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A natural promoter variation of *SlBBX31* confers enhanced cold tolerance during tomato domestication

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Summary

Cold stress affects crop growth and productivity worldwide. Understanding the genetic basis of cold tolerance in germplasms is critical for crop improvement. Plants can coordinate environmental stimuli of light and temperature to regulate cold tolerance. However, it remains unknown which gene in germplasms could have such function. Here, we utilized genome-wide association study (GWAS) to investigate the cold tolerance of wild and cultivated tomato accessions and discovered that increased cold tolerance is accompanied with tomato domestication. We further identified a 27-bp InDel in the promoter of the CONSTANS-like transcription factor (TF) *SlBBX31* is significantly linked with cold tolerance. Coincidentally, a key regulator of light signalling, SlHY5, can directly bind to the *SlBBX31* promoter to activate *SlBBX31* transcription while the 27-bp InDel can prevent S1HY5 from transactivating *SlBBX31*. Parallel to these findings, we observed that the loss of function of *SlBBX31* results in impaired tomato cold tolerance. *SlBBX31* can also modulate the cold-induced expression of several *ERF* TFs including *CBF2* and *DREBs*. Therefore, our study has uncovered that *SlBBX31* is possibly selected during tomato domestication for cold tolerance regulation, providing valuable insights for the development of hardy tomato varieties.

Introduction

Tomato (*Solanum lycopersicum*) is one of the most important crops in the world due to its enormous industrial and nutritional values (Tomato Genome Consortium, 2012; Zhu *et al*., 2018). Cold stress is a major concern for the global tomato industry as cold stress adversely influences plant growth (Ding and Yang, 2022; Zhu, 2016). Two ranges of low temperatures, chilling (0–15 °C) and freezing (<0 °C), are used to characterize the type of cold stress. These temperatures drastically affect the plant metabolism and transcriptomes, causing impaired plant membrane fluidity, poor germination, arrested seedling development and chlorosis (Gong *et al*., 2020; Sanghera *et al*., 2011; Zhang *et al*., 2022; Zhu, 2016). In Arabidopsis, a number of transcription factors (TFs), including CBFs (C-REPEAT/DRE BINDING FACTOR) and ICE1 (INDUCER OF CBF EXPRESSION 1), and protein kinases and phosphatases have been well characterized for their roles in the cold stress response (Chong *et al*., 2022a; Ding *et al*., 2020; Guo *et al*., 2018; Shi *et al*., 2018). Recently, a tomato study reported that the nucleotide variation in the key W-box of *SlWRKY33* promoter interrupted its self-transcriptional activation and protein accumulation, thereby attenuated cold signalling pathways activation in cultivated tomato (Ailsa Craig) in comparison to wild tomato varieties (Guo *et al*., 2022). Furthermore, the crosstalk between light and cold signalling pathways has also been known in plants but the genetic basis of cold tolerance in tomato, in particular, which gene(s) may coordinate light and temperature to confer cold tolerance in germplasms remains largely unclear.

In recent years, genome-wide association study (GWAS) has been utilized as a powerful tool for dissecting the genetic basis of tomato traits. GWAS was shown to be useful for determining the key gene (*SlMYB12*) that controls fruit peel colour in 360 tomato varieties (Lin *et al*., 2014). The genetic basis of tomato flavour was also unfolded through a GWAS analysis of 398 tomato varieties (Tieman *et al*., 2017). In 2018, a GWAS approach was utilized to analyse more than three thousand fruit metabolites in 442 accessions (Zhu *et al*., 2018). More recently, *SlHAK20* and *SlSOS1* were found as key regulators of salt tolerance during tomato domestication using GWAS (Wang *et al*., 2020a,b).

Here, we employed GWAS and uncovered that a natural promoter variation of a cold-induced TF, *SlBBX31*, contributes to

the cold tolerance of a natural tomato population. *SlBBX31* was found to positively regulate cold tolerance and a 27 bp InDel in the *SlBBX31* promoter of natural wild tomato species can influence the plant's response to cold. Coincidentally, SlHY5, a key regulator of light signalling, was discovered to activate the *SlBBX31* promoter. Introducing the 27 bp InDel in the *SlBBX31* promoter partially impairs the SlHY5-mediated activation of *SlBBX31*. Furthermore, SlBBX31 was demonstrated to directly bind to the promoters of cold-responsive *CBFs* to activate their transcription. These findings offer insights into a germplasms gene that is selected during domestication. More importantly, our results reveal new information about the tomato cold response pathway, which may help with the engineering of tomato with improved cold tolerance.

Results

Cold tolerance was positively selected during tomato domestication

A quantitative phenotypic analysis of cold response-related traits was conducted in 317 tomato germplasms consisting of 44 *S. pimpinellifolium* (PIM) accessions, 109 *S. lycopersicum* var. *cerasiforme* (CER) accessions, 160 *S. lycopersicum* (BIG) accessions and 4 wild species (Figure 1a). CER is a domestication line originated from PIM, and BIG is an improved line from CER. After subjecting tomato plants to cold temperature (4 °C), we performed electrolyte leakage (EL) measurement and assigned each plant with a score from 1 to 10 to indicate the plant's cold tolerance level. The average EL value from the PIM group was found to be significantly greater than that of the CER and BIG groups (Figure 1b). Consistently, the average cold tolerance score was lower in the PIM group relative to the CER and BIG groups (Figure 1c). These two sets of quantitative data showed a strong correlation (R^2 = 0.82, P = 2.2 × 10^{-16}), indicating that our screening method for cold tolerance phenotypes is reliable (Figure 1d). Moreover, the phenotype differences between PIM and CER were more evident than those of CER and BIG, indicating that the cold tolerance trait was likely selected during the domestication stage.

As illustrated in Figure 1e, TS-183 (BIG accession) is more tolerant to cold stress compared to TS-123 (PIM accession). In fact, several tomato accessions from the BIG group (TS-1, TS-48 and TS-183) displayed a greater sign of enhanced cold tolerance

