C017519) and chloride channel protein (MELO3C029513) (Table S1). To identify the candidate gene expression levels, sarcocarp samples from 15, 20 and 25 DAP in parental lines were subjected to qRT-PCR analysis. None of the candidate genes except for MELOC3C029513 showed a significant difference in expression between the parental lines in the different fruit development stages. Although MELO3C017513 and MELO3C017523 showed no difference in expression between M2-10 and ZT091 at 25 DAP (Fig. 5 a and b), the other genes all showed significant differences in expression at this time point. According to the results for most of the genes, 20 DAP was a key point in fruit development. For example, in the hard-fruit line M2-10, the MELOC3C017518, MELOC3C017523, and MELOC3C029507 genes (of unknown function) all showed decreased gene expression at 20 DAP but highly increased gene expression at 25 DAP (Fig. 5 b-d); the same expression trend was observed for MELOC3C029519, MELOC3C029520, MELOC3C029522, and MELOC3C029506 (Fig. 5 e-h). In the soft-fruit line ZT091, these genes showed interesting patterns of expression. For MELOC3C017519, MELOC3C017522, MELOC3C017523, MELOC3C029506, and MELOC3C017507, gene expression was higher at 20 DAP than at 15 DAP but significantly decreased at 25 DAP. Opposite trends were observed in M2-10 and ZT091 for MELO3C017518 and MELO3C017519, as shown in Fig. 5c and e. There were no significant differences detected between 20 and 25 DAP in the soft-fruit parent ZT091 (Fig. 5c and e). For MELOC3C029508, significant gene expression levels were detected at 15, 20 and 25 DAP, and a decreasing trend was found in M2-10(Fig. 5i). For MELOC3C029720, gene expression was increased in M2-10 at 15, 20 and 25 DAP, but both increasing and decreasing trends of expression were detected in ZT091 (Fig. 5j). For MELOC3C029720 (ARF gene), there were no differences at 15, 20 or 25 DAP in the hard-fruit line M2-10, but a significant difference was detected at 25 DAP in ZT091. In all of the different fruit development stages, significant differences were detected between M2-10 and ZT091 (Fig. 5f). For MELOC3C0217519 (adenylyl-sulphate kinase gene), there were significant differences in gene expression levels among the three fruit development stages in each parental lines and between M2-10 and ZT091 at 15, 20 and 25 DAP (Fig. 5e). For MELOC3C0217522 (cell division cycle 5-like protein related gene), there were no significance differences between 15 and 25 DAP in either M2-10 or ZT091, but a significant decrease in M2-10 and a significance increase in ZT091 were found (Fig. 5g). For each fruit development stage, gene expression was higher in ZT091 than in M2-10, and a significant difference was detected. For the Movement protein gene MELO3C029506, significant gene expression levels were detected at 25 DAP in M2-10 and ZT091, and the same was true for the L-allo-threonine aldolase gene (MELO3C017520) (Fig. 5 g and j).

Discussion

Genetic map construction is a useful method for investigating QTLs and qualitative traits of plants. However, the examined marker numbers and plant generations can influence its efficiency. With the development of sequencing technology and advanced bioinformatics tools, the application of sequencing techniques with the BSA method has become a rapid approach for identifying the genetic regions controlling various traits, including fruit traits (Michelmore et al. 1991; Yang et al. 2021). BSA has been used for rapid primary mapping and the investigation of QTLs/gene(s) in numerous horticultural crops, including watermelon (Dong et al. 2018; Sun et al. 2020; Yang et al. 2021), melon (Li et al. 2017a, b; Liu et al. 2019; Zhang et al. 2019), cucumber (Zhang et al. 2015), and tomato (Zhao et al. 2016). With the development of sequencing technology, the resequencing and assembly of target regions can improve genome assembly. Within a narrow mapped candidate region, it is easier to identify and analyse the candidate genes for target traits. In recent years, molecular technology has also been applied for analysis during melon fruit ripening and to determine QTLs and candidate genes related to fruit quality (Galpaz et al. 2018; Saladié et al. 2015; Zhang et al. 2016; Mohamed et al. 2020).

