Crosstalk of PIF4 and DELLA modulates CBF transcript and hormone homeostasis in cold response in tomato

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Summary
The ability to interpret daily and seasonal fluctuations, latitudinal and vegetation canopy variations in light and temperature signals is essential for plant survival. However, the precise molecular mechanisms transducing the signals from light and temperature perception to maintain plant growth and adaptation remain elusive. We show that far-red light induces PHYTOCHROME-INTERACTING TRANSCRIPTION 4 (SlPIF4) accumulation under low-temperature conditions via phytochrome A in Solanum lycopersicum (tomato). Reverse genetic approaches revealed that knocking out SlPIF4 increases cold susceptibility, while overexpressing SlPIF4 enhances cold tolerance in tomato plants. SlPIF4 not only directly binds to the promoters of the C-REPEAT BINDING FACTOR (SICBF) genes and activates their expression but also regulates plant hormone biosynthesis and signals, including abscisic acid, jasmonate and gibberellin (GA), in response to low temperature. Moreover, SlPIF4 directly activates the SIDELLA gene (GA-INSENSITIVE 4, SIGA4) under cold stress, and SIGA4 positively regulates cold tolerance. Additionally, SIGA4 represses accumulation of the SlPIF4 protein, thus forming multiple coherent feed-forward loops. Our results reveal that plants integrate light and temperature signals to better adapt to cold stress through shared hormone pathways and transcriptional regulators, which may provide a comprehensive understanding of plant growth and survival in a changing environment.

Introduction
As sessile organisms, plants must integrate multiple environmental and endogenous signals to adjust their growth patterns and developmental transitions to withstand adverse environments and minimize damage. Low temperature is a major environmental stress that severely impairs plant growth and productivity and affects the geographical distribution of plants. To survive under cold stress, plants trigger a suite of sophisticated physiological and biochemical processes. Previous studies have revealed that the ICE-CBF/DREB1 regulatory pathway plays a critical role in cold stress response (Chinnusamy et al., 2007; Thomashow, 1999). The cold stress-induced CBF transcription factors directly activate the expression of downstream COLD-REGULATED (COR) genes and enhance plant cold tolerance. Knocking out all three CBF genes leads to an extreme sensitivity to cold stress (Jia et al., 2016; Zhao et al., 2016), while overexpressing CBFs leads to constitutively enhanced cold tolerance (Gilmour et al., 2000; Jaglo-Ottosen et al., 1998).

Light and temperature are not completely independent under natural plant growth conditions; they provide vital immediate and predictive cues for plants to ensure optimal growth and development (Franklin, 2009; Legris et al., 2017). It has been reported that light is essential for the development of cold acclimation in plants (Kim et al., 2002). Furthermore, the circadian clock, photoperiod and light quality also regulate plant cold tolerance (Dong et al., 2011, Franklin and Whitelam, 2007, Lee and Thomashow, 2012; Li et al., 2016b; Wang et al., 2016, 2018, 2019). CIRCADIAN CLOCK-ASSOCIATED 1 (CCT1)-mediated and LATE-ELONGATED HYOCOTYL (LHY)-mediated outputs from the circadian clock positively regulate plant cold tolerance through the CBF pathway in Arabidopsis (Dong et al., 2011). Meanwhile, the CBF pathway is actively repressed by PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) and PIF7 during the warm long-day season in Arabidopsis (Lee and Thomashow, 2012). Blue light and low temperature-induced COR27 and COR28 negatively regulate freezing tolerance in Arabidopsis (Li et al., 2016b), whereas a low/red-far-red light ratio (L/R/FR) induces cold tolerance in both Arabidopsis and Solanum lycopersicum (Franklin and Whitelam, 2007; Wang et al., 2016, 2018). Intriguingly, recent work has demonstrated that Arabidopsis phytochrome B (phyB) acts as a thermosensor (Jung et al., 2016, Legris et al., 2016), and it negatively regulates cold tolerance in both Arabidopsis and tomato (Franklin and Whitelam, 2007, Wang et al., 2016, 2018). Although we found that phytochrome A (phyA) and phyB function antagonistically to regulate cold tolerance in tomato (Wang et al., 2016), whether phyA or other photoreceptors are sensors for low temperature remains to be investigated. LONG HYOCOTYL 5 (HY5), a bZIP transcription factor, acts downstream of phytochromes and integrates light and cold signalling to optimize plant survival under cold stress (Catalá et al., 2011; Wang et al., 2018, 2019). Therefore, plants have evolved a delicate system that perceives light and temperature signals, allowing them to exquisitely detect and predict changes in the natural environment (Franklin, 2009). PIFs are basic helix–loop–helix transcription factors and have key roles in...
light-regulated plant development and plant responses to multiple environmental signals (Leivar and Monte, 2014; Leivar and Quail, 2011; Pham et al., 2018). PIFs, particularly PIF4, have emerged as a central signalling hub controlling the thermosensory activation of flowering (Kumar et al., 2012) and thermosensory growth in Arabidopsis (Delker et al., 2014; Gangappa and Kumar, 2017). However, the evening-expressed clock component AtTOC1 interacts with and inactivates AtPIF4 to suppress thermoresponsiveness in the evening, which may serve to increase fitness by matching thermoresponsiveness with the day-night cycles of fluctuating temperature and light conditions (Zhu et al., 2016). ATPFs also coordinate light and temperature to regulate the transcription of photosynthesis and photoprotection genes (Toledo-Ortiz et al., 2014). In the natural environment, there is a significant drop in the R/FR ratio during shade and twilight periods in autumn months (Casal and Questa, 2018; Ross et al., 1986). L-R/FR stabilized the AtPIF4 protein during shade avoidance (Lorrain et al., 2008), and we previously demonstrated that L-R/FR induced cold tolerance in tomato shade leaves (Wang et al., 2018). Therefore, whether PIF4 is the central signalling hub that integrates light and temperature to regulate cold tolerance in tomato remains to be explored.

