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PpWRKY19-like regulates peach fruit browning during cold storage by activating *PpLAC7* expression

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Abstract

Cold storage is widely used postharvest to preserve fruits; however, extended storage can trigger chilling injury (CI), manifesting as internal browning (IB) in peaches (*Prunus persica*), which severely affects marketability. The physiological and molecular mechanisms underlying this phenomenon remain unclear. In this study, we compared two peach cultivars with contrasting storage phenotypes: 'Zhongtao No. 14', which exhibited severe IB, and 'Zhongyou No. 13', which did not show any IB symptoms. Comparative physiological analysis revealed significant differences in malondialdehyde (MDA) accumulation, total phenol content, antioxidant capacity and laccase enzymatic activity between the cultivars. We identified a laccase gene, *PpLAC7*, and a divergent *WRKY* transcription factor, *PpWRKY19-like*, whose expression levels were strongly correlated with browning severity. Dual-luciferase and yeast one-hybrid assays confirmed that *PpWRKY19-like* directly binds to the *PpLAC7* promoter. Functional validation through overexpression in fruit flesh further demonstrated that *PpWRKY19-like* activates *PpLAC7* expression, significantly enhancing laccase activity and promoting browning. This study elucidates a novel regulatory module, *PpWRKY19-like–PpLAC7*, providing significant insights into the enzymatic mechanisms of chilling injury and offering genetic targets for improving postharvest peach quality.

Keywords: Cold storage, Browning, Peach fruit, *PpWRKY19-like*, *PpLAC7*

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Introduction

The peach (*Prunus persica* [L.] Batsch) is an economically important fruit belonging to the Rosaceae family. It is extensively grown in China, Spain, Italy, the United States, and Greece (United Nations Food and Agriculture Organization Statistics Division)[1]. Although these nations are the primary producers, many other countries that do not cultivate peaches import substantial quantities. As a perishable fruit, peaches face significant issues with spoilage and postharvest losses, which play a major role in global food waste[2]. To minimize spoilage and prolong the shelf life of peaches to approximately 14–21 d, they are usually stored at temperatures ranging from 0 to 5°C[3]. However, storing peaches at these temperatures for 2–3 weeks can result in chilling injury (CI)[4].

Fruits from sensitive species exposed to low temperatures during CI undergo various physiological, biochemical, and genetic changes that lead to metabolic disruptions[5,6]. These alterations result in disorders, such as internal browning (IB) or flesh browning, surface pitting, mealiness, failure to ripen, flavor loss, and decay[7]. IB is the most prevalent disorder observed in peaches during cold storage[5,8]. Low-temperature-induced browning of peaches negatively affects their flavor and nutritional value, leading to significant losses in the commercial sector[9,10]. Therefore, understanding the mechanisms of browning during cold storage is essential for improving the quality of peach fruits.

The intricate composition of browning mechanisms in various plant species has been extensively documented. Browning results from a series of physiological and biochemical processes that occur in fruits under conditions of senescence or stress, and is categorized as either enzymatic or nonenzymatic[11]. Enzymatic browning is the most prevalent phenomenon observed in fruits and vegetables. Polyphenol oxidase (PPO) is a key enzyme involved in such browning. It catalyzes the oxidation of aromatic substrates to produce brown by-products[12]. PPO activity is positively correlated with the browning of peaches at low temperatures[13]. Furthermore, recent research has demonstrated that the browning of fruit during storage is caused by another PPO known as laccase[14,15]. Laccases (LACs; benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are a large group of blue multicopper oxidases (MCOs) that are prevalent in plants, insects, bacteria, and fungi. These enzymes are responsible for oxidizing a wide variety of substrates, such as polycyclic aromatic hydrocarbons, aromatic amines, and phenols[16,17], thus contributing to various biological processes. The *Arabidopsis thaliana* mutant, transparent testa10/12/16 (TT10/12/16), which generates an enzyme similar to LAC, delays the browning of its seed coat during development. TT10/12/16 encodes a protein, known as LAC-PPO, located in the developing testa, where it is linked with flavonols and proanthocyanidins[18]. Previous research has suggested that a disruption in the expression of genes encoding LACs may cause a shift in substrate flow within the phenylpropanoid pathway[19] Furthermore, an increase in the expression of LAC-encoding genes (*MdLAC7*) was observed during apple (*Malus domestica*) peel browning[20]. The pericarp of litchi (*Litchi chinensis*) contains a LAC enzyme known for its ability to degrade anthocyanins (*ADE/LAC*), which plays a vital role in fruit browning after harvest[21]. However, the role of LACs in the browning of peach fruit during cold storage remains unexplored.

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Cold tolerance in peaches is associated with transcriptional modifications in stress-responsive genes and the redox metabolism[22,23]. Transcription factors (TFs), including WRKYGQK (WRKY), Myeloblastosis (MYB), and NAC, significantly influence plants' growth, development, and stress responses[24,25]. *PuMYB21* and *PuMYB54* in 'Nanguo' pears (*Pyrus ussuriensis*) accelerate lipid breakdown, enhance browning, and activate the transcription of *PuPLDβ1* (phospholipase D)[26]. In plants, most WRKY TFs bind to the W-box (TTGAC) and target genes to control growth, development, and secondary metabolism[27]. Numerous studies have shown that abiotic stresses such as drought, oxidative stress, soil salinity, cold, and heat stress are controlled by WRKY TFs[28–30]. In banana (*Musa acuminata*) fruit, *MaWRKY26* can trigger the expression of jasmonic acid biosynthesis genes (*MaLOX2*, *MaAOS3*, and *MaOPR3*) to regulate cold tolerance[31]. Oxidative stress increases the expression of *SlWRKY75* and triggers the development of tissue browning in tomato (*Solanum lycopersicum*)[32]. Lipoxygenase gene transcription is regulated by *MaWRKY70* in banana fruits during the development of CI, which is associated with membrane lipid degradation[33]. Despite this progress, the specific function of WRKY TFs in chilling-induced browning of peaches remains unknown.

In this study, we examined the effects of various physiological characteristics of two different peach cultivars during cold storage. We assessed the expression levels of several LAC family genes and WRKY TFs by reverse transcription–quantitative polymerase chain reaction (RT-qPCR). Consequently, we identified a divergent WRKY TF (*PpWRKY19-like*) that regulates a LAC family gene (*PpLAC7*) using dual luciferase and yeast one-hybrid assays. We investigated the functions of *PpWRKY19-like* and *PpLAC7* by overexpressing them in fruit flesh. Our findings offer valuable insights into the mechanisms underlying the chilling-induced browning of peaches during cold storage.

