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SlGRAS4 mediates a novel regulatory pathway promoting chilling tolerance in tomato

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Summary

Tomato (*Solanum lycopersicum* L.) plants are cold-sensitive, and the fruit are susceptible to postharvest chilling injury when stored at low temperature. However, the mechanisms underlying cold stress responses in tomato are poorly understood. We demonstrate that *SlGRAS4*, encoding a transcription factor induced by low temperature, promotes chilling tolerance in tomato leaves and fruit. Combined genome-wide ChIP-seq and RNA-seq approaches identified among cold stress-associated genes those being direct targets of SlGRAS4 and protein studies revealed that SlGRAS4 forms a homodimer to self-activate its own promoter. SlGRAS4 can also directly bind tomato *SlCBF* promoters to activate their transcription without inducing any growth retardation. The study identifies the SlGRAS4-regulon as a new cold response pathway conferring cold stress tolerance in tomato independently of the ICE1-CBF pathway. This provides new track for breeding strategies aiming to improve chilling tolerance of cultivated tomatoes and to preserve sensory qualities of tomato fruit often deteriorated by storage at low temperatures.

Introduction

Tomato (*Solanum lycopersicum* L.) is known as a cold-sensitive crop which greatly limits the geographical areas where this important crop plant can be cultivated and shorten the period of its growing seasons. Chilling injury also represents a major issue with regard to the loss of sensory quality during postharvest storage and transportation of tomato fruit. Storage at low temperature is the most common method to limit postharvest losses and deterioration, but many fruit species including tomato are sensitive to temperatures below 12 °C as they develop chilling injury resulting in a number of physiological disorders such as uneven ripening, pitting and most importantly flavour deterioration (Zhang *et al.*, 2016). In addition, low temperature has been reported to impact cell membrane conformation and structure, resulting in attenuated vegetative growth and reduced crop yield and quality (Sevillano *et al.*, 2009). Plants from temperate regions such as Arabidopsis (*Arabidopsis thaliana* L.), wheat (*Triticum aestivum* L.) and *Brassica napus* L. exhibit freezing tolerance when they are pre-exposed to temperatures in the range of 0 to 12 °C, a phenomenon called cold acclimation. By contrast, many tropical plants such as maize (*Zea mays* L.), rice (*Oryza sativa* L.) and tomato suffer from chilling injury when exposed to cold acclimation temperatures (Zhang *et al.*, 2004). An important step towards deciphering the mechanisms underlying cold acclimation has been the discovery that the C-repeat (CRT) binding factor (CBF), a transcriptional regulator, plays important roles in this process in Arabidopsis. Subsequently, the so-called 'CBF regulon' has been further clarified, revealing that CBF proteins can directly bind to the CRT element in the promoter regions of *COLD-RESPONSIVE* (*COR*) genes to activate their expression under cold stress, thus contributing to freezing tolerance *via* enhancement of cryoprotective substances production (Gilmour *et al.*, 1998; Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999; Liu *et al.*, 1998; Stockinger *et al.*, 1997; Thomashow, 2001; Yamaguchi-Shinozaki and Shinozaki, 1994). The expression of *CBF* is also rapidly induced by multiple transcription factors under cold stress, including inducer of CBF expression 1 (ICE1) (Chinnusamy *et al.*, 2003; Kim *et al.*, 2015), calmodulin-binding transcription activator 3 (CAMTA3) (Doherty *et al.*, 2009; Kidokoro *et al.*, 2017), brassinazole-resistant 1/brassinosteroid-insensitive 1-EMS-suppressor 1 (BZR1/BES1) (Li *et al.*, 2017b), CESTA (Eremina *et al.*, 2016) and circadian clock-associated 1/late elongated hypocotyl (CCA1/LHY) (Dong *et al.*, 2011). On the other hand, the expression of *CBF* is repressed by MYB15 (Agarwal *et al.*, 2006; Kim *et al.*, 2017), phytochrome-interacting factors (PIFs) (Jiang *et al.*, 2017), ethylene insensitive 3 (EIN3) (Shi *et al.*, 2012) and suppressor of overexpression of constans 1 (SOC1) (Seo *et al.*, 2009), as reviewed recently by Shi *et al.* (2018). ICE1 is regarded as the most important regulator of *CBF* expression and was shown to undergo multiple post-translational modifications that are essential for its functionality including a ubiquitination process mediated by the high expression of responsive gene 1 (HOS1) (Dong *et al.*, 2006) and a sumoylation mediated by SIZ1 (Miura *et al.*, 2007). It was reported that OPEN STOMATA 1 (OST1) suppresses HOS1-mediated ICE1 degradation under cold stress, through its phosphorylation which enhances its stability and potentiates its transcriptional activity (Ding *et al.*, 2015). On the other hand, it was shown that MPK3 and MPK6 interact with and phosphorylate ICE1 to promote its degradation, thus attenuating the freezing tolerance (Li *et al.*, 2017a; Zhao *et al.*, 2017).

The CBF pathway associated with cold response is highly conserved in flowering plants, and not limited to those displaying cold acclimation, such as *Brassica napus* L. and barley (*Hordeum vulgare* L.), but also operates in plants unable to acclimate to cold stress, such as rice and tomato (Choi *et al.*, 2002; Dubouzet *et al.*, 2003; Jaglo *et al.*, 2001; Zhang *et al.*, 2004). In tomato, there are three CBF homologues, and overexpression of *LeCBF1* in Arabidopsis stimulates the expression of CBF-target genes and increases freezing tolerance, indicating that tomato *CBF1* encodes a functional homologue of the Arabidopsis CBF proteins, supporting the idea that tomato has a complete CBF cold response pathway although the tomato CBF family members have been reported to display less diversified function than Arabidopsis CBFs (Zhang *et al.*, 2004). Of particular note, it was reported that at least 28% of the cold-responsive genes were not regulated by CBFs in Arabidopsis, suggesting the existence of additional, but yet unveiled, low-temperature regulons (Fowler and Thomashow, 2002). More recently, it was shown that *SlICE1*-overexpressing tomato plants exhibit higher antioxidant activity and enhanced chilling tolerance associated with increased *SlCBF1* expression (Miura *et al.*, 2012a, 2012b). Also, SlICE1a, an ICE1-like transcription factor, was reported to bind to the MYC-recognition elements on the promoters of *SlCBF1* and *SlCBF3*, and to confer cold tolerance in transgenic tobacco (Feng *et al.*, 2013). Overall, these data support the notion that responses to low temperature rely on the intervention of diverse types of transcription factors, most of which remain unknown in the case of tomato, a species of major economic importance but highly sensitive to chilling injury.

The GRAS gene family encodes plant-specific transcription factors reported to play critical roles in plant growth and development, and remarkably, several GRAS genes are highly inducible by different abiotic stresses (Huang *et al.*, 2015, 2017; Lee *et al.*, 2008). Some DELLA proteins, belonging to the GRAS sub-family, are involved in abiotic stress resistance via increasing the expression level of genes encoding enzymes that detoxify reactive oxygen species (ROS), thus reducing ROS levels, delaying cell death and promoting tolerance (Achard *et al.*, 2008a). We previously reported that overexpression of *SlGRAS40* in tomato enhances drought and salt resistance by regulating auxin and gibberellin homeostasis (Liu *et al.*, 2017). Interestingly, among the 53 GRAS genes present in the tomato genome, only *SlGRAS4* (Solyc01g100200) exhibits substantial expression increase under low-temperature stress (Huang *et al.*, 2015), yet, it remains to be elucidated whether this GRAS gene is involved in responses to low-temperature stress. In the present study, we show that SlGRAS4 promotes cold tolerance in tomato mainly through direct regulation of many genes participating in the adaptation to low temperature as well as in inducing the expression of *SlCBF* genes. The outcome of the study uncovers a novel cold response mechanism in which SlGRAS4 promotes chilling injury resistance in tomato via multiple biological pathways and at least partly through the CBF pathway.

Results

SlGRAS4 expression is induced by low temperature

We previously identified 53 GRAS members in the tomato genome (Huang *et al.*, 2015), but only *SlGRAS4* was significantly induced by low-temperature stress, raising the hypothesis of its potential role in tomato responses to cold treatment. To gain insight on the putative involvement of *SlGRAS4* in cold stress responses, we first investigated its expression pattern at the transcript level in tomato leaves and fruit under low-temperature treatment. In tomato leaves, *SlGRAS4* transcripts undergo rapid and massive accumulation (more than 100-fold increase) starting 1 h after placing the plants at 4 °C (Figure S1). The same cold treatment applied to mature green fruit also induced transcript accumulation but significantly later (24 h) and at much lower amplitude (five fold increase) than in leaves (Figure S1). This suggests that *SlGRAS4* may act via different modes in tomato leaves and fruit subjected to cold stress.

SlGRAS4 plays a positive role in controlling cold tolerance in tomato plants

To address the functional significance of *SlGRAS4,* tomato plants (*Solanum lycopersicum* L. cv. Micro-Tom) overexpressing (OE) and down-regulated (RNAi) lines were generated. More than 10 independent lines were obtained for each construct among which three phenotypically representative lines were selected for subsequent physiological and molecular characterization. Transcript levels assessed by q-RT-PCR were 61–75 times higher in OE leaves than in WT, and the increase in transcript levels was between 31 to 42 times higher in OE mature green fruit compared to WT fruit at the same stage. In RNAi lines, *SlGRAS4* transcript levels represented 33%–41% the amount in WT leaves and 55%–62% that in WT mature green fruit.

