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RESOURCE

A *Solanum lycopersicoides* reference genome facilitates insights into tomato specialized metabolism and immunity

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SUMMARY

Wild relatives of tomato are a valuable source of natural variation in tomato breeding, as many can be hybridized to the cultivated species (*Solanum lycopersicum*). Several, including *Solanum lycopersicoides*, have been crossed to *S. lycopersicum* for the development of ordered introgression lines (ILs), facilitating breeding for desirable traits. Despite the utility of these wild relatives and their associated ILs, few finished genome sequences have been produced to aid genetic and genomic studies. Here we report a chromosome-scale genome assembly for *S. lycopersicoides* LA2951, which contains 37 938 predicted protein-coding genes. With the aid of this genome assembly, we have precisely delimited the boundaries of the *S. lycopersicoides* introgressions in a set of *S. lycopersicum* cv. VF36 × LA2951 ILs. We demonstrate the usefulness of the LA2951 genome by identifying several quantitative trait loci for phenolics and carotenoids, including underlying candidate genes, and by investigating the genome organization and immunity-associated function of the clustered *Pto* gene family. In addition, syntenic analysis of R2R3MYB genes sheds light on the identity of the *Aubergine* locus underlying anthocyanin production. The genome sequence and IL map provide valuable resources for studying fruit nutrient/quality traits, pathogen resistance, and environmental stress tolerance. We present a new genome resource for the wild species *S. lycopersicoides*, which we use to shed light on the *Aubergine* locus responsible for anthocyanin production. We also provide IL boundary mappings, which facilitated identifying novel carotenoid quantitative trait loci of which one was likely driven by an uncharacterized lycopene β-cyclase whose function we demonstrate.

Keywords: *Solanum lycopersicoides*, genome, carotenoids, anthocyanin, disease resistance, drought.

INTRODUCTION

Tomato (*Solanum lycopersicum*) is one of the most widely consumed fruit crops with great worldwide value (fao.org). It is rich in essential nutrients, particularly provitamin A, folate, vitamin C, vitamin E and vitamin K, and calcium (Ali et al., 2020). Tomato yield, however, is often reduced significantly by losses caused by adverse environmental conditions, disease, pest damage, and post-harvest processes (Panno et al., 2021). The narrow germplasm base currently deployed in most breeding programs limits the potential for tomato improvement (Bauchet & Causse, 2012). Fortunately, close wild relatives present opportunities to add enormous genetic diversity to tomato breeding programs and the means to identify and study genes that underpin useful novel variation. At least 14 wild relatives can be crossed to the cultivated tomato, and have been used for decades in breeding programs (Grandillo et al., 2011). Breeding with *Solanum lycopersicoides* was until recently prevented by strong crossing barriers and hybrid sterility. While introgression lines (ILs) are now available that can assist with identification of alleles and mapping of loci conferring beneficial traits from *S. lycopersicoides* (Canady et al., 2005), few loci have been cloned due, in part, to a lack of a reference genome for this species. Despite the importance of wild accessions in tomato breeding, only two such species, *Solanum pennellii* (Bolger et al., 2014a; Schmidt et al., 2017) and *Solanum pimpinellifolium* (Wang et al., 2020), have reference-quality genome assemblies. Three species, *Solanum galapagense* (Strickler et al., 2015), *Solanum arcanum* (The 100 Tomato Genome Sequencing Consortium et al., 2014), and *Solanum habrochaites* (The 100 Tomato Genome Sequencing Consortium et al., 2014) have draft *de novo* assemblies with small contig size and no gene annotation, whereas *Solanum sitiens* (Molitor et al., 2021) and *S. lycopersicum* var. *cerasiforme* (Takei et al., 2021) have been scaffolded using similarity to reference genomes of related species. Of the biparental tomato IL populations developed to date, the *S. lycopersicum* cv. VF36 × *S. lycopersicoides* LA2951 ILs (Canady et al., 2005) represents one of the widest crosses possible, because *S. lycopersicoides* belongs to a group that is sister to the tomato clade. Because of the lack of a *S. lycopersicoides* genome, introgression boundaries of these lines are typically defined based on the published tomato reference genome (Tomato Genome Consortium, 2012). Genetic mapping resolution depends on these boundaries being well-defined. Traditionally, boundaries have been defined by DNA markers dispersed across the genome, but more recent approaches increase precision by using single nucleotide polymorphisms (SNPs) derived from resequencing or RNA-sequencing (RNA-Seq) data (Gonda et al., 2019); (Chitwood et al., 2013). However, basing introgression coordinates on the "Heinz 1706" reference genome and annotation in wide crosses may lead to important genomic features in the non-reference genome being overlooked. Indeed, while many differences between genomes are accounted for by repetitive elements, gene gain and loss are also important to consider, as considerable variation in genes involved in tomato improvement, including fruit quality and stress tolerance, was observed in the tomato pan genome (Gao et al., 2019) as well as in efforts to mine structural variation in tomato and wild relatives (Alonge et al., 2020; Wang et al., 2020).

Wild relatives and ILs have been used to investigate flavor and nutritional quantitative trait loci (QTL) (Klee & Tieman, 2018; Lee et al., 2012; Tohge et al., 2020). Remarkably, under favorable conditions, *S. lycopersicoides* produces anthocyanins, which are potentially health beneficial (Martin et al., 2011), and result in a purple or black fruit, a trait not seen in cultivated tomatoes or their close relatives, but which is also expressed to a lesser degree in *Solanum cheesmaniae, Solanum chilense*, and *Solanum peruvianum* (Bedinger et al., 2011; Rick et al., 1994). The *Aubergine* (*Abg*) locus from chromosome 10 of *S. lycopersicoides* has been introgressed into cultivated *S. lycopersicum* resulting in anthocyanin production, but the identity of the underlying gene remains unknown.

Solanum lycopersicoides has also been investigated for its response to biotic and abiotic stressors. Some *S. lycopersicoides* accessions are susceptible to three nematode species (*Meloidogyne incognita*, *Meloidogyne javanica*, and *Meloidogyne hapla*) and to the oomycete responsible for the Irish potato famine, *Phytophthora infestans* (Phills et al., 1977b). Interestingly, no symptoms were observed in *S. lycopersicoides* upon inoculation with several viruses, including cucumber mosaic virus and tobacco mosaic virus, even though the plants were systemically infected and carried a high titer of the virus (Phills et al., 1977a; Zhao et al., 2005). Previous studies have demonstrated resistance of *S. lycopersicoides* to the necrotrophic fungal pathogen, *Botrytis cinerea*, and identified resistance to another necrotrophic pathogen *Alternaria solani* (Guimarães et al., 2004; Davis et al., 2009; Smith et al., 2014; Phills et al., 1977b; Egashira et al., 2000). Investigation of the genetic basis of resistance to *Botrytis* revealed five QTLs, with four decreasing the frequency of the infection and one reducing lesion diameter (Davis et al., 2009). Other studies identified resistance in *S. lycopersicoides* to the biotrophic fungal pathogens *Cladosporium fulvum* and *Fusarium oxysporum* sp. *lycopersici* although genetic analysis of the resistance has not been reported (Phills et al., 1977b; Zhao et al., 2005). Recently the *Ptr1* locus was identified in *S. lycopersicoides*, which mediates recognition of the bacterial type III effector AvrRpt2 (Mazo-Molina et al., 2019). This effector is conserved in race 1 strains of the bacterium *Pseudomonas syringae* pv. *tomato* for which no other genetic resistance has been available. The *Ptr1* gene encodes a coiled-coil

Table 1 Summary statistics for the *Solanum lycopersicoides* assembly, as well as numbers for "Heinz 1706" SL4.0 and *Solanum pennellii* "LA0716"

	S. lycopersicoides	*S. pennellii* v2	*Solanum lycopersicum* Heinz v 4.0
No. of pseudomolecules	12	12	12
Longest sequence (Mbp)	133.5	109.3	90.9
Contig N50 (bp)	253 764	60 347	6 007 830
Total length (Mbp)	1152	926	782.5
Expected genome size (Mbp)	1200	942	781
Total size (bp) of unanchored contigs (% of assembly)	135 089 793 (10.5%)	63 101 713 (6.4%)	9 643 250 (1.2%)

nucleotide binding leucine rich repeat (CC-NLR) protein (Mazo-Molina et al., 2020). In addition, *S. lycopersicoides* has been shown to exhibit enhanced cold tolerance (Zhao et al., 2005) and adaptation to arid environments (Peralta et al., 2008), and the IL population has been used to identify QTLs affecting salt tolerance in seedlings (Li et al., 2011).

To improve understanding of the genes responsible for these agriculturally important traits and increase the utility of the ILs, we generated a chromosome-scale, reference genome for *S. lycopersicoides*. We compared the genome with those of *S. pennellii* and *S. lycopersicum* to identify unique features that may be key to the understanding of the genetics of abiotic and biotic stress tolerance in *S. lycopersicoides*. Using RNA-Seq, we mapped the introgressions of the ILs to both parental genomes to refine the genetic map of the population. We use the genome and IL maps to explore the evolution of tomato fruit color and immunity. The genome and associated map will facilitate the understanding of genes responsible for agriculturally important traits, enabling their targeted introduction into tomato breeding lines.

RESULTS

Genome assembly and feature prediction

Solanum lycopersicoides LA2951 was selected for sequencing due to its genetic distance from the cultivated tomato and the existence of ILs previously generated using this accession as the donor parent (Canady et al., 2005). We assembled approximately 17 million PacBio reads with an average length of 6.29 kb totaling 107 Gb using the two different long-read genome assembly programs, Canu (Koren et al., 2017) and Falcon (Chin et al., 2016). Canu produced an assembly with 17 507 contigs and an N50 length of 139 475 bp, which was more contiguous and the assembly captured a larger proportion of the BUSCO set with only 48 missing genes versus 246 for Falcon (Waterhouse et al., 2018). Thus, the Canu assembly was selected for further scaffolding using Dovetail Chicago and Hi-C. The final assembly is 1.2 Gb in length (Table 1) and captures 97% of the BUSCO set (Table S1). The genome assembly is larger than the *S. lycopersicum* assembly is, in line with previous observations (Ji et al., 2004; Menzel, 1962; Rick et al., 1986). The final assembly had an N50 scaffold size of 93.9 Mb and 90% of the assembly was assigned to the 12 chromosomes. Approximately 1% of sites were found to be heterozygous.

Figure 1 shows the 12 pseudochromosomes, along with repeat density and gene feature density. About 68% of the sequence contained in the pseudochromosomes consists of repeats (Table S2). Genome annotation predicted a total of 37 939 putative genes with a mean length of 4388 bp. Genes had an average of 5.2 exons and a coding sequence length of 1232 bp. We were able to identify 96% of the BUSCO set in the predicted proteins (Table S1).