PIFs are emerging as integrators of signals from different hormone pathways during growth and development (Leivar and Monte, 2014). Recent studies have demonstrated that AtPIF4/PIFs induces ethylene and abscisic acid (ABA) signalling in leaf senescence (Sakuraba et al., 2014). Hormones in the gibberellin (GA) and brassinosteroid (BR) classes are also involved in AtPIF4-mediated light and temperature signalling (Franklin et al., 2014). DELLA proteins are the key repressors of almost all GA responses (Ueguchi-Tanaka et al., 2007). DELLA interact with PIFs and have a dual role in modulating PIFs by both sequestration and degradation (Li et al., 2016a). It has been demonstrated that plant hormones, such as ABA, jasmonate (JA), BR and GA, are involved in plant cold tolerance (Achard et al., 2008; Li et al., 2017; Wang et al., 2016; Zhou et al., 2017). Therefore, plants enhance the capacity of perception and prediction of seasonal changes by the multiple integration of light and temperature signals with hormone-signalling pathways and transcriptional regulators (Franklin, 2009).

In this study, we show that far-red light (FR) induces SSI4F accumulation dependent on phyA under low temperature conditions in Solanum lycopersicum. SSI4F positively regulates plant cold tolerance in tomato by directly binding to the promoters of the SlCBF genes and activating their expression, while promoting ABA and JA signalling under cold stress. SSI4F also directly associates with the promoter sequence of SIGA44, which encodes a DELLA protein in tomato, and activates its expression under cold stress. Interestingly, when large amounts of SIGA44 protein accumulated during cold stress, it repressed SSI4F accumulation in a negative feedback manner. Thus, our results suggest that SSI4F is a pivotal component of light and temperature cues and integrates environmental stimuli with plant hormones to coordinate tomato plant growth with impending cold temperatures.

**Results**

Far-red light and low temperature induce SSI4F accumulation via a phytochrome-dependent pathway in tomato

To investigate the possible involvement of SSI4Fs in the plant response to cold stress, we identified the eight tomato SSI4F genes through phylogenetic analysis (Figure S1) and investigated the expression of these genes in tomato plants exposed to cold stress. We found that the expression of SSI4F was the highest among the eight SSI4F genes after the plants were exposed to 4 °C (~2-fold than other genes; Figure 1a). To clarify which SSI4F gene is the major gene in response to cold stress, we silenced SSI4F family genes by tobacco rattle virus-induced gene silencing (VIGS). After cold stress, the relative electrolyte leakage (REL) in SSI4F-silenced plants (pTRV-PIF4) was higher than those of the other SSI4F gene-silenced plants (Figure 1b), which further demonstrated that SSI4F was the predominant gene among the SSI4F family genes in response to cold stress. Since low temperature induced SSI4FYA gene expression, but inhibited transcripts of SSI4FYB1 and SSI4FYB2 compared with those in plants grown at 25 °C (Figure 1c), we then wanted to know whether the regulation of SSI4F by low temperature was dependent on phytochrome. The results showed that SSI4F expression was higher in tomato phyB mutants than in wild-type (WT) plants, while its expression was lower in tomato phyA and phyAB1B2 mutants than in WT plants under cold stress (Figure 1d), which indicated that low temperature regulated SSI4F via the phytochrome pathway. Since FR enhanced cold tolerance via phyA and R inhibited cold tolerance via phyB in tomato plants (Wang et al., 2016), we then asked whether SSI4F was regulated by light quality during cold stress. We examined the gene expression of SSI4F and its protein accumulation in tomato plants under different light conditions, such as white light (WL), red light (R), FR light and dark (D). Compared with plants grown at 25 °C, plants grown at a low temperature had markedly induced SSI4F gene expression and protein accumulation, especially in combination with FR conditions (Figure 1e,f). The gene expression and protein accumulation of SSI4F increased and decreased in plants under FR and R conditions, respectively, compared with those in plants under WL conditions at 4 °C (Figure 1e,f). These results suggest that R and FR function antagonistically to regulate SSI4F accumulation via a phytochrome-dependent pathway in tomato plants.

**SSI4F is a positive regulator in L-R/FR-induced plant cold tolerance and directly activates CBF gene expression**

Since there is a significant drop in the R/FR ratio during twilight periods in autumn months, we used white light supplemented with R and/or FR to obtain different R/FR ratios and examined the effects of different R/FR ratios on cold tolerance in tomato. We found that low R/FR light ratios (L-R/FR) could alleviate cold-induced leaf wilted, the increased REL and the decreased maximum photochemical efficiency of PSII (Fv/Fm) compared with high R/FR light ratios (H-R/FR; Figure S2). To determine the role of SSI4F in L-R/FR-induced cold tolerance, we generated pif4 mutant and SSI4F-overexpressing (SSI4F-OE) transgenic tomato plants. Two independent pif4 mutants (pif4#3 and pif4#10) and two independent overexpression lines of tomato (OE#B7 and OE#B9) were used for further analysis (Figure S3), along with the corresponding untransformed WT. We found that D reduced the tomato hypocotyl length compared with WL, but the hypocotyl length of pif4 mutant and SSI4F-OE was the same in tomato under WL and D conditions (Figure S4). However, we found that the pif4 mutant exhibited increased sensitivity to cold stress, while the SSI4F-OE plants exhibited decreased sensitivity to cold stress, as indicated by the changes in Fv/Fm and REL (Figures 2a,b and S5a). The Fv/Fm values and REL were lower and higher in the pif4 mutant, respectively, than those in WT, while the Fv/Fm values and REL in the SSI4F-OE plants were higher and lower,
Figure 1 SIF4 is regulated by both light and low temperature. (a) and (b) Expression of SIF4s in tomato wild-type (WT) plants (a) and REL in SIF4-silenced plants (b) grown at white light (120 µmol m⁻² s⁻¹) after exposure to 4 °C for 6 h and 7 days, respectively. (c) Expression of PHYA, PHYB1 and PHYB2 in tomato plants after exposure to 25 °C or 4 °C for 6 h. (d) Expression of SIF4 in tomato WT plants and phytochrome mutants (phyA, phyB1B2 and phyAB1B2) after exposure to 25 °C or 4 °C for 6 h under white light conditions (120 µmol m⁻² s⁻¹). (e) Expression of SIF4 in WT plants after exposure to 25 °C or 4 °C for 6 h, which grown under dark (D), white light (WL), red light (R) or FR light conditions. The light intensity is 120 µmol m⁻² s⁻¹. Data are presented as the means of three biological replicates (±SD). Different letters indicate significant differences (P < 0.05) according to Tukey’s test.

respectively, than those of the WT plants after cold stress (Figure 2a,b). Meanwhile, the leaves were more wilted in the pif4 mutant, but less wilted in the SIF4-OE plants, respectively, than those in WT after cold stress (Figure S5a). These results indicate that SIF4 positively regulates cold tolerance in tomato plants. In addition, we found L/RFR decreased REL and increased the Fv/Fm values in both the WT and SIF4-OE plants under cold stress, but these positive effects on cold tolerance were almost abolished in the tomato pif4 mutant. Meanwhile, L-RFR induced the transcription of SICBF1 and SICOR413-like in the WT and SIF4-OE plants was mostly abolished in the tomato pif4 mutant plants (Figures 2c and S5b). These results indicate that L-RFR-induced cold tolerance is partially dependent on SIF4.

