Construction of a Yeast Two-Hybrid cDNA Library from Rubber Tree Cambium Tissue and Screening for HbHDA6-Interacting Proteins (Postprint)

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Abstract

Laticifers are the sites for natural rubber synthesis and storage, derived from the division and differentiation of vascular cambium cells. The number of secondary laticifers is directly correlated with natural rubber yield, and this number depends on the frequency of cambium differentiation into secondary laticifers (laticifer differentiation capacity), which constitutes a primary indicator for rubber tree yield breeding. In previous studies, we found that the histone deacetylase (HDA) inhibitor trichostatin A (TSA) can induce laticifer differentiation in rubber trees, and that the histone deacetylase gene (HbHDA6) participates in the regulation of laticifer differentiation in rubber trees. Since the molecular mechanism by which histone acetylation modification regulates laticifer differentiation in rubber trees has not yet been elucidated, this study employed an experimental system wherein coronatine (COR) induces cambium differentiation to produce secondary laticifers in rubber trees, isolated cambium tissue as material, constructed a yeast two-hybrid cDNA library, used the HbHDA6 gene as bait to screen the yeast two-hybrid library, and identified proteins that interact with HbHDA6. The results showed: (1) A normalized yeast two-hybrid cDNA library of COR-induced rubber tree cambium tissue was constructed using Gateway technology. The primary library had a capacity of $6.3\times10^{6}, total single clones of 1.2\times10^{7}, and a recombination rate of 100\times10^{6}, total single clones of 1.5\times10^{7}$, and a recombination rate of 100%. The average insert fragment lengths of the primary and secondary libraries were 1.1 kb and 1.2 kb, respectively. (2) The pGBKT7-HbHDA6 bait vector for screening HbHDA6-interacting proteins was successfully constructed and confirmed to have no auto-activation activity. (3) Using this bait vector to screen the constructed yeast two-hybrid cDNA library, and after NCBI BLAST comparison and removal of duplicates, 22

proteins that interact with HbHDA6 were obtained, including CLP1, ERF3, ERF4, HSP82, LARP6a, APT5, PP2A, FBA6, etc. This research provides a theoretical foundation for elucidating the molecular mechanism by which histone acetylation modification regulates laticifer differentiation in rubber trees, offers candidate genes for transgenic improvement of rubber yield potential in rubber trees, and provides new clues for genetic improvement breeding of high-performance natural rubber.

Full Text

Construction of a Yeast Two-Hybrid cDNA Library from Rubber Tree Cambium Tissue and Screening for HbHDA6-Interacting Proteins

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Abstract: Laticifers are the sites of natural rubber synthesis and storage, differentiated from vascular cambium cells. The quantity of secondary laticifers directly correlates with natural rubber yield, depending on the frequency of differentiation from the cambium (laticifer differentiation capacity), which represents a primary target for yield breeding in rubber trees. Our previous studies demonstrated that the histone deacetylase (HDA) inhibitor trichostatin A (TSA) induces laticifer differentiation and that the histone deacetylase gene *HbHDA6* participates in this regulatory process. However, the molecular mechanism by which histone acetylation regulates laticifer differentiation remains unelucidated. This study employed a coronatine (COR)-induced secondary laticifer differentiation system in rubber tree cambium to construct a yeast two-hybrid cDNA library, using *HbHDA6* as bait to screen for interacting proteins. The results showed: (1) A normalized yeast two-hybrid cDNA library from COR-induced rubber tree cambium was successfully constructed using Gateway technology, with primary library capacity of 6.3$×10^{6}$ cfu·mL^{-1}, total clone number of 1.2$×10^{7}, and recombination rate of 100×10^{6}$ cfu·mL^{-1}, total clone number of 1.5$×10^{7}$, and recombination rate of 100%. Average insert lengths were 1.1 kb and 1.2 kb for primary and secondary libraries, respectively. (2) The pGBKT7-*HbHDA6* bait vector was successfully constructed and confirmed to lack auto-activation activity. (3) Screening the yeast two-hybrid cDNA library with this bait vector identified 22 proteins interacting with HbHDA6, including CLP1, ERF3, ERF4, HSP82, LARP6a, APT5, PP2A, and

FBA6, after NCBI BLAST analysis and duplicate removal. These findings provide a theoretical foundation for elucidating the molecular mechanism of histone acetylation-mediated regulation of laticifer differentiation, offer candidate genes for genetically improving rubber yield potential, and present new insights for breeding high-performance natural rubber.

