Cloning of the HDR Gene from Masson Pine and Its Response to Drought and Salt Stress Post-print

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Abstract

Drought and soil salinization are critical factors constraining the sustainable development of forestry. When plants suffer from biotic or abiotic stress, they release volatile compounds such as terpenes in their leaves. 1-hydroxy-2-methyl-2-E-butenyl-4-diphosphate reductase (HDR) is the terminal active enzyme of the MEP pathway, playing a crucial role in providing terpenoid precursors and serving as a primary rate-limiting factor. To investigate whether the HDR gene from Masson pine (Pinus massoniana) participates in stress responses under drought and salt conditions, this study cloned the open reading frame of the Masson pine HDR gene and preliminarily analyzed its bioinformatic characteristics, tissue-specific expression levels, and functional properties. The results demonstrated: (1) The PmHDR coding region spans 1,458 bp, encoding a protein of 485 amino acids that contains the core sequence of the LytB/IspH gene superfamily and the PLN02821 multifunctional domain, classifying it as a member of the 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase family. (2) PmHDR exhibits weak codon usage bias, preferring codons ending in A/U, with tobacco, Arabidopsis thaliana, and Saccharomyces cerevisiae being suitable heterologous expression hosts. (3) qRT-PCR analysis revealed that PmHDR expression is highest in old leaves of Masson pine, followed by young leaves, young stems, and old stems, with the lowest expression in roots. (4) The gene expression vector pBI121-PmHDR was constructed and transformed into Arabidopsis thaliana, with transgenic Arabidopsis exhibiting enhanced stress resistance to drought and salt stress. These findings indicate that PmHDR is involved in the response and regulation of plant drought and salt stress, providing theoretical support for stress-resistance breeding in Masson pine.

Full Text

Cloning of the HDR Gene in *Pinus massoniana* and Its Response to Drought and Salt Stress

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Abstract

Drought and soil salinization are critical constraints on the sustainable development of forestry. When plants encounter biotic or abiotic stress, they release volatile compounds such as terpenoids from their leaves. 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR) is the terminal active enzyme in the MEP pathway, playing a crucial role in providing terpenoid precursors and acting as a major rate-limiting factor. To investigate whether the HDR gene in *Pinus massoniana* participates in stress responses under drought and salt conditions, this study cloned the open reading frame of the *PmHDR* gene and conducted preliminary analyses of its bioinformatics characteristics, tissue-specific expression patterns, and functional properties.

The results showed that: (1) The coding region of *PmHDR* is 1,458 bp, encoding 485 amino acids. The encoded protein contains the core sequence of the LytB/IspH gene superfamily and the PLN02821 multifunctional domain, placing it within the 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase family. (2) *PmHDR* exhibits weak codon usage bias, preferring codons ending in A/U, with tobacco, *Arabidopsis*, and *Saccharomyces cerevisiae* being suitable heterologous expression hosts. (3) qRT-PCR analysis revealed that *PmHDR* expression was highest in old needles, followed by young needles, young stems, and old stems, with the lowest expression in roots. (4) The expression vector pBI121-*PmHDR* was constructed and transformed into *Arabidopsis thaliana*, and the transgenic plants demonstrated enhanced tolerance to drought and salt stress. These findings indicate that *PmHDR* is involved in plant responses to drought and salt stress, providing theoretical support for stress-resistance breeding in *Pinus massoniana*.

Keywords: *Pinus massoniana*, *PmHDR*, terpenoids, gene cloning, stress response

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Introduction

Pinus massoniana is a tall tree species in the Pinaceae family, an ancient native conifer in China and one of the country's characteristic fast-growing species. It

is widely distributed across 17 provinces, autonomous regions, and municipalities south of the Qinling-Huaihe line and east of the Yunnan-Guizhou Plateau, representing the most extensively distributed coniferous forest species in China (Li et al., 2008). As global warming intensifies, water scarcity has become increasingly severe, with large-scale persistent drought and soil salinization posing serious threats to agricultural and forestry production in China. These stresses limit plant growth, alter organic compound composition, and affect both yield and quality (Pichler & Oberhuber, 2007; Zhang et al., 2021). Consequently, enhancing tree resistance to drought and salt stress has become a primary objective in forest cultivation. As an important timber, resin-producing, and pioneer species for afforestation on barren hills (Xu & Ding, 2006), *P. massoniana* possesses strong stress resistance, yet severe drought significantly inhibits seedling height and biomass accumulation, severely impacting its growth and reproduction (Quan & Ding, 2017). Therefore, investigating the mechanisms underlying *P. massoniana*'s adaptation to drought and salt stress is crucial.

