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Dissection of major QTLs and candidate genes for seedling stage salt/drought tolerance in tomato

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Abstract

Background As two of the most impactful abiotic stresses, salt and drought strongly affect tomato growth and development, especially at the seedling stage. However, dissection of the genetic basis underlying salt/drought tolerance at seedling stage in tomato remains limited in scope.

Results Here, we reported an analysis of major quantitative trait locus (QTL) and potential causal genetic variations in seedling stage salt/drought tolerance in recombinant inbred lines ($n=201$) of *S. pimpinellifolium* and *S. lycopersicum* parents by whole genome resequencing. A total of 5 QTLs on chromosome 1, 3, 5, 7 and 12 for salt tolerance (ST) and 15 QTLs on chromosome 1, 3, 4, 8, 9, 10, 12 for drought tolerance (DT) were identified by linkage mapping. The proportion of phenotypic variation explained (PVE%) by these QTLs ranged from 4.91 to 15.86. Two major QTLs *qST7* and *qDT1-3* were detected in both two years, for which two candidate genes (methionine sulfoxide reductase SlMSRB1 and brassinosteroid insensitive 1-like receptor SlBRL1) and the potential functional variations were further analyzed. Taking advantage of the tomato population resequencing data, the frequency changes of the potential favorable QTL allele for seedling stage ST/DT during tomato breeding were explored.

Conclusions These results will be beneficial for the exploration of salt/drought tolerance genes at seedling stages, laying a foundation for marker-assisted breeding for seedling stage salt/drought tolerance.

Keywords Tomato, Salt tolerance, Drought tolerance, QTL

Background

Tomato (*S. lycopersicum* L.) is one of the most important vegetables with approximately 189 million tons grown on 5.16 Mha worldwide (FAOSTAT, 2023, https://www.fao.org/faostat/en/). Cultivated tomatoes grown in many countries are consumed mainly as fresh or processed food products; the former type is mainly grown under controlled environments (e.g., greenhouses and plastic houses) in some countries, and the latter type is usually grown under field conditions. In recent years, intensive agronomic practices and climate change have resulted in a wide variety of limiting factors disrupting tomato

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yield and fruit nutritional quality. Among these factors, two prevalent abiotic stresses to which tomatoes are subjected are salinity and drought [1–3]. Thus, genetic improvements of ST and DT remain two of the major goals in tomato breeding. However, limited knowledge about functional molecular markers and causative genes has greatly slowed this progress. Therefore, dissection of the genetic bases of these two kinds of abiotic stresses is essential for tomato tolerance breeding.

Due to the narrow genetic basis of human selection during breeding, cultivated tomato plants are moderately sensitive to salt and/or drought stresses throughout the period of growth and development. The seedling growth stage of tomato may be more sensitive to salt and/or drought stresses because tolerance generally increases with plant age [4]. During the past few decades, many QTLs related to ST/DT have been identified from wild germplasms such as *S. pennellii*, *S. pimpinellifolium* and other wild relatives [4, 5]. Although many QTLs for ST/DT were mapped on all tomato chromosomes based on biparental populations, the number and location of QTLs were not exactly the same, which may indicate the complicated genetic and interaction effects [4, 6–8].

Compared to commonly used F_2 or backcross populations, recombinant inbred lines (RILs) have apparent advantages in excavating environmentally stable QTLs considering their reliable phenotypic characteristics for long-term repeated trials [9]. Recently, high-resolution single nucleotide polymorphisms (SNPs) from genome resequencing have greatly improved the mapping resolution, which prompted the combination of linkage analysis and genome-wide association study (GWAS) for analyzing complex traits [10, 11]. Furthermore, QTL mapping integrated with polymorphism studies has been widely used in candidate mining for complex quantitative traits, such as those related to biotic and abiotic stresses [8, 12–14].

Plants adapt to salt stress through a multitude of biochemical and molecular mechanisms, mainly including osmotic tolerance, ion exclusion and tissue tolerance [15], while the mechanisms of DT can be mainly summarized as escape, avoidance or tolerance via the activation of stress-responsive signaling pathways [16]. The reported tolerance mechanisms to salt/drought stress provide great convenience for homologous cloning of related tolerance genes. Recently, the transcription factor *SlWRKY57*, a salt-induced gene, was shown to act as a negative regulator of the salt stress response by directly attenuating the transcription of salt-responsive genes and an ion homeostasis gene [17]. *SlOST1*, the ortholog of Arabidopsis *AtOST1*, which encodes a protein kinase, positively regulates DT but also promotes flowering under drought stress conditions by physically interacting with the NAC-type transcription factor *SlVOZ1* [18]. Virus-induced gene silencing of the abscisic acid receptor *SlPYL4* decreased the DT of tomato plants [19]. In summary, the identification of key genes through homology analysis may benefit the discovery of functional variation and facilitate marker-assisted breeding in tomato.

S. pimpinellifolium is the closest wild relative to cultivated tomato and has long been considered a reservoir of favorable genes for improving abiotic stress tolerance. To dissect the genetic basis of seedling stage ST/DT of wild and cultivated tomatoes, *S. pimpinellifolium* 'PI365967' (PI) and *S. lycopersicum* 'Moneymaker' (MM) were selected to generate a stable RIL population based on their obvious variation in survival rate under seedling stage salt/drought stress. Through linkage analysis, five QTLs for ST and fifteen QTLs for DT at seedling stage were identified respectively, including two stable major QTLs *qST7* conferring ST and *qDT1-3* conferring DT. Furthermore, we identified two candidate genes for these two stable QTLs and analyzed the potential functional variations. We explored the pyramiding effect of the potential favorable alleles and their frequency change during tomato breeding. These seedling stage QTLs for ST/DT in our study may lay a foundation for improving salt/drought tolerance throughout the ontogeny of tomato by pyramiding breeding in the future.

Results

Phenotype analysis of ST and DT

The ST phenotypes of the PI/MM RIL population were evaluated for two years. The parent PI was obviously much more tolerant than the MM to seedling stage salt stress (Fig. 1A). We employed the salinity scale classes described by Dasgan et al. [20] for population evaluation (Fig. 1B), which were then transformed to the survival rate (%) in the following analysis. Statistical analysis revealed that the survival rate of PI was approximately 50% on average (33.33%-66.67%), which was greater than that of MM (33.33% on average, 16.67%-66.67%; Fig. 1C). The correlation coefficient (*R*) of the two-year salt tolerance phenotypes in the RIL population was 0.71, with a median survival rate of approximately 45.38% of the population (Fig. 1C; Table S1).