Flesh firmness is a quantitative inherited trait that is influenced by multiple genes, including some major genes. It is affected by many factors, such as the structure of the cell wall, swelling pressure (Saladié et al. 2007), and the characteristics of the cuticle (ChaïB et al. 2007). Similar to most fruit quality traits, flesh firmness is a typical quantitative trait that may be regulated by multiple genes and metabolic networks, and the genotype and environment both affect fruit firmness (Brummell et al. 2001). Because of the typical fruit diversity of melon, this species has been proposed as an alternative model for understanding fruit-related traits (Pereira L et al. 2020). Research on fruit firmness based on the analysis of ethylene biosynthesis during melon fruit ripening (Zarid et al. 2021) indicated that a QTL for flesh firmness (*FFQV6.3*, LOD = 2.7) was present in the same region on chr. 6 as the major QTL for fruit ripening. Moreno et al. (2008), using the nonclimacteric parents PI 161375 and PS to identify QTLs for flesh firmness, and five QTLs were detected (*ff2.2*, *ff3.5*, *ff8.2*, *ff8.4* and *ff10.2*). In the present study, SNPs and small InDels were used to detect major QTLs based on BSA sequencing. Two methods (the ΔSNP and ED methods) were used to analyse the candidate associated regions. According to SNP analysis, the combination of the two analysis methods indicated 3 candidate regions (on chr. 2, 5 and 10) to be investigated, whereas in the InDel analysis, 6 candidate regions (on chr. 1, 2, 5, 7, 11 and 12) were investigated. By combining the results of the two methods, two overlapping QTLs were identified on chr. 2 and chr. 5, which were further investigated. QTL *ff2.1* was mapped to a region spanning genomic positions 18996333- 23870331 based on SLAF-BSA sequencing. This is a new QTL according to a review of previous research and indicates that a portion of the long arm of chr. 2 is a major candidate region for fruit firmness. SSR markers in associated regions were used to construct a genetic map of chr. 2 and chr. 5. *ff2.1* was located between CmSSR07709 and SNP22228 and covered 106 Kb of the melon genome, in which 10 candidate genes were annotated. According to qRT-PCR analysis, most of the candidate gene showed significant differences in expression between the parental lines in different fruit development stages. The results for seven genes showed an inflection point in their expression at 20 DAP in both parental lines, which indicated that 20 DAP was a significant day for fruit development. In our study, hard-fruit M2-10 and soft-fruit ZT091 matured at 28 and 25 DAP, respectively, and they showed similar anthesis days. The analysis of fruit phenotypes showed that the fruit pericarp became yellow and soft in ZT091 and turned dark green to light green in M2-10.

For *ff2.1* ten candidate genes were investigated. Among these genes, MELO3C029720, annotated as an ARF, exhibited significant differences in expression in different fruit development stages between the parental lines. In the hard-fruit parental line M2-10, the ARF gene showed no difference in expression in the fruit pericarp at 15, 20, and 25 DAP. However, a significant difference was found in ZT091, and at every stage, ARF expression levels were higher in ZT091 (soft) than in M2-10 (hard) (Fig. 5f). ARF is a transcription factor whose function is specific to auxin signal transduction pathway regulation in plant growth and development (Wen et al. 2019). Molecular studies have revealed that *ARFs* regulate the expression of auxin-responsive genes (Weijers et al. 2005), and they play various roles in root development, shoot growth, and fruit ripening (Luo et al. 2018). Research on the regulation of ARF on fruit development has mainly focused on tomato. To date, a total of 21 ARFs have been identified in *Solanum lycopersicum*, and Goetz et al. (2007) found that the fruit diameter of parthenocarpic mutants in which *SlARF8* expression was inhibited was significantly greater than that of the wild type. It is speculated that *SLARF8* may be an important negative regulator of tomato fruit diameter, and these results have been verified in *Arabidopsis thaliana* and *Solanum melongena* L. In addition, some authors have speculated that *SLARF4* is associated with the tomato cell wall structural development.