Keywords: *Hevea brasiliensis*, secondary laticifer differentiation, vascular cambium, yeast two-hybrid, HbHDA6

Introduction

Natural rubber is a crucial industrial raw material and strategic resource for national economies, with the vast majority of global supply derived from the rubber tree (*Hevea brasiliensis*) (Tian et al., 2015). Secondary laticifers in the trunk bark of rubber trees serve as the primary sites for natural rubber synthesis and storage, originating from the differentiation of fusiform initial cells in the vascular cambium (Gomez, 1982; Hao & Wu, 2000; Chao et al., 2023). As these laticifers differentiate from the cambium, they gradually migrate outward, progressing through juvenile, mature, senescent, and dead stages. To maintain laticifer populations in the rubber-producing tissues, the cambium must continuously differentiate new secondary laticifers. The quantity of these secondary laticifers directly correlates with natural rubber yield, and their number depends on the frequency of differentiation from the vascular cambium—termed laticifer differentiation capacity—which represents a key trait for yield breeding in rubber trees (Tian et al., 2015; Chao et al., 2023).

Our research team was the first to demonstrate that exogenous jasmonic acid (JA) and its precursor linolenic acid (LA) can induce cambium cells to differentiate into secondary laticifers (Hao & Wu, 2000; Liu et al., 2001; Tian et al., 2003). We subsequently discovered that coronatine (COR), a molecule structurally similar to the active form of jasmonic acid (JA-Ile) (Ichihara et al., 1977), exhibits superior efficacy compared to methyl jasmonate (MeJA) in inducing secondary laticifer differentiation (Zhang et al., 2011; Zhang & Tian, 2015). This led us to establish a stable experimental system using COR to induce secondary laticifer differentiation in rubber tree sprouts (Zhang & Tian, 2015; Zhang et al., 2015, 2016; Wu et al., 2016, 2023; Zhang et al., 2018). More recently, we found that trichostatin A (TSA), an inhibitor of histone deacetylase (HDA), can also induce secondary laticifer differentiation in rubber trees (Zhang et al., 2016). Furthermore, COR treatment significantly enhances histone acetylation levels in the cambium region while affecting HDA activity and content. Based on these findings and considering that histone acetylation represents a critical mechanism for transcriptional regulation, we hypothesize that JA-induced secondary laticifer differentiation may be mediated through histone acetylation-mediated transcriptional control of genes involved in this process.

Yeast two-hybrid technology is a classical method for studying protein-protein

interactions, originally developed by Fields and Song during their investigations of eukaryotic gene transcription regulation (Fields, 1993). With advances in molecular biology, several derived techniques have emerged, including yeast one-hybrid, membrane yeast two-hybrid, and yeast three-hybrid systems, enabling the study of protein-protein, protein-DNA/RNA, and protein-ligand interactions. These tools have become indispensable in functional genomics and interactomics research. The yeast two-hybrid system enables rapid and direct analysis of interactions between known proteins while also facilitating the discovery and isolation of unknown interaction partners. It finds broad applications in investigating antigen-antibody interactions, discovering novel proteins and protein functions, screening drug target sites and assessing drug effects on protein interactions, and constructing genome-wide protein linkage maps. Compared with in vitro protein interaction techniques such as pull-down assays, electrophoretic mobility shift assay (EMSA), and surface plasmon resonance (SPR), yeast two-hybrid technology better mimics the intracellular environment, providing a more authentic representation of protein-protein interactions (Phizicky & Fields, 1995). The system is founded on the principles of eukaryotic transcription regulation, which requires trans-acting transcriptional activators. These activators contain two distinct domains: a DNA-binding domain (DNA-BD) and a transcriptional activation domain (AD) that can function independently without mutual interference. Neither domain alone can activate transcription; only when brought into close spatial proximity do they constitute a functional transcriptional activator capable of driving downstream gene expression. This characteristic makes yeast two-hybrid technology a powerful tool for identifying unknown proteins that interact with a protein of interest and for elucidating molecular regulatory networks (Paiano et al., 2019).

