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Generating Novel Tomato Germplasm Using the Ancestral Wild Relative of *Solanum pimpinellifolium*

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Abstract: To create novel tomato (*Solanum lycopersicum*) germplasm, a wild tomato relative, *S. pimpinellifolium* (a red-fruited LA1585 accession), was used as the male parent to cross with the yellow-fruited tomato mutant, *e9292* (*S. lycopersicum*). Forty-four morphological characteristics were examined in the present study; 22 *S. pimpinellifolium* (LA1585) traits and six *S. lycopersicum* (*e9292*) traits were dominant in the hybrids (first generation, F_1); 10 were intermediate types, and the remaining six resulted in a nonmorphological difference. Ten representative lines were chosen from 1338 line of the second generation of the hybrid (*e9292* × LA1585). The ascorbic acid content was higher in three F_2 hybrids than in LA1585, which had higher parental levels, as well as the lycopene content in two of the hybrid lines. The fructose and glucose contents were higher in five F_2 hybrids compared to *e9292*, and the sugar/acid value was higher in three hybrid lines. The broad-sense heritability values ranged from 75.06% for total soluble solids to 99.92% for ascorbic acid, and the average value was 92.66% for 15 quality traits. When seedlings were exposed to NaCl and mannitol, the tolerance of high salt concentrations and drought was enhanced in two hybrid lines (F_2-266 and F_2-299) compared with else tomatoes (*e9292*, LA1585, F_1 hybrids, and F_2-332). We created novel tomato germplasm resources with horticulturally desirable quality traits and abiotic stress tolerance, thus offering a methodology for novel tomato germplasm creation and evaluation.

Keywords: *Solanum lycopersicum*; *S. pimpinellifolium*; genetic improvement; quality traits; germplasm resources

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1. Introduction

Tomato (*Solanum lycopersicum*) is an economically important crop, reflecting its horticultural significance, value to human health as a dietary component, and potential as a model plant targeted for scientific research [1–4]. According to the FAO (Food and Agriculture Organization), tomato production reached 186.82 million tons, worth USD 92.83 billion, in 2020 (http://www.fao.org/faostat/en/#data/QV (accessed on 1 September 2022)). Tomato is used as a model in several scientific disciplines such as genetics, developmental biology, and molecular biology, especially with regard to studies on the regulation of ethylene signaling transduction in fruits that exhibit climacteric ripening [5–7]. Tomato fruit contains a spectrum of nutrients that are beneficial for human health, such as ascorbic acid, amino acids, minerals, and carotenoid pigments. Carotenoids in particular serve as antioxidants that protect against certain degenerative diseases, such as macular

degeneration of the eye, a leading cause of age-related blindness, as well as cardiovascular and cerebrovascular diseases, and cancers, such as prostate cancer [8–11].

To date, tomato production has met worldwide consumer demand largely through breeding-driven improvements in yield, shelf-life, and tolerance to biotic and abiotic stresses [12–18]. However, it is more difficult to satisfy consumer demand related to tomato quality, as this involves diverse traits, including taste, aroma, and flavor associated with sugars, organic acids, volatile substance, and secondary metabolites. The quality improvement of the cultivated tomato has been limited by germplasm diversity, which declined during domestication and dispersal from the New World to the Old World, as well as during modern breeding over the last 100 years [19]. Wild tomato relatives show ample genetic diversity and have the potential to improve tomato quality [20–22], although there are limitations due to reproductive barriers in interspecific hybridizations, hybrid progeny with severe separation, and linkage drag [23,24]. In contrast to other wild tomato relatives, *S. pimpinellifolium* is the wild progenitor of the cultivated tomato; it inhabits the coastal regions of Ecuador, Peru, and northern Chile, and it is fully cross-compatible with cultivated tomato (*S. lycopersicum*) [25]. As an immediate ancestor species, *S. pimpinellifolium* was successively domesticated in *S. lycopersicum* var. *cerasiforme* and is a cultivated tomato in the Andean region and Mesoamerica [25]. Furthermore, *S. pimpinellifolium* is also regarded as a largely unexploited genetic reservoir for tomato improvement. *Pseudomonas syringae* pv. *tomato* (*Pto*) was the first resistance (*R*) gene isolated from *S. pimpinellifolium* using a map-based cloning technique in tomato clade, and it has been introgressed into cultivated tomatoes to protect against tomato bacterial speck [26–28]. Later, an encoding coiled-coil nucleotide-binding leucine-rich repeat (CC-NBS-LRR) protein *Ph-3* gene was isolated from *S. pimpinellifolium*, conferring resistance to *Phytophthora infestans* in tomato [29]. The genomic data revealed that *S. pimpinellifolium* has the potential to improve the tolerance of the cultivated tomato to abiotic or biotic stresses such as diseases and salinity, as well as important quality traits such as lycopene accumulation and flavonoid biosynthesis [29–33]. Therefore, *S. pimpinellifolium* has been frequently used as an important germplasm donor in modern tomato breeding. Several important genes associated with fruit quality and resistance to abiotic or biotic stresses in *S. pimpinellifolium* have been identified or predicted by genomic sequencing for introduction into cultivars. However, the inadequate development of elite germplasms has limited the improvement of cultivated tomato using *S. pimpinellifolium.* In particular, *S. pimpinellifolium* has the potential to improve tomato qualities such as the contents of carotenoids, total soluble solids, and ascorbic acid [34–37]. These characteristics can provide a valuable germplasm to satisfy the demand from both breeders and consumers for tomato quality improvement. To create a new interspecies germplasm resource, the red-fruited accession of *S. pimpinellifolium* LA1585 was used as the male parent, while the *yft3* (*yellow-fruited tomato 3*, *e9292*) mutant, generated via ethyl methane sulfonate (EMS) treatment of *S. lycopersicum* M82 seeds, served as the female parent in a cross to produce a first generation (F_1, *yft3* × LA1585) population. The yellow-fruited *yft3* has the phenotype of lycopene accumulation deficiency, which allows easy distinction from its wild relative LA1585 based on lycopene accumulation. Individuals of the F_1 population were used to generate an F_2 segregating population through self-pollination. The phenotype, quality traits, and abiotic stress tolerance of the F_2 lines were analyzed and compared among the three different generations (parental, F_1, and F_2 lines), with a focus on the carotenoid content and the expression of key genes potentially involved in quality traits.

2. Materials and Methods

2.1. Creation of an Interspecific Cross Population

The red-fruited *S. pimpinellifolium* accession LA1585 was used as the male parent, in a cross with the *e9292* mutant (*yellow-fruited tomato 3*, *yft3*) as the female parent, which was derived from EMS mutagenesis of *S. lycopersicum* M82 [38]. LA1585 and *yft3* were provided by Professor Roger T. Chetelat (Tomato Genetics Resource Center, University

of California, Davis) and Professor Dani Zamir (Hebrew University of Jerusalem, http://zamir.sgn.cornell.edu/mutants (accessed on 1 September 2022)), respectively. One day before anthesis, *yft3* flower buds were emasculated and then pollinated on the same day using LA1585 pollen. F_1 generation (*yft3* × LA1585) seeds were collected from ripe fruit derived from the manually pollinated flowers, sown and grown in a greenhouse, and the resulting plants were self-pollinated to produce the population of the F_2 generation (total 1338 lines). All F_2 seeds were sown, and the resulting plants were grown in a greenhouse; then, five representatives each of yellow- or red-fruited plants were selected, and the associated seeds were sown and grown in a greenhouse to generate ten F_3 populations, which were then used to assess the phenotypes.

2.2. Growth Conditions and Identification of Hybridization Attributes

Tomato seeds from 13 genotypes, i.e., *e9292*, LA1585, F_1 (*e9292* × LA1585), and ten F_2 lines (*e9292* × LA1585, five yellow and five red-fruited), were stirred in water that was initially 50 °C and then left until room temperature was reached, before germinating in a constant-temperature and -humidity incubator (Tencheng Laboratory Instrument Co., Ltd., Shanghai, China) in the dark at 25 °C. The germinated seeds were sown in plastic plug flats with 60 cells (Qihang Horticultural Experimental Farm, Shanghai, China) and moist seedling soil (Pinshi Tuopu Horticultural nurseries Co., Ltd., Suqian, China). The seedlings were grown in a helio-greenhouse at the Shanghai Jiao Tong University experimental farm at Pujiang (121°30′10.89″ E, 31°3′5.20″ N, altitude 5 m) until they had 4–5 true leaves and were then transplanted to the field and cultivated using standard field management practices.