The behaviour of down-regulated and overexpressing lines in response to cold stress was assessed using 45-day-old plants placed at 4 °C for 4 days. In contrast to WT plants that displayed severe wilting symptoms, OE lines exhibited remarkable cold tolerance, with only very few leaves showing slight wilting (Figure S2). When subjected to the same cold treatment, RNAi plants observed similar wilting damage than WT plants. Reducing the duration of cold treatment to 1 day revealed higher sensitivity to low temperature of under-expressing lines, with WT plants displaying only slight wilting symptoms, whereas RNAi plants exhibiting more severe damages (Figure S2). The damages induced by low-temperature treatment were further investigated by assessing malondialdehyde (MDA) content. It is known that under cold stress, the production of reactive oxygen species causes cellular oxidative damage, and the accumulation of MDA, as product of ROS attacking the lipids, reflects the embodiment of the membrane oxidative damage. Remarkably, cold-treated leaves of OE lines accumulated lower amount of MDA than WT (Figure S2), indicating that overexpression of *SlGRAS4* results in lower oxidative damage. On the contrary, suppression of *SlGRAS4* reduces the plant capacity to control the negative effects induced by low-temperature stress as indicated by the higher accumulation of MDA content in RNAi leaves (Figure S2). These results support the idea that SlGRAS4 promotes chilling tolerance in tomato plants at least partly via controlling oxidative damage.

SlGRAS4 positively regulates chilling injury resistance in tomato fruit

Tomato fruit are known as sensitive to cold-induced physiological disorder which depreciates their sensory and commercial qualities. This, together with the increased expression of *SlGRAS4*, under low temperature in tomato fruit (Figure S1) prompted the investigation of the potential involvement of *SlGRAS4* in fruit tolerance to chilling injury. WT and transgenic fruit picked at mature green stage were stored at 5 °C for 14 days and then brought back to 25 °C for 14 days. When subjected to this

chilling treatment, WT fruit exhibited damage symptoms that can be visually observed including pitted skin and uneven ripening (Figure 1a). More severe injury symptoms were observed in RNAi fruit, with all fruit being severely damaged with pitted skin and impaired ripening as indicated by the presence of yellow and light orange colour (Figure 1a). By contrast, no obvious injury symptoms were detected in the OE fruit subjected to the same cold treatment, and once moved to room temperature (25 °C), the fruit ripen normally with no sign of pitting on the skin (Figure 1a). Consistently, fruit overexpressing *SlGRAS4* showed much lower CI index than WT and RNAi ones (Figure 1b). Assessing the 'a' colour parameter by a colorimeter indicated that OE fruit were more towards the red colour than WT after CI treatment, whereas RNAi fruit were more towards the green (Figure 1c). In lines with the displayed colour differences, -carotene content in OE fruit was higher than in WT and RNAi fruit, while total chlorophyll content was higher in RNAi fruit (Figure S3). Fruit firmness of cold-treated OE fruit was lower than WT fruit, whereas RNAi fruit showed higher firmness and contained more pectin and cellulose in pericarp tissue (Figure S3). Water loss in OE fruit was also lower than in WT and RNAi fruit after cold treatment (Figure S3). These data are indicative of a marked slowdown of the ripening process in *SlGRAS4* down-regulated fruit.

No significant differences in total sugar content and titratable acidity were observed between WT and transgenic fruit after chilling injury-inducing treatment (Figure S3). By contrast, assessing phenolics, known to contribute to the antioxidant capacity, revealed higher total phenolics and total flavonoid content in OE compared to WT cold-treated fruit, and total flavonoid content exhibited lower level in RNAi fruit (Figure 1d,e). We then performed DPPH (1,1-Diphenyl-2-picrylhydrazyl) and ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6)-sulphonic acid) assays to monitor free radical scavenging capacity and FRAP (ferric ion reducing antioxidant power) assay to assess total antioxidant capacity (Figure 1f–h). The DPPH, ABTS and FRAP abilities were significantly higher in OE fruit than in WT under chilling injury condition, suggesting the overexpression of *SlGRAS4* increases antioxidant capacity in a broad way.

SlGRAS4-target genes identified by combined ChIP-seq and RNA-seq

To gain insight on the mechanisms by which SlGRAS4 confers enhanced chilling injury tolerance, we investigated the putative SlGRAS4-binding sites at the genome-wide level by a ChIP-seq approach. Up to 5245 peaks were detected (Data S1) and the analysis of their genome-wide distribution revealed that 18% of the SlGRAS4 binding sites were enriched in the gene promoter regions, 1 kb upstream of the coding regions (Figure 2a). Enriched GO categories (Figure 2b) of the putative SlGRAS4-binding genes (Data S1) suggested that SlGRAS4 participates in multiple processes and *de novo* motif prediction performed with the SlGRAS4-binding regions identified revealed four putative DNA-binding motifs (Table S1). However, combining ChIP-seq and RNA-seq data revealed that motif 4 is by far the most abundant in the promoter of DEGs which guided the study towards the role of this specific motif in regulating the expression of selected DEGs (Figure 2c, Appendix S1). Further analysis performed by yeast-one hybrid and dual-luciferase assays revealed that motif 4 is efficient in directing SlGRAS4-mediated gene expression, although the transcriptional activity varies from strong to mild depending on the target promoter (Figure 3b,c).

Global transcriptomic profiling performed by RNA-seq on OE, WT and RNAi fruit samples at mature green stage identified differentially expressed genes in these fruit with reference to WT fruit subjected to the same treatment. The samples analysed included untreated fruit the day of harvest (0d), cold-treated for 14 days after harvest (CI 14d), and cold-treated for 14 days and then placed at room temperature (25 °C) for 14 days (CI 14d + RT 14d). The complete lists of differentially expressed genes screened by pairwise comparison at different time points are given in Data S2–S4. In the absence of cold treatment (0d), up to 995 DEGs were found when cumulating RNAi and OE lines compared to their expression level in WT. Using the WT reference samples, 1861 DEGs were found in cold-treated group (CI 14d) and 4564 DEGs in cold-treated followed by storage at room temperature CI 14d + RT 14d (Figure 2d, Data S2–S4). Of particular note, the most important changes in gene expression (highest number of DEGs) were observed when the fruit were put back at room temperature following a storage at low temperature, regardless of the nature of the sample taken into consideration. Crossing the genome-wide transcriptomic data with the ChIP-seq data revealed that 125, 192 and 714 genes are both differentially expressed and direct binding targets of SlGRAS4 in 0d, CI 14d, and CI 14d + RT 14d samples, respectively (Figure 2d, Data S2–S4).

SlGRAS4 regulates the promoter activity of genes participating in multiple biological processes

Genes that belong to both the ChIP-seq and DEG groups were regarded as best candidates to be direct targets of SlGRAS4 and therefore to contribute to the cold tolerance mechanism (Figure 2d, Data S2–S4). Several among these overlapping genes were selected to further confirm their regulation by SlGRAS4, and these included genes known to be involved in antioxidant capacity like *peroxidase* (Solyc02g094180), *glutathione S-transferase/peroxidase* (Solyc07g056480), *L-ascorbate peroxidase* (Solyc02g083620), *lipoxygenase* (Solyc08g014000) and *glutaredoxin* (Solyc10g008150). As well as *calcium-transporting ATPase* (Solyc02g064680) and *calmodulin-binding protein* (Solyc07g040710) are related to calcium signalling. *Ribulose bisphosphate carboxylase* (Solyc02g077860) participating in photosynthesis, and *phosphoenolpyruvate carboxykinase* (Solyc04g076880) and *malate dehydrogenase* (Solyc01g090710) involved in energy metabolism were also investigated. Interestingly, multiple putative SlGRAS4-binding sequence motifs (Figure 2c) were identified by *in silico* search in the promoter region of all these target genes (Figure 3a), and the ability of SlGRAS4 to directly bind to their promoters was demonstrated by yeast-one hybrid assay (Figure 3b). Furthermore, dual-luciferase assay revealed that SlGRAS4 can directly activate these promoters (Figure 3c), supporting the conclusion that SlGRAS4 regulates the transcription of genes involved in multiple biological processes including antioxidant capacity, calcium signalling, photosynthetic activity and energy metabolism pathways. However, whether or not these genes and the related processes contribute to the chilling injury resistance mediated by SlGRAS4 remains to be elucidated.