Another interesting candidate gene encoded a cell division cycle 5-like protein (MELO3C017522) (Fig. 5 g). A key inflection point was observed in both ZT091 and M2-10 at 20 DAP, but the major differences were found in the hard-fruit line M2-10. Gene expression decreased at 20 DAP relative to 15 DAP and then increased at 25 DAP in M2-10, but the soft-fruit line ZT091 showed the opposite trends. For most flesh fruit, cell division regulators were identified related to fruit weight in tomato (Renaudin et al. 2017; Mu et al. 2017; Su et al. 2021), and the related genes are well understood, but how cell division regulators modulate fruit firmness is still not clear. In tomato, important cell division regulators, including *FAS* (fasciated), *LC* (locule number), *OVATE* and *SUN,* were found to be involved in the regulation of cell division (Van der Knaap et al. 2014). Whether and how the cell division cycle 5-like protein regulates fruit firmness should be deeply studied in the future.

We also found that a major QTL(*ff5.1*) for fruit firmness was located on chr. 5, for which the candidate region covered 28.7 Mb based on SLAF-BSA sequencing. Although SSR markers were used to reconstruct the genetic map of chr. 5, the candidate

region still covered 7.5 Mb. Because the results indicated that *ff5.1* was a significant QTL for melon fruit firmness, the fine mapping of *ff5.1* should be conducted, and candidate gene functional analysis should be performed in the future. Previous research indicated that traits/genes related to fruit ripening, including C*mACO* and *CmETR* (Moreno et al. 2008), and abscission (ABS) (Pereira et al. 2020) were located near the *ff5.1* region on chr. 5. Generally, fruit ripening-related traits and especially abscission are highly relevant to fruit firmness. Several QTLs related to fruit firmness have been identified in melon linkage group (LG) X (Pitrat et al. 2017; Castro et al. 2017). Recently, Pereira et al. (2018) obtained two additional QTLs for quality traits in LG X using a RIL population and a genotype-by sequencing-based genetic map, and a transcriptomic analysis of melon NILs indicated flesh firmness. In a recent study, we performed SFLA-seq in another F_2 population of the thin-skinned melon ssp. *conomon* (Thunb.), which identified fruit firmness loci on chr. 5 and chr. 10 that explained 8.3% and 13.93% of the observed phenotypic variance. These loci were related to fruit firmness genes including *CmTrpD*, *CmNADH1*, *CmTCP15*, *CmGDS* Lesterase/lipase, *CmHK4-like* and *CmNAC18* in the candidate regions (not published).

Two genes, encoding adenylyl sulphate kinase and L-allothreonine aldolase (MELO3C017519 and MELO3C01720), were found to be significantly differentially expressed in the present study. In fleshy fruit, related enzymes could affect fruit firmness (Cappai et al. 2018). MELO3C017519 (adenylyl-sulphate kinase) is an important gene in hydrogen sulphide biosynthesis (GO:0070814), and H_2S interacts with ethylene and regulates fruit ripening (Liu et al. 2020). MELO3C017720 participates in cellular amino acid metabolism (GO:0006520), which includes pathways involving amino acids and carboxylic acids containing one or more amino groups. Based on the qRT-PCR results, gene expression levels were increasing at 15, 20 and 25 DAP in the hard-fruit line, but in the soft-fruit parental line, peak expression was observed at 20 DAP and then decreased significantly at 25 DAP. The regulatory mechanism of fruit firmness is complex, and the investigation of gene expression on additional DAP should be considered as a next step.

Fruit firmness is always a key target of melon breeding, as it is associated with shelf life and economic value in melon. However, the precise mechanism determining fruit softening remains elusive. The SLAF-BSA method was used to investigate two major QTLs for fruit firmness in this study, and a new QTL (*ff2.1*) spanning 106 Kb and containing 10 candidate genes was identified in the melon genome.

Declarations

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Author Contributions

YS designed this experiment. DD performed the research and wrote the manuscript. SZ and FL conducted data analysis. LW and PJ collected phenotypic data in field trials. HL gives specific modification opinions and constructive suggestion for submission. All co-authors reviewed and approved the manuscript before submission.

Compliance with ethical standards

Conflflict of interest

The authors declare that they have no conflflict of interest.