In addition, the DT of this population was evaluated for three seasons over two years. The two parents exhibited obviously different tolerances to seedling stage drought stress treatment (Fig. 1D). Similarly, we classified drought scale classes I to VI according to the degree of damage in the RIL population (Fig. 1E). The survival rate of PI was approximately 71.43%, which was more than twice the survival rate of MM (~35.71%). The correlation coefficients (*R*) of the two-year drought-tolerant phenotypes in the RIL population were 0.45 and 0.60, with a median survival rate of 45.38% (Fig. 1F; Table S1). The

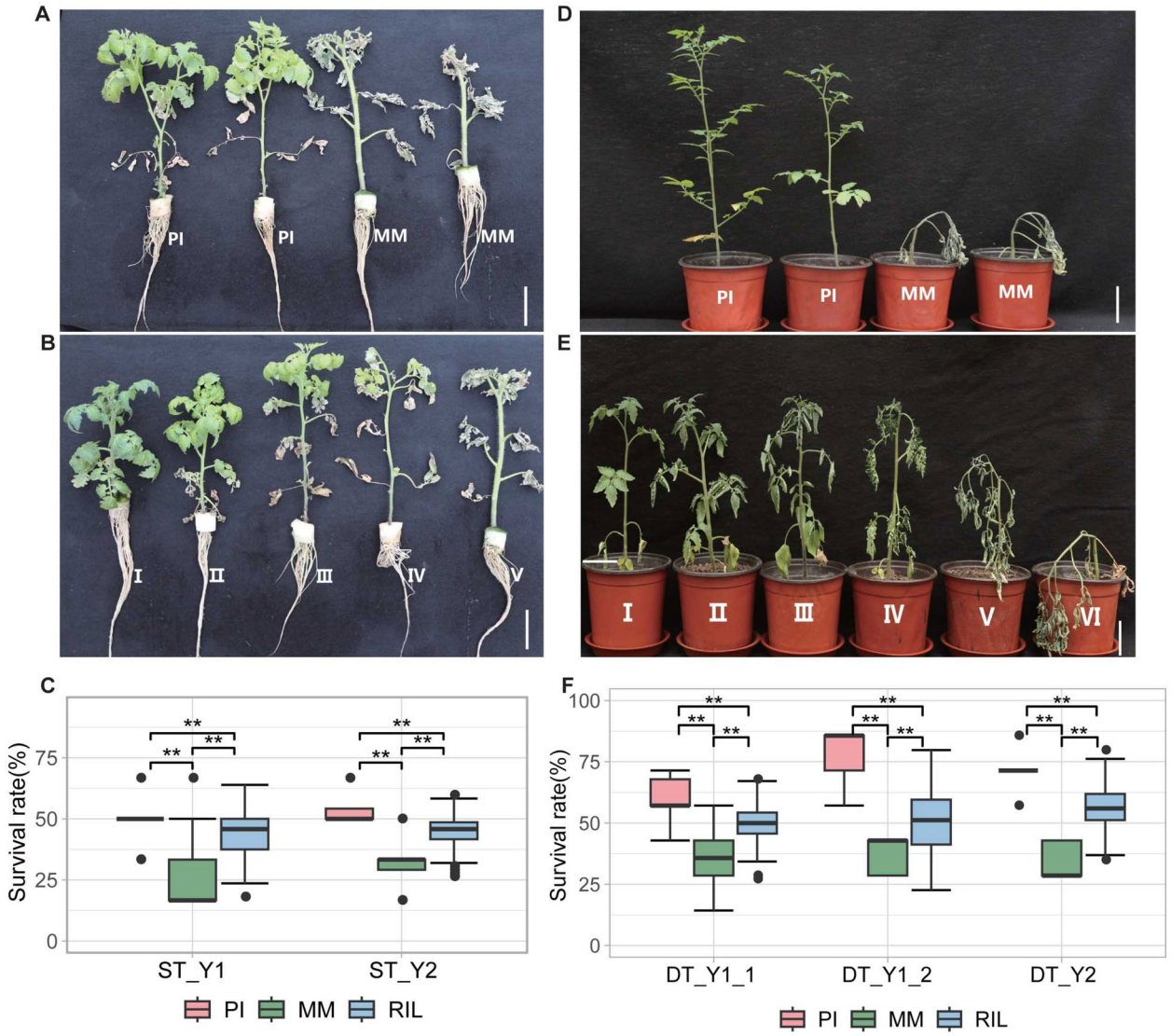

Fig. 1 Phenotypes of the RIL population after salt/drought treatment. **A** Phenotypic characteristics of PI and MM after salt treatment. **B** Properties of the ST in the RIL population were classified on a class I-V scale in the same way as Dasgan et al. [20]. **C** Phenotypic statistics of salt treatment in two trials. **D** Phenotypic characteristics of PI and MM after drought treatment. **E** Properties of DT in the RIL population were classified on a scale of I-VI in the same way as reported [7]. **F** Phenotypic statistics of drought treatment in three trials. Bar, 5 cm

approximately normal distribution of the survival rate of the RILs showed the characteristics of multiple gene-controlled quantitative traits (Fig. S1).

Identification of QTLs for ST

To take advantage of the RIL population in QTL detection, we combined linkage analysis and GWAS to identify genomic regions regulating ST at the seedling stage in tomato. Considering the limited recombination events in the RIL population, we constructed genotype bin maps based on SNP genotype data across the population. The total genetic distance of the linkage map was 1239.56 cM, and the map contained 1550 bin markers with a recombinant bin > 250 kb, as previously reported (Table S2, Fig. S2). In the QTL analysis with bin markers, a total of five QTLs distributed on chr1 (*qST1*), chr3 (*qST3*), chr5 (*qST5*), chr7 (*qST7*) and chr12 (*qST12*) were detected (Fig. 2; Table S3). Among them, the major QTL *qST3* was detected with the highest likelihood of odds (LOD = 4.90) and phenotypic variance explained (PVE% = 12.51) in ST_Y1. The stable QTL *qST7* was detected in both years and the best linear unbiased prediction (BLUP); the PVE% ranging from 7.98 to 10.77 (Fig. 2; Table S3). Interestingly, according the positive additive effect, the