Method and materials

Plant materials and fruit treatments

In this study, we used two peach cultivars, 'Zhongtao No. 14' ('Zhongtao14') and 'Zhongyou No. 13' ('Zhongyou13'). These cultivars were harvested at commercial ripeness from the peach breeding orchard at the Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Sciences, Henan, China (34°74′ N 113°62′ E). Approximately, 150 fruits without mechanical damage, disease, or pests were selected for the postharvest process. IB symptoms and physiological characteristics were assessed before storage on Day 0. The fruits were subsequently stored at 4 °C for 42 d, and each parameter was evaluated every 14 d. After the observations were complete, the flesh was cut into small pieces. These pieces from multiple fruits were then combined within the same batch, quickly frozen in liquid nitrogen, and stored in an ultra-low-temperature refrigerator for future testing.

Analysis of IB index and physiological characteristics

The IB index was assessed on the basis of the internal browning area of the peach fruit, using a scale ranging from 0 to 4: 0 = no browning, 1 = 1%–25%, 2 = 26%–50%, 3 = 51%–75%, and 4 = 76%–100% browning[34]. At each sampling timepoint, three biological replicates were used, with each replicate comprising nine fruits.

The formula: Internal browning index = [(internal browning score) × (number of fruits with this internal browning score)]/(4 × total number of fruits in each treatment). The results were expressed as percentages.

The malondialdehyde (MDA) content, antioxidant capacity, total phenol content and LAC activity were determined using commercial biochemical assay kits purchased from Suzhou Grace Biotechnology Co., Ltd. (Suzhou, China). For all assays, 0.1 g of peach mesocarp tissue was homogenized in 1 mL of the corresponding ice-cold extraction buffer, kept on ice for 10 min, and centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatant was collected for subsequent physiological measurements, and the soluble protein concentration of the extract was quantified via the Bradford assay for unit normalization. Three biological replicates were utilized for each time point.

MDA content was evaluated via the thiobarbituric acid (TBA) heating method. The absorbance of the reaction mixture was recorded at 532 nm and 600 nm, and the final value was calculated using the difference in absorbance (A_{532} – A_{600}) and are expressed as nmol·mg^{-1} protein.

Total phenol content was determined via the Folin–Ciocalteu method, with absorbance recorded at 760 nm, and the results are expressed as mg·mg^{-1} protein of gallic acid equivalents.

Antioxidant capacity was evaluated using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging method at a wavelength of 734 nm and is expressed as U·mg^{-1} protein.

LAC activity was measured by monitoring the kinetic oxidation rate of the substrate, ABTS, at 420 nm, and is expressed as U·mg^{-1} protein.

RNA extraction, cDNA synthesis, and RT-qPCR

RNA was extracted using a Huayueyang Mini Kit (Beijing Huayueyang Biotechnology Co., Ltd.). Each sampling time point had three biological replicates. For cDNA synthesis and gDNA removal, we used the HiScript® III RT SuperMix for the qPCR kit (+gDNA wiper) from Vazyme (Nanjing, China). Primers were designed using the Primer5 program for quantitative reverse transcription–polymerase chain reaction (qRT-PCR) (Supplementary Table S1). Primer specificity was confirmed by sequencing the polymerase chain reaction (PCR) products and performing a melting curve analysis. The *PpACT* gene from peaches was used as an internal control[35]. PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) was used for qRT-PCR on an ABI PRISM 7500 Fast Sequence Detection System 160. The $2^{-\Delta\Delta C_T}$ method was used to assess gene expression levels[36].

Phylogenetic analysis of *WRKY TFs* and *PpLAC7*

The protein sequences of *PpWRKYs* were analyzed through a BLAST search against the *Arabidopsis thaliana* database, TAIR (www.arabidopsis.org), to find similar sequences. In addition, the protein sequences of *PpLAC7* were analyzed through a BLAST search against the *Prunus persica*, *A. thaliana*, and *Malus domestica* libraries. The phylogenetic trees were constructed using the neighbor joining method in MEGA11 software, with a bootstrap analysis of 1,000 replicates to assess branch support.

Subcellular localization of *PpWRKY19-like*

The complete *PpWRKY19-like* sequence, excluding the stop codon, was amplified and fused into a pSAK277 vector containing green fluorescent protein (GFP)[37]. All primers are listed in Supplementary Table S2. The resulting *PpWRKY19-like*–pSAK277(GFP)

construct was introduced into *Agrobacterium tumefaciens* GV3101 and subsequently infiltrated into tobacco (*Nicotiana benthamiana*) leaves[38]. After 3 d, the fluorescence in the tobacco leaves was examined using a Nikon AIR HD25 laser scanning confocal microscope (Nikon, Japan).

Dual luciferase assay

Full-length TF Prupe.5G110700 (*PpWRKY19-like*) sequences and the promoter Prupe.6G267700 (*PpLAC7*) were used to construct the pGreen II 62-SK and pGreen II 0800-LUC vectors. The primers are listed in Supplementary Table S2. These constructs were introduced into *A. tumefaciens* GV3101 containing a p-soup via electroporation. Dual luciferase assay was performed on tobacco leaves (*N. benthamiana*), following a previously described method[39]. Briefly, to achieve an optical density at 600 nm (OD_{600}) of 0.75, *A. tumefaciens* cells with the constructs were suspended in a solution of ddH_2O, 150 μM acetosyringone, 10 mmol·L^{-1} 2-(*N*-morpholino)ethanesulfonic acid (MES), and 10 mmol·L^{-1} $MgCl_2$ at pH 5.6. *A. tumefaciens* cultures containing the TF and promoter constructs were mixed in a 10:1 ratio for infiltration into tobacco leaves. Firefly and Renilla luciferases (LUC and REN, respectively) were quantified using a spark multimode microplate reader (Tecan, Switzerland) with dual luciferase assay reagents (Promega, USA) after 3 d of infiltration. An empty vector (SK) containing the promoter was set as a control.

Yeast one-hybrid assay

To conduct the yeast one-hybrid (Y1H) assay, the −2,000-bp region of the *PpLAC7* promoter was extracted from peach genomic DNA and then inserted into the pAbAi vector. The yeast strain Y1HGold was co-transformed with the recombinant plasmids pGADT7-*PpWRKY19-like* and pAbAi-*PpLAC7*-pro following the guidelines provided by the manufacturer (Clontech). An AD empty vector (pGADT7) with the *PpLAC7* promoter was used as a negative control. Transformants were cultured in SD/−Ura drop-out medium (SD without Uracil). Once colonies were selected and diluted in sterile double-distilled (dd) H_2O to achieve an OD_{600} of 0.5, a 3 μL suspension was applied to SD/−Leu drop-out medium (SD without Leucine) containing the antibiotic Aureobasidin A (AbA_{400}) and incubated at 30 °C. All primers are listed in Supplementary Table S2.

