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PpWRKY4 regulates chilling injury in peach fruit by activating *PpVIN2* and accelerating sucrose metabolism

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ABSTRACT

Among the vacuolar invertase (VIN) genes involved in sucrose metabolism within peach fruit, only *PpVIN2* is sensitive to cold stress and is closely associated with both sucrose metabolism and cold stress. In this study, the *PpWRKY4* gene, which responded to cold stress, was screened through cluster analysis of WRKY genes in peach fruit at cold stress and Reverse transcription - Quantitative Polymerase Chain Reaction (RT-qPCR) analysis of methyl jasmonate (MeJA)-treated fruit. Subcellular localization revealed that PpWRKY4 functions as a nuclear-localization protein. Further investigations using yeast one-hybridization (Y1H), electrophoretic mobility shift assay (EMSA), and dual-luciferase reporter (DLR) assay demonstrated that PpWRKY4 acts as a transcriptional activator for *PpVIN2* promoter (*PpVIN2pro*). Transient transformation experiments of peach fruit showed that overexpression of *PpWRKY4* not only increased the expression level of *PpVIN2* but also enhanced VIN enzyme activity, thereby facilitating sucrose decomposition. To further explore the role of PpWRKY4, overexpressing *PpWRKY4* transgenic *Arabidopsis* plants were constructed. These transgenic plants exhibited higher levels of *AtVIN2* expression compared to wild-type (WT) plants, accompanied by reduced sucrose content. Interestingly, following exposure to low-temperature stress, the survival rate of the transgenic *Arabidopsis* was significantly lower than that of WT plants. Collectively, these findings suggested that PpWRKY4 influences sucrose metabolism and regulates the cold tolerance of peach fruit by activating *PpVIN2*.

1. Introduction

Peaches (*Prunus persica* (L.) Batsch) are widely cherished by consumers worldwide for their distinctive flavor and texture (Zhou et al., 2025). Peach fruit ripen in the hot and rainy summer and are highly perishable after being harvested. Low- temperature storage is a common employed commercial method for preserving fruit and vegetables (Hong et al., 2024). However, peach fruit is particularly susceptible to chilling injury (CI) during refrigeration. Previous research has identified that 5 ℃ is the CI temperature of peach fruit (Crisosto et al., 1999; Wang et al., 2013). The CI symptoms of peach fruit primarily include juice loss, browning, lignification, etc., which seriously impaired its commercial appeal (Lurie and Crisosto, 2005). Moreover, more severe CI symptoms tend to appear in the shelf-life period (Zhang et al., 2021). Therefore, the study of cold tolerance in postharvest peach fruit is an issue that deserves ongoing attention.

The accumulation of sugar has been documented to exhibit a significant correlation with plant cold tolerance (Li et al., 2024), especially sucrose (Wang et al., 2022a). Sucrose is not only recognized as a cryoprotectant capable of reducing cell membrane damage caused by low temperatures (Oluwatosin et al., 2022), but also acts as an essential energy substance, providing plants with necessary energy reserves (Ruan, 2014). Additionally, sucrose influences oxidative balance by activating antioxidant systems to eliminate free radicals, thus playing a protective role in plant stress response (Van den Ende and Valluru, 2009). For instance, when exogenous sucrose was applied to cucumber seedlings, the levels of superoxide radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) under low-temperature stress was declined (Cao et al., 2014). In peach fruit, soluble sugar primarily consists of sucrose, which accounts for about 75 % of the total sugar content, alongside glucose and fructose (Aubert et al., 2014). Compared with control group, chilling-injured peach fruit exhibited sharply increased membrane permeability and

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lower sucrose content, indicating that higher sucrose content is conducive to membrane stability and enhanced cold tolerance (Wang et al., 2013). Consistent with this finding, it is discovered that the increase of sucrose concentration in 'Yulu' fruit is positively correlated with cold tolerance (Zhang et al., 2020). Exogenous treatments such as hot air, methyl jasmonate (MeJA) (Yu et al., 2016), 1-methylcyclopropene (1-MCP) (Yu et al., 2017), and salicylic acid (SA) (Zhao et al., 2021) have all been shown to increase sucrose content and enhance the cold tolerance of peach fruit. Zhao et al. (2019) also demonstrated that near-freezing temperatures (NFT) (-1.4 ± 0.1 ℃) could induce cold tolerance in nectarine fruit by reducing enzyme activities related to sucrose metabolism, thereby increasing sucrose content.

The metabolic pathways of sucrose are highly intricate, with invertase being one of the most extensively studied enzymes involved in these processes. Invertase is capable of breaking down sucrose into glucose and fructose, thereby playing a pivotal role in carbohydrate transport, sugar signaling, and plant stress response (Roitsch and González, 2004). Based on their optimal pH value, invertase can be categorized into neutral invertase (NI), which operates optimally at a pH range of 7.0–7.8, and acid invertase (AI), functioning best within a pH range of 3.5–5.5 (Wan et al., 2018). Since the majority of intracellular sucrose is stored in vacuoles, AI plays an important role in modulating sucrose metabolism and participating in stress resistance (Zhao et al., 2022). AI can further be subdivided into cell wall invertase (CWIN) and vacuolar invertase (VIN). Among the AI genes in peach fruit, only *PpVIN2* showed significant sensitivity to cold stress (He et al., 2018). Research conducted by Deng et al. (2025) revealed that *PpVIN2* was strongly induced by cold stress in both 'Yulu' and 'HujingMilu' peach varieties, indicating a close relationship between PpVIN2 and sucrose decomposition as well as cold tolerance in peach fruit. It was also demonstrated that regulating PpVIN2 could significantly impact the cold tolerance of peach fruit. Previous studies have showed that PpINH1, an invertase inhibitor, enhanced the cold tolerance of peach fruit by interacting with PpVIN2 protein (Wang et al., 2020). Cao et al. (2021) found that PpCBF6 negatively regulates *PpVIN2*, thereby reducing VIN enzyme activity and increasing sucrose content, ultimately improving the cold tolerance of peach fruit. The regulation of PpVIN2 appears to be central to understanding the correlation between sucrose metabolism and cold tolerance in peach fruit.

Cold stress is one of the typical abiotic stresses of plants. In the regulation of abiotic stress in plants, transcription factors play an important role. Researches illustrated that strawberry R2R3-MYB transcription factor, FvMYB44 and FvMYB114 enhanced salt and cold tolerance in transgenic *Arabidopsis thaliana* (Li et al., 2023a; Li et al., 2023b). In apple, MbMYBC1 could respond to cold and hydropenia signals, and regulated the cold and drought tolerance of *Malus baccata* through the CBF and ABA pathways (Liu et al., 2023). ICE transcription factor MbICE1 and MbICE3 can also regulate the tolerance to cold and drought of *M. baccata* (Duan et al., 2022; Wei et al., 2023). Furthermore, ERF, bHLH, NAC and CBF etc. family transcription factors have been reported to be involved in abiotic stress in a variety of plants (Kazan and Kemal, 2015; Mei et al., 2023). These indicated that the research on the stress resistance regulation of plants by transcription factors is of great significance.

WRKY is regarded as one of the most important transcription factors family in plants, playing a critical role in plant growth, development, and stress resistance. Research has demonstrated that WRKY transcription factors can respond to plants cold stress. In *Arabidopsis thaliana,* both *AtWRKY25* and *AtWRKY33* have been shown to be induced by low-temperature stress (Chen et al., 2012). In grapes, *VvWRKY24* was identified as a cold-specific response gene, being inducing at all time points of cold treatment (Wang et al., 2014). When *Malus baccata* WRKY40 (*MbWRKY40*) was overexpressed in *Arabidopsis*, the transgenic plants exhibited higher cold tolerance (Han et al., 2023). WRKY transcription factors influence plant cold tolerance through various pathways. For instance, pollen-specific AtWRKY34 participated in the CBF signaling cascade within mature pollen, negatively regulating the cold sensitivity of mature *Arabidopsis* pollen (Zou et al., 2010). In cucumber, CsWRKY46 can modulate the cold signaling pathway in an abscisic acid (ABA)-dependent manner, thereby enhancing cold tolerance (Zhang et al., 2016). In tomatoes, it has been found that SlWRKY50 enhances cold tolerance by activating the jasmonic acid (JA) signaling pathway, while SlWRKY51 mediates proline synthesis to improved cold tolerance (Wang et al., 2023; Wang et al., 2024).