When plants experience biotic or abiotic stress, they release volatile substances from leaves, including terpenes and fatty acid derivatives, which play direct and indirect roles in signal regulation (Laothawornkitkul et al., 2008). These compounds promptly activate the synthesis and signal transduction pathways of plant hormones such as abscisic acid (ABA), methyl jasmonate (MeJA), and brassinosteroids (BR), regulating relevant gene expression to mitigate or adapt to stressful environments (Yan et al., 2007; Obata & Fernie, 2012). Terpenoids show particularly strong responses to drought stress and play important roles in plant resistance to adverse conditions (Rastogi et al., 2019). As the most abundant and structurally diverse class of plant secondary metabolites, terpenoids are built from isoprene units with common precursors isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Two biosynthetic pathways produce IPP and DMAPP in vivo: the mevalonate (MVA) pathway and the methylerythritol phosphate (MEP) pathway (Chen, 2018). The MVA pathway primarily participates in sesquiterpene synthesis, while the MEP pathway is mainly involved in monoterpene, diterpene, and carotenoid synthesis (Rohmer, 1999). 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR) is the terminal active enzyme in the MEP pathway, catalyzing the reduction of 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate (HMBPP) to generate a 5:1 mixture of IPP and DMAPP, thereby providing terpenoid precursors and serving as a major rate-limiting enzyme (Wang et al., 2014).

Early studies found that the HDR gene limits the MEP pathway in *Escherichia coli* (Cunningham et al., 2000). HDR transcript levels increased significantly during tomato fruit ripening and *Arabidopsis* seedling de-etiolation, processes that produce large amounts of carotenoids, suggesting a relationship between HDR expression and carotenoid accumulation (Botella-Pavía et al., 2004). Research on ginkgo (*Ginkgo biloba*) showed that HDR-transformed plants had significantly higher total lactone content compared to wild-type plants (Zhang et al., 2008). Studies on *Rauvolfia verticillata* found that elicitors such as MeJA, acetylsalicylic acid (ASA), ABA, and UV light could induce upregulation of

HDR gene transcription (Chen et al., 2010). In *Dendrobium officinale*, HDR gene expression was regulated by salicylic acid (SA) and ABA but not by MeJA (Wu et al., 2015). These studies collectively demonstrate that the HDR gene plays an important regulatory role in the MEP pathway, is closely related to the synthesis of terpenoids such as carotenoids and lactones, and is induced by plant hormones like ABA and SA that regulate abiotic stress responses, suggesting its potential involvement in plant responses to abiotic stress.

In *P. massoniana*, the upstream MEP pathway genes *PmDXS* (encoding 1-deoxy-D-xylulose-5-phosphate synthase) and *PmDXR* (encoding 1-deoxy-D-xylulose-5-phosphate reductoisomerase) have been successfully cloned and functionally analyzed. Studies on *PmDXS* showed that after treatment with six types of abiotic stresses or hormones including mechanical wounding, 15% osmotic agent, H_2O_2, ethephon, MeJA, and SA, *PmDXS* expression was upregulated within a short period. Additionally, under abiotic stress conditions including 400 $mmol \cdot L^{-1}$ NaCl, 800 $mmol \cdot L^{-1}$ D-mannitol, pH 5.0, and pH 9.0, the recombinant strain TransB/pET28a-PmDXS showed significantly better growth than the control, indicating a positive regulatory effect of *PmDXS* under drought and salt stress (Li, 2021). These results demonstrate that *PmDXS* participates in abiotic stress responses and plays a role in *P. massoniana*'s stress adaptation. DXR catalyzes the second step of the MEP pathway and is a key rate-limiting enzyme. Research on *PmDXR* showed that compared to wild-type *Arabidopsis*, *PmDXR* transgenic *Arabidopsis* exhibited less growth inhibition under various concentrations of NaCl, SA, MeJA, and D-mannitol stress (Zhu, 2021), indicating that *PmDXR* also participates in *P. massoniana*'s abiotic stress responses. Therefore, as the gene encoding the terminal rate-limiting enzyme HDR in the MEP pathway, *PmHDR* is highly likely to be involved in *P. massoniana*'s response to abiotic stresses such as drought and salt.

