Bioinformatics and Expression Analysis of PmWRKY2 and PmWRKY6 Genes in Masson Pine Postprint

Authors: Huang Jinlong, Chen Hu, Chen Yinghao, Zhangqi Yang

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Abstract

The WRKY family is one of the largest transcription factor families in higher plants. Previous studies by our research group have revealed that WRKY family genes play important roles in masson pine's responses to insect pests, growth and development, drought stress, and other aspects. To further investigate the biological functions of WRKY family genes and their responses to exogenous hormones, this study analyzed the bioinformatics characteristics of PmWRKY2 and PmWRKY6 genes and examined their expression patterns under treatments with exogenous hormones including ABA, SA, MeJA, and GA, as well as calcium ions using qPCR technology. The results showed that: (1) The PmWRKY2 and PmWRKY6 proteins encode 667 and 575 amino acids, respectively, are subcellularly localized in the nucleus, both contain a highly conserved WRKYGQK heptapeptide motif at the N-terminus and a zinc finger structure at the C-terminus, belonging to the WRKY transcription factor family. (2) In evolutionary terms, the PmWRKY2 and PmWRKY6 proteins are most closely related to PtXG20020.1 and Pt2G29990.1 proteins from Chinese pine (Pinus tabuliformis), another gymnosperm species. (3) Compared with CK, all four hormone treatments and the corresponding Ca2+ addition treatments significantly induced the expression of PmWRKY2 and PmWRKY6 genes, with expression levels peaking at the later stage of treatment; under Ca2+ treatment alone, both genes exhibited an expression pattern of initial decrease followed by increase, wherein the early stage of Ca2+ treatment significantly induced PmWRKY2 gene expression but showed no significant difference from the control at the later stage, while the expression level of PmWRKY6 gene remained significantly higher than the control throughout the entire treatment period. These results indicate that both PmWRKY2 and PmWRKY6 genes can respond to different exogenous signaling molecules, but their expression patterns differ.

Full Text

Bioinformatics Analysis and Expression of PmWRKY2 and PmWRKY6 in Pinus massoniana

Jinlong Huang[1],[2], Hu Chen[2], Yinghao Chen[2], Zhangqi Yang[2]*

[1]College of Life Sciences, Guangxi Normal University, Guilin 541006, Guangxi, China
[2]Forestry Research Institute, Engineering Technology Research Center of Masson Pine of State Forestry and Grassland Administration, Engineering Technology Research Center of Masson Pine of Guangxi, Guangxi Key Laboratory of Superior Timber Trees Resource Cultivation, Nanning 530002, China

Abstract: The WRKY family represents one of the largest transcription factor families in higher plants. Previous research by our group has demonstrated that WRKY family genes play crucial roles in Pinus massoniana's response to insect pests, growth and development, and drought stress. To further investigate the biological functions of WRKY family genes and their responses to exogenous hormones, this study analyzed the bioinformatics characteristics of PmWRKY2 and PmWRKY6 and examined their expression patterns under treatments with exogenous hormones (ABA, SA, MeJA, and GA) and calcium ions using qPCR technology. The results revealed: (1) PmWRKY2 and PmWRKY6 proteins encode 667 and 575 amino acids, respectively, with subcellular localization in the nucleus. Both genes contain a highly conserved WRKYGQK heptapeptide structure at the N-terminus and a zinc finger structure at the C-terminus, classifying them as members of the WRKY transcription factor family. (2) Phylogenetically, PmWRKY2 and PmWRKY6 proteins are most closely related to PtXG20020.1 and Pt2G29990.1 proteins from Pinus tabuliformis, another gymnosperm species. (3) Compared with the control, all four hormone treatments and their corresponding Ca^{2+} supplementation treatments significantly induced the expression of PmWRKY2 and PmWRKY6, with expression levels peaking during the later stages of treatment. Under Ca^{2+} treatment alone, both genes exhibited a trend of initial decline followed by upregulation. Ca^{2+} treatment significantly induced PmWRKY2 expression during the early stage, with no significant difference observed in the later stage compared to the control, whereas PmWRKY6 expression remained significantly higher than the control throughout the entire treatment period. These findings indicate that both PmWRKY2 and PmWRKY6 can respond to different exogenous signaling molecules, though their expression patterns differ.