SlGRAS4 increases antioxidant capacity

Because SlGRAS4 is shown here to activate the promoter of genes involved in antioxidant capacity, and given that *SlGRAS4* expression is significantly induced in WT tomato leaves sprayed with H_2O_2 (Figure 4a), we therefore performed oxidative stress test by

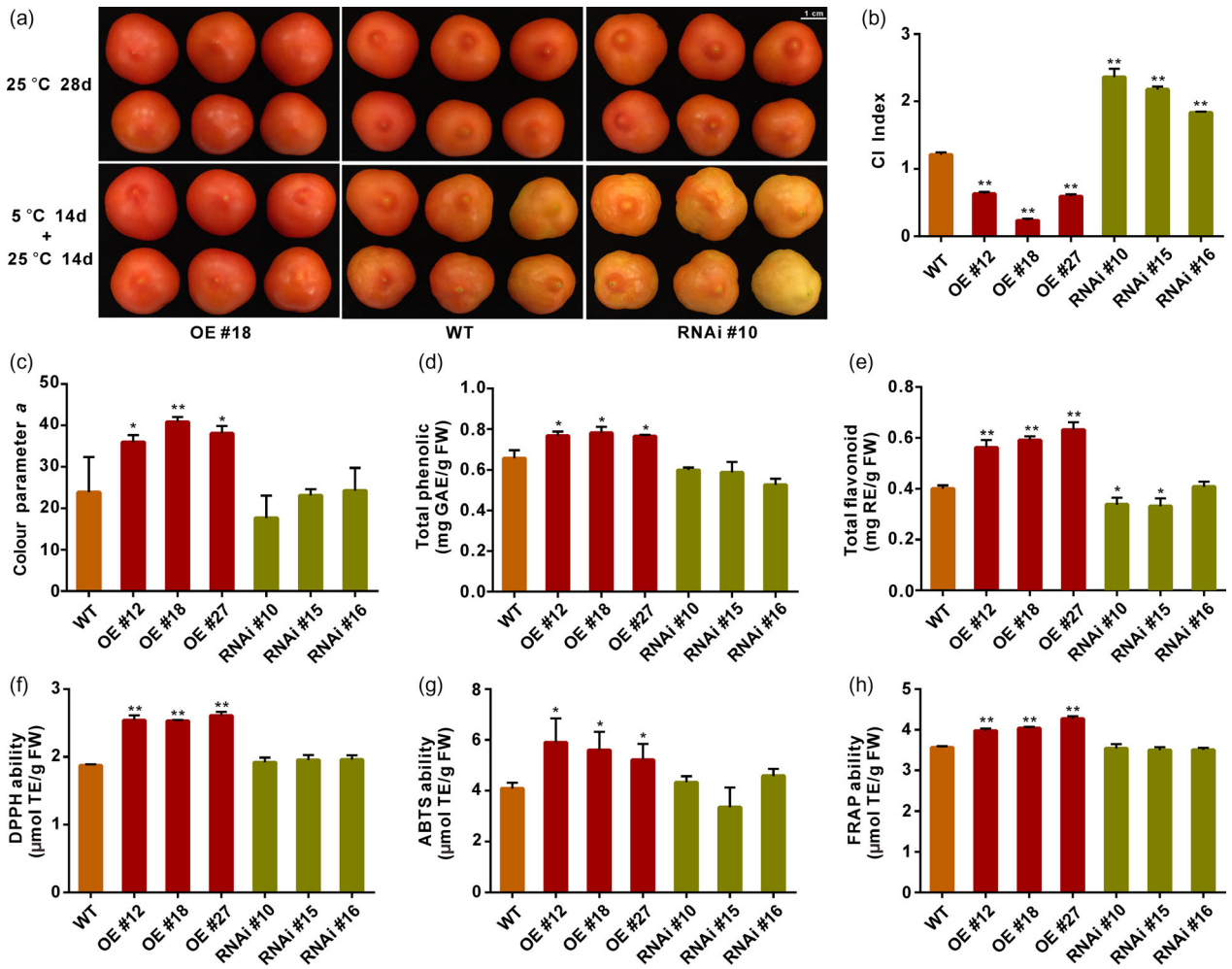

Figure 1 SlGRAS4 positively regulates chilling injury tolerance of tomato fruit. (a) Chilling injury symptoms in WT and transgenic tomato fruit following cold treatment. Transgenic and WT fruit harvested at mature green stage were either stored at 25 ˚C for 28 days (upper panel), or treated 14 days at 5 ˚C and then replaced at 25 ˚C for 14 days. (b–e) Chilling injury (CI) index (b), colour parameter *a* (c), total phenolic content (d), and total flavonoid content (e) of WT and transgenic tomato fruit subjected to cold stress as described above. Total phenolic content and total flavonoid were expressed as mg GAE/g FW and mg RE/g FW, respectively. (f–h) DPPH ability (f), ABTS ability (g) and FRAP ability (h) of WT and transgenic fruit subjected to cold stress as described above. The antioxidant capacities were expressed as mol TE/g FW. In (b) and (d) to (h), data are the mean values of three independent replicates and error bars show the s.d. In (c), three independent repeats were performed showing similar results, and the data showed here are the mean values of one replicate and error bars show s.d. (n = 6). In all cases, asterisks indicate significant differences between wild-type and transgenic lines (two-tailed Student's *t*-test, $*P < 0.05$, $**P < 0.01$).

spraying 45-day-old WT and transgenic plants with 100 μM MV (methyl viologen) once a day for 3 days, and then illuminating the plants for 4 days. In response to the intense oxidative stress applied, WT and RNAi plants exhibited more severe withering than OE plants (Figure 4b,c) and total chlorophyll content in leaves was significantly lower than in OE (Figure 4d), indicating that *SlGRAS4* expression attenuates oxidative stress damages. Moreover, *SlGRAS4* overexpression also enhanced oxidative stress tolerance during seed germination and seedling growth (Figure 4e–g). Together, these data support the idea that *SlGRAS4* promotes oxidative stress tolerance in tomato plants.

SlGRAS4 forms a protein homodimer that directly binds and activates its own promoter

Several SlGRAS4-binding motifs (Figure 2c) were found in the *SlGRAS4* promoter (Figure 5a), consistent with the identification of *SlGRAS4* among the target genes revealed by ChIP-seq assay (Figure 5b, Data S1). The ability of SlGRAS4 to bind its own promoter was validated by both yeast-one hybrid and dual-luciferase assays (Figure 5c,d). In addition, yeast transcriptional activity test, using either full-length or truncated SlGRAS4 proteins, restricted to the transcriptional activation domain located corresponding to the N-terminal region, indicating that SlGRAS4 works as transcriptional activator (Figure 5e). Subsequently, a deletion series of the N-terminal part of the protein identified the transactivation domain in a region encompassing amino acid residues 150–200 (Figure 5e). The truncated SlGRAS4 ΔN5 (200–667) and SlGRAS4 ΔN6 (286–667) proteins lacking the transcriptional activation domain were thereafter used in protein–protein interaction assays by yeast-two hybrid approach, to demonstrate the ability of SlGRAS4 proteins to self-dimerize (Figure 5f). The ability for homo-dimerization was further confirmed by bimolecular fluorescence complementation (BiFC) assay (Figure 5g). Taken together, these data suggest