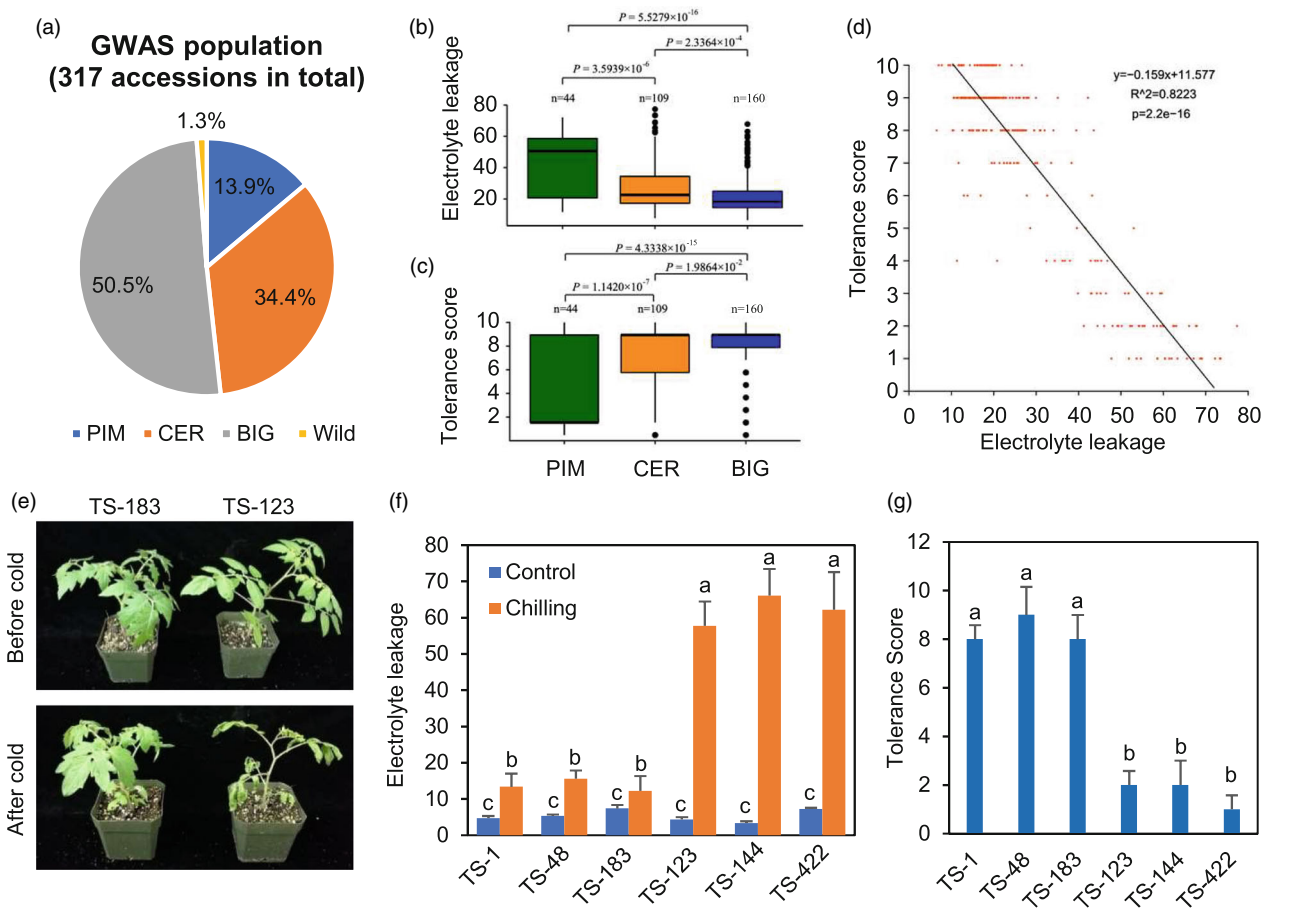

Figure 1 Tomato domestication was accompanied with increased cold tolerance. (a) Distribution of four wild tomato accessions, PIM (*S. pimpinellifolium*), CER (*S. lycopersicum var. cerasiforme*) and BIG (*S. lycopersicum*) accessions in the 317 tomato GWAS population. (b) Boxplot showing the average electrolyte leakage (EL) values of the three tomato groups after cold treatment. (c) Boxplot showing the average cold tolerance scores of the three tomato groups. (d) Correlation between the EL data and cold tolerance scores. (e) Representative plant phenotypes of TS-183 (BIG) and TS-123 (PIM) before and after cold treatment. (f) EL data of the selected tomato accessions BIG (TS-1, TS-48, and TS-183) and PIM groups (TS-123, TS-144, and TS-422) under control and cold conditions. (g) The cold tolerance score of the selected tomato accessions in f. Data represent mean ± SD from three biological replicates. Different letters represent significant differences, as determined by two-way ANOVA with Tukey's post hoc test (P < 0.05).

than the tomato accessions from the PIM group (TS-123, TS-144 and TS-422) as indicated by the relatively smaller EL values and higher cold tolerance scores found in the BIG accessions in comparison to the PIM accessions (Figure 1f,g).

A 27-bp variation in *SlBBX31* promoter is associated with cold tolerance and is selected during domestication

We next used GWAS analysis to uncover the genetic basis of cold tolerance in tomato. The two cold stress-related parameters tolerance score, EL and 2 316 472 SNPs were used for statistical analysis. The *P*-values of 1.7×10^{-6} were set as the significance threshold after Bonferroni-adjusted correction. As illustrated in Figure 2a,b, the signal corresponds to the most significant cold tolerance score and EL was found on chromosome 7. The two most prominent SNPs associated with cold tolerance score and EL (07_58916699 and 07_58916852) were identified in the 5′UTR and exon region of *Solyc07g053140*, which encodes a B-Box protein SlBBX31 (Figure 2c). Thus far, we know that SlBBX31 contains two SNPs (C/T and T/G) in the tomato population (Figure 2d). Most tomato accessions harbouring C/Ts were uncovered from the PIM group while accessions harbouring T/Gs mostly presented in the CER and BIG groups (Figure 2e). Interestingly, accessions with C/Ts had significantly lower tolerance scores and higher EL values, which are in agreement with observed signs of cold tolerance (Figure 2f,g).

To confirm whether *SlBBX31* was the candidate gene for improving germplasm's cold tolerance, we first examined its gene expression in response to cold stress. We selected several tomato accessions (including cold-tolerant and cold-sensitive accessions) from the three groups for cold treatment at 0 h, 6 h and 24 h and we then determined their *SlBBX31* expression. As shown in Figure 2h, it was revealed that cold treatment could significantly induce the expression of *SlBBX31*. Interestingly, this induction was considerably lower in the cold-sensitive accessions (TS-23 and TS-123) than in the cold-tolerant accessions (TS-1 and TS-183). The expression of *SlBBX31* was significantly different between the cold-tolerant and cold-sensitive accessions, indicating that there may be a causative variation in the gene regulatory region. Thus, we sequenced the promoter region of *SlBBX31* in several cold-sensitive and cold-tolerant accessions. We next found a 27-bp insertion in the promoter region (about 400 bp upstream of the initiation site) of cold-sensitive accessions, while cold-tolerant accessions were found without such insertion (Figure 3a). To further determine whether this variation was selected during domestication, we performed an in-depth assessment of this variation in all tomato accessions. We observed that most BIG accessions had the same genotype without a 27-bp insertion (Hap1). In contrast, more than half of the PIM accessions contained the homozygous insertion and heterozygous genotype (Hap2). The frequencies of this insertion for PIM, CER and BIG were 52.78%, 4.71% and 0.82%, respectively, suggesting that the allele for increased cold tolerance was selected in CER. It was also indicated that domestication may be accompanied by this 27 bp natural variation (Figure 3b).

In addition, the nucleotide sequence diversity, including *SlBBX31* and its flank sequence, was calculated. The highest diversity value appeared at the site of the 27-bp insertion for both PIM (1.59×10^{-3}) and CER (0.76×10^{-3}). Meanwhile, there was a noticeable decline from PIM to CER and from CER to BIG. The allelic frequency and nucleotide sequence diversity values for the 27-bp insertion in these three groups suggested that the cold-tolerant allele was possibly selected during the domestication stage (Figure 3c). To further confirm this, we also examined the accessions containing Hap1 and Hap2. We found that Hap1 accessions had higher tolerance scores but smaller EL values relative to those accessions with Hap2 (Figure 3d,e), further supporting the preferred selection of this 27 bp natural variation during domestication.