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Tables

Table 1. primer sequence and information of SSR markers

ID	Chr ID	Formards sequence	Reverse sequence	Genome start	Eezyme
CmSSR07381	Chr 2	CAATGCATGGTTGTTTGAGG	GCGTGAAGCTGAGGTTGTCT	18936841	
CmSSR07388	Chr 2	TCCATCTTTACCGTACATTTTGG	TTCATAACTTTACGCTTACATGCTG	18989954	
SNP19005	Chr 2	CCCTCGAATTTGACTCTCCA	TTGTCAGCGATCGTTTAATACA	19005820	PvuI
CmSSR07402	Chr 2	GGAAAAGGGAAACCCAAAAA	CTCTCTCTGCATGGGGTCTC	19091102	
CmSSR07688	Chr 2	AAGGAATTCAGGTTCATGCG	CCCAGCAGCTATTCTGGAAG	21921679	
SNP21922	Chr 2	GTTGGCAGGTCGAGAAGAAT	GCTTTGCTTAAACCATTGTGG	21922113	MseI
CmSSR07709	Chr 2	GGAGTGGCTTGCAAATTATGT	CGTCGATACACTCAAAATCCAA	22122424	
SNP22134	Chr 2	TTTCAATCCCTAATTATCCCGA	TTGCCATGATTTTAGGGAGG	22134803	MseI
SNP22228	Chr 2	CGACTCGTTTCACTTTTGCC	ATGGATTCTGAAGTGCTGGG	22228642	Hin6I
SNP22274	Chr 2	GCTAAAGGATTGCTTTCCAAGA	GCAAGAAGCCACCAAAACTT	22274899	MseI
CmSSR07919	Chr 2	CGATGCAGTGACGATGTTTT	TCTCCTAAGTAAACACGCACGA	23867283	
CmSSR12829	Chr 5	AGTTTGGCACATGGCTCTTT	TCTTCCCTTCCCAACTTCCT	395501	
CmSSR12854	Chr 5	AGGTGAGGAGATGGATAGGGA	GACCAAAATTGAACCATGCC	522715	
CmSSR13308	Chr 5	CTGCCTAAGTAAGGGCGTTG	CTTGGTTGAAAGGCAAAGGA	5002471	
CmSSR13423	Chr 5	GTTGGGTTTCTATCCATTAAACCAT	GCTTCCCTCTCTCCCAAGAA	7486090	
CmSSR13509	Chr 5	CAGAGATAGCCAAAATGTCTCG	CTTGAGGGCCACTGAACTTG	10503296	
CmSSR13911	Chr 5	AATTTCATGCCCAACCAAAA	TGCATGCTACGAAACAAAAGA	18014796	
SNP24007	Chr 5	GCGAAATCGATGTGGGTTAT	TTATTTGCCTAATTTATTGTGTGG	24007072	MseI
SNP24550	Chr 5	GGCCATCTCCCTCAATCATA	CACTCCCCATCGTTTGTCTT	24550885	BfaI
CmSSR14362	Chr 5	AGTGCGAAAGGAAAGTGAAAA	GGCTGAAGATTCAACCCAAA	25027285	

Table 2. Fruit firmness of P_1, P_2 and generations.

Traits	Mean of parent value		Mean of other generation		
	P1	P2	F1	F2	F3
Fruit firmness	10.8±1.24	3.2±0.32	8.7±1.15	5.3±1.12	5.93±0.89

Table 3. The information of sequencing data

	Raw Read	Clean Reads	Q30 (%)	GC (%)	Mapped (%)	Properly mapped(%)	Ave depth	Cov ratio 10X(%)
M2-10	42 916 190	42 680 203	93.03	36.94	95.84	88.8	26	96.15
ZT091	45 759 537	45 492 988	92.88	38.03	93.75	86.88	27	96.28
Hard firmness Bulk	31 658 078	31 658 000	91.27	37.32	95.46	85.45	19	92.88
Soft firmness Bulk	29 713 886	29 713 764	93.58	37.85	94.18	84.65	16	87.98

Table 4. Associate region based on SNP and INDEL analysis by SLAF-BSA sequencing .