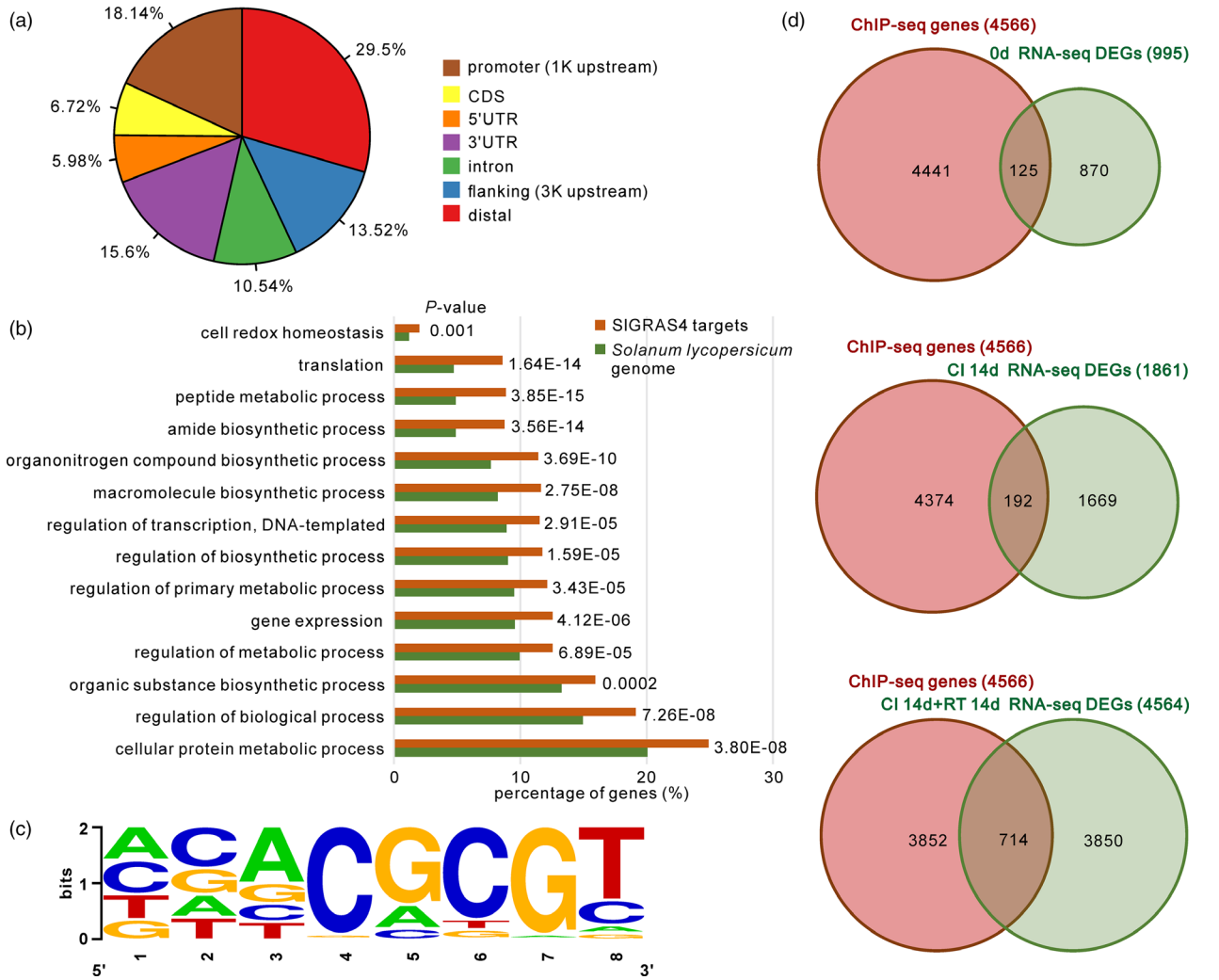

Figure 2 SlGRAS4 target genes identified by combined genome-wide ChIP-seq and RNA-seq approaches. (a) Genome-wide distribution analysis of the SlGRAS4-binding peaks. (b) Gene ontology (GO) categorization of SlGRAS4-binding genes. Enriched GO categories of SlGRAS4 compared with all *Solanum lycopersicum* L. genes are shown. A false discovery rate (FDR) cut-off was implemented on the basis of a *P*-value <0.001. Numbers indicate *P*-values. (c) The putative DNA-binding motif of SlGRAS4 was determined based on the occurrence with the highest frequency in the promoter region of the target genes identified by ChIP-seq. (d) Venn diagram showing the overlapping genes between the SlGRAS4-binding targets revealed by ChIP-seq (red circle) and the differentially expressed genes (DEGs, green circle) identified by RNA-seq in *SlGRAS4*-overexpressing and down-regulated lines. DEGs refer to those showing differential expression either after 14 days of cold treatment or 14 days of cold treatment followed by 14 days at room temperature when the chilling injury symptoms are observed. The complete lists of overlapping genes are given in Data S2–S4.

that the expression of *SlGRAS4* is at least partly under self-regulation.

SlGRAS4 can directly bind and activate the promoters of *SlCBF1*, *SlCBF2* and *SlCBF3*

Multiple SlGRAS4-binding motifs (Figure 2c) present in the promoters of *SlCBF1*, *SlCBF2* and *SlCBF3* genes (Figure 6a) and the ChIP-seq data indicated that SlGRAS4 has the ability to bind the *SlCBF1* and *SlCBF3* promoters (Figure 6b, Data S1). The ability of SlGRAS4 to bind and activate the promoters of *SlCBF1*, *SlCBF2* and *SlCBF3* was further confirmed by yeast-one hybrid and dual-luciferase assays (Figure 6c–e). And the expression levels of *SlCBF1*, *SlCBF2* and *SlCBF3* in *SlGRAS4*-OE fruit were higher than that in WT during chilling treatment (Figure 6f), suggesting that SlGRAS4 contributes to the higher expression of *SlCBF1*, *SlCBF2* and *SlCBF3* observed in *SlGRAS4*-OE fruit subjected to cold stress.

On the other hand, there were no continuous high expression levels of *SlCBFs* in *SlGRAS4*-OE fruit under cold stress and exhibited the similar expression patterns to low temperature to that of the wild type, and three *SlCBFs* were also seriously decreased in OE fruit after treated 1 day (Figure 6f). These results indicate that other CBF regulators also participate in regulating chilling tolerance in tomato fruit. This raises the hypothesis that *SlGRAS4* may enhance chilling injury tolerance in tomato, through both *SlCBF*-dependent and *SlCBF*-independent pathways.

Discussion

Uncovering the mechanisms and factors underlying responses to cold stress is instrumental to the future design of efficient strategies to improving cold tolerance of important crop species.

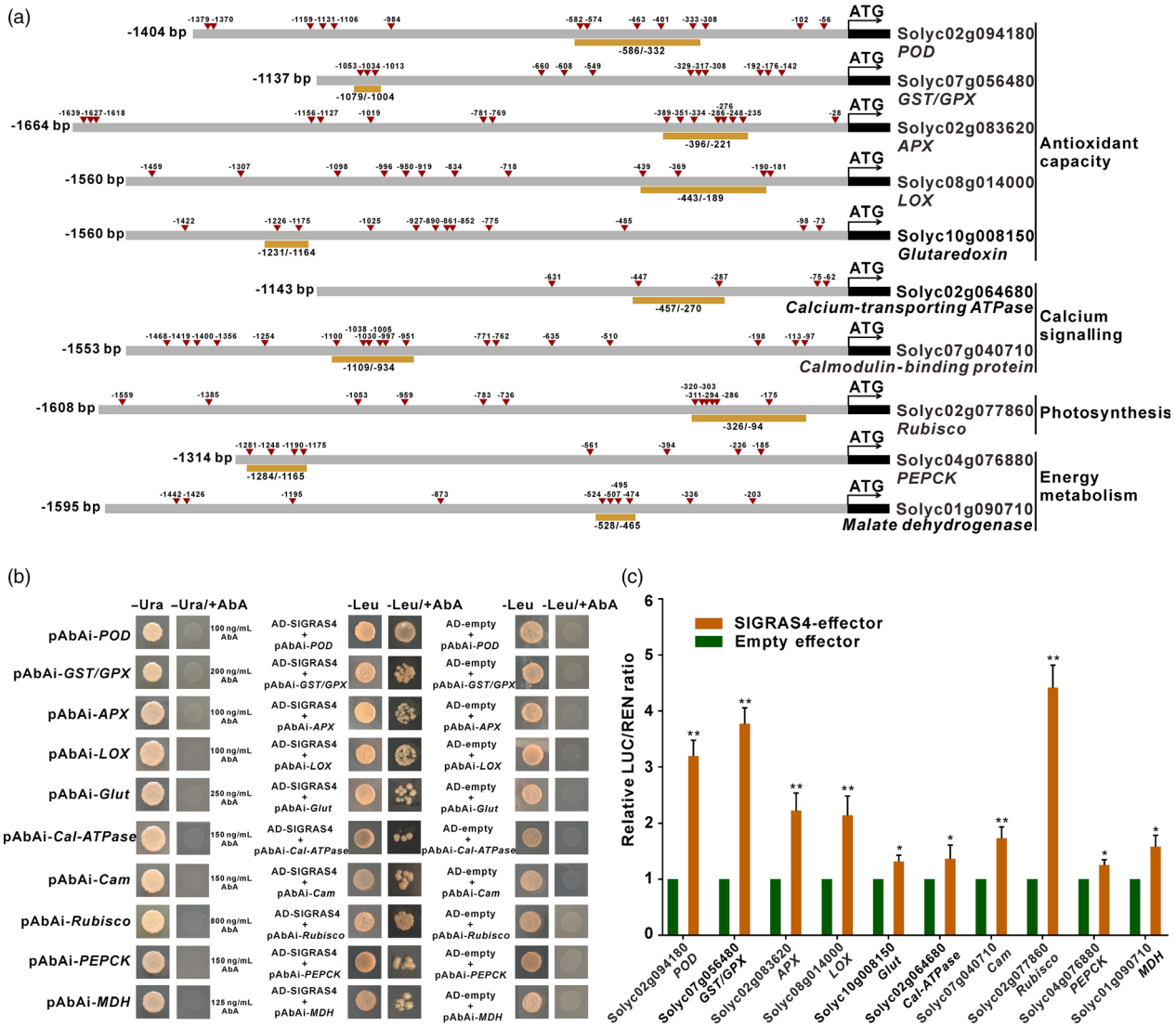

Figure 3 SlGRAS4 directly binds to and activates promoters of several target genes participate in multiple biological processes. (a) The promoter structure of SlGRAS4 target genes. The red triangles indicate the position of SlGRAS4-binding sites corresponding to motif 4 present in the promoter of target genes. The displayed length of each promoter was amplified and cloned into reporter vectors for dual-luciferase assays. The regions underlined in orange indicate promoter fragments used for yeast-one hybrid assays. (b) Interaction of SlGRAS4 with fragments of target gene promoters assessed by yeast-one hybrid assays. (c) The transcription activation ability of SlGRAS4 tested on the promoters of target genes by dual-luciferase assays. The LUC/REN ratio of empty effector plus the promoter reporter was used as calibrator (set as 1). Data are the mean values of five independent replicates, and error bars show the s.d. Asterisks indicate significant differences between SlGRAS4-effector group and control empty vector (two-tailed Student's *t*-test, $*P < 0.05$, $**P < 0.01$). In all cases, *POD* indicates *peroxidase*, *GST/GPX* indicates *glutathione S-transferase/peroxidase*, *APX* indicates *L-ascorbate peroxidase*, *LOX* indicates *lipoxygenase*, *Glut* indicates *glutaredoxin*, *Cal-ATPase* indicates *calcium-transporting ATPase*, *Cam* indicates *calmodulin-binding protein*, *Rubisco* indicates *ribulose bisphosphate carboxylase*, *PEPCK* indicates *phosphoenolpyruvate carboxykinase*, and *MDH* indicates *malate dehydrogenase*.