Yeast two-hybrid cDNA library construction has been widely applied in rubber tree molecular biology research, including studies establishing latex yeast two-hybrid cDNA expression libraries (Yang et al., 2013), normalized latex yeast two-hybrid cDNA libraries (Yu et al., 2016), leaf and latex cDNA libraries (Ouyang et al., 2016), normalized aboveground and belowground tissue yeast two-hybrid cDNA libraries (Chen et al., 2017), latex libraries from virgin and tapped trees (Chao et al., 2021), and membrane system yeast two-hybrid cDNA libraries (Nie et al., 2022). Given that the molecular mechanism of histone acetylation-mediated regulation of laticifer differentiation remains unelucidated, and considering that yeast two-hybrid library construction and screening can generate comprehensive protein interaction data, this approach offers significant advantages for constructing molecular regulatory networks underlying secondary laticifer differentiation. In this study, we utilized a COR-induced secondary laticifer differentiation system in rubber tree sprouts, isolated cambium tissue as starting material, constructed a yeast two-hybrid cDNA library, and screened it using *HbHDA6* as bait to identify interacting proteins. Our objective was to isolate genes or transcription factors involved in histone acetylation-mediated regulation of secondary laticifer differentiation, thereby providing a theoretical foundation for understanding the molecular regulatory network, offering candi-

date genes for genetically improving rubber yield potential, and presenting new insights for breeding high-performance natural rubber.

Materials and Methods

1.1 Plant Materials and Reagents

Plant materials consisted of one-year-old sprouts from the rubber tree (*Hevea brasiliensis*) clone Reyan 7-33-97, cultivated in the propagation nursery of the Rubber Research Institute, Chinese Academy of Tropical Agricultural Sciences. These sprouts were annually pruned at the base, with new shoots regenerating from latent buds, producing 5–6 extension units (EUs) within a single growing season (Zhang et al., 2011).

The main reagents and consumables used in this study included: RNAprep Pure Polysaccharide and Polyphenol Plant Total RNA Extraction Kit (DP441), Agarose Gel DNA Recovery Kit (DP204), Plasmid Mini-Prep Kit (DP103), and Yeast Plasmid Extraction Kit (DP112) from Tiangen Biotech (Beijing); FastTrack® MAG mRNA Isolation Kit (K158002), LR Clonase™ II Enzyme Mix (11791-020), UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) (15593-031), 5 M Ammonium Acetate (AM9070G), PureLink® HiPure Plasmid Filter Midiprep Kit (K2100-15), and ElectroMAX™ DH10B™ T1 Phage Resistant Cells (12033-015) from Thermo Fisher Scientific (China); Carrier DNA, YPDA medium, YPD plus medium, SD/Trp, SD/Leu, SD/His/Leu/Trp (TDO), SD/Ade/His/Leu/Trp (QDO), Aureobasidin A, and X-α-Gal from Clontech; FastPfu DNA Polymerase (AP221-01) from TransGen Biotech (Beijing); coronatine (COR), DMSO, acetosyringone, MES, $MgCl_2$, PEG, TE, LiAc, and other reagents from Sigma-Aldrich. Additional reagents were of analytical grade from domestic suppliers, and pipette tips and centrifuge tubes were Axygen products.

1.2 Coronatine (COR) Treatment of Plant Materials

One-year-old rubber tree sprouts at the third extension unit (EU3) stage, characterized by long internodes and robust stems, were selected as experimental material. At the middle portion of the EU3 stem, a 2 cm × 4 cm area of epidermis and partial cortex was removed using a single-edged blade. The treated area was covered with sterile dust-free paper slightly larger than the wound, saturated with 6 $g \cdot mL^{-1}$ COR solution, and sealed with plastic parafilm. Treatment durations were 0.5 h, 1 h, 2 h, 4 h, 8 h, 1 d, 2 d, and 3 d. Following treatment, the parafilm and paper were removed, and bark samples (including partial xylem) were excised, immediately cut into small pieces, placed in 2 mL centrifuge tubes, and snap-frozen in liquid nitrogen. Cambium tissue samples from each time point were pooled from five individual rubber tree sprouts, with three biological replicates per time point, totaling 15 sprouts per time point.