Next, we investigated whether SIF4 directly regulated the transcription of SICBF1. A previous study showed that the PIF proteins recognize the G-box, E-box and PBE-box motifs (Kim et al., 2003), and promoter analysis revealed the presence of G-box, E-box and PBE-box motifs in the SICBF1 gene promoter (Figure 2d). Thus, we performed electrophoretic mobility shift assays (EMSA) to test whether SIF4 could directly bind to these fragments of the SICBF1 gene promoter in vitro. EMSA showed that the His-SIF4 protein bound directly to the biotin-labelled G-box-containing probe (G2-box) of the SICBF1 promoter (nucleotides −2041 to −16) and caused a mobility shift (Figure 2e, f). Mutation of the core sequence of the G-box motif in the SICBF1 probes (SICBF1-G2-mut) resulted in the loss of the capacity of SIF4 to bind the probes. Then, we performed chromatin immunoprecipitation (ChIP) assays to test whether SIF4 was associated with the CBF promoters in vivo. The qPCR data showed that the promoters of SICBF1, SICBF2 and SICBF3 were significantly enriched in the 35S:SIF4-HA samples compared with the WT control, whereas the IgG control was not enriched (Figures 2g and S6). These results indicate that SIF4 directly binds to the G-box motifs in the SICBF1 gene promoters. Collectively, these data demonstrate that SIF4 positively regulates cold tolerance by directly binding to the promoters of the SICBF1 genes and activating their transcription in response to cold stress.

SIF4 promotes ABA and JA biosynthesis but inhibits GA biosynthesis in response to cold stress

Abscisic acid and JA have been shown to enhance plant cold tolerance, while GA inhibits plant cold tolerance (Achard et al., 2008; Wang et al., 2016). We found that the transcription levels of genes involved in ABA biosynthesis (SINCE6) and signalling (SIAREB), and JA biosynthesis (SAOS2) and signalling (SICO11) were not significantly different between the WT, pif4 mutant and SIF4-OE plants at 25 °C (Figures 3a,c and S7). In contrast, these genes were markedly up-regulated after cold stress, especially in the SIF4-OE plants. The transcript levels of these genes were lower in the pif4 mutant plants than in the WT plants under cold stress. We also noted greater increases in ABA and JA accumulation in the leaves of the WT and SIF4-OE plants than the pif4 mutant plants after cold stress, especially in the SIF4-OE plants.
In contrast, the transcript levels of GA biosynthesis genes (SlGA3ox2 and SlGA20ox1) and the levels of active GAs (GA1, GA3 and GA4) and their precursors (GA9, GA19 and GA20) significantly decreased after cold stress (Figure 4). Meanwhile, the transcript levels of these GA biosynthesis genes and the accumulation levels of these GAs were higher in the pif4 mutant plants and lower in the SlPIF4-OE plants than in the WT plants at 4 °C (Figure 4). These results indicate that SlPIF4 enhances cold tolerance in tomato plants partially by inducing ABA and JA, and repressing GA biosynthesis.

SlPIF4 directly binds the promoter of SlGAI4 and activates its transcription during cold stress

DELLA proteins are the key repressors of almost all GA responses (Achard et al., 2016) and play critical roles in the plant cold response (Achard et al., 2008; Zhou et al., 2017). There are ten DELLA genes (GA-INSENSITIVE, SIGA1s) in tomato, which were identified by phylogenetic analysis of tomato DELLA family genes (Figure S8a). VIGS experiments showed that the REL in SIGA1-silenced plants (pTRV-GAI4) was higher than those of the other SIGA1-silenced plants (Figure 5a), which demonstrated that SIGA1 was the predominant gene among the SlGAI family genes responsible for plant cold tolerance. To determine whether SlPIF4 participated in the regulation of SIGA1, we analysed the expression levels of the SlGAI4 gene in the WT, pif4 mutant and SlPIF4-OE plants at 25 and 4 °C (Figure 5b). The transcription of SlGAI4 was induced by low temperatures, especially under L-R/FR conditions, with the pif4 and the SlPIF4-OE plants exhibiting lower and higher transcript levels of SlGAI4, respectively, than the WT plants (Figure 5b). Promoter analysis revealed that there were two G-box-, two E-box- and two PBE-box-containing fragments in the promoter of the SlGAI4 gene (Figure 5c). EMSA showed
activated the promoter of SlPIF4 significantly during cold stress. Figure 5c). Dual-luciferase assays indicated that SlPIF4 bound to the promoter sequence of SlGAI4, which showed that the SlGAI4 promoter was lost when the promoter was mutated in the G-box elements (GA14-G1/2-mut; Figure 5c). Dual-luciferase assays indicated that SlPIF4 significantly activated the promoter of SlGAI4 under low-temperature conditions (Figures 5d and S8b). The results were further verified with ChIP assays, which showed that the SlGAI4 promoter sequence was significantly enriched in the 35S:SlPIF4-HA (SlPIF4-OE) samples pulled down by the anti-HA antibody compared with the WT control samples. No enrichment of the IgG control was observed (Figure 5e). Therefore, SlPIF4 directly binds to the promoter sequence of SlGAI4 and activates its gene expression under cold stress.