Keywords: *Pinus massoniana*, PmWRKY2, PmWRKY6, bioinformatics analysis, expression analysis

Introduction

Transcription factors (TFs), also known as trans-acting factors, play vital roles in plant growth, development, and environmental responses by binding to cis-acting elements of downstream target genes to activate or suppress their expression (Porto et al., 2014). Plants contain numerous transcription factor families, among which the WRKY family is one of the largest and most important families unique to plants discovered in recent years.

The WRKY gene family is characterized by a conserved domain of 60 amino acids, featuring a highly conserved WRKYGQK heptapeptide structure at the N-terminus and a zinc finger structure at the C-terminus (Rushton et al., 2010). Based on the number of conserved WRKYGQK domains and the type of zinc finger structure, WRKY transcription factors are classified into groups I, II, and III (Eulgem et al., 2000). Ishiguro and Nakamura (1994) first isolated the SPF1 (Sweet-Potato Factor-1) gene encoding a WRKY protein from sweet potato (*Ipomoea batatas*). With continuous advances in molecular biology techniques, WRKY family genes have been identified in many plant species, including 105 genes in poplar (*Populus trichocarpa*) (He et al., 2012), 43 in Masson pine (*Pinus massoniana*) (Sun et al., 2022), and 44 in Chinese fir (*Cunninghamia lanceolata*) (Zeng et al., 2019).

The WRKY transcription factor family plays important roles in regulating plant growth and development and in responding to biotic or abiotic stresses (Eulgem & Somssich, 2007; Chen et al., 2021). For example, the *GmWRKY54* gene enhances drought resistance in soybean (*Glycine max*) by activating abscisic acid (ABA) and Ca^{2+} signaling pathways to promote stomatal closure and reduce water loss (Wei et al., 2019). *AcWRKY40* regulates kiwifruit (*Actinidia chinensis*) fruit ripening by modulating the expression of genes related to ethylene (ET) biosynthesis (Gan et al., 2021). Both *OsWRKY53* and *OsWRKY70* positively regulate the content of trypsin inhibitors in rice (*Oryza sativa*), thereby enhancing resistance to the striped stem borer (*Chilo suppressalis*) (Hu et al., 2015; Li et al., 2015). In pine species, WRKY family genes play important roles in lateral bud development in Masson pine (Chen et al., 2021a) and in terpenoid and flavonoid biosynthesis (Mao et al., 2021). WRKY genes also participate in responses to abiotic stress in pines; for instance, *LoWRKY1* from Larix olgensis is significantly induced by drought and exogenous ABA (Wang et al., 2022). Overexpression of *PmWRKY30* and *PmWRKY164* from Masson pine enhances drought and phosphorus tolerance in transgenic tobacco, respectively (Wang et al., 2019; Sun et al., 2022). Regarding biotic stress, WRKY genes are significantly upregulated in Japanese red pine (*Pinus densiflora*) after infection with the pine wood nematode (*Bursaphelenchus xylophilus*), suggesting their involvement in nematode stress responses (Lee et al., 2024). Chen et al. (2021b) found that the *PmWRKY31* gene improves plant insect resistance by promoting terpenoid volatile content. Additionally, signaling pathways involving JA, SA, ABA, and Ca^{2+} have been shown to play important roles in plant defense against insects (Walling, 2000). For example, exogenous ABA

treatment significantly enhances rice resistance to the brown planthopper (*Nilaparvata lugens*; BPH) (Liu et al., 2017). WRKY2 and WRKY6 genes can regulate plant insect resistance by participating in these signaling pathways. In *Nicotiana attenuata*, *WRKY3* and *WRKY6* enhance plant resistance to insects by maintaining or increasing JA levels during continuous insect attack (Skibbe et al., 2008). The *NtWRKY6* gene is significantly induced by salicylic acid (SA), and overexpression enhances tobacco resistance to whitefly (*Bemisia tabaci*) (Yao et al., 2021). Four WRKY genes (*WRKY2*, *WRKY14*, *WRKY28*, *WRKY51*) in canary palm are significantly induced after infestation by the red palm weevil (*Rhynchophorus ferrugineus*), indicating their potential importance in insect resistance responses (Verde et al., 2019).

