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RESEARCH ARTICLE

FUNCTIONAL GAIN OF FRUIT NETTED-CRACKING IN AN INTROGRESSION LINE OF TOMATO WITH HIGHER EXPRESSION OF THE *FNC* GENE

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KEYWORDS

fine mapping, fruit netted-cracking, introgression line, transcript level

HIGHLIGHTS

- A novel netted-cracking fruit phenotype was discovered in tomato introgression line IL4-4.
- A single dominant gene (*FNC*) determined the fruit netted-cracking phenotype.
- The high transcript level of *FNC* results in the functional gain of fruit netted-cracking and it was found to be a common mechanism in a diverse range of plant species.

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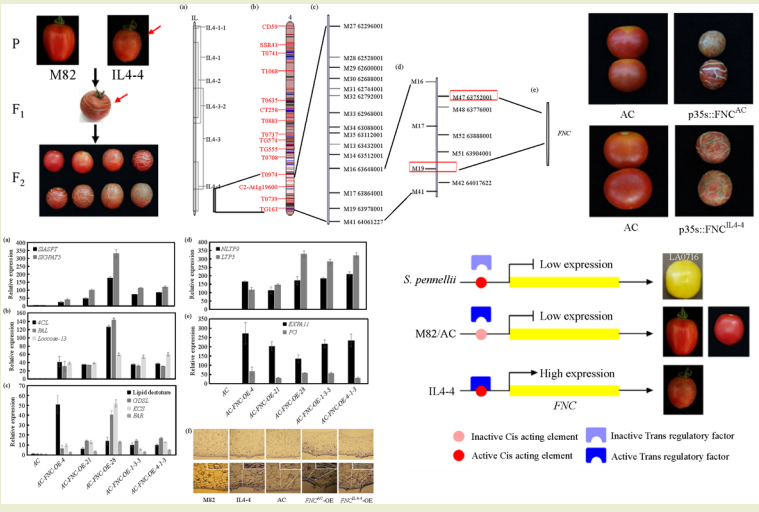
GRAPHICAL ABSTRACT



ABSTRACT

Fruit cracking is a major disorder that affects the integrity of fruit and reduces the commercial value of tomato and other fleshy fruit. Here, we have found a novel fruit 'netted-cracking' (FNC) phenotype in tomato introgression line IL4-4 which is present in neither the donor parent (LA0716) nor the receptor parent (M82). An F_2 population was generated by crossing IL4-4 with M82 to genetically characterize the *FNC* gene and this showed that a single dominant gene determined fruit netted-cracking. Further map-based cloning narrowed down the *FNC* locus to a 230 kb region on chromosome 4. Sequencing and annotation analysis show that *FNC* (Solyc04 g082540) was the most likely candidate gene. Functional characterization of *FNC* by overexpressing FNC^{AC} and FNC^{IL4-4} resulted in the fruit netted-cracking phenotype, suggesting that the *FNC* transcript level results in the functional gain of fruit netted-cracking. These findings were further confirmed by *FNC* ortholog in netted-cracking pepper and melon, indicating a

common regulatory mechanism in different plant species. Furthermore, cytoplasm and nucleus-localized *FNC* indicates increased expression of genes involved in suberin, lignin, lipid transport and cell wall metabolism. These findings provide novel genetic insights into fruit netted-cracking and offer a way to promote molecular improvement toward cracking resistant cultivars.

1 INTRODUCTION

Fruit cracking is a physiological disorder the occurrence of which is a physical process of mechanical fracture[1]. The cracking appears at all stages of fruit development in medium and large tomatoes, but in cherry tomatoes it more frequently occurs during ripening and after harvest[2]. Fruit cracking is usually related to the cracking of the fruit epidermis or stratum cuticle. External conditions such as mineral nutrition, temperature, water, light and plant growth regulators, can affect fruit cracking[3–7]. Although careful cultivation techniques can reduce the economic loss caused by fruit cracking, it cannot fundamentally solve fruit cracking. Breeding for novel cracking resistant cultivar is therefore a more feasible solution.

The cracking of fruit is a quantitative trait which is controlled by multiple genes. Quantitative trait locus (QTL) mapping and genetic analysis of fruit cracking traits provide valuable information when studying the genetic mechanisms and breeding of fruit cracking resistant cultivars. Fruit cracking is also closely related to the effects of the external environment and there are several reports on the QTL mapping of fruit cracking in horticultural plants. Previous studies show that two significant loci control tomato radial cracking. Fruit cracking resistance is correlated with several other tomato fruit traits including pink fruit, the small transverse diameter of fruit and a small number of fruit ventricles. Earlier, investigations identified the main QTLs controlling tomato fruit cracking using tomato recombinant inbred lines[2]. Five loci related to tomato cracking were identified using the F_2 population constructed by two tomato accessions with different fruit cracking susceptibility[2]. Pericarp firmness, pericarp anatomical structure and cell wall metabolism are also related to fruit cracking. Liao et al. found an indel of 11 bp and a neighboring SNP in ethylene-responsive transcription factor 4 (*ClERF4*) which was associated with variation in rind hardness of watermelon using a fine-mapping approach[8]. Due to the complex genetic regulatory mechanism of fruit cracking and the significant influence of environmental factors, the experimental results differ in different horticultural plants. The relationship between fruit cracking and pericarp structural characteristics does not follow the same pattern in different horticultural plant species.

The cuticle is the interface between the plant and the environment and has an important impact on normal plant development and the mechanical properties of the pericarp. Some genes related to the regulation of fruit cuticle composition have already been identified. For example, knockdown of *TAGL1* by RNAi changed mechanical properties such as thickness and stiffness of the cuticle in tomato fruit and its composition also changed[9]. Silencing of *SlSHN3* can reduce the cutin and wax contents of tomato fruit cuticles[10]. *SlMIXTA-like* is a downstream factor of *SlSHN3* and can affect the water permeability of tomato pericarp and resistance against pathogens by positively regulating the cuticular polymerization and deposition monomers and the formation of epidermal cells[11]. A gene, *CWP1*, has been identified that can control the cuticular micro-fissures and the dehydration of fruit from the introgression line population of *Solanum habrochaites*[12]. Low temperatures strongly induced the expression of *CWP* and increased the severity of stratum cuticle microcracks[13]. Also, it has been found that a locus, ER4.1, controlled the epidermal reticular structure of green tomato fruit, and its phenotype had a higher water loss rate than wild type fruit. Nevertheless, this function remains to be verified by the transgenic methods[14].

Unlike cuticle, suberin can be deposited in specific parts of the cell wall, both in internal and external tissues, during plant development. Muskmelon (*Cucumis melo* var. *reticulatus*) is characterized by exquisite reticulation on the surface of its pericarp. The net pattern formed by suberin makes its appearance more ornamental and can reduce the impairment caused by mechanical damage during harvest[15]. There have also been some studies on the QTL location of muskmelon reticular characteristics. For example, a study found 16 loci that control the formation of muskmelon reticulation[16]. Using an F_2 segregated population, QTLs that control reticular density and reticulate width (two highly correlated traits) were found to be co-located[17].