WRKY transcription factors can bind to the W-box (TTGACC/T) element, with a core motif of TGAC, located in the promoter region of downstream target genes (Eulgem et al., 2000). This binding activating or inhibiting gene transcription with highly conserved property. In barley, HvWRKY46 can bind to the sugar-responsive cis-element SURE (TAAAGATTACTAATAGGAA) and participate in the sugar signal transduction (Mangelsen et al., 2008). Nevertheless, the connection between WRKY transcription factors and sucrose metabolism remains largely uninvestigated, with limited research focusing on how the WRKY affects plant cold tolerance via the regulation of sucrose metabolism.

To investigate the mechanism by which WRKY transcription factors regulate cold tolerance in peach fruit, 61 WRKY transcription factors in 'Yulu' fruit were selected for low-temperature sensitivity in this study. Among these, the *PpWRKY4* gene, which responds to cold stress, was further screened using peach fruit treated with MeJA. The transcriptional regulation of PpWRKY4 on *PpVIN2* was explored through yeast one-hybridization (Y1H), electrophoretic mobility shift assay (EMSA), and dual-luciferase reporter (DLR) assay. Additionally, the cold tolerance function of PpWRKY4 and its relationship with sucrose metabolism were analyzed using both the transient overexpression system in peach fruit and transgenic *Arabidopsis* plants. This comprehensive study lays the foundation for the regulation of sucrose metabolism in peach fruit by WRKY transcription factors and contributes to enriching the regulatory network of sugar metabolism that impacts CI of postharvest peach fruit.

2. Materials and methods

2.1. Plant materials and treatments

Peach fruit (*Prunus persica* (L.) Batsch. 'Yulu'), which were commercially mature, disease-free, uniform in size, and free of mechanical damage, were harvested from Ningbo Fenghua Peach Orchard (Zhejiang, China) in 2021. After harvest, the peaches were immediately transported to the laboratory and stored at 5 ℃ with 92–95 % relative humidity. Samples were collected at 0, 24, and 36 h of refrigeration. The mesocarp was sliced to a thickness of approximately 1 cm, frozen in liquid nitrogen and stored at −80 ℃. Three biological replicates were prepared for each time point, with 8 peaches/replicates.

For the MeJA-treated peach experiment, commercially mature 'Yulu' peaches were harvested in 2020 and randomly divided into two groups. The treatment group was fumigated with 10 μmol L^{-1} MeJA (Beijing Solarbio Science & Technology Co., Ltd.) in a sealed container at 5 ℃ for 24 h. The control group (without MeJA treatment) was kept under identical conditions in a separate sealed container at 5 ℃ for 24 h. Subsequently, both groups of peaches were stored at 5 ℃ with 92–95 % relative humidity. Samples were taken at 0, 24, and 36 h of refrigeration. Three biological replicates were prepared for each time point, with 8 peaches/replicates.

2.2. RNA isolation, cDNA synthesis, and RT-qPCR

Approximately 0.1 g of frozen peach tissue was weighed and thoroughly ground under liquid nitrogen, the peach tissue powder was transferred to a 1.5 mL nuclease-free centrifuge tube (He et al., 2018). Total RNA extraction was performed using the Eastep® Spuer total RNA extraction kit (Promega Biotechnology Co., Ltd. Beijing). For cDNA synthesis, the HiScript II Q RT SuperMix for qPCR (Vazyme Biotech Co., Ltd. Nanjing) was employed. Primers for Reverse transcription -

Quantitative Polymerase Chain Reaction (RT-qPCR) analysis were designed using Primer Premier 5 software, and their sequences are provided in Supplementary Table S1. The reference genes used were translation elongation factor 2 (*TEF2*) for peach (Tong et al., 2009) and ubiquitin 4 (*UBQ4*) for *Arabidopsis* (Zhang et al., 2023a). RT-qPCR amplification was carried out according to the qPCR kit ChamQ™ Universal SYBR® qPCR Master Mix (Vazyme Biotech Co., Ltd. Nanjing) and the results were analyzed using the $2^{-\Delta\Delta Ct}$ method. Cluster heat map generating was performed using TBtools software.

2.3. Subcellular localization of PpWRKY4

The subcellular localization vector was constructed using the Golden Braid cloning technique. The primers used for vector construction in this study were listed in Supplementary Table S2. The full-length CDS (without termination codon) of *PpWRKY4* was inserted between the *KpnI* and *XbaI* restriction sites of the pCAM35S-GFP-TNos vector, generating the recombinant vector pCAM35S-PpWRKY4-GFP-TNos (Chen et al., 2022). Both the recombinant plasmids pCAM35S-PpWRKY4-GFP-TNos and pCAM35s-GFP-TNos (positive control) were separately transformed into *Agrobacterium tumefaciens* strain GV3101 (pSoup-p19). Four-week-old *Nicotiana benthamiana* leaves were then infiltrated with the *Agrobacterium* suspension via agroinfiltration. After a normal culture for 48 h, the infected tobacco leaves were observed by confocal laser scanning microscopy.

2.4. Y1H assay

The yeast recombinant expression bait vector pAbAi-*PpVIN2pro* has been constructed in our laboratory. Notably, the self-activation of yeast driven by *PpVIN2pro* was successfully inhibited when the AbA concentration reached 150 ng/mL (Cao et al., 2021). The full-length CDS of *PpWRKY4* was cloned into the pGADT7 vector to construct pGADT7-PpWRKY4. Yeast co-transformation was performed following the protocol provided in the yeast colony rapid transformation kit (Kulaibo Biotechnology Co., Ltd, Beijing). The strain carrying pGADT7-PpWRKY4 + pAbAi-*PpVIN2pro* served as the experimental group, while the strain containing pGADT7–53 + pAbAi-53 acted as the positive controls. Additionally, the strain with pGADT7-Empty + pAbAi-*PpVIN2pro* served as the negative controls. The interaction was verified using yeast defect medium SD-Leu and SD-Leu^{+ABA}.

2.5. EMSA test

Refer to the method of Li et al. (2025) with some minor modifications. The acid sequences (30 bp) adjacent to the W-Box element in the *PpVIN2pro* sequence were selected as probe sequences, and corresponding cold probe and mutant probe were designed based on the biotin-labeled probe sequences. These probes were synthesized by Weiao Gene Technology Changzhou Co., Ltd. EMSA detection was carried out according to the instructions provided in the LightShift™ Chemiluminescence EMSA Kit (Thermo Scientific, USA).

2.6. DLR assay

The DLR assay was performed as described by Gao et al. (2024) with slight modifications. The full-length CDS of *PpWRKY4* was cloned into the pGreenII 62-SK effector vector to construct pGreenII 62-SK-PpWRKY4, while the empty pGreen II 62-SK vector served as the control effector. The *PpVIN2* was cloned into the pGreenII 0800-LUC reporter vector to construct pGreenII 0800-LUC-*PpVIN2*. Recombinant plasmids were separately transformed into *Agrobacterium tumefaciens* strain GV3101 (pSoup-p19), and then transiently expressed in tobacco leaves. Each group included six biological replicates. After a 12 h dark culture and 48 h normal growth, the LUC and REN activity were detected using the dual luciferase reporter assay system (Promega, USA) on a Modular luminometer detector (Promega, GloMax ® 96, USA).