Inserting *SlBBX31* promoter with a 27 bp variant affects the activation by SlHY5

The light signalling pathway has been known to play a role in the plant's ability to respond to cold (Guo *et al.*, 2018). A master regulator in the light signalling pathway, ELONGATED HYPOCOTYL 5 (HY5), has been reported to interact with several BBXs and bind to the *BBXs* promoter in plants (Li *et al.*, 2021; Zhang *et al.*, 2020). To determine whether BBX could integrate light and temperature factors to perform cold stress response in tomato, we subsequently examined the protein–protein interaction of SlHY5 and SlBBX31. Our yeast two-hybrid (Y2H) and luciferase complementation imaging (LCI) assays (Figure S1) demonstrated that there was no direct interaction between SlHY5 and SlBBX31. As a bZIP-type TF, HY5 has been reported to bind to ACE motif (ACGT) (Lee *et al.*, 2007). We have successfully identified four ACE motifs in the promoter sequence of *SlBBX31* (Figure 4a). To confirm whether SlHY5 can directly bind to the *SlBBX31* promoter, we first conducted yeast one-hybrid (Y1H) assay. Our results showed that SlHY5 can activate two haplotypes of *SlBBX31* promoter reporters (Figure 4b). To further investigate whether the 27-bp variation affects the activation of *SlBBX31* by SlHY5, we co-expressed SlHY5-GFP (effector) with two types of *SlBBX31pro:LUC* reporters (hap1 and hap2) in dual-luciferase reporter assays (Figure 4c). As shown in Figure 4d, the expression of *SlBBX31pro:LUC* (Hap 1) or *SlBBX31pro*(+27 bp)*:LUC* (Hap 2) only resulted in weak and detectable LUC activities. When SlHY5-GFP was co-infiltrated with these constructs, the LUC activities of both *SlBBX31pro:LUC* and *SlBBX31pro*(+27 bp)*:LUC* were significantly enhanced. Importantly, *SlBBX31pro:LUC* (Hap1) activities were higher relative to the *SlBBX31pro*(+27 bp)*:LUC* (Hap2). Because an equal amount of SlHY5 protein was detected in immunoblot analysis (Figure 4e), the results suggested that *SlBBX31* is a target of SlHY5 and the 27 bp insertion in the *SlBBX31* promoter interferes with the transcriptional activation by SlHY5.

In addition, we examined the cold tolerance phenotype of *slhy5* mutants, which were generated by CRISPR-Cas9 as described previously (Wang *et al.*, 2021b). The two alleles of *slhy5* mutant plants (*slhy5-13* and *slhy5-29*) were evidently more sensitive to cold stress compared to the WT. Consistently, the *slhy5* mutants had much higher EL values than the WT (Figure 4f, g), implying a crucial role of *SlHY5* in tomato cold tolerance regulation.

SlBBX31 positively regulates tomato cold tolerance

In order to study the biological function of *SlBBX31* in cold tolerance, we first investigated its tissue expression pattern. As shown in Figure 5a, RT-qPCR results showed that *SlBBX31* is expressed in most tissues with greater expression present in developing buds and mature flowers. To validate the function of *SlBBX31* in the tomato cold response, we used CRISPR-Cas9 to generate two independent mutant alleles of *SlBBX31* in the Ailsa Craig cultivar. As demonstrated in Figure 5b, two *slbbx31* mutant

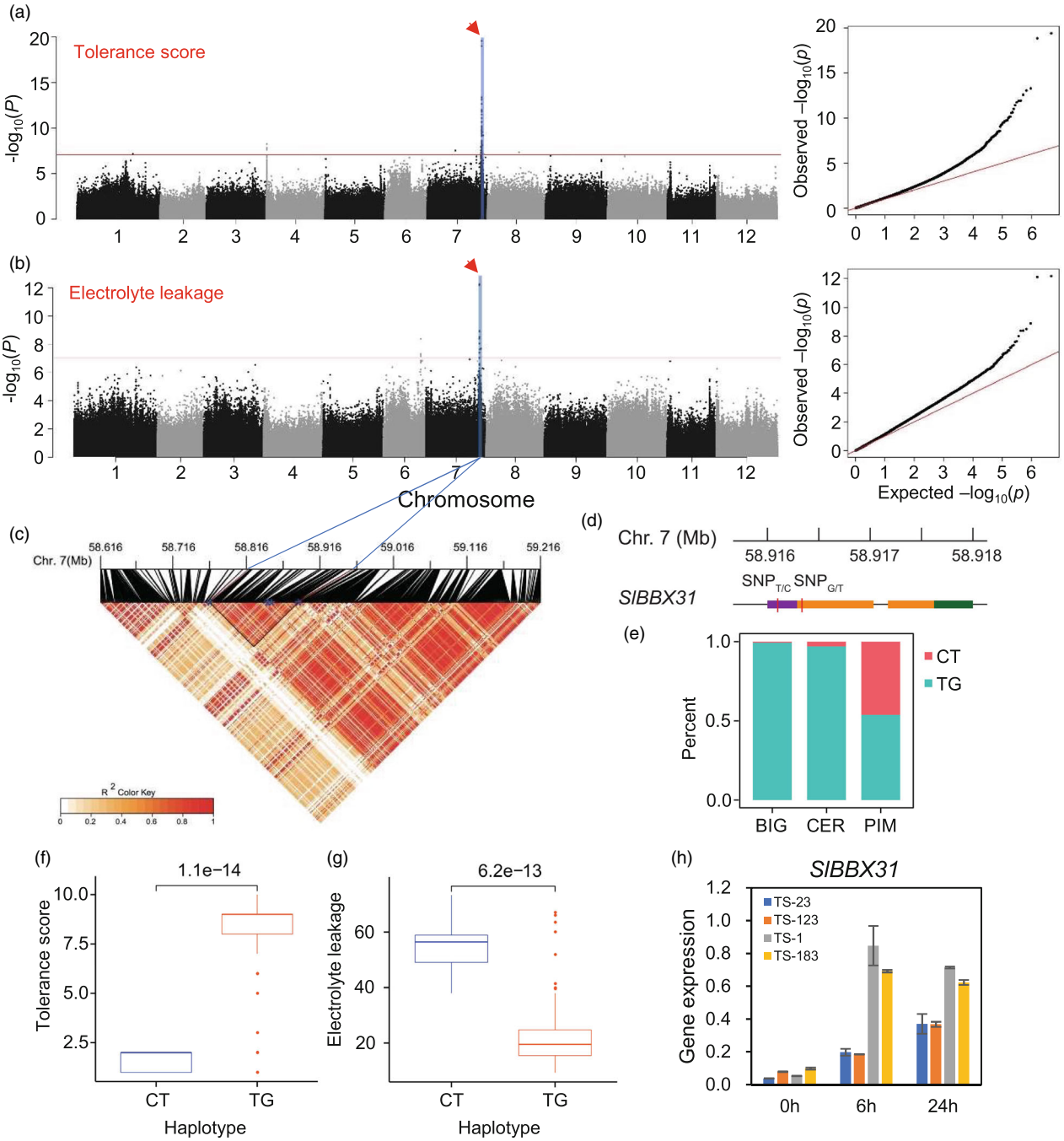

Figure 2 The variation in *SlBBX31* promoter is associated with cold tolerance. (a) Manhattan plot representing the SNPs associated with the cold tolerance score and its quantile-quantile plot (QQ-plot). (b) Manhattan plot representing the SNPs associated with EL and its quantile-quantile Plot (QQ-plot). (c) The top two SNPs and the pairwise LD analysis. (d) The location of two lead SNPs on chromosome 7. (e) The distribution of two haplotypes of lead SNPs in tomato PIM, CER and BIG groups. (f,g) The boxplots showing the average tolerance score and EL value of tomato accessions harbouring the indicated type of SNPs. (h) RT-qPCR showing the cold-induced expression of *SlBBX31* in the cold tolerant and sensitive tomato accessions. Tomato *Actin 7* was used as a control. Data represent means ± SD of the three biological repeats.

allele plants were created, one allele with 1 bp insertion and another allele with 2 bp deletion. After cold treatment, both alleles of *slbbx31* mutant plants displayed obvious cold tolerance compromise compared to the WT (Figure 5c), as indicated by the darker leaves stained by 3,3′-diaminobenzidine (DAB) and by the increased EL values (Figures 5d,e). These data suggested that *SlBBX31* can positively regulate cold tolerance.