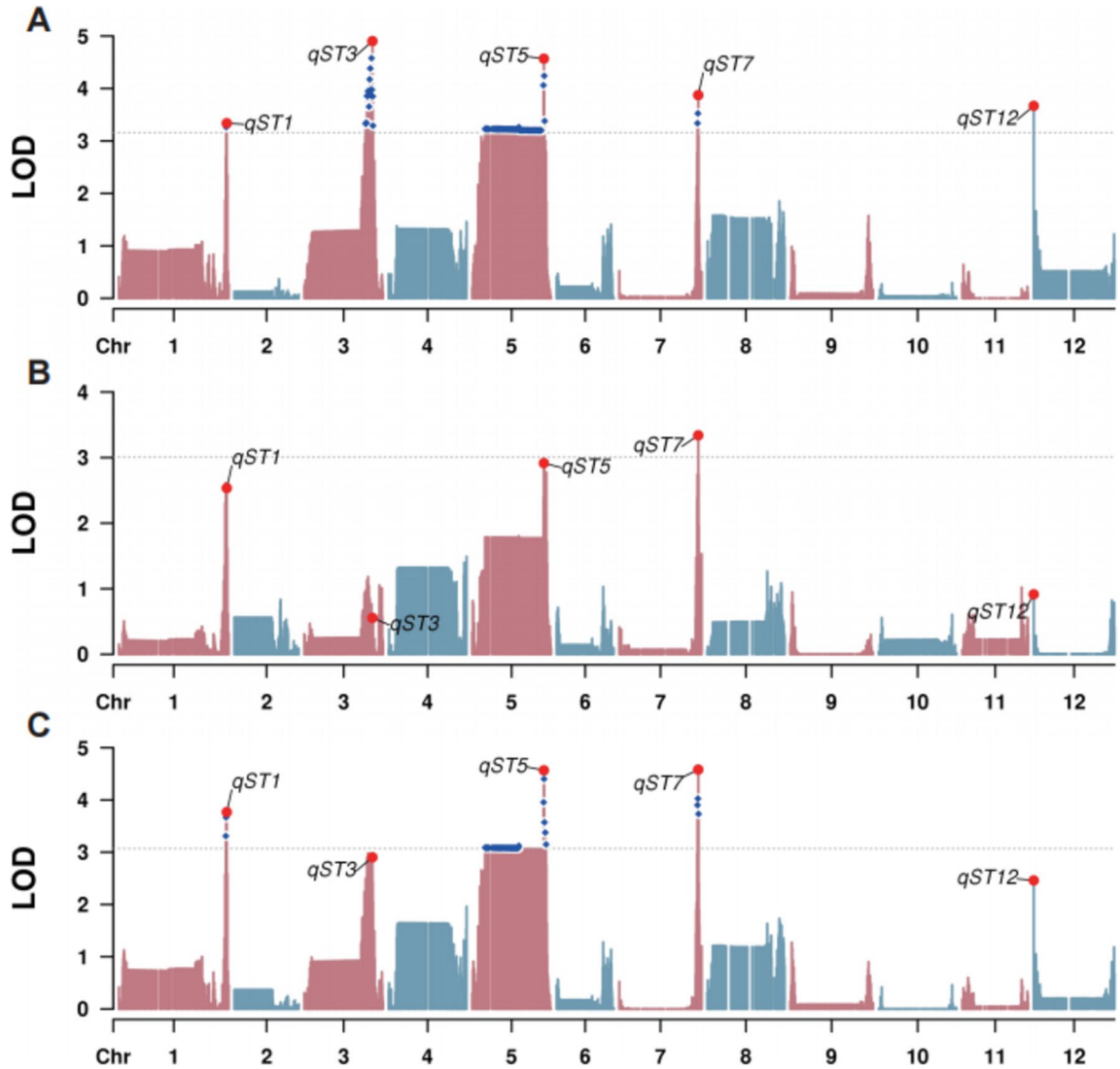

Fig. 2 QTLs identified for seedling stage ST in the RIL population in ST_Y1 (**A**), ST_Y2 (**B**) and ST_BLUP (**C**). Grey dashed lines represent the significant threshold of QTLs in each environment, as determined by 1000 permutations at $\alpha = 0.05$. The blue diamonds indicate the markers passed the threshold. The red points indicate the peak bin of each QTL

favorable allele of *qST1*, *qST3* and *qST12* for increasing seedling stage ST were derived from parent PI, and the MM provided the favorable allele of *qST5* and *qST7*. Several reported genes associated with the response to seedling stage salt stress were found to be located in our QTL region, such as the transcription factor SlbHLH22 [21] in *qST3*, VQ motif-containing protein SlVQ16 [17] in *qST7* and *SlHAK5* [22] in *qST12*.

Identification of QTLs for DT

Using the same linkage map, fifteen QTLs for DT were detected on chr1 (*qDT1-1, qDT1-2* and *qDT1-3*), chr3 (*qDT3-1, qDT3-2* and *qDT3-3*), chr4 (*qDT4*), chr8 (*qDT8-1, qDT8-2,* and *qDT8-3*), chr9 (*qDT9-1*, *qDT9-2*), chr10 (*qDT10-1* and *qDT10-2*) and chr12 (*qDT12*) (Fig. 3; Table S3). Among these QTLs, six were detected in a one year and DT_BLUP; four were detected with more than 10 PVE% in at least one environment (Table S3). We noticed that *qDT1-3* and *qDT8-1* were detected in different environments of the two years (Fig. 3; Table S3). The *qDT1-3* was a major QTL with the largest 7.56 LOD and 15.8 PVE% in DT_Y2. The other major QTL *qDT8-1* accounted for 11.40 PVE% and the LOD was 5.77 in DT_BLUP. The wild parent PI provided all the favorable QTL alleles for increasing DT based on their positive additive effect (Table S3). We also found several reported genes

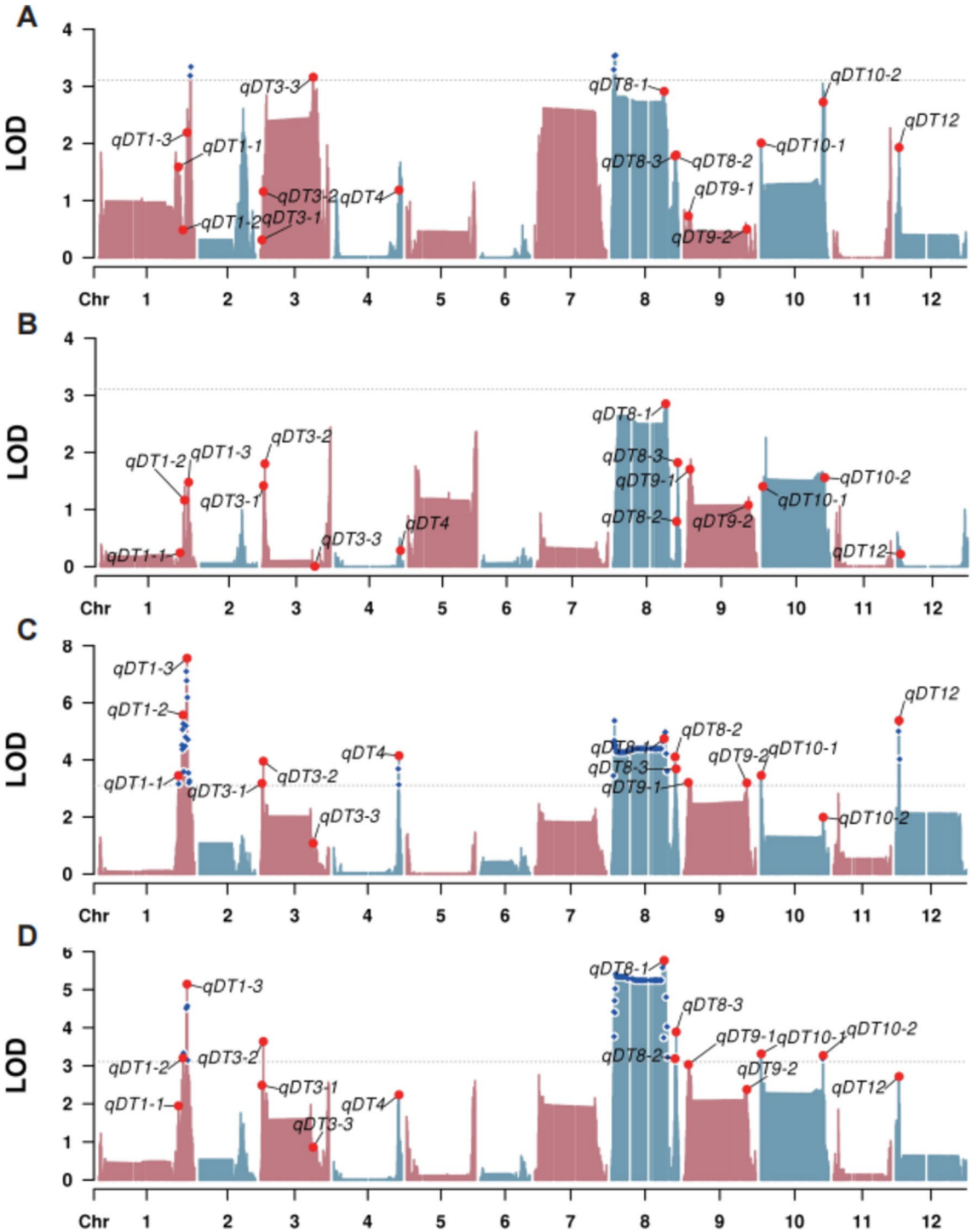

Fig. 3 QTLs identified for seedling stage DT in the RIL population in DT_Y1_1 (**A**), DT_Y1_2 (**B**), DT_Y2 (**C**) and DT_BLUP (**D**). Grey dashed lines represent the threshold of QTLs in each environment, as determined by 1000 permutations at $\alpha = 0.05$. The blue diamonds indicate the markers passed the threshold. The red points indicate the peak bin of each QTL

involved in the drought response in the QTL region, such as the late embryogenesis abundant gene *SlLEA6* [23] in *qDT1-2*, transcription factor *SlGRAS4* [24] in *qDT1-3*, and the transcription factor *SlGRAS40* [25] in *qDT8-2*.