On the basis of the TOMATO-EXPEN 2000 (https://solgenomics.net/ (accessed on 1 September 2022)) tomato genomic resource database, the TG294 DNA sequence located on chromosome 8 was chosen to design a CAPS (cleaved amplified polymorphic sequence) marker. Tomato leaf genomic DNA was extracted as described [39], and two specific primers, TG294F (5′−attggctgcaatgatggatt−3′) and TG294R (5′−ctaagcaggacggccatcta−3′), were used for PCR amplification. The sequencing results of the PCR products allowed for the design of restriction endonuclease sites, such as *Xmn*I. Electrophoretic separation of the enzymatically digested PCR products was used to identify parents and hybridization properties of different *e9292* × LA1585 generations.

2.3. Phenotypes and Heritability

The morphological phenotypes, including 19 qualitative and 25 quantitative traits, were investigated and measured at the RR/YR (red/yellow ripening) stage of the first fruit from the second truss of 13 genotypes, i.e., *e9292*, LA1585, F_1, and 10 F_2 generation lines, as previously described [40]. Ten biological replicates were used for the examinations. The broad-sense heritability (hB) and coefficient of variation (*Cv*) for key morphological phenotypes were calculated using the SPSS (Statistical Product and Service Solutions) software package (Chicago, IL, USA), according to Equations (1) and (4). As a function of the morphological phenotypes, a heatmap based on a cluster analysis was drawn using the heatmap.2 function in the gplots (R 3.2.2 version) software package (http://mirror.bjtu.edu.cn/cran/ (accessed on 1 September 2022) and help file http://docs.ggplot2.org/current/index.html (accessed on 1 September 2022)).

$$hB^2 = \frac{Vg}{Vg + Ve} \times 100\%, \quad (1)$$

$$Vg = \frac{MSg - MSe}{n0}, \quad (2)$$

$$n0 = \frac{Total\ number\ of\ observations}{The\ number\ of\ materials}, \quad (3)$$

where *MSg* is the mean squares between groups, *MSe* is the mean squares within a group, n_0 is the replication number of effective observations, *Vg* is the genetic variance, and *Ve* = *MSe* is the environmental variance.

$$Cv = \frac{\sigma}{\mu} \times 100\%, \quad (4)$$

where C_v is the coefficient of variation, σ is the standard error of the mean, and μ is the average value.

2.4. Quality Traits

Tomato fruit pericarp was sampled at RR/YR (red/yellow ripening). The ascorbic acid (vitamin C, Vc) content of tomato fruit was determined using the 2, 6-dichloroindophenol titration method [41]. The total soluble solid (TSS) content was measured using a portable refractometer (PAL-1, ATAGO, Japan). Tomato fruit firmness (TFF) was examined using a texture analyzer (model TA.XT Plus, SMSTA, UK) fitted with a 6 mm cylindrical plunger (model SMS P/5). Each tomato fruit was examined at five points: the top, the pedicle, and three points with 120° intervals around the equatorial zone (n = 5). Each measuring point was compressed to 11 mm at a speed of 100 mm/min with a trigger force of 0.3 N. The firmness value was determined as the maximum force developed during the test. The carotenoid content was determined using HPLC (high-performance liquid chromatography) as described in [42]. Lastly, the contents of sugars, such as glucose, fructose, and sucrose, and organic acids, including citric acid, malic acid, quininic acid, and succinic acid, were determined using GC–TOF-MS (gas chromatography/time-of-flight mass spectrometry) as described in [43]. Three biological replicates were used for those experiments, except for TSS, where five biological replicates were used.

2.5. Expression of Genes Involved in Carotenoid Synthesis

Total RNA was extracted from ripening equatorial zone pericarp (breaker stage, BR) of *e9292*, LA1585, F_1 (*e9292* × LA1585), and F_2 (*e9292* × LA1585) using a plant RNA extraction kit (Tiangen Bio-technology Co., Ltd., Beijing, China) in three biological replicates. In total, 1 μg of RNA was used to synthesize complementary DNA (cDNA) using 5× PrimeScript RT Master Mix (TAKARA, Dalian, China), and a 50-fold dilution of the reverse transcription product was used as the template to conduct real-time (RT) quantitative (q)PCR analysis. According to the method described [42], the expression of genes involved in carotenoid synthesis (*CRTISO* (*CAROTENOID ISOMERASE*), *CYCB* (*LYCOPENE β-CYCLASE*), *DXR* (*1-DEOXY-D-XYLULOSE 5-PHOSPHATE REDUCTOISOMERASE*), *DXS* (*1-DEOXY-D-XYLULOSE 5-PHOSPHATE SYNTHASE*), *HDR* (*4-HYDROXY-3-METHYLBUT-2-ENYL DIPHOSPHATE REDUCTASE*), *IDI1* (*ISOPENTENYL DIPHOSPHATE Δ-ISOMERASE 1*), *LCYE (LYCOPENE E-CYCLASE*), and *PSY1* (*PHYTOENE SYNTHASE 1*)) was analyzed using *ACTIN* (GenBank accession: BT013524) as the endogenous reference gene. All primers are listed in Table S1.

2.6. Response to Drought and Salt Stresses

Seeds collected from six tomato genotypes, i.e., *e9292*, LA1585, F_1, F_2-266, F_2-299, and F_2-332, were sterilized by immersion in 75% ethanol (*v*/*v*) for 1 min and then 20% bleach (sodium hypochlorite, *w*/*v*) for 10 min, before rinsing five times with ddH_2O to remove trace sodium hypochlorite. Seeds were then placed on moist sterile 3 M filter paper overlaying MS solid medium (4.41 g/L MS, 30% sucrose, and 0.26% Phytagel, pH = 5.8) for germination. Germinated seeds with a similar appearance were placed in square petri dishes with MS solid medium, containing different mannitol (0 mM, 50 mM, 100 mM, and 150 mM) and NaCl (0 mM, 50 mM, 100 mM, and 150 mM) concentrations. Five biological replicates were analyzed. The square petri dishes were placed vertically in an illuminating incubator (16 h light/8 h dark) at 25 °C, and the lengths of the hypocotyls and roots were measured using ImageJ software at the same timepoint for six consecutive days. Images were taken using a Cannon EOS 800D (Canon, Japan) camera.

3. Results

3.1. Identification of Hybridization between e9292 and LA1585

The female parent (yellow-fruited tomato mutant *e9292*) and male parent (red-fruited LA1585) produced a red-fruited F_1 (*e9292* × LA1585) generation, while there was color segregation in the F_2 generation (Figure 1a). To identify the nature of the interspecific hybrids between *e9292* and LA1585, a CAPS marker was designed corresponding to the TG294 DNA segment on chromosome 8. The resulting PCR products exhibited a single-nucleotide polymorphism (SNP) between *e9292* (G) and LA1585 (C) when amplified using the TG294F and TG294R primer set (Figure 1b), and distinct *e9292* and LA1585 bands were produced when the PCR product was digested by *Xmn*I (Figure 1c). This CPAS maker was competent to distinguish all interspecific hybrids of both F_1 and F_2 and confirmed their hybrid nature.

