

Identification and expression analysis of the bHLH gene family in pecan (*Carya illinoensis*)

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Abstract: **【Objective】** *Carya illinoensis* is a significant nut tree species that holds commercial importance, as well as an important timber and greening species, with comprehensive economic, ecological and social benefits. However, *C. illinoensis* is vulnerable to various biotic and abiotic stresses during its long growth period. Although bHLH transcription factors have been extensively studied in plant growth and metabolism, there are limited reports of studies on their response to abiotic stresses, particularly the response to iron deficiency stress. Therefore, in this study, we used bioinformatics methods to identify the bHLH gene family in *C. illinoensis* at a genome-wide level and then analyzed various aspects of the bHLHs, including gene structure, conserved motifs, domains, and cis-acting elements, to provide a more comprehensive understanding of the characteristics of the bHLHs. Through expression analysis in different tissues, we focused on genes that had high levels of expression in diverse tissues. In order to reveal the potential functions of these candidate genes, we performed qRT-PCR test under stress treatments and analyzed the molecular properties of the candidate genes by subcellular localization, transcriptional activity, yeast two-hybrid (Y2H), bimolecular fluorescence complementation (BiFC) and luciferase complementation imaging (LCI) assays. These results are helpful to explore the regulation mechanism of *C. illinoensis* on iron deficiency stress, further improve *C. illinoensis* stress resistance, and give full play to the potential of *C. illinoensis* in ecological and economic value. **【Method】** The bHLH gene family was identified and then categorized into 18 distinct subfamilies. Furthermore, in order to comprehend the characteristics of bHLHs genes, we conducted an analysis of the motif, gene structure, and domains of the members of the bHLH family. Additionally, the evolutionary rate of the bHLH gene family was examined using Ka/Ks and multiple sequence comparison analysis. Moreover, analysis of cis-acting elements in promoter was employed to forecast the expression and probable roles of bHLHs genes. Subsequently, heat maps were generated to analyze the transcriptome data of various tissues, aiming to get deeper insights into the expression patterns of bHLH in *C. illinoensis* stems, leaves, peel and embryo. Following that, the candidate genes were confirmed by qRT-PCR under iron deficiency stress. To further understand the molecular properties of the candidate genes, we performed transcriptional activity analysis. Based on the online website prediction and existing literature reports, we verified the interacting protein of the candidate genes by Y2H, BiFC and LCI experiments. **【Result】** A total of 156 bHLHs were identified in *C. illinoensis* and named *CibHLH1-CibHLH156* according to their gene scaffold positions. According to both evolutionary tree branches and classification of *Arabidopsis thaliana* evolutionary tree in the literature, the 156 bHLHs were categorized into 18 subfamilies. These analyses of gene structure, conserved motifs, domains and cis-acting elements illustrated that the bHLH gene family had undergone selection of species for purification, resulting

ng in the gradual development of distinct domain patterns. The promoter cis-element analysis results revealed that the 156 CibHLHs contained 35 light-responsive elements, 14 hormone-responsive elements, 11 biotic and abiotic stress-responsive elements, and 9 growth and development-responsive elements. The study results indicated that the Ka/Ks ratio for all homologous gene pairs was significantly less than 1, suggesting that the bHLH gene family had undergone purifying selection and exhibited a high degree of conservation. By analyzing the expression patterns of CibHLHs in different tissues, we found that *CibHLH128* and *CibHLH152* were highly expressed in four tissues. The results of qRT-PCR demonstrated that under iron deficiency treatment, *CibHLH128* and *CibHLH152* had significant expression differences in leaves and roots. These facts suggested that the *CibHLH128*-N and *CibHLH152*-N regions possess transcriptional activation activity, whereas the *CibHLH128*-C and *CibHLH152*-C regions lack transcriptional activity. Transcriptional activity analysis confirmed the interactions of *CibHLH128*//*152* and *CibHLH36* within plant. Y2H, BiFC and LCI experiments demonstrated that both *CibHLH128* and *CibHLH152* interact with the *CibHLH36* protein. 【Conclusion】 To sum up, this study carried out the whole genome identification of the *C. illinoensis* gene family. 156 CibHLHs were identified from *C. illinoensis* and divided into 18 subfamilies according to the phylogenetic relationship. Molecular physicochemical analysis and protein subcellular localization prediction of 156 bHLH genes were conducted through online websites. We found that CibHLH proteins have basic and helix-loop-helix domains and are both located in the nucleus. Gene structure analysis showed that the same subfamily genes had similar gene structure, whereas different subfamily genes showed differences. Evolutionary rate analysis and multiple sequence alignment indicated that bHLH family genes had undergone purifying selection during species evolution. Promoter cis acting element analysis revealed that CibHLH gene family members contain an enormous number of cis-acting elements related to abiotic stresses, such as MYB/MBS in response to drought stress, LTR in reaction to low temperature, STRE responded to Stress, etc. Combined with qRT-PCR experiments, it was found that *CibHLH128* and *CibHLH152* had obvious response under iron deficiency condition. Therefore, these two transcription factors were cloned from *C. illinoensis*, and their localization and transcriptional activity were investigated through subcellular localization and transcription self-activation experiments. Yeast Two-hybrid and Luciferase Complementation Assay were used to verify the interacting proteins, which further investigating the roles of *CibHLH128* and *CibHLH152* in iron homeostasis regulation. This provides a basis for in-depth research on the regulatory mechanism of *C. illinoensis* in response to iron deficiency stress and for molecular breeding of *C. illinoensis* for stress resistance.