Genome-wide identification of cold-responsive genes regulated by *SlBBX31*

To gain insights into the mechanism underlying the roles of *SlBBX31* in regulating cold tolerance, we conducted RNA sequencing to identify *SlBBX31*-regulated cold-responsive (*COR*) genes in tomato using the following criteria: genes up- or

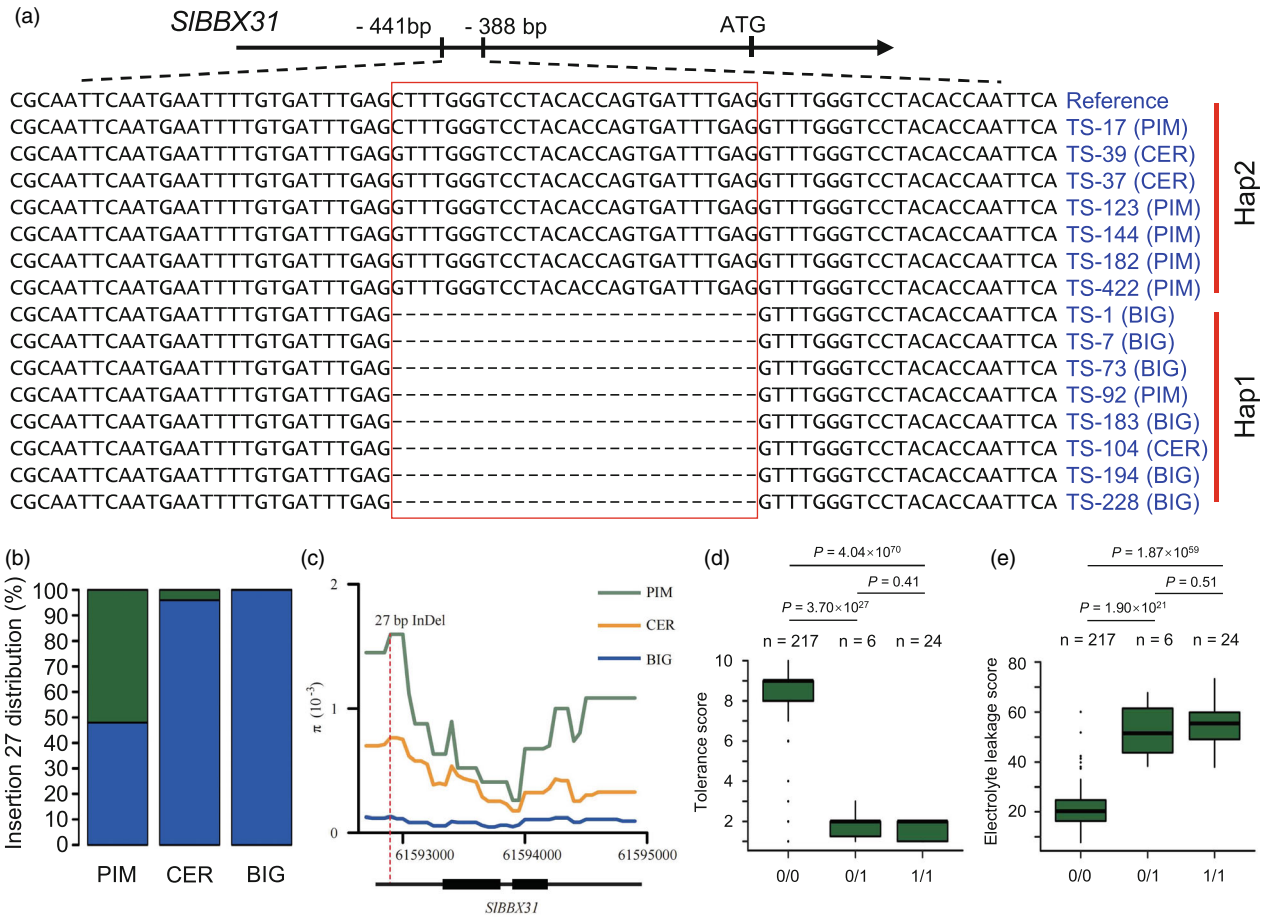

Figure 3 The 27-bp variation of *SlBBX31* promoter is accompanied with domestication. (a) The promoter variation of *SlBBX31* (two haplotypes) in cold tolerant and sensitive accessions. (b) The *SlBBX31* promoter allele distribution in the tomato natural population. (c) The nucleotide diversity (Pi) of *SlBBX31* genomic region in PIM, CER and BIG accessions. The 27-bp InDel position in the promoter is marked with red vertical line. (d) Boxplot showing the correlation between allele variation and cold tolerance score. *n* indicates the number of accessions. (e) Boxplot showing the correlation between allele variation and electrolyte leakage. *n* indicates the number of accessions.

downregulated more than fourfold change by cold treatment with adjusted *P* value <0.05; and genes differentially expressed in *slbbx31* mutants compared to WT after cold treatment. As illustrated in Figure 6a, a total of 623 genes and 2550 genes were identified as *COR* genes in wild-type tomato after 3 h and 24 h cold treatment, respectively. Among these 2817 *COR* genes identified in the WT, 842 *COR* genes were regulated by *SlBBX31* because they exhibited differential expression pattern in *slbbx31* mutants compared to WT, representing about 30% of all *COR* genes, which reinforced the important role of *SlBBX31* in regulating tomato cold response (The *COR* genes identified from RNA-seq were listed in Table S1). Gene Ontology (GO) enrichment analysis revealed that the *SlBBX31*-regulated *COR* genes were mainly involved in TF activity, protein serine/threonine kinase activity, iron ion binding and oxidoreductase activity processes (Figure 6b). The heatmap generated using *SlBBX31*-regulated *COR* genes indicated that *SlBBX31* may be a positive regulator as the expression of most cold-induced *COR* genes was reduced in *slbbx31* mutants (Figure 6c). We further analysed the *SlBBX31*-regulated TFs and found that the cold induced expression of many ERF/DREB and WRKY-type TFs was lower in *slbbx31* mutants (Figure 6d), implying that *SlBBX31* may regulate their transcription in response to cold stress.

To better detect the direct SlBBX31-binding motifs and target genes in tomato, we subsequently performed DAP-seq experiments. We identified 930 and 910 potential SlBBX31-binding peaks from two biological replicates, with 446 overlapping peaks correspond to 310 genes (Table S2). As shown in the pie chart of Figure 6d, nearly 70% of all peaks were located in the 3 kb promoter regions. By analysing the binding peaks, we found that SlBBX31 shows a preferential binding to the promoters that contain two types of motifs, both of which contain G-box element (Figure 6e). GO enrichment analysis of these potential SlBBX31 target genes revealed that they are mainly enriched in the functional roles of photosynthesis, translation, transcription and ATP synthesis coupled electron transport (Figure S2).