Tomato is an important fruit and economic crop and its appearance directly impacts market competitiveness. Fruit cracking affects fruit integrity and decreases the yield, and brings considerable losses to production. Studies have made

some progress in understanding the genetics and physiology of tomato fruit cracking, but there are few reports on the molecular mechanism of reticulation by healing response after fruit cracking. Here, we have found that fruit of IL4-4, which is an introgression line of the wild tomato species *Solanum pennellii* LA0716 with *Solanum lycopersicum* M82 as the background, had a novel phenotype, with fruit netted-cracking (FNC) appearing all over the pericarp. Still, neither LA0716 nor M82 has this cracking phenotype. To reveal the underlying mechanism of this functional gain, here, we fine-mapped and cloned the *FNC* gene that controls fruit reticulation formation using an F_2 genetic segregation population from IL4-4 × M82. Also, the molecular mechanism of regulating tomato netted-cracking was subjected to preliminary analysis to provide a theoretical basis for the breeding of cracking-resistant tomato cultivars.

2 MATERIALS AND METHODS

2.1 Plant materials

Using *S. lycopersicum* cv. M82 and *S. pennellii* introgression line IL4-4 (with M82 as introgression background) as parent materials, 6500 individuals from the F_2 segregation population were used to fine-map the gene controlling the fruit netted-cracking trait. The tomato cultivar Ailsa Craig (AC) was chosen as the transgenic background because of its higher genetic transformation efficiency.

2.2 Fine mapping of the *FNC* gene using F_2 segregation population

According to the DNA marker information published on the Solanaceae Genomics Network, we found an overlapping bin-region between IL4-3 and IL4-4. At the same time, the fruit of IL4-3 did not have the reticular crack phenotype, indicating that the candidate locus leading to the cracking phenotype was located in the given bin-region of IL4-4. We then used the existing DNA markers in the genetic map for analysis, selected the markers evenly distributed across the chromosome segment of IL4-4, and 39 polymorphic indel markers were designed using the genomic sequences of tomato M82 and *S. pennellii* to fine-map the netted-cracking traits (Table S1).

2.3 Gene expression

The expression of the *FNC* gene was analyzed in different tomato tissues, namely buds, ovaries and fruit at 7, 14, 21, 28 and 35 days post anthesis (DPA) from M82 and IL4-4 plants. The expression of *FNC* was also analyzed in the fruit of non-netted-cracking and netted-cracking peppers and muskmelons. Total RNA was extracted by Trizol one-step method and reverse transcribed into cDNA, and then gene expression was detected by cDNA template and *β-actin* as control. The derived measurement data were numerically calculated using the $2^{-\Delta\Delta Ct}$ method. The expression of transgenic plants was determined by the shoots of transgenic plants, and the method was the same as above.

The expression of genes related to the *FNC* regulatory pathways was analyzed. The qPCR primer sequences of the corresponding gene were designed using Primer 5.0 (Table S2). The pericarp tissues at the breaker stage from well-growing plants of overexpressing lines and cultivar AC were used for expression detection, and the method was the same as above.

2.4 Subcellular localization

The open reading frame (ORF) of *FNC* without the stop codon was amplified using the primers listed in Table S2 and ligated into 101YFP transient expression vector by homologous recombination method. The positive plasmid was transferred into *Agrobacterium tumefaciens* GV3101 competent cell by electroporation. The vector was injected into tobacco leaves by the transient transformation for two to three days. The injection spot of tobacco leaves was cut, and the fluorescence location was observed with a confocal microscope.

2.5 Construction of expression vector and genetic transformation

The full-length sequences of *FNC* were amplified using the gDNA of AC and IL4-4 as templates (primers listed in Table S2). Then the gene was ligated into pHellsgate8 overexpression vector by the homologous recombination method. Genetic transformation of tomato was mediated by *A. tumefaciens*. The transformed plants were detected by PCR using the primers p35S and FNC-OE-RV. The positive control template was corresponding plasmid and negative control template was aseptic ddH_2O. The positive transgenic plants were selected for the subsequent experiments.

2.6 Microscopic section and scanning electron microscopic observation of pericarp

To reveal the differences in tomato pericarp with or without cracking, cytological observations were made in the breaker

stage pericarps of M82, IL4-4, AC, and transgenic lines 14, 21, 28 and 35 DPA. The pericarp was cut into square pieces and immersed in FAA fixing solution. After dyeing and decolorization, the sample was placed in chloroform with broken wax. Paraffin slices with a thickness of 8–12 μm were cut and baked at 37°C. The samples were sealed with gum, observed and photographed under a microscope.

For scanning electron microscopic (SEM) analysis, pericarp slices were prepared from fruit of IL4-4 and M82 at 21, 28 and 35 DPA. The tissues were fixed, dried using a critical point drier (LEICA EM CPD030; Leica, Wetzlar, Germany) and examined by SEM (JEOL T 330A, JEOL, Tokyo, Japan) as described by Yasuzumy and Sugihara[18].

3 RESULTS

3.1 Fine mapping of the fruit netted-cracking gene in tomato

We investigated the fruit of 50 ILs covering the whole genome of *S. pennellii* in M82 background and found that only IL4-4 had netted-cracking which gradually increased during fruit development (Fig. 1(a)). The netted-cracking in the fruit epidermis started to appear at the fruit expansion stage and the cracks increased with the fruit development. Irregular netted-cracks formed at the mature green stage of fruit were observed under a light microscope (Fig. 1(b)) and SEM (Fig. 1(c)) 28 DPA.

All fruit from the cross between M82 and IL4-4 developed netted-cracking in the F_1 generation and segregated in F_2 progenies with netted-cracking and non-netted-cracking, at a ratio of 3:1 indicative of a single dominant gene (Fig. 1(d)). These F_2 progeny were used for subsequent fine mapping.

We conducted preliminary determination of the unique fragment of IL4-4 between the markers T0974 and TG163 in the genetic map (Fig. 2(a,b)). The polymorphisms of the designed molecular markers were screened using available genomic data for *S. pennellii* and M82, and the molecular markers with polymorphisms between two parents in the physical region of the introgression line IL4-4 were screened for mapping analysis (Fig. S1 and Table S1) using the DNA of F_1, M82, IL4-4 and IL4-3 as templates. The results show that the