The present study shows that overexpression of *SlGRAS4* in tomato confers chilling tolerance in both leaves and fruit resulting in minimal cellular damage compared to WT (Figures 1 and S2). Several cold stress-associated genes involved in antioxidant activity, calcium signalling, photosynthesis and energy metabolism are directly regulated by SlGRAS4 (Figure 3). And the expression of these genes is also induced by chilling injury in wild-type fruit in fact (Figure S4), suggesting that SlGRAS4 increases chilling tolerance in tomato fruit through these pathways. Indeed, the promoters of several genes encoding antioxidant enzymes exhibited high activation intensity mediated by SlGRAS4 (Figure 3c), consistent with the enhanced antioxidant capacity exhibited by *SlGRAS4*-overexpressing lines (Figure 4). These data suggest that SlGRAS4 confers chilling tolerance in tomato at least partially by increasing antioxidant capacity. However, it has been reported that heterologous expression of the Arabidopsis *CBF1* in tomato enhanced chilling tolerance via increasing antioxidant enzyme activities (Hsieh *et al.*, 2002; Singh *et al.*, 2011; Zhang *et al.*, 2011), and considering the up-regulation of *CBF* genes in *SlGRAS4*-overexpression lines (Figure 6f), it cannot be ruled out that *CBFs* also contribute to mediating the increased antioxidant capacity in *SlGRAS4*-OE plants.

The outcome of our study supports a working model (Figure 7) where two pathways operate in tomato during chilling tolerance,

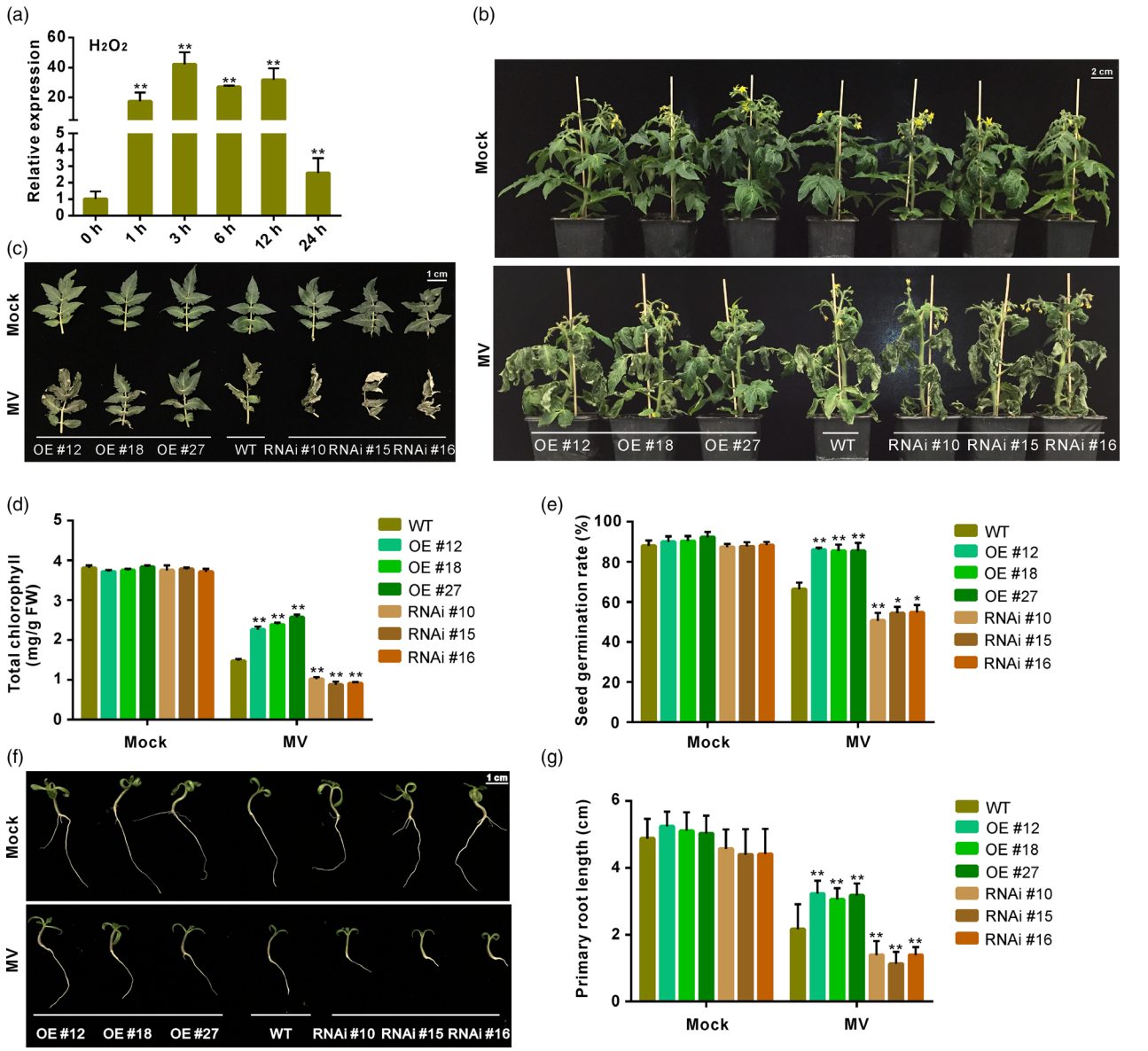

Figure 4 *SlGRAS4* overexpression enhances antioxidant capacity. (a) The response of *SlGRAS4* to oxidative stress in WT leaves following spraying the whole plants with 100 mM H_2O_2 for 0, 1, 3, 6, 12h and 24 h. (b–c) Representative plants (WT, OE and RNAi lines) after treatment for 7 days with 100 M MV (methyl viologen) compared to untreated plants (mocks). (d) Total chlorophyll content of WT and transgenic leaves treated or not (mock) with 100 M MV for 7 days. (e) Seed germination rate of WT and transgenic lines treated or not (mock) with 10 M MV for 7 days. (f) Phenotypes of WT and transgenic seedling treated or not (mock) with 10 M MV for 14 days. (g) Primary root length of WT and transgenic seedling treated or not (mock) with 10 M MV for 14 days. In (a), *SlGRAS4* transcript levels determined by q-RT-PCR were represented as the values relative to at time 0 h of the treatment, and the transcript level at 0 h was set as 1. Data are the mean values of three independent replicates, and error bars show the s.d. Asterisks indicate significant differences relative to the transcript level at 0 h (two-tailed Student's *t*-test, $^{**}P < 0.01$). In (d) and (e), data are the mean values of three independent replicates and error bars show the s.d. In (g), three independent replicates were performed showed similar results, and the data showed here are the mean values of one replicate and error bars show the s.d. (n = 14). Asterisks indicate significant differences between wild-type and transgenic lines (two-tailed Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$).

one mediated by 'CBF-regulon' and a second one based on the 'SlGRAS4-regulon'. In the proposed model, the SlGRAS4 pathway intersects the ICE1/CBF pathway down-stream of the ICE1 step, given the absence of interaction between SlGRAS4 and ICE1 proteins and considering that *ICE1* expression is not affected in *SlGRAS4* OE and RNAi lines. Although *SlCBFs* can be regulated by either SlGRAS4 or ICE1, the expression of *SlCBFs* in tomato is not strictly dependent on SlGRAS4 as indicated by their high expression levels in *SlGRAS4* down-regulated lines under cold stress (Figure S2). In a recent study, Wang *et al.* (2019) revealed the crosstalk of SlPIF4 and SlDELLA modulating *SlCBF* transcript and hormone homeostasis in cold response in tomato, the high expression level of *SlCBFs* in *SlGRAS4*-RNAi leaves under low temperature, presumably via a SlGRAS4-independent pathway. Strikingly, *SlGRAS4*-down-regulated plants exhibit higher sensitivity to cold stress than WT in despite of the strong expression of

