Postprint: Analysis of MYBL2 Gene Expression Profile in Fraxinus mandschurica

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Abstract

The MYB transcription factor family is one of the largest transcription factor families in plants, participating in all stages of plant growth, reproduction, and metabolism. Research has demonstrated that MYB transcription factors can be involved in plant stress tolerance through multiple mechanisms. In this study, the FmMYBL2 gene was cloned from Fraxinus mandshurica, its structure and expression characteristics were analyzed using bioinformatics, and a phylogenetic tree of the FmMYBL2 protein was constructed. Fraxinus mandshurica seedlings were subjected to low temperature stress, salt stress, and hormone induction treatments (including ABA, IAA, GA3, JA, and SA). Samples were collected at 0, 1, 3, 6, 12, 24, and 48 h. Real-time quantitative PCR was employed for quantitative analysis of the FmMYBL2 gene in the treated samples, and the spatiotemporal expression characteristics of FmMYBL2 were analyzed. The results indicated that the cloned FmMYBL2 gene had a full length of 762 bp, encoding 253 amino acids. The FmMYBL2 protein is hydrophilic, and amino acid sequence alignment revealed that it is closely related to cotton. Fluorescence quantitative analysis demonstrated that the FmMYBL2 gene responds to both low temperature stress and salt stress, while ABA, IAA, GA3, JA, and SA jointly regulate its expression. The expression level of FmMYBL2 peaked at 1 h under low temperature treatment and at 48 h under salt stress. Following hormone induction, expression levels fluctuated continuously but could respond rapidly within a short period. The FmMYBL2 gene was expressed in roots, buds, flowers, and seeds, with the highest expression observed in male flowers. These findings establish a foundation for further investigation into MYBL2 gene function and the regulation of stress tolerance in Fraxinus mandshurica.

Full Text

Preamble

Characteristic Analysis of FmMYBL2 Gene Expression in *Fraxinus mandshurica*

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Abstract

The MYB transcription factor family represents one of the largest transcription factor families in plants, participating in all stages of plant growth, reproduction, and metabolism. Previous studies have demonstrated that MYB transcription factors contribute to plant stress resistance through multiple mechanisms. In this study, we cloned the *FmMYBL2* gene from *Fraxinus mandshurica* and analyzed its structural and expression characteristics using bioinformatics approaches, constructing a phylogenetic tree of the FmMYBL2 protein. *F. mandshurica* seedlings were subjected to low-temperature stress, salt stress, and hormonal induction treatments (including ABA, IAA, GA_3, JA, and SA), with samples collected at 0, 1, 3, 6, 12, 24, and 48 hours. Real-time quantitative PCR was employed to quantify *FmMYBL2* expression in these treated samples, and both temporal and spatial expression patterns were analyzed. The results revealed that the cloned *FmMYBL2* gene spans 762 bp and encodes 253 amino acids. FmMYBL2 is a hydrophilic protein, and amino acid sequence alignment indicated close homology with cotton. Fluorescence quantification demonstrated that *FmMYBL2* responds to both low-temperature and salt stress, while being co-regulated by ABA, IAA, GA_3, JA, and SA. The highest expression level occurred after 1 hour of low-temperature treatment and 48 hours of salt stress. Following hormone induction, expression fluctuated continuously but showed rapid responsiveness within short timeframes. The gene was expressed in roots, buds, flowers, and seeds, with the highest expression observed in male flowers. These findings establish a foundation for further investigation into MYBL2 gene function and the regulation of stress resistance in *F. mandshurica*.

Keywords: *Fraxinus mandshurica*, MYBL2 transcription factor, gene cloning, stress resistance, bioinformatics

Introduction

In recent years, as global environmental degradation has intensified, the number of plants subjected to adverse conditions has increased dramatically. Environ-

mental stress causes massive cellular dehydration and membrane system dysfunction in plants, severely impairing growth. Consequently, investigating plant stress resistance pathways has become an urgent priority. The MYB transcription factor family is closely associated with plant stress resistance and represents one of the largest transcription factor families in plants, participating in various processes throughout plant growth and reproduction. The *F. mandshurica* MYBL2 protein belongs to the R2R3-MYB protein class, whose structural domain functions as a telomere-binding protein involved in plant stress responses and hormone induction. These proteins can also interact with bHLH and WDR transcription factor complexes to regulate anthocyanin gene expression.