2.7. Agrobacterium-mediated transient overexpression of PpWRKY4 in peach fruit

Following the method described by Zhang et al. (2023b) with a slight modification. The full-length CDS of *PpWRKY4* was cloned into the pBI121 vector to construct pBI121-*PpWRKY4*, while the empty pBI121 vector served as the control. The above plasmids were separately transformed into *Agrobacterium tumefaciens* strain GV3101. An infiltration solution was prepared and injected into peach fruit using a sterile syringe. The treated peaches were subsequently stored in an incubator at 20 ℃ with a relative humidity of 85–90 %. Samples were collected on the 1st and 3rd d after storage, quickly frozen in liquid nitrogen and stored at −80 ℃ for further analysis.

2.8. VIN activity and sucrose content measurement

The determination of VIN activity was referred to the method of Wang et al. (2020). Weigh 1 g frozen peach tissue into 5 mL extraction buffer [100 mM sodium phosphate buffer (pH 7.5), 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.1 % Triton X-100 (v/v), and 2 % polyvinylpyrrolidone (PVPP, m/v)] and homogenized on ice. The homogenate was centrifuged at 12,000×g for 20 min at 4 ℃ using a high-speed centrifuge (H1650R, Hunan Xiangyi Laboratory Instrument Development Co., Ltd, China). The supernatant was collected and dialyzed against 0.1 × extraction buffer (without PVPP) to remove soluble sugar. VIN activity was detected using the aforementioned method. The amount of enzyme required to produce 1 μmol of glucose per hour was defined as one VIN activity unit, and the result was expressed as U g^{-1} VIN.

The sucrose content was determined following the method described by Shao et al. (2013) with minor modifications. Take 2 g frozen peach tissue and mix well with 0.3 mL solution I [5.48 %(w/v) zinc acetate: glacial acetic acid (97:3)] and 0.3 mL solution II [2.65 %(w/v) potassium ferricyanide]. Subsequently, 3.4 mL deionized water was added, and the mixture was homogenized for 1 min by a homogenizer (model D-130, WIGGENS, Germany). The homogenate was diluted 4 times with deionized water and centrifuged at 10,000 × g for 10 min using a high-speed centrifuge. The supernatant was filtered through a 0.22 μm aqueous membrane, and 20 μL aliquot of the filtrate was injected into the HPLC system (model 2695, Waters, USA) equipped with an X BrigeTM amide column (Kromasil®100 A, Switzerland) and differential refractive index detector (Model 2414, Waters, USA). The mobile phase consisted of acetonitrile-water (80:20, v/v), with a flow rate of 1 mL/min and a column temperature of 40 ℃. The sucrose content was calculated by the retention time and peak area of the sucrose standard, and the result was expressed as g/kg FW.

2.9. Cold tolerance of transgenic Arabidopsis plants

Refer to the description of Zhang et al. (2023b). The *PpWRKY4* transgenic *Arabidopsis* was obtained using *Agrobacterium tumefaciens*-mediated inflorescence infiltration. The infiltration solution was prepared by GV3101/pBI121-*PpWRKY4* (as described in Section 2.7). *Arabidopsis* plants at the full flowering stage were selected for the experiment. The fruit pods were cut off, and the inflorescences were submerged in the infiltration solution for 1 min. Following this, the plants were cultured in a sealed plastic bag for 24 h before being returned to normal growth conditions until seed maturation and harvest. Seeds obtained from these infected plants were designated as the T0 generation. Subsequent generations were grown and harvested continuously until the T3 homozygous line was obtained. Genomic DNA was extracted from transgenic *Arabidopsis* leaves using the TransDirect Plant Tissue PCR Kit (TransGen Biotechnology Co., Ltd., Beijing), and positive transformants were screened accordingly.

The T3 transgenic *Arabidopsis* from three different lines and wild-type (WT) *Arabidopsis* were seeded on 1/2 MS medium, respectively. After being cultured in the incubator for 1 week, the seedlings were divided into two groups. One group was cultured at 25 ℃ for 6 d, while the other group was subjected to cold stress at 5 ℃ for 2 d and then recovered at 25 ℃ for 4 d. The phenotype of the *Arabidopsis* plants was observed and calculated the survival rate.

3,3′-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) staining were employed to visually analyze reactive oxygen species (ROS) changes in transgenic *Arabidopsis* plants (Cao et al., 2023). Four-week-old plants were exposed to 5 ℃ for 2 d, and then the leaves were taken for staining. The leaves were immersed in either a 1 mg/mL DAB solution (pH 3.8) or a 0.1 mg/mL NBT solution, respectively. Staining was performed overnight in the dark. Subsequently, the leaves were transferred to 95 % ethanol and boiled for 10 min to thoroughly remove chlorophyll. Finally, the leaves were observed and photographed.

Four-week-old plants were subjected to cold stress at 5 ℃, and samples were collected at 0 and 48 h for analysis of relevant indicators. The malondialdehyde (MDA) content was determined by thiobarbituric acid reaction method (Mei et al., 2023). The H_2O_2 content was measured with the hydrogen peroxide content detection kit (Beijing Solarbio Science & Technology Co., Ltd.). The sucrose content in *Arabidopsis* was quantified using a plant sucrose content detection kit (BC2460, Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's instructions.

2.10. Statistical analysis

The data were presented as mean values with standard deviations (mean ± standard deviation). Statistical analysis was performed using IBM SPSS Statistics Version 25.0 (SPSS Inc., Chicago, IL, USA). Differences among groups were assessed with one-way ANOVA or Student's *t*-test, and a p-value of less than 0.05 was considered statistically significant. Figures were generated using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

3. Result

3.1. Expression and cluster analysis of PpWRKYs at low temperature

The heat map of WRKY gene expression at 5 ℃ for 36 h was presented in Fig. 1. There are 18 *WRKY* genes showed up-regulation within 24 h under cold stress, including *PpWRKY1/2/4/19/21/22/25/26/30/31/42/46/48/51/55/58/60/61*. Among these, *PpWRKY4* (XM_007208228), *PpWRKY42* (XM_007208140), *PpWRKY48* (XM_007219434), and *PpWRKY51* (XM_007214189) exhibited relatively higher expression levels. These results indicated that *PpWRKY4/42/48/51* were sensitive to cold stress and might be involved in regulating the cold response of peach fruit.

3.2. Postharvest MeJA treatment down-regulates PpWRKY4 in peach fruit

As shown in Fig. 2A, the expression level of the cold-responsive marker genes *PpCBF1*, *PpCBF5*, and *PpCBF6* were upregulated during cold storage. At low-temperature treatment for 36 h, the gene expression levels of *PpCBF1/5/6* in peach fruit with MeJA treatment were remarkably up-regulated by 639-, 21-, and 35-fold, respectively. It was verified that MeJA treatment significantly reduced the chilling damage