Masson pine is a typical native conifer species in southern China, characterized by strong adaptability and rapid growth, making it an important timber species with comprehensive utilization value (Yang, 2015). Masson pine growth is threatened by various biotic and abiotic stresses that affect wood yield and ecological environment, with pests, diseases, and seasonal drought being important factors affecting forestry production (Wu et al., 2019). Our previous research found that transcription factor families such as WRKY and AP2/ERF respond to insect resistance processes in Masson pine (Yang et al., 2016). Subsequently, we found that *PmWRKY2* and *PmWRKY6* from the WRKY transcription factor family were significantly highly expressed in insect-resistant varieties of Masson pine (Chen et al., 2021b), but whether they are regulated by hormone and Ca^{2+} signals remains unclear. This study analyzed the protein physicochemical properties, phylogenetic evolution, and other bioinformatics characteristics of *PmWRKY2* and *PmWRKY6*, as well as their expression patterns under different exogenous signaling molecule treatments, to provide a theoretical foundation for exploring the hormone regulatory mechanisms of WRKY genes in Masson pine.

Materials and Methods

1.1 Experimental Materials This experiment utilized six-month-old full-sibling Masson pine seedlings (family line: 17-243) cultivated at the Masson pine improved variety breeding nursery of Guangxi Forestry Research Institute. Plump Masson pine seeds were selected and sown in yellow soil for germination. When sprouts reached 5 cm in height, they were transplanted into non-woven fabric nursery cups (12–15 cm) containing a light substrate composed of 45%–60% peat or coconut coir, 20%–30% carbonized rice husk, 8%–9.5% perlite, 1% superphosphate, and 10%–15% peat soil. When seedlings reached six months of age, healthy seedlings with uniform growth were selected as experimental materials.

1.2 Experimental Treatments Ten treatments were designed, including nine treatment groups and one control group. Four treatment groups received

foliar sprays of 75 mg · L^{-1} abscisic acid (ABA), 50 mg · L^{-1} salicylic acid (SA), 100 mg · L^{-1} methyl jasmonate (MeJA), and 150 mg · L^{-1} gibberellin acid (GA), respectively. Four additional treatments consisted of each hormone solution supplemented with 100 mg · L^{-1} $CaCl_2$ (with 1% Tween-20 added to each solution). One treatment group received 100 mg · L^{-1} $CaCl_2$ solution alone, and the control group (CK) was sprayed with an equal amount of distilled water. Each treatment included six seedlings with three replicates. Solutions and distilled water were sprayed at 9:00 AM daily, with 200 mL applied per treatment for five consecutive days. Needles from the same position of Masson pine seedlings were collected on days 1, 3, and 5 after treatment cessation. Samples from the same treatment were mixed in equal amounts and rapidly frozen in liquid nitrogen for preservation (Chen et al., 2021).

1.3 RNA Extraction and Reverse Transcription Total RNA was extracted from Masson pine needle samples using the Polysaccharide and Polyphenol Plant Total RNA Extraction Kit [PD441, Tiangen Biotech (Beijing) Co., Ltd.]. RNA integrity was detected using 1% agarose gel electrophoresis, and concentration was measured using a UV spectrophotometer. After obtaining qualified samples, RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase (D2639A, TaKaRa) following the manufacturer's instructions. The final sample concentration was diluted to 50 ng · L^{-1}.

1.4 Bioinformatics Analysis Protein molecular weight, isoelectric point, and other physicochemical properties were analyzed using the ExPASy online server (https://web.expasy.org/) (Séverine et al., 2021). Protein transmembrane domains were predicted using TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM) (Moller et al., 2001). Subcellular localization was predicted using WoLF PSORT Prediction (http://psort1.hgc.jp/form.html) (Horton et al., 2007). Protein secondary structure was predicted using SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_{automat}.pl?page=npsa_{sopma}.html) (Geourjon & Deleage, 1995). Protein phosphorylation sites were analyzed using NetPhos 3.1 (http://www.cbs.dtu.dk/services/NetPhos/) (Blom et al., 1999). Homologous protein sequences of *PmWRKY2* and *PmWRKY6* were identified using the NCBI Blastp tool (https://www.ncbi.nlm.nih.gov/cdd/). WRKY genes from *Arabidopsis*, poplar, and other species were downloaded, and protein sequences were aligned using ClustalW. A phylogenetic tree was constructed using the Neighbor-Joining (NJ) method in MEGA 7 software with the P-distance model and 1,000 bootstrap replicates.