1.3 Total RNA Extraction and mRNA Purification

Cambium tissue from rubber tree bark was collected using frozen section tangential cutting. Total RNA was extracted using the RNAprep Pure Polysaccharide and Polyphenol Plant Total RNA Extraction Kit (Tiangen, DP441). RNA concentration and purity were determined using a micro-UV spectrophotometer, and integrity was assessed via 1% agarose gel electrophoresis. Qualified total RNA samples were stored at –80°C for subsequent use. Total RNA from cambium tissues treated with COR for different durations was mixed in equal quantities. mRNA was then isolated and purified from the pooled total RNA using the magnetic bead-based FastTrack® MAG mRNA Isolation Kit (Invitrogen, K158096). The purified mRNA samples were subsequently mixed in equal amounts for yeast two-hybrid library construction.

1.4 Construction of Yeast Two-Hybrid cDNA Library

A yeast two-hybrid cDNA library was constructed using Gateway recombination technology with the CloneMiner™ II cDNA Library Construction Kit. The procedure involved reverse transcription of the purified and pooled mRNA to synthesize first-strand cDNA, followed by second-strand synthesis. The resulting double-stranded cDNA was size-fractionated and collected. BP recombination reactions were performed using LR Clonase™ II Enzyme Mix, and the products were electroporated into ElectroMAX DH10B competent cells using a BTX exponential decay wave electroporator (ECM630). Transformed cells were recovered in shaking culture at 37°C and 220 rpm for 1 h. A portion of the culture was plated to determine primary library capacity, recombination rate, and insert fragment length. The remaining culture was supplemented with glycerol to a final concentration of 20% and stored at –80°C as the primary cDNA library stock from COR-induced rubber tree cambium tissue.

The validated primary cDNA library was used for plasmid extraction with the PureLink® HiPure Plasmid Filter Midiprep Kit (Life, K2100-15). The extracted primary library plasmid was diluted to 300 ng $\cdot$ L^{-1} and subjected to LR recombination using LR Clonase™ II Enzyme Mix. The recombination products were extracted and transformed into ElectroMAX DH10B competent cells via electroporation as described above. Following transformation, 10 L of the culture was diluted 1,000-fold, and 50 L of the diluted culture was plated on LB agar containing streptomycin, followed by overnight incubation at 37°C. Secondary library capacity, recombination rate, and insert fragment length were then assessed. The remaining culture was mixed with glycerol to 20% final concentration and stored at –80°C as the secondary cDNA library stock. The total bacterial colony-forming units (CFU) were calculated using the formula: cfu $\cdot$ mL^{-1} = (colony count/50 L) $\times$ 1,000 $\times$ 1,000 L; total library CFU = cfu $\cdot$ mL^{-1} $\times$ total library volume (mL).

1.5 Construction, Identification, and Auto-Activation Detection of HbHDA6 Bait Plasmid

To construct the HbHDA6 bait plasmid, the HbHDA6 gene was PCR-amplified from its vector and digested with Sfi I to obtain the HbHDA6 insert fragment. The yeast two-hybrid bait vector pGBKT7 was linearized with Sfi I, and the HbHDA6 fragment was ligated into the linearized vector. The specific procedure was as follows: Based on the HbHDA6 cDNA sequence, primers were designed with Sfi I restriction sites added to both ends for amplification of the HbHDA6 cDNA fragment. The primer sequences were: HbHDA6-F: 5 -aaggccattacggccATGGGCGACACAACCGGTGGT-3 and HbHDA6-R: 5 -ccggccgaggcggccTCAAGAACGCGGATGCTCTTCTCTCT-3 . PCR amplification was performed using the FastPfu DNA Polymerase (AP221-01) high-fidelity enzyme system. Both the HbHDA6 amplicon and pGBKT7 vector were digested with Sfi I, and the digested products were purified using an Axygen gel extraction kit. The recovered fragments were ligated and transformed into *E. coli* Top10, followed by overnight incubation at 37°C.

Four transformants were randomly selected from the ligation transformation plates (containing streptomycin resistance) and inoculated into LB liquid medium, then cultured at 37°C with shaking at 220 rpm for 16 h. PCR verification was performed using pGBKT7 universal primers: pGBKT7-F: 5 -TAATACGACTCACTATAGGGC-3 and pGBKT7-R: 5 -TAAGAGTCACTTTAAAATTTGTAT-3 . PCR products were analyzed by 1% agarose gel electrophoresis. Positive clones were subjected to plasmid extraction (Axygen Plasmid Mini-Prep Kit) and sequencing. Clones with verified correct insert sequences were used for subsequent experiments. Various plasmids were constructed and different types of plates were prepared according to Table 1 , and transformed into the yeast recipient strain AH109 for observation and analysis.