Previous research has shown that cotton *GhMYB73* and Arabidopsis *AtMYB2* genes can enhance plant salt tolerance by improving osmotic stress resistance, while simultaneously increasing the transcription levels of abiotic stress-induced genes involved in ABA pathways. Overexpression of *AtMYB44* increases sensitivity to ABA-induced stomatal closure, thereby enhancing tolerance to drought and salt stress. Conversely, *AtMYB60* expression is inhibited under salt stress and ABA treatment, restricting plant growth. MYB transcription factors also participate in rice defense against environmental stresses. Additionally, the MYB family regulates anthocyanin synthesis as a stress resistance mechanism. In bHLH mutant Arabidopsis, the BoMYB2 transcription factor was found to cooperate with BoHLHs in regulating anthocyanin synthesis. Anthocyanins enhance chilling-induced resistance by increasing the activities of superoxide dismutase, ascorbate oxidase, and glutathione reductase. Red mangoes with higher anthocyanin accumulation exhibited stronger resistance to chilling injury compared to green mangoes with similar maturity parameters after three weeks of cold storage. In wild-type Arabidopsis, exogenous ABA induced continuous anthocyanin accumulation in shoot tips, a phenomenon dependent on *AtMYB75* and other ABA-inducible genes.

Salicylic acid (SA) and jasmonic acid (JA) also play crucial roles in plant stress resistance. SA and JA can induce plants to synthesize more defense compounds by strengthening their own defense signals, thereby enhancing stress resistance. Studies on the SA-binding protein LcSABP in wolfberry revealed that LcSABP enhances stress resistance by increasing endogenous SA content, promoting reactive oxygen species scavenging, and regulating stress-related transcription factor expression. JA can also extend the shelf life of fruits and vegetables and plays an important role in enabling plants to adapt to combined high-light and high-temperature stress.

Fraxinus mandshurica is a deciduous tree in the Oleaceae family, representing a valuable hardwood species in Northeast China and a nationally protected wild plant. Since comprehensive studies on multiple stress resistance pathways remain limited, this study cloned and analyzed the *FmMYBL2* gene in *F. mandshurica*, investigating its stress resistance capacity, hormone-induced expression, and expression patterns in different tissues. These findings provide a basis for understanding the mechanisms underlying *F. mandshurica* adaptation to en-

vironmental stress and hold significant theoretical and practical value for the development and optimization of *F. mandshurica* resources.

Materials and Methods

Plant Material and Stress Treatments One hundred fifty uniformly growing *F. mandshurica* seedlings, cultivated from seeds in a greenhouse under constant temperature, humidity, and light intensity for 45 days, were selected for stress induction experiments. Due to their tender nature and low secondary metabolite content, these seedlings were ideal for detecting gene expression changes under induced stress. The seedlings were subjected to 4°C low-temperature stress and 200 mmol $\cdot$ L^{-1} NaCl salt stress, as well as hormonal signal induction using 100 mol $\cdot$ L^{-1} ABA, GA_3, IAA, JA, and SA. The control group received no treatment. Both experimental and control groups were sampled at 0, 1, 3, 6, 12, 24, and 48 hours. All samples were frozen in liquid nitrogen and stored at -80°C.

Tissue-Specific Sampling Roots, stems, and leaves were collected from five uniformly growing seedlings cultivated for 60 days. Male flowers, female flowers, buds, and seeds were collected from mature, uniformly growing *F. mandshurica* trees in the Northeast Forestry University forest farm across different months (samples from at least five trees, with three replicates). Materials were disinfected in a laminar flow hood for subsequent *MYBL2* expression detection. The control group received disinfection only. All disinfected samples were frozen in liquid nitrogen and stored at -80°C.

