PpCBF6 Is Involved in Phytosulfokine α-Retarded Chilling Injury by Suppressing the Expression of PpLOX5 in Peach Fruit

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The involvement of PpCBF6 in phytosulfokine α (PSKα)-ameliorated chilling injury (CI) by suppressing the expression of lipoxygenase 5 (LOX5) in peach fruit was revealed. The peaches were immersed in distilled water and PSKα solution. PSKα application inhibited the progression of CI index and weight loss, and the reduction of firmness and total soluble solids content in peaches. The endogenous PSKα accumulation and gene expression of PSK receptor 1 (PSKR1) and PSKR2 were upregulated by PSKα application. The superoxide anion (O$_2^-$) production rate, hydrogen peroxide (H$_2$O$_2$) production and reactive oxygen species (ROS) content decreased by PSKα application. Furthermore, PSKα application reduced the gene expression of 12 PpLOXs and LOX activity. The gene expression of 6 PpCBFs was enhanced by PSKα application. Importantly, after PSKα application, among 12 PpLOXs, the decrease in gene expression of PpLOX5 was the lowest, and among 6 PpCBFs, the increase in gene expression of PpCBF6 was the highest. Further results suggested that PpCBF6 bound to the C-repeat/dehydration responsive element (CRT/DRE) motif in PpLOX5 promoter, and repressed its transcription. Thus, PpCBF6 was involved in the PSKα-retarded CI by inhibiting the expression of PpLOX5 in peaches.

Keywords: phytosulfokine α, chilling injury, lipoxygenase 5, PpCBF6, peaches

INTRODUCTION

Refrigeration is commonly employed to maintain fruit quality and to extend storage period (Belay et al., 2020). However, peaches are susceptible to chilling injury (CI) when subjected to cold stress (Nilo et al., 2010). The main CI symptom in peaches is internal browning (Chen et al., 2019), which causes certain economic losses. Thus, it is vital to hunt for potent approaches to relieve CI in peaches.

Phytosulfokine α (PSKα), a plant sulfonated pentapeptide growth regulator, has been verified to motivate many biochemical activities (Aghdam and Luo, 2021a). PSKα perception by leucine-rich repeat PSK receptor (PSKR) kinase in plasma membrane is vital for triggering a series of physiological reactions (Aghdam et al., 2021b). Accordingly, PSKα application promoted energy status and scavenged reactive oxygen species (ROS) overproduction, and thus retarded senescence in broccoli florets throughout cold storage (Aghdam and Luo, 2021b). PSKα treatment boosted...
PSK extending vase life of cut rose flowers (Aghdam et al., 2021a). PSK treatment retarded senescence in strawberries by inducing the phenylpropanoid pathway throughout cold storage (Aghdam et al., 2021b). However, whether PSK treatment could relieve CI in peaches remains to be explored.

Cold storage would result in the elevation of lipoxygenase (LOX) level in postharvest fruit (Sheng et al., 2016). LOX catalyzes the hydroperoxidation of unsaturated fatty acids to produce hydroperoxide and a lot of ROS, leading to cell membrane damage and browning. Therefore, to inhibit CI in cold sensitive fruit, it is critical to apply potent measures to reduce LOX level. Accordingly, PSK treatment could retard the increase in gene expression and activity of LOX in broccoli florets (Aghdam and Flores, 2021c), indicating that PSK application is an effective technology to relieve cell membrane damage in postharvest vegetable. However, the effects of PSK treatment on LOX level in peaches remain to be revealed.

Besides, the motivation of transcriptional factors (TFs) is an important defense response to cold stress in postharvest fruit (Peng et al., 2020; Jiao, 2021). C-repeat binding factors (CBFs) are the most widely investigated cold resistance TFs (Xiao et al., 2010; Liang et al., 2013). Transcription level of CBF1 in tomatoes was positively correlated with chilling tolerance, and negatively correlated with CI severity, suggesting that CBFs can effectively reflect chilling tolerance in fruit (Arae et al., 2017). Moreover, CBFs could be activated by lots of exogenous applications in plants. Methyl jasmonate treatment promoted the gene expression of CBF6, and thus enhanced the chilling tolerance in peaches (Cao et al., 2021). NO treatment enhanced the tolerance to cold stress by up regulating CBF1 expression in kiwifruit (Jiao, 2021). However, the modulation of CBFs by PSK treatment remains to be explored.