	Chromosome ID	Start	End	Size (Mb)	Gene Number
SNP linked region	chr02	16222964	23945729	7.72	533
	chr05	2414	29301403	29.3	2019
	chr10	23044	186480	0.16	15
Indel linked region	chr01	6434041	28015662	21.58	1169
	chr02	18996333	23870331	4.87	348
	chr05	49380	28751254	28.7	1930
	chr07	3133852	23804269	20.67	1164
	chr11	27042	33601568	33.57	2418
	chr12	4875391	24127339	19.25	1170
Final linked region	chr02	18996333	23870331	4.87	348
	chr05	49380	28751254	28.7	1930

Table 5. QTL analysis by SSR markers based on associate region

QTL Detected	Chr.	LOD	R2	Add	2.0 Lod Interval	
					Left (cM)	Right (cM)
ff2.1	2	3.8	28.14	1.82	CMSSR07709	SNP22228
ff5.1	5	17.44	38.44	0.85	CMSSR13509	CMSSR13423

Figures

Figure 1

As observed in the field, M2-10 ripened at ~28 DAP, with a fruit firmness of 10.8 ± 1.24, and ZT091 ripened at ~25 DAP, with a fruit firmness of 3.2 ± 0.32. There was a significant difference between the parental lines (Fig. 1). F1 fruit ripened ~30 DAP, with a fruit firmness of 8.7 ± 1.15, and the average fruit firmness values of the F2 population and F3 families were 5.3 ± 1.12 and 5.93 ± 0.89, respectively.

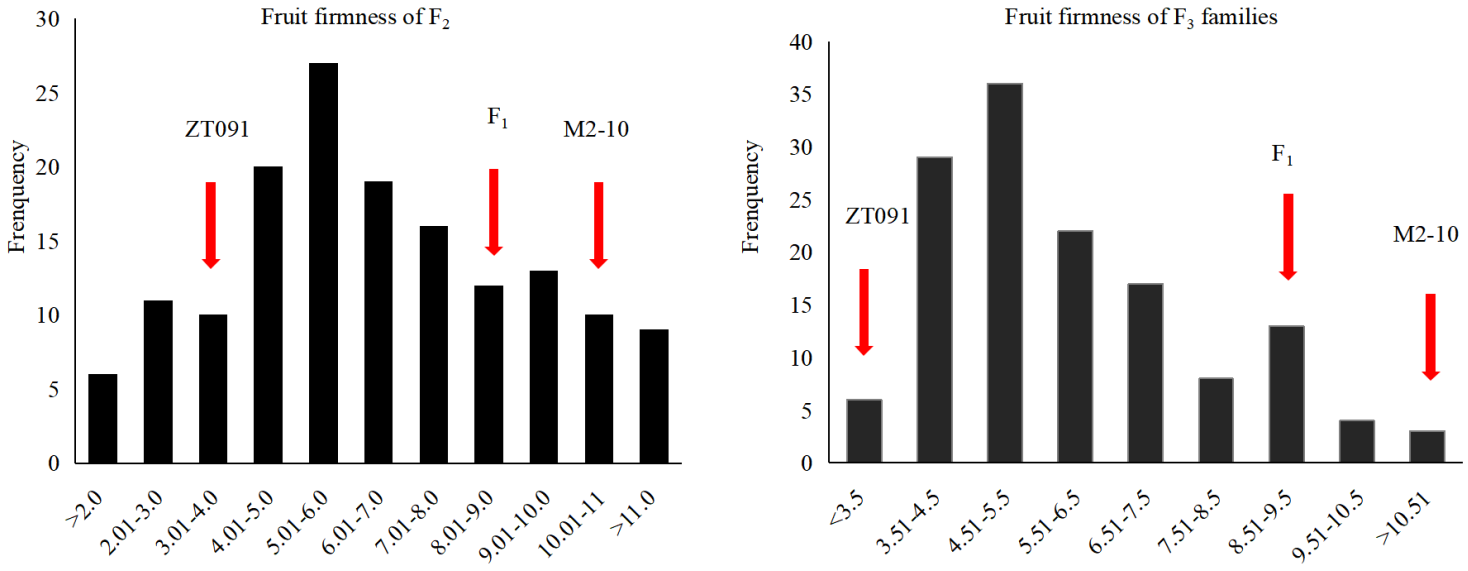

Figure 2

The fruit firmness of each population was continuously distributed, reflecting the fact that it is a quantitative trait controlled by multiple genes (Fig. 2).