Of all repeat classes, the long terminal repeat retrotransposons (LTR-RTs) constituted the greatest percentage (approximately 57%) of the *S. lycopersicoides* genome, as was the case with *S. lycopersicum* and *S. pennellii* genomes (Table S2). Among the LTR-RT elements, *Gypsy*-type elements were proportionately more abundant than *Copia*-type elements in all three genomes (Table S2) (Bolger et al., 2014a). *Solanum lycopersicoides* also had a greater proportional abundance of younger LTR-RTs (with insertion times <1 million years ago) compared with the other two genome assemblies (Figure S1), consistent with evidence of the expansion of the repetitive fraction based on GISH cytology (Ji et al., 2004).

Mapping of *Solanum lycopersicoides* introgressions in IL accessions

RNA-Seq data from 71 unique *S. lycopersicum* cv. VF36 × *S. lycopersicoides* LA2951 IL accessions were used to assemble genotype maps of the population. The map based on the current domesticated tomato reference genome (SL4.0) provides the coordinates delimiting the introgression boundaries within the reference parent background (Tables S3, S4, Figure S2). An additional map based on the present *S. lycopersicoides* reference provides the coordinates, which define the introgressed regions within the wild donor parent (Figure 2, Table S5). In total, 87% of the *S. lycopersicoides* LA2951 genome is represented across these 71 IL accessions. Our analysis revealed numerous unidentified introgressed segments and

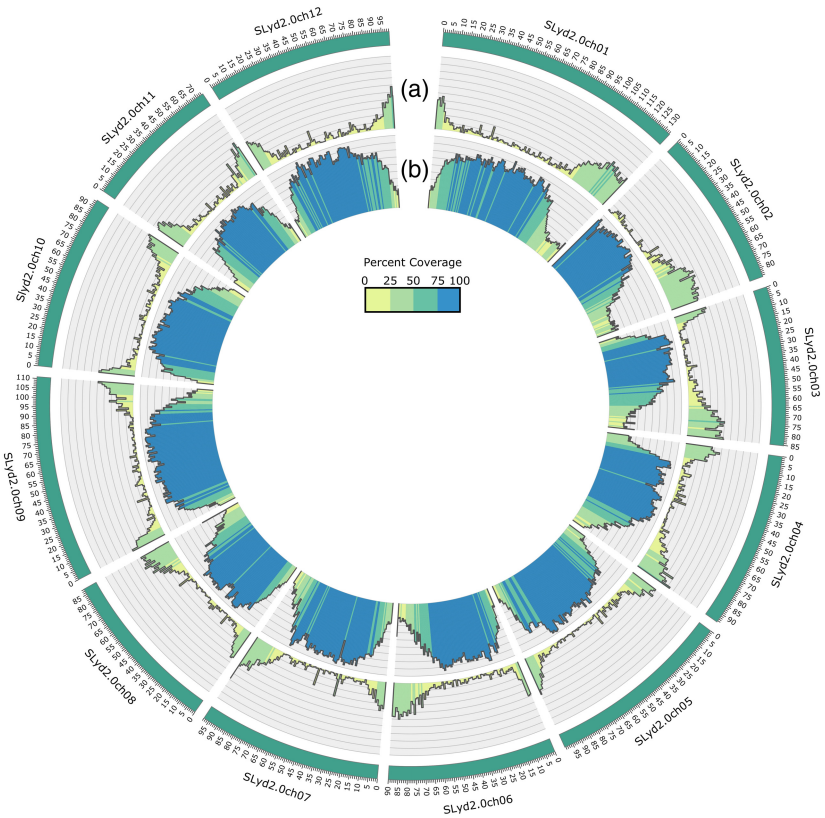

Figure 1. Circos diagram of the 12 *Solanum lycopersicoides* chromosomes.
(a) Gene densities.
(b) Repeat densities.
Densities are calculated for 1-Mb non-overlapping windows and range from 0 to 100%. Different colors are used for values falling within 0–25%, 25–50%, 50–75%, and 75–100% ranges.

chromosomal features when the Heinz 1706 genome was used as the reference. For example, the IL line LA4245 is believed to harbor a single introgression on chromosome 4 (Canady et al., 2005). Our results show that LA4245 harbors an additional approximately 400 kb introgressed region at the distal end of the short arm of chromosome 4. However, although this introgression appears at the end of the chromosome within LA4245, it is derived from a region closer to the centromere of chromosome 4 in *S. lycopersicoides*. This discovery prompted us to explore structural variation between the parental genomes. Alignment and visualization of paired chromosomal segments of at least 8 kb and 92% sequence identity showed high degrees of synteny between *S. lycopersicoides* and both *S. lycopersicum* and *S. pennellii*. Syntenic dot plots performed at a finer scale identified inversions between LA2951 and the domesticated tomato genome (Figure S3). One inversion, found on chromosome 10 at approximately 87–94 Mb based on domesticated tomato genome coordinates, is supported by a previously characterized inversion on chromosome 10 in *S. lycopersicoides* relative to *S. lycopersicum* and *S. pennellii* (Pertuzé et al., 2002). This inversion was not detected cytologically using BAC probes (Szinay et al., 2012). These findings demonstrate the risks of low-resolution genotype mapping in missing identification of introgressed segments and highlight the potential for error in downstream analyses. In addition, it shows that reference-quality genomes of the introgression donors help shed light on introgression fragments as they aid in deciding on the provenance of regions. Without adequate genotyping, QTL mapping may be misleading. The present genome allowed for high-resolution genotype mapping of associated ILs, providing a resource for the community to map QTLs more efficiently and to characterize the variation available from *S. lycopersicoides*. In addition, we used both the *S. lycopersicum* and *S. lycopersicoides* reference genomes and the introgression coordinates from the IL map to develop in-silico hybrid reference genomes specific to each IL where the introgressed part is derived from the *S. lycopersicoides* sequence and the rest from *S. lycopersicum* sequence as an optimal RNA-Seq mapping reference. This minimizes expression anomalies resulting from mapping to a reference other than that from which a transcript was derived (Figure S4).

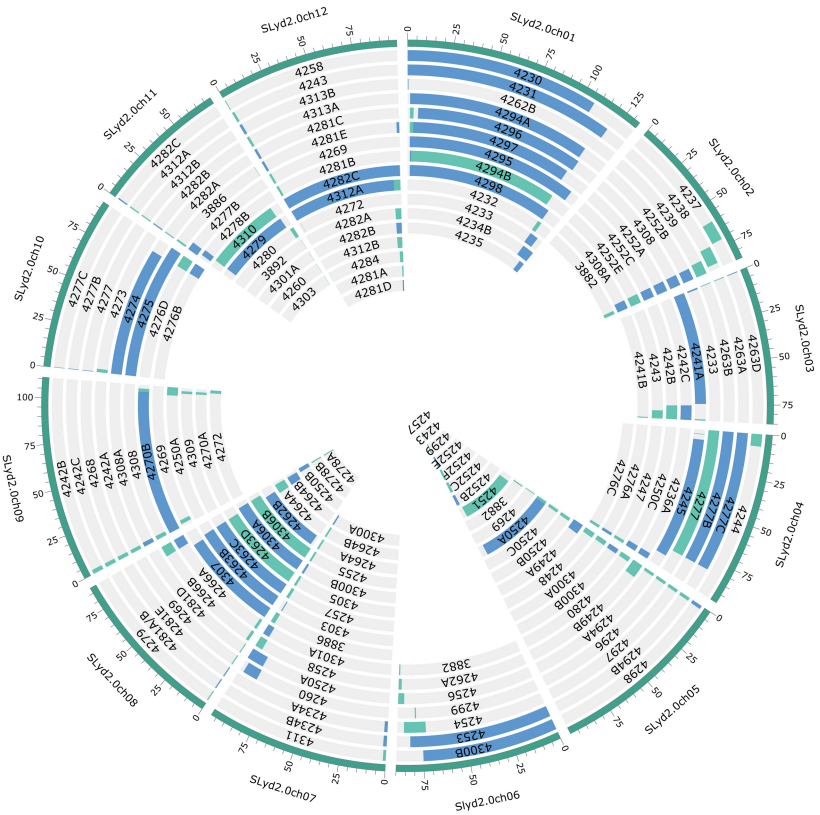

Figure 2. Introgressed segments in the *Solanum lycopersicoides* introgression lines on the genome of *S. lycopersicoides*.
Green indicates homozygous introgressed region and blue indicates heterozygous region. Detailed maps for individual chromosomes are depicted in Figure S4.

Solanum lycopersicoides ILs provide insight into the evolution of fruit color in tomato

Carotenoigenic biosynthesis regulation during tomato fruit ripening is mainly governed by transcriptional regulation of the coding genes for structural enzymes along the biosynthesis pathway (Figure 3). During fruit ripening, the accumulation of lycopene, the predominant carotenoid in ripe tomato fruit, is mediated through transcriptional upregulation of coding genes for enzymes upstream of lycopene in the biosynthesis pathway, including PSY1, ZISO, and CRTISO. In addition, there is transcriptional downregulation of genes coding for enzymes downstream of lycopene, CRTL-E and CRTL-B, for which two coding genes were described in tomato: CRTL-B1 (Pecker et al., 1996), CRTL-B2 (BETA), which was predominantly associated with chloroplast-containing tissues (Ronen et al., 2000).

Variation in fruit carotenoid accumulation mediated by *S. lycopersicoides* allelic diversity in the ILs was obvious in mature fruits (Figure S5). IL fruits were generally lighter in color and shifted from red (lycopene) to orange (β-carotene) pigment. Analysis of mature IL fruit carotenoid content revealed 15 potentially associated loci (Tables S6 and S7). For instance, IL4232 shows a significant decrease in lycopene content (Table S6 associated with c1m18 BIN QTL (Figure S6 and Table S8).

To pinpoint underlying genes we next analyzed gene expression in mature fruits of different ILs. This analysis revealed that *S. lycopersicoides* alleles of key carotenoid pathway genes upstream of lycopene were expressed at a substantially lower level in ILs containing the *S. lycopersicoides* alleles than in those harboring the *S. lycopersicum* alleles (Figure S7). One notable *S. lycopersicoides* allele with reduced expression found in BIN c3m3 encodes phytoene synthase 1 (PSY1), exhibiting a three-fold reduced mRNA accumulation as compared with its ortholog in the cultivated tomato.