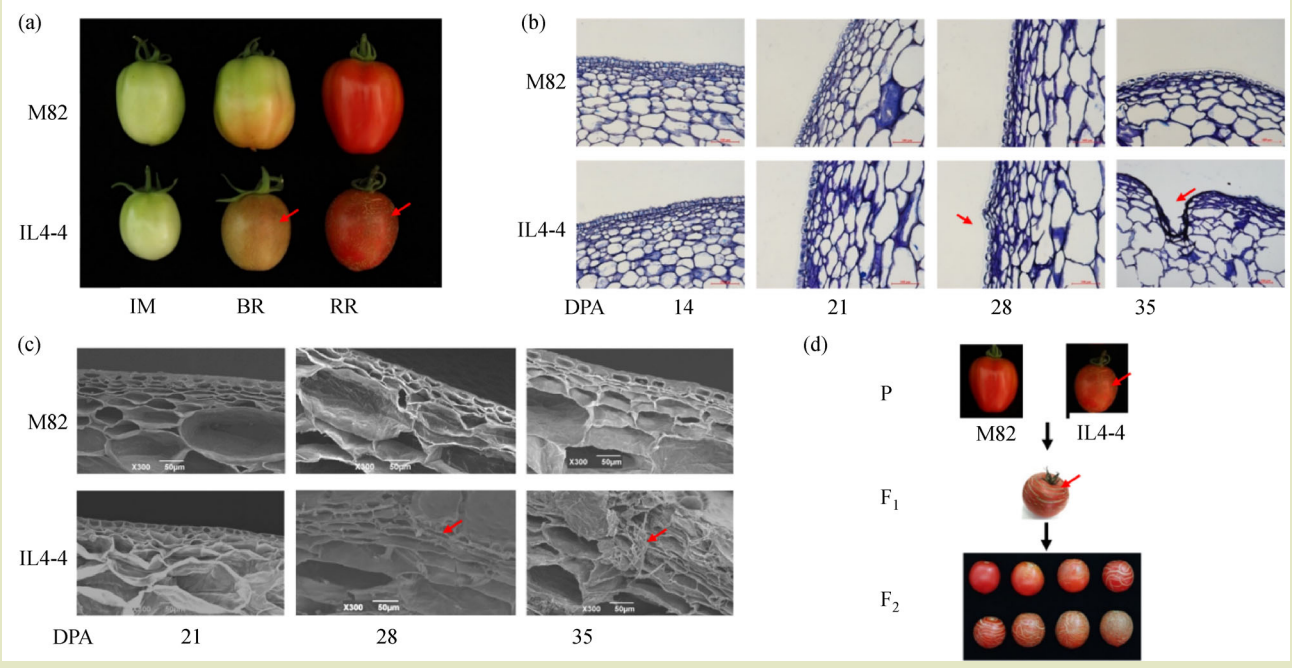


Fig. 1 Tomato fruit netted-cracking phenotypes (red arrow) of IL4-4. (a) The fruit cracking of different ripening stages at immature (IM), breaker (BR) and red ripe (RR) in IL4-4 compared with M82. (b) The netted-cracking structures of pericarps in IL4-4 and M82 fruit at 14, 21, 28 and 35 days post anthesis (DPA). (c) The ultrastructures of cracking tissues in IL4-4 and M82 fruit at 21, 28 and 35 DPA observed under scanning electron microscope. (d) Constructing F_2 population from selfing F_1 crossed with M82 and IL4-4.

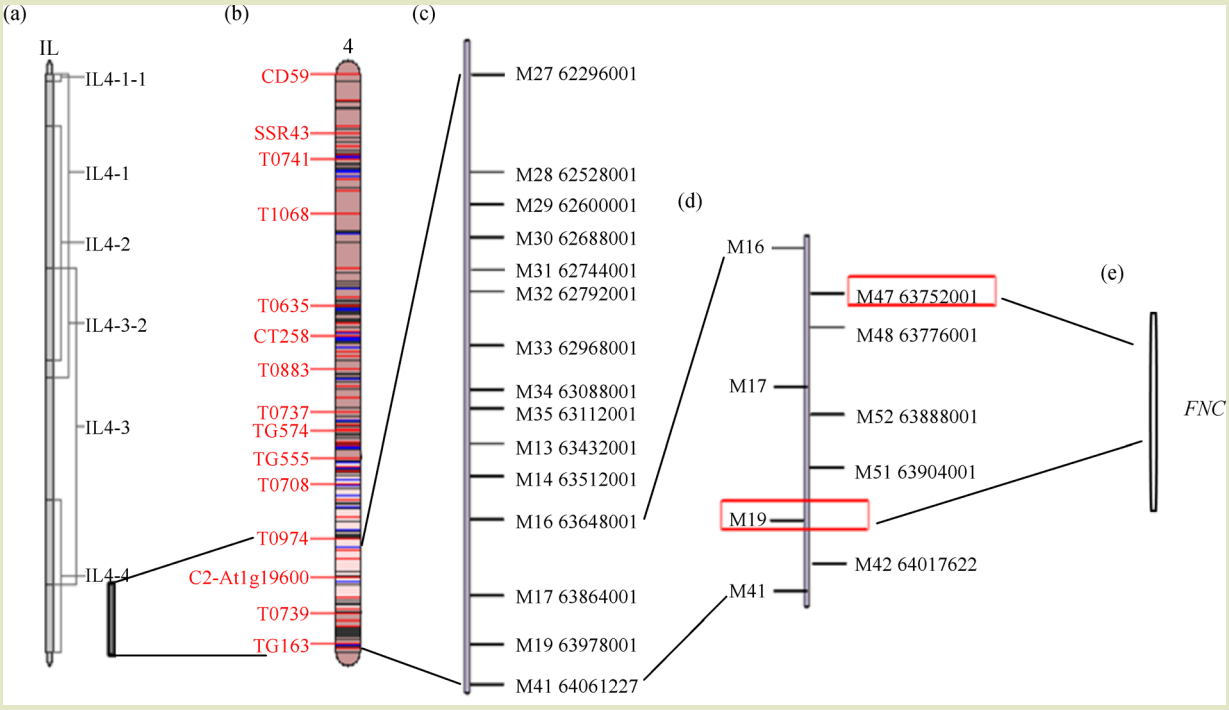


Fig. 2 Fine mapping of the fruit netted-cracking gene (*FNC*) of tomato. (a) The locus of netted-cracking phenotype is located in the specific region of IL4-4. (b,c) Mapping the *FNC* gene to the telomeric portion of chromosome 4 between the markers of T0974 and TG163 (b) and further mapped in the specific region of IL4-4 between M27 and M28 (c). (d,e) The candidate interval determined between markers M47 and M19 (d), and the physical location of the genome at 63.75–63.98 Mb, and the localized candidate *FNC* gene is Solyc04 g082540 (e).

front boundary of the specific region of IL4-4 was between M27 and M28 (Fig. 2(c)). We then designed molecular markers for fine mapping in the physical segment after M27 (62.29 Mb). Among 6500 plants of F_2 segregated groups we selected the recessive plants to screen recombinants by molecular markers. Finally, 70 recombinants were identified. Further, the polymorphic markers were developed and the candidate interval was narrowed to between M47 and M19 by evaluating the number of recombinants, and the physical location of the genome was 63.75–63.98 Mb (Fig. 2(d)).

We searched the tomato reference genomes on Solanaceae Genomics Network website and found 38 genes located in the candidate region (Table S3). According to the genomic sequence variation and annotation of candidate genes, *ORF5* (Solyc04 g082540.1.1) was selected as the candidate gene to regulate pericarp reticulation traits. We found that this gene was identified from the introgression line population of wild *S. habrochaites*[12]. However, sequence analysis showed that there were many polymorphisms in the CDS region of *S. habrochaites* and *S. pennellii* (Fig. S2). The fruit of the introgression line of *S. habrochaites* did not have the same netted-cracking phenotype as *S. pennellii*.