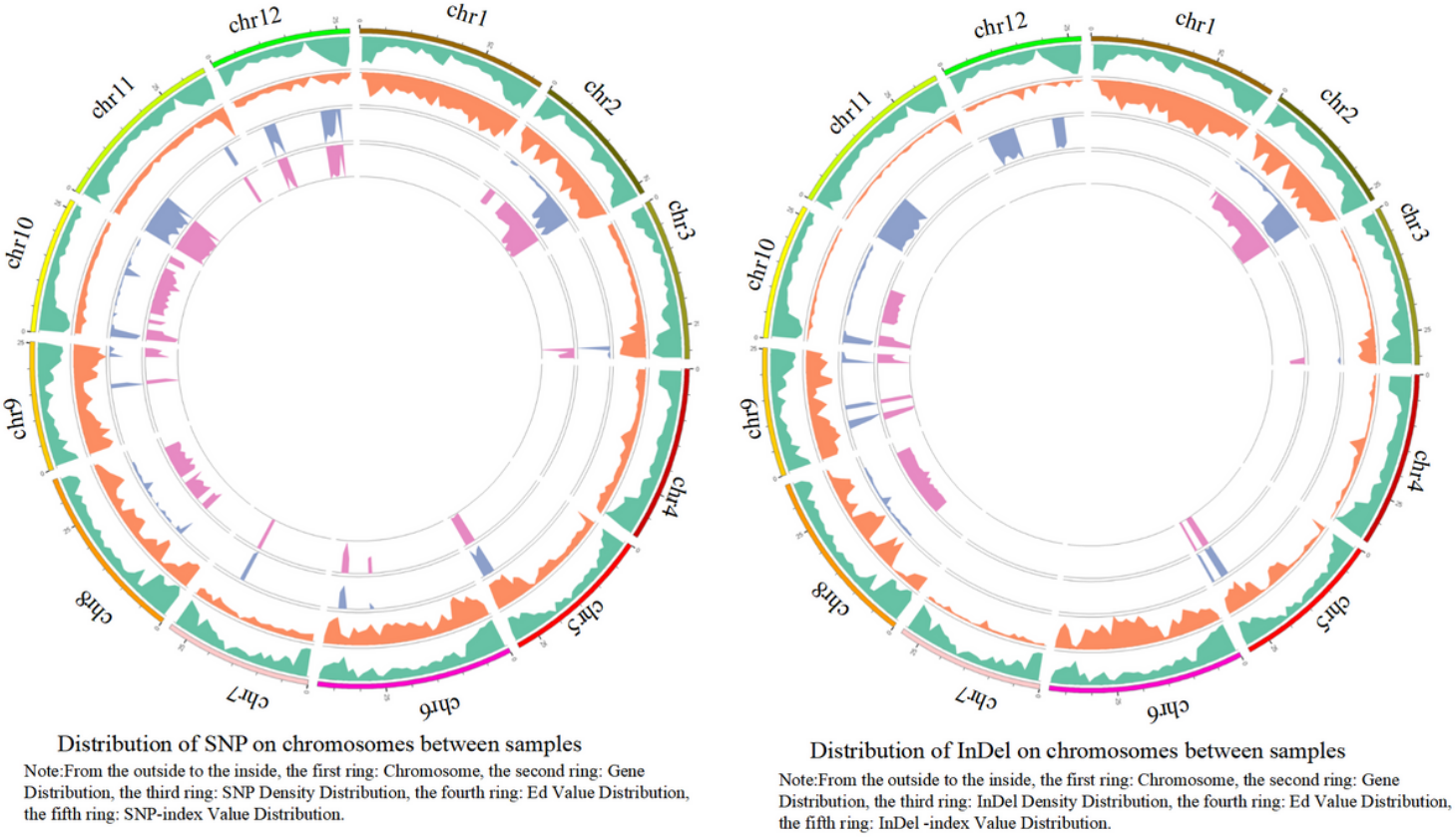

Figure 3

Based on these SNPs, the results obtained for the associated regions via the two association analysis methods overlapped on chr. 2 chr. 5 and chr. 10 (Table 3 and Fig. 3).