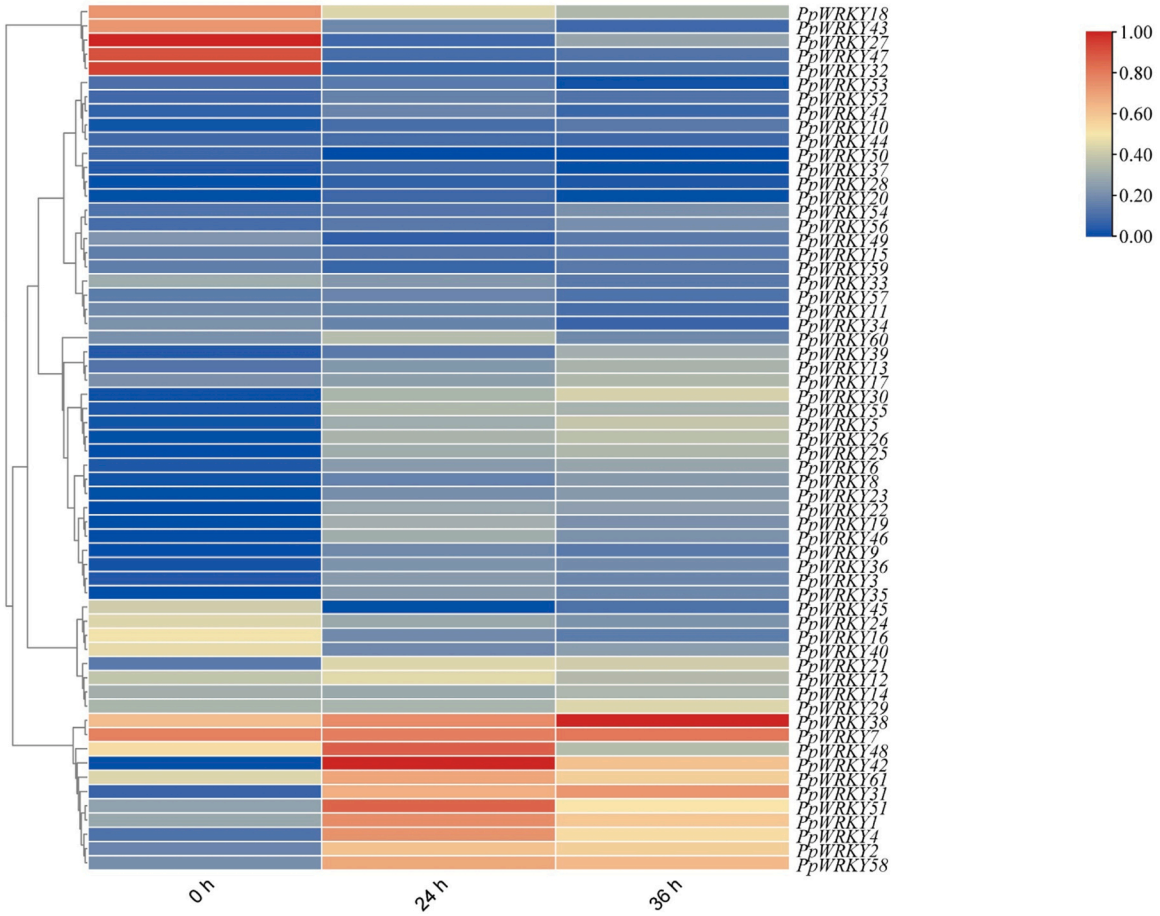

Fig. 1. Expression profiles of *WRKY* genes in peach fruit during cold storage at 5 ℃.

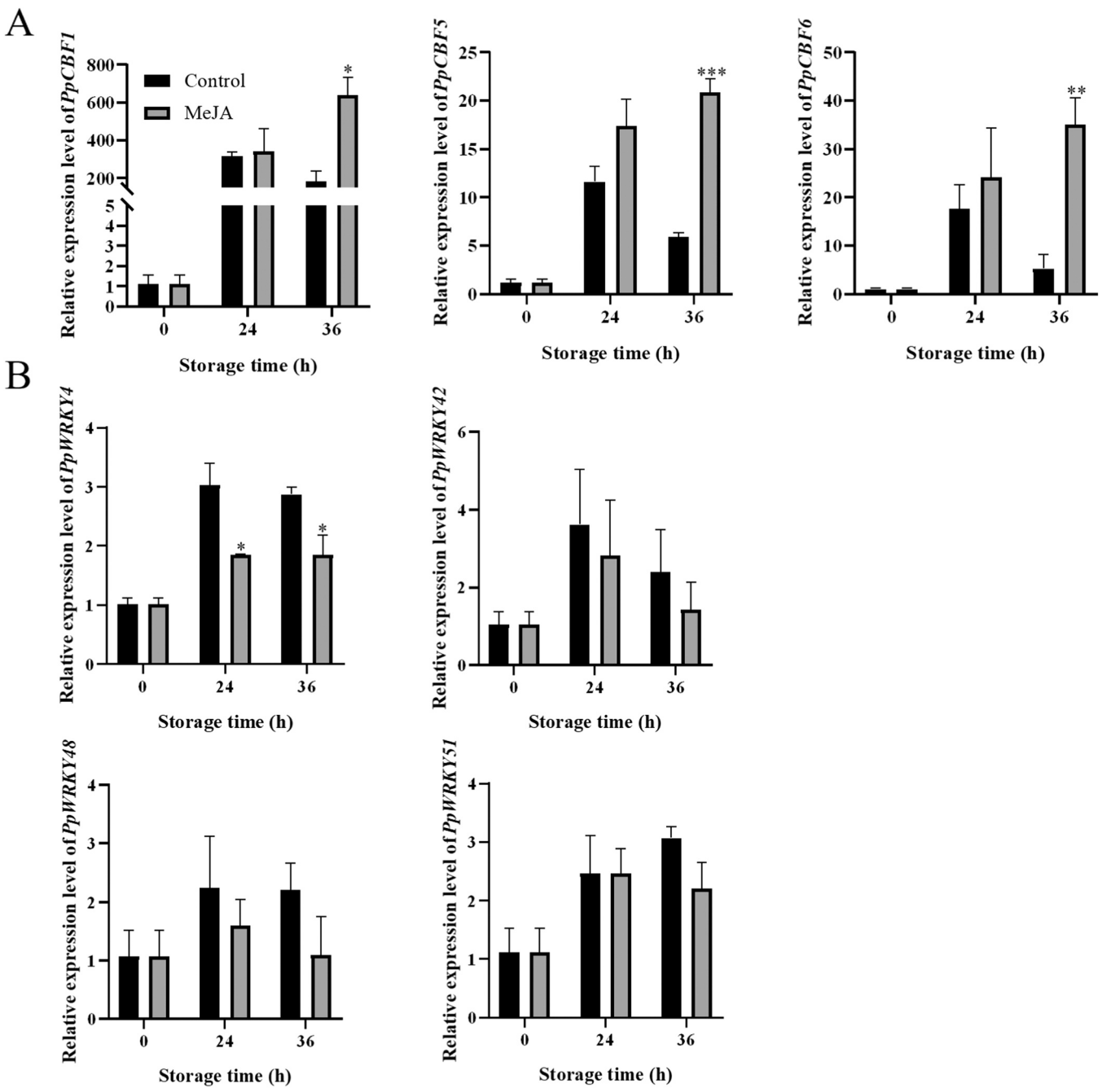

Fig. 2. Effect of MeJA treatment on the expression of *PpCBFs* and *PpWRKYs in* postharvest peach fruit during cold storage at 5 ℃. Expression of (A) *PpCBF1*, *PpCBF5*, *PpCBF6*. (B) *PpWRKY4*, *PpWRKY42*, *PpWRKY48*, and *PpWRKY51*. "*", "**", and "***" represent significant differences at $p < 0.05$, 0.01, and 0.001, respectively.

of peach fruit. Among the aforementioned four candidate WRKY transcription factors, compared to control group, the gene expression level of *PpWRKY4* in MeJA-treated fruit was significantly downregulated at both 24 and 36 h, but *PpWRKY42/48/51* did not exhibit a marked response to MeJA treatment. These results indicated that PpWRKY4 may play a role in regulating cold tolerance in MeJA-treated peach fruit.

3.3. Subcellular localization of PpWRKY4

When PpWRKY4-GFP was expressed as a fusion protein, green fluorescence appeared in the nucleus of tobacco leaf cells, while green fluorescence of positive control was detected in the nucleus, cell membrane, and cytoplasm (Fig. 3). These results elucidated that PpWRKY4 is a nuclear-localization protein.

3.4. PpWRKY4 binds to the promoter of PpVIN2 and positively regulates its expression

As presented in Fig. 4A, the growth of yeast [pABAi-*PpVIN2pro* + pGADT7-PpWRKY4] on SD/-Leu/AbA^{150} defective medium was similar to that of positive control. In contrast, the negative control failed to grow on this medium. These results demonstrated that PpWRKY4 could bind to the *PpVIN2*.