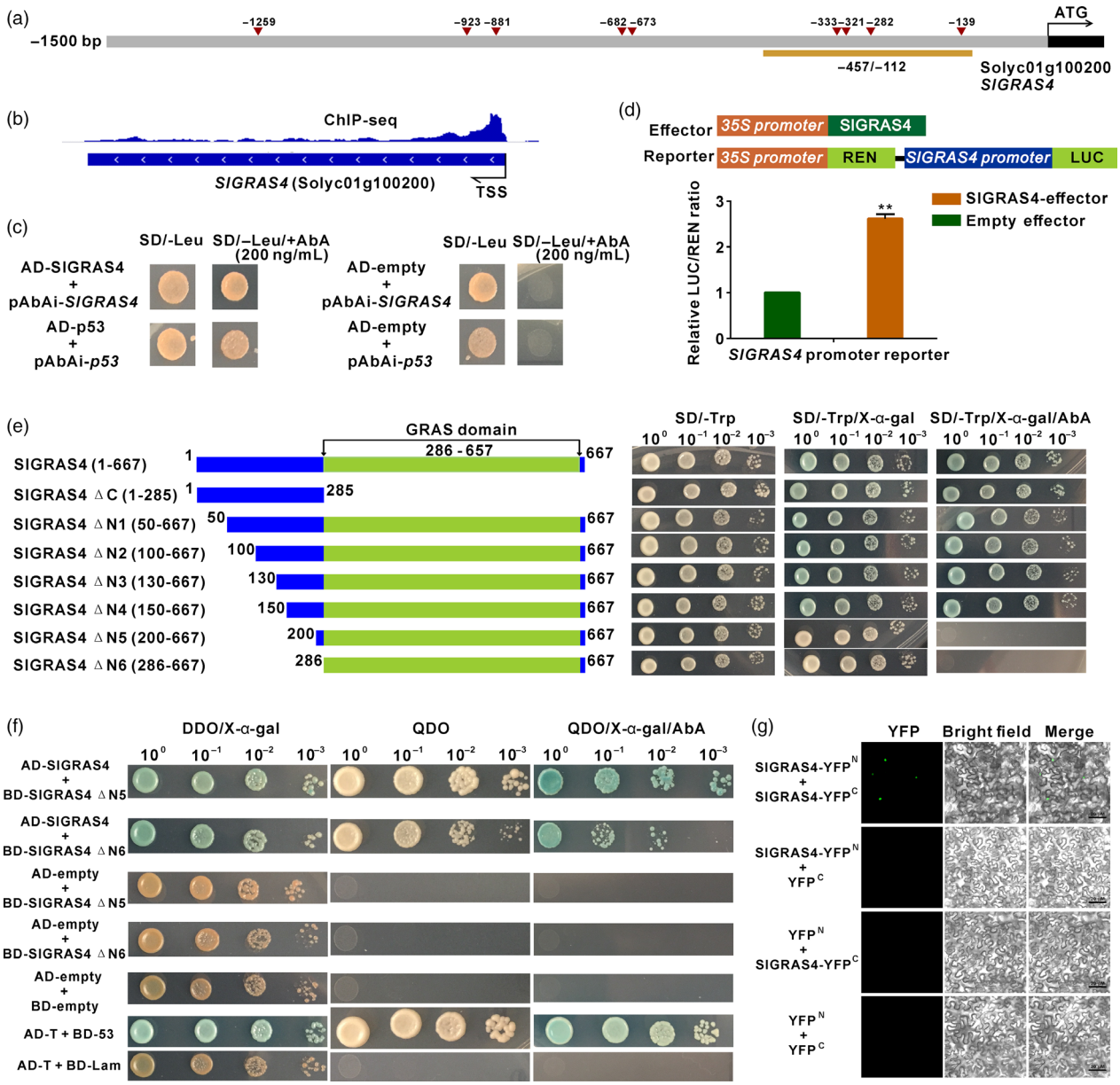

Figure 5 SlGRAS4 directly binds to and activates its own promoter and forms a homodimer by protein–protein interaction. (a) The promoter structure of *SlGRAS4*. The red triangles indicate the position of SlGRAS4-binding sites corresponding to motif 4 present in its own promoter, the 1500 bp length of promoter was amplified and cloned into reporter vector used for dual-luciferase assay, and the regions underlined in orange indicate promoter fragment used for yeast-one hybrid assay. (b) Integrative Genomics Viewer (IGV) image of the *SlGRAS4* gene in ChIP-seq reads. (c) Interaction of SlGRAS4 with its own promoter fragment by yeast-one hybrid assay. (d) Dual-luciferase assay to test the transcription activation ability of SlGRAS4 on its own promoter. The LUC/REN ratio of empty effector plus *SlGRAS4* promoter reporter was used as the calibrator (set as 1). Data are the mean values of five independent replicates, and error bars show the s.d. Asterisks indicate significant differences between SlGRAS4-effector group and empty group (two-tailed Student's *t*-test, $^{**}P < 0.01$). (e) Determining the transactivation domain of SlGRAS4 by deletion series and transcriptional activation test in yeast. C and N indicate deletion of the C and N terminus of SlGRAS4 protein, respectively, and numbers in bracket indicate the amino acid composition of the native and truncated SlGRAS4 proteins. (f) SlGRAS4 homo-dimerization tested by protein–protein interaction in yeast. DDO indicates SD/-Leu/-Trp medium, and QDO indicates SD/-Ade/-His/-Leu/-Trp medium. AD-T + BD-53 is the positive control, and AD-T + BD-Lam is the negative control. (g) SlGRAS4 forms a homodimer as assessed by protein–protein interaction in *Nicotiana benthamiana* L. leaves.

SlCBF1. These data support the notion that 'SlGRAS4-regulon' plays an important role in promoting cold tolerance in tomato plants and seems to operate, at least partially, independently of the *SlCBF* pathway.

On the other hand, the transcriptional levels of *SlCBFs* are increased rapidly in wild-type fruit under chilling treatment and seriously decreased after 12 h (Figure 6f), whereas an significant induction of *SlGRAS4* is observed until treated for 1 day (Figure S1), suggesting the responses of *SlCBFs* to low temperature in wild-type fruit may not dependent on SlGRAS4. Furthermore, there are no persistent high levels of *SlCBFs* in *SlGRAS4*-OE fruit under chilling stress; meanwhile, their

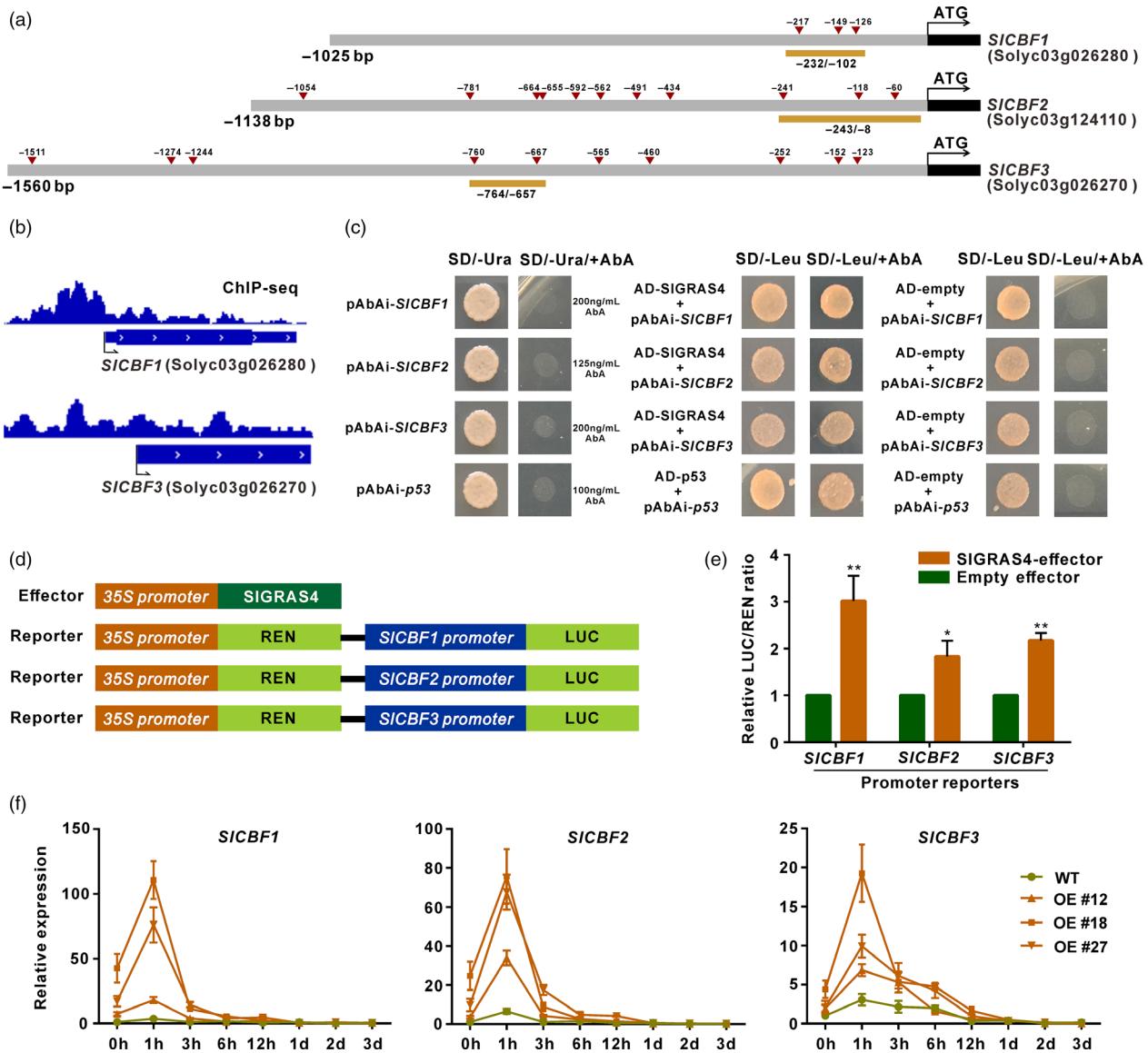

Figure 6 *SlCBF1*, *SlCBF2* and *SlCBF3* are under direct regulation of SlGRAS4 expression. (a) The promoter structure of *SlCBF1*, *SlCBF2* and *SlCBF3*. The red triangles indicate the position of SlGRAS4-binding sites corresponding to motif 4 present in the promoter of *SlCBF1*, *SlCBF2* and *SlCBF3*. The displayed length of promoters was amplified and cloned into reporter vectors used for dual-luciferase assays, and the regions underlined in orange indicate promoter fragments used for yeast-one hybrid assays. (b) Integrative Genomics Viewer (IGV) image of the *SlCBF1* and *SlCBF3* genes as revealed by SlGRAS4 ChIP-seq reads. (c) Interaction of SlGRAS4 with *SlCBF1*, *SlCBF2* and *SlCBF3* promoter fragments assessed by yeast-one hybrid assays. (d) Effector and reporters constructs used for dual-luciferase assays. (e) The transcription activation ability of SlGRAS4 tested on *SlCBF1*, *SlCBF2* and *SlCBF3* promoters by dual-luciferase assays. The LUC/REN ratio of empty effector plus the promoter reporter was used as calibrator (set as 1). (f) Expression pattern of *SlCBF1*, *SlCBF2* and *SlCBF3* in WT and *SlGRAS4*-OE mature green fruit in the absence (0 h) or presence of chilling stress treatment. In (e), data are the mean values of five independent replicates and error bars show the s.d. Asterisks indicate significant differences between SlGRAS4-effector group and empty group (two-tailed Student's *t*-test, $*P < 0.05$, $**P < 0.01$). In (f), the transcript levels of *SlCBF1*, *SlCBF2* and *SlCBF3* in WT and transgenic fruit at different treatment points were relative to WT 0 h, and data are the mean values of three independent replicates and error bars show the s.d.

expression is also seriously decreased in OE fruit after treated 12 h (Figure 6f). These data also suggest that other CBF regulators participate in regulating chilling tolerance in tomato fruit. SlGRAS4 may enhance chilling tolerance through both *SlCBF*-dependent and *SlCBF*-independent pathways in tomato.