PSY1 encodes the first committed step in the carotenoid biosynthesis pathway and is highly induced at ripening initiation in red tomatoes. Loss of function of PSY1 is based on the yellow-flesh mutation (*r*) resulting in pale yellow and low carotenoid fruit (Fray & Grierson, 1993). Pathway inhibition in the ILs was not as severe as in the yellow-flesh mutant due to heterozygosity of the *PSY1* gene maintained in these ILs (Figure S7). Fruit from overlapping chromosome 12 introgressions harboring a low expression

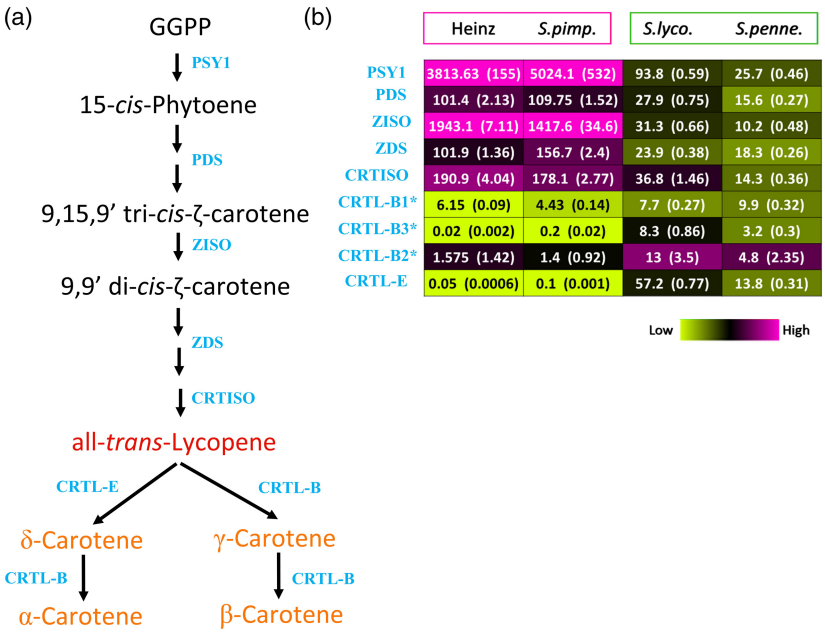

Figure 3. Transcriptional regulation of carotenoid flux in cultivated tomato and non-lycopene accumulating wild tomato fruit.
(a) Carotenoid biosynthesis pathway with pathway genes indicated in blue.
(b) Gene expression (RPKM) in ripe fruit of lycopene accumulating *Solanum lycopersicum* (cultivated) and *Solanum pimpinellifolium* (wild progenitor of cultivated tomato) and non-lycopene accumulating wild species *Solanum lycopersicoides* and *Solanum pennellii*. Fold-changes in fruit versus leaves are shown in parenthesis. Background color represents differences of log ratio of fold-change. The gene list in (b) follows the pathway as represented in (a), except CRTL-B three homologs which are marked by asterisks.

allele of the ζ -carotene isomerase (ZISO), which isomerizes 9,15,9′ tri-cis-ζ-carotene to 9,9′ di-cis-ζ-carotene (a precursor of prolycopene), also had low lycopene levels (Figures S6 and S7g). Lycopene is the red carotenoid predominant in ripe *S. lycopersicum* tomatoes. VIGS-mediated silencing of this gene in tomato resulted in similarly pale-red low lycopene fruit (Fantini et al., 2013). Elevated expression of these genes in the red cultivated tomato implies enhanced flux toward carotenoid synthesis. Downstream of lycopene, lycopene ε-cyclase (CRTL-E) was elevated in expression in lines harboring the *S. lycopersicoides* allele implying that fruit containing low lycopene could be due to redirection of carotenoid flux away from lycopene and toward lutein (Figures S6 and S7f). This is similar to the previously described gene from *S. pennellii* whose corresponding allele is termed *Delta* (*Del*) (Ronen et al., 1999; Ronen et al., 2000) for elevated delta carotene accumulation.

Lycopene β-cyclase is a key enzyme influencing lycopene accumulation as it catalyzes the conversion of lycopene to β-carotene. Expression of the fruit *lycopene β-cyclase 2* (*CRTL-B2*) gene is repressed in cultivated tomatoes by ethylene during ripening, facilitating the accumulation of lycopene via reduced conversion to β-carotene (Alba et al., 2005). The *S. lycopersicoides* allele, however, is not repressed during ripening (Figure 3), resulting in the conversion of lycopene to β-carotene leading to orange fruit in IL4254 (Figures S5 and S6). As with the gene encoding the ε-cyclase, the *S. lycopersicoides* allele is similar to the previously described *Beta* allele of *S. pennellii* (Ronen et al., 2000) in that it drives flux away from lycopene accumulation due to enduring high *lycopene* β-cyclase expression. A second *CRTL-B* gene, *CRTL-B1*, resides in an introgression conferring low lycopene on chromosome 4, and showed an elevated expression of this allele compared with *S. lycopersicum* VF36, driving flux from lycopene toward β-carotene (Figures S6 and S7b). Chromosome 10 harbors two carotenoid accumulation loci, of which one (c10m5) includes both *CRTR-E*, responsible for ε-hydroxylation of α-carotene toward the synthesis of lutein, and a putative lycopene β-cyclase, *CRTL-B3*, which has not been functionally characterized. Like *CRTL-B1* and *B2*, the *S. lycopersicoides* allele of *CRTL-B3* is more highly expressed than the *S. lycopersicum* allele (Figure S7d), resulting in a shift in the lycopene to β-carotene ratio of almost 6:1 in the VF36 control to 1:1 in the IL (Figure S8). As this gene has not been previously characterized, we demonstrated its product's ability to convert lycopene to β-carotene by in-vitro expression in *Escherichia coli* (Figure S8). Phylogenetic analysis of the *CRTL-B* genes indicates a duplication resulting in the creation of *CRTL-B3*, which occurred between the separation of tobacco and petunia (Figure S9), and evolution of red, lycopene accumulating fruit is accompanied by silencing of this gene during ripening (Figure S10).

In summary, analysis of the effects of *S. lycopersicoides* carotenoid QTLs is consistent with the fact that green-fruited wild tomatoes can remain photosynthetic (Cipollini & Levey, 1991; Kilambi et al., 2017). The red-fruited tomatoes, however, show a comparable induction of genes leading to the red pigment lycopene and repression of downstream genes involved in lycopene catabolism during ripening (Figure 3).

Flavonoid pathway

Solanum lycopersicoides LA2951 fruit accumulated about 200-fold more polyphenols (phenylpropanoids, chlorogenic acids, and flavonoids) compared with the VF36 cultivated control (Table S9). Under high light intensity conditions, *S. lycopersicoides* produces anthocyanins in its otherwise green fruit. This prompted us to analyze the region on chromosome 10 harboring the R2R3MYB transcription factors belonging to subgroup 6, known to regulate anthocyanin biosynthesis in plants (Muñoz-Gómez et al., 2021). On chromosome 10 of *S. lycopersicoides* there were three clustered genes encoding subgroup 6 R2R3MYB transcription factors, in a region syntenic with a cluster of five R2R3MYB subgroup 6 genes in *S. lycopersicum* (*AN2*, *AN2-like*, *AN2-like2*, *ANT1*, and *ANT1-like*) on chromosome 10 (Figure 4b). In addition, another three R2R3-MYB subgroup 6 genes were identified by searching the *S. lycopersicoides* genome sequence in a contig not anchored to the genome. These are likely the result of heterozygosity in the *S. lycopersicoides* genome. Indeed, IL10-2 (LA4275) and IL10-4 (LA4276), which both contain the clustered R2R3-MYB transcription factors, have been reported as available only as heterozygotes for the *S. lycopersicoides* interval from chromosome 10 (Canady et al., 2005), supporting the view that the additional copies detected in the parental genome are alternative alleles of the annotated genes. If this entire region were heterozygous on chromosome 10 in the sequenced *S. lycopersicoides* genome, it would affect all the genes in the cluster, which is exactly what was observed.

A phylogenetic tree of the R2R3MYB subgroup 6 genes from *S. lycopersicum*, *S. lycopersicoides*, *S. pennellii*, potato (*S. tuberosum*), and petunia (*Petunia axillaris*, *Petunia inflate*, and *Petunia hybrida*), showed that the major gene duplications (in fact a five-fold multiplication) occurred before the divergence of the *Solanum* family, but independently of the gene multiplications that occurred in petunia (Figure 4a).

A syntenic map was constructed based on the genomic regions harboring subgroup 6 R2R3MYB genes from four species in the Solanaceae family (*S. lycopersicum*, *S. pennellii*, *S. lycopersicoides*, and the diploid *S. tuberosum*) (Figure 4b). In general, the five clustered genes encoding R2R3-MYB transcription factors belonging to subgroup 6 (*ANT1*, *ANT1-like*, *AN2*, *AN2-like*, and *AN2-like2*) are present in different *Solanum* species, derived from the ancestor of the genus *Solanum* (and represented by six genes encoding proteins in the different clades in potato). One clade comprising genes encoding ANT1 and ANT1-like in tomato has been lost in *S. lycopersicoides* (Figure 4a,b) leaving just three genes encoding R2R3-MYB subgroup 6 proteins. Based on the genome annotation, in one of the other three clades (*AN2*, *AN2-like*, and *AN2-like2*), a gene (*AN2-like2*) appears to have been lost in tomato but was present in potato, *S. lycopersicoides*, and *S. pennellii*. However, our analysis of synteny showed that a sequence in the tomato genome (*Solyc10g086300.1*), lying closer to the telomere than the other four genes in the subgroup 6 MYB cluster and annotated as an "unknown protein," was syntenic to the *AN2-like2* genes, *SpAN2-like2*, *SlydAN2-like2*, and *StAN2-like2*. Consequently, we renamed the genomic region containing *Solyc10g086300.1* as *SlAN2-like2*. Furthermore, the sequence upstream of *Solyc10g086300.1* has a high similarity with the sequences upstream of the coding sequences of the *SlydAN2-like2* and *StAN2-like2* genes (Figure 4c). We found that the predicted AN2-like2 protein in *S. pennellii* is much shorter than the predicted AN2-like2 proteins from *S. lycopersicoides* and *S. tuberosum* and it is truncated at its 5′ end encoding the N-terminal DNA binding domain. However, the sequence upstream of *SpAN2-like2* (marked as "SpAN2-like2 upstream") also aligned well with the upstream regions of the *SlydAN2-like2* and *StAN2-like2* genes (Figure 4c). These results suggested that, in this clade, the *AN2-like2* gene from the ancestor of the genus *Solanum* (represented by *StAN2-like2*) and *SlydAN2-like2* encode intact MYB proteins, which may also be functional. However, during evolution, polymorphisms causing a premature stop codon resulted in the loss of function of the corresponding AN2-like2 proteins in *S. lycopersicum* and *S. pennellii*. For the other two *Solanum* R2R3-MYB subgroup 6 subclades comprising *AN2* and *AN2-like*, respectively, there are single copies in each subclade in *S. lycopersicum*, *S. pennellii*, and *S. lycopersicoides*, with phylogenetic distances that support the *S. lycopersicoides* genes being sister to the *S. lycopersicum* and *S. pennellii* sequences.

From the tissue-specific RNA-Seq of *S. lycopersicoides*, we found that *SlydAN2*-like was predominantly expressed in fruit, while no transcripts were detected for *SlydAN2* or *SlydAN2*-like2 in fruit (Table S10 and online database).