In Fig. 4B, the PpWRKY4 protein was co-incubated with biotin-labeled probes containing the W-Box motif, along with unlabeled cold probes. The observed bands appeared in lanes containing both the PpWRKY4 protein and biotin-labeled probes. The binding signal intensity weakened proportionally with the addition of increasing amounts of cold probes, while the addition of mutant probes had no

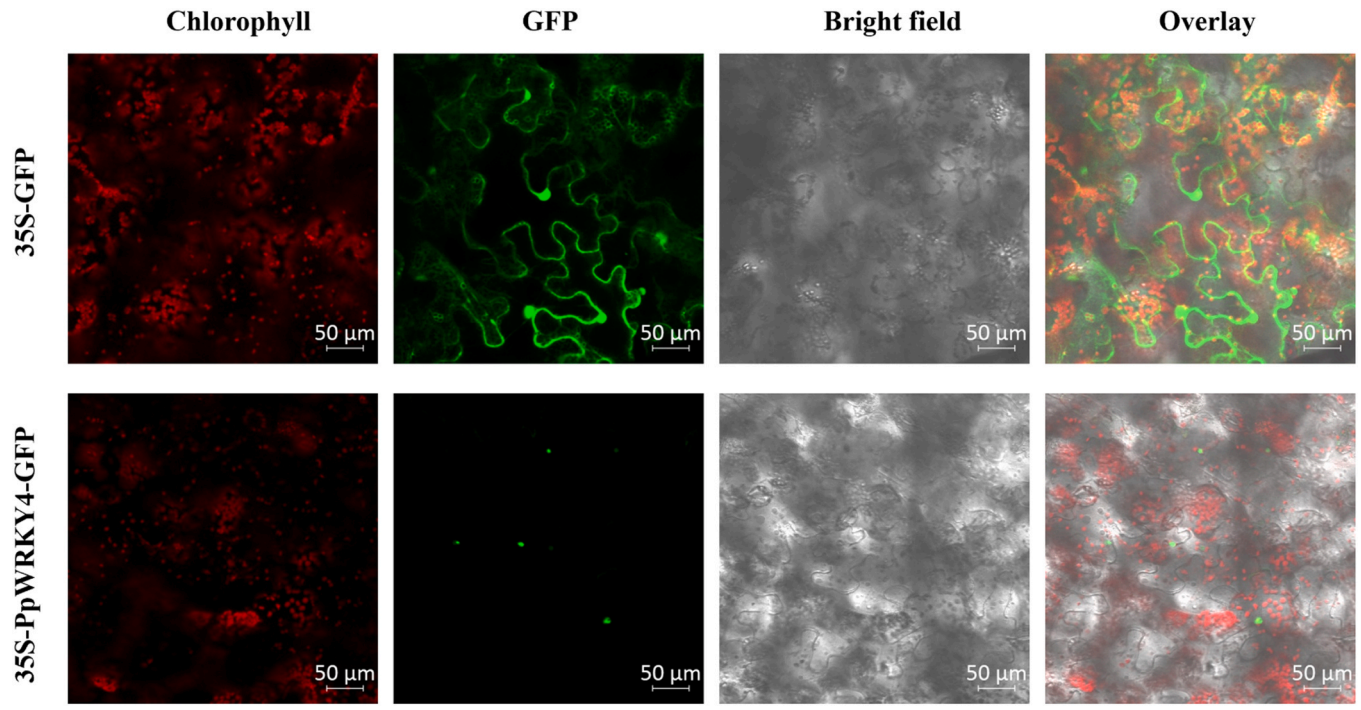

Fig. 3. Subcellular localization of PpWRKY4 in tobacco leaf. 35S-PpWRKY4-GFP: subcellular localization results of PpWRKY4, 35S-GFP: subcellular localization results of positive control, Chloroplast: chloroplast autofluorescence, GFP: green fluorescent protein, Bright field: brightfield image, Overlay: fluorescence overlay field, Bar 50 μm.

effect on the band strength, suggesting that the binding was specific. These results verified that PpWRK4 specifically interacts with the W-Box element of *PpVIN2* in vitro.

Furthermore, as illustrated in Fig. 4C, PpWRKY4 transcriptionally activates the expression of *PpVIN2*, with a relative Luc/Ren ratio of 2.23, which was prominently higher than that of the control group. Collectively, the above results elucidated that PpWRKY4 binds to *PpVIN2* and positively regulated its expression.

3.5. Transient overexpression of PpWRKY4 in peaches increased PpVIN2 expression and enzymatic activity, and decreased sucrose content

Fig. 5A confirmed that the transient overexpression of *PpWRKY4* in peach fruit was successful. Compared to the control group, the expression level of *PpVIN2* gene in OE-*PpWRKY4* group increased by 1.57 times after 1 d of infection (Fig. 5B), accompanied by an enhancement in VIN enzyme activity (Fig. 5C). These findings indicated that PpWRKY4 positively regulated gene expression and enzyme activity by binding to *PpVIN2* in peach fruit. Following the transient overexpression of *PpWRKY4*, the sucrose content of peach fruit continuously decreased during 3 d of storage and was significantly lower than that in the control group (Fig. 5D).

3.6. Overexpression of PpWRKY4 enhances cold tolerance in transgenic Arabidopsis thaliana

As shown in Fig. 6A, both WT and three independent transgenic *Arabidopsis* lines exhibited healthy growth under normal culture conditions (25 ℃), with no significant differences observed. However, after exposure to 5 ℃ for 2 days followed by recovery at 25 ℃ for 4 days, some transgenic plants displayed leaf yellowing and death. In contrast, the majority of WT plants retained green leaves and demonstrated a superior survival state compared to the transgenic lines. Furthermore, the survival rate of WT plants was 58.86 %, whereas that of the three transgenic *Arabidopsis* lines (L1, L2, and L3) were 32.46 %, 36.59 %, and 32.53 %, respectively (Fig. 6B). These findings elucidated that the overexpression of *PpWRKY4* reduced the survival rate and cold tolerance of *Arabidopsis* under low temperature stress.

As presented in Fig. 6C, regardless of DAB and NBT staining, the staining intensity in the leaves of transgenic *Arabidopsis* was more pronounced than that in WT plants (Fig. 6C). Indicating that low-temperature stress elevated the production rate of H_2O_2 and O_2^- in *Arabidopsis* tissues, and the overexpression of *PpWRKY4* further enhanced the accumulation of H_2O_2 and O_2^-.

For H_2O_2 content (Fig. 6D), no significant difference was observed between WT and the three overexpression lines prior to cold stress. However, under low-temperature stress, the H_2O_2 content increased compared to WT plants, especially in L1 line. As illustrated in Fig. 6E, there was no significant difference in MDA content between WT and overexpression lines before cold stress, except that the L2 line was significantly lower than other groups. Cold stress induced an increase in MDA content, particularly in the L1 and L3 lines, which were remarkably higher than those of WT plants. The above results indicated overexpressing *PpWRKY4* transgenic *Arabidopsis* plants experienced greater oxidative stress damage.

3.7. Effects of low-temperature stress on AtVIN2 gene expression and sucrose content of transgenic Arabidopsis thaliana

As illustrated in Fig. 7A-C, prior to low-temperature stress, no significant difference appeared in *AtCBF* genes expression between transgenic and WT *Arabidopsis*. Upon exposure to low-temperature stress, the expression of *AtCBF* genes markedly increased in transgenic *Arabidopsis*. Notably, in the L3 line, the expression levels of *AtCBF1/2/3* were outstandingly higher than that of WT, which indicated that the overexpression of *PpWRKY4* in *Arabidopsis* effectively activates the expression of cold stress-related genes.