Transient overexpression of *PpWRKY19-like* and *PpLAC7* in peach flesh

The enzyme EcoRI was used to amplify and insert the *PpWRKY19-like* and *PpLAC7* gene sequences into the pSAK277 vector. Detailed information about the primers are provided in Supplementary Table S2. The newly constructed vectors, pSAK277-*PpWRKY19-like* and pSAK277-*PpLAC7*, were introduced into the *A. tumefaciens* strain GV3101. Subsequently, these vectors were used to treat the sliced flesh of 'Zhongyou13' which did not show browning symptoms during cold storage. Peach fruits with peel were immersed in a 5% (w/v) sodium hypochlorite solution for 20 min. To treat the peach flesh, the *Agrobacterium* strains containing the vectors were mixed with a solution as described for the dual luciferase assay. The sliced flesh was immersed in this solution for 10 min, followed by rinsing with ddH_2O three or four times. The samples were then dried using filter paper. The slices were cultured on Murashige and Skoog (MS) medium, which contained 6.5 g agar, 30 g sucrose, and 4.43 g M519 (MS basal medium with vitamins). Before adding agar, the pH was adjusted to 5.8, and 1 mL of timentin (2,000 mg/mL) was added. The samples were kept under light conditions at 25 °C for 3 d.

Statistical analysis

All statistical analyses were performed using Microsoft Excel (Microsoft, Redmond, WA, USA). Student's t-tests were used for analyzing the significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). We used GraphPad Prism 9.01 to draw the figures.

Results

IB symptoms, IB index, MDA content, antioxidant activity, and total phenol content in different peach cultivars during cold storage

The primary indicator of CI is internal browning[34]. Of these two cultivars, 'Zhongtao14' began to exhibit IB symptoms after 14 d, with the IB index reaching 54% at 28 d and 80% at 42 d. In contrast, 'Zhongyou13' showed no IB symptoms during cold storage (Fig. 1a, b). MDA damages the cell membrane during cold storage, contributing to fruit browning[35]. In 'Zhongtao14', MDA content significantly increased from 0.80 to 2.98 nmol·mg^{-1} protein over the cold storage period. Conversely, 'Zhongyou13' did not show a significant rise in MDA content (Fig. 1c). 'Zhongtao14' exhibited fluctuations in total phenol content throughout the cold storage period, whereas 'Zhongyou13' demonstrated a reduced total phenol content (Fig. 1d). Antioxidant activity in 'Zhongyou13' increased significantly; however, 'Zhongtao14' displayed a minor increase at 14 d, followed by a substantial decline (Fig. 1e).

Analysis of the relative expression of LAC family genes and LAC activity

We identified two genes from the LAC family, *PpLAC7* (Prupe.6G267700) and *PpLAC9* (Prupe.8G048100), from our previous transcriptome examination of a browning cultivar, 'Zhongtao No. 9' ('Zhongtao9')[40], which exhibited increased expression during cold storage (Supplementary Fig. S1a). Subsequently, RT-qPCR analysis indicated that the expression levels of *PpLAC7* and *PpLAC9* varied among the cultivars during cold storage (Fig. 2a, b). In 'Zhongtao14', *PpLAC7* expression was 213.96 times higher at 42 d compared with 0 d. In contrast, 'Zhongyou13' did not show a significant increase in *PpLAC7* expression. Similarly, *PpLAC9* expression in 'Zhongtao14' was 21.94 times higher at 42 d compared with 0 d, whereas 'Zhongyou13' did not exhibit an upregulation of *PpLAC9*. LAC activity in 'Zhongtao14' increased significantly from 13.14 to 123.84 U·mg^{-1} protein over the cold storage period. However, 'Zhongyou13' did not show a significant increase in LAC activity during cold storage (Fig. 2c).

Expression profiles of *WRKY* TFs and sequence features, proteins, and subcellular localization of *PpWRKY19-like*

To identify WRKY transcription factors, we used our previous transcriptome data from 'Zhongtao9', a cultivar showing browning during cold storage[40]. We screened 14 WRKY TFs and among these, eight WRKY TFs, namely *PpWRKY65* (Prupe.4G066400), *PpWRY19-like* (Prupe.5G110700), *PpWRKY74* (Prupe.6G345100), *PpWRKY75* (Prupe.1G223200), *PpWRKY48* (Prupe.1G114800), *PpWRKY47* (Prupe.3G002300), *PpWRKY40* (Prupe.3G098100), and *PpWRKY71* (Prupe.3G174300), exhibited upregulated expression levels during cold storage (Supplementary Fig. S1b). We validated the expression levels of these eight TFs in 'Zhongtao14' and 'Zhongyou13' using RT-qPCR (Fig. 3). In 'Zhongtao14', all TFs showed significant