Genetic variation analysis of candidate genes

Methionine sulfoxide reductase (MSR) is an antioxidant enzyme that plays important roles in stress resistance by scavenging free radicals and repairing oxidized

proteins [26, 27]. Interestingly, a top SNP ($-1517^{G/A}$) located in the promoter of *MSRB1* was significantly associated with ST ($p = 2.83 \times 10^{-7}$), which was also located in the region of the stable QTL *qST7* (Fig. 4A, B). Interestingly, this variation changed the number of CAAT-box cis-acting elements in the promoter of *MSRB1* from two to one (Fig. 4C). Moreover, two other SNPs ($+124^{C/T}$ and $+2639^{C/G}$) that caused amino acid substitutions in exons were also significantly associated with ST ($p = 7.59 \times 10^{-6}$ and 8.12×10^{-6}). These three SNPs comprised two major haplotypes, Hap_GCC (MM haplotype) and Hap_ATG (PI haplotype). To our surprise, the lines with the MM haplotype (*SlMSRB1*^{MM}) had a significantly greater survival rate after seedling stage salt stress treatment (Fig. 4D). Subsequently, a phylogenetic tree was constructed using homologous proteins to evaluate the evolutionary relationship of *MSRB1* in different species (Fig. 4E). Frequency analysis of the haplotypes indicated that Hap_GCC was extensively selected during tomato domestication and almost fixed in modern tomato breeding (Fig. 4F). The expression of *SlMSRB1*^{MM} decreased after seedling stage salt stress treatment (Fig. S6A).

A high-salt environment causes plant metabolic disorders and the accumulation of toxic substances. Maintaining intracellular K^+/Na^+ homeostasis plays an important role in improving ST. Tomato high-affinity potassium transporter 5 (*SlHAK5*) is a major system for root K^+ uptake required for plant growth in the presence of salinity [22]. We detected a significant association signal for ST in the region of *qST12*. The most significantly associated SNP, SNP_365326, downstream of the top signal was located 6,766 bp upstream of *SlHAK5*, which exhibited high linkage (Fig. S3A). The lines harboring SNP_365326_T from PI had a greater survival rate in the seeding stage salt stress trial (Fig. S3B). Variation analysis of *SlHAK5* revealed eight SNPs in the coding region

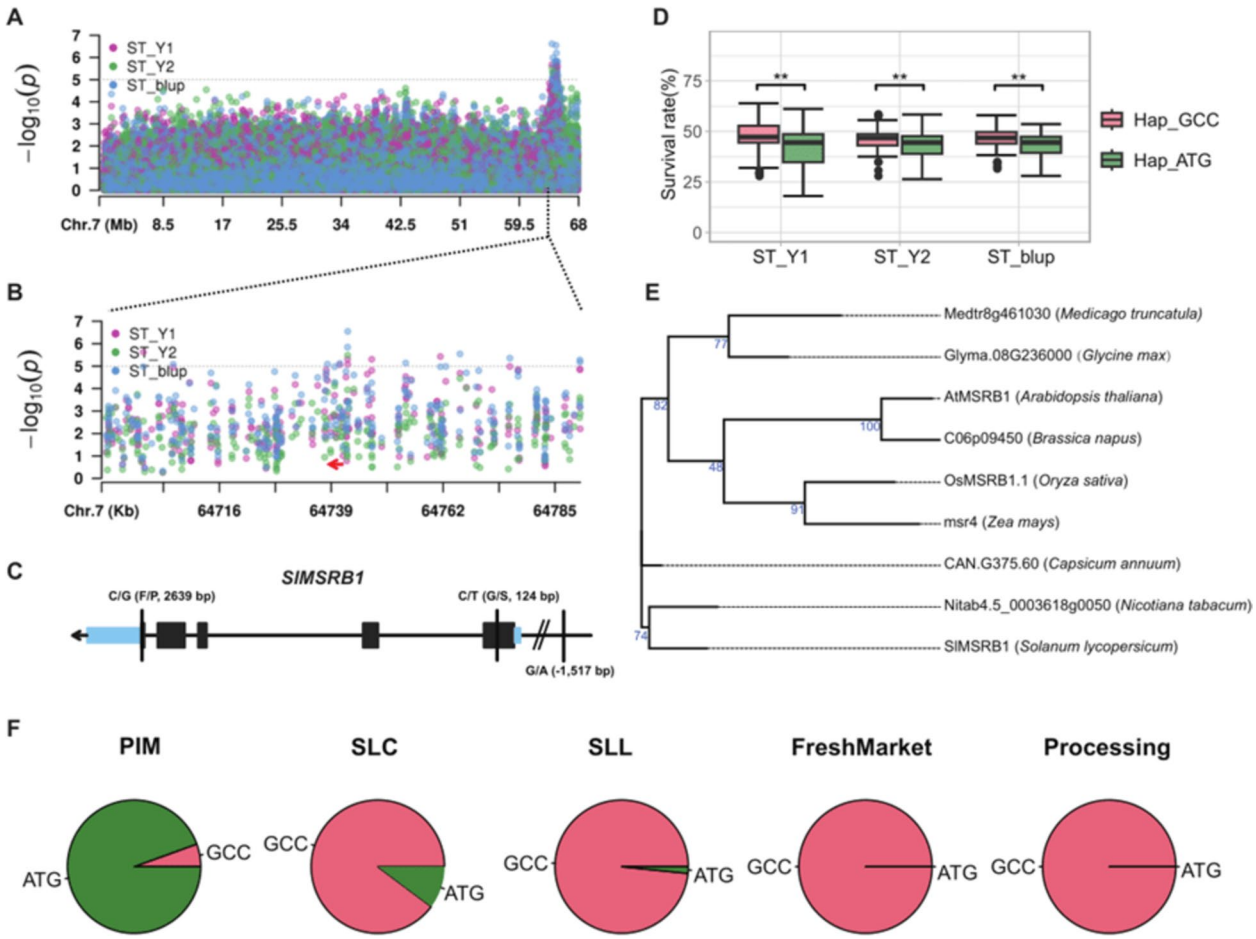

Fig. 4 Identification of candidate gene *SlMSRB1* in *qST7* region. **A** Manhattan plot for loci of ST on chr7. **B** Local Manhattan plot surrounding *SlMSRB1*. The position of the candidate gene is shown as a red arrow. **C** Gene structure and polymorphisms in *SlMSRB1*. **D** Phenotype of two major haplotypes of *SlMSRB1* in the seedling stage salt stress trial. **E** Phylogenetic analysis of MSRB1 proteins in the Dicots PLAZA 5.0 database. **F** Changes in frequency of *SlMSRB1* haplotypes during tomato breeding

and one that caused an amino acid change (Fig. S3C). Using a publicly available database, we constructed an interaction network of *SlHAK5*, which provided a better understanding of its gene biological function (Fig. S3D). Frequency analysis indicated that the putative favorable SNP_365326_T allele was progressively lost during tomato breeding (Fig. S3E).