SlGAI4 is a positive regulator in L-R/FR-induced plant cold tolerance

To substantiate the role of SlGAI4 in cold tolerance, we obtained SlGAI4-silenced tomato plants (pTRV-SlGAI4) and SlGAI4-overexpressing plants (SlGAI4-OE#54, SlGAI4-OE#56) and analysed the expression levels of SlGAI4 in these plants by qRT-PCR. The results showed that SlGAI4 gene transcription was suppressed by 62% and induced by ~20-fold in SlGAI4-silenced plants and SlGAI4-overexpressing plants, respectively (Figure S9). Then, the SlGAI4-silenced plants (pTRV-SlGAI4), pTRV plants, SlGAI4-overexpressing plants and WT plants were exposed to cold stress under different light conditions (H-R/FR or L-R/FR). No differences in REL were observed between the pTRV-SlGAI4 and pTRV plants and between the WT and SlGAI4-overexpressing plants grown under optimal growth conditions (Figure 6a,d). However, the pTRV-SlGAI4 plants showed increased sensitivity to cold stress at 4 °C, with wilted leaf phenotypes, and L-R/FR-induced cold tolerance was also significantly decreased at 4 °C in the pTRV-SlGAI4 plants compared with the pTRV plants, as indicated by the increased REL and decreased Fv/Fm values (Figures 6a,b and S10a). In contrast, the REL of the SlGAI4-overexpressing plants decreased significantly, and Fv/Fm values increased consistently compared with those of the WT plants at 4 °C (Figures 6d,e and S10a). These results demonstrate that SlGAI4 is a positive regulator in L-R/FR-induced cold tolerance in tomatoes.

We further examined the expression levels of the cold stress-responsive genes, such as SlCBF1 and SlCOR413-like, in the SlGAI4-silenced plants (pTRV-SlGAI4), SlGAI4-overexpressing plants and the WT plants (pTRV and WT) via qRT-PCR. The expression levels of SlCBF1 and SlCOR413-like were significantly higher in the WT and SlGAI4-overexpressing plants under cold stress, especially in the SlGAI4-overexpressing plants, whereas those of SlCBF1 and SlCOR413-like were lower in the pTRV-SlGAI4 plants than in the pTRV plants (Figures 6c,f and S10b,c). Furthermore, L-R/FR-induced SlCBF1 and SlCOR413-like gene expression levels in the WT and SlGAI4-overexpressing plants were markedly decreased in the SlGAI4-silenced plants. These results indicate that SlGAI4 positively regulates the CBF-pathway genes under cold stress.

SlGAI4 acts downstream of SlPIF4 to positively regulate ABA and JA biosynthesis and signalling under cold stress

The results described above suggested that SlPIF4 enhanced cold tolerance in tomato plants by directly activating SlGAI4 and inducing ABA and JA biosynthesis. We then asked whether SlGAI4 regulated the levels of ABA and JA under cold stress. To this end, we examined the ABA biosynthesis (SlNCED6) and signalling (SlAREB and SlRD22-like) genes, JA biosynthesis (SlLOXD and SlAOC) and signalling genes (SlCO1), and the levels of ABA and JA in WT plants (WT-pTRV), SlGAI4-silenced plants (WT-pTRV-SlGAI4) and SlGAI4-overexpressing plants (OE-SlGAI4-pTRV) under cold stress. The results showed that low temperature induced the transcription of ABA- and JA-related genes and their contents (Figures 7 and S11). Meanwhile, the levels of ABA, JA and their related gene expression in the SlGAI4-overexpressing
plants (OE-SIGAI4-pTRV) increased significantly compared with those of the WT plants (WT-pTRV) at 4 °C. In contrast, their levels and related gene expression decreased significantly in SIGAI4-silenced plants (WT-pTRV-SIGAI4) compared with the WT plants (WT-pTRV) at 4 °C. Therefore, SIGAI4 might be involved in cold signalling partially by positively regulating ABA and JA.

To further explore the exact role of GA signalling in SIF4-regulated cold tolerance in tomato plants, we tested whether the cold tolerance of the WT, pif4 mutant and SIF4-OE plants was affected by altered GA levels. We observed that exogenous GA3 significantly repressed cold tolerance, with wilted leaves, decreased Fv/Fm values and CBF-pathway gene (CBF1, CBF3, COR47-like and COR413-like) transcript levels and increased REL in the SIF4-OE and WT plants at 4 °C (Figures 8a–c and S12). In contrast, application of the GA biosynthesis inhibitor paclobutrazol (PAC) dramatically enhanced the cold tolerance of both the WT and pif4 mutant plants, with increased Fv/Fm values and CBF-pathway gene (CBF1, CBF3, COR47-like and COR413-like) transcript levels and decreased REL. These results suggest that GA signalling functions downstream of SIF4 in the cold response in tomato plants.

To establish whether the actions of GA and ABA in the cold response occur in a linear sequence, we analysed the changes in cold tolerance after foliar application of exogenous GA3 and PAC in WT and ABA-deficient mutant (notabilis, not) tomato plants. We observed that PAC clearly enhanced the cold tolerance of WT plants, with increased Fv/Fm values and CBF-pathway gene (CBF1, CBF3, COR47-like and COR413-like) transcript levels and decreased REL (Figures 8d–f and S13). However, this induction of plant cold tolerance by PAC was significantly inhibited in the not mutant. These results suggest that GA signalling functions upstream of ABA in the cold response in tomato plants. Collectively, these results indicate that SIGAI4, a key repressor of GA signalling, acts downstream of SIF4 to promote cold tolerance in tomato plants partially through activating ABA and JA signalling.

SIGAI4 negatively regulates SIF4 at transcriptional and post-translational levels under cold stress

It was previously reported that DELLAs interact with PIFs and block their activities by sequestering transcription factors from binding to their targets in Arabidopsis (Feng et al., 2008; de...
Lucas et al., 2008). To investigate whether tomato DELLAs also promoted SIPIF4 degradation under cold stress, 35S:SIPIF4-HA plants were treated with GA$_3$ or PAC under optimal temperature conditions and cold stress, and the SIPIF4-HA protein levels were determined. As shown in Figure 9a, the SIPIF4-HA protein accumulated at increased levels at 4 °C, especially after GA$_3$ application. In contrast, the SIPIF4-HA protein abundance significantly decreased when PAC was applied compared with the effect of mock treatment at 4 °C (Figure 9a). These data indicate that DELLAs promote the degradation of the SIPIF4 protein. Next, we tested the transcription of SIPIF4 and its protein abundance in SIGAI4-silenced plants and SIGAI4-overexpressing plants at 4 °C under H-R/FR or L-R/FR conditions. The results showed that SIPIF4 transcription and its protein levels were higher in the SIGAI4-silenced plants and lower in the SIGAI4-overexpressing plants than in the WT plants (pTRV or WT) during cold stress (Figures 9b, c and S14). These results demonstrate that SIGAI4 negative feedback regulates SIPIF4 at both transcriptional and post-translational levels under cold stress.