It is worth noting that *CBF* overexpression in Arabidopsis, potato and *B. napus* L. resulted in a 'stunted' growth phenotype (Gilmour *et al.*, 2000; Jaglo *et al.*, 2001; Liu *et al.*, 1998; Pino *et al.*, 2008) and that overexpression of *AtCBF1* in tomato also exhibited growth retardation with reduced fruit and seed number (Zhang *et al.*, 2004). By contrast, *SlGRAS4* overexpression have no detrimental effect on tomato growth which display normal plant height, leaf size, fruit set and fruit size, and seed numbers. Moreover, unlike the situation resulting from the overexpression *CBF1* in Arabidopsis which results in the inactivation of the GA signalling pathway and the associated growth defects (Achard *et al.*, 2008b), the expression level of genes involved in GA metabolism is not affected in *SlGRAS4*-OE lines. These data argue

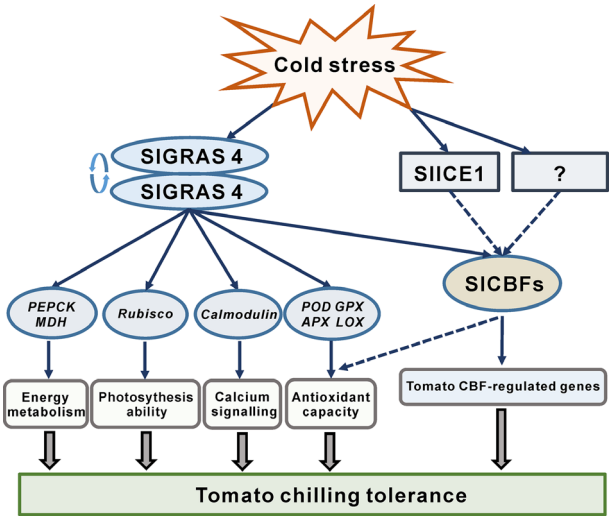

Figure 7 Proposed model for SlGRAS4-dependent regulation of chilling injury tolerance in tomato. Our study uncovers a SlGRAS4-regulated pathway underlying chilling injury tolerance. Deciphering the mechanism of this new pathway revealed that SlGRAS4 forms a homodimer that activates its own expression. SlGRAS4 is able to bind and activate the promoters of target genes involved in antioxidant capacity, calcium signalling, photosynthesis ability and energy metabolism. In this way, SlGRAS4 seems to operate independently of the 'ICE1/CBF' regulon. SlGRAS4 can also directly regulate *SlCBF1*, *SlCBF2* and *SlCBF3* expression by binding to their promoters. Therefore, the up-regulation of *SlCBFs* may contribute to the higher chilling tolerance of *SlGRAS4*-overexpressing lines. We provide a mechanism that SlGRAS4 positively regulates chilling injury resistance in tomato mainly by increasing antioxidant capacity and at least partially by CBF pathway.

for the existence of a new cold stress pathway in tomato and sustain the idea that the newly uncovered 'SlGRAS4-regulon' plays a more prominent role than the 'CBF-pathway' in conferring cold stress tolerance to tomato fruit and plants. The SlGRAS4 pathway provides new targets for novel breeding strategies aiming to enhance tomato tolerance to low temperature and to improve sensory qualities of tomato fruit that are often deteriorated by storage in temperature below 15°C.

Experimental procedures

Plant material growth conditions and generation of transgenic tomato lines

To generate *SlGRAS4* overexpression (OE) plants, the ORF of *SlGRAS4* without the stop codon was cloned into modified plant binary vector K303 under the CaMV 35S promoter (Liu *et al.*, 2017). The *SlGRAS4* RNA-interference (RNAi) construct was generated by cloning a 320-bp sequence fragment amplified by PCR into modified plant binary vector pCambia 1301 under the CaMV 35S promoter. *Agrobacterium tumefaciens* strain GV3101 was used to transform wild-type tomato plants (*Solanum lycopersicum* L. cv. Micro-Tom) following standard methods. Positive transgenic lines were screened by kanamycin (100 mg/L) selection and then confirmed by PCR, and the relative expression level was confirmed by q-RT-PCR using homozygous lines from T2 or T3 generations. All plants were grown in greenhouse in controlled conditions (18-h light/6-h dark cycles, 25 °C day/18 °C night, and 60% relative humidity).

Low-temperature stress treatment

To assess the impact of low-temperature stress on gene expression, 30-day-old wild-type plants treated at 4 °C condition and leaves (5th from cotyledon) were harvested after 1-, 3-, 6-, 12-, and 24-h cold treatment, and untreated leaves were used as control. For each sample, leaves from six different plants were mixed and the treatments were performed in three independent experiments at different times. Wild-type and transgenic tomato fruit at mature green stage were harvested and washed by distilled water, and then treated at 4 °C for 1, 3, 6, 12 h, 1, 2 and 3 days, and untreated fruit were used as control. For each sample, six fruits were mixed and all treatments were performed three times in three independent replicates. All samples were frozen in liquid nitrogen and stored at −80 °C for RNA extraction and q-RT-PCR.

To assess low-temperature tolerance of tomato plants, 45-day-old WT and transgenic plants grown under normal conditions were transferred to 4 °C condition. For physiological assessments, OE plants were treated for 4 days and RNAi plants for 1 day before collecting leaves, with leaves from plants not subjected to cold treatment used as control. At least 10 OE plants and 10 RNAi plants each corresponding to independent transformation events were used for low-temperature stress treatment, and three independent repeats were performed.

For fruit chilling injury test, fruit at mature green stage were harvested and washed by distilled water, and divided into two lots, one placed at 5 °C for 14 days, and then restored to 25 °C condition for 14 days, the other group used as control was placed 25 °C for 28 days. Cold-treated fruit were sampled at each time point and frozen in liquid nitrogen and then stored at −80 °C for further experiments. At least thirty fruits from ten plants of each transgenic line were used for chilling injury treatment, and three independent repeats from different plants were performed. The CI index (chilling injury index) was used to determine the chilling injury tolerance for fruit as subjective evaluation in this study (Cruz-Mendívil *et al.*, 2015). Briefly, CI index = (ILU + ILW)/2, ILU indicates injury as the level of uneven ripening (a five-point scale based on the ripening stage for each criterion (0 = red, 1 = orange, 2 = yellow, 3 = breaker and 4 = green)), and ILW indicates injury as the level of pitting (a five-point scale based on the percentage of tissue affected for each criterion (0 = no injury, 1 10%, 2 = 11%–25%, 3 = 26%–40% and 4 40%)). Fruit firmness was performed by GY-4 digital fruit sclerometer (Aiwoshi, China). Colour parameter was measured by a colorimeter (Lovibond, Germany).

Oxidative stress treatment

To determine *SlGRAS4* transcript accumulation in response to oxidative stress, 30-day-old wild-type plants were sprayed with 100 mM hydrogen peroxide, and leaves (5th from cotyledons) were harvested after 1-, 3-, 6-, 12- and 24-h treatment, while untreated leaves were used as control. For each sample, leaves from six plants were mixed and all treatments were performed three independent times. All samples were frozen in liquid nitrogen and stored at −80 °C until RNA extraction for q-RT-PCR.

For oxidative stress test, 45-day-old WT, OE and RNAi plants were sprayed with 100 μM MV (methyl viologen) once a day for three days, and placed thereafter in continuous illumination condition for 4 days until the leaves display symptoms of wilting and desiccation, and then, leaf samples (5th and 6th from cotyledon) were harvested for total chlorophyll measurement.

For oxidative stress test at germination stage, seeds of WT, OE and RNAi lines were sterilized and sown on ½ MS alone and

½ MS containing 10 μM MV, and incubated under 18-h light (25 °C)/6-h dark (18 °C) cycle conditions. Seed germination rate was assessed after 7 days, and the lengths of primary roots were measured after 14 days. The treatment was performed three independent times, and at least 30 seeds were used for each treatment.