The *Anthocyanin in fruit* (*Aft*) allele of *SlAN2* (*SlAN2-like*^{Aft}) from *S. chilense* also promotes anthocyanin production under appropriate light conditions. SlAN2-like^{Aft} shares high similarity in its protein sequence with SlydAN2-like (89%), compared with SlydAN2 (62%) or SlydAN2-like2 (59%). Therefore, the *SlydAN2-like* gene introgressed into cultivated tomato very likely contributes to the Aubergine phenotype. It has been reported that the

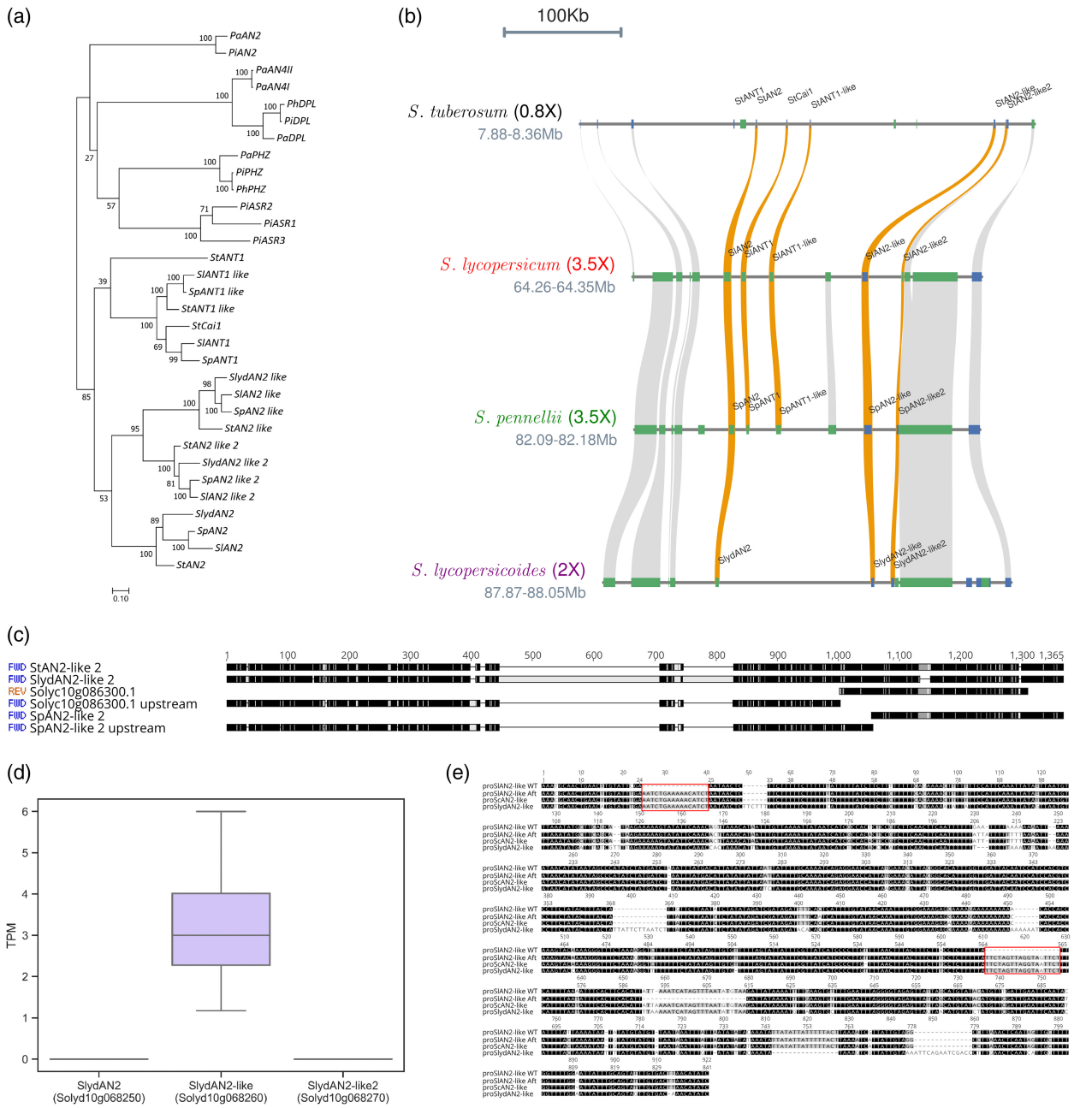

Figure 4. Analysis of *Aubergine* Locus.
(a) Phylogenetic tree of subgroup 6 R2R3MYB genes from *Solanum lycopersicum* (Sl), *Solanum lycopersicoides* (Slyd), *Solanum pennellii* (Sp), *Solanum tuberosum* (St), *Petunia axillaris* (Pa), *Petunia hybrida* (Ph), and *Petunia inflata* (Pi). The maximum-likelihood method was applied with 1000 replicate bootstrap support, following MUSCLE alignment of corresponding genomic sequences.
(b) Syntenic analysis of the R2R3MYB subgroup 6 gene clusters in the genus *Solanum*.
(c) Identity between the *AN2-like2* genes from *S. tuberosum*, *S. lycopersicum*, *S. pennellii*, and *S. lycopersicoides*. Black indicates identical nucleotides, white indicates difference.
(d) Transcript levels of *SlydAN2*, *SlydAN2-like*, and *SlydAN2-like2* in fruit of *S. lycopersicoides*.
(e) Comparison of promoter sequences of *SlAN2-like* in WT tomato, *Aft* locus in tomato, *ScAN2-like* in *Solanum chilense*, and *AN2-like* in *S. lycopersicoides* candidate for *Abg* locus. Red boxes show sequences common to fruit-making anthocyanin but absent in wild-type tomato gene.

expression level of *SlAN2-like*^{Aft} is significantly higher than *SlAN2-like*^{WT} in fruit (Colanero et al., 2020b), so we compared promoter regions of *SlAN2-like*^{WT}, *SlAN2-like*^{Aft}, *ScAN2-like*, and *SlydAN2-like* genes (Figure 4e). There were two blocks of sequence conserved in the *AN2-like* alleles conferring fruit anthocyanin production but absent

from the WT allele from *S. lycopersicum*. The gene-proximal sequence includes an AC box on the negative strand, which is a recognition motif for R2R3-MYB proteins, suggesting that fruit-specific expression of *SlydAN2-like* might be subject to autoregulation as has been reported for *MdMYB10* in apples (Espley et al., 2009). The presence of this sequence may cause the elevated transcript levels of *AN2-like* genes in fruit, particularly in response to light, although these *in silico* analyses need further verification with experimental testing of WT and mutant promoters in fruit.

When *S. lycopersicoides* was grown in our growth chambers (which is not conducive to the light-dependent phenotype as it lacks ultraviolet components inducing anthocyanins; (Colanero et al., 2020a) only green ripe fruit without visible anthocyanin production were produced, although we did note substantial variation in phenolic compounds (Table S9). Hence, we used the IL population to identify QTLs impacting the accumulation of phenolic compounds. On chromosome 5 (IL4252 and IL4299) we identified a metabolic QTL (mQTL) positively influencing chlorogenic acid contents (Figure 5, Table S11, and Figure S11), which was correlated to reduced *CHS2* expression.

Another mQTL on chromosome 10 resulted in up to five-fold elevation of total phenylpropanoids (Figure 5, Table S11, and Figure S11). *PAL5* resides at this locus as it does for a *Solanum neorickii* phenylalanine mQTL (Brog et al., 2019). In contrast to cultivated tomato, wild tomato species accumulate significant phenolics in fruit pericarp (Willits et al., 2005) as do lines with the *S. lycopersicoides* allele of this mQTL locus (Figure S12).

Differences in flavonoids might also be due to differences in the genes encoding the enzymes of flavonoid synthesis and modification, as we detected an expansion of these genes. While the gene encoding dihydroflavonol 4-reductase was triplicated (Solyd02g073780, Solyd02g073830, and Solyd02g073850) in *S. lycopersicoides* compared with the domesticated tomato and *S. pennellii*, the gene encoding flavonol-3-*O*-glycoside-rhamnosyltransferase exists in two copies on chromosomes 3 and 5 in the genome of domesticated tomato, *S. pennellii* shows a tandem duplication of the gene on chromosome 3 of *S. pennellii* and a (near) tandem duplication on both chromosomes 3 and 5 of *S. lycopersicoides* (Solyd03g076070, Solyd03g076130, Solyd05g056030, and Solyd05g056040).

Immunity-associated genes in the LA2951 genome

The LA2951 genome sequence was examined for genes encoding potential immunity-associated proteins. Considering only full-length gene models in LA2951 as compared with Heinz 1706, there are more Nod-like receptors (NLRs; 254 versus 123), but comparable numbers of receptor-like kinases (203 versus 214) and receptor-like proteins (24 versus 30) (Table S12). A search in LA2951 for homologs of other genes potentially associated with effector- or pattern-triggered immunity, identified homologs, most of which had sequence identities >95% (Table S13). In several cases, two closely related genes occur in both LA2951 and Heinz 1706 indicating these gene duplications occurred in a common ancestor (e.g., *Bti9a/Bti9b*, *Fls2.1/Fls2.2*, and *Pti1a/Pti1b*) (Roberts et al., 2020; Schwizer et al., 2017; Zeng et al., 2012).

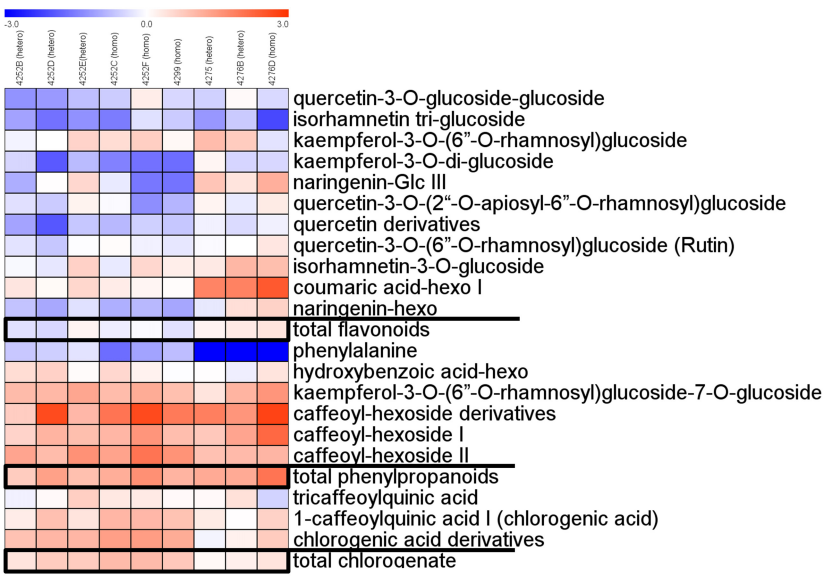

Figure 5. Polyphenol accumulation across the introgression line individuals underlying the metabolic quantitative trait loci. Data are shown as a heatmap as a $\log_2$ fold-change relative to the VF36 control.