1.5 Real-Time Fluorescence Quantitative PCR Using the *PmCYP* gene as an internal reference, qPCR primers for *PmWRKY2*, *PmWRKY6*, and *PmCYP* were designed using Primer Premier 5 software (Table 1). Real-time quantitative PCR reactions were performed according to the SYBR Premix Ex Taq II kit (TaKaRa, Dalian) instructions to detect the expression levels of

PmWRKY2, *PmWRKY6*, and *PmCYP* under hormone treatments (ABA, SA, MeJA, and GA) and combined hormone and calcium treatments, with three biological replicates per treatment. The 2^(-ΔΔCt) method (Livak & Schmittgen, 2001) was used for relative gene expression analysis. Variance analysis was performed using SPSS software, and expression analysis bar charts were generated using Origin software.

Table 1 qRT-PCR primer sequences of the genes

Gene name	Forward sequence (5 -3)	Reverse sequence (5 -3)
PmWRKY2	GGTTCAGGATGATGCTCGGATGTG	AGTGCCAGTCGTTGTTGGTTCAG
PmWRKY6	CACCATGTCACCGTGCTGTCC	GCTGCTGCTGTAGTGGATGCC
PmCYP	GGCAAACTTCTCGCCGTA	GCCAATGCCATCAATGAAGCTCTG

Results

2.1.1 Physicochemical Properties Analysis of PmWRKY2 and PmWRKY6 Genes Bioinformatics prediction of protein molecular weight, isoelectric point, and hydrophobicity revealed that PmWRKY2 and PmWRKY6 proteins encode 667 and 575 amino acids, respectively, with molecular weights of 72.97 and 62.60 kDa. Their theoretical isoelectric points are 6.02 and 6.64, classifying them as acidic proteins. The average hydrophobicity values are -0.795 and -0.684, with instability coefficients of 50.75 and 51.0, indicating they are unstable hydrophobic proteins. Both proteins are localized in the nucleus without transmembrane structures, suggesting these genes function within the nucleus. Phosphorylation site analysis revealed that both genes contain serine, threonine, and tyrosine phosphorylation sites, with serine sites being most abundant, followed by threonine. PmWRKY2 has more tyrosine sites, while PmWRKY6 has only one. Secondary structure prediction showed that both proteins are dominated by random coils (76.50% and 60.24%, respectively). In PmWRKY2, β-turns constitute the smallest proportion, while in PmWRKY6, extended strands are minimal (Table 2).

Table 2 Bioinformatics analysis of PmWRKY2 and PmWRKY6 proteins

Protein	Amino number	Protein (kDa)	Isoelectric point	Subcellular localization	Transmembrane	Kinase phosphorylation	Secondary structure (%)
							Alpha helix
PmWRKY2	667	72.97	6.02	Nucleus	No	Yes	-
PmWRKY6	575	62.60	6.64	Nucleus	No	Yes	-

2.1.2 Phylogenetic and Multiple Sequence Alignment Analysis of PmWRKY2 and PmWRKY6 Homologous amino acid sequences of PmWRKY2 and PmWRKY6 proteins were identified using NCBI BlastP. Fourteen sequences with high homology to PmWRKY2 were obtained, including from *Taxus chinensis*, *Cryptomeria japonica*, and *Pinus monticola*. Fifteen sequences with high homology to PmWRKY6 were obtained, including from *Pinus taeda* and *Picea abies*. Multiple sequence alignment and phylogenetic tree construction revealed that Masson pine clustered with gymnosperms such as *Pinus tabuliformis*, indicating a closer genetic relationship than with other species. PmWRKY2 and PmWRKY6 showed the highest sequence similarity with *Pinus tabuliformis* at 93% and 99.83%, respectively (Figure 1 [Figure 1: see original paper]).

Multiple sequence alignment showed that in PmWRKY2 protein, ginkgo, Norway spruce, and macadamia contain only one WRKY conserved domain at the C-terminus, belonging to group IIc members (Figure 2 [Figure 2: see original paper]). Masson pine and other species contain one WRKY conserved domain each at the C- and N-termini, with a C2H2 (C-X4-C-X23-H-X-H) type zinc finger structure, belonging to group Ic members. PmWRKY6 protein and the other 15 sequences all contain one highly conserved WRKYGQK heptapeptide domain at the N-terminus and a C2H2-type zinc finger structure at the C-terminus with the ligand pattern C-X5-C-X23-H-X-H, classifying them as group IIb subfamily members (Figure 3 [Figure 3: see original paper]).

Figure 1 Phylogenetic analysis of PmWRKY2 (A) and PmWRKY6 (B)

Transverse line indicates conserved WRKY amino domains; indicates the C2H2 zinc finger structure. The same below.