Reference Gene and Quantification Method The housekeeping gene TU, which shows relatively stable expression in *F. mandshurica*, was used as an internal reference to ensure accurate quantification and facilitate data normalization. The 2^(-ΔΔCT) method was employed for expression analysis.

Gene Cloning of *FmMYBL2* Total RNA was extracted from roots, stems, leaves, buds, male flowers, female flowers, and seeds (0.5 g each) using the Tris-CTAB method. CTAB binds to proteins and polysaccharides to form complexes that can be separated from nucleic acids using organic solvents, with subsequent precipitation yielding RNA from different samples. The extracted RNA was reverse-transcribed into cDNA using a reverse transcription kit. Primers were designed based on the *F. mandshurica* transcriptome data generated in our laboratory (Table 1) to clone the full coding sequence of *MYBL2* (Figure 1 [Figure 1: see original paper]).

Multiple PCR reactions were performed (20 L system: 2 L template cDNA, 1 L each of forward and reverse primers, 1.6 L dNTP, 0.2 L rTaq, 2 L Buffer, 12.2 L ddH_2O). Products were purified by gel recovery, ligated into pMD18-T vector, transformed into JM109 competent cells, and cultured on antibiotic-

containing medium. Single colonies were selected for verification, and positive clones were sent for sequencing.

Expression Analysis of *FmMYBL2* Under Stress and Hormone Signals Quantitative real-time PCR primers were designed based on the *FmMYBL2* gene sequence (Table 1). Fluorescence quantitative PCR was used to analyze *FmMYBL2* expression under 4°C low-temperature stress, NaCl stress, and induction by IAA, ABA, GA_3, JA, and SA. All samples were analyzed in triplicate, and expression levels were calculated using the 2^(-ΔΔCT) method.

Table 1 Primer sequences for *FmMYBL2* gene cloning and quantitative real-time PCR

Primer name	Primer sequence (5'$→$3')	Remark
FmMYBL2-F	TTGGCTCAATGGGAAGGTCTCC	Gene cloning
FmMYBL2-R	TTATTTCATTTCCAGGCCTCTGTAGC	Gene cloning
qFmMYBL2-F	TGCTGGCGTTCGCTTCCTAA	Quantitative
qFmMYBL2-R	GAGACCATTTGTTACCAAGAAGACTG	Quantitative

Results

Gene Cloning

The *FmMYBL2* gene sequence was successfully cloned (Figure 1 [Figure 1: see original paper]), revealing a full length of 762 bp encoding 253 amino acids.

Sequence Alignment and Phylogenetic Analysis

Homology sequence alignment of FmMYBL2 was performed using Protein BLAST in NCBI. A phylogenetic tree constructed using highly similar homologous sequences (Figure 2 [Figure 2: see original paper]) showed high amino acid sequence similarity with cotton, almond, cacao, and plum, indicating close phylogenetic relationships.

Physicochemical Properties and Hydrophobicity Prediction of FmMYBL2

ProtParam online tool analysis revealed that FmMYBL2 comprises 253 amino acids, including 29 negatively charged residues (Asp + Glu) and 38 positively charged residues (Arg + Lys). The instability index of 49.94 classifies it as an unstable protein. Protein hydrophobicity significantly impacts protein conformation, stability, and function. Proscale online tool analysis (Figure 3 [Figure 3: see original paper]) identified 21 hydrophilic regions and 10 hydrophobic regions, with a grand average of hydropathicity of -0.660, confirming FmMYBL2 as a hydrophilic protein. The hydrophobic regions facilitate protein folding to form secondary structures and domains, promoting α-helix formation and ensuring

stability. Compute pI/Mw analysis indicated an isoelectric point of 9.02, suggesting minimal solubility and maximal precipitation at pH 9.02, with minimal viscosity, osmotic pressure, expansibility, and conductivity.

Expression Patterns of *FmMYBL2* Under 4°C and NaCl Stress

Under both stress treatments, *FmMYBL2* expression was upregulated compared to the control (TU), with significant temporal fluctuations (Figure 4 [Figure 4: see original paper]). During 4°C treatment, expression showed an initial decline followed by an increase, peaking at 1 hour (7-fold higher than control). Expression at 6 and 12 hours fell below control levels, with 6-hour expression at 0.54-fold and 12-hour expression at the lowest level (0.10-fold). Under NaCl stress, expression also exhibited a decline-then-increase pattern, reaching 1.20-fold at 1 hour, dropping below control at 3, 6, and 12 hours, and peaking at 48 hours (2.41-fold).