Moreover, CBFs modulate the expression of downstream genes by binding to the C-repeat/dehydration responsive element (CRT/DRE) motif (CCGAC) in their promoters (Cao et al., 2021). Accordingly, CBF3 bound to the CRT/DRE motif in the promoter of ureidoglycate amidohydrolase, and enhanced its expression in rice (Li et al., 2015). CBF6 suppressed the expression of vacuolar invertase by interacting with the CRT/DRE-binding site in its promoter, and thus retarded CI in peaches (Cao et al., 2021). However, the regulation of LOXs by CBFs in peaches remains to be investigated.

The aims of this research were to explore the effects of PSK application on the decrease in CI severity and the induction of PpLOX5 and PpCBF6, and the modulation of PpLOX5 promoter by PpCBF6 in peaches.

**MATERIALS AND METHODS**

**Plant Material and Postharvest Applications**

Peaches (*Prunus persica* Batsch cv. ‘Yuhua No. 3’) were obtained at 80% ripeness from the orchard in Nanjing, China. The chosen uniform peaches absence of visual defects were divided into two groups each of three biological replicates at random. For each biological replicate of each group, 400 fruit were used.

1. Control (CK): The peaches were immersed in distilled water.
2. PSKα: The peaches were immersed in 300 nM PSKα.

The PSKα concentration was determined according to my preliminary experiments (Supplementary Figure 1). The aforementioned fruit were immersed for 10 min and air dried for 40 min thereafter. The peaches were stored at 4 ± 1°C for 35 days under 80–90% relative humidity afterward. For each biological replicate, 30 peaches were used.

**TABLE 1 | The primers for qRT-PCR tests.**

| Gene | Gene ID (LOC) | Primer name | Primer sequences (5’→3’) | Amplicon size (bp) |
|------|--------------|-------------|--------------------------|--------------------|
| PpPSKR1 | 18793371 | Sense GGTAAACAGGCTTTCCGGGAT | 102 |
| PpPSKR2 | 18779076 | Sense CAAGTCCACGCGCCATCCTGG | 116 |
| PpLOX2-1 | 18773985 | Sense ATTCACGATGGAGTCCGCA | 100 |
| PpLOX2-1 | 18787524 | Sense CAACGGCCTTGGTGAAGCTCA | 120 |
| PpLOX2-1 | 18787140 | Sense AGAAGACCTGGAAATGACTG | 123 |
| PpLOX3-1 | 18781374 | Sense TGATGGGACGGAGCTCAGAT | 110 |
| PpLOX3-1 | 18783171 | Sense TGCGACCAGAATGCTCACCC | 112 |
| PpLOX4 | 18793349 | Sense ACCGCCATCCATGTTAAGTG | 109 |
| PpLOX5 | 18774887 | Sense ACCGGTGTAGCCGCTTGGC | 113 |
| PpLOX5 | 18773395 | Sense CAGAGAACACCCCAAGATG | 104 |
| PpLOX5 | 18774870 | Sense AAAGACCAGAACTTGAGGCCA | 112 |
| PpLOX5 | 18774983 | Sense ACTCTCTGAGTTACAGCACG | 109 |
| PpLOX5 | 18775056 | Sense CCATGGTCATGGATGGGCTT | 113 |
| PpCBF1 | 18778067 | Sense TTCATAAGGCCTTATGCAACTGT | 110 |
| PpCBF2 | 18776669 | Sense AGGCTGTAAGTTCACGAGG | 113 |
| PpCBF3 | 18776409 | Sense GCCGGAGTGAGTATTGAGG | 103 |
| PpCBF4 | 18776400 | Sense GCCGGAGTGAGTATTGAGG | 103 |
| PpCBF5 | 18777414 | Sense CCGTTCCTTGCCCTGCACTG | 109 |
| PpCBF6 | 18787317 | Sense TGGCCAATCATCAGAGGGG | 106 |
| β-actin | 18797908 | Sense GTATTTCCCTTGGAGTCAGTC | 109 |
to determine the CI degree, weight loss, firmness, and total soluble solids content every 7 days. For each biological replicate, 20 peaches were used to detect the physiological indicators ($O_2^-$ production rate, $H_2O_2$ production, ROS content, LOX activity, and gene expression of PpLOXs and PpCBFs) every 7 days, which were preserved at $-80^\circ$C. For CI severity calculation, peaches were removed to 20°C for 3 days after each time point. Other indicators were evaluated immediately after each time point.