Figure 2 Multiple sequences alignment of PmWRKY2 and homological proteins

Figure 3 Multiple sequences alignment of PmWRKY6 and homological proteins

2.2 Expression Analysis of PmWRKY2 and PmWRKY6 Genes Quantitative real-time PCR was used to analyze the expression patterns of PmWRKY2 and PmWRKY6 under different exogenous hormone treatments and corresponding Ca^{2+} supplementation. Under ABA treatment, the expression of both PmWRKY2 and PmWRKY6 increased with treatment duration and was significantly higher than the control, indicating that ABA induces expression of these genes. Under CK+Ca^{2+} treatment, PmWRKY2 expression was significantly higher than the control on day 1 but showed no significant difference on days 3 and 5. PmWRKY6 exhibited a trend of initial decline followed by increase, with expression significantly higher than the control throughout the process, suggesting Ca^{2+} has an inductive effect on both genes. Under ABA and ABA+Ca^{2+} treatments, both genes showed upregulated expression, reaching maximum expression on day 5 that was significantly higher than the control (Figure 4 [Figure 4: see original paper]A). These

results suggest that ABA and ABA+Ca^{2+} treatments may positively regulate PmWRKY2 and PmWRKY6 expression.

Under SA treatment, both PmWRKY2 and PmWRKY6 were upregulated with increasing treatment time, reaching maximum expression on day 5. Under CK+Ca^{2+} treatment, PmWRKY2 expression was significantly higher than the control on day 1 but showed no significant difference on days 3 and 5, suggesting that Ca^{2+} treatment can induce PmWRKY2 expression during the early stage. PmWRKY6 expression showed a trend of initial decline followed by increase, remaining significantly higher than the control throughout the process, indicating Ca^{2+} induces PmWRKY6 expression. In the SA+Ca^{2+} treatment, PmWRKY2 was upregulated throughout the process and was significantly higher than CK+Ca^{2+} on days 3 and 5, significantly higher than SA treatment on day 3, but significantly lower than SA treatment on day 5. PmWRKY6 showed a trend of initial decline followed by increase, with expression significantly higher than both the control and SA treatment on day 5 (Figure 4B). These results demonstrate that SA and Ca^{2+} may positively regulate expression of both genes, with Ca^{2+} treatment significantly inducing PmWRKY2 expression during the early stage.

Under MeJA treatment, the expression of both PmWRKY6 and PmWRKY2 increased with treatment duration. On day 1, PmWRKY2 showed no significant change compared to the control, while PmWRKY6 was significantly lower than the control. As treatment extended to days 3 and 5, both genes were significantly higher than the control, indicating that MeJA accumulation induces PmWRKY2 and PmWRKY6 expression. Under CK+Ca^{2+} treatment, PmWRKY2 expression was significantly higher than the control on day 1 but showed no significant difference on days 3 and 5, suggesting that Ca^{2+} treatment can induce PmWRKY2 expression during the early stage, though this effect may diminish over time. PmWRKY6 showed a trend of initial decline followed by increase, with expression significantly higher than the control throughout the process (Figure 4C). These results indicate that both MeJA and MeJA+Ca^{2+} treatments significantly induce PmWRKY2 and PmWRKY6 expression.

Under GA treatment, PmWRKY2 and PmWRKY6 expression showed no significant difference from the control on day 1 but were significantly higher on days 3 and 5. Under CK+Ca^{2+} treatment, PmWRKY2 was significantly higher than the control and other treatments on day 1 but showed no significant difference from the control on days 3 and 5, while PmWRKY6 expression remained significantly higher than the control throughout the process, indicating Ca^{2+} treatment induces expression of both genes. Under GA+Ca^{2+} treatment, both PmWRKY2 and PmWRKY6 showed higher expression than the control, displaying an upregulated expression trend (Figure 4D). These results demonstrate that both GA and GA+Ca^{2+} treatments can induce PmWRKY2 and PmWRKY6 expression.

Figure 4 Expression analysis of PmWRKY2 and PmWRKY6 under different hormone treatments

Different lowercase letters indicate significant differences among different treatments on the same day ($P < 0.05$).