When we identified the candidate genes responsible for DT, the highest peak in *qDT1-3* (LOD = 7.56) and the most significant top SNP ($p = 2.70 \times 10^{-10}$) located in this QTL region attracted our attention. The previously reported drought-responsive gene *SlGRAS4* was in this QTL region (Fig. S4A). The lines carrying the SNP_82529246_A allele in the promoter of *SlGRAS4* had a greater survival rate after seedling stage drought stress treatment (Fig. S4B). Sequence alignment of *SlGRAS4* revealed two SNPs in the coding region, one of which caused an amino acid change (Fig. S4C). We constructed a gene association network for *GRAS4* via the STRING database to gain a better understanding of the functions of genes involved in the response to stress (Fig. S4D). Frequency analysis indicated that the putative favorable SNP_82529246_A allele was gradually lost during domestication and improvement (Fig. S4E). However, modern processed tomato varieties had a greater frequency of the putative favorable alleles than did fresh market tomato varieties (Fisher's exact test, $p < 0.05$). The expression of $SlGRAS4^{MM}$ decreased after seedling stage drought stress treatment ($p = 1 \times 10^{-3}$), but there was no significant difference in the expression of $SlGRAS4^{PI}$ (Fig. S6B).

To further explore the new DT candidates, we searched the genes in the QTL region and found a BRI-like receptor homolog, BRL1, in which a nonsynonymous mutation (SNP_592) was significantly ($p = 5.54 \times 10^{-6}$) associated with DT in DT_Y2 and DT_blup (Fig. 5A, B). Taking advantage of the 33 released high-quality tomato genomes [28, 29], we conducted multiple sequence alignment of *SlBRL1* and genotyped a total of 17 variations in the coding region (Fig. S5; Table S4). We found that *S. pimpinellifolium* accessions (hereafter PIM) tended to have more variations in the coding region than *S. lycopersicum* L. accessions (hereafter SLL), and there were seven SNPs and one insertion between PI and MM (Fig. 5C; Fig. S5; Table S4). These potential functional variations formed two main haplotypes, and the cultivated tomato lines harboring the ancestral haplotype Hap_TGATGCA had a greater survival rate than did those harboring the Hap_CATAATT haplotype after seedling stage drought stress treatment (Fig. 5D).

To further decipher the vital variation potentially affecting protein function, we conducted a sequence analysis of SlBRL1 homologous proteins in different species. Interestingly, we detected four amino acid substitutions at the conserved positions (198th, 455th, 696th, and 746th positions) of SlBRL1 in the cultivated tomato MM, which could be responsible for the drought-sensitive phenotype (Fig. 5E, F). Frequency analysis of the tomato resequencing populations revealed that Hap_GTGA was highly prevalent in the PIM population but was progressively lost during domestication and improvement. However, we found a significantly greater frequency of putative favorable haplotype in the processing tomatoes than in the fresh-market tomatoes, which might be due to the common drought stress environment in processing tomato cultivation (Fig. 5G). The expression of $SlBRL1^{MM}$ decreased after seedling stage drought stress treatment ($p = 2.17 \times 10^{-6}$), but there was no significant difference in the expression of $SlBRL1^{PI}$ (Fig. S6C).

Frequency change of potential favorable QTLs for seedling stage ST/DT in tomato breeding

To investigate the potential pyramiding effect of favorable QTLs for seedling stage ST/DT in our study, we checked the favorable QTL number of each line in RIL based on the genotype of top bin-marker. As a result, significant positive correlations were observed in linear regression analysis in each stress trial (Fig. 6A, B), which revealed a large additive effect in the seedling stage ST/DT QTLs.

Domestication has resulted in reduced favorable alleles for ST/DT in tomato [30–32]. To explore the QTLs identified in this study potentially lost during breeding, we performed frequency analysis in a population consisting of 828 tomato accessions with majority of released genome resequencing data [33–37]. Twenty tag SNPs with the most significant association signal in each QTL region were selected to conduct frequency change analysis. The potential favorable allele of *qST3*, *qST5* and *qST7* rapidly increased in frequency during domestication and improvement. Interestingly, significant differences in the frequency of potential favorable alleles were found in *qST1*, *qST3* and *qST5* between fresh market tomato and processing tomato (Fisher's exact test, $p < 0.05$), and the former was higher (Fig. 6C).

In addition, the frequency of potential favorable QTLs identified in the seedling stage drought stress trial were analyzed. The favorable alleles of all the 12 QTLs for DT were derived from PI. The frequency change pattern showed that all the potential favorable DT alleles in our study were almost lost during domestication and improvement and existed at an extremely low frequency among the modern tomato varieties (Fig. 6C).

Discussion

Comparison of QTLs for ST/DT

Foolad [4] suggested that salinity tolerance is a stage-specific and developmentally regulated phenomenon, while

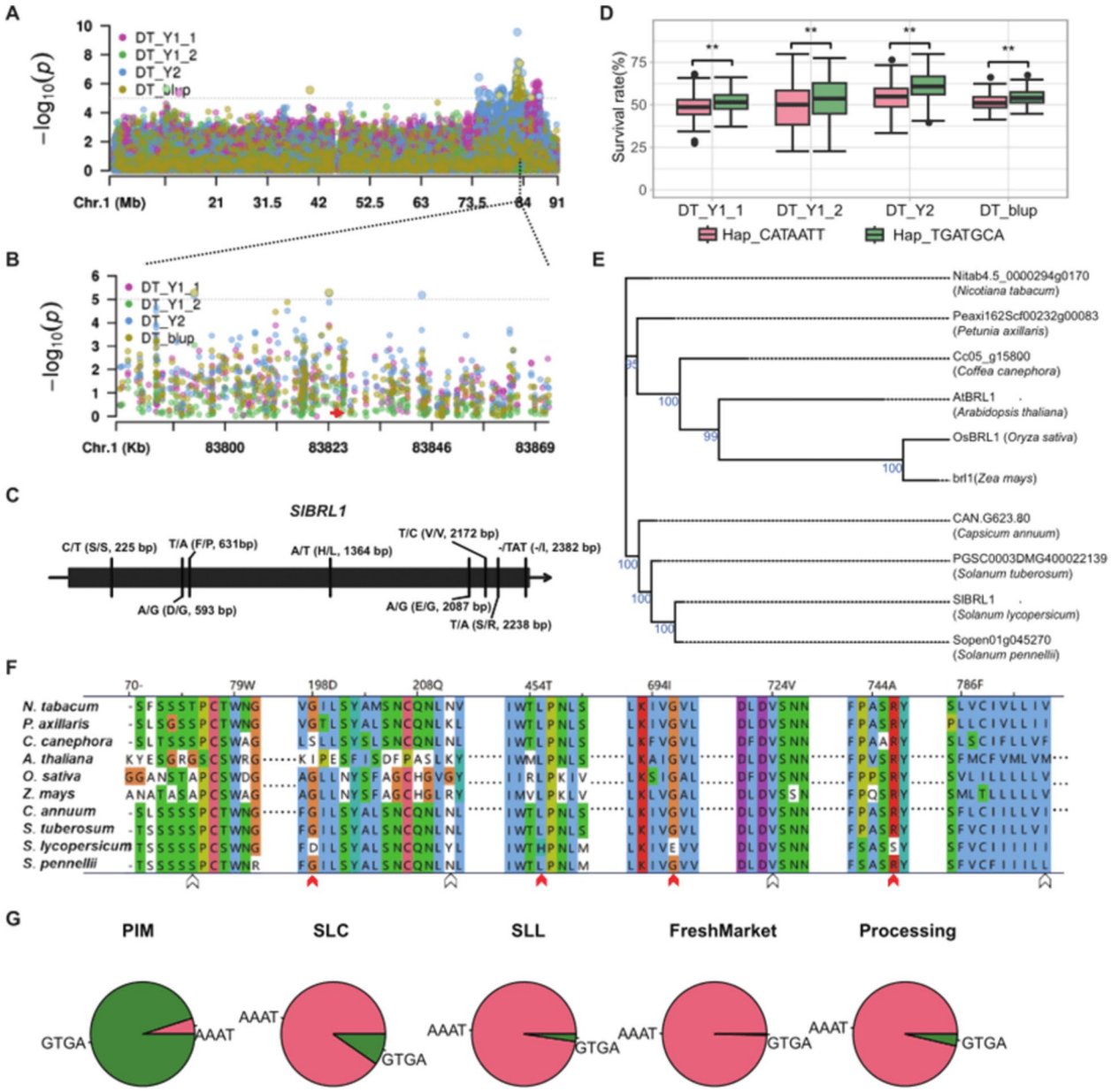

Fig. 5 Identification of candidate gene *SlBRL1* in *qDT1-3* region. **A** Manhattan plot for the DT loci on chr1. **B** Local Manhattan plot surrounding *SlBRL1*. The position of the candidate gene is shown as a red arrow. **C** Gene structure and polymorphisms in *SlBRL1*. **D** Phenotype of two major haplotypes of *SlBRL1* in seedling stage drought stress trials. **E** Phylogenetic analysis of BRL1 proteins in the Dicots PLAZA 5.0 database. **F** Display of the variations in amino acid sequences; the red arrows indicate the four conserved positions. **G** Changes in the frequencies of the *SlBRL1* haplotypes resulting from the four conserved variations during tomato breeding