**Discussion**

Light and temperature are arguably two of the most important environmental factors that coordinate control plant growth and survival. Light and temperature signals change with daily and seasonal fluctuations and according to latitudinal and vegetation canopy variations. For example, the R/FR ratios are reduced...
naturally with vegetative shading and twilight durations at northern latitudes in cool seasons (Franklin and Whitelam, 2007). We previously found that L-R/FR ratios induced plant cold tolerance via a phyA-dependent pathway in tomato (Wang et al., 2017). OsPIF14 directly bound the promoter of the CBF gene and activating its transcription in Oryza sativa (Wang et al., 2017), and enhanced the degradation of GA genes and regulates phytohor- mone homeostasis. We observed that GA₃ significantly repressed cold tolerance in the SİPİF4-Œ and WT plants at 4 °C (Figures 8a–c and S12); in contrast, application of PAC dramatically enhanced the cold tolerance in tomato plants (Figure 10). First, FR induced SİPİF4 accumulation at low temperatures via phyA (Figure 1d–f). Second, the L-R/FR-induced transcript levels of CBF₁ under cold stress decreased in pİF4 mutant plants, which displayed impaired cold tolerance, but its transcript levels increased in the SİPİF4-overexpression lines, which exhibited enhanced cold tolerance (Figures 2a–c and S5). Third, SİPİF4 directly bound to the G-box of the CBF promoters in vitro and was associated with the promoters of the CBF genes in vivo (Figures 2d–g and S6). Finally, SİPİF4 promoted ABA and JA accumulation in tomato plants under cold stress (Figures 3 and S7), which were positive regulators in cold stress (Wang et al., 2016), and enhanced the degradation of GA (Figures 3 and S7), which was a negative regulator in cold tolerance (Achard et al., 2008; Zhou et al., 2017). Collectively, our results indicate that SİPİF4 works as a positive regulator of L-R/FR-induced cold tolerance in tomato plants that directly activates the transcription of CBF genes and regulates phytohor- mone homeostasis.

We observed that GA₃ significantly repressed cold tolerance in the SİPİF4-Œ and WT plants at 4 °C (Figures 8a–c and S12), in contrast, application of PAC dramatically enhanced the cold
tolerance of both the WT and pit4 mutant plants. These results suggest that GA signalling functions downstream of SlPIF4 in the cold response in tomato plants. Previous studies showed that PHYTOCHROME-INTERACTING FACTOR3-LIKES (PIFL5) inhibited seed germination by directly binding the promoters of two DELLA genes (GAI and RGA) and promoting their transcription in Arabidopsis (Oh et al., 2007). Consistent with these observations, our ChIP analyses and EMSA showed that SlPIF4 directly and specifically bound G-box elements of the SlGAI4 promoters in vitro and in vivo (Figure 5c,e). Dual-luciferase assays and transcript analyses in the pit4 mutant and SlPIF4-overexpressing plants further confirmed that SlPIF4 directly activated SlGAI4 gene expression under cold stress, especially under L-R/FR conditions (Figures 5b,c and S8b). In addition, we provided evidence that SlGAI4 was a positive regulator that modulates cold tolerance in tomato plants. We showed that multiple transgenic lines overexpressing SlGAI4 displayed a decrease in REL and increases in Fv/Fm values and CBF-pathway gene (CBF1 and COR413-like) expression under cold stress (Figures 6d,e and S10a,c). However, L-R/FR failed to induce Fv/Fm values and CBF-pathway gene (CBF1 and COR413-like) expression in the SlGAI4-silenced plants (Figures 6b,c and S8b). Moreover, it has been reported that CBF and DELLA proteins positively regulate each other in response to low temperature in Arabidopsis (Achard et al., 2008; Zhou et al., 2017). Taken together, these results demonstrate that SlPIF4 also directly activated SlGAI4, which acts as a positive regulator in the regulation of CBF1 gene expression and L-R/FR-induced cold tolerance in tomato plants during cold stress.

DELLA proteins serve as integrators of various hormonal and environmental signals (Achard et al., 2006; Daviere and Achard, 2016); thus, we investigated the levels of ABA-related genes and their contents in the SlGAI4-silenced and SlGAI4-overexpressing plants under cold stress. Our results indicated that SlGAI4 positively regulated the levels of ABA-related genes and ABA under cold stress (Figures 7a,b and S11a,b). Foliar application of exogenous GA3 and PAC significantly decreased and increased cold tolerance in tomato plants, respectively, while foliar application of PAC failed to fully rescue the changes in CBF-related gene transcription and cold tolerance in the ABA-deficient nrt plants (Figures 8d,f and S13). These results suggest that low temperature induces a decrease in GA levels and promotes SlGAI4 gene expression, which may result in an increase in ABA levels in tomato plants. Consistent with this finding, DELLA proteins interact with ABI3 and ABI5 and form DELLA/ABI3/ABI5 complexes under unfavourable conditions (e.g. high or low temperature), which positively regulate ABA biosynthesis in Arabidopsis (Kim et al., 2008; Lim et al., 2013; Park et al., 2011). Conversely, a previous report showed that ABA decreased GA levels by repressing GA biosynthetic genes in seeds (Seo et al., 2006). Thus, it appears that ABA and GA antagonistically regulate each other. In addition, recent studies have shown that jasmonate ZIM-domain proteins (JAZs), major repressors in JA signalling, directly target ICE1 to inhibit the activation of CBFs, while DELLA competitively bind to JAZs to release MYC2 and activate the JA response (Hou et al., 2010; Hu et al., 2013; Wild et al., 2012). Meanwhile, MYC2 also interacts with ICE1 to enhance CBF gene transcription in cold conditions (Zhao et al., 2012). Here, we showed that the levels of JA-related gene transcription and JA decreased in the SlGAI4-silenced plants but increased in the SlGAI4-overexpressing plants under cold stress (Figures 7c,d and S11c,d). Thus, SlGAI4 could promote ABA and JA accumulation under cold stress (Figures 7 and S11), which would positively regulate plant cold tolerance via CBF-dependent and CBF-independent pathways (Eremina et al., 2016; Hu et al., 2013; Shinozaki and Yamaguchi-Shinozaki, 2000; Wang et al., 2016).