This study clones the *PmHDR* gene sequence, performs bioinformatics and tissue-specific expression analyses, constructs an expression vector for *Arabidopsis* transformation, and compares growth differences between transgenic and wild-type *Arabidopsis* under drought and salt stress to address: (1) the physicochemical properties, structure, gene family affiliation, and evolutionary relationships of PmHDR protein; (2) the tissue-specific expression pattern of *PmHDR*; and (3) whether *PmHDR* participates in stress responses under abiotic stresses such as drought and salt. These investigations aim to elucidate the potential biological functions of key enzyme genes in terpenoid biosynthesis in *P. massoniana* and provide references for stress-resistance breeding.

Materials and Methods

Plant Materials and Reagents

Two-year-old *P. massoniana* seedlings and *Arabidopsis thaliana* seeds were obtained from the Key Laboratory of Forest Genetics & Biotechnology of the

Ministry of Education (Nanjing Forestry University). *Escherichia coli* Trelief™ 5α competent cells and pClone007 Blunt vector were purchased from Beijing Tsingke Biotechnology Co., Ltd. *Agrobacterium tumefaciens* GV3101 competent cells were purchased from Shanghai Weidi Biotechnology Co., Ltd. Plant Total RNA Isolation Kit, Gel DNA Extraction Mini Kit, Plant DNA Isolation Kit, ChamQ SYBR Color qPCR Master Mix, and ClonExpress II One Step Cloning Kit were purchased from Nanjing Vazyme Biotech Co., Ltd. BamH I, Sac I, and QuickCut Buffer were purchased from Takara. PCR Master Mix and 1st Strand cDNA Synthesis SuperMix were purchased from Yeasen Biotechnology (Shanghai) Co., Ltd. DNA ladder, Loading buffer for Agarose Gels, and TAE Buffer were purchased from Shanghai Generay Biotech Co., Ltd. All primers were synthesized and sequenced by Nanjing Tsingke Biotechnology Co., Ltd. and Shanghai Jieli Biotechnology Co., Ltd.

Experimental Methods

1.2.1 Cloning of *PmHDR* Gene Total RNA was extracted from two-year-old *P. massoniana* using the FastPure® Plant Total RNA Isolation Kit (Vazyme) according to the manufacturer's instructions. RNA concentration and purity were measured using a Nanodrop One microvolume UV spectrophotometer, and RNA integrity was assessed by agarose gel electrophoresis. High-quality, intact RNA was reverse-transcribed into cDNA using the 1st Strand cDNA Synthesis SuperMix for qPCR and stored at -20°C.

Based on the homologous gene sequence from loblolly pine (*Pinus taeda*), specific primers for *PmHDR* were designed using DNAMAN software: *PmHDR*-F (5 -ATGCCTTCGAGCCTCAGCTTTGC-3) and *PmHDR*-R (5 -TACCGTCTGCAACGCCTCCTCAT-3). PCR amplification was performed using the reverse-transcribed *P. massoniana* cDNA as template in a 50 L reaction containing 20 L high-fidelity enzyme, 1 L each of forward and reverse primers, 2.5 L cDNA, and 25.5 L ddH_2O. The PCR program consisted of initial denaturation at 95°C for 3 min; 35 cycles of 95°C for 15 s, 60°C for 45 s, and 72°C for 1 min; and final extension at 72°C for 5 min. PCR products were detected by agarose gel electrophoresis, purified using the Gel DNA Extraction Kit, ligated into the pClone007 cloning vector, and transformed into *E. coli* Trelief™ 5α competent cells. Positive clones were selected by colony PCR and sent to Tsingke Biotechnology for sequencing.