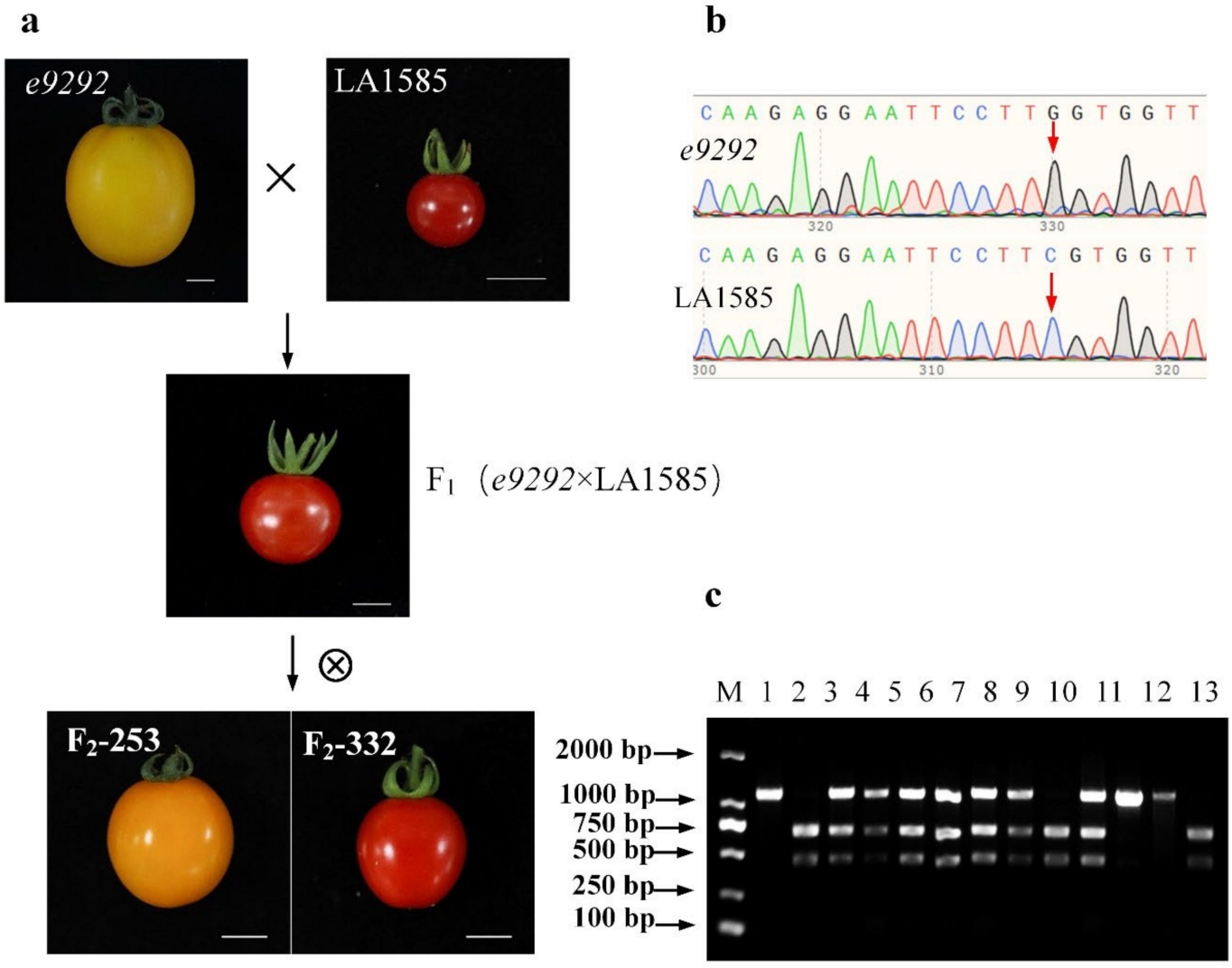

Figure 1. Creation of populations of different generations resulting from distant hybridization and identification of features caused by hybridization. (**a**) Fruit color and size of the *Solanum lycopersicum e9292* mutant used as the pistil parent and *S. pimpinellifolium* LA1585 used as the pollen parent, along with subsequent generations (scale bar = 1 cm). (**b**) The SNP (single-nucleotide polymorphism) difference found in TG 294 at chromosome 8 between *e9292* and LA1585. (**c**) Identification of genomic DNA features caused by hybridization using a CAPS marker. Lane M, DL2000 DNA ladder. Lanes 1–13: *e9292*, LA1585, F_1 (*e9292* × LA1585), F_2-259, F_2-280, F_2-292, F_2-318, F_2-332, F_2-253, F_2-266, F_2-299, F_2-328, and F_2-330, respectively.

3.2. Genetic Analysis of Phenotypes

After assessing 44 phenotypes/morphological traits in different genetic backgrounds (*e9292*, LA1585, F_1, and 10 F_2 lines (F_2-253, F_2-259, F_2-266, F_2-280, F_2-292, F_2-299, F_2-318, F_2-328, F_2-330, and F_2-332)), we found that 38 phenotypes presented a morphological difference, while the remaining six phenotypes (leaf color, leaf vein color, inflorescence type, flower cluster, corolla color, and pedicel abscission layer) did not. Twenty-two out of the 38 morphological traits in *S. pimpinellifolium* (LA1585) were dominant in the hybrids: plant type, growth type, leaf attitude, style type, pubescence on fruit surface, fruit apex shape, flesh color, fruit color, fruit shape, fruit surface furrow, plant height, leaf length, leaf width, anther number, anther length, petal number, calyx number, calyx length, fruit shape index, locule number, seed length, and seed thickness. Six phenotypic traits of *S. lycopersicum* (*e9292*) were dominant in the hybrids: leaf shape, number of leaves under the first inflorescence, number of leaves under the second inflorescence, petal length, flower number in single inflorescences, and pedicel length from the abscission layer. The remaining 10 phenotypes were intermediate between *e9292* and LA1585: leaf division, glandular hairs on the leaf/steam, stem diameter, style length, fruit weight, pericarp thickness, fruit longitudinal diameter, fruit transverse diameter, seed width, and 1000-seed weight. Lastly, we found four phenotypes with traits superior to the parent with the highest/best trait values (plant height, number of leaves under the first inflorescence, number of leaves under the second inflorescence node, and pedicel length from the abscission layer) and five phenotypes with lower values than the parent with the lowest values (leaf length, average flower number per inflorescence, anther length, petal length, and seed length) in the present evaluation (Tables S2 and S3). Details of the different categories are given in the table legends. These results showed that a novel tomato germplasm was created via an interspecific approach using cultivated tomato and a wild relative.

On the basis of the polymorphisms of the 37 phenotypic characteristics derived from the 13 genotypes, cluster analysis was performed, and a heatmap was created. The 13 genotype tomatoes were divided into two groups: the first comprised *e9292*, F_2-328, F_2-318, F_2-253, and F_2-266, while the second comprised the remaining nine genotypes (Figure 2). In the F_1 hybrids, 22 of the 37 polymorphic traits were predominantly associated with *S. pimpinellifolium* LA1585, while six traits were similar to those of *S. lycopersicum* *e9292*, and the remaining nine phenotypes were of an intermediate type (Table S3).

3.3. Tomato Quality Traits

We found that the various quality traits of the progeny of the interspecific hybrid (*e9292* × LA1585) were significantly greater than those of either parent. Among all 13 genotypes, the Vc content ranged from 14.21 mg/100 g FW (*e9292*) to 55.28 mg/100 g FW (F2-280), and the average value (33.05 mg/100 g FW) of the F1 (e9292 × LA1585) population was closer to that of LA1585 (43.93 mg/100 g FW) than to that of e9292, while it even appeared superior to the parent, LA1585, with the highest levels in F2-280, F2-299, and F2-332 (Table 1). The TSS content ranged from 4.44° Brix (*e9292*) to 8.12° Brix (LA1585), and the mean value in the F1 generation was 6.86° Brix, which was closer to the LA1585 value, and ranged from 4.52 Brix (F2-259) to 7.22 Brix (F2-299) in F2 lines (Table 1). Fruit firmness (fruit apex, 11.57 N; equatorial plane 11.02 N) in F1 tomatoes was similar to that of the e9292 parent (fruit apex, 11.31 N; equatorial plane 10.71 N), while the F2 generation showed considerable diversity, ranging from 4.63 N (F2-299) to 13.02 N (F2-318) at the fruit apex and from 3.91 N (F2-330) to 8.90 N (F2-253) at the equatorial plane (Table 1).

