Construction of a Three-Frame cDNA Expression Library in Dongzao Jujube and Screening for Upstream Regulators of the ZjRWD40 Gene (Post-Print)

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Abstract

Winter jujube is an important fresh fruit with medicinal properties. RWD40 modulates downstream gene expression and regulates fruit development by altering DNA methylation levels, thereby influencing fruit flavor quality. As a key protein in the methylation regulatory pathway, the upstream regulators of RWD40 in winter jujube remain to be elucidated. This study employed yeast one-hybrid screening to preliminarily identify candidate upstream regulatory factors of the ZjRWD40 gene. The results demonstrated: (1) The winter jujube cDNA three-frame expression library had a titer of 4$×$10^9 CFU·mL^-1 and a recombination rate of 100%. (2) Stress-responsive elements ABRE, MBS, and TGACG-motif were identified in the promoter region of the winter jujube ZjRWD40 gene family, and bait vectors were constructed using their respective sequences, designated as Bait1-ABRE, Bait2-MBS, and Bait3-TGACG-motif. (3) Yeast one-hybrid screening revealed that Bait1-ABRE exhibited self-activation, whereas Bait2-MBS and Bait3-TGACG-motif retrieved 11 gene sequences, five of which were directly associated with plant stress responses. These factors may regulate ZjRWD40 expression by interacting with MBS and TGACG-motif elements, thereby modulating DNA methylation levels in winter jujube and providing insights into the molecular network mechanism underlying ZjRWD40-mediated regulation of fruit development.

Full Text

Construction of a Three-Frame cDNA Expression Library for Winter Jujube and Screening of Upstream Regulatory Factors of the ZjRWD40 Gene

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Abstract

Winter jujube (*Zizyphus jujuba* cv. 'Dong zao') is an important fresh fruit with medicinal properties. The RWD40 protein influences fruit development and flavor quality by regulating DNA methylation levels and downstream gene expression. As a key component of the methylation regulatory pathway, RWD40 proteins play crucial roles in plant stress responses. To identify upstream proteins that regulate ZjRWD40 expression in winter jujube, this study employed yeast one-hybrid screening to identify candidate regulatory factors. The results demonstrated: (1) The titer of the three-frame cDNA expression library reached 4$×10^{9}$ CFU·mL^{-1} with a recombination rate of 100%. (2) Three stress-defense elements—ABRE, MBS, and TGACG-motif—were identified in the promoter region of the ZjRWD40 gene family and used to construct bait vectors named Bait1-ABRE, Bait2-MBS, and Bait3-TGACG-motif. (3) Yeast one-hybrid screening revealed that Bait1-ABRE exhibited self-activation, while Bait2-MBS and Bait3-TGACG-motif retrieved 11 gene sequences, five of which were directly related to plant stress responses. These transcription factors may regulate ZjRWD40 expression by interacting with MBS and TGACG-motif elements, thereby modulating DNA methylation levels in winter jujube under stress conditions. These findings provide valuable insights into the molecular network mechanism by which ZjRWD40 regulates fruit development and offer a theoretical foundation for drought-resistant breeding in winter jujube.

Keywords: Winter jujube (*Zizyphus jujuba* cv. 'Dong zao'), RWD40 gene, cDNA library, Yeast one-hybrid, cis-acting element

Introduction

Winter jujube (*Zizyphus jujuba* cv. 'Dong zao'), a member of the Rhamnaceae family, is a unique late-maturing fresh jujube variety in China, characterized by tender flesh, abundant juice, and minimal residues [Chen, 2011]. China produces approximately 2.5$×10^{8}$ kg of winter jujube annually across about 60,000 hectares, making it one of the country' s most popular fruits [Huang et al., 2023]. The fruit development period from flowering to full ripening spans approximately three and a half months, which can be divided into three stages based on peel color: white-ripe, half-red, and full-red. Previous studies have shown that fruit flavor quality is primarily influenced by environmental factors such as water, light, and temperature. However, drought stress severely impacts fruit quality and yield, reducing fruit set when occurring during flowering, limiting fruit size during early development, and diminishing quality during later growth stages.

Under drought stress, plants rapidly adjust their gene expression patterns to adapt to environmental changes. DNA methylation, as a crucial epigenetic modification, plays a key role in regulating gene expression. Research has identified RWD40 as one of the most significantly affected genes in response to drought stress, with rice plants lacking RWD40 showing heightened drought sensitivity, including early leaf wilting and severe growth inhibition [Eryong et al., 2022]. Drought stress affects heritable traits by altering DNA methylation levels, leading to variations in fruit quality [Zhang, 2013]. However, the specific upstream factors regulating RWD40 expression in winter jujube under drought and other stresses remain unclear, making investigation of its molecular regulatory mechanism essential for drought-resistance breeding.