Expression Patterns of *FmMYBL2* Under Hormone Induction

Hormone treatments induced distinct expression patterns (Figure 5 [Figure 5: see original paper]). ABA treatment caused significant fluctuations, with expression suppressed below normal levels at most time points except 6 hours (1.05-fold), reaching the lowest level at 1 hour (0.19-fold). IAA treatment produced remarkable changes, peaking at 24 hours (11.80-fold), with all time points except 1 hour (0.65-fold) exceeding control levels. GA_3 treatment peaked at 3 hours (2.68-fold), with expression above control at 3, 6, and 24 hours, but below control at 1, 12, and 48 hours. JA treatment (Figure 6 [Figure 6: see original paper]) peaked at 3 hours, declined from 3-6 hours, then increased again with continuous fluctuations. SA treatment reached maximum expression at 1 hour, subsequently fluctuating but remaining within 10-fold of control.

Tissue-Specific and Monthly Expression Analysis of *FmMYBL2*

Tissue-specific expression analysis (Figure 7 [Figure 7: see original paper]) used the *F. mandshurica* tubulin gene TU as internal reference. The relative expression algorithm was 2^(-ΔCT) (ΔCT = gene expression - reference expression). *FmMYBL2* showed differential expression across tissues, with minimal expression in stems and leaves (control set to 1), very low expression in roots, and high expression in flowers. Specifically, expression was 1.38-fold in roots, 13.38-fold in female flowers, and remarkably 20.78-fold in male flowers. Monthly expression analysis (Figure 8 [Figure 8: see original paper]) also used TU as reference, revealing highest expression in August (10-fold), gradually decreasing in September.

Discussion

Bioinformatic analysis of the *FmMYBL2* gene sequence confirmed it encodes a hydrophilic protein without signal peptides. Amino acid sequence alignment

showed high similarity with cotton and other plants, suggesting close phylogenetic relationships and potentially similar functional mechanisms. We investigated relative expression of *MYBL2* under various conditions, focusing primarily on cold stress and hormone induction.

Cold stress reduces defense-related protein content through cold shock responses, adversely affecting plants. Our quantitative analysis revealed *FmMYBL2* expression peaked at 1 hour and reached its lowest level at 12 hours under cold stress. This dramatic expression change indicates responsiveness to cold stress, similar to the cold stress-induced fluctuations observed in Arabidopsis *MYB15* expression. Under NaCl stress, *FmMYBL2* expression showed a decline-then-increase pattern, peaking at 48 hours, demonstrating the gene's capacity to enhance salt tolerance in *F. mandshurica*.

Hormone induction by ABA, IAA, GA_3, JA, and SA differentially affected *FmMYBL2* expression, with the most pronounced response to IAA (11.80-fold at 24 hours). Significant fluctuations under ABA and GA_3 further indicate *FmMYBL2* responsiveness to hormonal signals, enabling it to assist in stress resistance when hormone levels change during stress responses. Since SA and JA regulate stomatal closure and wound signals increase their content, the elevated *FmMYBL2* expression under JA and SA induction suggests coordinated defense against stress-induced cellular damage.

Tissue-specific expression analysis revealed highest *FmMYBL2* levels in flowers and buds, with minimal expression in stems and leaves, indicating tissue-specific expression patterns. The 6-fold expression in May likely correlates with the flowering period and anthocyanin accumulation, while the July-August increase and August-September decrease may reflect reduced need for stress-related metabolites during optimal growth temperatures. In conclusion, *FmMYBL2* participates in both stress response and hormone signaling in *F. mandshurica*, playing a crucial role in development and stress resistance whether through expression level changes or modulation of anthocyanin content. Elucidating this mechanism is significant for *F. mandshurica* breeding and cultivation.

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