some QTLs are conserved across species. In contrast to this study, Li et al. [6] found some QTLs for ST in two other wild relatives at the seedling stage. In the *S. pennellii* LA716 introgression line population, four major QTLs were discovered on chr6 (*Stpq6*), chr7 (*Stpq7a, Stpq7b*) and chr11 (*Stpq11*). In the *S. lycopersicoides* LA2951 introgression line population, six major QTLs were identified on chr4 (*Stlq4*), chr6 (*Stlq6*), chr9 (*Stlq9a*, *Stlq9b*), and chr12 (*Stlq12a*, *Stlq12b*). Compared to the above QTLs, we found that *qST7* and *qST12* in our study were colocalized with *Stpa7a* and *Stlq12a*, respectively. Furthermore, *qST1*, *qST3* and *qST12* might colocalize with reported QTLs conferring ST during seed germination or the fruiting stage according to the physical position of flanking markers [38, 39]. In addition, *qDT1-1*, *qDT1-3*, *qDT4*, *qDT8-1*, *qDT10-2* and *qDT12* identified in this

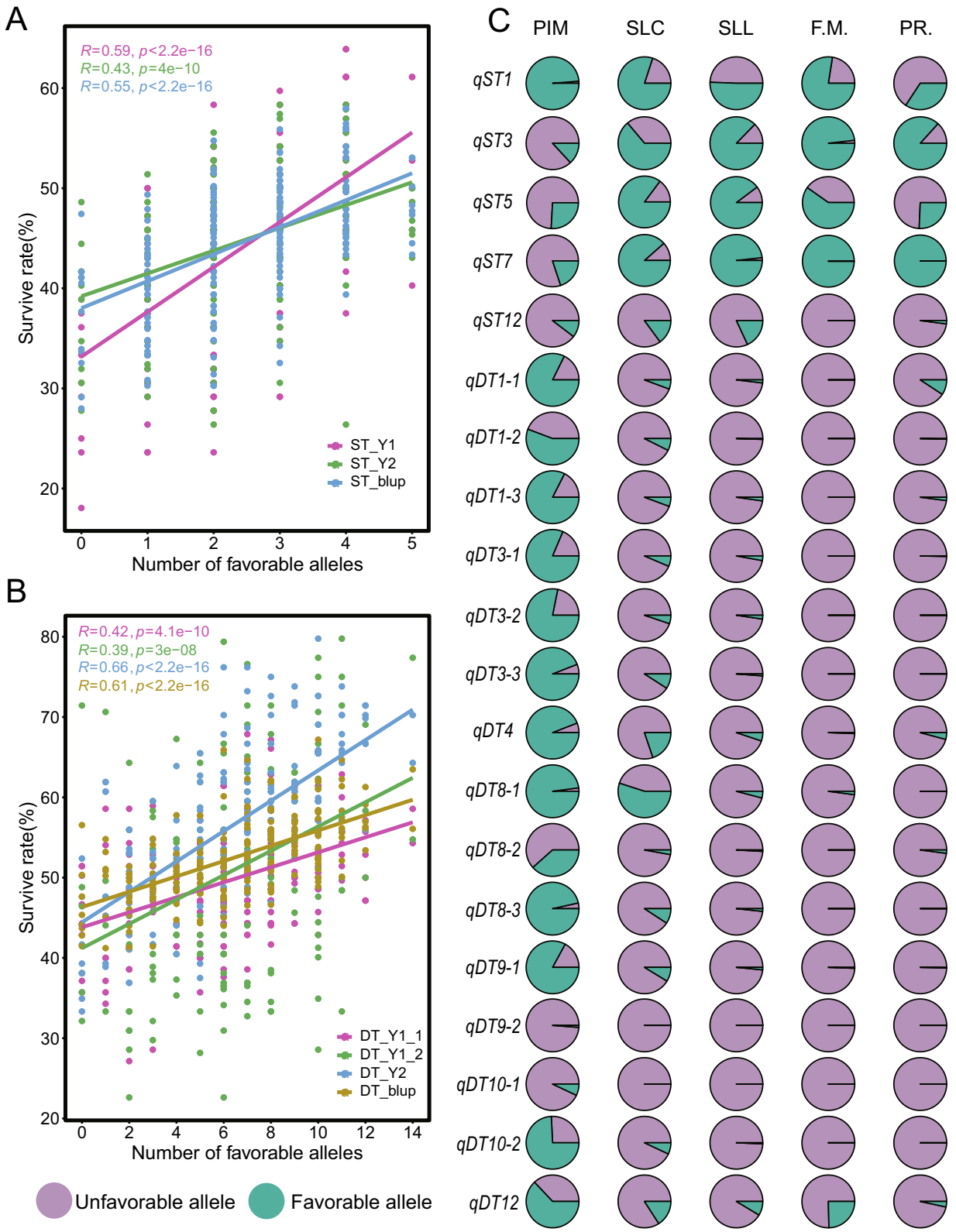

Fig. 6 The potential pyramiding effect and frequency change of favorable QTLs. **A**, **B** The potential pyramiding effect of favorable QTLs in seedling stage salt stress (**A**) or drought stress (**B**) trials, respectively. **C** Changes in frequency of potential favorable alleles in this study during the different breeding stages. PIM: *S. pimpinellifolium*. SLC: *S. lycopersicum var. cerasiforme*. SLL: *S. lycopersicum* L. F.M: modern fresh market tomato. PR.: modern processing tomato

study were also found to be colocalized with previously reported QTLs for DT [7]. Comparison of the number, chromosomal location and derived population of QTLs will facilitate pyramiding of QTLs through marker-assisted selection to develop tomatoes with improved ST/DT throughout the cultivation process.

Salt and drought stresses often occur simultaneously or sequentially in protected cultivation or arid/semi-arid areas. Zhu [1] investigated the complex regulation of salt- or drought-responsive genes. To understand the interactions between salt and drought stresses and their combined influence on plant growth, yield, and quality, there is a need to study these two abiotic stresses in isolation first. Recent studies demonstrated that the effects of salinity and drought were similar in the early stage because both can result in physiological water deficit in plants [1, 15, 40]. However, we did not find any overlapping QTLs in the linkage analysis between the seedling stage salt stress and drought stress trials, and no SNPs were found significantly associated ($p < 1 \times 10^{-5}$) with these two stresses simultaneously in our study. This was probably due in part to the complexity of these two abiotic stresses; compared with drought stress, the toxic ion effect and the nutritional imbalance caused by salt stress had more prominent adverse impacts on plants. In this study, we examined the seedling stage salt/drought stress in pot and tray-grown seedlings, more evaluation indicators (such as chlorophyll and photosynthesis, antioxidant enzyme activity and leaf dry matter accumulation) and more growth stages (such as the adult-plant stage and fruiting stage) in field conditions are needed to assess the ST/DT more comprehensively.