Previous studies demonstrated that DELLA interact with AtPIF3 and AtPIF4 and inhibit their activities by sequestering their DNA-recognition domains, ultimately results in the inhibition of
Excitingly, recent studies have revealed that DELLA proteins negatively regulate PIF accumulation by inducing rapid degradation of PIFs through the 26S proteasome pathway (Li et al., 2016a; Pham et al., 2018). Indeed, our work showed that GA3 promotes SlPIF4 protein accumulation, while PAC inhibits SlPIF4 protein accumulation (Figure 9a). Furthermore, we found that the SlPIF4 protein and its transcription increased in the SlGAI4-silenced plants, while its protein abundance and gene expression decreased in SlGAI4-overexpressing plants compared with WT plants (Figures 9b,c and S14). These results support a role for SlGAI4 in the negative feedback regulation of SlPIF4 at both transcriptional and post-translational levels under cold stress.

Therefore, SlPIF4 acts as a central hub that integrates light and temperature signals to orchestrate the regulation of the transcriptional network that drives multiple facets of downstream cold response. During cold stress, low temperature and FR signals induced the accumulation of SlPIF4, which directly activated SlCBFs and SlGAI4 to enhance cold tolerance. SlGAI4-induced ABA and JA signalling enhanced plant cold tolerance by regulating CBF-dependent or CBF-independent pathways. Since increased expression of CBFs and SlGAI4 would result in plant growth cessation, SlGAI4 forms a negative feedback loop with SlPIF4. This feedback modulation and redundant cold response pathways likely contribute to maintain appropriate levels of SlPIF4 to balance plant growth and cold tolerance.

Figure 8 The effects of GA3 and PAC on cold tolerance in tomato WT, pif4 Mutant, SlPIF4-OE and not mutant plants. (a) Fv/Fm in tomato wild-type (WT), pif4 mutant (pif4) and SlPIF4-overexpressing plants (SlPIF4-OE) after exposure to 4 °C under low R/FR (L-R/FR, 0.5) light conditions for 7 days, which pretreated with water (H2O), GA3 (50 µM) or PAC (GA biosynthesis inhibitor, 25 µM) for 12 h prior to exposure to cold conditions at 4 °C. The false-colour code depicted at the bottom of the image ranges from 0 (black) to 1.0 (purple), representing the level of damage in the leaves. (b) and (c) REL (b) and SlCBF1 gene expression (c) in tomato WT, pif4 and SlPIF4-OE plants after exposure to 25 °C or 4 °C under L-R/FR light for 7 days and 6 h, respectively, which pretreated with H2O, GA3 or PAC for 12 h prior to exposure to cold conditions at 4 °C. (e) and (f) REL (e) and SlCBF1 gene expression (f) in tomato WT and not plants after exposure to 25 °C or 4 °C under L-R/FR light for 7 days and 6 h, respectively, which pretreated with H2O, GA3 or PAC for 12 h prior to exposure to cold conditions at 4 °C. Data are presented as the means of three biological replicates (±SD). Different letters indicate significant differences (P < 0.05) according to Tukey’s test.

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Conclusions

In summary, we propose a model that illustrates how light and temperature signals are integrated to regulate cold tolerance in tomato plants (Figure 10). Briefly, L-R/FR and low temperature induce SlPIF4 accumulation via a phyA-dependent pathway under cold stress. SlPIF4 not only directly activates CBF expression but also associates with the promoter of the SlGAI4 gene and activates its transcription, promoting ABA and JA biosynthesis and CBF expression. Thus, SlPIF4 is a positive regulator in L-R/FR-induced cold tolerance in tomato. SlGAI4, a DELLA protein, acts downstream of SlPIF4 and positively regulates L-R/FR-induced cold tolerance. Interestingly, when large amounts of SlGAI4 protein accumulate during cold stress, it represses SlPIF4 accumulation in a negative feedback manner.

Figure 9 DELLAs negatively regulate SlPIF4 protein abundance at low-temperature condition. (a) Levels of SlPIF4-HA proteins in 3SS:PIF43-HA tomato plants grown at 25 °C or 4 °C for 24 h. 3SS:PIF43-HA seedlings were pretreated with water (H2O), GA3 (50 µM) or PAC (GA biosynthesis inhibitor, 25 µM) for 12 h before exposure to cold stress. (b) Levels of SlPIF4 proteins in tomato wild-type (pTRV) and SlGAI4-silenced plants (pTRV-GAI4) after exposure to 4 °C under high R/FR (H-R/FR, 2.5) light or low R/FR (L-R/FR, 0.5) light conditions for 24 h. (c) Levels of SlPIF4 proteins in tomato wild-type (WT) and SlGAI4-overexpressing plants (OE#54, OE#56) after exposure to 4 °C under H-R/FR or L-R/FR conditions for 24 h. For light-quality treatments, plants were maintained at R conditions (120 µmol m⁻² s⁻¹) and supplemented with different intensities of FR. Rubisco was used as a loading control.

Figure 10 A proposed model for SlPIF4 positively regulating tomato cold tolerance by integrating light and temperature signals. Briefly, L-R/FR and low temperature induce SlPIF4 protein accumulation via a phyA-dependent pathway under cold stress. SlPIF4 not only directly activates CBF expression but also associates with the promoter of the SIGAII gene and activates its transcription, promoting ABA and JA biosynthesis and CBF expression. Thus, SlPIF4 is a positive regulator in L-R/FR-induced cold tolerance in tomato. SIGAII, a DELLA protein, acts downstream of SlPIF4 and positively regulates L-R/FR-induced cold tolerance. Interestingly, when large amounts of SIGAII protein accumulate during cold stress, it represses SlPIF4 accumulation in a negative feedback manner.