**Chilling Injury Severity, Weight Loss, Firmness, and Total Soluble Solids Content Calculation**

For CI index calculation, the severity of internal browning in each fruit was recorded: 0 = none, 1 $\leq$ 5%, 2 = 6–25%, 3 = 26–50%, 4 $\geq$ 50%. CI index = $\sum$(CI severity $\times$ number of peaches at the CI severity)/(5 $\times$ total number of peaches in the replicate).

For weight loss assay, the peaches were weighed before and after each time point.

Firmness was determined using the firmness analyzer (FT327, Effegi, Alfonzine, Italy) with a probe of a 7.5 mm penetration depth.

For total soluble solids content determination, 5.0 g peach samples were ground, and centrifuged at 9,000 g for 15 min. The collected supernatant was assayed using WYT-4 hand-held refractometer (Shanghai Precision & Scientific Instrument Co., Ltd., Shanghai, China) afterward.

**Endogenous PSK$\alpha$ Accumulation Assay**

Five gram of peach samples were incubated with 8 M urea for 60 min at 4°C as described by Song et al. (2017). After pH was adjusted to 2.0–3.0 using 1 M HCl, the homogenate was incubated for 15 min at 4°C, followed by a centrifugation at 12,000 g for 30 min at 4°C. The endogenous PSK$\alpha$ accumulation was detected using the protocol of Aghdam et al. (2021b).

**$O_2^-$ Production Rate, $H_2O_2$ Production, and ROS Content Assay**

For $O_2^-$ production rate detection, 5.0 g peach samples were homogenized in 50 mM phosphate buffer (pH 7.8), followed by a centrifugation at 11,000 g for 20 min at 4°C thhereafter. The $O_2^-$ production rate was measured following the protocol of Wang et al. (2020). The data were expressed as mmol/kg on a fresh weight (FW) basis.

For $H_2O_2$ production measurement, 5.0 g peach samples were homogenized using cold acetone, followed by a centrifugation at 11,000 g for 20 min at 4°C thereafter. The $H_2O_2$ production was assayed using the protocol of Yang et al. (2016). The data were expressed as $\mu$mol/kg on a FW basis.

![FIGURE 1](image-url) | PSK$\alpha$ application reduced the CI degree (A) and weight loss (B) and maintained the firmness (C) and total soluble solids content (D) in peaches. The CI degree (A) was analyzed after peaches were removed to 20°C for 3 days after each time point. The weight loss (B), firmness (C), and total soluble solids content (D) were detected immediately after each time point. For each biological replicate, after each time point, 30 peaches were used to determine the CI degree (A) and weight loss (B) and maintained the firmness (C) and total soluble solids content (D). Values represent the mean $\pm$ standard deviation. Values not with the same letter are significantly different at $p < 0.05$. 
Frontiers in Plant Science | www.frontiersin.org 3 April 2022 | Volume 13 | Article 874338
The ROS content was assayed using fluorescence spectrophotometer (Cary Eclipse, VARIAN, United States) using the protocol of Jing et al. (2016). The maximum excitation and emission wavelengths were 485 and 530 nm, respectively. The slit width was 5 nm. The data were expressed as a.u./mg on a FW basis.

**Transcriptomic Analysis**

The total RNA was extracted according to the protocol of MiniBEST Plant RNA Extraction Kit (Takara Bio Inc.,

![Figure 2](image-url)  
**FIGURE 2** | PSKα application elevated the endogenous PSKα accumulation (A) and gene expression of PSKR1 (B), and PSKR2 (C) in peaches. The endogenous PSKα accumulation (A) and gene expression of PSKR1 (B) and PSKR2 (C) were determined immediately after each time point. For each biological replicate, after each time point, 20 peaches were used to detect the endogenous PSKα accumulation (A) and gene expression of PSKR1 (B) and PSKR2 (C). Values represent the mean ± standard deviation. Values not with the same letter are significantly different at ρ < 0.05.