1.2.2 Bioinformatics Analysis of *PmHDR* Online software tools were used for bioinformatics analysis of *PmHDR* and its encoded protein (Table 1). Homology alignment of PmHDR protein was performed using DNAMAN software, and a phylogenetic tree was constructed using the neighbor-joining method in MEGA 7.0.

1.2.3 Synonymous Codon Usage Bias Analysis of *PmHDR* Codon usage characteristics of *PmHDR* were calculated using CodonW 1.4.2 software,

including A3s, U3s, C3s, G3s, relative synonymous codon usage (RSCU), effective number of codons (ENC), codon adaptation index (CAI), frequency of optimal codons (FOP), GC content, and GC content at the third codon position (GC3s). Codon usage frequency was calculated using the CUSP online tool from EMBOSS. Genome-wide codon usage frequency data for *Arabidopsis thaliana*, tobacco (*Nicotiana tabacum*), *E. coli*, and *S. cerevisiae* were obtained from the Codon Usage Database (http://www.kazusa.or.jp/codon).

1.2.4 Expression Analysis of *PmHDR* Samples were collected from five tissues of two-year-old *P. massoniana*: roots, young needles, old needles, young stems, and old stems. Each sample was ground in liquid nitrogen, and total RNA was extracted. RNA concentration and quality were assessed by agarose gel electrophoresis and Nanodrop One. Qualified RNA was reverse-transcribed into cDNA, which was diluted to 10 ng· L^{-1} after concentration determination.

Based on the cloned *PmHDR* sequence, qPCR primers were designed using Primer 5.0: q-*PmHDR*-F (5 -AGAAATCGCAGAGCAGAA-3) and q-*PmHDR*-R (5 -CAACGCCTCCTCATCCTT-3). The *TUA* gene (GenBank: KM496535.1) served as the internal reference. Real-time fluorescence quantitative PCR was performed using SYBR Green in a 10 L reaction containing 1 L cDNA template, 0.2 L each of forward and reverse primers, 5 L SYBR Green Real-time PCR Master Mix (2×), and 3.6 L sterile water. qPCR was conducted on an ABI StepOne Plus system using a two-step program: 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 30 s. Three biological and three technical replicates were performed, followed by melting curve analysis. Data were analyzed using SPSS 26 software, and Duncan's multiple comparison test was used to analyze significant differences in expression among tissues.

1.2.5 Vector Construction The pBI121-GUS plasmid was used as the eukaryotic expression vector. Based on the sequenced *PmHDR* gene, recombinant primers were designed to remove the stop codon and include restriction sites: *PmHDR*-BamHI-F (5 -ACGGGGGACTCTAGAGGATCCATGCCTTCGAGCCTCAGCTT-3) and *PmHDR*-SacI-R (5 -CGATCGGGGAAATTCGAGCTCCCGTCTGCAACGCCTCCT-3). PCR amplification was performed using *P. massoniana* cDNA as template. The product was verified by agarose gel electrophoresis, gel-purified, and ligated into pBI121-GUS plasmid that had been double-digested with BamH I and Sac I. The ligation product was immediately transformed into *E. coli* Trelief™ 5α, plated on LB solid medium containing kanamycin, and incubated overnight. Positive clones were identified by PCR, cultured, and sent to Tsingke Biotechnology for sequencing. The recombinant plasmid pBI121-*PmHDR* was returned for further experiments.

1.2.6 Genetic Transformation of *Arabidopsis* and Stress Treatment The recombinant plasmid was transformed into *Agrobacterium* by freeze-thaw method, and positive colonies were used for floral dip transformation of *Arabidopsis*. Transgenic seeds were collected, sterilized sequentially with

75% ethanol and 20% sodium hypochlorite, and sown on MS solid medium containing 50 mg · mL^{-1} kanamycin for resistance screening. Vigorous seedlings were transplanted to nutrient soil for further cultivation. Genomic DNA was extracted from leaves of wild-type and transgenic *Arabidopsis* using the FastPure® Plant DNA Isolation Kit (Vazyme). PCR amplification using primers *PmHDR*-BamHI-F and *PmHDR*-SacI-R was performed to screen for *PmHDR* transgenic plants. After two rounds of screening, seeds were collected for subsequent experiments.