3.2 Expression and evolution analysis of the *FNC* gene

FNC (*Solyc04 g082540.1.1*) encodes a Ser/Thr-rich protein and is a member of the NRED family[19]. The full-length *FNC* ORF is 813 bp, including a long conservation domain TANGO2 (Fig. 3(a)). In addition, to predict possible involvement in protein secretion and Golgi apparatus assembly in eukaryotes[18,20], there is little substantive reporting about TANGO2.

Given that the expression of *FNC* in tomato vegetative organs was extremely low, we specifically explored the expression profiles of this gene at different developmental stages of fruit in M82 and IL4-4. Considerably higher *FNC* expression was measured at all fruit development stages of IL4-4 than in M82, indicating a possible explanation for fruit netted-cracking (Fig. 3(b)). Also, we detected and confirmed higher expression of *FNC* in pepper and muskmelon with netted-cracking fruit than non-netted-cracking fruit. However, the expression of *FNC* was higher in muskmelon than in pepper (Fig. S3(a,b)).

To explore the conservation of the *FNC* gene during evolution and its possible function in plants, the homolog genes with

TANGO2 domain were extracted from Ensembl Plants. The constructed phylogenetic tree of genes with TANGO2 domain, and tomato *FNC*, potato *TANGO2* homolog, and wild tobacco OIT03940 and OIT18796 belong to the same evolutionary branch. *FNC* had the closest genetic relationship with potato *TANGO2* homolog and the farthest genetic association with *Selaginella moellenodorffil* EFJ36398 and EFJ35804 (Fig. 3(c)).

3.3 Fruit netted-cracking is a functional gain through higher expression of the *FNC* gene in tomato

To determine whether the netted-cracking phenotype was related to amino acid variation in *FNC*, we aligned the genomic DNA and amino acid sequences of *FNC* in the non-netted-cracking accessions AC and M82 and the netted-cracking accession IL4-4. It was found that the genomic DNA was identical in FNC^{AC} and FNC^{M82}, but had five amino acid differences compared to $FNC^{IL4\text{-}4}$ (Fig. S4(a)). From the predicted three-dimensional structure of the protein, it was found that there were three additional β-folding structures in the $FNC^{IL4\text{-}4}$ protein (netted-cracking fruit) than in the FNC^{AC} protein (non-netted-cracking fruit) (Fig. 4(a)).

Transgenic verification was subsequently conducted. The overexpression vectors with gDNA sequences of *FNC* from IL4-4 ($FNC^{IL4\text{-}4}$) and AC (FNC^{AC}) were transferred into AC. It was found that the expression of $FNC^{IL4\text{-}4}$ and FNC^{AC} in overexpressing lines was significantly higher than that in AC (Fig. 4(b). All overexpressing lines showed the phenotype of netted-cracking phenotype. This finding confirmed that the higher expression of $FNC^{IL4\text{-}4}$ and FNC^{AC} was the cause of the netted-cracking phenotype. The cracks spread throughout the pericarp and were filled with suberin in the recesses of cracks (Fig. 4(c)).

We also found that the formation process of tomato cracks was a dynamic process similar to that in muskmelon. A small number of cracks were observed during the fruit expansion stage, and the reticular cracks in the pericarp increased with the expansion of the fruit (Fig. S4(b)).

3.4 FNC is localized in both cytoplasm and nucleus

To investigate the subcellular localization of FNC, we constructed a subcellular localization vector 35S::FNC-YFP. The 35S::YFP empty vector was used as a control and ERF-101YFP was used as a nuclear localized marker. The transient transformation was performed in tobacco and the expression location of yellow fluorescent protein was observed by confocal microscopy. The results show that FNC was located in both the cytoplasm and the nucleus (Fig. 5).

3.5 Increased *FNC* gene expression affects the several metabolic pathways of the pericarp

Given that fruit netted-cracking involves the metabolism of pericarp components, we analyzed gene expression related to pericarp metabolic pathways at the breaker stage of over-

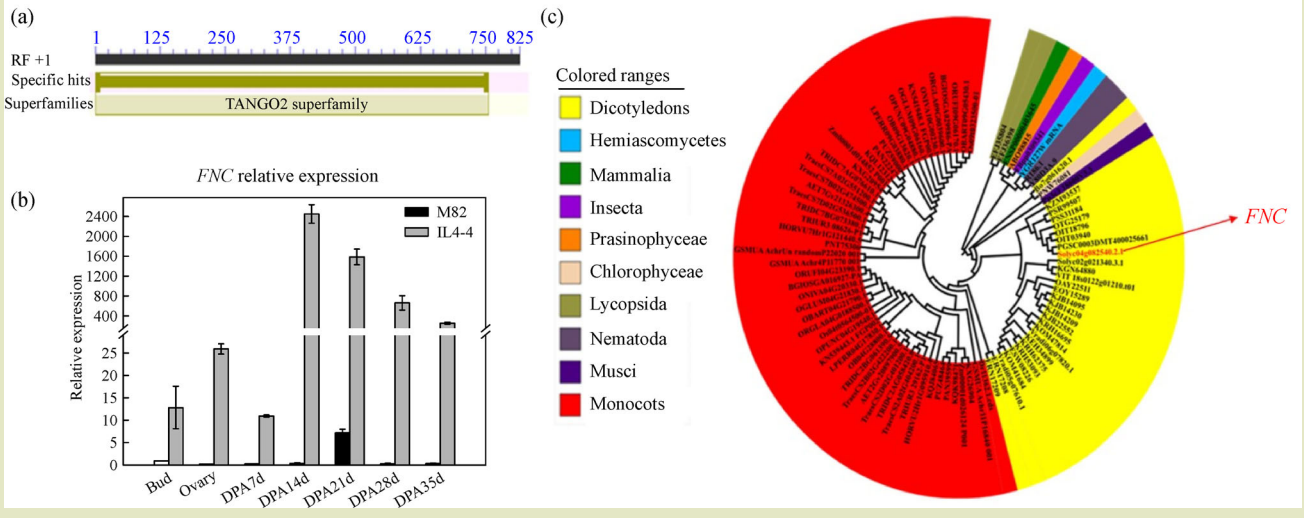


Fig. 3 *FNC* gene expression and evolution analyses. (a) The analysis of conserved domain of the *FNC* gene. (b) The expression of the *FNC* gene in IL4-4 and M82 at different days post anthesis (DPA). (c) Phylogenetic analysis of the *FNC* gene. Diverse colors represent different species, *FNC* and potato PGSC0003DMT400025661 are classified into one group (yellow).