![Figure 3](image-url)  
**FIGURE 3** | PSKα application retarded the elevation of the O₂⁻ production rate (A), H₂O₂ content (B) and ROS accumulation (C) in peaches. The O₂⁻ production rate (A), H₂O₂ content (B), and ROS accumulation (C) were assayed immediately after each time point. For each biological replicate, after each time point, 20 peaches were used to detect the O₂⁻ production rate (A), H₂O₂ content (B), and ROS accumulation (C). Values represent the mean ± standard deviation. Values not with the same letter are significantly different at ρ < 0.05.
After purified, the total RNA was used for cDNA library construction. The clean reads were mapped to peach genome. The read numbers were transformed to FPKM (Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced) value for gene expression quantification. The differentially expressed genes was analyzed using edgeR with following criteria: False discovery rate (FDR) < 0.05 and \(|\log_2^{fold\ change}\) \(\geq 1\). Three biological replicates were included for each assay.

**Lipoxygenase Activity Assay**

Five gram of peach samples were extracted in 0.1 M phosphate buffer (pH 6.8) containing 1% (w/v) polyvinylpyrrolidone, and centrifuged at 10,000g for 15 min at 4°C thereafter. LOX activity was determined using the protocol of Yao et al. (2019). The data were expressed as U/g on a FW basis.

**Gene Expression Assay**

Total RNA in peach samples was acquired following the protocol of E.Z.N.A.™ Plant RNA Kit (Omega, United States). The first-strand cDNA was synthesized using the protocol of Jiao (2021) thereafter. The primers in quantitative real-time polymerase chain reaction (qRT-PCR) tests for PpPSKRI, PpPSK2, 12 PpLOXs, 6 PpCBFs, and \(\beta\)-actin were designed (Table 1). The gene expression was assayed according to the protocol of Jiao (2021). Three replicates were included for each assay.

**Yeast One-Hybrid Assay**

A yeast one-hybrid (Y1H) assay was performed using the protocol of the Clontech™ Matchmaker® one-hybrid system. The three tandem copies of the CRT/DRE-binding site (CCGAC) (-405 to -401 bp) and adjacent nucleotides in the PpLOX5 promoter (Supplementary Text 1) were ligated into pAbAi vector. The PpLOX5-AbAi and p53-AbAi were introduced into the Y1H Gold strain thereafter. Positive yeast cells were transformed with the PSK to bind to PpLOX5 promoter was judged by the growth status of co-transformants on SD/-Leu medium in the presence of AbA.

**Dual Luciferase Reporter Assay**

The sequences of PpLOX5 promoter were inserted into the pGreen II 0800-LUC vector. The CDS of PpCBF6 were inserted into the pGreen II 62-SK vector. The plasmids were introduced into Agrobacterium tumefaciens strain GV3101, and transiently expressed in tobacco thereafter. After 3 days, LUC and REN were determined using the dual luciferase reporter (DLR) assay system (Promega). Nine independent replicates were conducted for each combination. The data were expressed as the relative LUC/REN ratio.

**Statistical Analysis**

Statistical analysis was performed using SPSS 22.0 (SPSS Inc., Chicago, IL, United States). Each data was analyzed with one-way analysis of variance. The significant differences at \(p < 0.05\) were determined using Duncan test.

**RESULTS**

**PSK\(\alpha\) Application Reduced the Chilling Injury Degree and Weight Loss and Maintained the Firmness and Total Soluble Solids Content in Peaches**

The CI disorder in peaches was initially found at 14 days. The CI index and weight loss continuously increased following control treatments throughout storage. PSK\(\alpha\) treatment caused the decrease in CI index and weight loss by 35 and 23% (Figures 1A,B).