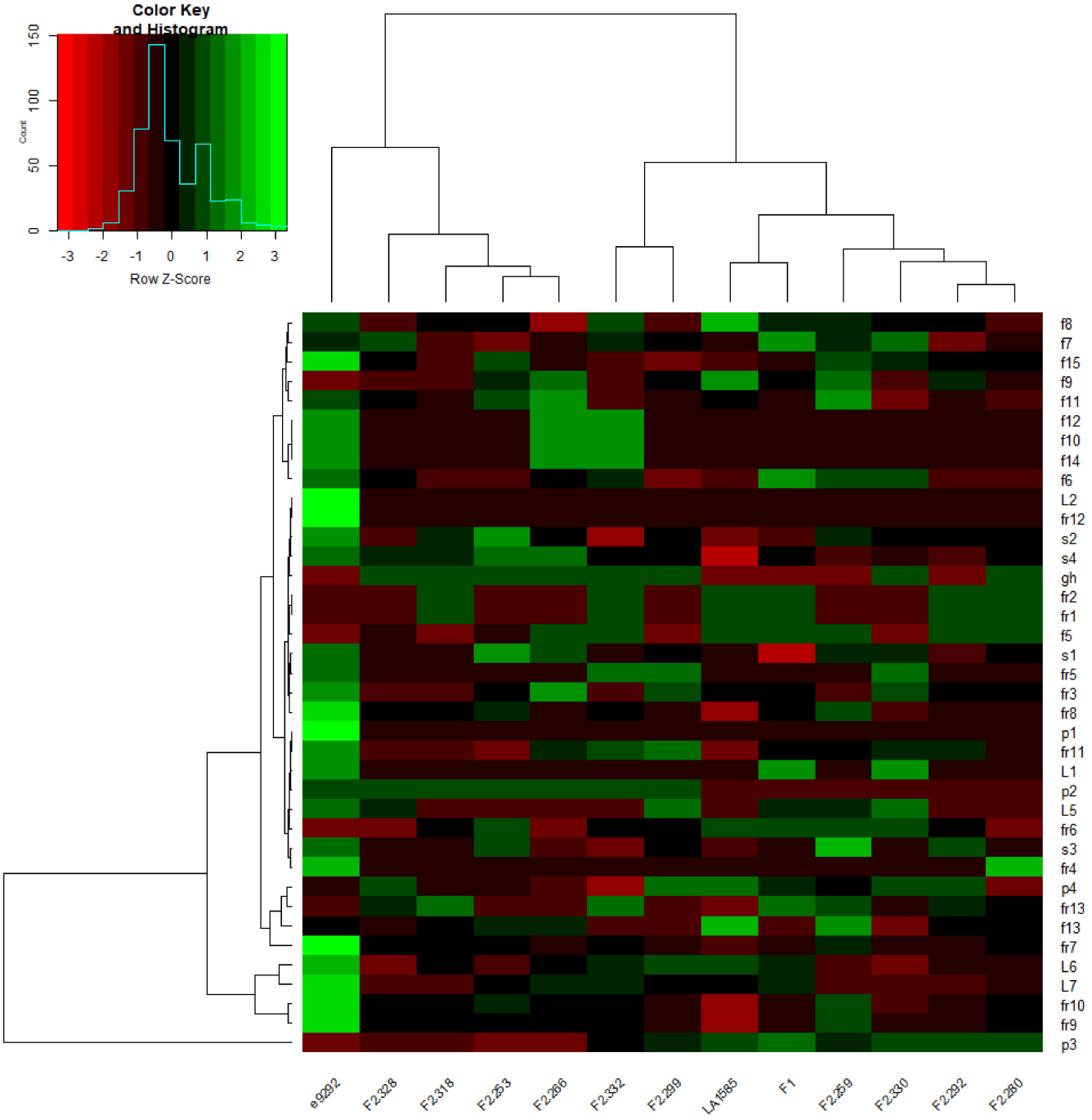

Figure 2. Heatmap visualizing data from a cluster analysis based on morphological markers from 13 tomato accessions. The different tomatoes are indicated at the bottom of the heatmap. The codes on the right side of the heatmap indicate the total of 38 polymorphic phenotype traits where differences were observed between the lines. P: plant: p1, plant type, 1-vine, 2-semi-vine, 3-straight; p2, growth type, 1-indeterminate, 2-determinate; p3, plant height (cm); p4, stem diameter (mm). L, leaves: L1, leaf shape, 1-simple odd-pinnately compound leaf, 2-bipinnate odd-pinnately compound leaf; L2, leaf attitude, 1-semi-erect, 2-horizontal, 3-drooping; L5, leaf division, 0-none, 1-lobate, 3-parted, 4-divided; L6, leaf length (cm); L7, leaf width (cm); gh, glandular hairs on the leaves or steams, 0-none, 1-thin short, 2-thick short, 3-thin long, 4-thick long. F: flower: f5, style type, 1-shorter than the stamen, 2-nearly the same length as the stamen, 3-longer than the stamen; f6, number of leaves under the first

inflorescence; f7, number of leaves under the second inflorescence; f8, flower number per inflorescence; f9, style length (mm); f10, anther number; f11, anther length (mm); f12, petal number; f13, petal length (mm); f14, calyx number; f15, calyx length (mm). Fr: fruit; fr1, fruit color, 1-light yellow, 2-yellow, 3-pinkish red, 4-red; fr2, flesh color of pericarp, 1-light yellow, 2-yellow, 3-pinkish red, 4-red; fr3, fruit apex shape, 1-dented, 2-slightly dented, 3-flat, 4-salient, 5-pointed; fr5, fruit shape, 1-flat, 2-oblate, 3-round, 4-high round, 5-prolate round; fr6, pubescence on fruit surface, 0-none, 1-sparse, 2-intermediate, 3-dense; fr7, fruit weight (g); fr8, pericarp thickness (mm); fr9, fruit longitudinal diameter (mm); fr10, fruit transverse diameter (mm); fr11, fruit shape index; fr12, locule number; fr13, pedicel length from abscission layer (mm); fr14, fruit surface furrow, 0-none, 1-little, 2-intermediate, 3-prominent. S: seed: s1, seed length (mm); s2, seed width (mm); s3, seed thickness (mm); s4, 1000-seed weight (g).

The mean lycopene content of the F_1 fruit (109.51 μg/g FW) was between the values for *e9292* (27.84 μg/g FW) and LA1585 (174.39 μg/g FW), whereas the content in F_2-280 and F_2-332 fruit reached 203.55 μg/g FW and 251.30 μg/g FW, respectively. However, the lycopene contents of F_2-299 and F_2-253 were 34.71 μg/g FW and 34.84 μg/g FW, respectively, which were lower than that of the parent with the lower content, *e9292*. The α-carotene level in *e9292* (12.59 μg/g FW) was significantly higher than that in LA1585 (4.85 μg/g FW), while the F_1 tomatoes (6.76 μg/g FW) exhibited mid-parental levels. Interestingly, the α-carotene content in seven F_2 lines (F_2-253, F_2-259, F_2-266, F_2-299, F_2-328, F_2-328, and F_2-300) was higher than that in *e9292*, while F_2-280 (1.37 μg/g FW) had lower levels than the LA1585 parent. The β-carotene content in the cultivated *e9292* tomato (5.29 μg/g FW) was significantly lower than that in the wild LA1585 tomato (18.55 μg/g FW), while the F_1 hybrid had levels between those of *e9292* and LA1585, albeit nearer to the *e9292* level, and four F_2 lines (F_2-266 (18.79 μg/g FW), F_2-280 (27.00 μg/g FW), F_2-299 (22.54 μg/g FW), and F_2-330 (24.76 μg/g FW)) had higher levels than that of LA1585. Interestingly, an F_2 line, F_2-318, had a lower content (4.21 μg/g FW) than the parent with lower amounts, i.e., *e9292*. The total carotenoid levels in the F_1 hybrid (*e9292* × LA1585, 127.11 μg/g FW) were between the values for *e9292* (51.93 μg/g FW) and LA1585 (200.48 μg/g FW), while the total carotenoid levels in F_2-280 (235.45 μg/g FW) and F_2-332 (267.63 μg/g FW) were higher than that in the parent with the higher level, i.e., LA1585 (Table 1).

Soluble sugars in ripening tomato fruit mainly consist of fructose and glucose, while the sucrose content is relatively low. In *e9292* fruits, the fructose and glucose contents (18.10 mg/g FW and 12.12 mg/g FW, respectively) were significantly higher than those in LA1585 (9.45 mg/g FW and 9.48 mg/g FW, respectively), while F_1 fruit had values between those of the parents, albeit closer to the LA1585 level. The fructose and glucose levels in five F_2 lines (F_2-280, F_2-292, F_2-299, F_2-328, and F_2-332) were higher than those in *e9292* (Table 1).