For auto-activation detection, six yeast colonies from AH109 co-transformed with pGADT7 and pGBKT7-HbHDA6 were randomly selected. The detection included three reporter genes: HIS3, ADE2, and MEL1. Reporter gene activity was assessed using a spot plate assay. Transformants were spotted onto SD-TL+X-α-Gal and SD-TLHA plates and incubated at 30°C for 4 days to observe growth. For MEL1 detection, 200 L of X-α-Gal solution was applied to pre-prepared SD-TLHA plates. Once the X-α-Gal solution was fully absorbed, transformant cultures were spotted and incubated.

1.6 Screening of Yeast Library with pGBKT7-HbHDA6 Bait Plasmid

Yeast competent cells were prepared using AH109 transformants harboring the sequence-verified pGBKT7-HbHDA6 bait plasmid as recipient cells. The cDNA primary library plasmid from COR-treated rubber tree cambium tissue was transformed into the yeast competent cells and plated onto SD-Trp-Leu-His medium supplemented with 5 mmol $\cdot$ L^{-1} 3-amino-1,2,4-triazole (3AT) for incu-

bation. A single colony was selected from SD-Trp plates, inoculated into SD-Trp liquid medium, and cultured at 30°C with shaking at 220 rpm for 18 h, then transferred to YPDA medium for further culture. The initial culture density was measured by UV spectrophotometry at OD_{600} = 0.2. Cultures were then incubated at 30°C with shaking at 220 rpm for 4–5 h, with periodic monitoring of OD_{600} until reaching 0.6.

The culture was centrifuged, the supernatant discarded, and the cell pellet resuspended. Reagents were added in order of decreasing volume: 9.6 mL of 50% PEG3350, 1.44 mL of 1 M LiAc, 300 L of ssDNA (10 $mg \cdot mL^{-1}$), and 25 g of library plasmid DNA, followed by thorough mixing. The mixture was incubated in a water bath at 30°C for 30 min, heat-shocked at 42°C for 25 min, then recovered at 30°C for 1 h. The recovered cells were centrifuged and resuspended. To assess transformation efficiency, 20 L of the resuspended culture was gradient-diluted and plated onto three SD-TL plates. The remaining culture was plated onto SD-TLH + 5 $mmol \cdot L^{-1}$ 3AT plates (200 L per plate, 40 plates total) and incubated upside-down at 30°C for 3–4 days. Transformation results were observed, recorded, and analyzed to determine efficiency.

1.7 Identification and Sequence Analysis of Positive Clones

Positive clones from the pGBKT7-HbHDA6 bait screening plates were selected and restreaked onto SD-TL dropout medium plates, followed by incubation at 30°C for 2–3 days. Initial positive transformants growing on SD-TL plates were diluted with sterile water and spotted onto both SD-TL and SD-TLHA+X-α-Gal dropout plates to assay the HIS3, ADE2, and MEL1 reporter genes, with incubation at 30°C for 3–4 days.

The reporter gene assays revealed that positive controls grew normally on both SD-TL and SD-TLHA+X-α-Gal dropout plates, forming blue colonies on SD-TLHA+X-α-Gal medium. Negative controls, which cannot activate HIS3 and ADE2, grew on SD-TL dropout plates without turning blue and failed to grow on SD-TLHA+X-α-Gal medium lacking histidine and adenine. Therefore, initial positive clones capable of growing on SD-TLHA+X-α-Gal plates and forming blue colonies were considered to have activated all three reporter genes (HIS3, ADE2, and MEL1).

To identify the genes corresponding to the positive clones, each clone was inoculated into SD-TL dropout liquid medium and cultured at 30°C with shaking at 220 rpm for 16 h. Yeast plasmids were extracted and purified using the Yeast Plasmid Extraction Kit (Tiangen, DP112). The purified yeast plasmids were transformed into *E. coli* Top10 competent cells for amplification, followed by culture at 37°C with shaking at 220 rpm for 16 h. Plasmids were extracted from *E. coli* using the Plasmid Mini-Prep Kit (Tiangen, DP103) and sent to Sangon Biotech (Shanghai) Co., Ltd. for DNA sequencing. Sequencing results were analyzed by BLAST comparison against the NCBI GenBank database to obtain information on genes interacting with HbHDA6.