As shown in Fig. 7D, except for the L3 line, there was no significant difference in *AtVIN2* expression between the transgenic and WT lines before low-temperature stress. Cold stress up-regulated the expression of *AtVIN2*, with the expression level in the L2 transgenic line being remarkably higher than that in the WT plants. Prior to cold stress, no

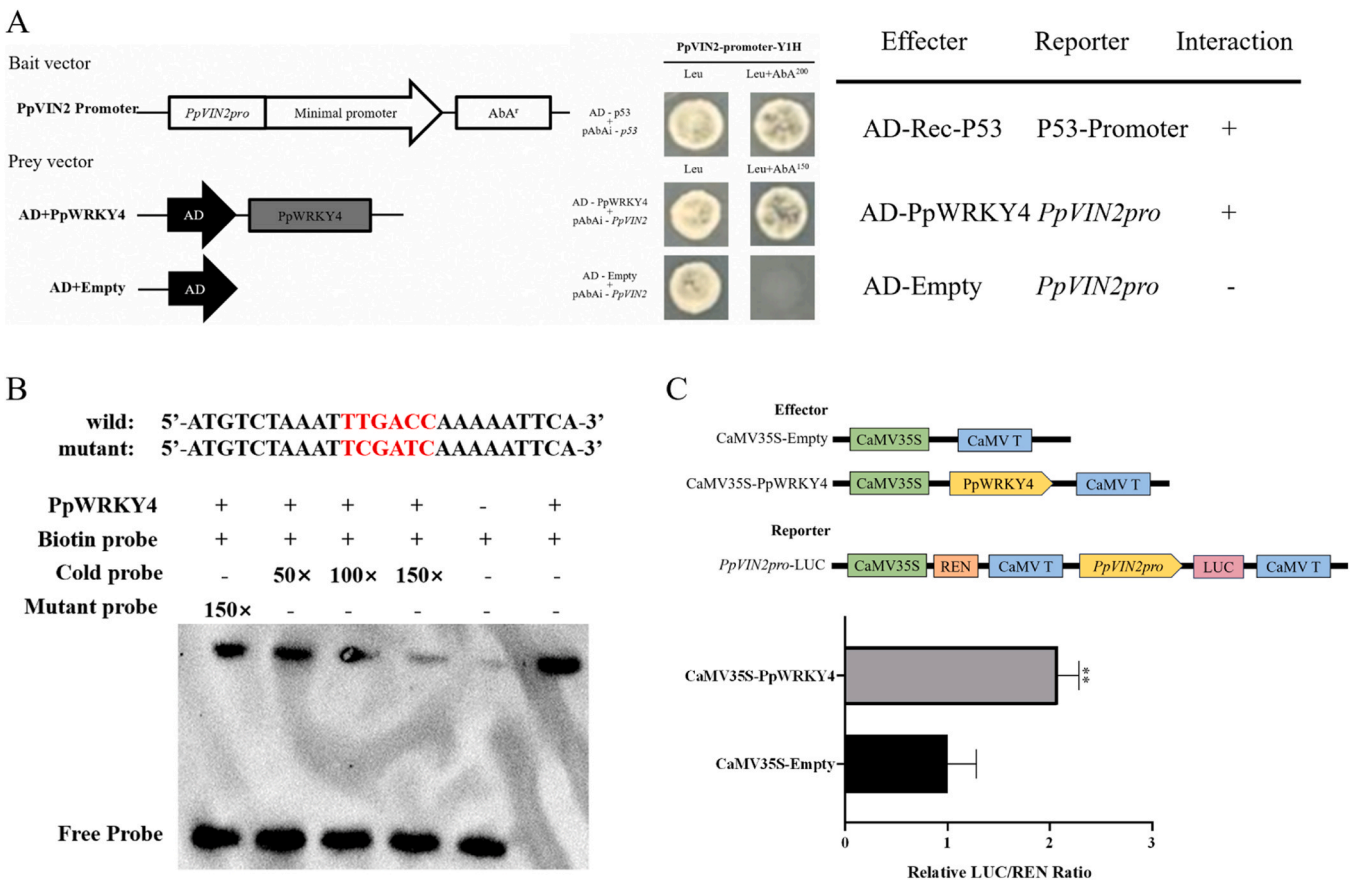

Fig. 4. PpWRKY4 binds to the promoter of *PpVIN2* and acts as a promoter of *PpVIN2* expression. (A) Y1H assay. Yeast co-transformed by pABAi-53 and pGADT7–53 were used as positive controls. Yeast transformed by pAbAi-*PpVIN2pro* and pGADT7 were used as negative controls. (B) EMSA of PpWRKY4-His and *PpVIN2pro* in vitro interaction. PpWRKY4 was incubated with biotin-labeled, unlabeled, and mutant *PpVIN2pro* probes. The symbols "+ " and "-" indicate presence or absence, respectively. (C) Dual-luciferase reporter assay in tobacco leaf. Top: Schematic of the reporter and effector structure, bottom: Transcriptional activation activity of PpWRKY4. The LUC/REN ratio of pGreenII 62-SK reporter was normalized as 1. "**" represents significant differences at $p < 0.01$.

prominent difference of sucrose content was exhibited between the transgenic and WT plants (Fig. 7E). In response to cold stress, the sucrose content in WT plants increased, whereas it decreased in all three transgenic lines. In particular, the sucrose content of L2 line was significantly lower than that in the WT plants. These results showed that the overexpression of *PpWRKY4* accelerated sucrose metabolism under low-temperature stress.

4. Discussion

Under abiotic stress, free radicals will continuously accumulate in cells. Once the accumulation exceeds the plant's own ability to eliminate them independently, it will cause harmful effects on the plant. Therefore, transcription factors are crucial for the regulation of abiotic stress in plants. WRKY is an important family of transcription factors that respond to cold stress and are closely associated with cold tolerance in plants. Both tomato ShWRKY55 (Zhang et al., 2024a) and tea tree CsWRKY21 (Mi et al., 2024) were activated by cold signal, and enhanced the cold tolerance of wild tomatoes and tea plants by transcriptionally regulating key genes. MaWRKY21 responded promptly to the cold stress of bananas and enhanced the cold tolerance in bananas by regulating ROS accumulation (Chen et al., 2025). In peach fruit, PpWRKY2 responsive to cold stress, positively regulated the cold tolerance by activating the expression of *PpMLP10* (Ma et al., 2024). In this study, the expression of 61 WRKY family genes in peach fruit under cold stress was analyzed, *PpWRKY4/42/48* and *PpWRKY51* were identified to be highly sensitive to cold stress (Fig. 1). It is well-known that MeJA treatment can alleviate plants CI. Zhou et al. (2021) revealed that tomato fruits treated with MeJA had higher levels of sucrose accumulation, thus effectively alleviating CI. Studies have demonstrated that MeJA enhanced the cold tolerance of peach fruit by regulating α-linolenic acid metabolism, JA signaling pathway (Huan et al., 2022), as well as activating mitochondrial energy metabolism (Wang et al., 2022b). In our previous research, MeJA treatment maintained higher sucrose content of peach fruit and heightened the cold tolerance (Yu et al., 2016). In this study, MeJA treatment upregulated the expression of cold-responsive marker genes *PpCBF1/5/6* (Fig. 2A). Among the cold-sensitive *PpWRKY* genes (*PpWRKY4/42/48/51*), only *PpWRKY4* showed a prominent response to MeJA treatment (Fig. 2B). Indicated that PpWRKY4 was closely related to the cold tolerance regulation of peach fruit.

