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Overexpression of a repressor MdMYB15L negatively regulates anthocyanin and cold tolerance in red-fleshed callus

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ABSTRACT

The cold-induced metabolic pathway and anthocyanin biosynthesis play important roles in plant growth. In this study, we identified a bHLH binding motif in the MdMYB15L protein using protein sequence analyses. Yeast two-hybrid and pull-down assays showed that MdMYB15L could interact with MdbHLH33. Overexpressing *MdMYB15L* in red-fleshed callus inhibited the expression of *MdCBF2* and resulted in reduced cold tolerance but did not affect anthocyanin levels. Chip-PCR and EMSA analysis showed that MdMYB15L could bind the type II cis-acting element found in the *MdCBF2* promoter. Overexpressing *MdMYB15L* in red-fleshed callus overexpressing *MdbHLH33* also reduced cold tolerance and reduced MdbHLH33-induced anthocyanin biosynthesis. Knocking out the bHLH binding sequence of MdMYB15L (LBSMdMYB15L) prevented LBSMdMYB15L from interacting with MdbHLH33. Overexpressing LBS*MdMYB15L* in red-fleshed callus overexpressing *MdbHLH33* also reduced cold tolerance and reduced MdbHLH33-induced anthocyanin biosynthesis. Together, these results suggested that an apple repressor MdMYB15L might play a key role in the cold signaling and anthocyanin metabolic pathways.

1. Introduction

Plants have evolved a metabolic regulatory network to respond to cold stress that affects plant growth [1,2]. This network regulates both physiological changes but also biochemical and molecular changes [3,4]. Anthocyanin, as a secondary metabolite, functions in cold stress resistance.

The ICE1-CBF-COR metabolic pathway is important in the regulation of cold tolerance in *Arabidopsis thaliana* [5,6]. Members of the CBF or DREB transcription factor families can bind the C-repeat/dehydration-responsive element (CRT/DRE) found in the promoter of *COR* genes [7–10]. AtICE1, a MYC type bHLH transcription factor, specifically binds to the MYC recognition site in the *AtCBF3* promoter and initiates the expression of *AtCBF3* during cold stress [11]. VvICE1 and MdClbHLH1 in grape and apple were found to be similar to AtICE1 [12,13]. In anthocyanin biosynthesis, the structural genes *ANS* and *UFGT* play key roles in regulating anthocyanin accumulation [14,15] and their transcription is controlled by an interactive MYB-bHLH-WD40 complex [16–18].

MdbHLH3 and MdbHLH33 promote anthocyanin accumulation with the former responding to low temperature and the latter promoting *MdCBF2* expression to increase cold tolerance [19,20]. Conversely, AtMYB15 is a repressor of *CBF* genes and may, therefore, reduce cold tolerance [21]. However, the relationship between *CBF*-induced cold tolerance and anthocyanin accumulation regulated by the MBW complex in apple remains unclear. In our study, a repressor MdMYB15L was identified that could inhibit the expression of *MdCBF2* by binding its promoter and reducing cold tolerance in transgenic red-fleshed callus. Furthermore, MdMYB15L could weaken MdbHLH33-induced anthocyanin accumulation by interacting with MdbHLH33.

Abbreviations: ANS, anthocyanin synthase; UFGT, UDP-glucose:flavonoid 3-glucosyltransferase; GFP, green fluorescent protein; GST, glutathione S-transferase; His, histidine; Anti, antibody; MS, Murashige and Skoog; AD, activation domain; BD, binding domain; CDS, coding DNA sequence; bHLH, basic helix-loop-helix; EMSA, Electrophoretic mobility shift assays; Chip-PCR, Chromatin immunoprecipitation-Polymerase Chain Reaction; ICE, Inducer of CBF expression; CBF, C-repeat-binding factor; Y2H, Yeast two hybrid.

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2. Materials and methods

2.1. Plant materials

The red-fleshed callus was cultured using a published procedure [22]. The calli used for genetic transformations and other analyses were all grown for 2 w. They were cultured in MS + 1 mg/l 6-benzylaminopurine + 0.3 mg/l 1-naphthylacetic acid.

2.2. Anthocyanin extraction and absorbance measurements

Anthocyanin extraction was carried out according to Ji et al. [22] with minor modifications. Approximately 1 g of plant material was ground into a powder in liquid nitrogen and subjected to extraction in 40 ml of 1% (v/v) HCl-methanol at 4 °C in the dark for 24 h. After centrifugation at 12,000 rpm min $^{-1}$ for 10 min, the absorbance of the supernatant was measured at 530 nm using a UV spectrophotometer.

2.3. Total RNA extraction and qRT-PCR

RNA was extracted according to the method of Xu et al. [23] with reagents purchased from TransGen Biotech (Beijing, China). The Bole CFX96 system (Bio-Rad) was used for qRT-PCR analysis according to the manufacturer's instructions and each sample was repeated three times. *MdActin* was used as the internal control and was amplified simultaneously for each gene amplification. Ct values were read under default conditions and the $2^{-\Delta\Delta CT}$ method was used for data analysis [24].