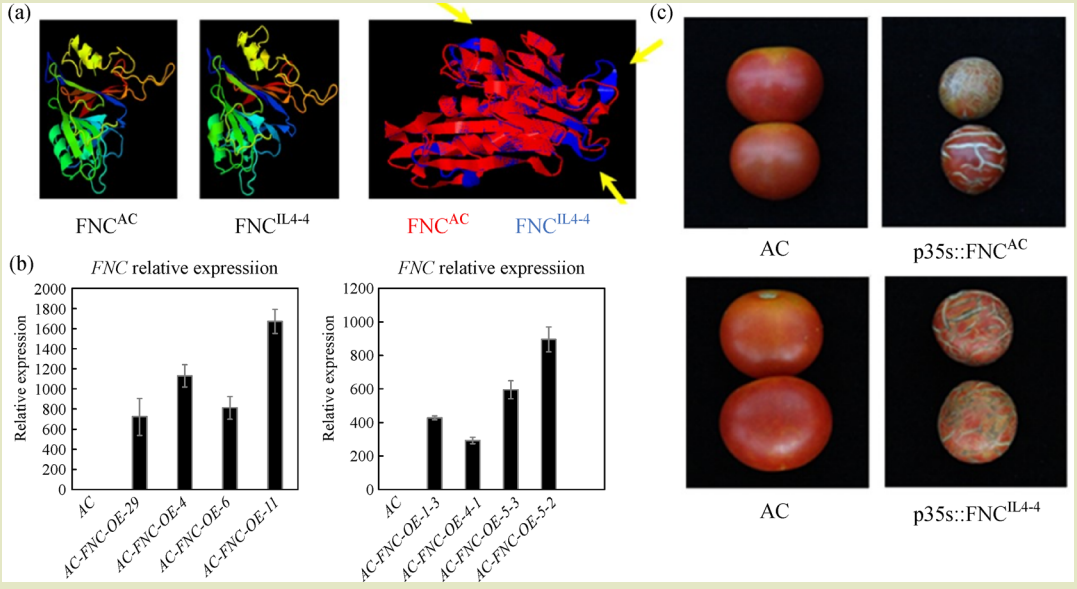


Fig. 4 Overexpressing the *FNC* gene in tomato leads to the fruit netted-cracking phenotype. (a) FNC^{IL4-4} protein has three more β-fold structures than FNC^{AC}. Red (FNC^{IL4-4}), Blue (FNC^{AC}). The yellow arrows indicate three of the β-fold structures. (b) The transcript levels of overexpressing FNC^{IL4-4} and FNC^{AC}, Ailsa Craig (AC) as a control cultivar, (OE) overexpression. (c) Overexpression of FNC^{IL4-4} and FNC^{AC} in a non-cracking AC resulted in a netted-cracking on the fruit surface.

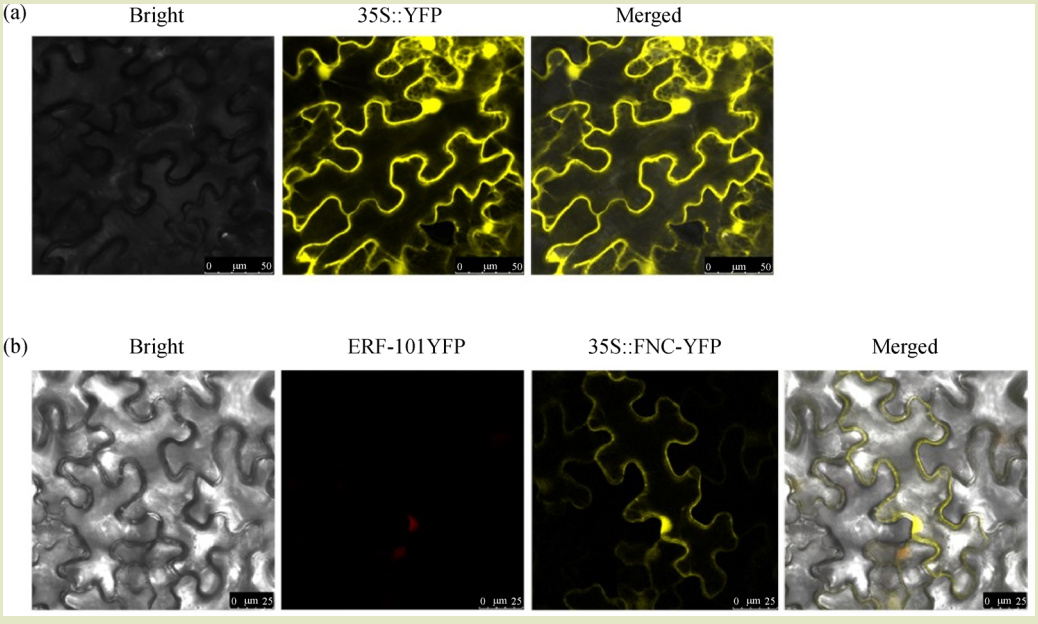


Fig. 5 Subcellular localization of the FNC protein observed by fluorescence confocal microscopy. The transient expressions of 35S::YFP (a) as control and 35S::FNC-YFP (b) in tobacco leaves.

expressing plants and AC. The expression of *ASFT* and *GPAT5* in the suberin metabolism pathway was upregulated in *FNC* overexpressed lines (Fig. 6(a)). Similar transcript abundance of several genes involved in the lignin metabolism pathway (Fig. 6(b)), lipid metabolism (Fig. 6(c)), lipid transport (Fig. 6(d)) and cell wall metabolism (Fig. 6(e)) was observed.

To evaluate the abnormal fruit epidermis development cytologically, we observed the epidermis of tomato fruit by paraffin section in M82, AC, IL4-4 and overexpressing lines during the breaker stage. It was found that the epidermal cell layer of IL4-4 and overexpressing lines had discontinuous fracturing but no in that of M82 and AC. The morphology of the cells near the outermost layer changed, the shape of the cells was flatter and more slender, and the arrangement was irregular (Fig. 6(f)). Cell deformation may be caused by internal pressure to reach the bearing limit of the epidermis. Also, the cracks were protruding on the surface of pericarp cells, and this may be why the epidermis cracks induced a large number of divisions and proliferation of pericarp cells, resulting in the accumulation of cell wall embolized cells and the formation of healed tissues.

4 DISCUSSION

4.1 Phenotypes of fruit cracking varied in different backgrounds

Fruit cracking is a common phenomenon in fruit production and is associated with some physiological, environmental and