MS solid media containing 50 mmol · L^{-1} NaCl or 5, 10, 50, and 100 mmol · L^{-1} D-mannitol were prepared. Wild-type and transgenic *Arabidopsis* seeds were sterilized and sown on each medium (20 seeds per plate). After 10 days of culture, germination and growth were observed. Ten successfully germinated seedlings from each plate were selected, and radicle length was measured and recorded. Root length data were analyzed using RStudio software.

Results

2.1 Cloning and Sequence Alignment of *PmHDR*

The intermediate fragment of *PmHDR* was amplified with a length of 1,458 bp, consistent with agarose gel electrophoresis results (Figure 1 [Figure 1: see original paper]). Blastn analysis against NCBI revealed five plant gene sequences with >85% similarity to *PmHDR*: *Pinus densiflora*, *P. taeda*, *Picea glauca*, *P. sitchensis*, and *Larix kaempferi*. Notably, the IDS1 genes from *P. densiflora* and *P. taeda* showed >98% sequence similarity with *PmHDR*.

Blastp analysis of the PmHDR amino acid sequence showed highest similarity ($ $97%) with *P. densiflora* and *P. taeda*, and >78% similarity with *P. sitchensis*, *L. kaempferi*, *Ginkgo biloba*, *Morus notabilis*, *Macadamia integrifolia*, *Juglans regia*, and *Ananas comosus*, indicating that HDR is evolutionarily conserved. Amino acid sequence alignment of PmHDR with highly similar species using DNAMAN revealed four conserved cysteine residues that may participate in iron-sulfur bond formation during catalysis (Lu et al., 2008) (Figure 2 [Figure 2: see original paper]).

2.2 Bioinformatics Analysis of *PmHDR*

2.2.1 Primary Structure and Physicochemical Properties The PmHDR-encoded protein has a molecular formula of $C_{2415}H_{3844}N_{654}O_{744}S_{19}$, a theoretical molecular mass of 54.55 kDa, and a theoretical isoelectric point of 5.98. Composed of 485 amino acids, it contains 71 negatively charged and 65 positively charged residues. Lysine is the most abundant amino acid (9.3%), while cysteine is the least abundant (1.4%) (Figure 3A [Figure 3: see original paper]). The protein has an aliphatic index of 80.99 and a grand average of hydropathicity of -0.447, indicating hydrophilicity (Figure 3B). The instability coefficient of 28.98 suggests that the protein is stable.

2.2.2 Conserved Domain Prediction and Analysis Conserved domain prediction revealed that PmHDR contains the PLN02821 multifunctional domain from amino acid residues 60 to 483, belonging to the LytB/IspH gene superfamily (Accession: cl19123). Members of this family convert HMBPP to IPP and DMAPP, suggesting that PmHDR possesses HMBPP reductase activity (Figure 4A [Figure 4: see original paper]).

2.2.3 Subcellular Localization Prediction Online tools Cell-PLoc2.0 and IncLocator predicted with high reliability that the PmHDR-encoded protein localizes to chloroplasts.

2.2.4 Transmembrane Structure and Signal Peptide Analysis TMHMM Server v.2.0 predicted no transmembrane structures, with the entire protein located outside the membrane (Figure 4B). SignalP-5.0 Server analysis revealed no signal peptide, indicating that PmHDR is a non-secretory protein (Figure 4C).

2.2.5 Phosphorylation and Glycosylation Site Analysis NetPhos 3.1 Server predicted 47 phosphorylation sites: 23 serine (S), 20 threonine (T), and 4 tyrosine (Y) residues (Figure 4D). YinOYang 1.2 and NetNGlyc 1.0 Server predicted 4 O-glycosylation sites at positions 4(S), 34(S), 58(T), and 236(S) (Figure 4E), and 3 N-glycosylation sites at positions 129, 172, and 397 (Figure 4F).

2.2.6 Secondary Structure Analysis Secondary structure prediction revealed that the PmHDR-encoded protein contains 211 α-helices (43.51%), 164 random coils (33.81%), 78 extended strands (16.08%), and 32 β-turns (6.60%) (Figure 5 [Figure 5: see original paper]).