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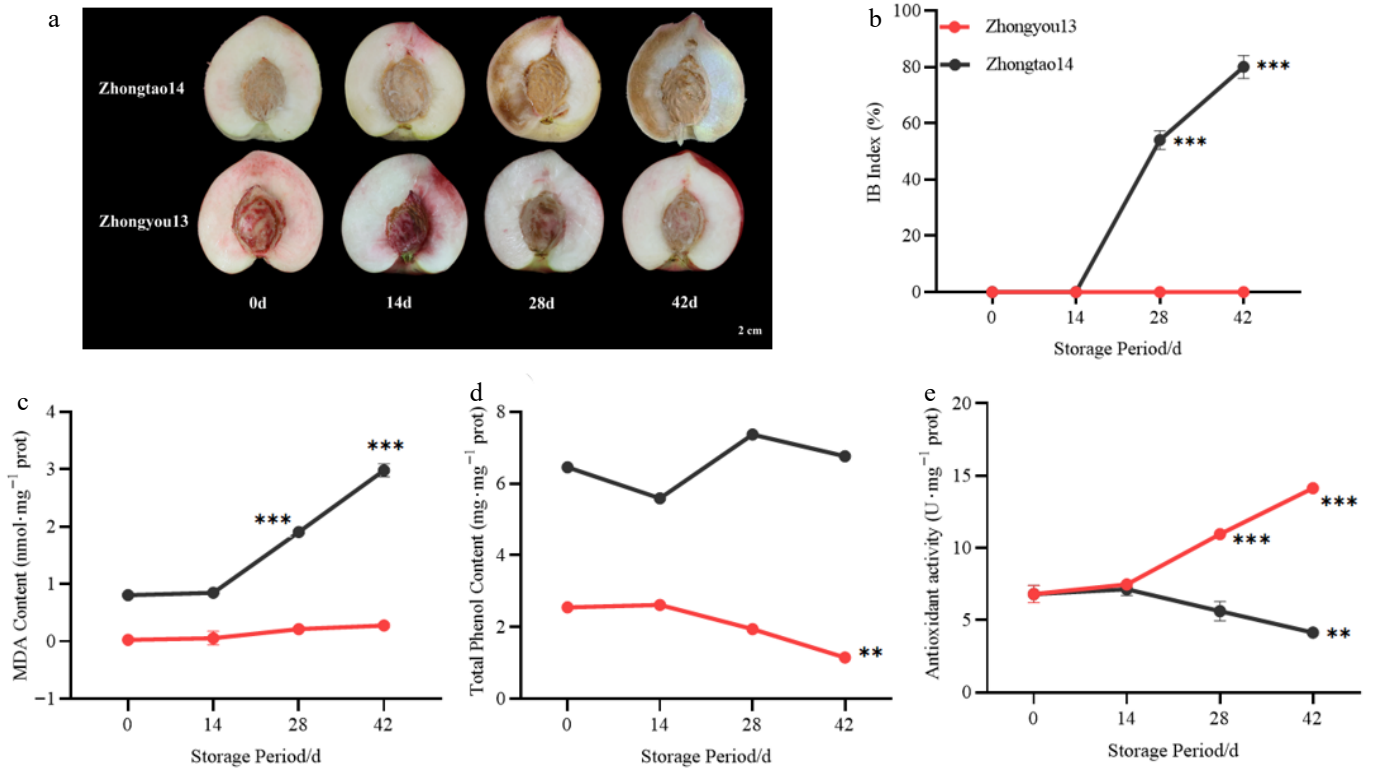

Fig. 1 Phenotypic characteristics, MDA content, total phenol content, and antioxidant activity in different peach cultivars. (a) Internal browning symptoms in two different peach cultivars. Scale bar = 2 cm, (b) IB index, (c) MDA content, (d) antioxidant activity, and (e) total phenol content. Error bars indicate the means ± standard error (SE) from three biological replicates. * indicates significant differences between the control (0 d) and the different cold storage treatment timepoints (14, 28, and 42 d) based on Student's *t*-tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

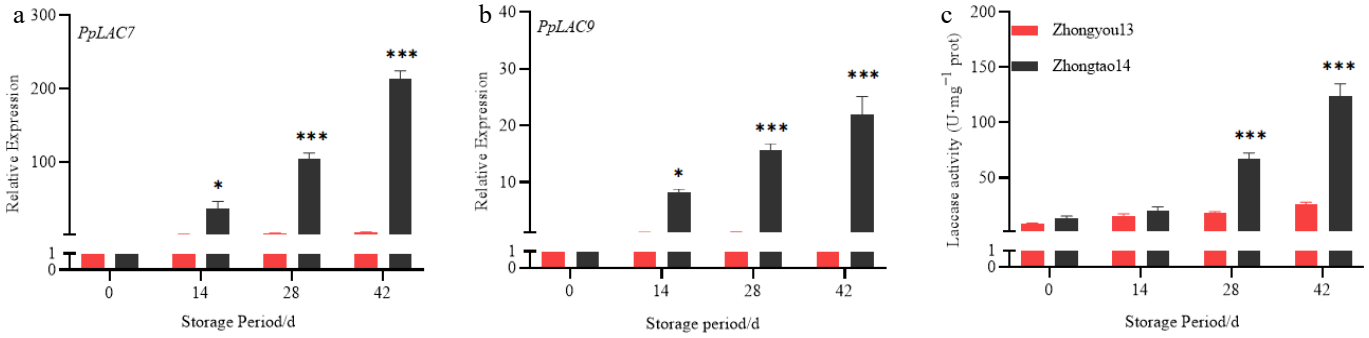

Fig. 2 Effect of relative expression of LAC family genes and LAC activity in different peach cultivars. (a) Analysis of the relative expression of *PpLAC7*, (b) *PpLAC9*, and (c) LAC activity. Error bars indicate the means ± SE from three biological replicates. * indicates significant differences between the control (0 d) and the different cold storage treatment timepoints (14, 28, and 42 d) based on Student's *t*-tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

upregulation compared with Day 0, whereas 'Zhongyou13' did not show a notable increase throughout the cold storage period. Interestingly, *PpWRKY19-like* exhibited a 198.58-fold increase in 'Zhongtao14', which was the highest expression level among all the candidate TFs. Therefore, we selected *PpWRKY19-like* for further analysis. Although it was initially identified as a WRKY family member via transcriptome annotation, sequence analysis and AlphaFold-based structural modeling revealed that *PpWRKY19-like* possesses an atypical architecture compared with canonical WRKY TFs. It lacks the WRKYGQK heptapeptide, and the protein contains a tandem array of six C2H2-type zinc finger domains (Fig. 4b, c). These domains exhibit the conserved *C-*X_2*-C* and *H-*X_3*-H* spacing characteristic of the transcription factor IIIA (TFIIIA) - type zinc finger family, which is known for high-affinity DNA binding. This structural divergence suggests that *PpWRKY19-like* has undergone a neofunctionalization event, where the expanded C_2H_2 domains have compensated for the loss of the canonical WRKY domain, maintaining the ability to recognize and transactivate the W-box elements in the promoter region. Despite this structural divergence, its strong phylogenetic clustering with *AtWRKY40* suggests that it is a divergent WRKY-related transcription factor (Fig 4a). To determine its subcellular localization, we transiently expressed the PpWRKY19-like-pSAK277(GFP) fusion construct and pSAK277(GFP) (as a control) in tobacco leaves. The GFP signal was observed in the cell membrane, cytosol, and nucleus of the control leaves expressing pSAK277(GFP). In contrast, leaves expressing PpWRKY19-like-pSAK277(GFP) showed GFP signals exclusively localized in the nucleus (Supplementary Fig. S2). This finding suggests that PpWRKY19-like is a nuclear-localized protein.