Results

2.1 Isolation of Cambium Tissue, Total RNA Extraction, and mRNA Purification

Cambium tissue from the bark of one-year-old COR-treated rubber tree sprouts was isolated using frozen section tangential cutting [Figure 1: see original paper]A. Microscopic observation revealed that the isolated cambium tissue was free of contaminants, with the vast majority of cells exhibiting elongated fusiform morphology and dense arrangement, containing minimal other cell types [Figure 1: see original paper]B.

Total RNA extracted from cambium tissue using the RNAprep Pure Polysaccharide and Polyphenol Plant Total RNA Extraction Kit (DP441) showed high concentration, averaging 1,642.98 ng· L^{-1}, with A_{260}/A_{280} and A_{260}/A_{230} ratios averaging 2.11 and 2.13, respectively. Agarose gel electrophoresis (1%) revealed clear 28S and 18S rRNA bands with an intensity ratio of approximately 2:1, indicating intact RNA without degradation [Figure 1: see original paper]C. The total RNA extracted from cambium tissues treated with COR for various durations exhibited good quality and was suitable for subsequent experiments.

Total RNA from cambium tissues treated with COR at eight time points was pooled in equal amounts. mRNA was then isolated and purified from the pooled total RNA using the magnetic bead-based FastTrack® MAG mRNA Isolation Kit (Invitrogen, K158096). Electrophoresis of the purified mRNA on 1% agarose gel showed a smeared, evenly distributed band pattern [Figure 1: see original paper]D, confirming good mRNA quality for downstream applications.

2.2 Construction and Characterization of Primary and Secondary cDNA Libraries

Following plating and culture of the primary cDNA library from COR-treated rubber tree cambium tissue [Figure 2: see original paper]A, we assessed library capacity, recombination rate, and insert fragment length. The primary cDNA library transformation plates (diluted 1:1,000) yielded 6.3$×10^{6}$ cfu·mL^{-1} (calculated as 317 colonies/50 L × 1,000 × 1,000 L), exceeding the required threshold of 1$×10^{6}$ cfu·mL^{-1}. The total clone number was 1.2$×10^{7}$ (6.3$×10^{6}$ cfu·mL^{-1} × 2 mL), also surpassing the standard requirement of 1$×10^{7}$. PCR analysis of 24 randomly selected colonies from the primary library revealed 100% insertion efficiency, with insert fragments ranging from 0.6–1.2 kb and an average length of 1.1 kb [Figure 2: see original paper]C.

Similarly, the secondary cDNA library from COR-treated cambium tissue [Figure 2: see original paper]B was evaluated. Transformation plates (diluted 1:1,000) showed 7.7$×10^{6}$ cfu·mL^{-1} (386 colonies/50 L × 1,000 × 1,000 L), exceeding the 1$×10^{6}$ cfu·mL^{-1} requirement. Total clone number reached 1.5$×10^{7}$ (7.7$×10^{6}$ cfu·mL^{-1} × 2 mL), above the 1$×10^{7}$ standard. PCR screening of 24 random clones from the secondary

library demonstrated 100% insertion efficiency, with inserts ranging from 1.0–1.6 kb and an average length of 1.2 kb [Figure 2: see original paper]D.

In summary, the yeast two-hybrid cDNA library constructed from COR-treated rubber tree cambium tissue exhibited high quality, with adequate capacity, recombination rate, and insert fragment length, making it suitable for screening and identification of HbHDA6-interacting proteins.

2.3 Construction, Identification, and Auto-Activation Detection of HbHDA6 Bait Plasmid

PCR amplification of the HbHDA6 gene yielded a band of approximately 1.7 kb [Figure 3: see original paper]A, which was confirmed by sequencing to match the HbHDA6 coding sequence. The HbHDA6 fragment was obtained by SfiI digestion and ligated with SfiI-linearized pGBKT7 bait vector to generate the yeast two-hybrid bait plasmid pGBKT7-HbHDA6. Colony PCR screening showed bands of 1.7 kb for all clones [Figure 3: see original paper]B, and sequencing verification confirmed correct insertion, enabling subsequent auto-activation assays.