SlBBX31 activates the transcription of *SlCBFs*

To validate the altered expression of those TFs identified from RNA-seq analysis, we performed RT-qPCR in WT and *slbbx31* mutants under normal and cold treatment. Our results showed that the cold induced expression of tomato *CBF1*, *CBF2*, *CBF3* and *DREB1-like* was compromised in *slbbx31* mutant plants compared to WT (Figure 7a). In addition, we also checked the expression of two WRKY type TFs *SlWRKY22* and *SlWRKY40*, and our results revealed that cold induced expression of *SlWRKY22* and

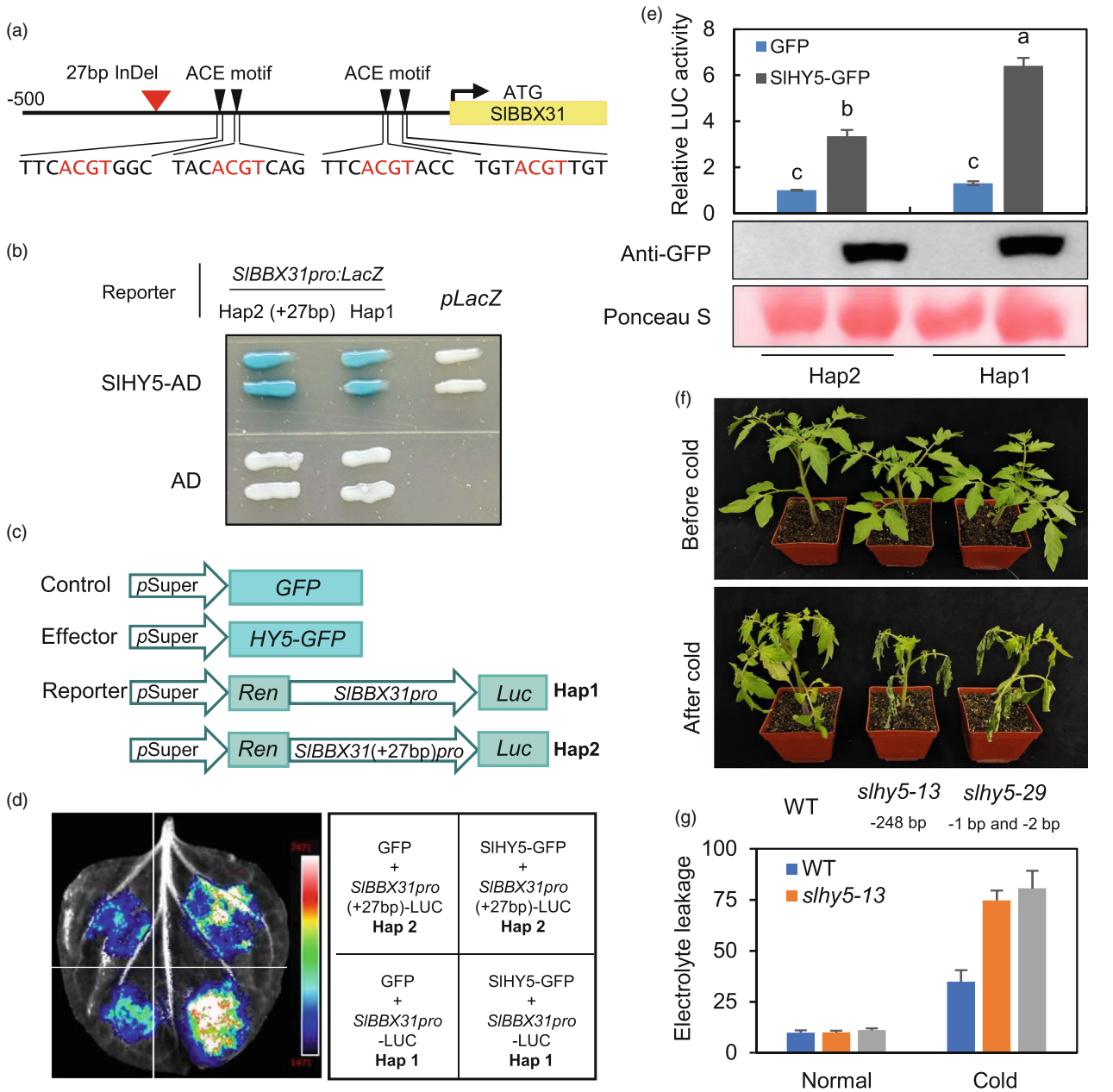

Figure 4 The 27-bp variation of *SlBBX31* interferes its transactivation by SlHY5. (a) The distribution of ACE motifs in the SlBBX31 promoter. (b) SlHY5 directly activates *SlBBX31* promoters in Y1H assays. (c) The schematic diagram of SlHY5-GFP (effector) and two haplotypes of SlBBX31pro-LUC reporter constructs used for the transactivation assay. (d) SlHY5 promotes the transcription of *SlBBX31* promoter in the transactivation assay. (e) The quantification of LUC activities for d. The protein expression of SlHY5 was detected by Western blot using anti-GFP antibodies. (f) *slhy5* mutants are hypersensitive to cold. (g) The electrolyte leakage values of WT and *slhy5* mutants under normal and cold treatment conditions. Data represent means ± SD of three technical repeats. The experiments were repeated at least three times independently with similar results.

SlWRKY40 was also suppressed by *SlBBX31* mutation (Figure S3), confirming the positive role of *SlBBX31* in regulating the expression of *COR* genes.

It has been reported that BBX proteins can bind to CCAAT and G-box motifs (An *et al.*, 2021; Plunkett *et al.*, 2019), our DAP-seq data also confirmed that SlBBX31 can be enriched at G-box element. Thus, we analysed the promoter sequence of *SlCBFs*, given that they are the most recognized cold-responsive genes. Interestingly, *SlCBF1* and *SlCBF2* promoters contain multiple CCAAT and G-box motifs, while *SlCBF3* only contains one CCAAT motif (as illustrated in Figure 7b). We further performed transactivation assay to study if SlBBX31 can activate the promoters of *SlCBF2* in Arabidopsis protoplasts. Our results indicated that the both *SlCBF1pro-LUC* and *SlCBF2pro-LUC* reporters, rather than *SlCBF3pro-LUC* reporter, were significantly activated by SlBBX31 (Figure 7c).

Discussion

Cold stress poses serious threat to tomato plant growth and yield. Many important crops from the tropical areas such as tomato are also sensitive to cold. However, the molecular mechanisms