In *Arabidopsis*, RWD40 serves as an adaptor that forms a WD40 complex with the ROS1 demethylase and RMB1 or RHD1, regulating active DNA demethylation [Liu et al., 2021; Wang et al., 2024]. This complex influences the DNA methylation status under drought stress. While numerous studies have demonstrated WD40 involvement in drought and salt stress responses, most have focused on model plants like *Arabidopsis thaliana* [Hua et al., 2015; Yang et al., 2015]. For instance, research on *Arabidopsis* mutants revealed that WD40-repeat proteins AtARCA and AtAGB1 participate in drought stress signal transduction [Yan, 2005]. In cotton, overexpression of GhWD40 reduced tolerance to salt and drought, indicating its negative regulatory role [Li, 2013].

WD40 genes are vital for plant growth and development, influencing anthocyanin synthesis, meristem formation, seedling development, floral development, and light signal transduction [Ji et al., 2022]. In *Meconopsis horridula*, the WD40 promoter region contains numerous stress-responsive elements, suggesting roles in growth, development, and secondary metabolite accumulation [Ren et al., 2022]. In pepper, silencing of CaMYBA and CaWD40 genes caused similar reductions in anthocyanin content [Ohno et al., 2020], while in tomato, overexpression of SlWDR204 increased stem length [Zhang et al., 2021]. WD40 pro-

teins can sense histone modifications and transmit signals to DNA methylation-related proteins, triggering methylation or demethylation reactions [Valentina et al., 2012]. When histone H3 lysine residues are methylated, WD40 proteins may recruit DNA methyltransferases or demethylases to adjust methylation levels, thereby affecting gene expression and plant development [Zhang et al., 2022]. Therefore, investigating the molecular regulation of RWD40 is crucial for understanding winter jujube' s response to drought stress and its involvement in DNA methylation regulation.

While epigenetic regulation of fruit ripening has been extensively studied, the mechanisms of DNA methylation controlling winter jujube growth and development remain poorly understood. The specific molecular mechanisms by which RWD40 influences DNA methylation under drought stress require further investigation. This study constructed a full-length cDNA library from winter jujube leaves and fruits and employed yeast one-hybrid technology using three cis-acting elements from the ZjRWD40 promoter region as baits to screen for upstream regulatory proteins. These results provide a foundation for elucidating ZjRWD40 molecular mechanisms and their role in fruit development, while establishing a theoretical basis for drought-resistant breeding in winter jujube.

1.1 Plant Materials

Three-year-old winter jujube trees grafted onto sour jujube (*Ziziphus jujuba* var. *spinose*) rootstocks were grown at the Biological Research Institute of North Minzu University (106°10 E, 38°29 N) under a 12 $h \cdot d^{-1}$ photoperiod with light intensity of 150 $mol \cdot m^{-2} \cdot s^{-1}$. Leaf and fruit tissues were collected at 10-day intervals from May to September 2023. Young leaves, mature leaves, and fruits at the white-ripe, half-red, and full-red stages were pooled separately with three biological replicates and stored at -80 °C.

1.2 Methods

1.2.1 cDNA Synthesis and Normalization Approximately 1 g of total RNA was used for first-strand cDNA synthesis using the SMART method. Double-stranded cDNA was obtained through PCR amplification, and 5 L was analyzed by 1.5% agarose gel electrophoresis to verify synthesis efficiency. The purified cDNA was normalized, followed by PCR amplification and electrophoresis to obtain normalized cDNA. After digestion with restriction enzyme *Sfi* I, the cDNA was processed through a gel filtration resin column (Tris-EDTA Buffer) to remove short fragments, then treated with phenol:chloroform:isoamyl alcohol (25:24:1) [Zhang et al., 2023]. The purified cDNA was dissolved in ddH_2O and verified by electrophoresis.

1.2.2 cDNA Library Construction and Plasmid Extraction The column-purified cDNA was ligated with the pGADT7 three-frame vector overnight at 12 °C. The ligation mixture was purified to obtain the primary cDNA library. One microliter of ligation product was electroporated into *HST08* competent cells (1.8 kV, 200 Ω, 25 F), plated on LB medium containing ampicillin (Amp), and incubated overnight at 37 °C. Colony counts were used to determine primary library titer. Random colonies were selected for colony PCR using pGADT7 primers to assess insert length [Zhang, 2022]. The primary three-frame library ligation mixtures were combined, electroporated into *HST08* cells, and plated on LB medium. Amplified colonies were collected, and plasmids were extracted. One microliter was analyzed by electrophoresis to verify library plasmid quality (expected size 2,500–5,000 bp), and 40 mL of glycerol stock was preserved.