For auto-activation detection, six yeast colonies from AH109 co-transformed with pGADT7 and pGBKT7-HbHDA6 were randomly selected and tested for three reporter genes: HIS3, ADE2, and MEL1. Transformants were spotted onto SD-TL+X-α-Gal and SD-TLHA dropout plates and incubated at 30°C for 4 days. Control strains grew normally on SD-TL dropout plates, while only the positive control pGADT7-largeT/pGBKT7-p53 grew on SD-TLHA+X-α-Gal plates. The six randomly selected pGADT7+pGBKT7-HbHDA6 transformants failed to grow on SD-TLHA+X-α-Gal plates, showing the same growth pattern as the negative control pGADT7-LargeT/pGBKT7-LaminC, with MEL1 assay results also matching the negative control. These results confirmed the absence of auto-activation [Figure 3: see original paper]C.

The yeast two-hybrid cDNA library from COR-treated rubber tree cambium was transformed into competent cells prepared from AH109 harboring the correct pGBKT7-HbHDA6 bait plasmid. To evaluate library transformation efficiency, 20 L of yeast culture was gradient-diluted and plated onto three SD-TL plates [Figure 3: see original paper]D. Colony counting revealed a total of 7.06$×10^{6}$ transformants [(3,978/20 + 2,038/2 + 286/0.2) × 1/3 × 8,000], corresponding to a transformation efficiency of 2.82$×10^{5}\cdot\mu g$-1$ (7.06$×10^{6}$/25 g).

Reporter gene assays showed that positive controls grew normally on both SD-TL+X-α-Gal and SD-TLHA dropout media, forming blue colonies, while negative controls grew on SD-TL without blue coloration but failed to grow on SD-TLHA medium lacking histidine and adenine. Therefore, the 24 initial positive clones obtained in this study, which grew on SD-TLHA and produced blue colonies, were confirmed to have activated all three reporter genes (HIS3, ADE2, and MEL1) [Figure 3: see original paper]E.

2.4 Identification of Positive Clones and Analysis of Interacting Proteins

To identify the genes corresponding to positive clones screened with the pGBKT7-HbHDA6 bait plasmid, each clone was cultured in SD-TL liquid medium overnight. Yeast plasmids were extracted and purified, then transformed into fresh *E. coli* Top10 competent cells for amplification. Top10 transformants harboring positive clones were cultured in LBA liquid medium, and plasmids were extracted for DNA sequencing. Sequencing results were analyzed by BLAST comparison against the NCBI GenBank database. The analysis identified 22 distinct protein-coding genes that interact with HbHDA6, after removing duplicates, as shown in Table 2 .

Based on UniProt protein information, the 22 HbHDA6-interacting proteins could be classified into seven functional categories: oxidoreductases, hydrolases, protein/DNA/RNA binding proteins, transporters, isomerases, chromatin regulators, and glycoproteins. Hydrolases, oxidoreductases, and protein/DNA/RNA binding proteins were the most abundant categories. Subcellular localization predictions indicated that these 22 proteins were distributed throughout various cellular compartments, with relatively high representation in the plasma membrane, chloroplast, mitochondrion, and nucleus.

Discussion and Conclusions

High-quality total RNA and purified mRNA extracted from animal or plant tissues and cells serve as the starting material for constructing yeast two-hybrid cDNA libraries, and obtaining such high-quality nucleic acids is a prerequisite for building high-quality libraries (Gao et al., 2014). In this study, the vascular cambium tissue from rubber tree bark is located between the phloem and xylem, composed of several to dozens of layers of tightly arranged fusiform cambial cells, making it difficult to obtain intact and pure cambium tissue. Previous research revealed a clear pattern in cambial cell layer numbers in rubber tree sprouts: during spring shoot growth (March–April) in Hainan, the cambium region initiates division and differentiation activities with the fewest cell layers (approximately 4 layers) (Wu et al., 2002), whereas during the hot and humid conditions of July–August, which favor sprout growth and cambial cell division, the cambium reaches its maximum thickness (10–11 layers) (Zhang et al., 2011).