**TABLE 2** The identification and quantification of PpLOXs in postharvest peaches after PSK\(\alpha\) treatment using transcriptome.

| Gene ID (LOC) | Gene description | 7 days \(\log_2^{fold\ change}\) | 14 days \(\log_2^{fold\ change}\) | 21 days \(\log_2^{fold\ change}\) | 28 days \(\log_2^{fold\ change}\) | 35 days \(\log_2^{fold\ change}\) |
|--------------|------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| 18773995     | Linoleate 13S-lipoxygenase 2-1 | -0.22 ± 0.02 | -0.45 ± 0.06 | -0.47 ± 0.04 | -0.37 ± 0.04 | -0.49 ± 0.05 |
| 18787524     | Linoleate 13S-lipoxygenase 2-1 | -0.73 ± 0.05 | -0.38 ± 0.04 | -0.51 ± 0.06 | -0.37 ± 0.04 | -0.79 ± 0.08 |
| 18787140     | Linoleate 13S-lipoxygenase 2-1 | -0.26 ± 0.02 | -0.26 ± 0.05 | -0.69 ± 0.03 | -0.20 ± 0.04 | -0.31 ± 0.04 |
| 18781374     | Linoleate 13S-lipoxygenase 3-1 | -0.73 ± 0.05 | -0.84 ± 0.04 | -0.44 ± 0.05 | -0.48 ± 0.07 | -0.39 ± 0.04 |
| 18783171     | Linoleate 13S-lipoxygenase 3-1 | -0.28 ± 0.03 | -0.37 ± 0.04 | -0.59 ± 0.06 | -0.62 ± 0.06 | -0.48 ± 0.05 |
| 18793349     | Lipoxigenase 6 | -0.24 ± 0.03 | -0.42 ± 0.04 | -1.18 ± 0.05 | -0.39 ± 0.04 | -1.02 ± 0.04 |
| 18766622     | Linoleate 9S-lipoxygenase 6 | -0.83 ± 0.05 | -1.12 ± 0.08 | -1.25 ± 0.09 | -1.35 ± 0.07 | -1.27 ± 0.09 |
| 18774987     | Probable linoleate 9S-lipoxygenase 5 | -1.36 ± 0.13 | -1.27 ± 0.15 | -1.42 ± 0.15 | -1.25 ± 0.16 | -1.49 ± 0.15 |
| 18773359     | Probable linoleate 9S-lipoxygenase 5 | -1.47 ± 0.14 | -1.52 ± 0.20 | -1.62 ± 0.15 | -0.99 ± 0.09 | -1.37 ± 0.16 |
| 18774870     | Probable linoleate 9S-lipoxygenase 5 | -1.99 ± 0.14 | -2.26 ± 0.27 | -2.27 ± 0.15 | -2.69 ± 0.14 | -2.37 ± 0.16 |
| 18774983     | Probable linoleate 9S-lipoxygenase 5 | -1.38 ± 0.09 | -1.40 ± 0.07 | -1.19 ± 0.09 | -1.62 ± 0.15 | -1.38 ± 0.09 |
| 18775056     | Probable linoleate 9S-lipoxygenase 5 | -1.44 ± 0.08 | -1.57 ± 0.19 | -1.35 ± 0.16 | -1.57 ± 0.09 | -1.14 ± 0.11 |

\(|\log_2^{fold\ change}\) \(\geq 1\) represents up-regulation, while 0 < \(|\log_2^{fold\ change}\) < 1 represents no statistical difference.
FIGURE 4 | Effects of PSKα application on the gene expression of PpLOX2-1 (LOC18773995) (A), PpLOX2-1 (LOC18787524) (B), PpLOX2-1 (LOC18787410) (C), PpLOX3-1 (LOC18781374) (D), PpLOX3-1 (LOC18783171) (E), PpLOX6 (LOC18793349) (F), PpLOX6 (LOC18766622) (G), PpLOX5 (LOC18774987) (H), PpLOX5 (LOC18773559) (I), PpLOX5 (LOC18774870) (J), PpLOX5 (LOC18774983) (K), and PpLOX5 (LOC18775056) (L) in peaches. The gene expression of PpLOXs were determined immediately after each time point. For each biological replicate, after each time point, 20 peaches were used to detect the gene expression of PpLOXs. Values represent the mean ± standard deviation. Values not with the same letter are significantly different at $p < 0.05$. 
The firmness continuously decreased in peaches following control and PSKα treatments throughout storage. PSKα treatment markedly delayed the decrease in firmness. Following 35 days of storage, PSKα treatment boosted the firmness by 44% (Figure 1C).

The total soluble solids content increased firstly and decreased thereafter in peaches following control and PSKα treatments throughout storage. PSKα treatment markedly retarded the decrease in total soluble solids content. Following 35 days of storage, PSKα treatment caused the elevation of total soluble solids content by 38% (Figure 1D).