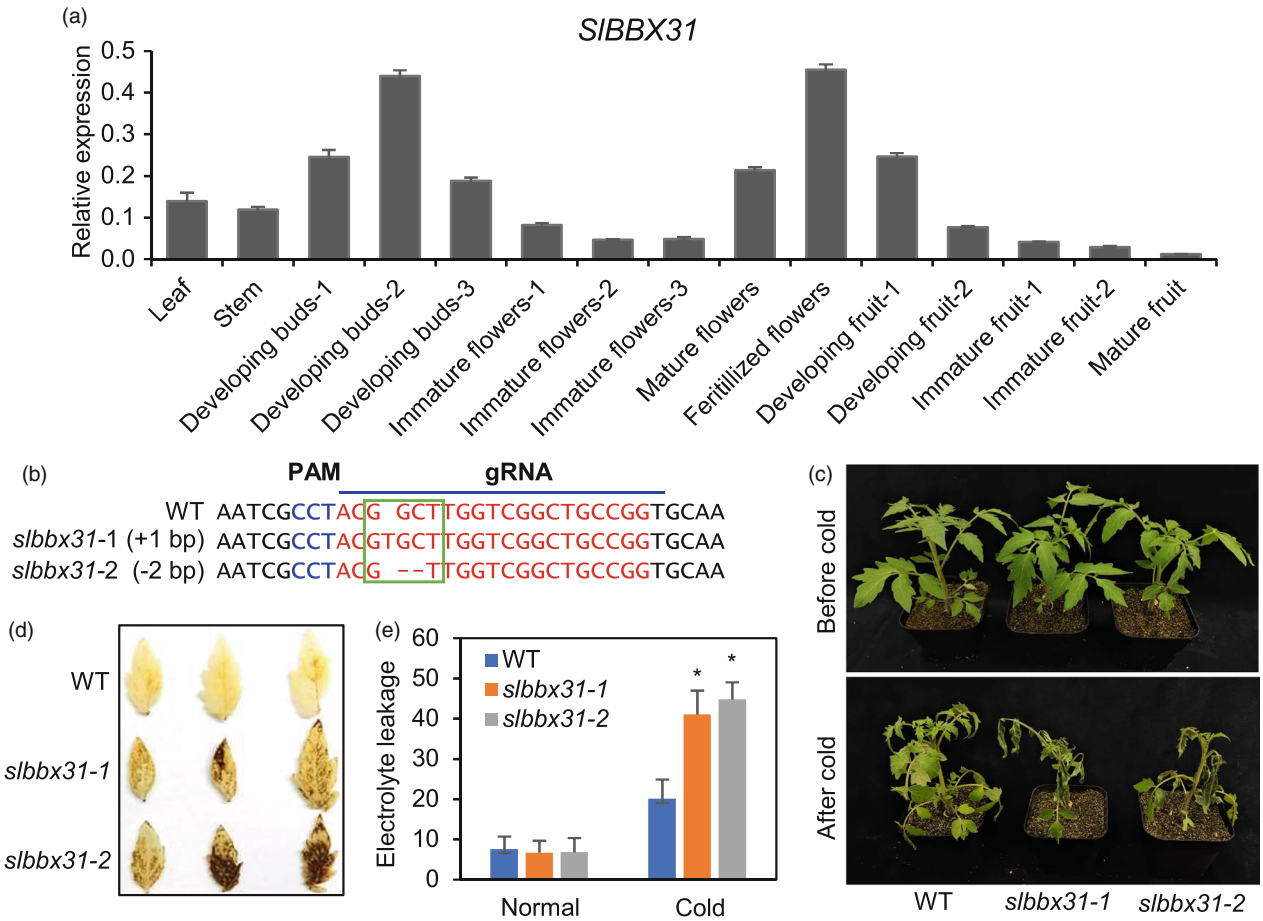

Figure 5 *SlBBX31* positively regulates cold tolerance. (a) RT-qPCR showing the *SlBBX31* expression in different tomato tissues and developing stages. Data represent means ± SD of three technical repeats. (b) The two mutation types of *slbbx31* mutant lines in tomato via CRISPR-Cas9 gene editing. (c) The reduced cold tolerance of *slbbx31* mutants relative to WT. Representative phenotypes of the wild type and *slbbx31* mutant plants before and after cold treatment. (d) DAB staining of the leaves detached from the WT and *slbbx31* mutant plants after cold treatment. (e) Electrolyte leakage data for c. Data represent means ± SD of three technical repeats. The experiments were repeated at least three times with similar results. *$P < 0.01$, Student's *t* test, relative to WT.

underlying tomato cold response are unclear. In this study, we utilized GWAS to investigate the cold-related phenotypes of 317 tomato natural accessions at seedling stage and uncovered an increase in cold tolerance during tomato domestication. This is in contrast with our previous observation of domestication causes reduced tomato's salt tolerance (Wang *et al.*, 2020b). We identified that a 27-bp variation in the *SlBBX31* promoter is tightly associated with cold tolerance in the tomato population. We also discovered that the 27-bp sequence in the *SlBBX31* promoter is absent in BIG, while many PIM accessions in our study contain the 27-bp insertion; implying that this genetic variation may be involved in domestication. Furthermore, we found that the expression of *SlBBX31* is highly induced by cold stress, and that this 27-bp insertion in its promoter can interfere with its expression. Our genetic data validated the positive role of *SlBBX31* in regulating cold tolerance. More importantly, we showed that the key regulator of light signalling, SlHY5, promotes the transcriptional activation of *SlBBX31* while this function is interrupted by the 27-bp variant inserted into the *SlBBX31* promoter (Figure 7d). In addition, we have identified *SlBBX31*-regulated *COR* genes and its binding motifs by RNA-seq and DAP-seq analyses to shed light on the molecular mechanism of tomato cold tolerance.

The light signalling pathway plays a crucial role in plant cold tolerance. Several components of the light signalling pathway have been reported to be involved in the cold response of Arabidopsis and tomato. Light receptors, Phytochromes (PhyA and PhyB) and Cryptochromes (CRYs), as well as TFs phytochrome-interacting factors (PIFs) have been demonstrated to positively regulate plant cold tolerance (Ding *et al.*, 2020; Guo *et al.*, 2018). HY5 is a bZIP TF which functions as a central regulator that integrates light and environmental signals (Gangappa and Botto, 2016; Wang *et al.*, 2021a; Xu, 2020). In tomato, HY5 has been demonstrated to regulate ion uptake, metabolite accumulation and abiotic stress tolerance (Dong *et al.*, 2021; Guo *et al.*, 2021b; Lee *et al.*, 2007; Yang *et al.*, 2022; Zhang *et al.*, 2020). BBX family proteins also contribute key roles in light-dependent development, circadian clock and adaptation to abiotic stress (Bu *et al.*, 2021; Gangappa and Botto, 2014; Song *et al.*, 2020). Interestingly, HY5 and BBXs are reported to function together in regulating hypocotyl elongation, anthocyanin accumulation and transcriptional regulation (Heng *et al.*, 2019; Xu, 2020; Zhao *et al.*, 2020). Our present work suggests that the genetic variation of *SlBBX31* promoter sequence is important for the SlHY5-mediated *SlBBX31* activation, supporting that light signalling is critical for cold