Discussion and Conclusion

WRKY transcription factors are one of the plant-specific transcription factor families that regulate multiple key biological processes, including plant growth, development, and stress responses, by activating or suppressing target gene expression (Gao et al., 2020). WRKY transcription factors related to insect resistance have been identified in various plants, but research on insect-resistant WRKY genes in Masson pine remains relatively limited. Mining and studying insect-resistant WRKY genes in Masson pine is of great significance for improving its pest resistance. This study analyzed the bioinformatics characteristics and expression patterns under exogenous signal treatments of *PmWRKY2* and *PmWRKY6* genes obtained in our previous research. Multiple sequence alignment revealed that both *PmWRKY2* and *PmWRKY6* contain the core domain of the WRKY gene family and a C2H2 zinc finger structure at the end of the domain, consistent with findings in peach (Chen et al., 2016) and pepper (Diao et al., 2019), indicating that WRKY family protein structures are conserved across different species. Phylogenetic analysis showed that these two genes have the highest sequence similarity with *Pinus tabuliformis* at 93% and 99.83%, respectively, suggesting that Masson pine WRKY genes are most closely related to gymnosperm conifers in evolution.

Ca^{2+} acts as a second messenger in cell signal transduction and plays important roles in plant responses to hormone signals and insect defense. Hormones also play crucial roles in plant responses to pest stress, with multiple hormones including JA, SA, and ABA, as well as Ca^{2+} signaling pathways, having important regulatory functions in plant defense against insects (Walling, 2000). This study found that exogenous spraying of ABA, GA, SA, MeJA, and Ca^{2+} treatments all significantly increased the expression of *PmWRKY2* and *PmWRKY6* in Masson pine, indicating that these genes may participate in resisting external stresses by regulating ABA, GA, and other signaling pathways in Masson pine. Exogenous ABA treatment significantly induces expression of the tea (*Camellia sinensis*) *CsWRKY2* gene (Wang et al., 2016). Rice *OsWRKY50* expression shows an initial upregulation followed by downregulation with increasing ABA treatment time, indicating that exogenous ABA treatment can induce WRKY gene expression (Huang et al., 2021), consistent with our results. Exogenous MeJA spraying can reduce damage from leaf-eating pests in Larix gmelinii by inhibiting the growth rate of gypsy moth (*Lymantria dispar*) larvae (Li et al., 2014) while also improving insect resistance in Larix olgensis (Meng et al., 2018). Liu et al. (2021) found that the *CsWRKY17* gene is significantly induced by exogenous ABA, JA, and other defense-related signaling molecules, as well as by *Ectropis oblique* larval feeding, suggesting that this gene may regulate tea plant resistance to geometric moths by participating in ABA and JA signaling

pathways. Therefore, we speculate that *PmWRKY2* and *PmWRKY6* may regulate Masson pine resistance by participating in ABA and JA signaling pathways. However, exogenous JA treatment significantly reduces *FaWRKY25* expression, suggesting this gene may negatively regulate strawberry resistance to *Botrytis cinerea* (Jia et al., 2021), indicating that JA treatment regulation of WRKY gene expression may differ across species and stress conditions. SA and GA treatments can induce WRKY gene expression; for example, the *GhWRKY70* gene shows a trend of initial decline followed by increase under SA treatment, indicating its involvement in cotton SA signaling pathway regulation (Xiong et al., 2019). SA treatment significantly increases *ShWRKY81* expression in tomato compared to the control (Wang et al., 2023). GA treatment significantly induces expression of foxtail millet (*SiWRKY36*) and tea plant (*CsWRKY17*) WRKY genes, with *SiWRKY36* expression upregulated 2.5-fold compared to the control (Zu et al., 2015; Liu, 2021). Our finding that SA and GA significantly induce *PmWRKY2* and *PmWRKY6* expression is consistent with these results. Furthermore, Chen et al. (2021b) found that *PmWRKY31* can regulate the expression of genes related to JA, GA, and SA synthesis, thereby promoting terpenoid synthase gene expression and increasing terpenoid content to enhance Masson pine resistance to pine caterpillars. Therefore, we speculate that *PmWRKY2* and *PmWRKY6* genes may play important roles in Masson pine stress defense processes.

In summary, *PmWRKY2* and *PmWRKY6* belong to the WRKY family, are localized in the nucleus, and are most closely related to the gymnosperm *Pinus tabuliformis* in evolutionary terms. Exogenous ABA, SA, MeJA, and GA hormones and Ca^{2+} treatment can all induce expression of *PmWRKY2* and *PmWRKY6*, indicating that these two genes may regulate Masson pine stress resistance by participating in hormone and calcium ion signaling pathways, though the specific regulatory mechanisms require further investigation.

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Note: Figure translations are in progress. See original paper for figures.