The *Pto* resistance gene was originally identified as a member of a clustered gene family on a segment of chromosome 5 derived from *S. pimpinellifolium* (Martin et al., 1993). Embedded within this cluster is *Prf*, which encodes a CC-NLR that acts in concert with the Pto kinase and another member of the Pto family, Fen, to confer resistance to race 0 strains of *P. syringae* pv. *tomato* (*Pst*) (Pedley & Martin, 2003). To test whether LA2951 has a functional *Pto* gene, we inoculated three ILs carrying the LA2951 *Pto/Prf* region in the background of VF36 with *Pst* DC3000 (Figure 6a). *Pst* DC3000 has the effectors AvrPto and AvrPtoB, both of which are recognized by the Pto/Prf complex. As expected, tomato line RG-PtoR, which has the *Pto/Prf* genes, developed no disease in response to DC3000, whereas related lines with mutations in *Pto* or *Prf* (RG-pto11 and RG-prf3) developed disease symptoms. All three ILs developed extensive disease symptoms on stems and leaves in response to DC3000. Another *Pst* strain, DC3000mut5, is recognized by *Fen* in lines lacking a *Pto* gene. An IL containing the *Pto/Prf* region of LA2951 was also susceptible to this *Pst* strain (Figure S13). Together these observations suggest that LA2951 lacks functional *Fen* and *Pto* genes or possibly lacks a functional *Prf* gene. Analysis of the LA2951 genome sequence spanning the *Pto/Prf* region revealed that orthologs of *PtoA* (96%) and *Fen* (93%) are present. *PtoD* is present but contains a premature stop codon. A homolog of *Prf* was also identified, but it contains an insertion with a mutation leading to a premature stop codon, likely rendering the gene nonfunctional (Figure 6b). The susceptibility of the ILs to DC3000 and DC3000mut5 is therefore likely due to the lack of functional *Fen*, *Pto*, and *Prf* genes in LA2951.

Expression analysis of drought resistance

To analyze the effect of drought stress on *S. lycopersicoides*, water was withheld and transcriptome profiling was conducted at two time points and in a control treatment. While the initial drought condition yielded several thousand differentially expressed genes (DEGs), sustained low water yielded fewer DEGs, but both sets exhibited a large overlap of genes similarly regulated. Interestingly, although a number of genes (172) that were upregulated at the earlier time point were downregulated later, many more targets (460) were downregulated first and upregulated later. This pattern is consistent with widespread downregulation of gene expression at the onset of drought followed by recovery under the low-water condition accompanied by sustained activity of a set of upregulated genes at both time points. The set of genes displaying sustained upregulation across both time points might play a protective role. Indeed, the most significant among these were two annexin homologs (Solyd04g071760 and Solyd04g071720), a protein family with well-established roles in drought stress response in tomato (Ijaz et al., 2017). Analyzing the drought response at a pathway level revealed a consistent downregulation of many LRR-III protein kinase family members at both time points. This family includes the *AtRDK1* gene in Arabidopsis, whose loss of function results in drought hypersensitivity (Kumar et al., 2017). In addition, many other genes encoding post-translational modifiers were upregulated at both time points, including clade A of the PPP Fe-Zn-dependent phosphatase families and glutaredoxins (Table S14).

To make the expression dataset readily accessible, we developed a web-based expression atlas for *S. lycopersicoides*, the Lycopersicoides Expression Atlas (http://lycopersicoides-ea.sgn.cornell.edu), which also includes gene expression in multiple tissues. The website allows users to select a project dataset and explore visualizations and analyses of the data using tools, including an expression cube and scatter plot viewer. Additional datasets contributed from the community can be included in the future.

DISCUSSION

The last few years have seen a tremendous advance in plant genomic sciences driven by technologies such as PacBio long-read and Oxford Nanopore sequencing (Bolger et al., 2019) as well as new technologies to obtain long-range chromatin information, including optical mapping and Hi-C data (Schreiber et al., 2018). Indeed, it has been shown that these technological advances can be used to develop chromosome-scale assemblies for large, complicated plant genomes (Belser et al., 2018; Li et al., 2021) and that they are particularly useful for unraveling genomes of wild relatives of important crops, providing information about exotic germplasm, and facilitating its use (Sun et al., 2020; Wu et al., 2018). However, while this would be particularly useful in the tomato clade, reference-grade assemblies exist only for the domesticated tomato (Razali et al., 2017; Tomato Genome Consortium, 2012), the red-fruited *S. pimpinellifolium* (Wang et al., 2020), and the wild species *S. pennellii* (Bolger et al., 2014a; Schmidt et al., 2017). Here, we present a novel high-quality chromosome-scale assembly for the genome of *S. lycopersicoides*, which is one of the most distantly related sexually compatible wild species to the cultivated tomato, representing an exotic germplasm donor that, together with *S. sitiens*, comprises the *Solanum* sect. *Lycopersicoides*, sister to the sect. *Lycopersicon* harboring domesticated tomato (Knapp & Peralta, 2016).

Using long-read sequencing technology and Dovetail scaffolding, we provide a genome with >90% of the sequence gathered in 12 chromosome-like scaffolds and very high gene content completeness. This is of particular importance as *S. lycopersicoides* has been used to establish an IL population (Canady et al., 2005) mapped for abiotic and biotic stress tolerance QTL underlying the enhanced resilience of this wild species. Thus, this genome

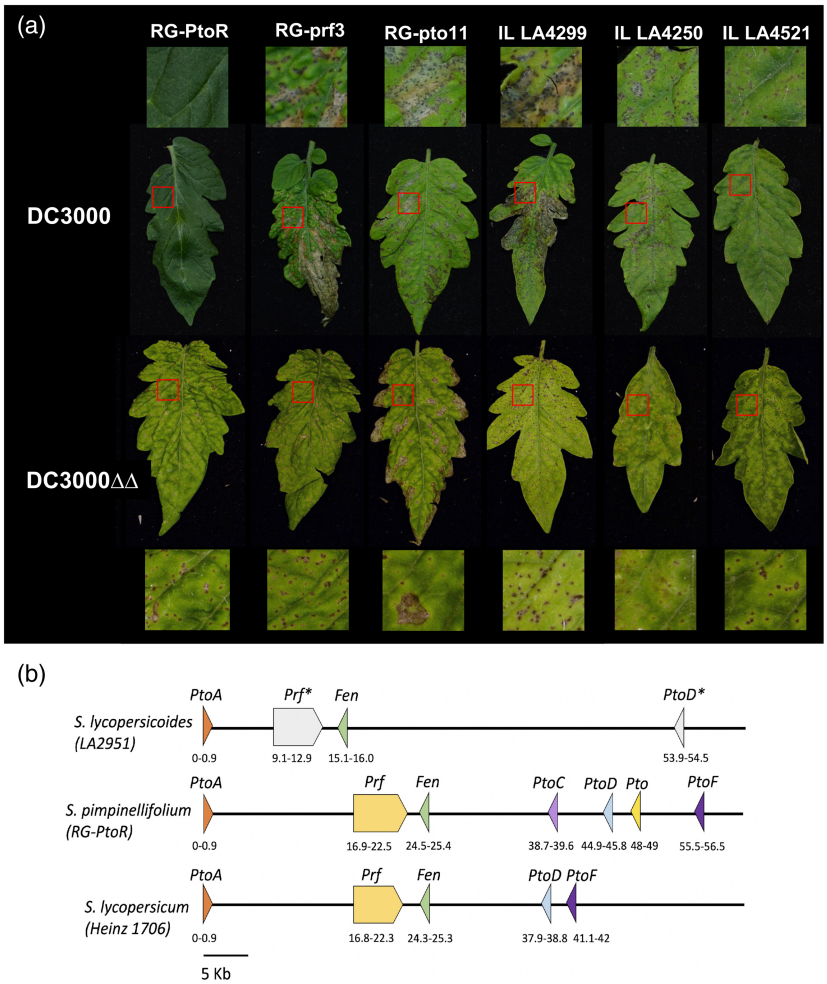

Figure 6. LA2951 does not recognize AvrPto or AvrPtoB in *Pseudomonas syringae* pv. *tomato*.
(a) Responses of introgression lines (ILs) having the Pto/Prf region from LA2951 to Pseudomonas syringae pv. tomato (Pst) DC3000 and DC3000ΔavrPtoΔavrPtoB (DC3000ΔΔ). Plants were vacuum infiltrated with 105 cfu ml−1 of the Pst strains and photographs of representative leaves were taken 7 days post-inoculation. Areas in the red boxes are shown in close-ups to visualize disease symptoms better. Controls are Rio Grande-PtoR (RG-PtoR), which expresses *Pto/Prf* and is resistant to DC3000 but susceptible to DC3000ΔΔ, and RG-prf3 and RG-pto11, which have mutations in the *Prf* and *Pto* genes, respectively, and are susceptible to both *Pst* strains. The three IL lines having the *Pto/Prf* region from LA2951 were susceptible to DC3000 indicating that LA2951 lacks a functional *Pto* and/or *Prf* gene.
(b) Genome organization of the *Pto/Prf* region in LA2951, RG-PtoR, and Heinz 1706. Open reading frames and orientation of the *Pto* gene family members and *Prf* are shown. Coordinates correspond to the genome sequence of each accession with the first nucleotide of *PtoA* set to 0 in each case. Note that *Fen* is also referred to as *PtoB* and *Pto* is also referred to as *PtoE* (Riely & Martin, 2001). *In LA2951, *Pto* is not present and *Prf* and *PtoD* each contain multiple mutations including one in each that leads to a premature stop codon.

provides a reference to facilitate the fine mapping and identification of causative genes underlying IL QTL. In line with previous marker-based data, we also observed an inversion on chromosome 10 of the cultivated tomato relative to the ancestral *S. lycopersicoides* configuration (Pertuzé et al., 2002). We also found an additional inversion between the cultivated tomato and *S. lycopersicoides* on chromosome 4, which had previously been speculated to be a hotspot for rearrangements (Albrecht & Chetelat, 2009). The complete genome sequence enabled understanding of the evolution of subgroup 6 R2R3-MYB genes in the genus *Solanum*, exemplifying that this genome sequence, together with extant *Solanum* genomes, could be applied to investigate the evolution of subgroup 6 R2R3-MYB genes in other species, as well as to understand the evolution of other gene families in the genus *Solanum*, and facilitate the verification of gene annotation in related plant species.

Fruit of domesticated tomato and its wild progenitor, *S. pimpinellifolium*, does not normally accumulate anthocyanins, either in the fruit flesh or in the peel. However, some green-fruited species produce purple anthocyanins in their fruit, notably *S. lycopersicoides* and *S. chilense*. In both species, purple anthocyanin pigmentation occurs principally in the fruit peel and is strongly dependent on incident light. The *Aubergine* (*Abg*) locus was identified in

S. lycopericum × *S. lycopersicoides* ILs and is responsible for anthocyanin production in purple-peeled fruit in *S. lycopersicum* (Rick et al., 1994). *Abg* has been mapped to chromosome 10 in *S. lycopersicoides*. However, a paracentric inversion from the cross between cultivated tomato and *S. lycopersicoides* prevents the introgression from being stable (Canady et al., 2006). Therefore, the genetic nature of the *Abg* locus has remained unclear. The phenotype of *Abg* lines is very similar to that of *Aft* (derived by introgression from *S. chilense*) in tomato. *Aft* has been shown to be conferred by the *S. chilense* allele of *SlAN2-like* (Colanero et al., 2020b; Yan et al., 2020). The two *S. lycopersicoides* genes evolutionarily closest to *SlAN2-like* (*SlydAN2-like* and *SlydAN2-like2*) are therefore the most likely candidates for *Abg* (Colanero et al., 2020b; Yan et al., 2020). RNA-Seq data from fruit of *S. lycopersicoides* showed elevated expression of *SlydAN2-like* alone among the genes encoding R2R3MYB subgroup 6 transcription factors on chromosome 10, supporting the view that the *SlydAN2-like* gene in *S. lycopersicoides* confers the *Abg* purple fruit phenotype.