Materials and methods

Plant materials and constructs

The tomato phyA, phyB1B2 and phyAB1B2 mutants were obtained from the Tomato Genetics Resource Center (http://tgrc.ucdavis.edu). Tobacco rattle virus (TRV)-based vectors (pTRV1/2) were used for VIGS of the SlPIF and SlGAI family genes, and VIGS was performed as described previously (Wang et al., 2016). The tomato pif4 mutant in the Ailsa Craig ecotype was obtained by using the CRISPR/Cas9 technique (Pan et al., 2011; Wang et al., 2018). The target sequence (AGGTCATCCAATGTGCAGCT) and its complementary sequence were annealed and inserted into the BbsI site of the AtU6-sgRNA-AtUBQ-Cas9 vector, and the AtU6-sgRNA-AtUBQ-Cas9 cassette was inserted into the HindIII and KpnI sites of the pCAMBIA1301 binary vector. Transgenic plants overexpressing HA-tagged SlPIF4 and SlGAI4 were generated by cloning the full-length SlPIF4 and SlGAI4 cDNAs into the pFGC1008-HA vector, which contains a CaMV 35S promoter, transforming the vectors into Agrobacterium tumefaciens strain.
EHA105, and then introducing them into tomato seeds of ecotype Ailsa Craig via a previously described method (Fillatti et al., 1987). All primers used for plasmid construction are listed in Table S1. Two independent homozygous lines of the F2 generation in SlPIF4- and SIG4-overexpressing plants and two independent pif4 lines, which were mutated at the first base of the protospacer adjacent motif (PAM) to stop translation immediately, were used for the study. In tomato pif4#3 and pif4#10 mutants, there was a single nucleotide (T) insertion at 3-bp upstream of the PAM sites and 2-bp (GC) deletion at 4-bp upstream of the PAM sites in the sgRNA, respectively, as showed in Figure S3a–c. The growth conditions for the tomato mutants and overexpressing seedlings were as follows: temperature of 25 °C/20 °C (day/night), 12-h light/dark cycles and photosynthetic photon flux density (PPFD) of 600 μmol m⁻² s⁻¹. VIGS plants were grown at 21 °C/20 °C under 12-h light/dark cycles.

Cold and light treatments

Plants at the 4-leaf stage were used for all experiments, which were carried out in controlled-environment growth chambers (Zhejiang Qiushi Artificial Environment Co., Ltd, China). Plants were grown under dark (D) or white light (WL), red light (R) and FR light conditions with an aerial temperature of 25 °C or 4 °C for 12-h cold treatment. The light intensities of WL, R and FR were 120 μmol m⁻² s⁻¹. For the different R/FR ratio (high R/FR ratios, 2.5, or low R/FR ratios, 0.5) treatments, R (λmax = 660 nm, Philips, Netherlands) light intensity was maintained at 120 μmol m⁻² s⁻¹, and FR (λmax = 735 nm, Philips, Netherlands) was added. The R/FR ratio was calculated as the quantum flux density from 655 to 665 nm divided by the quantum flux density from 730 to 740 nm. Plants remained under 12-h light/dark cycles while exposed to the cold treatment. The cold treatment at 4 °C lasted for 7 days, unless stated otherwise in the text.

GA₃ and PAC treatment

To unveil the relationship between GA and ABA in cold tolerance, 50 μM GA₃ or 25 μM PAC (GA biosynthesis inhibitor) was applied on WT and not plants 12 h prior to exposure to cold conditions at 4 °C under low R/FR (L-R/FR, 0.5) light conditions for 7 days. To determine the effect of DELLAs on SlPIF4 in cold tolerance, WT, pif4 and SlPFI4-OE plants were pretreated with 50 μM GA₃ or 25 μM PAC prior to cold treatment at 4 °C under L-R/FR light for 7 days. The GA₃ (Sigma-Aldrich, St. Louis, MO, USA) and PAC (Sigma-Aldrich) solutions were prepared by dissolving the solutes in ethanol followed by dilution with distilled water (ethanol : water [v/v] = 1 : 10 000), respectively. The cold tolerance of tomato plants was analysed after foliar application with 20 mL solution or water on each plant.

Cold tolerance and hypocotyl length assays

The REL, indicating the membrane permeability, was measured as described previously (Cao et al., 2007). The maximum quantum yield of PSII (Fv/Fm) in the leaves was assayed by using the Imaging-PAM set-up (IMAG-MAXI; Heinz Walz, Germany), as previously described (Wang et al., 2018). Hypocotyl length was measured after the germination seeding under white light (12-h light/12-h dark) or dark (24-h dark) conditions for 7 days.

Determination of ABA, JA and GA levels

Endogenous ABA and JA were extracted from tomato leaves and determined by LC/MS-MS on an Agilent 1290 Infinity HPLC system coupled to an Agilent 6460 Triple Quad LC-MS device (Agilent Technologies, Amstelveen, the Netherlands), as described previously (Wang et al., 2016). GA was extracted from 1-g samples of tomato leaves and quantified by a derivation approach coupled with nano-LC-ESI-Q-TOF-MS analysis as described previously (Chen et al., 2012; Wang et al., 2019). For the determination of GA levels, D₂-GA₁₉, D₂-GA₃, D₂-GA₄, D₂-GA₉, D₂-GA₁₉ and D₂-GA₄₀ were added to the extraction solution as internal standards.

Phylogenetic analysis

The amino acid sequences of the eight Arabidopsis thaliana canonical PIF proteins (Leivar and Quail, 2011) were used as queries to perform a BLAST search against Sol Genomics databases (https://solgenomics.net/). Sequence alignment and phylogenetic tree construction were determined with MEGA 6 software using the corrected Nei–Gojobori method. A consensus neighbour-joining tree was obtained from 1000 bootstrap replicates of aligned sequences. The percentage at the branch points represents the posterior probabilities of amino acid sequences.

Isolation of RNA and qRT-PCR

Total RNA was isolated using an RNAPrep Pure Plant Kit (Tiangen Biotech Co., Ltd., Beijing, China) from tomato leaves under different conditions as indicated in the figure legend. The extracted RNA was reverse-transcribed using a ReverTra Ace qPCR RT Kit with an enzyme for genomic DNA removal (Toyobo, Osaka, Japan). qRT-PCR was performed with SYBR Green PCR Master Mix (Takara, Japan) using a LightCycler 480 II detection system (Roche, Germany). The PCR procedure was described previously (Wang et al., 2018). The expression levels were normalized to the expression of tomato ACTIN2 gene, which was stably expressed in tomato plants under cold and light stress combined conditions by geNorm algorithm (Livak and Schmittgen, 2001; Lövdal and Lillo, 2009). Primers are listed in Table S2.