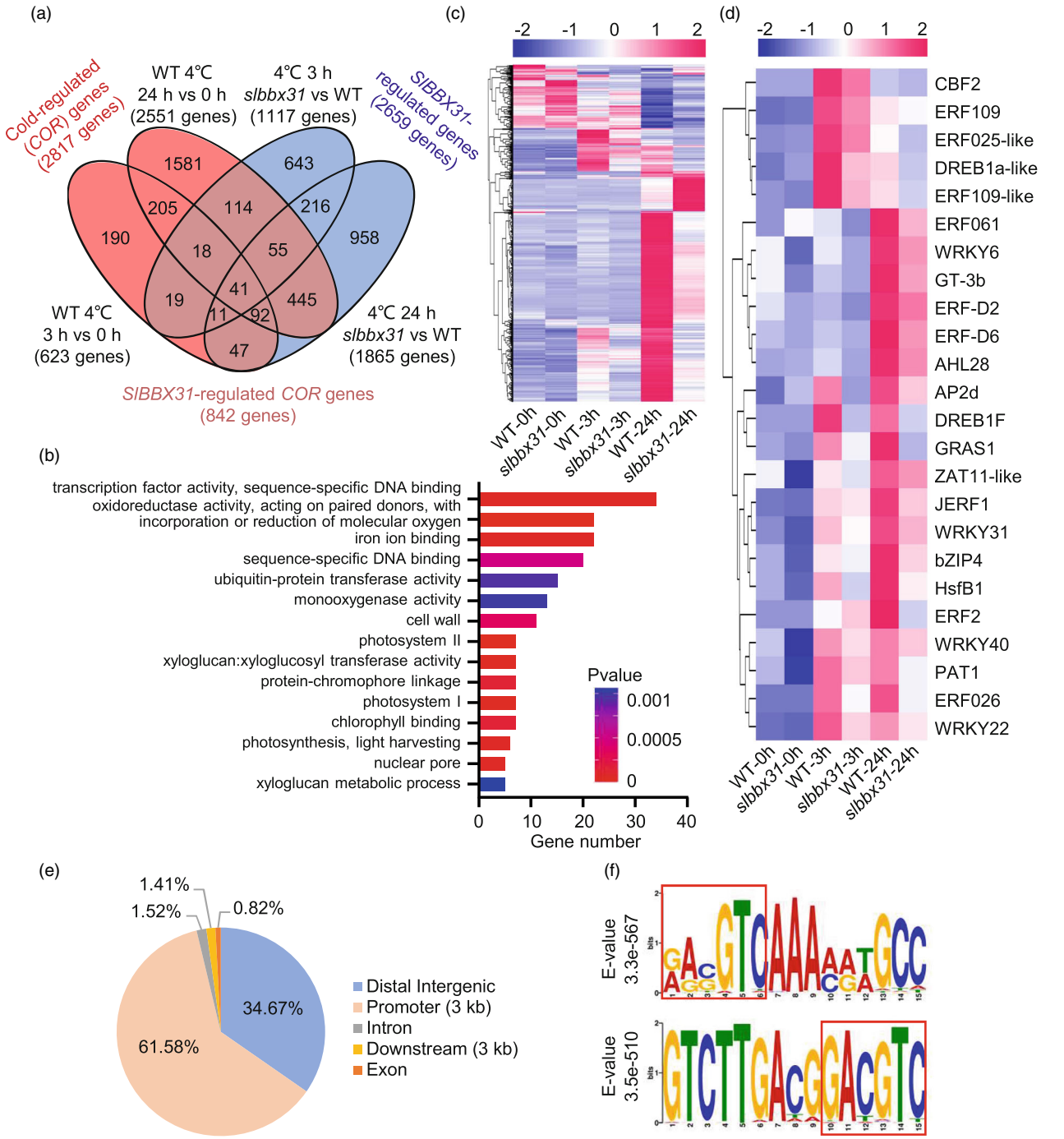

Figure 6 Transcriptomic analysis of *SlBBX31*-regulated *COR* genes. (a) Venn diagrams showing the number of *SlBBX31*-regulated *COR* genes. (b) GO enrichment analysis of *SlBBX31*-regulated *COR* genes. (c) The heatmap generated with *SlBBX31*-regulated *COR* genes. (d) The heatmap generated with selected *SlBBX31*-regulated TFs. (e) Distribution of SlBBX31-binding peaks in the tomato genome from DAPs-seq analysis. (f) The enriched binding motifs of SlBBX31 identified from DAP-seq.

tolerance in tomato. Moreover, our transcriptomic analysis revealed many *COR* genes, especially many cold-induced ERF/DREB type and WRKY TFs, are positively regulated by *SlBBX31*. It is well known that ERF/DREB TFs including CBFs play important roles in regulating the cold response pathway and the transcription of *COR* genes (Shi *et al*., 2018). Our DAP-seq analysis further uncovered two new SlBBX31-binding motifs, which are also present in the *SlCBF1/2* promoters. Moreover, we validated that SlBBX31 can directly bind to the promoters of *SlCBF1/2* to activate their transcription, indicating that SlBBX31 may act as a critical regulator for the transcriptional activation of cold-induced *SlCBFs*. In summary, our present work identified a natural variation in the *SlBBX31* promoter that confers tomato's cold tolerance, and it was possibly selected during tomato domestication based on our evidence. Furthermore, we not only found that *SlBBX31* has relevance with light but also can serve as a key