1.2.3 Prediction of cis-Acting Elements in the ZjRWD40 Gene Family The *AtRWD40* gene sequence was obtained from the *Arabidopsis* database, and the winter jujube genome and annotation files were retrieved from NCBI. TBtools was used for sequence alignment to identify the ZjRWD40 gene family. Promoter sequences (1,500 bp upstream of transcription start sites) were extracted and analyzed using Plant CARE to predict cis-acting elements. Three stress-related elements—ABRE, MBS, and TGACG-motif—were selected as bait sequences for subsequent yeast one-hybrid analysis.

1.2.4 Construction of Bait Yeast Strains and Minimum AbA Resistance Detection Each bait sequence was extended by two nucleotides at both ends and concatenated in triplicate, then cloned into the *Sac* I/*Sal* I sites of the pAbAi plasmid. Ligation products were verified by electrophoresis, and correctly sized plasmids were sequenced. The confirmed bait plasmids were linearized with *Bbs* I, gel-purified, and transformed into Y1HGold yeast strain. Transformants were plated on SD/-Ura medium to generate Bait1-ABRE, Bait2-MBS, and Bait3-TGACG-motif strains. For minimum AbA resistance determination, cultures were diluted to OD_{600} 0.002, and 100 L aliquots were plated on SD/-Ura medium containing various concentrations of Aureobasidin A (AbA).

1.2.5 Yeast One-Hybrid Screening for Interacting Proteins Twenty micrograms of library plasmid were transformed into Y1HGold-Bait yeast strains using the Yeastmaker kit (Takara), yielding 15 mL of suspension culture. Dilutions (1/10 and 1/100) were plated on SD/-Leu monitoring plates to calculate total screening coverage. The remaining suspension was plated on 150 mm SD/-Leu/AbA (300 $ng \cdot mL^{-1}$) plates and incubated at 30 °C for 5 days. Colonies that grew normally were transferred to fresh SD/-Leu/AbA (300 $ng \cdot mL^{-1}$) plates for confirmation.

1.2.6 Sequencing and Functional Analysis of Positive Clones Positive clones were subjected to colony PCR, and products were analyzed by elec-

trophoresis. Specific bands were excised and sequenced (some samples with two main bands were sequenced separately). Sequences were queried against NCBI using BLASTn, and after removing invalid sequences, functional annotation was performed using UniProt [Wang et al., 2024].

2.1 Total RNA Extraction and cDNA Synthesis

Total RNA from winter jujube leaves and fruits showed good quality [Figure 1: see original paper]A. The synthesized cDNA ranged from 500–5,000 bp [Figure 1: see original paper]B. After normalization and short fragment removal, the cDNA size distribution narrowed to 700–2,500 bp [Figure 1: see original paper]C, D, indicating effective elimination of fragments smaller than 500 bp.

2.2 cDNA Library Construction, Insert Detection, and Plasmid Extraction

The primary libraries for all three reading frames achieved titers of 1.5$×10^{6}$ CFU. Sixteen random colonies from each frame were analyzed by colony PCR. All clones showed single bands with inserts ranging from 400–2,000 bp (average ~1,000 bp) and a recombination rate of 100%, confirming high library quality [Figure 2: see original paper]. Colonies from the three primary libraries were pooled, and plasmids were extracted. Electrophoresis confirmed plasmid sizes between 2,500–5,000 bp, suitable for subsequent yeast one-hybrid screening.

2.3 Prediction of cis-Acting Elements in the ZjRWD40 Gene Family

Promoter analysis of the ZjRWD40 gene family revealed numerous cis-acting elements, including CAAT-box, TATA-box, light-responsive elements, hormone-responsive elements, growth and development elements, and stress-defense elements . Three elements were selected as bait sequences: ABRE (tgACGTGgc, abscisic acid-responsive element), MBS (agCAACTGca, drought-responsive element), and TGACG-motif (atTGACGaa, methyl jasmonate-responsive element).

2.4 Bait Strain Construction and AbA Resistance Self-Activation Detection

The three bait sequences, each concatenated in triplicate, were successfully cloned into the *Sac* I/*Sal* I sites of pAbAi. Linearized plasmids exceeded 5,000 bp, confirming correct insertion [Figure 3: see original paper]. These plasmids

were transformed into Y1HGold to generate Y1HGold-Bait1, Y1HGold-Bait2, and Y1HGold-Bait3 strains.

Minimum AbA resistance testing showed that Bait1 exhibited severe self-activation, with abundant colonies growing on all AbA concentrations, making it unsuitable for screening [Figure 4: see original paper]A. In contrast, Bait2 and Bait3 showed concentration-dependent growth. Bait2 produced >2,000 colonies on SD/-Ura and SD/-Ura with 100 ng · mL^{-1} AbA, but only one colony at 200 ng · mL^{-1} [Figure 4: see original paper]B. Bait3 showed >2,000 colonies on SD/-Ura and three colonies at 100 ng · mL^{-1} AbA [Figure 4: see original paper]C. Neither Bait2 nor Bait3 showed self-activation, and 300 ng · mL^{-1} AbA was selected as the screening concentration for both.