Sucrose metabolism plays a vital role in the CI of peach fruit, with higher sucrose content contributing outstandingly to enhancing the cold tolerance of peach fruit. Wang et al. (2025) and Zhao et al. (2022) demonstrated that hydrogen sulfide and ABA treatment alleviated the CI of peach fruit by inhibiting the decomposition of sucrose. Sucrose not only strengthens the stability and fluidity of cell membrane, but also regulates the osmotic pressure between the inside and outside of cells (Van den Ende and Valluru, 2009). Studies have reported that low temperature have reduced the sucrose content in melon fruit (Yang et al., 2025). Wang et al. (2013) revealed that the sucrose content of chilling-injured peach fruit reduced sharply while the reducing sugar level increased. Compared to non-chilling-injured peaches, the activity of enzymes involved in sucrose metabolism, such as AI, NI, sucrose synthase (SS), and sucrose phosphate synthase (SPS), as well as the expression levels of related genes, were higher in chilling-injured peach fruit (Yu et al., 2015). This indicated that the CI markedly reduced

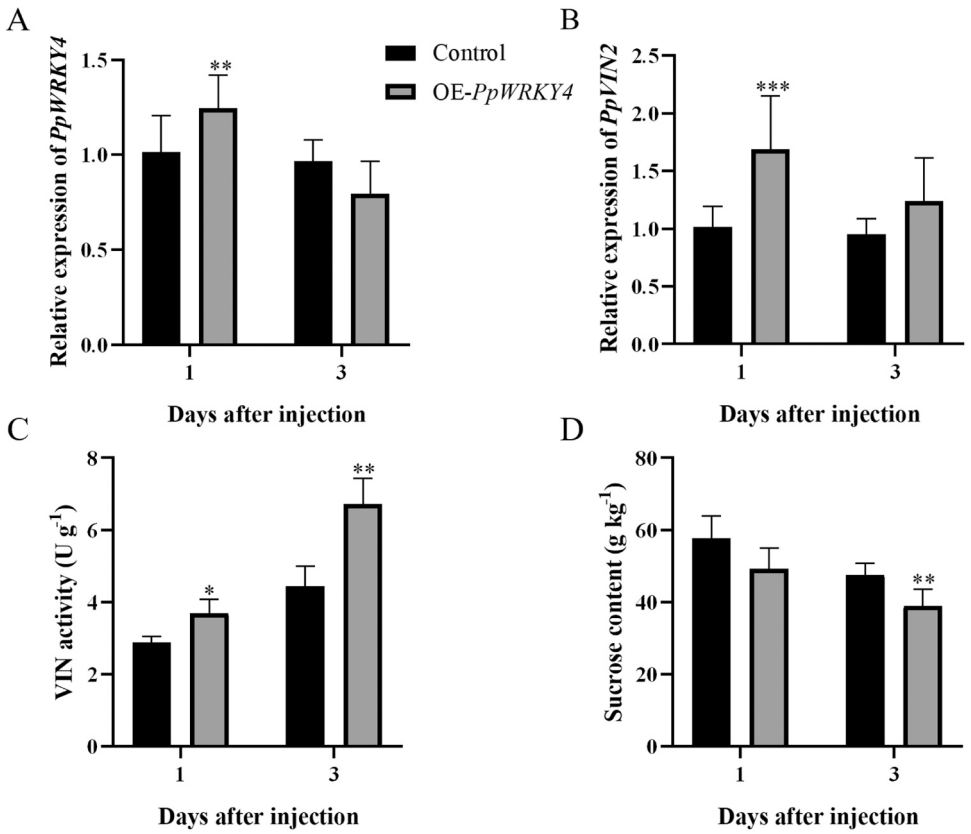

Fig. 5. Transient overexpression (OE) of *PpWRKY4* in peaches at 1st and 3rd d after infiltration with *Agrobacterium*. (A) The relative expression level of *PpWRKY4* and (B) *PpVIN2*. (C) VIN activity. (D) Sucrose content. "*", "**", and "***" represent significant differences at $p < 0.05$, 0.01, and 0.001, respectively.

sucrose levels by increasing sucrose decomposition activity. Moreover, previous studies have demonstrated that cold stress induced ROS accumulation and subsequent oxidative damage, which had adverse effects on seed germination and survival rate of *Arabidopsis* plants (Huang et al., 2010). Fei et al. (2022) found that overexpression of *KoWRKY40* decreased MDA and H_2O_2 levels, thereby enhancing the cold tolerance of transgenic *Arabidopsis* plants. Similarly, in wheat and soybeans, both TaWRKY19 and GmWRKY21 endowed cold stress tolerant to transgenic *Arabidopsis* plants (Niu et al., 2012; Zhou et al., 2008). VhWRKY44 enhanced the cold resistance of transgenic *Arabidopsis thaliana* by increasing the proline content and reducing the MDA content (Zhang et al., 2024b). In contrast, this study revealed that under cold stress, the survival rate of *PpWRKY4* transgenic *Arabidopsis* was markedly lower than that of WT plants (Fig. 6A and B), and transgenic *Arabidopsis* exhibited higher levels of O_2^-, MDA, and H_2O_2 (Fig. 6C, D, E), which indicated that PpWRKY4 acts as a negative regulator of cold tolerance.

Invertase holds a particularly crucial position in sucrose metabolism and VIN is closely related to cold tolerance in plants (Qian et al., 2018). In peach fruit, *PpVIN2* has been identified as the only VIN gene that responds to cold stress (He et al., 2018). In the prior study conducted by our group, it was discovered that PpBZR1 negatively regulated *PpVIN2*, thereby inhibiting sucrose degradation in peach fruit and enhancing cold tolerance (Zhang et al., 2023b). Furthermore, both PpRAP2.12 (Cao et al., 2023) and PpZAT10 (Chen et al., 2022) enhanced VIN enzyme activity and promoted the sucrose decomposition by regulating *PpVIN2*, reduced the cold tolerance of peach fruit. The aforementioned studies demonstrated that sucrose metabolism can be influenced through regulating *PpVIN2*, which in turn significantly impacts the cold tolerance of peach fruit. In the present study, PpWRKY4 was identified to bind to *PpVIN2* and played a transcriptional activation role (Fig. 4). Transient overexpression of *PpWRKY4* in peach fruit increased the *PpVIN2* expression level (Fig. 5B) and enhanced the VIN enzyme activity (Fig. 5C), thus reducing sucrose content within the fruit (Fig. 5D). These findings indicated that PpWRKY4 regulates the cold tolerance of peach fruit by activating *PpVIN2*, a key gene in sucrose metabolism. Similarly, Wu et al. (2022) also found CoWRKY are involved in the response to low-temperature stress by regulating sugar metabolism-related genes in oil tea buds. More intriguingly, it was discovered that the overexpression of *PpWRKY4* in *Arabidopsis* upregulated the *AtVIN2* expression level and decreased sucrose content under cold stress (Fig. 7D, E). Taken together, these results illustrated that PpWRKY4 transcriptionally activated *PpVIN2*, accelerating sucrose metabolism and thus regulating the cold tolerance of peach fruit.

5. Conclusion

This study identified the cold-sensitive gene PpWRKY4, which positively regulated the expression of *PpVIN2* and accelerated sucrose metabolism. The role of PpWRKY4 in cold tolerance was further substantiated by transgenic *Arabidopsis*, wherein it was discovered that the overexpression of *PpWRKY4* reduced the sucrose content of transgenic *Arabidopsis* under cold stress. This study revealed that PpWRKY4 accelerating sucrose metabolism by transcriptionally activating *PpVIN2*, thereby regulating the cold tolerance of peach fruit. The model of PpWRKY4 regulating the cold tolerance of peach fruit displayed in Fig. 8. Such findings provide novel insights into how sucrose metabolism can be modulated to enhance cold tolerance in peach fruit, potentially providing valuable strategies for improving crop resilience against adverse environmental conditions. However, the effect of cross-regulation in PpVIN2-mediated sucrose metabolism regulation network on the cold tolerance of peach fruit remains to be further investigated.