Citric acid is one of the most important organic acids that contribute to the taste of tomato. Here, we observed that the citric acid levels in the F_1 hybrid were slightly higher than those in the parent with the higher content, LA1585. The citric acid levels in most of the F_2 lines were between those in *e9292* and LA1585, with the exception of F_2-253, F_2-259, and F_2-266, of which F_2-253 and F_2-259 had lower levels than *e9292*, while F_2-266 had higher levels than LA1585 (Table 1).

The broad-sense heritability (hB2, Equation (1)) of 15 traits associated with tomato quality ranged from 75.06% (TSS) to 99.99% (Vc), with an average value of 92.66%. The coefficient of variation (Cv, Equation (4)) varied from 18% (TSS) to 68.82% (lycopene), with an average value of 41.73% (Table 2). These results implied that the quality traits were mainly regulated by genetic factors, while the effects derived from environmental factors were less important. There were differences in Cv values between the different genotypes, as well as between different generations, implying that ample genetic diversity existed between the parents and between different hybrid offspring. Taken together, the results showed the considerable potential for creating novel germplasm and improving quality traits using wild tomato relatives for breeding.

Table 1. Analysis of ripening fruit quality in different genotypes.

Genotype	AA (mg/100 g FW)	TSS (°Brix)	LF (N)	TF (N)	Lycopene (μg/g FW)	α-Carotene (μg/g FW)	β-Carotene (μg/g FW)	Lutein (μg/g FW)	TC (μg/g FW)	Fructose (mg/g FW)	Glucose (mg/g FW)	L-Malic Acid (mg/g FW)	CA (mg/g FW)	Quinic Acid (mg/g FW)	Sugar/Acid
e9292	14.21 ± 0.23	4.44 ± 0.24	11.31 ± 1.15	10.71 ± 1.04	27.84 ± 0.27	12.59 ± 1.47	5.29 ± 0.61	6.21 ± 0.61	51.93 ± 2.96	18.10 ± 0.93	12.12 ± 0.24	0.26 ± 0.01	2.80 ± 0.26	0.05 ± 0.01	9.70
LA1585	43.93 ± 0.28	8.12 ± 1.45	3.28 ± 0.27	3.13 ± 0.58	174.39 ± 1.70	4.85 ± 0.48	18.55 ± 0.84	2.69 ± 0.39	200.48 ± 3.41	9.45 ± 0.74	9.48 ± 0.16	0.42 ± 0.04	4.83 ± 0.44	0.04 ± 0.00	3.57
F_1	33.05 ± 0.26	6.86 ± 0.15	11.57 ± 0.25	11.02 ± 0.24	109.51 ± 2.75	6.76 ± 0.72	8.84 ± 0.45	2.00 ± 0.28	127.11 ± 4.20	8.98 ± 0.374	10.43 ± 0.70	0.31 ± 0.02	5.01 ± 0.27	0.04 ± 0.00	3.62
F_2-253	40.32 ± 0.33	6.04 ± 0.32	12.63 ± 0.83	8.90 ± 0.78	34.84 ± 2.55	17.70 ± 2.40	16.62 ± 2.88	3.07 ± 0.44	72.23 ± 8.27	16.39 ± 1.34	12.17 ± 1.04	0.27 ± 0.07	2.55 ± 0.34	0.04 ± 0.01	9.98
F_2-259	33.11 ± 0.37	4.52 ± 0.08	8.59 ± 0.76	5.54 ± 0.61	160.54 ± 1.18	20.98 ± 3.58	7.03 ± 1.72	0.47 ± 0.02	189.02 ± 6.50	19.73 ± 0.53	9.96 ± 0.44	0.28 ± 0.02	2.58 ± 0.11	0.02 ± 0.00	10.32
F_2-266	43.02 ± 0.30	6.24 ± 0.54	5.05 ± 0.77	4.83 ± 0.56	45.99 ± 2.70	20.32 ± 2.00	18.79 ± 2.85	2.00 ± 0.07	87.1 ± 7.62	21.65 ± 0.67	10.64 ± 0.08	0.32 ± 0.00	4.92 ± 0.12	0.02 ± 0.00	6.14
F_2-280	53.28 ± 0.39	6.64 ± 0.41	4.92 ± 0.54	4.84 ± 0.71	203.55 ± 6.28	1.37 ± 0.08	27.00 ± 5.89	3.53 ± 0.39	235.45 ± 12.64	20.43 ± 1.20	12.51 ± 0.48	0.22 ± 0.02	4.53 ± 0.62	0.02 ± 0.00	6.90
F_2-292	33.45 ± 0.14	6.64 ± 0.37	5.39 ± 0.66	4.14 ± 0.74	170.63 ± 4.93	5.37 ± 0.60	15.21 ± 0.86	2.57 ± 0.22	193.78 ± 6.61	26.48 ± 0.84	16.76 ± 1.45	0.27 ± 0.03	4.13 ± 0.04	0.05 ± 0.01	9.71
F_2-299	55.12 ± 0.50	7.22 ± 0.13	4.63 ± 0.69	4.66 ± 0.85	34.71 ± 2.03	21.82 ± 3.63	22.54 ± 3.27	3.46 ± 0.42	82.53 ± 9.35	21.93 ± 0.66	14.16 ± 0.04	0.24 ± 0.01	3.63 ± 0.19	0.00 ± 0.00	9.32
F_2-318	32.46 ± 0.17	5.42 ± 0.13	13.02 ± 0.82	8.34 ± 0.84	114.01 ± 3.90	16.79 ± 0.42	4.21 ± 0.16	nd	135.01 ± 4.48	17.78 ± 1.06	12.77 ± 0.27	0.34 ± 0.02	3.43 ± 0.31	0.15 ± 0.01	7.78
F_2-328	37.62 ± 0.29	6.50 ± 0.48	7.15 ± 0.77	7.47 ± 0.64	47.49 ± 4.21	20.76 ± 3.81	5.51 ± 1.65	3.01 ± 0.57	76.77 ± 10.24	21.76 ± 1.33	16.60 ± 1.19	0.16 ± 0.01	2.83 ± 0.32	0.06 ± 0.00	12.56
F_2-330	35.40 ± 0.23	7.04 ± 1.05	5.21 ± 0.28	3.91 ± 0.82	36.35 ± 0.85	17.89 ± 2.25	24.76 ± 2.79	6.08 ± 0.10	85.08 ± 5.99	10.14 ± 0.47	10.17 ± 0.42	0.13 ± 0.02	4.73 ± 0.57	0.07 ± 0.01	4.12
F_2-332	44.95 ± 0.22	5.82 ± 0.33	7.64 ± 0.99	6.09 ± 0.97	251.30 ± 6.23	5.45 ± 0.25	10.19 ± 1.85	0.69 ± 0.12	267.63 ± 8.45	26.86 ± 2.06	18.44 ± 0.76	0.26 ± 0.03	3.77 ± 0.39	0.08 ± 0.01	11.01

Note: Values shown in the table are the mean ± sd (standard deviation). Three biological replicates were used for the analysis of quality traits, except for TSS, where five biological replicates were used. Sucrose and succinic acid are not listed in the table due to a very low content that could not be detected. AA/Vc, ascorbic acid/vitamin C; CA, citric acid; FW, fresh weight; LF, longitudinal firmness; N, newton; nd, not detected; sd, standard deviation; TC, total carotenoid; TF, transverse firmness; TSS, total soluble solids.

Table 2. Broad-sense heritability and the coefficient of variation of quality traits in tomatoes with different genetic backgrounds.