Immunoblotting assays

Total proteins were extracted from tomato leaves by homogenization in extraction buffer as described previously (Wang et al., 2019). Protein concentrations were measured using Coomassie stain (Bradford, 1976). Equal amounts of total proteins from each sample were subjected to 15% SDS-PAGE and electrotransferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The proteins were blotted with antibodies against PIF4 (AS163955; Agrisera) or anti-HA (Cat. No. 26183; Pierce) and subsequently with horseradish-peroxidase-conjugated secondary antibody (anti-goat, Invitrogen, Sweden). The signals were visualized with enhanced chemical luminescence (ECL).

Electrophoretic mobility shift assay

The pET-32a-His-SlPIF4 vector was generated using the full-length coding region of SlPIF4 with the primers listed in Table S1. His-tagged SlPIF4 protein was expressed in Escherichia coli strain BL21 (DE3) and purified with the manufacturer’s instructions of the Novagen pET purification system. EMSA was performed using biotin-labelled probes and the LightShift Chemiluminescent EMSA Kit (Cat. no. 201418; Thermo Fisher Scientific). The SlPIF4 proteins and biotin-labelled probe were incubated together in binding buffer for 20 min at room temperature, the reaction
mixture was resolved by 6% non-denaturing polyacrylamide gel in Tris–glycine buffer and electrophoresed at 100 V, then transferred to a positive nylon membrane, and subjected to UV cross-linking. Finally, the protein-DNA signals were detected by chemiluminescence according to the instructions of the LightShift Chemiluminescent EMSA Kit. The sequences of the biotin-labelled are shown in Table S3.

Chromatin immunoprecipitation assay

ChIP-PCR assays were performed following the manufacturer’s instructions for the EpiQuik™ Plant ChIP Kit (Cat. No. P-2014; EpiGentek) as previously described (Wang et al., 2018). Approximately 1 g of leaf tissue was harvested from S1114-OE and WT plants, which were grown at 4 °C under L–R/FR conditions for 1 day and were treated with formaldehyde to cross-link the protein-DNA complexes. The chromatin complexes containing S1114-3HA fusion protein were immunoprecipitated with an anti-HA antibody (Cat. No. 26183; Pierce) and Protein A Agarose beads (GE). Goat anti-mouse IgG (Cat. No. AP124P; Millipore) was used as a negative control. The immunoprecipitated DNA was analysed by qPCR using gene-specific primers which are listed in Table S4.

Dual-luciferase assays

S1114 full-length and the GAI promoter fragment were cloned into the pGreenII 0029 62-SK and pGreenII 0800-LUC vectors, respectively (Figure S8b). The recombinant vectors were transformed into Agrobacterium GV3101. The pGreenII 0029 62-SK empty vector was used as a negative control, and the 35S promoter-driven Renilla luciferase was used as an internal control. Different combinations of strains were injected into the back of tobacco leaves. After infiltration, the tobacco plants were grown at 25 °C for 24 h, and then, one group of these plants was transferred to 4 °C for 24 h before taking samples. The tobacco leaves were ground, and the extraction solutions were incubated in buffer at a low temperature. LUC/Ren was detected with an enzyme standard instrument (SpectraMax iD5, Molecular Devices). Different strains were incubated in buffer at a low temperature, LUC/REN was formed into IC50.

Statistical analyses

Three biological replicates for each treatment were used with at least 6 plants for each replicate. The experiments were independently performed three times. To determine statistical significance, we employed Tukey’s least significant difference (LSD) test. The difference was considered significant at P < 0.05 and indicated by different letters.

Accession numbers

Sequence data from this article can be found in the Sol Genomics databases (https://solgenomics.net/) under the accession numbers listed in Tables S2, S3 and S4.

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Conflict of interest

The authors declare no conflict of interests.

Author contributions

Y.Z. and J.Y. designed the research, F.W., X.C., D.S., X.J. and L.W. performed the experiments. F.W. and X.C. analysed the data. F.W. and Y.Z. wrote 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** Phylogenetic analysis of tomato PIF family genes (SlPIFs).

**Figure S2** Low R/FR enhances the cold tolerance in tomato plants.

**Figure S3** Tomato pif4 mutant and SlPIF4-overexpressing plants.

**Figure S4** SlPIF4 has no effect on the hypocotyl length in tomato plants.

**Figure S5** Phenotypes (a) and expression of COR413-like gene (b) in tomato WT, pif4 mutants and SlPIF4-OE plants after exposure to 25 °C or 4 °C for 7 days or 6 h, respectively, under high R/FR or low R/FR light conditions.

**Figure S6** ChIP-qPCR assay shows the relative amount of SlCBF2 and SlCBF3 fragments in 35S: SlPIF4-HA and wild-type tomato plants.

**Figure S7** SlPIF4 positively regulates expression of ABA and JA signalling genes in response to cold stress.

**Figure S8** Phylogenetic analysis of tomato GAI family genes (SlGAs) and schematic diagram showing vectors construction in dual-luciferase assays.

**Figure S9** Expression of SlGAI4 gene in wild-type (WT/pTRV), SlGAI4-silenced plants (pTRV-GAI4) and SlGAI4-overexpressing plants (OE#54, OE#56).

**Figure S10** Phenotypes (a) and expression of COR413-like gene in tomato SlGAI4-silenced plants (pTRV-GAI4; b) and SlGAI4-overexpressing plants (OE#54, OE#56; c) after exposure to 25 °C or 4 °C for 7 days or 6 h, respectively, under high R/FR or low R/FR light conditions.

**Figure S11** SlGAI4 positively regulates expression of ABA and JA signalling genes in response to cold stress.

**Figure S12** The effects of GA3 and PAC on cold tolerance in tomato WT, pif4 mutant and SlPIF4-OE plants.

**Figure S13** The effects of GA3 and PAC on cold tolerance in tomato WT and not plants.

**Figure S14** Expression of SlPIF4 in tomato SlGAI4-silenced plants (a) and SlGAI4-overexpressing plants (b) after exposure to 25 °C or 4 °C under H-R/FR or L-R/FR conditions for 6 h.

**Table S1** PCR primer sequences used for vector construction

**Table S2** List of primer sequences used for qRT-PCR analysis

**Table S3** Probes used in the electrophoretic mobility shift assays (EMSA)

**Table S4** Primers used for ChIP-qPCR assays