SIGGRAS4 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 SIGGRAS4, 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 SIGGRAS4 and protein studies revealed that SIGGRAS4 forms a homodimer to self-activate its own promoter. SIGGRAS4 can also directly bind tomato CBF promoters to activate their transcription without inducing any growth retardation. The study identifies the SIGGRAS4-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/BE1S1) (Li et al., 2017b), CESTA (Eremaina 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) (See 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 phosphor- ylation 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 SICE1-overexpressing tomato plants exhibit higher antioxidant activity and enhanced chilling tolerance associated with increased SICBF1 expression (Miura et al., 2012a, 2012b). Also, SICE1a, an ICE1-like transcription factor, was reported to bind to the MYC-recognition elements on the promoters of SICBF1 and SICBF3, 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 SIGRAS40 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 SIGRAS4 (Soly01g00200) 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 SIGRAS4 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 SICBF genes. The outcome of the study uncovers a novel cold response mechanism in which SIGRAS4 promotes chilling injury resistance in tomato via multiple biological pathways and at least partly through the CBF pathway.

**Results**

**SIGRAS4 expression is induced by low temperature**

We previously identified 53 GRAS members in the tomato genome (Huang et al., 2015), but only SIGRAS4 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 SIGRAS4 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, SIGRAS4 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 SIGRAS4 may act via different modes in tomato leaves and fruit subjected to cold stress.

**SIGRAS4 plays a positive role in controlling cold tolerance in tomato plants**

To address the functional significance of SIGRAS4, 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, SIGRAS4 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 SIGRAS4 results in lower oxidative damage. On the contrary, suppression of SIGRAS4 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 SIGRAS4 promotes chilling tolerance in tomato plants at least partly via controlling oxidative damage.

**SIGRAS4 positively regulates chilling injury resistance in tomato fruit**

Tomato fruit are known as sensitive to cold-induced physiological disorder which deprecates their sensory and commercial qualities. This, together with the increased expression of SIGRAS4, under low temperature in tomato fruit (Figure S1) prompted the investigation of the potential involvement of SIGRAS4 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 SGRAS4 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 SGRAS4 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 after chilling injury condition, suggesting the overexpression of SGRAS4 increases antioxidant capacity in a broad way.

SGRAS4-target genes identified by combined ChiP-seq and RNA-seq

To gain insight on the mechanisms by which SGRAS4 confers enhanced chilling injury tolerance, we investigated the putative SGRAS4-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 SGRAS4 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 SGRAS4-binding genes (Data S1) suggested that SGRAS4 participates in multiple processes and de novo motif prediction performed with the SGRAS4-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 SGRAS4-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 comparing 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 SGRAS4 in 0d, CI 14d, and CI 14d + RT 14d samples, respectively (Figure 2d, Data S2–S4).

SGRAS4 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 SGRAS4 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 SGRAS4, and these included genes known to be involved in antioxidant capacity like peroxidase (Solyc02g094180), glutathione S-transferase/peroxidase (Solyc07g056480), L-ascorbate peroxidase (Solyc02g083620), lipoygenase (Solyc09g014000) 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 SGRAS4-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 SGRAS4 to directly bind to their promoters was demonstrated by yeast-one hybrid assay (Figure 3b). Furthermore, dual-luciferase assay revealed that SGRAS4 can directly activate these promoters (Figure 3c), supporting the conclusion that SGRAS4 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 SGRAS4 remains to be elucidated.

SGRAS4 increases antioxidant capacity

Because SGRAS4 is shown here to activate the promoter of genes involved in antioxidant capacity, and given that SGRAS4 expression is significantly induced in WT tomato leaves sprayed with H2O2 (Figure 4a), we therefore performed oxidative stress test by...
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 SIGRAS4 among the target genes revealed by ChIP-seq assay (Figure 5b, Data S1). The ability of SIGRAS4 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 SIGRAS4 proteins, restricted to the transcriptional activation domain located corresponding to the N-terminal region, indicating that SIGRAS4 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 SIGRAS4 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
that the expression of SIGRAS4 is at least partly under self-regulation.