2.5 Yeast One-Hybrid Screening for Upstream Interacting Proteins

Twenty micrograms of library plasmid were transformed into Y1HGold-Bait2 and Y1HGold-Bait3, yielding 15 mL of suspension culture each. Dilution plating on SD/-Leu monitoring plates [Figure 5: see original paper] indicated total screening coverage of 2.7 million clones for Bait2 [180 × 100 × 10 × 15] and 2.4 million for Bait3 [160 × 100 × 10 × 15]. Positive colonies from selection plates were transferred to fresh SD/-Leu/AbA (300 ng · mL^{-1}) plates for confirmation [Figure 6: see original paper]. Colony PCR of 48 positive clones showed insert sizes of 500–2,000 bp for Bait2 and 500–3,000 bp for Bait3 [Figure 7: see original paper], which were excised and sequenced.

2.6 BLAST Analysis of Sequencing Results

BLASTn analysis of positive clone sequences identified candidate interacting proteins involved in plant growth and development, signal transduction, and stress defense. These transcription factors may regulate RWD40 expression by interacting with MBS and TGACG-motif elements, thereby modulating DNA methylation levels in winter jujube under stress conditions.

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3 Discussion and Conclusion

Improving fruit taste and quality has become a prominent issue in winter jujube production. Fruit flavor formation is influenced by environmental factors (water, light, temperature) and genetic/epigenetic regulation of relevant genes. WD40 proteins extensively participate in abiotic stress responses, morphological development, and secondary metabolism regulation through molecular interactions [Zhao et al., 2022].

Analysis of the pearl millet PgWD40 gene family promoter revealed various hormone stress-responsive cis-elements, suggesting regulation of abiotic stress responses through different hormone signaling pathways [Yang et al., 2024]. Similarly, ZjRWD40 promoter analysis identified numerous growth and development-related elements (meristem expression, endosperm expression, palisade mesophyll differentiation) and hormone-related elements (methyl jasmonate, salicylic acid, abscisic acid), along with biotic and abiotic stress-responsive elements, indicating potential involvement in drought and disease responses [Wang et al., 2024].

To investigate ZjRWD40 function in fruit development and DNA methylation, three cis-elements (ABRE, MBS, TGACG-motif) were used as baits to screen upstream transcription factors. While Bait1 showed self-activation (possibly due to non-specific binding or overexpression of certain transcription factors), Bait2 and Bait3 screening yielded 11 gene sequences, five of which were stress-related.

Functional analysis of these five sequences revealed their potential roles in stress responses: Kinesin Light Chain-Associated Protein 3 (KLCAP3) participates in pollen tube growth and defense regulation. In cotton, GhKLCR1 (a kinesin light chain-related protein) is involved in stress responses [Li, 2018]. Under drought, KLCAP3 may regulate transcription factor activity or localization to control downstream stress genes, enhancing drought tolerance by facilitating synthesis of protective proteins and metabolites. Phosphatidylinositol:ceramide inositol phosphotransferase 2-like (PITPNC2L) may regulate defense-related programmed cell death (PCD) through sphingolipid metabolism [Song, 2022]. In winter jujube, this pathway could affect osmotic regulation and membrane stability under water deficit, maintaining cell turgor and physiological function [Yang et al., 2024]. Proline-rich receptor-like protein kinase PERK1 participates in endoplasmic reticulum stress and acts as a stress sensor. PERK1-overexpressing plants show higher proline content and better water retention under drought [Ren et al., 2018], suggesting PERK1 may help winter jujube maintain water balance by regulating proline metabolism. PTI1-like tyrosine-protein kinase At3g15890 is involved in disease resistance and immunity. Tyrosine kinases like Src and Abl participate in pathogen infection [Zhang et al., 2019], suggesting this kinase may phosphorylate and activate proteins related to antimicrobial compound synthesis when winter jujube is pathogen-challenged. Under drought, it may regulate osmotic adjustment or signal transduction to maintain cell turgor. Rho-N domain-containing protein 1, chloroplastic isoform X1, is a novel RNA-binding protein that supports processing of specific chloroplast RNAs. Under drought stress, this protein may maintain chloroplast stability and function, ensuring continued photosynthesis and enhancing survival.

This study successfully constructed a high-quality cDNA library from winter jujube leaves and fruits and used promoter cis-elements as baits in yeast one-hybrid screening to identify five transcription factors potentially involved in stress defense responses. These factors may regulate ZjRWD40 expression through interactions with MBS and TGACG-motif elements. Future research

will employ transcriptome sequencing to identify ZjRWD40 downstream target genes, completing the molecular pathway of drought stress regulation in winter jujube and providing experimental evidence for further validation.

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