

Genome-wide identification, molecular evolution, and expression analysis of the *bHLH* gene family in *Mesembryanthemum crystallinum*

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Abstract

The basic helix-loop-helix (*bHLH*) transcription factors (TFs) play crucial regulatory roles in plant responses to abiotic stress. However, their molecular evolution and functions in facultative crassulacean acid metabolism (CAM) plants, *Mesembryanthemum crystallinum* remain largely unexplored. In this study, 91 *bHLH* family members were systematically identified in *M. crystallinum* through genome-wide analysis. Phylogenetic and synteny analyses revealed that the evolution of this family was dominated by gene loss, with dispersed duplication serving as the primary driver for family expansion. Homologous gene pairs generally underwent purifying selection. *bHLH* members exhibited significant diversity in physicochemical properties, motif composition, and the distribution of stress-responsive *cis*-elements, suggesting their involvement in salt adaptation by integrating light signaling and hormonal pathways. Co-expression network and protein-protein interaction analyses further revealed that *bHLH* genes collaborate with glutathione metabolism, ion homeostasis, and salt-responsive hormonal signaling pathways (particularly abscisic acid and jasmonic acid), to form a multidimensional regulatory network. This study revealed the evolutionary trajectory of the *M. crystallinum bHLH* gene family and indicated the potential roles of *bHLH* under salt stress, providing some clues for salt-tolerance breeding of crops.

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Introduction

Mesembryanthemum crystallinum (2n = 18), commonly known as ice plant, belongs to the family *Aizoaceae*. Native to southern and eastern Africa, *M. crystallinum* is now cultivated globally, and possesses high nutritional value for humans due to its rich content of antioxidants, such as phenolic compounds^[1]. Studies indicate its nutritional components also contain unique bioactive substances like D-pinitol, myo-inositol, and flavonoids, which can regulate blood glucose levels, prevent metabolic diseases, and exert antioxidant effects^[2–4]. Furthermore, *M. crystallinum* is a facultative crassulacean acid metabolism (CAM) halophyte, requiring partial NaCl for optimal growth^[5]. Under salt or drought stress, its photosynthetic pathway can shift from C3 to facultative CAM, achieving a water-use efficiency 3–6 times higher than typical C3 plants^[6]. Additionally, the bladder cells on its stems and leaves effectively sequester Na ions from the cytoplasm by storing natural salts, significantly mitigating ionic toxicity and osmotic imbalance caused by salt stress, further supporting its adaptive survival in high-salinity environments^[5].

Currently, global salt-affected land area exceeds 900 million hectares (accounting for approximately 10.7% of the total global land area)^[7], posing a global environmental challenge that significantly inhibits seed germination, crop development, and yield formation^[8]. To cope with salt stress, plants have evolved multi-layered regulatory mechanisms^[9]. The core of these mechanisms lies in adaptive responses achieved through the regulation of gene

expression. In this process, transcription factors (TFs) play a central regulatory role at the transcriptional level by activating or repressing downstream target genes^[10].

In plants, basic helix-loop-helix (*bHLH*) proteins form a large TF family, second only to the MYB TF family in size^[11]. The *bHLH* family members are defined by the highly conserved *bHLH* domain, approximately 50–60 amino acids long, consisting of two functionally distinct sub-regions: an N-terminal basic region (10–15 amino acids) and a C-terminal helix-loop-helix (HLH) region (40–50 amino acids)^[12]. The basic region contains a conserved HER (His-Glu-Arg) motif that is required for binding to the E-box (CANNTG) element^[13]. Enriched in basic residues, the basic region directly recognizes and binds to DNA *cis*-elements like the E-box^[14] and G-box (CACGTG) target^[15]. The HLH region, composed of hydrophobic residues forming two amphipathic α -helices connected by a loop of variable length, mediates protein homo- or hetero-dimerization. This process is crucial for DNA-binding activity and functional specificity^[13].