**SIGRAS4 can directly bind and activate the promoters of SlCBF1, SlCBF2 and SlCBF3**

Multiple SIGRAS4-binding motifs (Figure 2c) present in the promoters of SlCBF1, SlCBF2 and SlCBF3 genes (Figure 6a) and the ChIP-seq data indicated that SIGRAS4 has the ability to bind the SlCBF1 and SlCBF3 promoters (Figure 6b, Data S1). The ability of SIGRAS4 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 SIGRAS4-OE fruit were higher than that in WT during chilling treatment (Figure 6f), suggesting that SIGRAS4 contributes to the higher expression of SlCBF1, SlCBF2 and SlCBF3 observed in SIGRAS4-OE fruit subjected to cold stress.

On the other hand, there were no continuous high expression levels of SlCBFs in SIGRAS4-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 SIGRAS4 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.
The present study shows that overexpression of SIGRAS4 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 SIGRAS4 (Figure 3). And the expression of these genes is also induced by chilling injury in wild-type fruit in fact (Figure S4), suggesting that SIGRAS4 increases chilling tolerance in tomato fruit through these pathways. Indeed, the promoters of several genes encoding antioxidant enzymes exhibited high activation intensity mediated by SIGRAS4 (Figure 3c), consistent with the enhanced antioxidant capacity exhibited by SIGRAS4-overexpressing lines (Figure 4). These data suggest that SIGRAS4 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 SIGRAS4-overexpression lines (Figure 6f), it cannot be ruled out that CBFs also contribute to mediating the increased antioxidant capacity in SIGRAS4-OE plants. The outcome of our study supports a working model (Figure 7) where two pathways operate in tomato during chilling tolerance, ...
one mediated by ‘CBF-regulon’ and a second one based on the ‘SIGRAS4-regulon’. In the proposed model, the SIGRAS4 pathway intersects the ICE1/CBF pathway down-stream of the ICE1 step, given the absence of interaction between SIGRAS4 and ICE1 proteins and considering that ICE1 expression is not affected in SIGRAS4 OE and RNAi lines. Although SICBFs can be regulated by either SIGRAS4 or ICE1, the expression of SICBFs in tomato is not strictly dependent on SIGRAS4 as indicated by their high expression levels in SIGRAS4 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 SIGRAS4-RNAi leaves under low temperature, presumably via a SIGRAS4-independent pathway. Strikingly, SIGRAS4-down-regulated plants exhibit higher sensitivity to cold stress than WT in despite of the strong expression of

Figure 4 SIGRAS4 overexpression enhances antioxidative capacity. (a) The response of SIGRAS4 to oxidative stress in WT leaves following spraying the whole plants with 100 μM H2O2 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 μM MV (methyl viologen) compared to untreated plants (mocks). (d) Total chlorophyll content of WT and transgenic leaves treated or not (mock) with 100 μM MV for 7 days. (e) Seed germination rate of WT and transgenic lines treated or not (mock) with 10 μM MV for 7 days. (f) Phenotypes of WT and transgenic seedling treated or not (mock) with 10 μM MV for 14 days. (g) Primary root length of WT and transgenic seedling treated or not (mock) with 10 μM MV for 14 days. In (a), SIGRAS4 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).
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...
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, SIGRAS4 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 6  SlCBF1, SlCBF2 and SlCBF3 are under direct regulation of SIGRAS4 expression. (a) The promoter structure of SlCBF1, SlCBF2 and SlCBF3. The red triangles indicate the position of SIGRAS4-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 SIGRAS4 ChIP-seq reads. (c) Interaction of SIGRAS4 with SlCBF1, SlCBF2 and SlCBF3 promoter fragments assessed by yeast-one hybrid assays. (d) Effector and reporter constructs used for dual-luciferase assays. (e) The transcription activation ability of SIGRAS4 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 SIGRAS4-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 SIGRAS4-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.
for the existence of a new cold stress pathway in tomato and sustain the idea that the newly uncovered ‘SIGRAS4-regulon’ plays a more prominent role than the ‘CBF-pathway’ in conferring cold stress tolerance to tomato fruit and plants. The SIGRAS4 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 SIGRAS4 overexpression (OE) plants, the ORF of SIGRAS4 without the stop codon was cloned into modified plant binary vector K303 under the CaMV 35S promoter (Liu et al., 2017). The SIGRAS4 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 homoyzogous 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-Mendivil et al., 2015). Briefly, CI index = (ILL - ILW)/2, ILU indicates injury as the level of uneven ripening (a five-point scale based on the ripening stage 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 SIGRAS4 transcript accumulation in response to oxidative stress, 30-day-old wild-type plants were sprayed with 100 μM 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 was measured as mg GAE (Gallic acid)/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 SIGRAS4 protein (SIGRAS4-ORF fused with GFP under 35S promoter) were used for chromatin immunoprecipitation, and the ChiP assay was 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.