Quality Traits	gv	ev	Mean	sd	hb	Cv
TSS	1.01	0.34	6.27	1.13	75.06%	18.10%
Fruit apex firmness	11.31	0.52	7.72	3.34	95.56%	43.19%
Equatorial plane firmness	6.71	0.56	6.43	2.62	92.29%	40.72%
AA	109.20	0.09	38.46	10.18	99.92%	26.46%
α-Carotene	54.39	4.52	13.28	7.49	92.33%	56.37%
β-Carotene	59.81	6.21	14.20	7.93	90.59%	55.85%
Lycopene	5877.49	12.78	108.55	74.71	99.78%	68.82%
Lutein	3.50	0.12	2.75	1.85	96.77%	67.39%
Succinic acid	ld	ld	ld	ld	89.12%	36.81%
Malic acid	0.01	ld	0.27	0.08	88.59%	28.68%
Citric acid	0.83	0.12	3.83	0.95	87.31%	24.91%
Quinic acid	ld	nd	0.05	0.04	97.10%	73.49%
Fructose	34.55	1.08	18.44	5.81	96.98%	31.54%
Glucose	8.26	0.49	12.78	2.88	94.36%	22.56%
Sucrose	ld	ld	ld	ld	ld	ld

Note: AA, ascorbic acid; Cv, coefficient of variation; ev, environmental variance; gv, genetic variance; ld, very low detection level; hb, broad-sense heritability; nd, not detected; sd, standard deviation; TSS, total soluble solids. Sucrose and succinic acid are not listed in the table due to a very low content that could not be detected.

3.4. Expression of Genes Associated with Carotenoid Synthesis

To reveal how carotenoid synthesis was regulated in the interspecific cross (*e9292* × LA1585), seven genotypes (*e9292*, LA1585, F_1, and four F_2 lines (yellow-fruited F_2-266 and F_2-299, and red-fruited F_2-318 and F_2-332)) were chosen to evaluate the transcription of the *DXS, DXR, HDR*, and *IDI1* genes involved in the methylerythritol 4-phosphate pathway (MEP) and the *PSY1*, *LCYE*, *CRTISO*, and *CYCB* genes involved in the carotenoid synthesis pathway (Figure S1).

DXR, *HDR*, and *IDI1* expressions in the MEP pathway were similar in *e9292* and LA1585 during the ripening stage (yellow/red ripening), while *DXS* expression was significantly lower in *e9292* than in LA1585. However, *DXS* and *DXR* expressions were substantially higher in F_1 fruits than in either of the parents (Figure 3). These results implied that the interspecific hybrid promoted MEP synthesis through the condensation and intramolecular rearrangement catalyzed by DXS and DXR [44]. We also found that *HDR* and *IDI1* expressions in F_2-332 were significantly higher than those in the remaining six genotypes (*e9292*, LA1585, F_1 hybrid, and three F_2 families, F_2-266, F_2-299, and F_2-318) (Figure 3). An increase in the expressions of *HDR* and *IDI1* in line F_2-332 would likely accelerate the catalytic process carried out by isopentenyl diphosphate (IPP)/dimethylallyl diphosphate (DMAPP) in the MEP pathway, a step that is located upstream from the carotenoid biosynthetic pathway (CBP), which provides it with precursors. The results showed that *HDR* and *IDI1* regulate CBP and affect the synthesis of secondary metabolites, such as lycopene, the content of which reached 251.30 μg/g FW in F_2-332, approximately 1.5-fold greater than that in LA1585 (174.39 μg/g FW) (Table 1). In the CBP itself, *PSY1*, *LCYE*, and *CYCB* expressions were significantly higher in F_1 fruits than in both parents. The lycopene content in F_1 fruits (109.51 μg/g FW) was between the *e9292* and LA1585 levels but closer to LA1585. Furthermore, F_2-332 *CRTISO* and *CYCB* expressions were significantly higher than those in the other six genotypes. The encoding enzymes promote the conversion of all-*trans*-lycopene from 7,7′,9,9′-tetra-*cis*-lycopene and *trans*-carotenoid cyclization, thereby increasing the lycopene content in F_2-332 fruit (Figure 3 and Table 1). These results indicated that the expression of genes resulting in improved lycopene synthesis was additive in F_2-332.

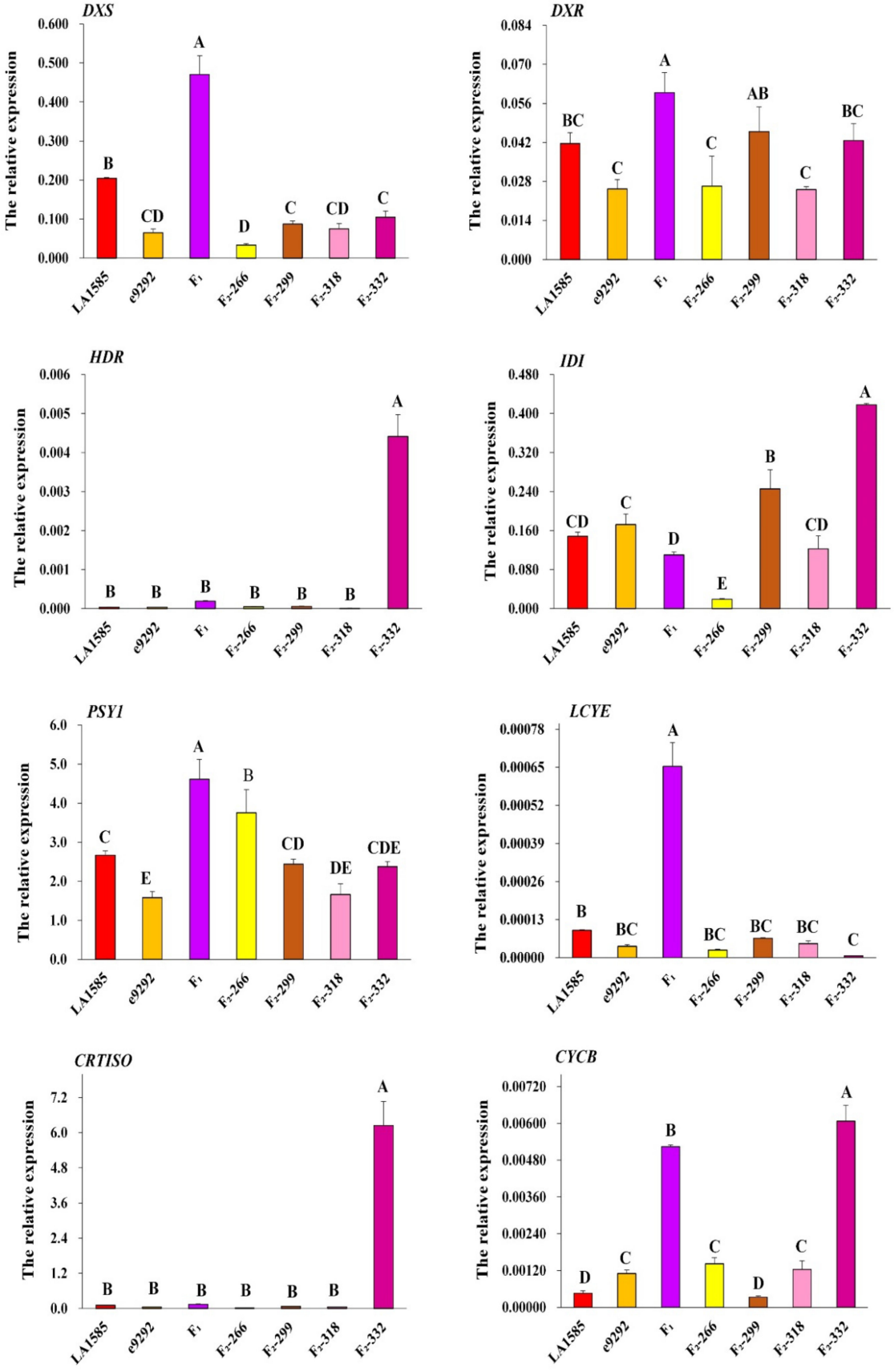

Figure 3. Expression levels of genes associated with the carotenoid synthesis and pathway. The capital letters indicate statistical significance at the $p < 0.01$ level based on Duncan's test.

3.5. Response to Drought and Salt Stresses

Germinated seeds of *e9292*, LA1585, F_1 hybrids, F_2-266, F_2-299, and F_2-332 were sown on MS_0 medium with mannitol (final concentration: 0 mM, 50 mM, 100 mM, and 150 mM) or NaCl (final concentration: 0 mM, 50 mM, 100 mM, and 150 mM), and then the root and hypocotyl lengths were measured every 24 h between the 24 h and 144 h timepoints (Tables S4 and S5).