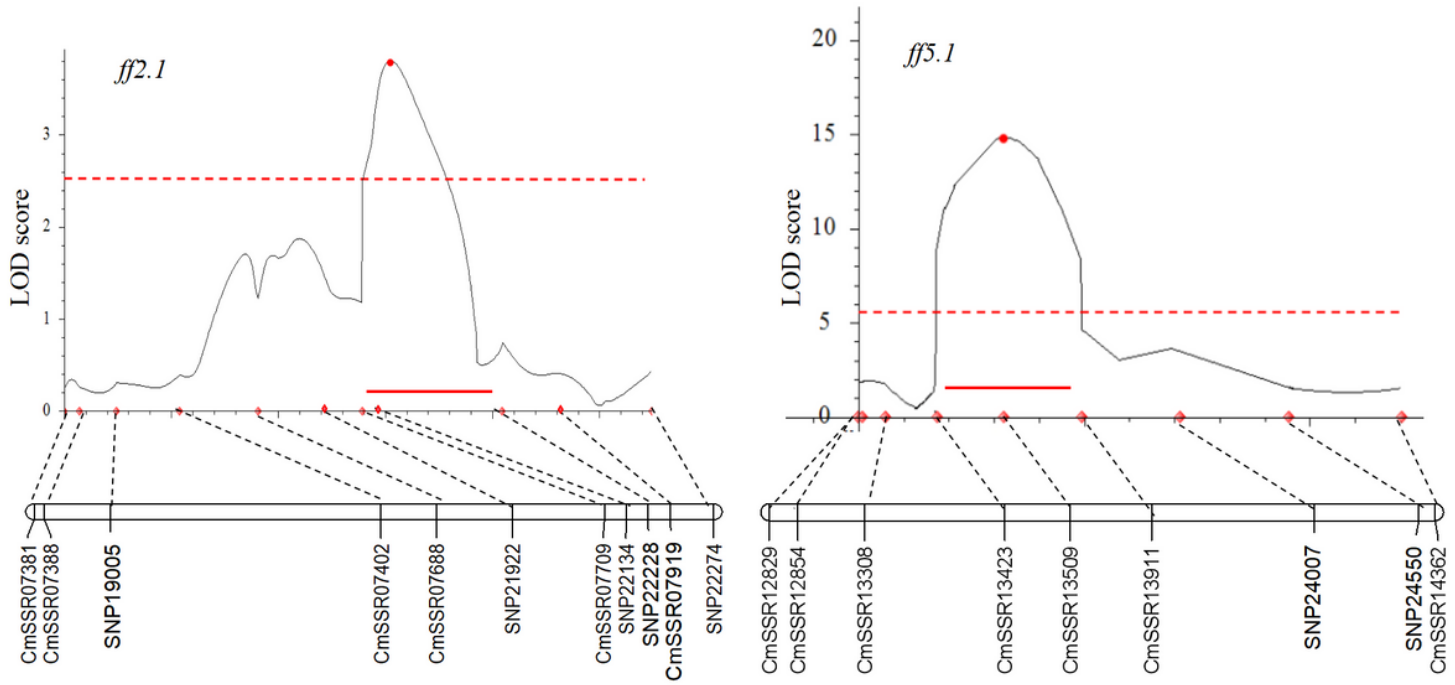

Figure 4

QTL ff2.1 was located on chr. 2 between CmSSR07709 and SNP22228 and explained 28.14% of the phenotypic variation; the LOD value of ff2.1 was 3.8. The candidate region spanned genomic positions 22122424 to 22228642, covering 106 Kb, and it contained 10 candidate genes (Fig. 4).