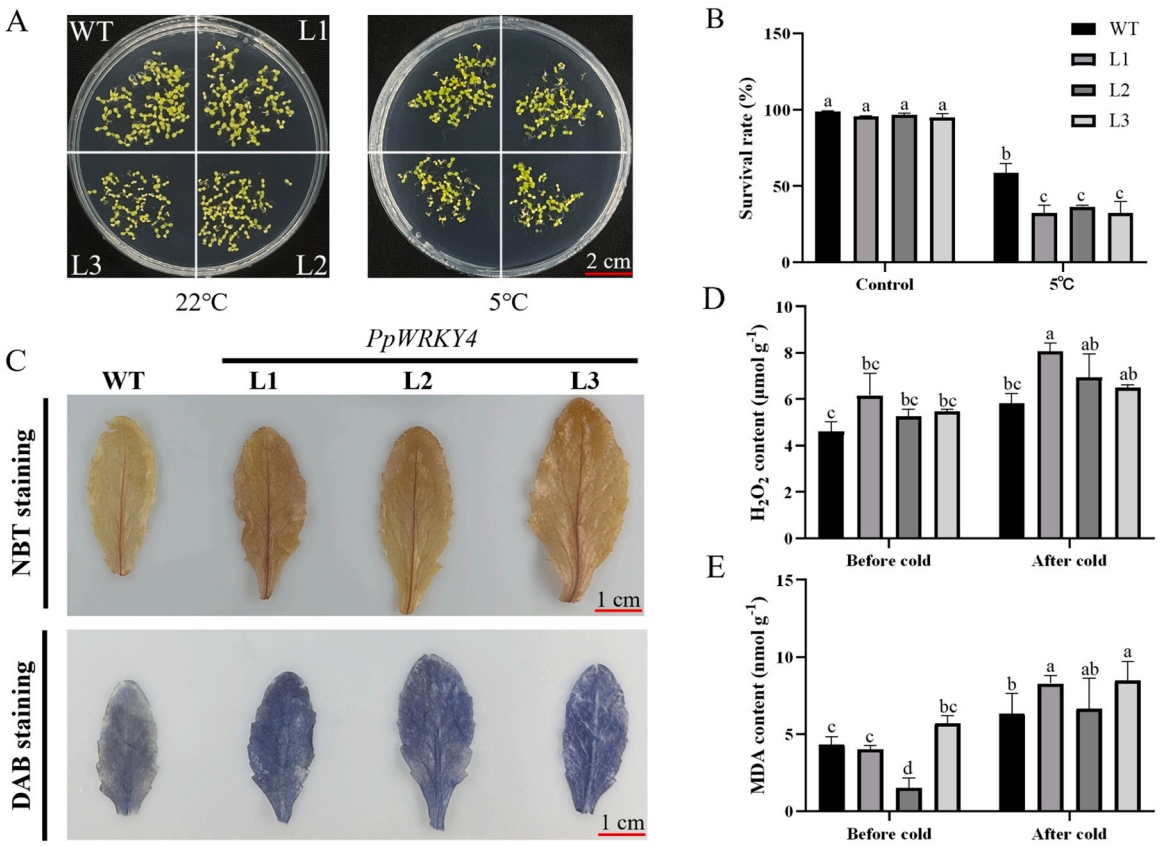

Fig. 6. Effect of cold stress on the survival state and oxidative stress in *PpWRKY4*-overexpressing transgenic *Arabidopsis*. (A) *Arabidopsis* phenotype of seed germination, WT and three transgenic *Arabidopsis* lines, scale bar 2 cm. (B) Survival rate. (C) DAB and NBT staining of *Arabidopsis*, scale bar 1 cm. (D) H_2O_2 content. (E) MDA content. Different letters indicate significant differences ($p < 0.05$).

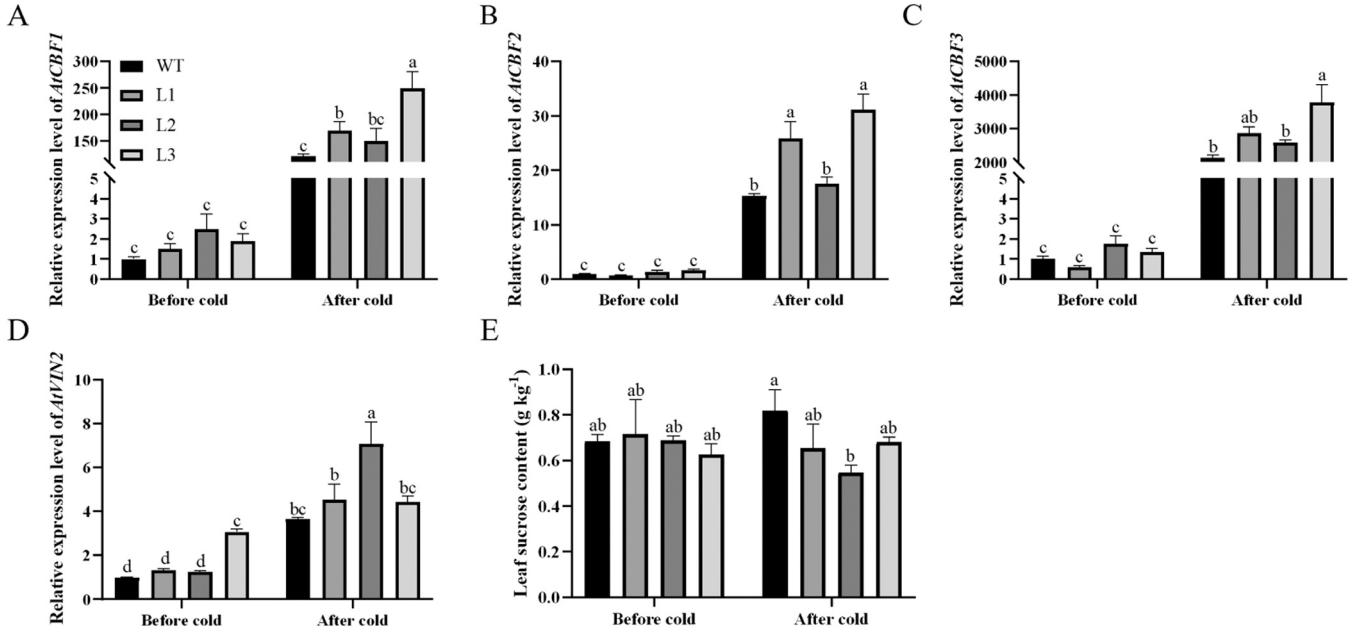

Fig. 7. Effect of *PpWRKY4* expression on the cold tolerance of *Arabidopsis*. (A-D) The relative expression levels of *AtCBF1*, *AtCBF2*, *AtCBF3*, and *AtVIN2*, respectively. (E) Leaf sucrose content. Different letters indicate significant differences ($p < 0.05$).

CRediT authorship contribution statement

Xingfeng Shao: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Yingying Wei:** Writing – review & editing, Supervision, Methodology. **Yi Chen:** Writing – review & editing, Supervision, Formal analysis. **Phebe Ding:** Investigation, Formal analysis. **Feng Xu:** Validation, Funding acquisition, Data curation. **Shiyun Zhang:** Software, Investigation, Formal analysis, Data

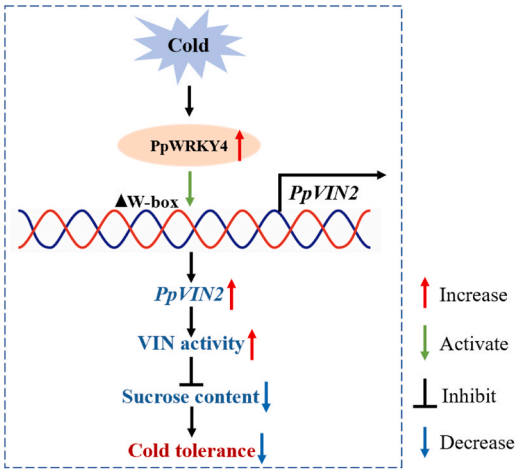

Fig. 8. The model of PpWRKY4 regulating the cold tolerance of peach fruit. In peach fruit, low temperature treatment up-regulated the expression of *PpWRKY4*, which activates the gene expression of *PpVIN2*. Enhanced the activity of VIN enzyme and accelerated the decomposition of sucrose, thus reducing the cold tolerance of peach fruit. Red arrow indicates increase compared to the control group, and blue indicates decrease compared to the control group. Green arrow indicates activation, and the black T-shaped line indicates inhibition.

curation. **Yongyan Wu:** Writing – original draft, Visualization, Validation, Investigation, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.postharvbio.2025.113729.

Data availability

Data will be made available on request.

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