2.4. Knockout of the MdMYB15L bHLH binding sequence by overlap PCR

The bHLH binding sequence of *MdMYB15L* (EAIIKLHEMLGNRWSAIAAR) was removed using the overlap PCR technique. The specific description is shown in Supplementary Fig. S1. The primers were designed as follows:

F1: 5′- ATGGGGAGAGCTCCTTGCT -3′
R1: 5′- CGTCCTGGTAACTCTTCTTCTCTTGTGAAGTTT -3′
F2: 5′- GAGAAGAAGAGTTACCAGGACGCACC -3′
R2: 5′- TCAAAATTCTGGTAATTCTGGC -3′

The *MdMYB15L-1* fragment was amplified using the F1 and R1 primers. The *MdMYB15L-2* fragment was amplified using the F2 and R2 primers. Equal amounts of *MdMYB15L-1* and *MdMYB15L-2* were then mixed and used as a template for amplification with the F1 and R2 primers. The amplified fragment was identified as *MdMYB15L* in which the bHLH binding sequence was removed and was, therefore, named the lost bHLH binding sequence of *MdMYB15L* (LBS*MdMYB15L*).

2.5. Yeast two-hybrid (Y2H) and pull-down analyses

The experiments were conducted according to the methods of Xu et al. [23]. The recombinant plasmids MdbHLH33-AD, MdMYB15L-BD, and LBSMYB15L-BD are shown in Supplementary Fig. S2 E and F. These were transformed into Y2H cells according to the instructions of the Yeastmaker™ Yeast Transformation System 2 Kit (Clontech). Initially, cells were cultured in selective media lacking Leu and Trp (−Leu/Trp, Clontech) and putative transformants were transferred to media lacking Ade, His, Leu, and Trp (-Ade/-His/-Leu/-Trp, Clontech). The substrate X-α-gal was added to media (-Ade/-His/-Leu/-Trp) for the detection of β-galactosidase activity. The recombinant plasmids MdMYB15L-His and MdbHLH33-GST are shown in Supplementary Fig. S2 C and D. The mixed proteins were column-purified using the His tag before the purified mixed proteins were detected by western blotting with anti-HIS or anti-GST antibodies (Abmart).

2.6. Red-fleshed callus transformation

The recombinant plasmids 35S:*MdMYB15L*-GFP, 35S:LBS*MdMYB15L*-GFP, and 35S:*MdbHLH33*-GFP are shown in Supplementary Fig. S2 A, B. These vectors were transformed into *Agrobacterium tumefaciens* LBA4404 that was then used to infect red-fleshed callus for 20 min followed by co-culturing on MS solid medium in the dark for 24–48 h at 24 °C. The co-cultured calli were then transferred to screening medium containing 250 mg/L carbenicillin and 50 mg/L kanamycin (Solarbio, Beijing, China).

2.7. Electrophoretic mobility shift assays (EMSA)

EMSA experiments were performed according to the manufacturer's instructions using an EMSA kit (Pierce, Rockford, IL, USA). *MdCBF2* promoter sequences were used as biotin-labeled probes (Sangon Biotech, Shanghai, China). First, 50 ng recombinant MdMYB15L-His was purified for the binding reactions. The binding reactions were conducted in volumes of 20 μl containing 4 mg poly(dI-dC), 17% glycerol, 25 mM HEPES-KOH (pH 7.5), 0.1 mM ethylenediaminetetraacetic acid, 1 mM DTT, 100 mM KCl, 1 pmol labeled probe, competitor DNA (25, 50, or 100 pmol), and 50 ng purified protein. These reaction mixtures were incubated at room temperature for 30 min. After a pre-run in 0.5% TBE buffer at 100 V for 1 h samples were electrophoresed on 6% acrylamide gels containing 0.5% TBE buffer and 3.6% glycerol for 2 h at 4 °C. Subsequently, the DNA was transferred onto the nylon membranes and chemiluminescent nucleic acid detection was used to detect the signal.

2.8. Chromatin immunoprecipitation (Chip-PCR) analysis

Chip-PCR experiments were performed according to the method of Xu et al. [23]. Cross-linking, removal of cross-linking, immunoprecipitation, and elution were performed using a Chip Kit (Upstate, Lake Placid, NY, USA) and GFP antibody (Abmart, Shanghai, China). PCR was used to detect the amount of immunoprecipitated chromatin. The experiment was repeated three times.

2.9. Data analysis, phylogenetic tree construction, and protein sequence alignment

All the results were based on the average of three parallel experiments. The statistical analysis was performed with appropriate methods using Duncan's new multiple range test. The significance tests are shown as i, ii, iii, and iv. Different lowercase letters on the chart columns indicate significant differences ($P < 0.05$). MEGA 5.0 was used to construct the phylogenetic tree and Clustal X was used to analyze related protein sequences.

3. Results

3.1. MdMYB15L could interact with MdbHLH33 via its own bHLH binding sequence

A bHLH binding sequence was found in the MdMYB15L protein sequence (Fig. S3). Interactive analyses between MdMYB15L and MdbHLH33 are shown in Fig. 1A. Y2H showed that the yeast strains co-transformed with MdMYB15L-BD and MdbHLH33-AD grew successfully on −T-L, -T-L-H-A, and -T-L-H-A selective media

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