

ZbERF6 and ZbbHLH2: Novel regulators of ZbIDI-mediated monoterpenoid synthesis in *Zanthoxylum bungeanum*

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Abstract: Monoterpenoid compounds give *Zanthoxylum bungeanum* its unique aroma and affect its sensory quality. These volatile substances not only directly affect the fragrance of the peel, but also impact human health. It is necessary to investigate the metabolic regulatory network underlying monoterpenoid biosynthesis and accumulation to encourage the cultivation of *Z. bungeanum* germplasm with strong or specific fragrances. This transcriptomics and bioinformatics study identifies two new transcription factors, ZbERF6 and ZbbHLH2, as positive regulators of monoterpenoid biosynthesis in *Z. bungeanum* peel and elucidates the primary molecular mechanisms. Overexpression of ZbERF6, ZbbHLH2, and ZbIDI (the gene expressing isoprene pyrophosphate isomerase) induced monoterpenoids accumulation in *Z. bungeanum* peels and callus, and tobacco. Dual-luciferase reporter, yeast one-hybrid, and electrophoretic mobility shift assays all indicated that ZbERF6 and ZbbHLH2 directly interact with the monoterpenoids promoter (isoprene pyrophosphate isomerase: ZbIDI) to activate its expression. This directs secondary metabolism towards the 2-C-methyl-derythritol 4-phosphate pathway, resulting in accumulation of monoterpenoids. The direct interaction between the ZbERF6 and ZbbHLH2 proteins that activates the expression of the isoprene pyrophosphate isomerase ZbIDI was confirmed by bimolecular fluorescence complementarity assay and co-immunoprecipitation with western blotting in *Nicotiana benthamiana* epidermal cells. This interaction promotes binding to the ZbIDI promoter, which, in turn, elevates expression of monoterpenoid biosynthesis genes. These findings help to elucidate the regulatory mechanisms underlying monoterpenoid accumulation and offer insights for improving the quality of *Z. bungeanum* peel.

Keywords: *Zanthoxylum bungeanum*, monoterpenoids biosynthesis, ZbIDI, ZbERF6, ZbbHLH2, transcription regulation, MEP pathway.

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