Due to the crucial roles of *bHLH* genes in diverse physiological and biological processes, they have been extensively studied across various species. It has been reported that *bHLH* TFs play important regulatory roles in the growth and development of various reproductive tissues, including petals^[16], tapetum, and pollen^[17]. Simultaneously, some *bHLH* proteins participate in multiple signal transduction processes, such as light^[18] and hormone signaling^[19]. Notably, *bHLH* proteins are involved in responses to various abiotic stresses, such as drought^[20], salt stress^[21], low temperature^[22], and high-temperature stress^[23]. Research has shown that *bHLH* TFs can

act as transcriptional activators in abscisic acid (ABA) signaling, regulating stomatal aperture^[19,23], while also functioning as *in vivo* kinase substrates for ABA, acting as stomatal opening enhancers^[24]. As a facultative CAM plant, nocturnal stomatal opening and diurnal closure are key characteristics of the CAM pathway in the *M. crystallinum* under salt stress. Moreover, existing studies have confirmed a close relationship between the remodeling of the photosynthetic pathway under salt stress and ABA in *M. crystallinum*. Therefore, in-depth research on the *bHLH* gene family provides an important theoretical basis for deciphering the mechanisms underlying photosynthetic pathway remodeling and salt stress tolerance in *M. crystallinum*.

Currently, the functions of the *bHLH* gene family have been extensively characterized in plants^[25], they however remain uncharacterized in *M. crystallinum*. Hence, this study identified 91 *M. crystallinum* *bHLH* family genes and conducted comprehensive bioinformatics analyses. The present analytical framework encompassed comprehensive characterization of the *bHLH* family, including family member identification, physicochemical properties, and subcellular localization prediction, phylogenetic tree construction, domain and motif analysis, gene structure analysis, gene family evolutionary tracking (gain and loss), chromosomal localization, *Ka/Ks* calculation, synteny analysis, co-expression network analysis, functional annotation, promoter *cis*-acting element analysis, expression profiling, and protein-protein interaction network analysis. The results provide a crucial reference for evolution and functional elucidation of the *bHLH* gene family in *M. crystallinum*.

Materials and methods

Collection of genomic data, identification, physicochemical properties, and subcellular localization of the *bHLH* gene family

Data files required for family identification of *M. crystallinum* and its related species *Chenopodium pallidicaule*, *Fagopyrum tataricum*, *Hylocereus undatus*, *Chenopodium quinoa*, *Atriplex hortensis*, *Amaranthus cruentus*, *Simmondsia chinensis*, *Beta vulgaris*, and *Vitis vinifera* were downloaded from PlantGIR (<http://plantgir.cn>). Simultaneously, relevant data files for *Spinacia oleracea*, *Arabidopsis thaliana*, and *Oryza sativa* were downloaded from Phytozome (<https://phytozome.jgi.doe.gov>). Identification was performed using HMMER 3.2.1 with the *bHLH* domain profile (Pfam, PF00010) to search for members containing the *bHLH* domain within the complete protein sequences of the 13 species (default parameters), using an *E*-value threshold of $< 10^{-5}$. The domain of each protein sequence was double-verified using the CDD and PFAM databases within the InterProScan online tool (www.ebi.ac.uk/interpro/about/interproscan). Proteins containing the *bHLH* domain were retained as the final *bHLH* family members.

Based on the obtained *bHLH* genes, their isoelectric point (pI), molecular weight, and other molecular characteristics were predicted using the 'Protein parameter Calc (ProtParam-based)' plugin in TBtools-II software (version 2.210)^[26]. Subcellular localization of the *bHLH* proteins in *M. crystallinum* was predicted using Plant-mPLOC^[27], and WoLF PSORT^[28].