**PSKα Application Elevated the Endogenous PSKα Accumulation and Gene Expression of PSKR1 and PSKR2 in Peaches**

The endogenous PSKα accumulation and gene expression of PSKR1 and PSKR2 increased firstly and decreased thereafter following control and PSKα treatments throughout storage in peaches. PSKα application promoted the endogenous PSKα accumulation and gene expression of PSKR1 and PSKR2. Following 35 d of storage, the endogenous PSKα accumulation and gene expression of PSKR1 and PSKR2 in PSKα-immersed peaches was 2.5, 2.4, and 1.6 times of the control (Figure 2).

**PSKα Application Reduced the O$_2^-$ Production Rate, H$_2$O$_2$ Production and ROS Accumulation in Peaches**

The O$_2^-$ production rate, H$_2$O$_2$ production and ROS accumulation continuously increased following control and PSKα applications throughout storage in peaches. PSKα application suppressed the elevation of O$_2^-$ production rate, H$_2$O$_2$ production and ROS content. Following 35 days of storage, the O$_2^-$ production rate, H$_2$O$_2$ production and ROS accumulation in PSKα-immersed peaches were suppressed by 22, 36, and 24% (Figure 3).

| Gene ID (LOC) | Gene description | Log$_2$ Fold change |
|---------------|------------------|--------------------|
| 18778067      | C-repeat binding factor 1 | 1.09 ± 0.08 |
| 18776669      | C-repeat binding factor 2 | 1.29 ± 0.08 |
| 18776409      | C-repeat binding factor 3 | 1.49 ± 0.06 |
| 18776409      | C-repeat binding factor 4 | 1.59 ± 0.09 |
| 18777414      | C-repeat binding factor 5 | 1.31 ± 0.09 |
| 18787317      | C-repeat binding factor 6 | 2.57 ± 0.15 |

| Gene ID (LOC) | Gene description | Log$_2$ Fold change |
|---------------|------------------|--------------------|
| 18778067      | C-repeat binding factor 1 | 1.35 ± 0.13 |
| 18776669      | C-repeat binding factor 2 | 1.03 ± 0.13 |
| 18776409      | C-repeat binding factor 3 | 1.84 ± 0.04 |
| 18776409      | C-repeat binding factor 4 | 1.48 ± 0.07 |
| 18777414      | C-repeat binding factor 5 | 1.18 ± 0.17 |
| 18787317      | C-repeat binding factor 6 | 1.97 ± 0.09 |

| Gene ID (LOC) | Gene description | Log$_2$ Fold change |
|---------------|------------------|--------------------|
| 18778067      | C-repeat binding factor 1 | 1.48 ± 0.18 |
| 18776669      | C-repeat binding factor 2 | 1.59 ± 0.09 |
| 18776409      | C-repeat binding factor 3 | 1.73 ± 0.17 |
| 18776409      | C-repeat binding factor 4 | 1.27 ± 0.14 |
| 18777414      | C-repeat binding factor 5 | 1.19 ± 0.08 |
| 18787317      | C-repeat binding factor 6 | 2.25 ± 0.11 |

| Gene ID (LOC) | Gene description | Log$_2$ Fold change |
|---------------|------------------|--------------------|
| 18778067      | C-repeat binding factor 1 | 1.59 ± 0.09 |
| 18776669      | C-repeat binding factor 2 | 1.73 ± 0.17 |
| 18776409      | C-repeat binding factor 3 | 1.27 ± 0.14 |
| 18776409      | C-repeat binding factor 4 | 1.41 ± 0.09 |
| 18777414      | C-repeat binding factor 5 | 1.19 ± 0.08 |
| 18787317      | C-repeat binding factor 6 | 2.39 ± 0.16 |

| Gene ID (LOC) | Gene description | Log$_2$ Fold change |
|---------------|------------------|--------------------|
| 18778067      | C-repeat binding factor 1 | 1.28 ± 0.12 |
| 18776669      | C-repeat binding factor 2 | 1.52 ± 0.06 |
| 18776409      | C-repeat binding factor 3 | 1.70 ± 0.06 |
| 18776409      | C-repeat binding factor 4 | 1.18 ± 0.16 |
| 18777414      | C-repeat binding factor 5 | 1.48 ± 0.18 |
| 18787317      | C-repeat binding factor 6 | 2.12 ± 0.15 |

*Log$_2$ Fold change* ≥ 1 represents up-regulation, while 0 < |Log$_2$ Fold change| < 1 represents no statistical difference.
peaches. PSKα treatment suppressed the LOX activity. The LOX activity after PSKα treatment was at the summit on 21 days, which decreased by 27%. Following 35 days of storage, the LOX activity in PSKα-immersed peaches decreased by 35% (Figure 5).