2.2.7 Tertiary Structure Prediction Homology modeling showed that the PmHDR-encoded protein shares 33% sequence similarity with the template c3dnfB [1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase] with 100% confidence (Figure 6 [Figure 6: see original paper]).

2.2.8 Phylogenetic Tree Construction A neighbor-joining phylogenetic tree based on PmHDR amino acid sequences clustered it with gymnosperms, showing highest homology with *P. densiflora* and *P. taeda* (Figure 7 [Figure 7: see original paper]).

2.3 Codon Usage Bias Analysis

2.3.1 GC, GC3s, ENC, CAI, and FOP Analysis CodonW 1.4.2 analysis of *PmHDR* yielded the following parameters: A3s = 0.400, U3s = 0.468, C3s = 0.272, G3s = 0.155, ENC = 47.77, CAI = 0.215, and FOP = 0.363. ENC values range from 20 to 61, with lower values indicating stronger codon bias; values

<35 represent significant bias (Mensah et al., 2019). The ENC value of 47.77 indicates weak codon usage bias in *PmHDR*. CAI and FOP values range from 0 to 1, with higher values indicating stronger bias and higher expression levels. The CAI of 0.215 and FOP of 0.363, both approaching 0, further confirm weak codon preference and potentially low expression levels. CUSP analysis revealed GC content and GC3s values of 39.92% and 34.77%, respectively, indicating a preference for A/U-ending codons.

2.3.2 Relative Synonymous Codon Usage Analysis Among the codons used in *PmHDR*, 28 had RSCU values >1.0, representing preferred codons (Table 2). Five codons—CCA, AGA, GGA, ACU, and CUU—showed RSCU values >2.0, indicating strong preference, with the arginine codon AGA having an extremely high RSCU of 2.70. In contrast, CCC, CCG (proline), and CGG (arginine) had RSCU values of 0, suggesting they may not participate in *PmHDR* translation. The predominance of A/U-ending codons among preferred codons further confirms weak codon bias.

2.3.3 Selection of Heterologous Expression Hosts Comparative analysis of *PmHDR* codon usage frequency with model organisms revealed minimal differences with plants: 16 and 14 codons showed significant frequency differences with *Arabidopsis* and tobacco, respectively, making both suitable heterologous expression hosts. In contrast, 24 codons differed significantly with *E. coli*, while only 14 differed with *S. cerevisiae*, indicating that yeast is more suitable than bacteria for microbial expression (Table 3).

2.4 Tissue-Specific Expression Analysis of *PmHDR*

qRT-PCR analysis of *PmHDR* expression in five tissues of adult *P. massoniana* showed lowest expression in roots and highest in old needles (122.2-fold higher than roots). Expression in young needles, young stems, and old stems was 21.2-, 6.5-, and 6.0-fold higher than in roots, respectively (Figure 8 [Figure 8: see original paper]).

2.5 Growth Observations of *Arabidopsis* Under Stress

Transgenic *Arabidopsis* seeds were screened on kanamycin-containing MS medium, and healthy seedlings were transferred to nutrient soil for 2–3 weeks before molecular identification (Figure 9 [Figure 9: see original paper]). Six of eight selected transgenic lines tested positive and were used for subsequent experiments.

Wild-type and transgenic *Arabidopsis* seeds were sown on MS medium containing D-mannitol (5, 10, 50, 100 mmol $\cdot$ L^{-1}) or NaCl (50 mmol $\cdot$ L^{-1}). After 10 days, growth was observed and radicle lengths were measured (Figure 10 [Figure 10: see original paper]). Under normal conditions, transgenic *Arabidopsis* showed slightly longer radicles than wild-type, though not significantly different.

Stress treatments inhibited growth to varying degrees: wild-type radicle length decreased by 35.49%, 39.26%, 53.59%, 77.08%, and 77.06% under 5, 10, 50, 100 mmol · L^{-1} D-mannitol and 50 mmol · L^{-1} NaCl, respectively, while transgenic plants showed reductions of only 11.03%, 7.40%, 21.88%, 59.09%, and 20.10%. Transgenic *Arabidopsis* maintained significantly longer radicles than wild-type under both D-mannitol and NaCl treatments, demonstrating enhanced stress tolerance (Figure 11 [Figure 11: see original paper]).