Candidate genes of seedling stage ST/DT

Salt stress can induce the accumulation of reactive oxygen species (ROS), which have toxic effects at high concentrations [41, 42]. MSR protects cells from oxidative damage mainly in two ways. On the one hand, it can repair oxidized methionine residues, whose oxidization usually leads to a decrease in or loss of protein biological activity. On the other hand, MSR also acts as an oxidative repair factor that participates in the scavenging of ROS in cells [26]. Cui et al. [27] demonstrated that *SlMsrB2* functions in DT and promotes chlorophyll accumulation by modulating ROS accumulation. Recent research has reported the role of SlMsrB5 in the tomato immune response to *Botrytis cinerea* [43, 44]. In this work, we first identified *SlMSRB1* as a candidate for *qST7*. Lines carrying the $SlMSRB1^{MM}$ allele exhibited a greater survival rate under seedling stage salt stress treatment, which demonstrated that MM also had favorable QTLs for ST. Previous results from Borsani et al. [45] revealed two loci responsible for ST in the tomato cultivar MM. In this study, we identified a SNP variation in the promoter region of *SlMSRB1* that might change the number of CAAT-box cis-elements.

Compared to that caused by salt stress, the problem caused by drought is even more pervasive and economically damaging [1]. Receptor-like kinases (RLKs) play an important role in plant responses to drought stress [46]. Brassinosteroid INSENSITIVE 1 (BRI1) is a receptor RLK family member on the plasma membrane that perceives BR. The overexpression of the BRI1-LIKE receptor BRL3 was reported to confer DT without penalizing overall growth in *Arabidopsis* [47]. However, genetic research on *BRL1* is only just beginning. In our work, *SlBRL1* was identified as a candidate for *qDT1-3* under seedling stage drought stress. Seven amino acid mutations were found in the protein sequence of this gene between MM and PI, of which four amino acid sites were relatively conserved in different species, as revealed by sequence alignment. Thus, we proposed that these four SNPs might be important in the process of response to seedling stage drought stress. In view of the situation of the growing world population, reducing arable land and the challenges of climate change, the candidate genes and potential functional variations identified in our study provide valuable information for further QTL fine mapping and causal gene discovery in seedling stage salt/drought stress research.

Possible divergent selection of potential favorable alleles for seedling stage ST/DT

The regression analysis showed the great potential of pyramiding favorable QTLs in tomato stress tolerance breeding. However, cultivated tomato have suffered severe bottlenecks during their breeding history; for example, domestication has resulted in the loss of favorable alleles, such as *SlHAK20*, *SlSOS1* and *SlBBX18* in cultivated tomato [30–32]. In our study, the potential favorable allele of *qST12* was lost during tomato breeding, while the potential favorable allele of *qST7* was extensively used in modern tomato breeding (Fig. S3, Fig. 4A, Fig. 6C). The potential favorable allele of *qST1*, *qST3* and *qST5* presented divergent selection between fresh market tomato and processing tomato during modern breeding (Fig. 6C), which might be related to the different growth habit and cultivation environments. Coincidentally, the majority of potential favorable alleles for seedling stage DT identified in our study were lost during tomato domestication and improvement. For example, the potential favorable allele of *qDT3-1* had a frequency of 95.02% in the wild tomato species (PIM), while only 8.89% of the early domesticated cherry tomatoes (SLC) and 2.56% of cultivated tomatoes (SLL). Even though, about 75% DT QTLs not lost completely in our study displayed significant higher favorable allele frequency in modern processing tomato than fresh market tomato (Fig. 6C). Thus, we supposed that there

was a possible divergent selection for the seedling stage ST/DT QTLs during modern breeding of tomato, while more QTLs and experimental evidences are needed for confirming this fact.

Conclusions

We identified five ST QTLs and fifteen DT QTLs at seedling stage by linkage mapping in a tomato RIL population. Genetic variation analysis revealed two potential candidate genes, namely, the methionine sulfoxide reductase SlMSRB1 in the major QTL *qST7*, and the brassinosteroid insensitive 1-like receptor SlBRL1 in the major QTL *qDT1-3*. We explored the frequency change of the potential favorable alleles for QTLs identified in our study during tomato breeding. These results may provide valuable resources for further deciphering the genetic basis of seedling stage salt/drought stress in tomato.

Methods

Plant materials and growth conditions

The wild *S. pimpinellifolium* PI365697 was more salt- and drought-tolerant than cultivar Moneymaker in our previous experiment. A population of 201 RIL was constructed by a single-seed descent method from a cross between Moneymaker (♀) and PI365967 (♂).. Seedlings of each line were grown in seedling trays with 2:1 peat-vermiculite (v/v) media before the salt and drought screening trials. The experiment was performed in a greenhouse at the northern experimental station of Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences in Beijing, China. The salt and drought screening trials were performed in a randomized complete block design with three replicates of each treatment and four plants each replicate. The temperature in the greenhouse was 22–24℃ during the day and 15–16℃ at night. The relative humidity in March and September was about 30–40% and 50–60%, respectively. The plants in the greenhouse were maintained in natural lighting conditions with an average photoperiod of 12.6 h light /7.9 h dark in March, 11.8 h light /7.4 h dark in September.

Salt treatment

Seedlings of each RIL were transferred to 25 L plastic containers with 0.5 × Hoagland solution at the two-leaf stage. The seedling roots wrapped with a sponge were fixed in a foam float board (24 plants per container). When the fourth true leaf expanded, the salt-treated plants were cultivated in a nutrient solution of 50 mM NaCl. The concentration of NaCl was then increased by 50 mM every other day up to 400 mM. The nutrient solution was replaced every 7 days and water was replenished every other day for both the seedlings in the control and salt treatment groups; the pH was adjusted by HCl to 6.0–6.5 for both groups each time. In addition, the ventilation pump operated for one hour every two hours every day. Seedling phenotypes were assessed after 10 days cultivated in 400 mM NaCl solution. The plants were rated for severity of salt susceptibility on a 1–5 scale as described by Dasgan et al. [20]. The scale class was converted into a survival rate: survival rate = 100% × (1-class/6) according to Li et al. [6]. The screening experiments were performed for two consecutive years (in September of the first year, referred to as ST_Y1; March of the second year, referred to as ST_Y2).

Drought treatment

At two-leaf stage, uniformly growing plants of each RIL were selected to be transplanted to pots (10 cm × 11 cm, one seedling per pot). The total weight of seedling and nutrient soil in each pot was controlled to 165 g. All plants were watered daily until the fifth true leaf unfolds to ensure seedlings were able to grow in soil with sufficient water. Before the drought treatment, all the pots were watered 285 ml water. Then, water was withheld from the treatment pots; the control pots were irrigated every day at the same time [5]. The drought-treated plants were then re-watered when 80% of the drought-sensitive parent MM exhibited permanent wilting. Then seedling phenotypes were assessed after 12 h. All the plants were rated for severity of drought susceptibility on a 1–6 scale as described [7]. The scale class was converted into a survival rate: survival rate = 100% × (1-class/7). Drought experiments were performed for two consecutive years (in March and September of the first year, hereafter DT_Y1_1 and DT_Y1_2; in March of the second year, hereafter DT_Y2).