**PSKα Treatment Enhanced the Gene Expression of PpCBFs in Peaches**

Six PpCBFs were identified using transcriptome. During storage, PSKα application enhanced the gene expression of six PpCBFs (Table 3).

To verify the results of transcriptome, qRT-PCR tests were carried out. Following 35 days of storage, the gene expression of PpCBF1 (LOC18778067), PpCBF2 (LOC18776669), PpCBF3 (LOC18776409), PpCBF4 (LOC18776400), PpCBF5 (LOC18777414) and PpCBF6 (LOC18787317) in PSKα-immersed peaches was 1.2, 1.4, 1.3, 1.2, 1.2, and 2.1 times of the control (Figure 6).

**The Suppression of PpLOX5 Promoter by PpCBF6**

The promoter sequences of PpLOX5 were characterized, and a putative CRT/DRE-binding site (CCGAC) was identified (Supplementary Text 1). Then, Y1H assay was performed to explore the interaction between PpCBF6 and PpLOX5 promoter.

![FIGURE 6](image_url)
the results in Y1H assay showed that yeast cells co-transformed with pGADT7-PpCBF6 and pAbAi-PpLOX5 promoter grew in the presence of 200 ng/ml AbA, indicating that PpCBF6 bound to the CRT/DRE motif in the PpLOX5 promoter (Figure 7).

Furthermore, as indicated from the DLR assay, compared with the control that was cotransfected with the empty vector, the relative LUC/REN ratio decreased when the promoter-LUC reporter construct was cotransfected with

**FIGURE 7** | The interaction between PpCBF6 and PpLOX5 promoter. The direct binding of PpCBF6 protein to PpLOX5 promoter was tested on the basis of the ability of Y1HGold [PpLOX5-AbAi] + PpCBF6-pGADT7 to grow on SD/-Leu in the presence of 200 ng/ml AbA.

**FIGURE 8** | The regulation of PpLOX5 promoter by PpCBF6. (A) Schematic of the reporter and effector constructs. (B) The inhibition of PpLOX5 promoter by PpCBF6 protein. Nine independent replicates were included for each combination. Transactivation was indicated by the LUC/REN ratio. Values represent the mean ± standard deviation. Values not with the same letter are significantly different at $p < 0.05$. 
PpCBF6, suggesting that PpCBF6 suppressed PpLOX5 expression. (Figure 8).

DISCUSSION

Phytosulfokine α application was verified to suppress the progression of CI degree and weight loss and the decrease in firmness and total soluble solids content (Figure 1), and to induce endogenous PSKα signaling (Figure 2), illustrating that PSKα treatment could be applied as an efficient approach to elevate tolerance to cold stress in peaches.