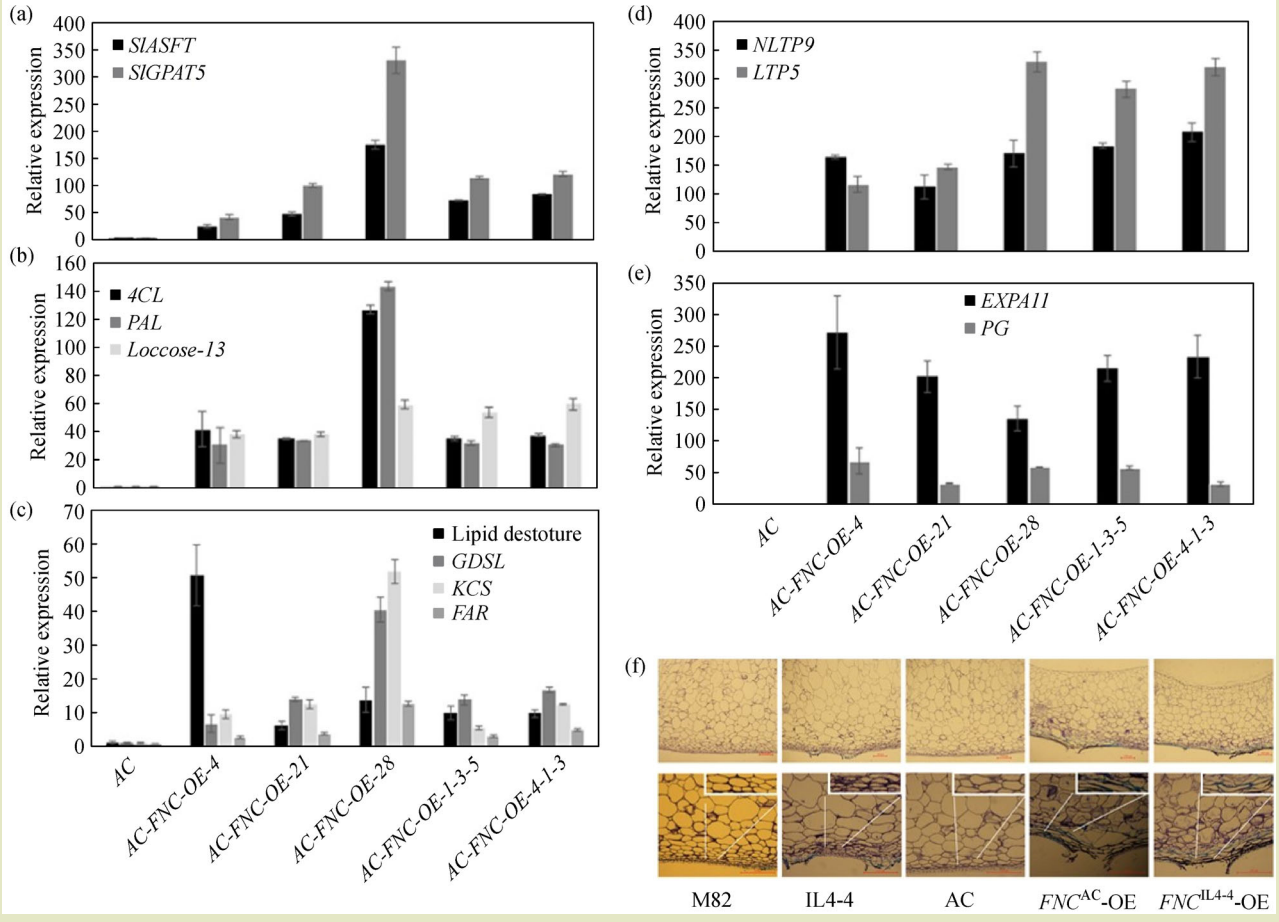


Fig. 6 Increased expression of the *FNC* gene can affect the metabolic pathway of pericarp of tomato. (a–e) The relative expression genes involved in various metabolism pathways such as in suberin metabolism pathway (a), in lignin metabolism pathway (b), in lipid metabolism pathway (c), in lipid transport pathway (d) and in cell wall metabolism pathway (e) in *FNC-OE* plants and AC. *SlGPAT5* (Solyc04 g011600), *SlASFT* (Solyc03 g097500), *PAL* (phenylalanine ammonia lyase, Solyc10 g086180), *4CL* (4-coumaric acid, CoA ligase, Solyc06 g068650), *Laccase-13* (laccase, Solyc06 g082240), *GDSL* lipase, *KCS* (β-ketoacyl CoA synthase), *FAR* (fatty acyl reductase), *NLTP9* (Solyc09 g082270), *LTP5* (Solyc09 g065430), *EXPA11* (Solyc04 g081870) and *PG* (Solyc08 g060970). (f) The ultrastructure of fruit epidermis in the overexpressing plants and the control cultivar during the breaker stage.

genetic factors, resulting in severe economic losses of fleshy fruit[21–24]. Previous studies have found that cracking susceptibility varies between different genetic backgrounds, indicating that genetic factors are involved in fruit cracking[25]. Capel and coworkers discovered that QTL with epistatic effects and environment interactions are involved in the genetic variation of the cracking trait[2]. Genetic complexity makes it challenging to fine-map the genes controlling fruit cracking. Here, we cloned a fruit netted-cracking gene, *FNC,* from the tomato introgression line IL4-4. We compared the phenotypes of *FNC* and found that they were distinguishable from different backgrounds. *FNC* from the introgression line population of *S. habrochaites* was found to control the dehydration of fruit with cuticular microcracks which is hard to observe with the naked eye[12]. More severe cracks appeared on the fruit surface of IL4-4 and the overexpressed *FNC* lines in the present study. The cracks were filled with suberin to form reticular cracks similar to muskmelon, which meant that the cracks were more profound and severe. ER4.1 locus was also found to control the epidermal reticular structure of green tomato fruit and water loss and identified four candidate genes. *FNC* is likely to be the causative gene[14], and this needs to be confirmed by transgenic functional verification. These results indicate that netted-cracking phenotypes are variated and complicated in different backgrounds, suggesting that there are unknown regulating factors involved in this development process.

4.2 Netted-cracking was generally related to the transcript level of the *FNC* gene

We found that both *S. pennellii* and M82/AC did not have a netted-cracking phenotype, but this phenotype occurred in their introgression line, IL4-4. After we mapped the candidate gene, *FNC*, we found that *FNC* had non-synonymous mutations in IL4-4 and M82/AC. The allelic form is considered crucial in determining the severity of the netted-cracking phenotype[14]. We therefore overexpressed the different alleles of *FNC* from IL4-4 and AC; a similar phenotype of netted-cracking was observed in overexpressing lines of $FNC^{IL4\text{-}4}$ and FNC^{AC}. This clearly indicates that differences in amino acids were not the determining factor of the netted-cracking phenotype in this case. We noticed that the expression level of *FNC* in IL4-4 was dramatically higher in M82 during all fruit development stages. This increased expression of *FNC* may be a major explanation for pericarp cracking. We analyzed the promoter sequence of IL4-4 and AC and found differences (Fig. S5). The binding ability of regulatory factors may be changed due to the difference between promoters but the key motifs remain to be identified. Notably, the expression of *FNC* in netted-cracking pepper and muskmelon was found to be higher than in non-netted-cracking genotypes. These results show that functional gain of fruit netted-cracking may be dependent on the expression abundances of *FNC*, and this phenomenon seems to be a common regulatory mechanism in different plant species.

4.3 *FNC* gene regulated cracking of pericarp by interfering with the multi-metabolic pathways

It was found that increased expression of *FNC* significantly upregulated the expressions of genes related to suberin metabolism, lignin metabolism, lipid metabolism, lipid transport and cell wall metabolism, thus regulating the formation of tomato cracks (Fig. 6).

The overexpression of *FNC* upregulated the expression of *EXPA11* (*Solyc04 g081870*) and *PG* (*Solyc08 g060970*), which are involved in the regulation of cell wall extensibility and fruit softening. Recently, a report showed that inhibiting *PG* and *EXP* gene expression can thicken the cell wall and waxy layer, increase fruit firmness, and then reduce the fruit cracking rate in tomato[24]. The expression of *EXP* was also upregulated in muskmelon pericarp[26]. These results are consistent with this experiment, thus we can say that increased expression of *FNC* promotes *PG* and *EXPA11* to induce fruit cracking by altering cell wall ductility and pericarp firmness.