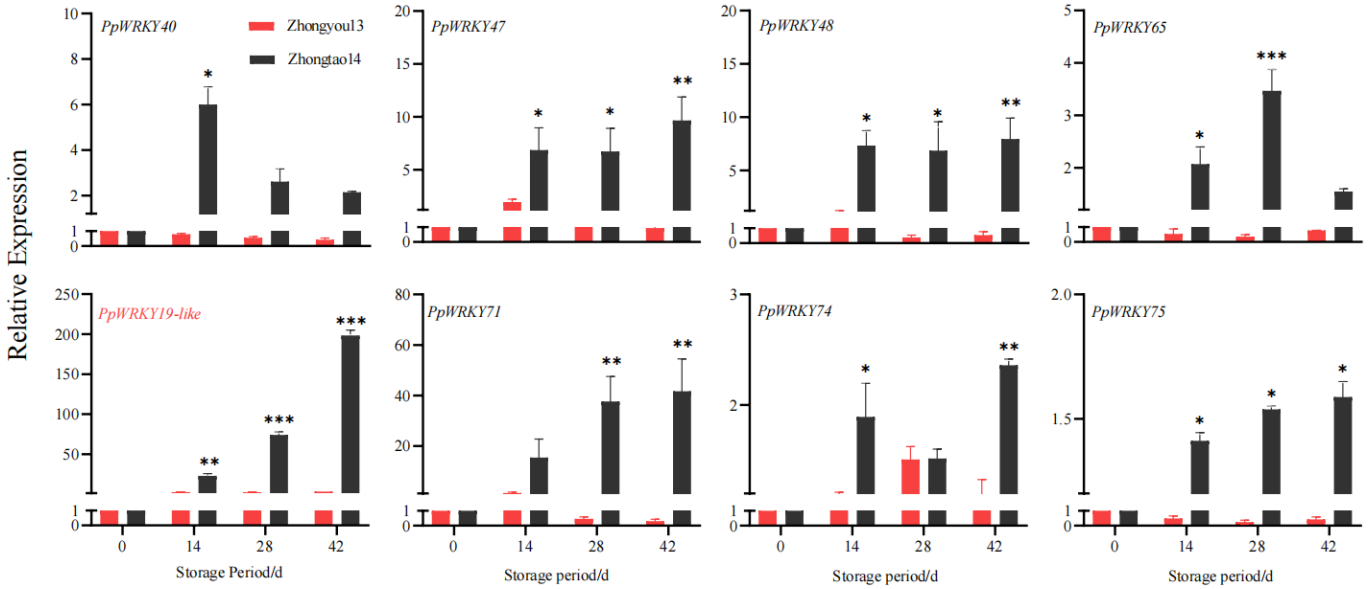

Fig. 3 Relative expression of *WRKY* TFs in different peach cultivars during cold storage. Error bars indicate the means ± SE from three biological replicates. * indicates significant differences between the control (0 d) and the different cold storage treatment timepoints (14, 28, and 42 d) based on Student's *t*-tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

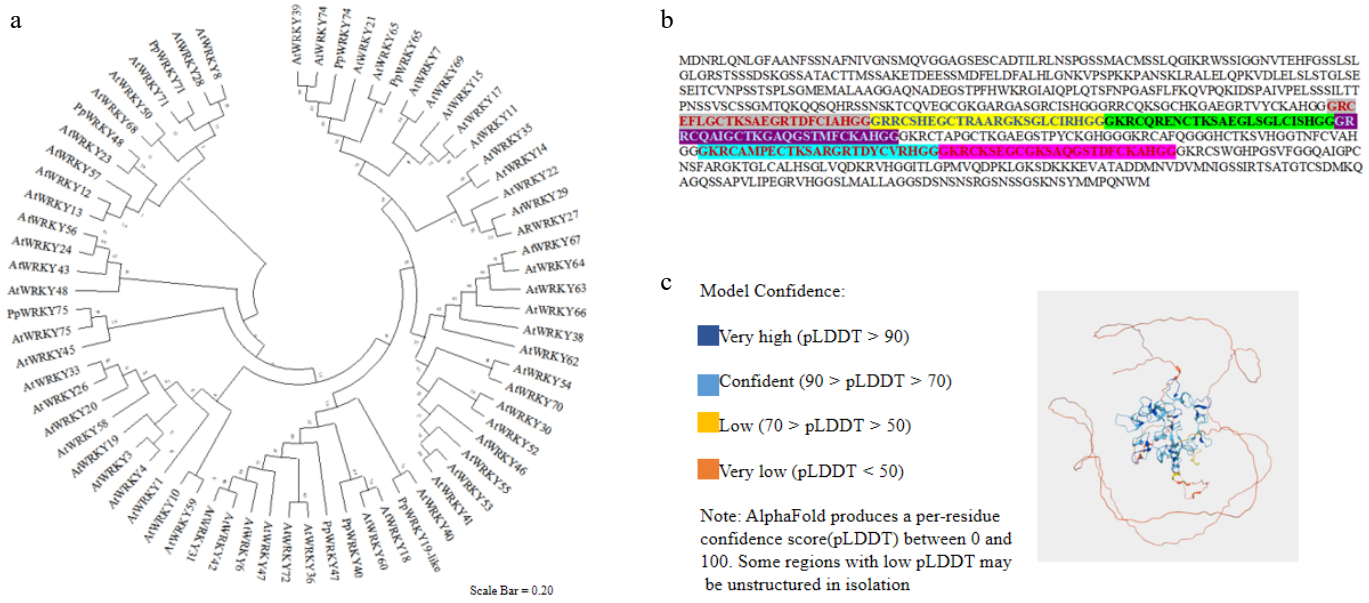

Fig. 4 Sequence features of *PpWRKY19-like* and its protein. (a) A rooted neighbor joining phylogenetic tree relating to *PpWRKY* TFs in *Arabidopsis thaliana*. (b) The deduced amino acid sequence of *PpWRKY19-like*. The colored sequences are the C_2H_2 zinc finger domains. (c) A hypothetical three-dimensional (3D) structural model of *WRKY19-like* as predicted by the AlphaFold model.

PpWRKY19-like binds to the promoter of *PpLAC7*

Analysis of the *cis*-acting elements within the gene promoter revealed the presence of WRKY-binding sites (TTGAC) in *PpLAC7* and *PpLAC9* (Fig. 5a). The potential regulatory effects of *PpWRKY19-like* on the expression of *PpLAC7* and *PpLAC9* were investigated using a dual luciferase assay. The results indicated that *PpWRKY19-like* transactivated the *PpLAC7* promoter, leading to an approximately five-fold increase in expression, whereas it did not activate the *PpLAC9* promoter's expression (Fig. 5b, c). Therefore, *PpLAC7* was considered as a potential candidate gene for further analysis. The Y1H assay showed that *PpWRKY19-like* interacted directly with the *PpLAC7* promoter (Fig. 5d). These findings provide that, despite lacking the canonical WRKYGQK motif, the multi-zinc finger architecture of *PpWRKY19-like* allows it to retain high affinity and functional specificity for W-box (TTGAC) elements, suggesting that *PpWRKY19-like* is a key regulator of *PpLAC7*-mediated browning.