Analysis of the domain, motif, and exon-intron structure of *bHLH* family

The domain composition of all *bHLH* protein sequences was analyzed using the CDD and PFAM databases within the InterProScan online tool. Then, motifs within all *bHLH* protein sequences were analyzed using the MEME website (number of motifs set to 10, other parameters default)^[29]. Next, the exon-intron structure of

all *bHLH* protein sequences was analyzed using CFVisual software (version 2.1.5)^[30]. Finally, the results were visualized using iTOL (version 7, <https://itol.embl.de>)^[31], and CFVisual software, respectively.

Phylogenetic tree, inference of gene gain and loss, and genotype classification of the *bHLH* gene family

Sequences were aligned using MAFFT-7.526^[32]. A phylogenetic tree of the *bHLH* family for *M. crystallinum* and *A. thaliana* was constructed using IQ-TREE2^[33]. Subsequently, a phylogenetic tree including the C3 plant *M. crystallinum*, the C4 plant *A. cruentus*, and the CAM plant *H. undatus* was constructed (default parameters, bootstrap number = 1,000).

To investigate the quantitative evolutionary process of the *bHLH* family, Notung software (version 2.9.1.5) was used to infer the history of gain and loss of *bHLH* family members from the ancestor to the extant species among the 13 species mentioned above, including *M. crystallinum*, based on previous methodologies^[34,35]. Based on previous research^[15], the *bHLH* family members of the study species *M. crystallinum* and *A. thaliana* were classified according to the classification, phylogenetic relationships, and topological structure established for *A. thaliana*.

Chromosomal localization and synteny analysis of the *bHLH* gene family

Chromosomal localization of the *M. crystallinum* *bHLH* gene family was performed and visualized using TBtools-II software. The *Ka* (nonsynonymous substitution rate) and *Ks* (synonymous substitution rate) values for homologous gene pairs within *M. crystallinum* were calculated using TBtools-II. Intra-species synteny analysis for *M. crystallinum* was performed using MCScanX^[36]. Additionally, two separate four-species inter-species synteny analyses were conducted: one involving *M. crystallinum* and the three model species *A. thaliana*, *V. vinifera*, and *O. sativa*; and another involving *M. crystallinum* and the three related species *S. oleracea*, *F. tataricum*, and *H. undatus*. Syntenic plots of homologous genes were generated.

Analysis of the co-expression network, functional annotation, promoter *cis*-acting element, and protein-protein interactions network of *bHLH* genes

Python scripts were used to perform Pearson correlation coefficient (PCC) analysis between the identified *M. crystallinum* gene set and RNA-seq expression data (FPKM values) from various sample groups (including control and salt-treated samples). Data with correlation coefficients greater than 0.95 or less than -0.95 were extracted, and co-expression network diagrams were drawn for visualization.

Functional annotation (including description, GO, nr, pfam, swissprot, and trembl) of the *M. crystallinum* *bHLH* gene family was performed using genome annotation data downloaded from TBGR (www.tbgr.org.cn). To further explore the regulatory network of *bHLH* genes, the 1500 bp nucleotide sequence upstream of the start codon was extracted for each *M. crystallinum* *bHLH* gene as its promoter region. Prediction of promoter *cis*-acting elements was then conducted using the PlantCARE website (default parameters)^[37]. Finally, the results were visualized using CFVisual software.

Furthermore, the protein-protein interaction network profile for the proteins encoded by the *M. crystallinum* *bHLH* genes was analyzed using the STRING database (Version: 12.0)^[38] (parameter setting: confidence score > 0.7).

Plant growth conditions and expression analysis

RNA-seq expression data for *M. crystallinum* were obtained based on sample data measured in previous literature^[6,39,40], and

experimental measurements conducted by the present research group. This dataset includes FPKM expression values for all *M. crystallinum* genes across experimental replicates under different time points, varying salt concentration treatments, and different tissue types (root, stem, and leaf) respectively. Python scripts were then written to extract expression data for all *M. crystallinum* bHLH genes, and heatmaps were generated for visualization.