In addition, increased expression of *FNC* can upregulate the expressions of *SlASFT* and *SlGPAT5* to accelerate the suberin accumulation in the cracks and protect the internal tissue of fruit. *GPAT5* (*Solyc04 g011600*) is responsible for the synthesis of multi-aliphatic components of suberin monomers, while *ASFT* (*Solyc03 g097500*) is responsible for the synthesis of polyphenol monomers[27,28]. Knocking down *DCR* (defective in cuticular ridges) causes the tomato fruit to have brown cracks[29], but the cracked phenotype is different from IL4-4, and we found that the expression of *GPAT5* and *ASFT* increased in *FNC* overexpressing lines. Moreover, it was found that there was no protein interaction between *FNC* and *DCR* in our yeast two-hybrid experiment, and the expression of *SlDCR* in *FNC* overexpressing lines made no significant change (data not shown). Therefore, it is speculated that *SlDCR* functions upstream of *FNC* or independently regulates the suberin metabolic pathway.

PAL (*Solyc10 g086180*), *4CL* (*Solyc06 g068650*) and *Laccase-13* (*Solyc06 g082240*) in the lignin pathway were upregulated in *FNC* transgenic plants. *PAL* and *4CL* are involved in the synthesis of flavonoids which can change the elasticity of cuticle and relate to suberin polyphenol synthesis[30,31]. The *GDSL* lipase gene is involved in the polymerization and crosslinking of horny monomers in lipid metabolism, *KCS* is involved in

prolonging acyl products in suberin, and horny biosynthesis is responsible for the catalytic reduction of long-chain fatty acyl-CoA to primary alcohol. The expression of lipid desaturase related to lipid desaturation was also upregulated in the present study. It has been reported that expression of *PAL*, *4CL,* and *GDSL* in tomato with reticular phenotype is higher than that in wild type tomato[14]; this is consistent with our results. Also, *NLP9* and *LTP* (*Solyc09 g082270*, *Solyc09 g065430*), which have the function of lipid transport, were also upregulated in the present study, which may affect the transport and deposition of synthetic suberin and stratum corneum components[32–34].

In summary, *FNC* can regulate the formation of netted-cracking in tomato pericarp by affecting the expression of genes related to the above metabolic pathways, but whether *FNC* directly acts on these downstream genes needs to be verified.

4.4 Proposed mechanism of crack formation

There have been few studies to explain the mechanism of fruit cracking from the perspective of molecular biology, and most of the related reports focus on the level of physiology, biochemistry or genetics. Based on the above analysis, we suggest that the netted-cracking fruit may be cooperatively regulated by two genetic loci which affect tomato fruit cracking by *cis-trans* regulation. There is an active *trans*-regulatory factor but a lack of an active corresponding binding motif in cultivated tomatoes. However, there is an active *cis*-element but the *trans*-regulatory factor is inactive in *S. pennellii*. Both active *trans*-regulatory elements and active *cis*-element are present in IL4-4 and the *trans*-element binds to the *cis*-element of the *FNC* promoter to activate its expression (Fig. S6). There are likely to be other mechanisms involved in this netted-cracking phenotype and further studies are needed.

5 CONCLUSIONS

Here, we have found a novel phenotype of fruit netted-cracking in the tomato introgression line IL4-4; genetic analysis indicates that it is controlled by a single dominant gene. By map-based cloning, *FNC* (Solyc04 g082540) was identified as the most likely candidate gene. Although five amino acid differences exist between FNC^{AC} and $FNC^{IL4\text{-}4}$ proteins, overexpression of both FNC^{AC} and $FNC^{IL4\text{-}4}$ had the fruit netted-cracking phenotype, suggesting that the transcript level of *FNC* determined the fruit netted-cracking. These findings were confirmed by *FNC* orthologs in the netted-cracking pepper and melon, indicating a general regulatory mechanism in different species. Overexpression of *FNC* also led to the upregulation of suberin, lignin, lipid and cell wall metabolism pathways. These results provide a novel genetic insight into the formation of fruit netted-cracking.

Supplementary materials

The online version of this article at https://doi.org/10.15302/J-FASE-2020374 contains supplementary materials (Figs. S1–S6; Tables S1–S3).

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Compliance with ethics guidelines

Chunli Zhang, Taotao Wang, Jing Li, Danqiu Zhang, Qingmin Xie, Shoaib Munir, Jie Ye, Hanxia Li, Yongen Lu, Changxian Yang, Bo Ouyang, Yuyang Zhang, Junhong Zhang, and Zhibiao Ye declare that they have no conflicts of interest or financial conflicts to disclose. This article does not contain any studies with human or animal subjects performed by any of the authors.

REFERENCES

1. Khadivi-Khub A. Physiological and genetic factors influencing fruit cracking. *Acta Physiologiae Plantarum,* 2015, **37**(1): 1718
2. Capel C, Yuste-Lisbona F J, López-Casado G, Angosto T, Cuartero J, Lozano R, Capel J. Multi-environment QTL mapping reveals genetic architecture of fruit cracking in a tomato RIL *Solanum lycopersicum* × *S. pimpinellifolium* population. *Theoretical and Applied Genetics,* 2017, **130**(1): 213–222
3. Islam M Z, Mele M A, Baek J P, Kang H M. Cherry tomato qualities affected by foliar spraying with boron and calcium. *Horticulture, Environment and Biotechnology,* 2016, **57**(1): 46–52
4. Knoche M, Peschel S. Water on the surface aggravates microscopic cracking of the sweet cherry fruit cuticle. *Journal*