Transient overexpression of *PpWRKY19-like* and *PpLAC7* enhances browning in peach flesh

To investigate the functions of *PpWRKY19-like* and *PpLAC7* in peach fruit browning during cold storage, we used the peach flesh of 'Zhongyou13', which did not exhibit browning symptoms during

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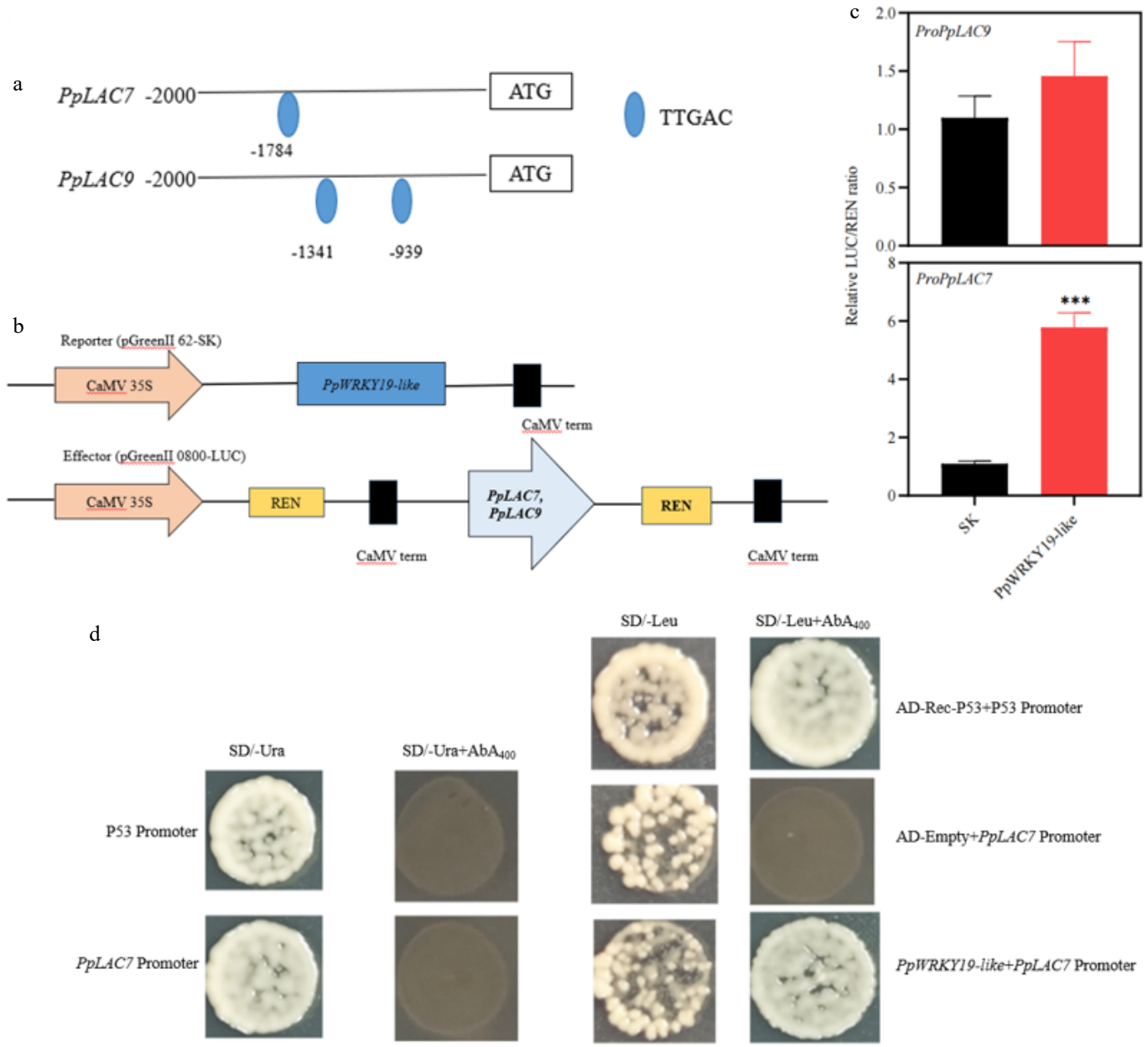

Fig. 5 *PpWRKY19-like* binds to the *PpLAC7* promoter. (a) Binding sites in *PpLAC7* and the *PpLAC9* promoter. (b) Reporter and effector vectors applied in the dual luciferase assay. (c) The fluorescence ratio of LUC/REN for the empty vector (SK) combined with the promoter, standardized to 1. (d) Examination of the interaction between *PpWRKY19-like* and the *PpLAC7* promoter using the Y1H assay. Autoactivation of the *PpLAC7* promoter was assessed on SD medium without Ura, supplemented with 400 ng mL^{-1} Aureobasidin A (SD/−Ura+AbA_{400}). The interaction was evaluated on SD medium lacking Leu with 400 ng mL^{-1} AbA (SD/−Leu+AbA_{400}). Positive control: p53 and its promoter; negative control: the AD empty vector + *PpLAC7* promoter. Error bars represent the mean ± SE from three replicates. * indicates significant differences between the SK (control) and the *PpWRKY19-like* (treatment) based on Student's *t*-tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

cold storage (Fig. 1a). After three days of infiltration, peach flesh overexpressing *PpWRKY19-like* (OE-*PpWRKY19-like*) and *PpLAC7* (OE-*PpLAC7*) showed visible browning compared with the empty vector (Fig. 6a). Quantitative PCR analysis revealed significant upregulation of *PpLAC7* and *PpWRKY19-like* in OE-*PpWRKY19-like* compared with the empty vector. Additionally, OE-*PpLAC7* showed significant upregulation of *PpLAC7* (Fig. 6b, c). LAC activity significantly increased in OE-*PpWRKY19-like* and OE-*PpLAC7* compared with the empty vector (Fig. 6d). These findings suggest that *PpWRKY19-like* and *PpLAC7* play significant roles in peach fruit browning during cold storage.