Fruits of the *S. lycopersicoides* ILs were generally lighter in color and shifted from red (lycopene) to orange (β-carotene) accumulation. Using an IL mapping strategy relying on the improved genome, we could show that *CRTL-B2* is not repressed during ripening in *S. lycopersicoides*, as is its cultivated tomato ortholog. Ripening-related *CRTL-B2* repression in cultivated tomato contributes to lycopene accumulation and the characteristic red tomato color, while CRTL-B2 activity catalyzes the conversion of lycopene to β-carotene (orange) and downstream metabolites. This was also the case for the previously uncharacterized *CRTL-B3*, underlying another QTL for carotenoid accumulation. We showed that *CRTL-B3* encodes a gene with lycopene β-cyclase activity, which together with somewhat reduced expression of *CRTL-B1*, indicates that transcriptional regulation plays a major role in determining carotenoid accumulation that could be deduced using the improved IL maps enabled by a high-quality *S. lycopersicoides* reference genome.

Many resistance genes used in tomato varietal improvement programs were originally identified in wild relatives and subsequently introgressed into breeding lines (Blanca et al., 2015). Resistance to several microbial pathogens has been reported in *S. lycopersicoides* and the genome sequence, in combination with the IL population, will now facilitate cloning of these and additional underlying genes. Testing the response of LA2951 ILs to two *Pst* strains and analysis of the *Pto/Prf* region in LA2951 showed that it lacks the *Pto*, *PtoC*, and *PtoF* genes. *Fen* is present although it is unable to detect the truncated AvrPtoB. At present, we cannot say whether this is because of its diverged sequence or because *Prf* is non-functional due to a premature stop codon. *Solanum lycopersicoides* may have an ancestral arrangement of the *Pto/Prf* region before multiple gene duplications occurred in this region. Based on molecular analyses of AvrPtoB, we hypothesized previously that *Fen* arose first to recognize variants of AvrPtoB that lack an E3 ligase domain and that *Pto* evolved later to overcome AvrPtoB variants with the E3 ligase domain (Mathieu et al., 2014; Rosebrock et al., 2007). The presence of *Fen*, even though it might be non-functional, and not *Pto* in LA2951 supports this hypothesis and it will be interesting to examine *Pto/Prf* genome organization in additional wild relatives of tomato to gain further insight into the evolution of this clustered gene family.

Finally, in addition to improving QTL analyses, we also demonstrated the importance of this genome in assessing gene expression data. Given the large evolutionary distance between *S. lycopersicoides* and the domesticated tomato, simply mapping *S. lycopersicoides* RNA-Seq data to the domesticated gene models showed a low mapping rate, resulting in expression results sometimes different from those obtained with the optimal reference. Mapping to closely related relatives has been used as a stop gap (Hekman et al., 2015) and was recommended as a better strategy than *de novo* transcriptome assemblies (Vijay et al., 2013). Our data show that this approach can be problematic particularly with wide interspecific crosses. While sophisticated bioinformatics pipelines that allow more flexible read alignment and iterative analysis of data and normalization (Zhou et al., 2019) can alleviate these problems, a high-quality reference genome provides the best solution. To improve gene expression analyses within ILs, we present a reference grafting strategy. Using the coordinates from the IL map and both parental reference genomes, we have assembled synthetic genomes specific for each IL. These grafted reference genomes dramatically improve RNA-Seq mapping efficiency within introgressed regions of the ILs and yield the most accurate transcriptomic outputs. IL populations can be powerful tools for both breeding and research communities and the wild reference genome improves their utility. The high-quality *S. lycopersicoides* genome presented here provides a tool for plant evolutionary analyses, characterization of QTLs derived from exotic and stress resilient tomato germplasm, gene discovery, and crop improvement.

EXPERIMENTAL PROCEDURES

Plant material

Seeds of *S. lycopersicoides* LA2951 and the corresponding IL line seeds were obtained from the Tomato Genetics Resource Center (TGRC; https://tgrc.ucdavis.edu/). After germination, they were transferred to soil and further cultivated in a greenhouse supplemented with artificial light to a light intensity of at least 200 μmol m^{-2} sec^{-1} generated using Philips hpi-t plus 400 w/645 metal-halide lamps for 16 h a day. To preserve the genotype of the used accession, one plant was chosen and propagated by

cuttings. Young fresh leaves were collected and used for DNA extraction. *Solanum lycopersicoides* plants, used for RNA-Seq and fruit metabolic analysis, were grown in a growing chamber, equipped with HID illumination, photoperiod of 10-h light, 14-h dark, in root-limiting conditions to induce flowering. Flowers were crossed using "mass sib" pollination. Fruit, flowers, and leaves were ground in liquid N_2 into very fine powder. An aliquot of 200 mg of each of these tissues was used to extract total RNA using Qiagen RNeasy Mini Kit (Hilden, Germany). RNA-Seq libraries were prepared following the method described in Zhong et al. (2011).

For a drought stress experiment, in total, 40 plants were grown in groups of eight with randomized positions in a phyto chamber with artificial light at 350 μmol m^{-2} sec^{-1} for 16 h per day. Humidity was maintained at approximately 70% and the temperature was maintained at 22°C and 18°C in day and night conditions, respectively. Approximately 8-week-old plants were transplanted into approximately 2-L pots and pruned apically at approximately 1 cm above the 11th node. At 10 days after transplanting and pruning, any emergent sucker branch >1 cm in length was removed and plants were recovered for a further 14 days before the start of the experiment. Third and fourth leaf tissues from the first and second apical branches of eight plants were harvested at 11–12 h after dawn (Timepoint in the diurnal cycle [ZT] 11–12) as a time point 0 control (day 0). Pots were all saturated with water at this time by soaking for 5 min and for the next 5 days the weight of control group pots was kept constant by adding measured volumes of water every 1–2 days while drought stress pots were not watered at all. The same tissues were then harvested at ZT 11–12 for time point 1 (day 5) from eight drought-stressed and eight control group plants. The remaining eight control plants were again soaked in water for 5 min and for the next 4 days their weight was again kept constant with measured volumes of water, while eight treatment plants were now supplied with 50 ml water each day. Tissues were then harvested at ZT 11–12 for time point 2 (day 9). Tissue was always pooled for two plants, hence four pools of two plants each representing eight different plants were analyzed. Tissues from all time points were first ground with liquid N_2 and stored at −80°C until further use. RNA was extracted from approximately 50 mg of tissue using a Zymo DirectZol Kit. Approximately 200 mg of the same ground tissue was used for metabolomic profiling (Giavalisco et al., 2009; Lisec et al., 2006).

Metabolite analysis

Secondary metabolites were profiled by the Waters Acquity ultra performance liquid chromatography (UPLC) system coupled to the Q Exactive Orbitrap mass detector according to the previously published protocol (Giavalisco et al., 2009). The UPLC system was equipped with a HSS T3 C18 reversed-phase column (100 × 2.1 mm i.d., 1.8-μm particle size; Waters, Milford, MA, USA) that was operated at a temperature of 40°C. The mobile phases consisted of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). The flow rate of the mobile phase was 400 μl min^{-1}, and 2 μl of sample was loaded per injection. The UPLC was connected to a Q Exactive Orbitrap (Thermo Fisher Scientific, Waltham, MA, USA) via a heated electrospray source (Thermo Fisher Scientific). The spectra were recorded using full scan mode of negative ion detection, covering a mass range from *m/z* 100 to 1500. The resolution was set to 25 000, and the maximum scan time was set to 250 msec. The sheath gas was set to a value of 60, while the auxiliary gas was set to 35. The transfer capillary temperature was set to 150°C, while the heater temperature was adjusted to 300°C. The spray voltage was fixed at 3 kV, with a capillary voltage and a skimmer voltage of 25 and 15 V, respectively. Mass spectrometry spectra were recorded from minute 0 to 19 of the UPLC gradient. Molecular masses, retention time, and associated peak intensities were extracted from the raw files using REFINERMS (version 5.3; GeneData), and XCALIBUR software (Thermo Fisher Scientific). Metabolite identification and annotation were performed using standard compounds, data-dependent method (ddMS2) fragmentation, the literature, and metabolomics databases (Alseekh et al., 2021).

RNA-sequencing

Extracted RNA was treated with Epicenter Baseline-ZERO DNAse. Sample quality and quantity were checked on a Nanodrop and a 1% agarose gel. Within each group, samples were combined in pools of two before sequencing library preparation. Libraries were prepared using the KAPA stranded RNA-Seq reagents and sequenced on an Illumina NextSeq at Oklahoma State University.

Genome sequencing and assembly

A single *S. lycopersicoides* LA2951 plant was chosen for all genome sequencing efforts because this species is self-incompatible and highly heterozygous (Albrecht et al., 2010). DNA was extracted from young fresh leaves as previously described in Bolger et al. (2014a) and sequenced using PacBio P6C4 chemistry at Weill Cornell. In addition, one Illumina Nextera library, three Illumina TruSeq PCR-based, and two Illumina PCR-free libraries were generated and sequenced on an Illumina MiSeq at Research Center Jülich following standard Illumina protocols. The PacBio sequence was assembled using Canu (Koren et al., 2017) (version 1.3, with the parameter "genomeSize = 1.3 g") and Falcon (Chin et al., 2016) assemblers (Table 1). The contigs assembled by Canu were polished using Quiver (https://github.com/PacificBiosciences/GenomicConsensus) with PacBio reads followed by three iterative rounds of correction using Pilon (Walker et al., 2014) with Illumina paired-end reads and then scaffolded with Illumina mate-pair sequences using SSPACE (Boetzer et al., 2011). The assembly was submitted to Dovetail Genomics for further scaffolding using Chicago and Hi-C technologies. Scaffolding gaps were filled with PBJelly (English et al., 2012) using PacBio reads. BUSCO (Waterhouse et al., 2018) was used to assess the quality of the assemblies. Heterozygosity was estimated by mapping Illumina reads from genomic DNA to the final assembly with HISAT2 (Kim et al., 2019) and calling SNPs with the GATK (McKenna et al., 2010) pipeline.