Read mapping and variant calling

Quality control of Illumina HiSeq 2500 resequencing raw reads of 201 RILs was performed using fastp (v0.21.0) with the parameters "–cut_front –cut_tail –cut_mean_quality 20 –length_required 50", which removed low-quality reads and potential adapters. The generated clean reads were aligned to the tomato reference genome (SL4.0, https://solgenomics.net/ftp/tomato_genome/assembly/build_4.00/) by BWA-MEM (0.7.17-r1188). The alignment results were filtered by SAMTools (v1.7) using the "view" function with the parameters "-bq 20" and then sorted using "sort" with the parameters "-O bam". The binary alignment file was marked for potential PCR duplicates using the "MarkDuplicates" function in Picard (v2.23.3). The "HaplotypeCaller" function in GATK (v.3.5) was used to call the raw variants of each accession with the parameter "–emitRefConfidence GVCF". Then, the RIL population variants were subjected to joint genotyping using the "GenotypeGVCFs" function to obtain a single VCF

file containing all the accessions. To obtain high-quality SNP variants, we applied a hard filter with the parameters "QD < 2.0 || FS > 60.0 || MQ < 40.0 || MQRankSum < -12.5 || ReadPosRankSum < -8.0 || SOR > 3.0". The high-quality InDels were exploded by the parameters "QD < 2.0 || FS > 200.0 || ReadPosRankSum < -20.0 || SOR > 10.0". Furthermore, we filtered potential low-quality variants using VCFtools (v 0.1.16) with the parameters "—max-missing 0.5 –maf 0.05". For further analysis, the missing genotypes were imputed with Beagle (v.5.2) with default parameters.

Functional annotation of genetic variationandhaplotype analysis

The annotation and functional effects of the identified variations were predicted by SnpEff (v5.0), and SnpSift (v5.0) was used to manipulate the annotated variants. Variants that caused amino acid changes or were located in the putative regulatory region (2 kb upstream or downstream of the gene) of candidate genes were selected to construct haplotypes. The survival rates of the different haplotypes were determined by two-tailed t-test.

Recombinant bin construction andlinkageanalysis

SNPbinner [48] was used to identify crossover events and to generate a high-resolution genetic map with a minimum recombinant bin size > 250 kb in length [11]. QTL mapping of the RILs was performed using the R/qtl package. We used the composite interval mapping (CIM) method for QTL analysis and a 1000 permutations test ($p < 0.05$) for defining the QTL logarithm (base 10) of the LOD threshold. The QTL confidence interval was finally defined by a 1.5 LOD decrease [49]. The QTL effect and phenotypic variation explained were analyzed via a linear model in R [49].

Genome-wide association study

We used a linear mixed model in the EMMAX package to conduct GWAS involving genome-wide high-quality SNPs. Both the survival rate of a single trial and the BLUP were used to perform GWAS. To determine the GWAS cutoff for classifying significant associations, we estimated the number of genome-wide effective SNPs by pruning SNPs as reported in PLINK –indep-pairwise 5000 100 0.2. After pruning, the number of effective SNPs was 565. We then selected 1×10^{-5} (Benjamini–Hochberg FDR < 0.05) as the genome-wide significance cutoff [50].

Candidate gene natural variation analysis

The sequences of the candidate genes were queried from 32 published genomes, including the genome of Moneymaker [28]. The genome sequence of PI365967 was downloaded from https://ngdc.cncb.ac.cn/gwh/ [29]. The gene sequences of the other genomes were determined by BLASTing the coding sequences of the genes in Heinz1706 against those of the other genomes. Multiple sequence alignment was performed in Jalview 2.11.3.2 (https://www.jalview.org/). The translated amino acid sequences were obtained from SnpGene 2.3.2 (https://www.snapgene.com/). The cis-regulatory elements of the promoter regions were analyzed using PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The protein interaction network was predicted with STRING (https://cn.string-db.org/).

Statistical analysis

The survival rates under salt or drought stress conditions across all trials were fit by a linear mixed model using the lme4 package in R according to the reported formula [50]. The best linear unbiased predictor (BLUP) values:

$$Y_{ij} = \mu + \text{Line}_i + \text{Env}_j + (\text{Line} \times \text{Env})_{ij} + (\text{Env} \times \text{Rep})_{jn} + \text{error}_{ijn}$$

In the above equation, μ is the mean survival rate of each accession, Line_i is the genotype effect of the i-th accession of RILs, Env_j is the effect of the j-th environment (i.e., ST_Y1 and ST_Y2 in ST trials; DT_Y1_1, DT_Y1_2 and DT_Y2 in DT trials), $(\text{Line} \times \text{Env})_{ij}$ is the genotype and environment interaction, $(\text{Env} \times \text{Rep})_{jn}$ is the environment and replication interaction, and error_{ijn} is the error of the j-th environment and the n-th replication.

RNA extraction and qRT-PCR analysis

The fresh leaves of the MM and PI plants in the stress treatment and control groups were collected in ST_Y2 and DT_Y2. Total RNA was isolated using a Quick RNA isolation kit (Huayueyang Biotechnology Co., Ltd., Beijing, China). Ten biological replicates were performed for each trial. Reverse transcription was performed using Hifair® III 1st Strand cDNA Synthesis SuperMix for qPCR (Yeasen Biotechnology Co., Ltd., Shanghai, China). Primers were designed using Primer 5.0 software and are listed in Table S5. qRT–PCR (quantitative reverse transcription polymerase chain reaction) was carried out on a Roche® Lightcycler 480 II platform (Roche Diagnostics Ltd., Shanghai, China). The reaction mixture included 5 μL of Hieff UNICON® Universal Blue qPCR SYBR Green Master Mix (Yeasen Biotechnology Co., Ltd., Shanghai, China), 0.2 μM of each primer, 1 μL of cDNA template (diluted 1:10), and sterile distilled water to make up a total volume of 10 μL. The PCR procedure consisted of a predenaturation step at 95 °C for 120 s, followed by 40 cycles with each

cycle consisting of a denaturation step at 95 °C for 10 s and an annealing/extension step at 60 °C for 30 s. This was followed by melt curve analysis. Three experimental replicates were performed for each sample. The final results were analyzed for relative quantification using the $2^{-\Delta\Delta Ct}$ method.

Abbreviations

BLUP	Best Linear Unbiased Prediction
BRI1	Brassinosteroid Insensitive 1
BRL1	BRI-like 1
CIM	Composite Interval Mapping
DT	Drought Tolerance
GWAS	Genome-wide Association Study
LOD	Likelihood of Odd
MSR	Methionine Sulfoxide Reductase
PVE	Phenotypic Variance Explained
QTL	Quantitative Trait Locus
RIL	Recombinant Inbred Line
RLKs	Receptor-like Kinases
ROS	Reactive Oxygen Species
SNP	Single Nucleotide Polymorphism
ST	Salt Tolerance

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions
J.L. and L.L. conceived and designed the research. X.L. (Xin Li), X.L. (Xiyan Liu) and F.P. performed the experiments, analyzed the data, and wrote the manuscript. J.H., Y.H., R.B. and C. Z (Chen Zhang) conducted the gene expression analysis. Y.L., Y.W., Z.L., C.Z. (Can Zhu), Y.G., and Z.H. performed the phenotypic evaluation. J.L., L.L., X.W., and Y.D. constructed the RIL population.

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Data availability
The raw Illumina sequencing reads of RILs are available in the NCBI Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra/) under accession number SRP063485. Resequencing data of the tomato population for frequency analysis are extracted from the NCBI (BioProjects: PRJNA259308, PRJNA353161, PRJNA454805, PRJEB5235, PRJNA557253, PRJEB5226, PRJEB5227, and PRJEB5228). Some of the tomato hybrids resequencing data used are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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