Discussion

Physiological responses in different peach cultivars during cold storage

Cold storage is commercially used to maintain fruits' freshness and transport them safely by slowing metabolism and respiration, thereby extending shelf life[41]. However, peaches are highly susceptible to CI, particularly flesh browning, which restricts preservation during storage[42]. In this study, the cultivar 'Zhongtao14' exhibited severe IB symptoms after 42 d at 4 °C, whereas 'Zhongyou13' did not

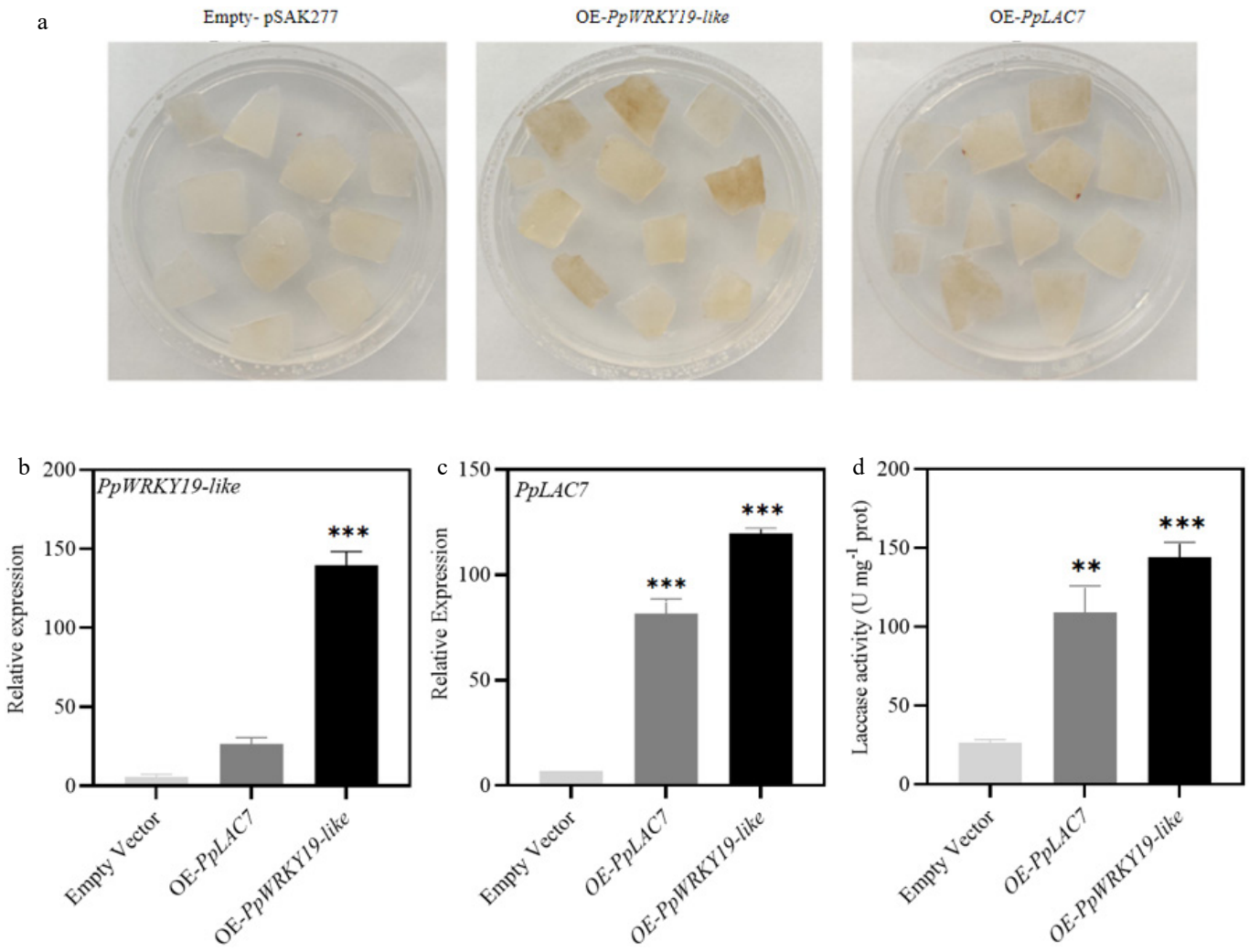

Fig. 6 Functional verification of *PpWRKY19-like* and *PpLAC7* in peach flesh. (a) Peach flesh showing the incidence of browning in OE-*PpWRKY19-like* and OE-*PpLAC7* samples after three days of infiltration. Relative expression of (b) *PpWRKY19-like*, (c) *PpLAC7*, and (d) LAC activity in OE-*PpWRKY19-like* and OE-*PpLAC7* compared with the empty vector (pSAK277). Error bars represent the mean ± SE from three replicates. * indicates significant differences between the control (empty vector) and treatments (OE-*PpLAC7* and OE-*PpWRKY19-like*) based on Student's *t*-tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

show IB symptoms (Fig. 1a, b). A previous report indicated that elevated MDA levels are primary indicators of membrane damage and development of CI[43]. The significant increase in MDA in 'Zhongtao14' suggests that membrane lipid peroxidation was associated with its increased browning incidence (Fig. 1c). Furthermore, the substantial increase in antioxidant activity in 'Zhongyou13' reflects an effective capacity for reactive oxygen species (ROS) scavenging, which mitigated oxidative stress. In contrast, the decline in antioxidant activity in 'Zhongtao14' after 14 d suggests an inadequate defense mechanism, allowing for the accumulation of oxidative damage[44]. The imbalance between stress-induced phenolic synthesis and antioxidant capacity in 'Zhongtao14' hastened cellular compartmentalization breakdown, providing substrates for enzymatic browning[45].

Regulation of *PpLAC7*-mediated browning during cold storage

PPOs are primarily responsible for fruit browning, and LACs are considered to be crucial variants of PPOs that significantly contribute to this process[20,46]. LAC activity has been implicated in peach fruit browning because of its insensitivity to the PPO inhibitor 4-hexylresorcinol[46]. Our findings showed that LAC activity increased significantly in the browning cultivar 'Zhongtao14', which correlated with the severity of IB symptoms (Figs. 1a, b, and 2c). *LcLAC* can be polymerized with polyphenols and is involved in callus browning[47]. *LcLAC7* enhances pericarp browning during storage by facilitating the breakdown of anthocyanin[14].We identified two genes, *PpLAC7* and *PpLAC9*, that showed a significant increase in 'Zhongtao14' (Fig. 2a, b). Notably, *PpWRKY19-like* specifically transactivated the *PpLAC7* promoter (Fig. 4). Given its phenotypic association and functional activation, *PpLAC7* was identified as the primary candidate for chilling-induced browning in peaches. Phylogenetic analysis grouped *PpLAC7* with *MdLAC7* (Supplementary Fig. S3), which was recently reported to directly enhance peel browning in apples[22], suggesting a conserved role for *LAC7* in fruit browning.