Discussion and Conclusion

Terpenoids are the most abundant and structurally diverse class of plant secondary metabolites, playing vital roles in plant growth, development, and stress resistance. HDR is the terminal active enzyme in the MEP pathway for terpenoid synthesis, providing precursors and serving as a major rate-limiting factor. To explore *P. massoniana*'s response to drought and salt stress, this study cloned and characterized *PmHDR*. Previous work has successfully cloned and analyzed the upstream MEP pathway genes *PmDXS* and *PmDXR*, both of which participate in abiotic stress responses (Li, 2021; Zhu, 2021).

The PmHDR protein likely promotes *P. massoniana* growth and stress responses. This study found that *PmHDR* spans 1,458 bp, encoding 485 amino acids containing the LytB/IspH superfamily core sequence and PLN02821 domain, confirming its role in terpenoid biosynthesis. Structural predictions revealed abundant α-helices and β-sheets forming a "cloverleaf" tertiary structure similar to other HDR proteins. Four conserved cysteine residues likely form iron-sulfur bonds at the catalytic center, participating in redox reactions (Liu et al., 2017).

Studies of HDR genes in different plants require species-specific analysis based on terpenoid types and functions. *PmHDR* was expressed in all tissues but at significantly higher levels in old needles, followed by young needles, stems, and roots—a pattern similar to *Dendrobium officinale* (Wu et al., 2015) and sweet potato (Wang et al., 2012). However, HDR expression was highest in flowers of *Rauvolfia verticillata* (Chen et al., 2010) and *Gentiana macrophylla* (Cen et al., 2015), while ginkgo and rubber showed highest expression in roots (Lu et al., 2008; Sando et al., 2008). These differences likely reflect variations in terpenoid types, quantities, and functions among species, necessitating tailored research approaches. For medicinal plants like *G. macrophylla*, *Artemisia argyi*, and *Salvia miltiorrhiza*, terpenoids are key active ingredients, while in grape (*Vitis vinifera*), terpenes are major contributors to aroma quality (Chen et al., 2023).

Codon bias analysis facilitates gene function studies and host selection for efficient heterologous expression. This systematic analysis revealed that *PmHDR* strongly prefers five codons (CCA, AGA, GGA, ACU, CUU) while rarely using CCC, CCG, and CGG. ENC, CAI, and FOP values collectively indicate weak codon bias and potentially low expression levels. The GC content (39.92%) and GC3s (34.77%) confirm a preference for A/T-ending codons, consistent with the

overall codon usage pattern in *P. massoniana* (Zhu et al., 2020).

Due to difficulties in establishing a *P. massoniana* transformation system, functional analysis relies on heterologous expression. Comparative analysis showed that yeast is more suitable than bacterial systems for *PmHDR* expression, while both *Arabidopsis* and tobacco are appropriate plant hosts, consistent with findings for *PmDXR* (Zhu et al., 2021). Therefore, this study used *Arabidopsis* as the transformation host to investigate *PmHDR* function by comparing phenotypic differences under stress.

Under normal conditions, transgenic *Arabidopsis* grew slightly better than wild-type, though not significantly. Under drought and salt stress, transgenic plants showed significantly less growth inhibition and enhanced stress tolerance, similar to results for upstream genes *PmDXS* (Li, 2021) and *PmDXR* (Zhu, 2021). Previous studies demonstrated that HDR expression in *R. verticillata* and *D. officinale* is regulated by ABA (Chen et al., 2010; Wu et al., 2015). As a stress hormone, ABA plays crucial roles in cold, drought, and salt resistance. ABA biosynthesis in higher plants occurs primarily via the carotenoid pathway (Yoshida et al., 2020), a downstream branch of the MEP pathway, suggesting that *PmHDR*'s involvement in drought and salt responses is likely ABA-mediated and may involve feedback regulation. These results provide candidate genes and references for molecular breeding of drought- and salt-resistant *P. massoniana*, though the specific regulatory mechanisms require further investigation.

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