Phytosulfokine α treatment inhibited the gene expression of PpLOXs and LOX activity (Table 2 and Figures 4, 5). Low-temperature storage would cause the progression of the LOX level in postharvest fruit (Sheng et al., 2016). LOX catalyzes the hydroperoxidation of unsaturated fatty acids. Meanwhile, this process produces lots of ROS (Porta, 2002). ROS overproduction participates in the peroxidation of cell membrane lipid, resulting in cell membrane damage and cell necrosis. These physiological processes would consequently lead to browning, a typical symptom of CI. PSKα application was shown to be an effective approach to reduce $\mathrm{O}_2^-$ production rate, $\mathrm{H}_2\mathrm{O}_2$ content and ROS accumulation in peaches (Figure 3), illustrating that PSKα treatment maintained redox equilibrium in peaches. Therefore, the PSKα treatment-suppressed LOX level may facilitate to alleviate redox stress, therefore relieving CI in peaches. Additionally, the gene expression of PpCBFs was promoted by PSKα treatment (Table 3 and Figure 6). CBFs are the clearest cold signal transduction pathway in plants (Álvaro et al., 2019). Accordingly, CBF1 promoted the expression and activity of catalase, and down regulated $\mathrm{H}_2\mathrm{O}_2$ content, thereby ameliorating oxidative damage and boosting chilling tolerance in transgenic tomato (Hsieh et al., 2002). Also, overexpression of CBFc from Prunus mume in Arabidopsis promoted the activity of superoxide dismutase and peroxidase, thereby boosting cold resistance (Peng et al., 2016). Therefore, the PSKα application-retarded gene expression of PpCBF6 (Table 3 and Figure 6) may function in chilling tolerance through avoiding ROS overproduction (Figure 3). In a word, this work proved that PSKα application regulated the ROS level by weakening the gene expression of PpLOX5 and LOX activity and boosting the gene expression of PpCBF6, and thus inhibited CI in peaches (Figures 1–6), which is consistent with a previous report suggesting that PSKα treatment enhanced the ROS scavenging capacity by elevating the expression of alternative oxidase and uncoupling protein in broccoli florets (Aghdam and Luo, 2021b). Thus, this work would provide new evidences to prove that PSKα application is an effective approach to maintain redox equilibrium in fruits and vegetables easy to suffer CI, and expand our horizon regarding the effects of PSKα on suppression of CI.

Moreover, the promotion of chilling tolerance following exogenous applications in postharvest fruit is mediated by endogenous signals (Jiao, 2021). Both of the results of transcriptome and qRT-PCR tests suggested that the down regulation of gene expression of PpLOX5 (LOC18774870) by PSKα treatment is the lowest (Table 2 and Figures 4, 5), and the up regulation of gene expression of PpCBF6 (LOC18787317) by PSKα treatment is the highest (Table 3 and Figure 6). Thus, I investigated the involvement of PpCBF6 in the PSKα-suppressed PpLOX5 in peaches afterwards. As seen from the Y1H assay, PpCBF6 recognized the CRT/DRE motif in the promoter of PpLOX5 (Figure 7). What's more, the negative regulation of PpLOX5 transcription by PpCBF6 was verified using the DLR assay (Figure 8). Accordingly, a previous study revealed that NF-YC transcription factor bound to LOX3 promoter in Arabidopsis thaliana (Breeze, 2014). Overexpression of EREBP1 (a APETALA2/ethylene responsive factor transcription factor) in rice elevated the expression of chloroplastic LOX (Jisha et al., 2015). This work would broaden our perceptions regarding the molecular mechanisms of the modulation of genes in membrane lipid metabolism by transcription factors. Based on the above results, it can be inferred that when the PSKα-immersed peaches were subjected to cold stress, PSKα perception in the plasma membrane by leucine-rich repeat PSKR1 and PSKR2 may be

![FIGURE 9 | Proposed model of the involvement of PpCBF6 in the PSKα application-retarded CI by inhibiting PpLOX5 expression in peaches.](image-url)
fundamental for the up regulation of gene expression of PpCBF6. Then, PpCBF6 bound to PpLOX5 promoter, and weakened its transcription. This suppression retarded ROS accumulation, thus relieving CI (Figure 9). Thus, a possible novel molecular mechanism underlying the PSKα treatment-relieved CI in peaches was elucidated in this work.

In conclusion, PSKα application reduced CI degree and weight loss, and maintained the firmness and total soluble solids content in peaches. The endogenous PSKα production and gene expression of PSK1 and PSK2 were enhanced by PSKα application. The elevation of O$_2^-$ production rate, H$_2$O$_2$ production and ROS content was delayed by PSKα application. Moreover, PSKα application weakened the gene expression of PpLOX5 and LOX activity. The gene expression of PpCBF6 was promoted by PSKα and LOX activity. The gene expression of PpLOX5 and LOX activity. The gene expression of PpLOX5 and LOX activity. The gene expression of PpLOX5 and LOX activity. The gene expression of PpLOX5 and LOX activity. The gene expression of PpLOX5 and LOX activity. The gene expression of PpLOX5 and LOX activity. The gene expression of PpLOX5 and LOX activity.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

**AUTHOR CONTRIBUTIONS**

CJ: conceptualization, formal analysis, investigation, methodology, data curation, writing, supervision, project administration, and funding acquisition.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.874338/full#supplementary-material

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