PpWRKY19-like acts as a divergent regulator of cold stress

WRKY TFs significantly regulate plants' growth and development, secondary metabolism, and responses to biotic and abiotic stressors[48]. Notably, *VvWRKY28*, a cold-induced WRKY gene in grapes (*Vitis vinifera*), regulates the expression of genes associated

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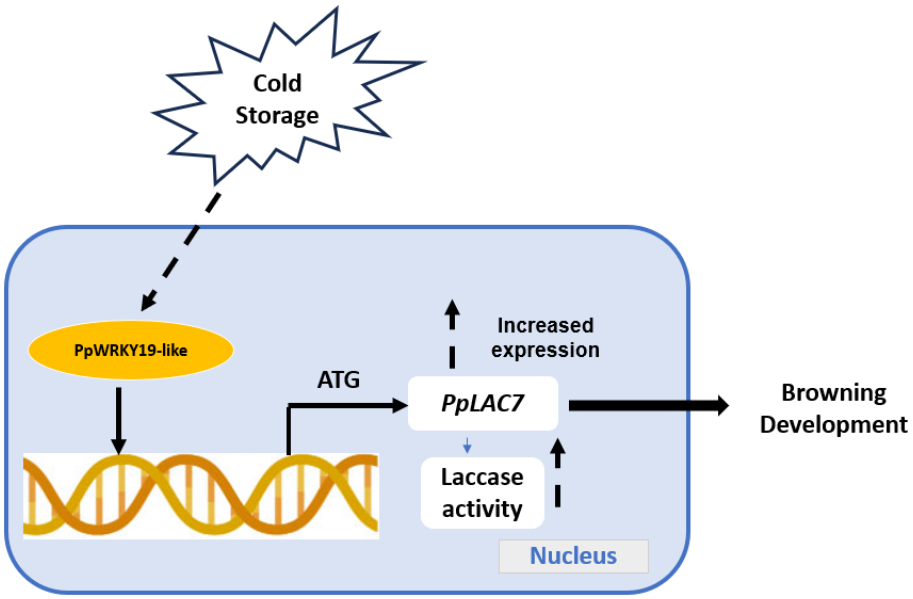

Fig. 7 A proposed model of how *PpWRKY19-like* regulates the expression of *PpLAC7* to promote the development of browning in peaches during cold storage.

with cold stress and cold stress resistance[49]. Similarly, *CsWRKY46* modulates cold stress-responsive genes in cucumbers (*Cucumis sativus*), leading to increased cold tolerance[50]. In our study, we found that the relative expression of *PpWRKY19-like* was positively correlated with the IB index and the expression of *PpLAC7* (Figs. 1a, b, 2a, and 3a). *PpWRKY19-like* promoted browning by directly binding to the *PpLAC7* promoter and activating its expression (Fig. 5). Furthermore, overexpression of *PpWRKY19-like* significantly upregulated *PpWRKY19-like* and *PpLAC7*, and overexpression of *PpLAC7* significantly upregulated *PpLAC7,* thereby enhancing laccase activity and promoting tissue browning (Fig. 6). Previous studies have reported that *WRKYs* are involved in the cold tolerance of fruits and are associated with browning symptoms during cold storage. *MaWRKYs* play a crucial role in the browning of banana peel by binding to W-box motifs in the *MaNCED1* and *MaNCED2* promoters[51]. *EjCML19* and *EjWRKY7* collaboratively enhance the transcription of genes associated with browning in loquats (*Eriobotrya japonica*) during cold storage[52]. The phylogenetic tree showed that *PpWRKY19-like* had a close genetic relationship with *A. thaliana AtWRKY40* (Fig. 4a). Genetically close homologous genes might share similar functions. A recent study reported that *A. thaliana WRKY40* is involved in osmotic and cold stress responses[53]. Thus, *PpWRKY19-like* may show similar function in cold stress. A study reported that WRKY members that exhibit a total or partial loss of the signature WRKY or zinc-finger motifs are categorized as Group IV[54]. Although these "incomplete" members were previously thought to be nonfunctional, recent studies suggest that such structural divergence can result from extensive evolutionary domain expansion or loss within the Rosaceae family[55]. We therefore classified *PpWRKY19-like* as a divergent member of WRKY family that has undergone extensive domain expansion while maintaining its functional ability to bind W-box elements. By integrating these structural insights into the PpWRKY19-like/*PpLAC7* pathway, we propose a schematic model illustrating the regulatory mechanism of cold-storage induced browning development in peach fruit (Fig. 7). Among other *PpWRKYTFs*, *PpWRKY74* clustered with *AtWRKY74* and *AtWRKY39*. *AtWRKY39* positively regulates hormonal signaling pathways that mediate stress responses[56]. *PpWRKY40* was clustered with *AtWRKY60* and *AtWRKY18*. *AtWRKY18* and *AtWRKY60* act as positive regulators of abscisic acid (ABA) sensitivity and abiotic stress responses[57]. *PpWRKY48* clustered with *AtWRKY23*. *AtWRKY23* is involved in ammonium-induced repression of primary root growth in *A. thaliana* under ammonium toxicity[58]. *PpWRKY71* clustered with *AtWRKY71*. *WRKY71* acts antagonistically against salt-delayed flowering in *A. thaliana*[59]. *PpWRKY65 and PpWRKY47* clustered with *AtWRKY65 and AtWRKY36*. There have been no relevant reports on *AtWRKY65* or *AtWRKY36* under abiotic stress. This comparative analysis highlighted the functional divergence of *WRKY* TFs under different abiotic stresses. However, functional validation was conducted using transient expression systems, and thus future research using stable transgenic peach callus transformation will be necessary to further confirm the long-term regulatory mechanisms of this pathway. In addition, further investigations are needed to explore the role of additional *WRKYs* in the browning of peach fruit during cold storage.

Conclusions

This study elucidates the physiological and molecular mechanisms underlying chilling-induced IB in peach fruit. We demonstrate that cultivar-specific sensitivity to cold storage is mediated by differences in membrane lipid peroxidation (MDA), antioxidant capacity, and phenolic metabolism. Our findings identify the LAC gene *PpLAC7* as a critical enzymatic regulator of browning that is directly activated by the divergent transcription factor *PpWRKY19-like*. We confirmed that *PpWRKY19-like* binds to the *PpLAC7* promoter to activate its transcription, thereby accelerating the development of enzymatic browning. These results provide novel insights into the regulatory networks governing CI and offer promising genetic targets for improving the postharvest quality and storage life of peach fruit.

Author contributions

The authors confirm their contributions to the paper as follows: study conception and design: Badrunnesa A, Zeng W; data

collection: Badrunnesa A, Zhan W, Li A; analysis and interpretation: Badrunnesa A, Zhan W, Meng J; draft manuscript preparation: Badrunnesa A, Duan W, Sun S; critical revision: Niu L, Pan L, Cui G, Zeng W. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analyzed in the current study are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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