Annotation

De novo repeats were predicted in the *S. lycopersicoides* genome assembly using RepeatModeler (Smit & Robert, 2008–2015). The predicted repeats containing known protein domains were removed and the remaining repeats were then used with RepeatMasker in conjunction with the Repbase library (Bao et al., 2015). For gene prediction, RNA-Seq reads were mapped to the genome with HISAT2 (Kim et al., 2019). Portcullis (Mapleson et al., 2018) and Mikado (Venturini et al., 2018) were used to process the resulting BAM files. PacBio IsoSeq data were also generated from a pool of leaves, fruits, and flowers, and corrected using the Ice pipeline. AUGUSTUS (Hoff & Stanke, 2019) and SNAP (Korf, 2004) were used for ab initio gene predictions, which were integrated with evidence from Iso-Seq transcripts, proteins from Swiss-Prot, and processed RNA-Seq data, to derive the final consensus gene models using the Maker (Campbell et al., 2014) pipeline. Functional annotation and classification was performed using the automated Mercator pipeline (Schwacke et al., 2019).

Repeat analysis

The genomes of *S. lycopersicoides*, *S. lycopersicum*, and *S. pennellii* were analyzed for LTR retrotransposons using LTRharvest (Ellinghaus et al., 2008) with the parameters "-seqids yes -minlenltr 100 -maxlenltr 5000 -mindistltr 1000 -maxdistltr 20000 -similar 85 -mintsd 4 -overlaps best." The genomes were then analyzed using LTR_FINDER (Xu & Wang, 2007) with parameters "-D 20000 -d 1000 -L 5000 -l 100 -p 20 -C -M 0.85 -w 0." LTR_retriever was then used to filter the LTR-RT candidates using default parameters, except that the neutral mutation rate was set at 1.0×10^{-8} using the -u parameter. This neutral mutation rate was selected as it has been used previously for tomatoes (Lin et al., 2014), assuming one generation per year (Beddows et al., 2017).

Candidate miniature inverted-repeat transposable elements (MITEs) were obtained using MITE-Hunter (Han & Wessler, 2010), with default parameters except for "-P 0.2." Output candidate MITEs were manually checked for their TSDs and TIRs as suggested in the MITE-Hunter manual. The candidate MITEs were also assigned to superfamilies based on best hits obtained by BLAST against the P-MITE database (http://pmite.hzau.edu.cn/download/), with an e-value cutoff of $1e^{-5}$. Any candidates that could not be unambiguously classified in this way were classified as unknowns.

To arrive at a comprehensive characterization of TEs, the genomes were then masked using the repeat libraries generated by LTR_retriever and MITE-Hunter using Repeatmasker. Additional repeats were then identified *de novo* in the genomes using RepeatModeler. These repeats were classified using BLASTx against the Uniprot and Dfam libraries and protein-coding sequences were excluded using the script ProtExcluder.pl (Campbell et al., 2014). The masked genomes were then re-masked with Repeatmasker and the corresponding repeat libraries generated by RepeatModeler.

We used TEffectR (Karakülah et al., 2019) to determine cases where expression levels of drought-responsive genes, determined through the differential expression analysis conducted (i.e., DEGs at a false discovery rate cutoff of 0.05), were correlated with the expression of neighboring LTR TEs (with no covariates and with a Bonferroni-adjusted $P < 0.05$). This analysis was done using DEGs from the comparison of Day 5 samples to Day 0, as well as from Day 9 to Day 0. The counts generated in the drought DEG analysis were used for the gene count levels in TEffectR. For TE expression levels, reads were mapped to the reference genome using hisat2 (Kim et al., 2019) and SAMtools (Li et al., 2009) was used to sort and merge BAM files corresponding to libraries from the same biological samples. For TE expression, multi- and unmapped reads were also removed from BAM files, as suggested previously (Karakülah et al., 2019).

Immunity gene analysis

NLR genes in the Heinz 1706 (SL4.0) and *S. lycopersicoides* genomes (v1.1) and transcriptomes (ITAG v4.2 and v1.1) were identified with NLR-annotator (Steuernagel et al., 2020). Receptor-like kinases and receptor-like proteins were selected from TAIR10 according to their protein functional descriptions. DIAMOND (Buchfink et al., 2015) was used to query this protein database with the Heinz 1706 and *S. lycopersicoides* protein sequences using an e-value cutoff of 10^{-10}. Structural domains were annotated according to Pfam (Mistry et al., 2021) and Phobius (Käll et al., 2007) was used to predict transmembrane domains and signal peptides.

The *Pto/Prf* region was identified in LA2951 and Heinz 1706 (SL4.0) by querying their genome sequences (Hosmani et al., 2019) with the coding sequences of the *Pto* family members and *Prf* from *PrfRG-PtoR* (NCBI: AF220602.1) using BLASTn (Altschul et al., 1990). A 100 kb region encompassing the identified *Pto/Prf* regions in LA2951 and Heinz 1706 was aligned to that in *RG-PtoR* using the nucmer tool in the MUMmer 3.23 package (Kurtz et al., 2004) with default parameters followed by filtering alignments below 95% identity. The specific coordinates of *PtoA*, *Prf*, *Fen* (*PtoB*), *PtoC*, *PtoD*, and *Pto* (*PtoE*) were determined by querying the 100 kb *Pto/Prf* region of LA2951 and Heinz 1706 with the coding sequence from RG-PtoR for each gene. Multiple sequence alignments of genes were performed with MAFFT version 7 (Katoh & Standley, 2013).

The LA2951 ILs were obtained from the Tomato Genetics Resource Center (https://tgrc.ucdavis.edu/) and consist of segments of LA2951 in the background of *S. lycopersicum* (cv. VF36). The lines for this study contained an *S. lycopersicoides* introgression containing the *Pto/Prf* region. Tomato varieties Rio Grande-PtoR (RG-PtoR; *Pto/Pto*, *Prf/Prf*) and Rio Grande-prf3 (RG-prf3; *prf3/prf3*) were included as the resistant and susceptible controls respectively. RG-Pto11 was also included as a control for the DC3000mut5 experiments. RG-PtoR carries the resistant *S. pimpinellifolium Pto/Prf* haplotype, and RG-prf3 has a non-functional *Prf* gene. *Solanum lycopersicum* cv VF36 and *S. lycopersicoides* LA2951 ILs were grown in Cornell Plus Mix (0.16 m^3 peat moss, 0.34 m^3 vermiculite, 2.27 kg lime, 2.27 kg Osmocote Plus15-9-12 and 0.54 kg Uni-Mix 11-5-11; Everris, Israeli Chemicals Ltd). Plants were grown in a greenhouse without supplemental lighting at 24°C day and 22°C nighttime temperatures. Four-week-old plants were vacuum infiltrated with *P. syringae* pv. *tomato* strains DC3000 or DC3000ΔΔ (lacking *avrPto* and *avrPtoB*), or DC3000mut5. Strains were grown on King's B medium for 48 h and suspended in 10 mM $MgCl_2$ for a final titer of 10^5 cfu ml^{-1} for vacuum infiltration. After inoculation, the plants were kept in a growth chamber (24°C day and 20°C night) for 7 days (10 days for the DC3000mut5 experiment) before photographing.

Genome visualizations, synteny, and structural variants

Circular genome visualizations were generated using Circos (Krzywinski et al., 2009). For *S. lycopersicoides*, gene and repeat densities were calculated by generating 1 Mb non-overlapping windows and calculating the percentage coverage for each feature type (either annotated gene features or repeat elements identified in the repeat analysis) using BEDTools (Quinlan, 2014; Quinlan & Hall, 2010). For synteny analysis, alignment of *S. lycopersicoides* to *S. lycopersicum* (SL4.0) and *S. pennellii* LA0716 v 2 (Bolger et al., 2014a) was conducted with nucmer using the parameters --maxgap = 500 --mincluster = 100, followed by delta-filter with parameters "-r -q -i 92 -l 8000 and show-coords."

Drought expression

RNA-Seq reads were trimmed using Trimmomatic (Bolger et al., 2014b). Pseudo-alignment was performed using Kallisto (100 bootstraps) to estimate the abundance of each target in each library (Bray et al., 2016). The quality of each library was initially examined in FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Clustering and quality control revealed three libraries that were not considered in further testing. Because of the sampling strategy where two branches were sampled from the same pair of source plants, there was some correlation expected between the two branch samples from a given pool within a treatment group. We first assessed the potential for an effect of the branch by performing likelihood ratio tests described in Sleuth (Pimentel et al., 2017). Including estimation

of a branch effect (i.e., approximately time.treatment + branch) provided no significant improvement to the performance of models for any target. Estimated counts were extracted using TxImport from DESeq2 for further processing (Love et al., 2014; Soneson et al., 2015). A minimum expression level across a minimum number of libraries was enforced and normalization factors (edgeR::calcNormFactors) were applied to generate normalized read counts (McCarthy et al., 2012; Robinson et al., 2010). The extent of correlation between branches from the same source plants was estimated using Limmas duplicateCorrelation function and accounted for in all subsequent testing in mixed linear models by blocking on pool ID and supplying the correlation value to lmFit in Limma (Ritchie et al., 2015). Contrasts were evaluated to estimate typical fold-change in response to drought at both time points. MapMan bins (Schwacke et al., 2019) were examined for enrichment in upregulated, downregulated, or differentially expressed targets in a Fisher's exact test on a contingency table per-bin versus a background of all detected targets. Adjustment of *P*-values to correct for false discovery rate was performed within each set (time point x DEG classification) according to the Benjamini–Hochberg procedure (Benjamini & Hochberg, 1995).

Expression atlas

The Lycopersicoides Expression Atlas uses the Tomato Expression Atlas (Fernandez-Pozo et al., 2017) as its template. The main code for the expression atlas is available from GitHub (https://github.com/solgenomics/Tea). The Perl scripts used to format and import data into the database are included in this repository. The code used for customizing the Lycopersicoides Expression Atlas is also publicly available (https://github.com/solgenomics/lycopersicoides_ea).

Two datasets were included in the Lycopersicoides Expression Atlas. To generate expression values in counts per million (CPM) for display in the atlas, reads from the two datasets were mapped to the reference genome using hisat2 (Kim et al., 2015), SAMtools (Li et al., 2009) was used to sort and merge files from the same biological samples where appropriate, and stringtie (Pertea et al., 2016) was used to count reads mapped to gene features. The CPM values were generated from the raw counts using the *cpm* function from the edgeR R package (Robinson et al., 2010). For the correlation values in the atlas, the raw reads were first transformed using the *vst* function from the DESeq2 R package (Love et al., 2014), and Spearman correlation values were calculated from the transformed counts, following the code included in the TEA repository. For the generation of both expression and correlation values, genes with no read counts in any samples were excluded.