Physiological measurement

Total chlorophyll content and MDA content were measured according to the method described in our previous study (Liu *et al.*, 2017). Total phenolic content and total flavonoid content were performed as described by Jian *et al.* (2019), total phenolic was expressed as mg GAE (Gallic acid)/g FW and total flavonoid was expressed as mg RE (Rutin)/g FW. DPPH ability, FRAP ability and ABTS ability were performed according to the protocol described previously with minor modification (Zhang *et al.*, 2014), and these antioxidant capacities were expressed as mol TE (Trolox)/g FW.

ChIP-seq assay

Mature green transgenic tomato fruit expressing a GFP-tagged SlGRAS4 protein (*SlGRAS4*-ORF fused with *GFP* under 35S promoter) were used for chromatin immunoprecipitation, and the ChIP assay was performed as described by Lü *et al.* (2018). Briefly, fruit tissues were fixed in 1x PBS with 1% formaldehyde for 15 min under vacuum and ground to fine power under liquid nitrogen. Subsequently, nuclei were isolated in nuclei extraction buffer, then sonicated the chromatin to 300- to 500-bp fragments with Covaris M220 in TE buffer containing 0.2% SDS and protease inhibitors, and diluted with low-salt wash buffer with 1% Triton X-100. The chromatin samples were first pre-cleared with empty Dynabeads protein A/G and incubated overnight with Dynabeads with anti-GFP antibody (Millipore). The beads were then washed twice with low-salt buffer, followed by two times high-salt and LiCl washing buffer. The ChIPmentation method was used for ChIP-seq library construction (ChIPmentation: fast, robust, low-input ChIP-seq for histones and transcription factors). Beads washed above were resuspended in 30 μL of the tagmentation reaction buffer (10 mM $MgCl_2$, 25 mM Tris pH 8.0, 10% DMF) containing 1 μL Tagment DNA Enzyme from the Nextera DNA Sample Prep Kit (Illumina) and incubated at 37 °C for 10 min in a thermocycler. The tagmented beads were then washed with low-salt, high-salt and TE washing buffers. At last, the samples were eluted in elution buffer for reverse cross-linking overnight. After purification, the final DNA was used for PCR and sequencing. Raw reads were mapped to tomato genome (http://solgenomics.net/) using *Bowtie2*. And the ChIP-seq datasets were supplied to *MACS2* for peak calling. Peaks were then associated to genes if they were located within the gene body or the region 1 kb upstream of the TSS. Transcription factor binding motifs were predicted with *HOMER*.

RNA-seq assay

Overexpression line OE #18 and down-expression line RNAi #10 were used for RNA-seq assay, with wild type used as control. Total RNA was extracted (RNeasy kit, QIAGEN, Germany) from mature green fruit (0d), mature green fruit treated 14 days at 5 °C (CI 14d) and mature green fruit treated 14 days at 5 °C then replaced at 25 °C for 14 days (CI 14d + RT 14d). cDNA libraries (Illumina) were then constructed for sequencing on the BGI-Seq 500 system (BGI Inc., China). For each sample, the summary of sequencing data is shown in Data S2–S4, respectively. *HISAT* was used to map clean reads to the reference genome of *Solanum lycopersicum* L. in the Tomato Sol Genomic Network database (http://solgenomics.net/), and *Bowtie2* was used to map clean reads to reference gene. And the homogenized data were used to calculate gene expression levels with *RSEM*. For 0d samples, differentially expressed genes (DEGs) were detected with *DEGseq* with the following parameters: fold change 2.00 and adjusted *P*-value (*Q*-value) 0.001. For the samples of CI 14d and CI 14d + RT 14d, DEGs were detected with *NOIseq* with the following parameters: fold change 2.00 and probability 0.8. Sequence data of RNA-seq from this article can be found in the Sequence Read Archive (SRA) database under accession number PRJNA594085.

Yeast-one hybrid assay

The promoters of target genes containing SlGRAS4-binding motif were amplified by PCR and cloned into pAbAi vector as the baits. Recombined bait–pAbAi plasmids were digested by *BstBI*, and the linearized plasmids were transformed into Y1HGold yeast strain according to Yeastmaker Yeast Transformation System 2 (Clontech). The positive Y1HGold [bait/AbAi] strains were confirmed by colony PCR using Matchmaker Insert Check PCR Mix 1 (Clontech) and then screened inhibitory concentration of aureobasidin A (AbA) to avoid self-activation by spreading gradient concentration SD/-Ura/AbA plates. The full length of *SlGRAS4* ORF lacking the stop codon was cloned into pGADT7 vector to construct the prey, the recombined SlGRAS4-pGADT7 plasmid was transformed into Y1HGold [bait/AbAi] strains and spread on SD/-Leu/AbA plates, and transformed empty pGADT7 plasmid was used as control. Protein–DNA interaction was determined based on growth ability of the transformed yeast cells on SD/-Leu/AbA medium following the manufacturer's protocol (Clontech).

Dual-luciferase assay

The full-length ORF of *SlGRAS4* was cloned into pGreenII 62-SK binary vector to generate an effector construct, and about 1.5-kb-length promoter fragment of target genes was amplified by PCR and cloned into pGreenII 0800-LUC binary vector as reporter constructs. The recombined plasmids were co-transformed with pSoup plasmid into *Agrobacterium tumefaciens* strain GV3101, transfected to tobacco (*Nicotiana benthamiana* L.) leaves for transient gene expression analysis. The transformed tobacco plants were incubated at 25 °C in dark for 16 h and then replaced at 25 °C in normal light cycles for 3 days, and then, the leaves were subjected to LUC assays using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Transactivation in yeast and yeast-two hybrid assays

The full-length ORF of *SlGRAS4* (Appendix S2) and the truncated *SlGRAS4* versions were cloned into pGBDT7 vector. The recombined SlGRAS4-pGBDT7 plasmids named SlGRAS4 (1-667), SlGRAS4 ΔC (1-285), SlGRAS4 ΔN1 (50-667), SlGRAS4 ΔN2 (100-667), SlGRAS4 ΔN3 (130-667), SlGRAS4 ΔN4 (150-667), SlGRAS4 ΔN5 (200-667) and SlGRAS4 ΔN6 (286-667) were transformed into Y2HGold strain and spread on SD/-Trp, SD/-Trp/X--gal and SD/-Trp/X--gal/AbA plates for transcriptional activation test. The SlGRAS4-pGADT7 recombined plasmid as prey and the truncated SlGRAS4 ΔN5 (200-667) and SlGRAS4 ΔN6 (286-667) have no transcriptional activation activity as baits were co-transformed into Y2HGold strain and spread on SD/-Leu/-Trp/X--gal (DDO/X--gal), SD/-Ade/-His/-Leu/-Trp (QDO), and

SD/-Ade/-His/-Leu/-Trp/X--gal/AbA (QDO/X--gal/AbA) plates for protein–protein interaction test following the manufacturer's protocol (Clontech).

Bimolecular fluorescence complementation (BiFC) assay

The vectors of BiFC (pXY104 and pXY106) were described previously (Liu and Howell, 2010; Yu *et al.*, 2008). The full-length ORF of *SlGRAS4* was cloned into pXY104 vector to generate a C-terminal YFP *fluorescent* fusion protein and into pXY106 vector to generate a N-terminal YFP *fluorescent* protein. The BiFC assay was performed as described by Luo *et al.* (2014). The recombined plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101, and tobacco leaves were used for transient expression. The transformed tobacco plants were incubated at 25 °C in the dark for 16 h and then replaced at 25 °C in the normal light cycles for 3 days, and then, fluorescence was observed by confocal laser scanning microscope (Leica, Germany).

Gene expression analysis

Total RNA was extracted using an RNeasy kit (QIAGEN, Germany), and first-strand cDNA was synthesized with PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (TAKARA, Japan). Quantitative real-time PCR was performed using SYBR Premix Ex Taq (Tli RNaseH Plus), and the PCR amplification cycles were set according to the instructions (TAKARA, Japan). Melting curve analysis was performed in the temperature ranging 60–95 °C to verify the specificity of the amplicon for each primer pairs. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative fold differences (Bio-Rad), using *SlActin* as an internal reference gene. All the primers used for q-RT-PCR are listed in Table S2.

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Conflict of interest

The authors have no conflicts of interest to declare.

Author contributions

Y.L. and Z.L. designed research. Y.L. and Y.S. performed most of the described experiments. N.Z. and S.Z. performed the ChIP assay. Y.L. and M.B. analysed data. Y.L. wrote the paper. M.B. and Z.L. revised the paper.

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Supporting information

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

Figure S1 *SlGRAS4* is significantly induced by low temperature.
Figure S2 SlGRAS4 promotes cold tolerance in tomato plants.
Figure S3 Other physiological phenotypes in WT and transgenic fruit after chilling injury treatment.
Figure S4 The response to chilling stress of SlGRAS4-targeted genes in wild type fruit.
Table S1 The potential SlGRAS4-binding motifs analysed based on ChIP-seq results
Table S2 Primers used in this study
Appendix S1 Nucleotide sequences of promoters of SlGRAS4-target genes

Appendix S2 *SlGRAS4* nucleotide sequence and encoded amino acid sequence
Data S1 SlGRAS4-binding peaks and SlGRAS4-binding genes identified from ChIP-seq analysis
Data S2 RNA-seq data of 0d group and overlapping ChIP-seq and RNA-seq genes
Data S3 RNA-seq data of CI 14d group and overlapping ChIP-seq and RNA-seq genes
Data S4 RNA-seq data of CI 14d + RT 14d group and overlapping ChIP-seq and RNA-seq genes