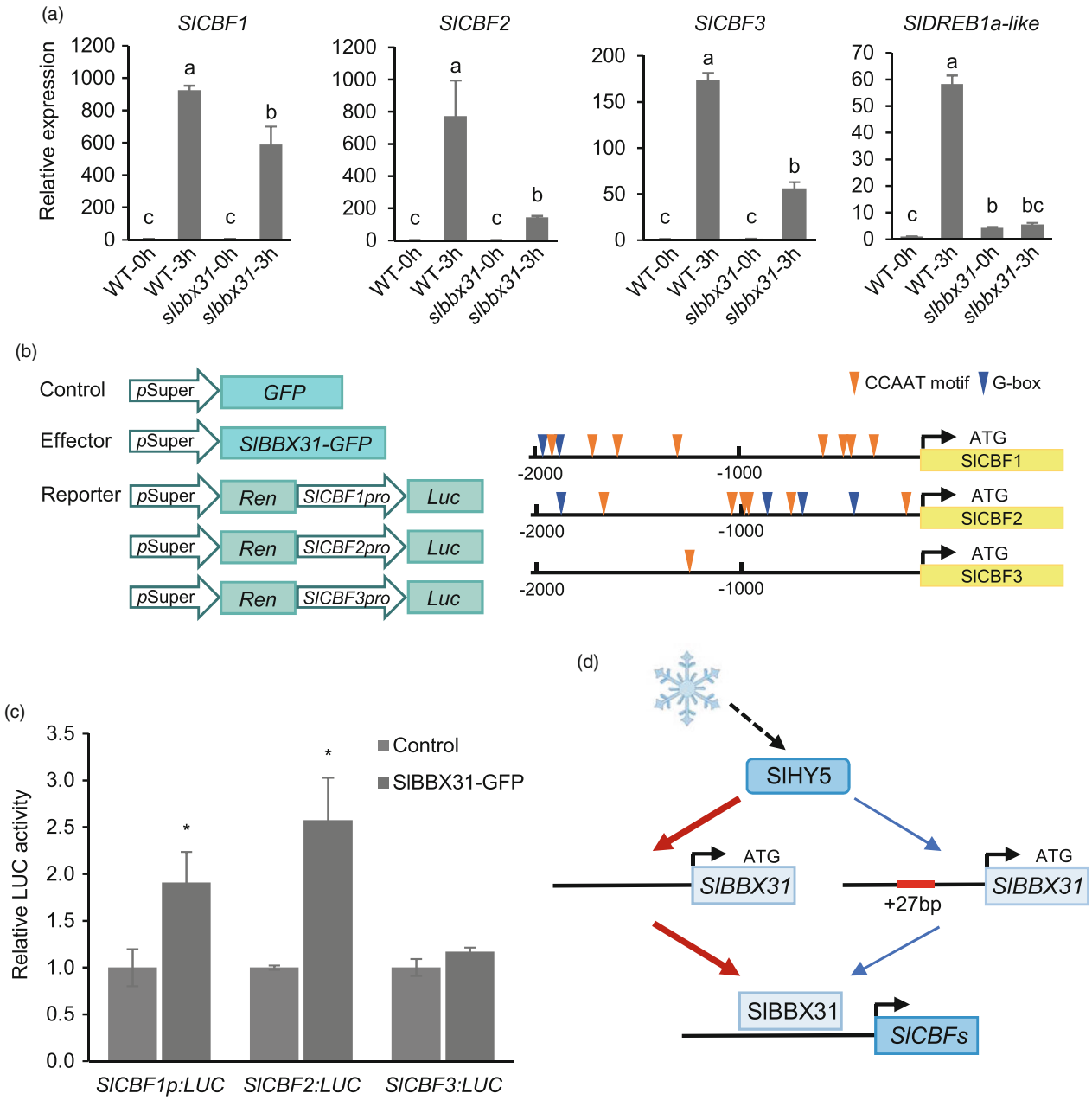

Figure 7 SlBBX31 is essential for the cold-induced transcription of *SlCBFs*. (a) The expression of *SlCBF1*, *SlCBF2*, *SlCBF3* and *SlDREB1a-like* in WT and *slbbx31* mutant plants under normal and cold treatment conditions. The expression of WT at normal temperature was set to 1. Tomato *actin 7* was used as a normalization control. Data represent mean ± SD from three biological replicates. Different letters represent significant differences, as determined using two-way ANOVA with Tukey's post hoc test ($P < 0.05$). (b) Illustration of potential bind motifs of SlBBX31 in the *SlCBFs* promoters and the effector and reporter construct used for the transactivation assay. (c) The activation of *SlCBFs-LUC* reporter by SlBBX31 in dual luciferase reporter assays. (d) The proposed model for SlBBX31-mediated cold signalling. Cold-activated SlHY5 directly binds to the promoter of *SlBBX31* to promote its transcription, which could further facilitate the activation of cold-induced *SlCBFs* to enhance the tomato cold tolerance.

regulator in the tomato cold signalling pathway, thereby providing useful information for the future design of a hardy tomato line.

Methods

Plant materials and growth conditions

All tomato accessions used in this study are described in (Lin *et al.*, 2014; Wang *et al.*, 2020b). Tomato seeds were first germinated in soil, and seedlings with identical growth were transferred to separate pots and grown in greenhouse with 16 h/8 h light–dark cycles.

Cold treatment and tolerance evaluation

At least three replications of 5-week-old tomato plants from each accession were moved into a cool room (4 °C) with 16/8 h of light/darkness for 5–7 days. The cold stress phenotyping was performed and repeated at four independent times. The cold

tolerance score was evaluated by the percentage of wilting leaves (1–10). 1 means extreme sensitivity (most leaves exhibited severe wilted symptoms) and 10 indicates extreme tolerance (none of the leaves showed obvious wilted symptoms).

The electrolyte leakage (EL) measurement

The electrolyte leakage (EL) value was measured as follows: fully expanded leaves from each accession were detached and immersed in 50 mL tubes with 25 mL distilled water. After gentle shaking overnight, the initial electrolyte conductivity (E1) was measured with the conductivity meter before autoclaving the samples. After cooling the samples to room temperature, a second electrolyte conductivity (E2) was gathered. The relative electrolyte leakage was calculated as: E1/E2 × 100. The EL measurements were repeated at three independent times.

GWAS analysis

A total of 317 accessions were used for cold tolerance investigation. The SNPs datasets were obtained in a previous study (Lin *et al*., 2014). To conduct GWAS for the cold-related traits, we used the EMMAX software (Efficient Mixed-Model Association eXpedited vbeta; https://genome.sph.umich.edu/wiki/EMMAX), with 2 316 472 SNP across the entire tomato genome (minor allele frequency >5% and missing ratio < 10%). The calculated genome-wide significance threshold value was 8.88×10^{-8}. Linkage disequilibrium (LD) heatmap was constructed using the R package 'LDheatmap' based on all the SNPs in the targeted genomic regions.

CRISPR vector construction and tomato transformation

The gRNAs were designed to target the tomato genomic sequence, the CRISPR vector was constructed as described in (Chong *et al*., 2022b). Tomato (Alisa Craig cultivates) transformation was conducted in Biogel company. Homozygous T3 mutant plants without Cas9 were used for phenotypical analysis. Primers are listed in Table S3.

RNA isolation and RT-qPCR

Total RNA of tomato leaf tissues was extracted with RNAiso (TaKaRa) following the instruction. 1 μg of RNA was used for first-strand cDNA synthesis (TaKaRa). RT-qPCR was conducted on QuantStudio 5 instrument (Applied Biosystems) with SYBR Green Master Mix (YEASEN). Gene expression was normalized to Act 7 as described in (Zhu *et al*., 2020). Primers used in this study are listed in Table S3.

Dual luciferase reporter transactivation assays

The 1–2 kb promoter sequence upstream of start codon was cloned into the pGreenII-0800-LUC vector as described in (Zhu *et al*., 2020). SlHY5 or SlBBX31 was cloned into 35S: GFP vector (pCAMBIA1300) or 35S: MYC vector as an effector. The transactivation assays were conducted in *N. benthamiana* leaves or Arabidopsis protoplasts as described in (Guo *et al*., 2021a).

RNA-seq analysis

Three biological replications of 5-week-old wild-type tomato (WT) and *slbbx31* mutant plants were treated with 4 °C or with mock treatment. Total RNA was extracted with Trizol reagent. The RNA-seq was conducted in BioMarker company. The clean reads were mapped to the reference tomato genome (https://phytozome-next.jgi.doe.gov/info/Slycopersicum_ITAG2.4) using Tophat2 tool software (Kim *et al*., 2013). Gene function was annotated based on the following databases: Nr (NCBI non-redundant protein sequences). Differentially expressed genes (DEGs) between two sample groups were analysed using the DESeq R package. The FDR <0.01 (false discovery rate) and FC ≥2 (fold change) were set as the thresholds for significant DEGs. GO enrichment analysis of the DEGs was implemented by the GOseq R package (Young *et al*., 2010). Heat maps were drawn using the online platform OmicShare tools (https://www.omicshare.com/tools) according to the FPKM values. Sequences have been deposited at the Sequence Read Archive of the National Center for Biotechnology under BioProject numbers PRJNA848126.

DAP-seq analysis

The DAP-seq and data analysis were performed as described (Cao *et al*., 2020; Chong *et al*., 2022b). In brief, SlBBX31-HaloTag bead mixture was incubated with tomato genomic DNA library. The binding DNA by SlBBX31 was eluted from the beads for sequencing. The DAP-seq reads were aligned to the tomato reference genome (iTAG2.4). SlBBX31-binding motifs were identified by MEME-ChIP (Machanick and Bailey, 2011).

Yeast one hybrid (Y1H)

The activation domain-fusion effectors and LacZ reporters were constructed and co-transformed into yeast strain EGY48 as described in (Xu, 2020). The detailed yeast transformation and liquid assay were conducted according to the Yeast Protocols Handbook (BD Clontech).

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Conflict of interest

The authors declare no competing interest.

Author contributions

YZ, LC, JKZ and SWH conceived the project. YZ, LC, RX and ZJ performed the biological experiments. GZ, JY and TL conducted the data analysis. YZ, LC and JKZ wrote the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 List of differentially expressed COR genes identified from RNA-seq.

Table S2 List of SlBBX31-enriched peaks identified from DAP-seq.

Table S3 Primers used in this study.

Figure S1 SlHY5 does not interact with SlBBX31 in Y2H (a) and LCI assays (b).
Figure S2 GO analysis of SlBBX31 enriched peaks from DAP-seq.
Figure S3 The expression of *SlWRKY22* and *SlWRKY40* in WT and slbbx31 mutants under normal and cold treatment.