Introgression line maps

RNA-Seq reads from the population were first processed with Trimmomatic-0.36 (Bolger et al., 2014b) to trim and filter low-quality reads. Using Bowtie2 (Langmead & Salzberg, 2012) the reads were then aligned to databases of ribosomal RNA (Quast et al., 2013) and plant virus sequences (Zheng et al., 2017) to filter out non-mRNA reads. The reads were then used to call SNPs following the best practices in the Genome Analysis Toolkit documentation (McKenna et al., 2010). Both *S. lycopersicum* (SL4.0) and *S. lycopersicoides* reference genomes were used to establish a set of SNP markers within the population that distinguish between background and introgressed regions. SNPs that did not match either reference genome were filtered out. Only loci that were homozygous for a single parent or heterozygous with one base from each parent were used. This high-density genotype marker map was then converted into a map of introgressions using SNPbinner (Gonda et al., 2019). Again, reference genomes of both *S. lycopersicum* and *S. lycopersicoides* were used to map the introgression boundaries within the context of the domesticated tomato background as well as to define the introgressed segments within *S. lycopersicoides*.

Carotenoid quantification and analysis

Carotenoids were extracted and quantified from 200 mg finely ground frozen pericarp tissue of ILs collected from the greenhouse 2015 experiment as previously described (McQuinn et al., 2018). For the 2015/2016 field experiments, the population was first genotyped (Tables S6, S7), extracted carotenoids were dried at reduced pressure and resuspended in ethyl acetate containing 50 $\mu g\ ml^{-1}$ of diindolylmethane as an internal standard. Samples were quantified using an Ultra Performance Convergence Chromatography (UPC 2) system, equipped with an HSS C 18 SB column (2.1 $\times$ 150 mm, 1.8 μm particle size; Waters), as described (Gonda et al., 2019). Logarithm of the odds score analysis was carried out in the R environment, using the R/qtl package (Arends et al., 2010). Genome scan was performed with a single QTL model, using linear marker regression method of phenotypes on marker genotypes. Carotenoid QTL analysis was performed using the field 2015 data set.

CRTLB 3 functional analysis

The CRTL-B3 coding sequence was obtained from cv. VF36, by PCR using the following primers, forward: ATGGATACATTGTTGAAAACCC; reverse: TTCTGTATCCTTTAACAAATTGTTAATCATAG. The amplicon was transferred to expression vector pEXP5-CT/TOPO (Thermo Fisher Scientific), following confirmation by Sanger sequencing, yielding pEXP-CRTLB3. This construct was transformed into the lycopene-accumulating *E. coli* strain, (pEBI) (Ronen et al., 1999). Positive colonies were incubated in 150 ml liquid LB medium supplemented with IPTG and incubated for 40 h (25°C, 280 r.p.m.). Cells were harvested, followed by carotenoid extraction and analysis as described previously (Chayut et al., 2017).

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AUTHOR CONTRIBUTIONS

BU and SRS managed the project. AFP, AF, JL, CM, LC, JJG, BU, MM, and SRS wrote the manuscript with help from all authors. AFP, AF, MHWS, AF, JL, CM, EMJ, SRH, GBM, ZF, JJG, BU, MM, and SRS designed the analysis. AF, MHWS, AV, EMJ, KD, MM, AD, and YX conducted the DNA and RNA preparation and sequencing and plant experiments. AFP, LC, MHS, DL, and AD contributed new reagents and analytical tools. SA and ARF conducted metabolic analysis and interpretation. AFP, LC, EMJ, LM, MM, AKD, GBM, ZF, SRS, and BU conducted the data analyses.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The genome and raw reads are available under NCBI BioProject ID PRJNA727176, PRJNA526255. In addition the genome its annotation and additional data are available through Solgenomics.net (https://solgenomics.net/organism/Solanum_lycopersicoides/genome). Expression profile data are available at the Lycopersicoides Expression Atlas (http://lycopersicoides-ea.sgn.cornell.edu). The genome and its annotations have been integrated into solgenomics.net. Seeds are available from the Tomato Genetics Resource Center where the IL number corresponds to the LA code.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Insertion age of repetitive elements. Figure shows estimated ages for Copia, Gypsy, and other repetitive elements for (A) *Solanum lycopersicoides*, (B) *Solanum lycopersicum*, and (C) *Solanum pennellii*.

Figure S2. Detailed IL map for the *Solanum lycopersicoides* × *Solanum lycopersicum* VF36 introgression population

Figure S3. Syntenic dot plot showing the alignment between *Solanum lycopersicoides* and *Solanum lycopersicum* Main figure shows the genomic dot plot and two breakouts show regions for chromosome 10 and chromosome 4, respectively.

Figure S4. Mapping efficiency of the *Solanum lyopersicoides* IL population.

Figure S5. Selected phenotypes of different IL fruits. Fruits are shown at breaker +7 stage from a greenhouse experiment.

Figure S6. Fifteen loci (IL bins) affecting carotenoid levels. Specific carotenoids in a QTL analysis are indicated on the left of each numbered section. The left part of each sub-panel shows carotenoid levels among all genotypes in the IL population. For each assessed carotenoid, the right subpanel shows LOD scores throughout the specified chromosome. In some cases, positions of specific candidate genes are indicated. Blue bars indicate the position of specific ILs associated with the trait. Red marks on the *x*-axis indicate the location of the bin. AA (VF36 homozygous), AB (heterozygous), BB (*Solanum lycopersicoides* homozygous).

Figure S7. Expression of candidate genes in genetic BINs. Expression data of selected candidate genes are shown in the ILs depending on the genotype.

Figure S8. Role of CRTL-B3. (a) Lycopene accumulating *Escherichia coli* cells. (b) Lycopene accumulating *E. coli* cells transformed with CmCRTL-B3. (c,d) Chromatograms of the elution profiles at 450 nm of (a,b) respectively. (e,f) Lycopene and beta-carotene absorbance spectra respectively. (g) Lycopene/beta carotene ratio in ILs harboring *Solanum lycopersicoides* CRTL-B3 allele from field 2015 experiment.

Figure S9. CRTL-B3 diversification within the Solanaceae family. Phylogenetic tree of the lycopene cyclase gene family proteins: CRTL-E (in gray, lycopene-epsilon cyclase), CRTL-B (in black lycopene-beta cyclase). Aco, *Ananas comosus*; At, *Arabidopsis thaliana*; CA, *Capsicum annuum*; Cm, *Cucumis melo*; Cs, *Citrus sinensis*; DCAR, *Daucus carota*; Md, *Malus domestica*; Migut, *Mimulus guttatus*; Nitab, *Nicotiana tabacum*; Os, *Orysa sativa*; Peaxi, *Petunia axillaris*; Pp, *Physcomitrella patens*; Sd, *Solanum lycopersicoides*; Sl, *Solanum lycopersicum*; SMEL, *Solanum melongena*; St, *Solanum tuberosum*; Vv, *Vitis vinifera*; Zm, *Zea mays*. Occurrence of two CRTL-B homologs in the genome is conserved between Embryophytes (*Physcomitrella*, black) to Angiosperms (*Ananas*; CRTL-B1/2- pink/green), Except grasses (corn, rice) and Arabidopsis, which lost the CRTL-B2 ortholog. The Asterids: wild carrot, Seep monkeyflower, and wild white petunia exhibit a single CRTL-B1 ortholog, while the transition between petunia and tobacco is accompanied with duplication of this gene (red asterisk), which was further diversified into two separate clades in tomato, pepper, eggplant, and potato (CRTL-B1/3 –red/blue).

Figure S10. Evolution of lycopene accumulating fruit is accompanied by silencing of *Sl*CRTL-B3 transcription during fruit ripening. While maintaining stable expression in both leaf, flower, and ripe fruit of *Solanum lycopersicoides*, expression of *CRTL-B3* in orange stage fruit of 397 lycopene accumulating accessions is downregulated. SP, *Solanum pimpinellifolium* (27 accessions); SLC, *Solanum lycopersicum* var *cerasiforme* (110 accessions); SLL, *S. lycopersicum* L. (258 accessions). In addition (not in the graph) *Solanum cheesmaniae* (LA0429): 0.04 RPKM; *Solanum galapagense* (LA0528): 0.23 RPKM.

Figure S11. IL population polyphenol compounds mQTLs in chromosome 5 (a), and chromosome 10 (b). By each compound: on the right LOD scores throughout the specified chromosome, on the left is compound accumulation in the corresponding bin, specified by allelic variation: AA (homozygous VF36), AB (heterozygous), BB (homozygous *Solanum lycopersicoides*).

Figure S12. Analysis of LA4276 fruit methanol extracts. (a,c) LC-MS total chromatogram spectra. In black are biological repetitions of control LA4276 segregated out from *Solanum lycopersicoides* insertion, in red are LA4276 harboring homozygous insertion to Chr.10. (a,b) Fruit peel. (c,d) Fruit flesh. (b,d) Significantly upregulated compounds associated with *S. lycopersicoides* introgression. (e) Total phenolic content estimation performed by OD 300 in comparison with ferulic acid standard. On the right peel, on the left fruit flesh of independent fruit out of segregating IL7276 population. In blue VF36 cultivated allele. In green are fruit derived from heterozygous plants, and in yellow are homozygous to *S. lycopersicoides* insertion.

Figure S13. An IL containing the *Pto/Prf* region of LA2951 is susceptible to DC3000mut5. Tomato plants were vacuum infiltrated with 10^5 cfu ml^{-1} of *Pseudomonas syringae* pv. *tomato* strain DC3000mut5 and representative leaves were photographed 10 days later. DC3000mut5 carries a version of AvrPtoB lacking its C-terminal E3 ligase domain. The truncated AvrPtoB is recognized

by Pto and by Fen. In RG-pto11 *Pto* is non-functional and the resistance in this line to DC3000mut5 is due to Fen. RG-prf3 lacks a functional *Prf* gene, which disables both Pto and Fen. IL4284, carrying the *Pto/Prf* region of LA2951, is susceptible to DC3000mut5, which was expected as its *Prf* gene has multiple mutations indicating a loss of function.

Table S1. Gene model statistics.

Table S2. Repetitive elements in *Solanum lycopersicoides*, *Solanum lycopersicum*, and *Solanum pennellii*.

Table S3. Insertion segregation in the ILs.

Table S4. Detailed IL map including coordinates.

Table S5. Introgression Bin Map created by SNPBinner, mapped to *Solanum lycopersicoides* genome.

Table S6. Fruit carotenoid accumulation, Subsets i/iii and ii/iii, which was further used for QTL analysis.

Table S7. Fruit carotenoid accumulation field 2016 iii/iii line subset.

Table S8. IL BINs after RNAseq SNP calling.

Table S9. LC-MS peak area of polyphenol compounds accumulation comparison between cultivated control ripe fruit (VF36, marked in red) and *Solanum lycopersicoides* ripe green fruit (Lyc, green).

Table S10. Comparison of polyphenol biosynthesis structural genes expression between VF36 ripe fruit and *Solanum lycopersicoides* ripe green fruit.

Table S11. Polyphenols accumulation in ripe fruit across the IL population.

Table S12. Immunity gene survey for NLR, RLK, and RPL genes in *Solanum lycopersicoides*, *Solanum lycopersicum*, and *Solanum pennellii*.

Table S13. Immunity-associated genes in the *Solanum lycopersicoides* genome.

Table S14. Enrichment and depletion analysis for drought stress time points 1 and 2 using MapMan Bins as classes.

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