Low mannitol concentrations (50 mM) promoted root elongation at early timepoints. Root elongation in F_2-226, in particular, was accelerated under supplied mannitol concentrations of 50 mM, 100 mM, and 150 mM compared with the control (0 mM supplement). However, the root elongation of F_1 and all F_2 lines was suppressed except for F_2-226, and this suppression was enhanced with the addition of mannitol, especially in F_1 and F_2-332 (Figure 4, Table S4). Hypocotyl elongation was inhibited with mannitol supplementation, and this effect increased with both concentration and over time. However, the hypocotyl elongation of F_2-299 was accelerated with supplementation of low mannitol concentrations, as well as 100 mM and 150 mM at the early timepoints (Figure 4 and Table S5).

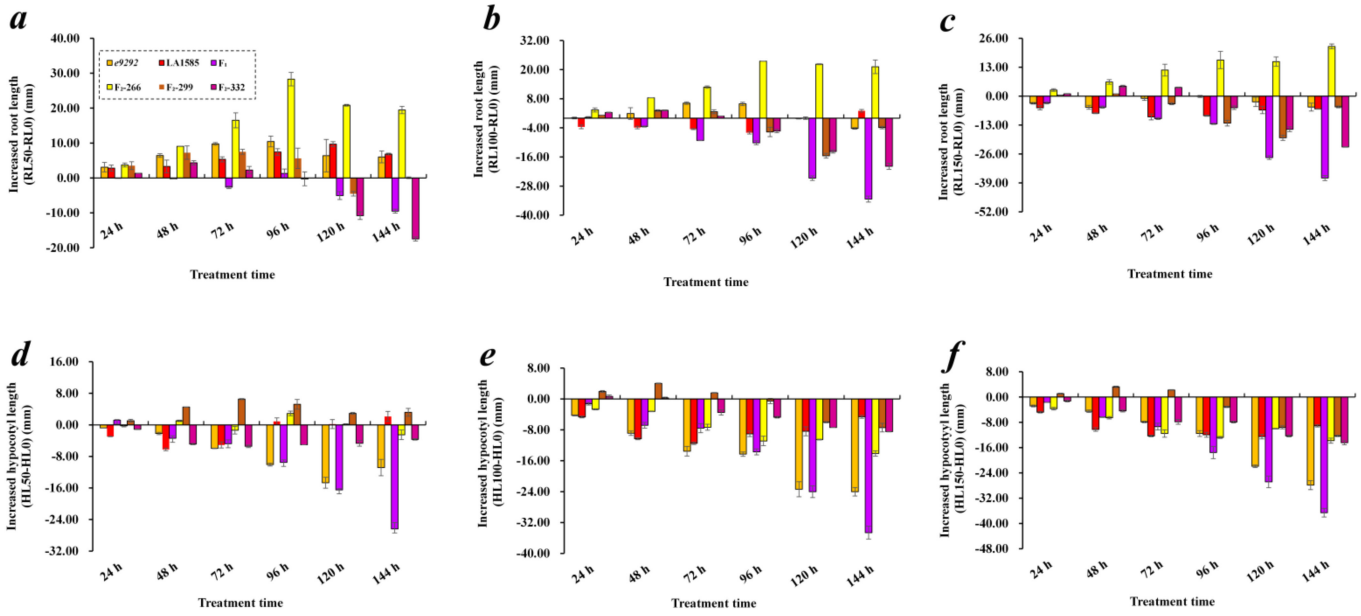

Figure 4. Mannitol stress affects the root and hypocotyl growth of tomato seedlings. (**a**–**c**) Differences in the root length of seedlings with different genetic backgrounds between mannitol treatments (50 mmol/L to 150 mmol/L) and the control (0 mmol/L). (**d**–**f**) Differences in the hypocotyl length of seedlings with different genetic backgrounds between mannitol treatments (50 mmol/L to 150 mmol/L) and the control (0 mmol/L).

In the salt stress assay, root elongation was promoted by supplementation of 50 mM and 100 mM NaCl compared with the control of 0 mM NaCl, with the exception of the F_2-332 line, where it was suppressed at these concentrations at later timepoints. Root elongation was suppressed in all six genotypes when supplemented with 150 mM NaCl (Figure 5 and Table S4).

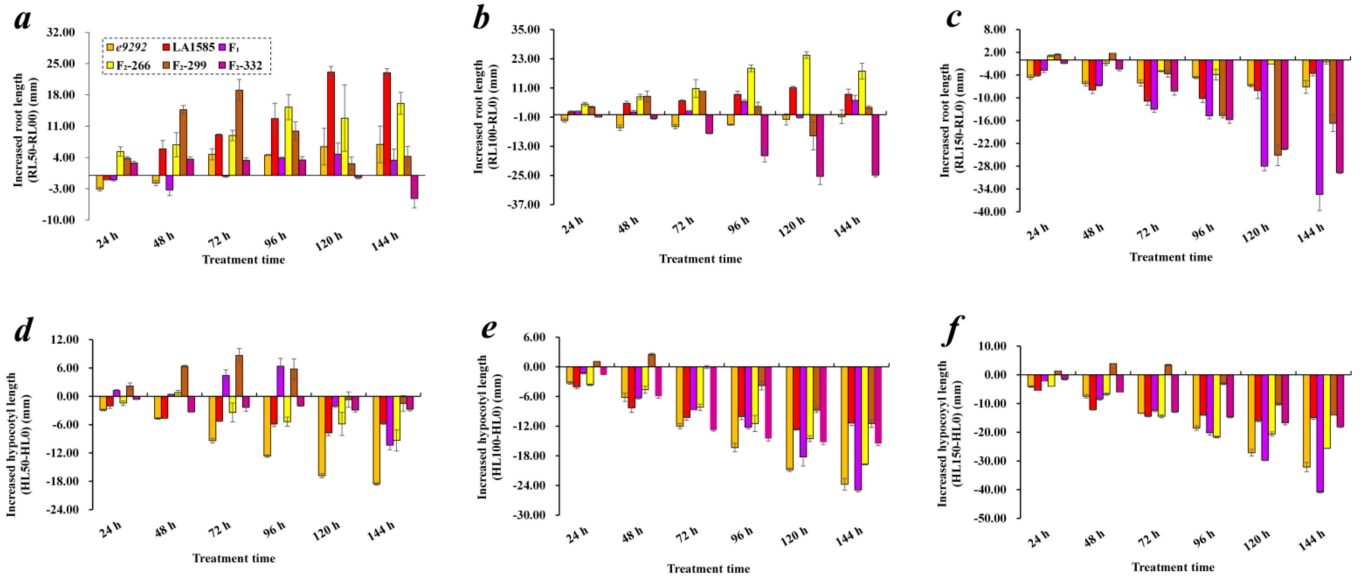

Figure 5. NaCl stress affects the root and hypocotyl growth of tomato seedlings. (**a**–**c**) Differences in the root length of seedlings with different genetic backgrounds between NaCl treatments (50 mmol/L to 150 mmol/L) and the control (0 mmol/L). (**d**–**f**) Differences in the hypocotyl length of seedlings with different genetic backgrounds between NaCl treatments (50 mmol/L to 150 mmol/L) and the control (0 mmol/L).

Salt stress inhibited hypocotyl growth in tomato seedlings, and this effect was exacerbated with NaCl concentration and over time. Hypocotyl growth was promoted with a low NaCl concentration of 50 mM in F_1 and F_2-299, while the hypocotyl growth of the *e9292* and F_1 plants was suppressed under high salt stress (100 mM and 150 mM NaCl). This effect was not observed for F_2-299, especially at the early timepoints of NaCl treatment (Figure 5 and Table S5).