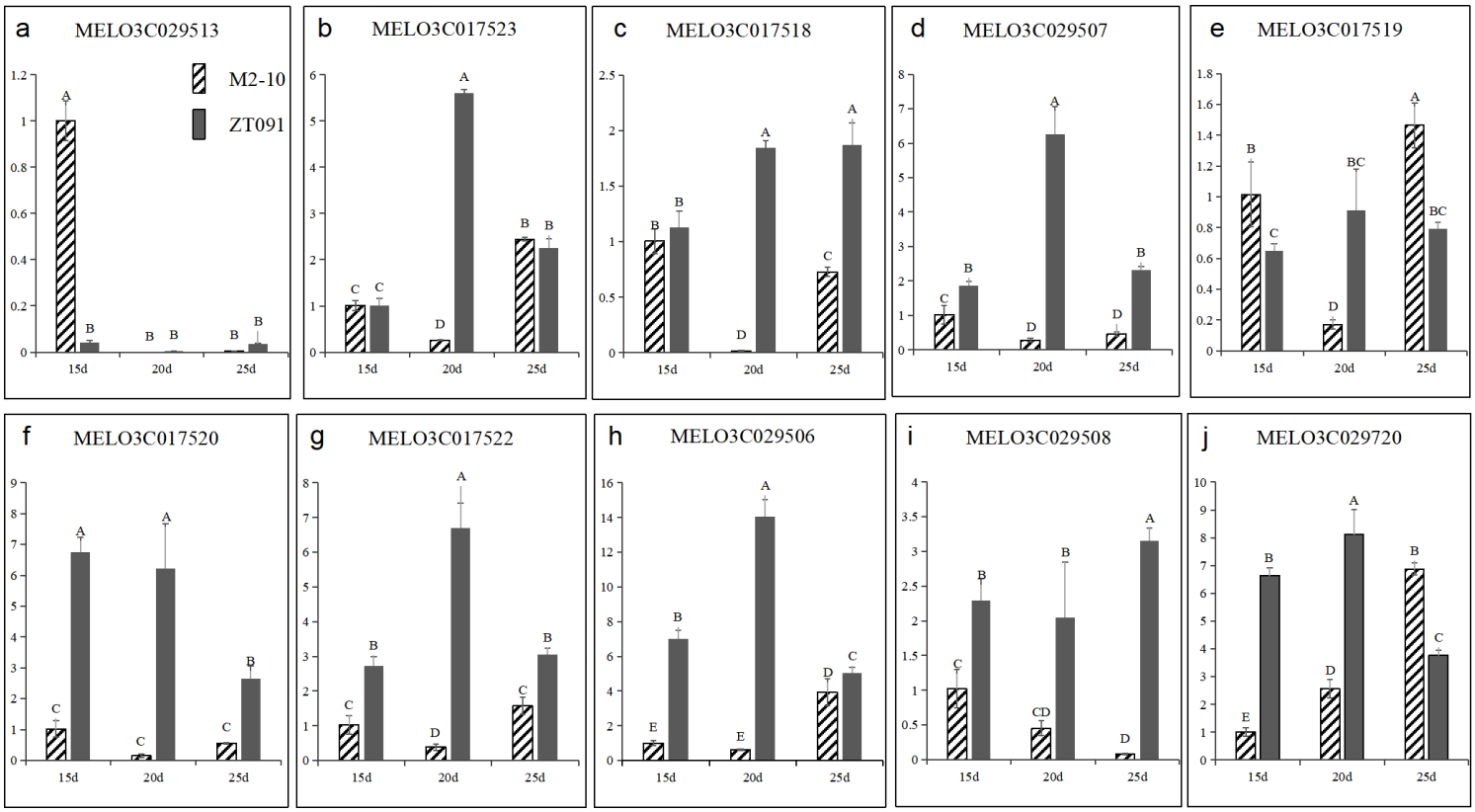

Figure 5

Although MELO3C017513 and MELO3C017523 showed no difference in expression between M2-10 and ZT091 at 25 DAP (Fig. 5 a and b), the other genes all showed significant differences in expression at this time point. According to the results for most of the genes, 20 DAP was a key point in fruit development. For example, in the hard-fruit line M2-10, the MELOC3C017518, MELOC3C017523, and MELOC3C029507 genes (of unknown function) all showed decreased gene expression at 20 DAP but highly increased gene expression at 25 DAP (Fig. 5 b-d); the same expression trend was observed for MELOC3C029519, MELOC3C029520, MELOC3C029522, and MELOC3C029506 (Fig. 5 e-h). For MELOC3C029508, significant gene expression levels were detected at 15, 20 and 25 DAP, and a decreasing trend was found in M2-10(Fig. 5i). a significant difference was detected. For the Movement protein gene MELO3C029506, significant gene expression levels were detected at 25 DAP in M2-10 and ZT091, and the same was true for the L-allo-threonine aldolase gene (MELO3C017520) (Fig. 5 g and j).

Supplementary Files

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- TableS1.xlsx