**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. Overexpression line OE #18 and down-expression line RNAi #10 were used for RNA-seq assay, with wild type used as control. Overexpression line OE #18 and down-expression line RNAi #10 were used for RNA-seq assay, with wild type used as control.

**ChIP-seq assay**

Mature green transgenic tomato fruit expressing a GFP-tagged SIGRAS4 protein (SIGRAS4-ORF fused with GFP under 35S promoter) were used for chromatin immunoprecipitation, and the ChiP assay was performed as described by Liu et al. (2018). Briefly, fruit tissues were fixed in 1x PBS with 1% formaldehyde for 15 min under vacuum and ground to fine powder under liquid nitrogen. Subsequently, nuclei were isolated in nuclei extraction buffer and 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 MgCl₂, 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.

**Yeast-one hybrid assay**

The promoters of target genes containing SIGRAS4-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 100 μM/25 μM/10 μM/5 μM/2.5 μM/1 μM 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 SIGRAS4 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 recombinated 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 SIGRAS4 (Appendix S2) and the truncated SIGRAS4 versions were cloned into pGBD7T7 vector. The recombinated SIGRAS4-pGBD7T7 plasmids named SIGRAS4 (1-667), SIGRAS4 ΔC (1-285), SIGRAS4 ΔN1 (50-667), SIGRAS4 ΔN2 (100-667), SIGRAS4 ΔN3 (130-667), SIGRAS4 ΔN4 (150-667), SIGRAS4 ΔN5 (200-667) and SIGRAS4 ΔN6 (286-667) were transformed into Y2HGold strain and spread on SD/-Trp, SD/-Ade/-His/-Leu/-Trp plates for transcriptional activation test. The recombinated SIGRAS4-pGBD7T7 recombinated plasmid as prey and the truncated SIGRAS4 D(N1) (50-667) and SIGRAS4 D(N2) (100-667) were transformed into Y2HGold strain and spread on SD/-Leu/-Trp/X-gal and SD/-Trp/X-gal/AbA plates for transcriptional activation test. The recombinated SIGRAS4-pGBD7T7 recombinated plasmid as prey and the truncated SIGRAS4 D(N5) (200-667) and SIGRAS4 D(N6) (286-667) have no transcriptional activation activity as baits.
SD/-Adel/-His/-Leur-TrpX--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 SIGRAS4 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−ΔΔCT method was used to calculate relative fold differences (Bio-Rad), using SΛActin 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 SIGRAS4 is significantly induced by low temperature.

Figure S2 SIGRAS4 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 SIGRAS4-targeted genes in wild type fruit.

Table S1 The potential SIGRAS4-binding motifs analysed based on CHIP-seq results.

Table S2 Primers used in this study.

Appendix S1 Nucleotide sequences of promoters of SIGRAS4-target genes.
Appendix S2  *SIGRAS4* nucleotide sequence and encoded amino acid sequence

Data S1  *SIGRAS4*-binding peaks and *SIGRAS4*-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