4. Discussion

The genetic improvement of yield, stress tolerance, and quantity is a critical consideration for consumers and tomato breeders [45]. However, the genetic diversity of the cultivated tomato has been narrowed due to loss during domestication and the spread from South America to Europe [25], as well as during the selection imposed by modern breeding. As a direct ancestor of cultivated tomato, cherry tomato (*S. lycopersicum* var. *cerasiforme*), was domesticated from *S. pimpinellifolium*, in the Andes region, before migrating into Mesoamerica, where the cherry tomato was domesticated into the cultivated tomato, and then transported back to Europe by Spanish colonists [19,46]. The genetic diversity of cultivated tomato markedly declined during the migration to Mesoamerica from the Peruvian and Ecuadorian Amazon region [25,30,46]. Moreover, this loss became more severe with selection stress during modern tomato breeding. However, the ancestral wild relative of cultivated tomato, *S. pimpinellifolium*, was naturally distributed in contrasting environments, such as the northern coastal Ecuadorian tropical rainforests and the Peruvian coastal desert, and it evolved into several ecotypes with genetic differentiation in response to the climate [25]. Accordingly, *S. pimpinellifolium* is regarded as a largely unexploited genetic reservoir, which has the potential to improve the cultivated tomato [20–22,47]. The reproductive barriers between tomato and its wild species severely limit the use of those wild relatives, including generated descendants, with severe separating and linkage drag. However, as the wild progenitor of cultivated tomato [2], *S. pimpinellifolium* (accession number, LA1585) is fully cross-compatible with cultivated tomato (*e9292*); hence, we selected the red-fruited LA1585 as a pollen parent to cross with yellow-fruited *e9292*. This allowed

us to track genetic improvement effects using *S. pimpinellifolium* and dissect the molecular mechanisms for quality improvement such as carotenoid synthesis in hybrid progenies.

As a progenitor species of cultivated tomato, *S. pimpinellifolium* is a potential genetic resource to improve tomato fruit quality related to the content of total solids [34], volatile compounds [31], fruit color [48], and resistance against bio-stress such as tomato leaf curl virus [49], *Alternaria solani*, *Fusarium oxysporum*, *Phytophthora infestans*, or *Cladosporium fulvum* [30].

In the present study, as representatives, 10 F_2 lines (five each for yellow/red fruited lines) were randomly selected to examine their phenotypes, and some were also used to assess possible improvements (four for quality and three for abiotic stresses) for tomato. The Vc content in three F_2 lines (*e9292* × LA1585) was higher than that in the LA1585 parent, which had a higher content than *e9292*. The TSS content in two F_2 lines was similar to that in LA1585 and 1.6-fold higher than that in *e9292*. In particular, lycopene contents in two F_2 lines (F_2-280 and F_2-332) were dramatically higher than in the LA1585 parent, and even ninefold higher than in *e9292* (Table 1). To dissect lycopene accumulation in the inter-specific hybrid, the expression of key genes associated with lycopene synthesis was examined, which showed that the distant interspecific hybridization between *e9292* and LA1585 resulted in elevated expressions of *DXS*, *DXR*, *PSY1*, *LCYE*, and *CYCB* in the F_1 generation, while the *HDR*, *IDI1*, *CRTISO*, and *CYCB* expression levels were higher in the F_2-332 line (Figure 3). These results implied that the interspecific hybridization event increased the expressions of genes associated with carotenoid synthesis and its upstream 2-C-methyl-D-*erythritol* 4-phosphate (MEP) pathway, as well as promoted lycopene accumulation in hybrid tomatoes, especially some lines in the F_2 generation.

Glucose and fructose are the principal components of the total soluble sugars associated with tomato flavor [31], and their contents in five of the F_2 lines were higher than those in the parent with a higher content, i.e., *e9292*, in present study (Table 1). For citric acid, F_1 and one F_2 lines had a higher content than LA1585, which had the higher content of the two parents. Among all 13 genotypes, the sugar–acid ratio ranged from 3.573 in LA1585 to 12.556 in the F_2-328 line. *e9292* had the higher levels of the two parents, while five F_2 lines had a higher ratio (Table 1). Both monosaccharides and organic acids are known to be associated with tomato flavor; hence, on the basis of the fruit quality data, we ranked the 13 genotypes (*e9292*, LA1585, F_1 hybrid, and 10 F_2 families) by flavor, from best (1) to worst (13). *e9292* and LA1585 were ranked 13 and 4, respectively, while the F_1 hybrid was placed at rank 11, and the F_2-332 line had the fruit with the best quality (Table 3). In the present study, a novel tomato genotype F_2-332 was successfully created, which had better-quality fruit than the parental cultivated tomato *e9292*, as well as some excellent quality traits of LA1585 related to the Vc and carotenoid contents. These results implied that *S. pimpinellifolium* has the potential to create novel valuable germplasm and improve fruit quality for cultivated tomato breeding.

In addition, *S. pimpinellifolium* also exhibited potential to tolerate abiotic stress. We found that both salt and mannitol stress substantially suppressed the elongation of roots and hypocotyls in both parent seedlings, while more tolerant lines were developed in the F_2 generation.

We successfully introgressed elite genes associated with fruit quality and resistance to abiotic stress into the cultivated tomato *e9292* from *S. pimpinellifolium*, and we created a series of valuable novel tomato resources through an interspecific cross approach. However, the remarkable linkage drag, e.g., the small fruit trait, is difficult to eliminate from the progenies of the interspecific hybrid. The average single fruit weight was 6.53 g in the F_1 hybrid and 8.51 g in the F_2 lines. The single-fruit weight was similar to that of the cherry tomato (*S. lycopersicum* var. *cerasiforme*), which is extensively planted around the world. Nevertheless, the small-fruited trait can still be improved by genetic manipulation/editing or backcrossing with large tomatoes. Previous studies revealed that the fruit weight trait of tomato is associated with genes such as *CELL SIZE REGULATOR* (*CSR*) [50], *EXCESSIVE NUMBER OF FLORAL ORGANS*(*ENO*) [51], and *fw2.2* [52,53], and these alleles exhibit

DNA fragment deletion in both *CRS* (1.4 kb) and *ENO* (85 bp in promoter region), while the recessive *fw2.2* allele in cultivated tomato is associated with a larger fruit compared with *S. pimpinellifolium* with a small fruit [33,52]. Therefore, the linkage drag of small fruit can be removed from interspecific progenies between *e9292* and LA1585 through genetic manipulation. Here, we must point out that only 10 F_2 lines were assessed in the present study, implying that a more desirable germplasm can potentially be obtained from a larger F_2 population upon assessing a broader range of characteristics.

Table 3. Quality ranking of tomatoes with different genetic backgrounds.

Genotypes	Vitamin C	Total Soluble Solids	Carotenoids	Sugar/Acid Ratio	Total Score	Ranking
e9292	13	13	13	6	45	13
LA1585	4	1	3	13	21	4
F_1	11	4	7	12	34	11
F_2-253	6	9	12	4	31	10
F_2-259	10	12	5	3	30	7
F_2-266	5	8	8	10	31	9
F_2-280	2	5	2	9	18	2
F_2-292	9	6	4	5	24	5
F_2-299	1	2	10	7	20	3
F_2-318	12	11	6	8	37	12
F_2-328	7	7	11	1	26	6
F_2-330	8	3	9	11	31	8
F_2-332	3	10	1	2	16	1

Note: The ranking was assigned on the basis of each quality content, whereby 1 is the highest and 13 is the lowest; the total score was aggregated using values derived from each quality trait, and the final ranking was estimated according to the total score.

Taken together, the results in the present study provide a valid approach to create novel germplasm for cultivated tomato improvement using wild tomato relatives and a practical evaluation method. Furthermore, valuable novel germplasm resources were created for the improvement of fruit quality and resistance to abiotic stresses in cultivated tomatoes using *S. pimpinellifolium*. In particular, this study provided insight into the creation of novel resources for tomato genetic improvement and breeding with wild relatives.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae9010034/s1, Figure S1. Pathway of lycopene biosynthesis and degradation in tomato; Table S1. Primers used in the present study; Table S2. Phenotypic traits of tomato with different genetic backgrounds; Table S3. Inheritance trend of each phenotypic trait in the F_1 (*e9292* × LA1585) generation; Table S4. Effect of root growth resulting from NaCl and mannitol in six tomato genotypes; Table S5. Effect of hypocotyl elongation resulting from NaCl and mannitol in six tomato genotypes.

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Data Availability Statement: CAPS (cleaved amplified polymorphic sequence) marker was designed according to the TOMATO-EXPEN 2000 (https://solgenomics.net/ (accessed on 1 September 2022)) tomato genomic resource database in present study.

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