of the American Society for Horticultural Science, 2006, **131**(2): 192–200

5. Ginzberg I, Stern R A. Strengthening fruit-skin resistance to growth strain by application of plant growth regulators. *Scientia Horticulturae,* 2016, **198**: 150–153
6. Jiang H K, Tian H M, Yan C S, Jia L, Wang Y, Wang M X, Jiang C J, Li Y Y, Jiang J Y, Fang L, Zhao Q A. RNA-seq analysis of watermelon (*Citrullus lanatus*) to identify genes involved in fruit cracking. *Scientia Horticulturae,* 2019, **248**: 248–255
7. Song Y Q, Li J, Fu L J, Li N, Li L L. Change of fruit surface characteristics and its relationship with water absorption and fruit cracking in ziziphus jujuba 'Huping'. *Scientia Silvae Sinicae,* 2018, **54**(12): 52–59 (in Chinese)
8. Liao N, Hu Z, Li Y, Hao J, Chen S, Xue Q, Ma Y, Zhang K, Mahmoud A, Ali A, Malangisha G K, Lyu X, Yang J, Zhang M. Ethylene-responsive factor 4 is associated with the desirable rind hardness trait conferring cracking resistance in fresh fruits of watermelon. *Plant Biotechnology Journal,* 2020, **18**(4): 1066–1077
9. Giménez E, Dominguez E, Pineda B, Heredia A, Moreno V, Lozano R, Angosto T. Transcriptional activity of the MADS box ARLEQUIN/TOMATO AGAMOUS-LIKE1 gene is required for cuticle development of tomato fruit. *Plant Physiology,* 2015, **168** (3): 1036–1048
10. Shi J X, Adato A, Alkan N, He Y, Lashbrooke J, Matas A J, Meir S, Malitsky S, Isaacson T, Prusky D, Leshkowitz D, Schreiber L, Granell A R, Widemann E, Grausem B, Pinot F, Rose J K C, Rogachev I, Rothan C, Aharoni A. The tomato SlSHINE3 transcription factor regulates fruit cuticle formation and epidermal patterning. *New Phytologist,* 2013, **197**(2): 468–480
11. Petit J, Bres C, Mauxion J P, Bakan B, Rothan C. Breeding for cuticle-associated traits in crop species: traits, targets, and strategies. *Journal of Experimental Botany,* 2017, **68**(19): 5369–5387
12. Hovav R, Chehanovsky N, Moy M, Jetter R, Schaffer A A. The identification of a gene (Cwp1), silenced during Solanum evolution, which causes cuticle microfissuring and dehydration when expressed in tomato fruit. *Plant Journal,* 2007, **52**(4): 627–639
13. Chechanovsky N, Hovav R, Frenkel R, Faigenboim A, Eselson Y, Petreikov M, Moy M, Shen S, Schaffer A A. Low temperature upregulates *cwp* expression and modifies alternative splicing patterns, increasing the severity of *cwp*-induced tomato fruit cuticular microfissures. *Horticulture Research,* 2019, **6**(1): 122
14. Cui L, Qiu Z, Wang Z, Gao J, Guo Y, Huang Z, Du Y, Wang X. Fine mapping of a gene (ER4.1) that causes epidermal reticulation of tomato fruit and characterization of the associated transcriptome. *Frontiers in Plant Science,* 2017, **8**: 1254
15. Keren-Keiserman A, Tanami Z, Shoseyov O, Ginzberg I. Peroxidase activity associated with suberization processes of the muskmelon (*Cucumis melo*) rind. *Physiologia Plantarum,* 2004, **121**(1): 141–148
16. Ramamurthy R K, Waters B M. Identification of fruit quality and morphology QTLs in melon (*Cucumis melo*) using a population derived from flexuosus and cantalupensis botanical groups. *Euphytica,* 2015, **204**(1): 163–177
17. Wang Y Z, Dai M S, Cai D Y, Zhang S J, Shi Z B. A review for the molecular research of russet/semi-russet of sand pear exocarp and their genetic characters. *Scientia Horticulturae,* 2016, **210**: 138–142
18. Yasuzumi G, Sugihara R. The fine structure of nuclei fixed by a double fixation procedure. *Experimental Cell Research,* 1964, **33** (3): 578–580
19. Monforte A J, Friedman E, Zamir D, Tanksley S D. Comparison of a set of allelic QTL-NILs for chromosome 4 of tomato: deductions about natural variation and implications for germplasm utilization. *Theoretical and Applied Genetics,* 2001, **102**(4): 572–590
20. Bard F, Casano L, Mallabiabarrena A, Wallace E, Saito K, Kitayama H, Guizzunti G, Hu Y, Wendler F, Dasgupta R, Perrimon N, Malhotra V. Functional genomics reveals genes involved in protein secretion and Golgi organization. *Nature,* 2006, **439**(7076): 604–607
21. Saladié M, Matas A J, Isaacson T, Jenks M A, Goodwin S M, Niklas K J, Xiaolin R, Labavitch J M, Shackel K A, Fernie A R, Lytovchenko A, O'Neill M A, Watkins C B, Rose J K C. A reevaluation of the key factors that influence tomato fruit softening and integrity. *Plant Physiology,* 2007, **144**(2): 1012–1028
22. Isaacson T, Kosma D K, Matas A J, Buda G J, He Y, Yu B, Pravitasari A, Batteas J D, Stark R E, Jenks M A, Rose J K C. Cutin deficiency in the tomato fruit cuticle consistently affects resistance to microbial infection and biomechanical properties, but not transpirational water loss. *Plant Journal,* 2009, **60**(2): 363–377
23. Hen-Avivi S, Lashbrooke J, Costa F, Aharoni A. Scratching the surface: genetic regulation of cuticle assembly in fleshy fruit. *Journal of Experimental Botany,* 2014, **65**(16): 4653–4664
24. Jiang F, Lopez A, Jeon S, de Freitas S T, Yu Q, Wu Z, Labavitch J M, Tian S, Powell A L T, Mitcham E. Disassembly of the fruit cell wall by the ripening-associated polygalacturonase and expansin influences tomato cracking. *Horticulture Research,* 2019, **6**(1): 17
25. Correia S, Schouten R, Silva A P, Gonçalves B. Sweet cherry fruit cracking mechanisms and prevention strategies: a review. *Scientia Horticulturae,* 2018, **240**: 369–377
26. Cohen H, Dong Y, Szymanski J, Lashbrooke J, Meir S, Almekias-Siegl E, Zeisler-Diehl V V, Schreiber L, Aharoni A. A multilevel study of melon fruit reticulation provides insight into skin lignosuberization hallmarks. *Plant Physiology,* 2019, **179**(4): 1486–1501
27. Pollard M, Beisson F, Li Y, Ohlrogge J B. Building lipid barriers: biosynthesis of cutin and suberin. *Trends in Plant Science,* 2008, **13**(5): 236–246
28. Lashbrooke J, Aharoni A, Costa F. Genome investigation suggests MdSHN3, an APETALA2-domain transcription factor gene, to be a positive regulator of apple fruit cuticle formation and an inhibitor of russet development. *Journal of Experimental Botany,* 2015, **66**(21): 6579–6589

29. Lashbrooke J, Cohen H, Levy-Samocha D, Tzfadia O, Panizel I, Zeisler V, Massalha H, Stern A, Trainotti L, Schreiber L, Costa F, Aharoni A. MYB107 and MYB9 homologs regulate suberin deposition in angiosperms. *Plant Cell,* 2016, **28**(9): 2097–2116
30. Xing Y L, Shi C X, Li H X, Ye Z B. Formation mechanism of tomato fruit cuticles. *Acta Horticulturae Sinica,* 2016, **43**(9): 1726–1734 (in Chinese)
31. España L, Heredia-Guerrero J A, Reina-Pinto J J, Fernández-Muñoz R, Heredia A, Domínguez E. Transient silencing of CHALCONE SYNTHASE during fruit ripening modifies tomato epidermal cells and cuticle properties. *Plant Physiology,* 2014, **166**(3): 1371–1386
32. Fich E A, Segerson N A, Rose J K C. The plant polyester cutin: biosynthesis, structure, and biological roles. *Annual Review of Plant Biology,* 2016, **67**(1): 207–233
33. Kolattukudy P E. Biopolyester membranes of plants: cutin and suberin. *Science,* 1980, **208**(4447): 990–1000
34. Pighin J A, Zheng H, Balakshin L J, Goodman I P, Western T L, Jetter R, Kunst L, Samuels A L. Plant cuticular lipid export requires an ABC transporter. *Science,* 2004, **306**(5696): 702–704