In this study, bark samples from EU3 internodes were collected during July–August when cambial cells were most abundant and secondary phloem had not yet differentiated secondary laticifers, enabling acquisition of the maximum amount of pure cambium tissue. Frozen section tangential cutting yielded cambium tissue free of contaminants, with the vast majority of cells displaying elongated fusiform morphology and dense arrangement. Consequently, even limited tissue material could yield high-quantity, high-quality total RNA. Subsequent mRNA isolation and purification using the established magnetic bead technology achieved excellent capture rates and success (Albretsen et al., 1990),

ensuring the high-quality total RNA and mRNA required for yeast two-hybrid cDNA library construction. cDNA synthesis from mRNA involved LD-PCR amplification both before and after normalization. This two-round LD-PCR amplification not only ensured sufficient cDNA yield for library construction from limited starting total RNA but also increased the representation of low-abundance genes, thereby enhancing the probability of identifying such genes during library screening (Yang et al., 2013; Yu et al., 2016).

Quality assessment of yeast two-hybrid cDNA libraries primarily relies on three key parameters: library clone number, recombination rate, and insert fragment length. Clone number reflects library completeness and coverage, while insert length indicates gene integrity (Van Criekinge & Beyaert, 1999). A clone number exceeding 1$\times$10^{6}$ is essential for successful library screening (Li et al., 2016). In this study, we constructed a normalized yeast two-hybrid cDNA library from jasmonic acid-induced cambium tissue undergoing secondary laticifer differentiation. The primary library capacity reached 6.3$\times$10^{6}$ cfu $\cdot$ mL^{-1} with 1.2$\times$10^{7}$ total clones, while the secondary library capacity was 7.7$\times$10^{6}$ cfu $\cdot$ mL^{-1} with 1.5$\times$10^{7}$ total clones. Both libraries achieved 100% recombination rates, surpassing the basic requirements for yeast two-hybrid cDNA library construction. Furthermore, the average insert lengths of 1.1 kb and 1.2 kb for the primary and secondary libraries, respectively, approximate the average CDS length reported for the rubber tree Reyan 7-33-97 genome sequence (Tang et al., 2016). These results demonstrate that our COR-treated rubber tree cambium yeast two-hybrid library meets high standards for clone number, recombination rate, and insert length, fulfilling the requirements for library screening.

Our research team first discovered that exogenous JA and its precursor linolenic acid (LA) can induce cambium cells to differentiate into secondary laticifers (Hao & Wu, 2000; Tian et al., 2003). COR, which is structurally similar to the active form of jasmonic acid (JA-Ile), can bind to the COI1 receptor to activate JA signaling pathways (Katsir et al., 2008) and demonstrates superior efficacy in inducing rubber tree laticifer differentiation compared to MeJA (Zhang et al., 2011). Based on this, we established a stable COR-induced secondary laticifer differentiation system in rubber tree sprouts, with induced laticifers observable within 5 days of treatment (Zhang & Tian, 2015; Zhang et al., 2015, 2016; Wu et al., 2016, 2023; Zhang et al., 2018).

More recently, we found that trichostatin A (TSA), an HDA inhibitor, can also induce secondary laticifer differentiation (Zhang et al., 2016), and that COR treatment significantly increases histone acetylation levels in the cambium region while affecting HDA activity and, particularly, HDA6 content and gene expression (unpublished data). Building upon these findings and considering histone acetylation as a crucial gene expression regulatory mechanism, we constructed a yeast two-hybrid library from COR-treated rubber tree cambium and screened it using the HbHDA6 bait vector, identifying 22 proteins that interact with HbHDA6. According to UniProt predictions, these proteins fall into

seven functional categories: oxidoreductases, hydrolases, protein/DNA/RNA binding proteins, transporters, isomerases, chromatin regulators, and glycoproteins, with hydrolases, oxidoreductases, and protein/DNA/RNA binding proteins being the most abundant. Subcellular localization predictions indicated distribution across various compartments, with relatively high representation in the plasma membrane, chloroplast, mitochondrion, and nucleus. In future work, we will perform pairwise yeast two-hybrid validation of HbHDA6 interactions with these 22 proteins to dissect their interactions, laying the foundation for identifying target proteins regulated by histone acetylation during secondary laticifer differentiation and providing a theoretical basis for constructing the molecular regulatory network of this process.

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