Ice plant seeds were imbibed at 4 °C for 2 d. They were then directly planted in soil (Klasmann-Deilmann 876) and grown at 23 ± 1 °C under 200 μmol·m⁻²·s⁻¹ white light with a 14 h/10 h day/night cycle. During the maintenance process of *M. crystallinum*, plants were irrigated with 0, 25, 50, 75, 100, and 125 mM NaCl every week. After 42 d, the second pair of mature leaves was collected for RNA sequencing and qRT-PCR examination.

For qRT-PCR analysis, total RNA was isolated using RNA Easy Fast Plant Tissue Kit (Tiangen, Tiangen Biotech [Beijing] Co. Ltd). First-strand cDNA was synthesized using the FastKing RT Kit (with gDNase, Tiangen, Tiangen Biotech [Beijing] Co. Ltd). qRT-PCR was performed using SuperReal PreMix Plus (SYBR Green, Tiangen, Tiangen Biotech [Beijing] Co. Ltd). One μL cDNA was used as a template. The qRT-PCR conditions were set as follows: 95 °C for 15 min, followed by 40 cycles of 95 °C at 10 s, and 60 °C at 30 s. FNR1 was used as an internal control. Primers used in the qRT-PCR are shown in [Supplementary Table S1](#). Three replicates were employed, and the relative expression levels were calculated using the 2^{-ΔΔCt} method. Statistical differences were determined by variance analysis. A *p*-value < 0.05 and an absolute fold change ≥ 2.0 signified differential expression.

Results

Identification and physicochemical properties of the bHLH gene family

A total of 91 non-redundant genes containing the typical bHLH domain were identified within the *M. crystallinum* genome. Comparative analysis with related species revealed 84, 82, 125, 90, 156, 153, 74, 94, and 90 bHLH genes identified in *S. oleracea*, *C. pallidicaule*, *F. tataricum*, *A. cruentus*, *C. quinoa*, *H. undatus*, *B. vulgaris*, *S. chinensis*, and *A. hortensis*, respectively. Additionally, 125, 115, and 158 bHLH genes were identified in the reference model species *O. sativa*, *V. vinifera*, and *A. thaliana*, respectively. Overall, the number of bHLH genes in *M. crystallinum* (91) falls within an intermediate range, being lower than quinoa, rice, *V. vinifera*, and *A. thaliana*, but higher than some related dicot species.

Further analysis showed that the 91 *M. crystallinum* bHLH proteins range in length from 135 to 702 amino acids. Their molecular weights range from 15.7 to 76.7 kDa, theoretical pI range from 4.47 to 9.8, instability indices range from 26.54 to 72.67, aliphatic indices range from 49.78 to 103.37, and grand average of hydropathicity (GRAVY) values range from -1.057 to -0.126, indicating a high degree of structural diversity. At the subfamily level, proteins belonging to subfamilies VII(a + b) generally possess longer amino acid sequences and larger molecular weights; conversely, subfamilies Ib(2) and IVc exhibit more similar characteristics. Notably, subfamily IIIf contains both the protein with the largest molecular weight (*Mc02G01577*) and the smallest molecular weight (*Mc03G02933*) within the entire genome, reflecting functional divergence within this subgroup ([Supplementary Table S2](#)).

Subcellular localization predictions indicated that the vast majority of *M. crystallinum* bHLH proteins are localized to the nucleus, consistent with their fundamental role as TFs. A small number of proteins were predicted to localize to the cell membrane,

chloroplasts, or mitochondria ([Supplementary Table S3](#)). These results not only demonstrate significant diversity in the physicochemical properties and structural composition of the *M. crystallinum* bHLH protein family but also provide important clues for subsequent functional classification and in-depth investigation of regulatory mechanisms.

Exon-intron structure, domain, and motif analysis of the bHLH gene family

The gene structure of *M. crystallinum* bHLH genes were analyzed to understand their diversity. Analysis revealed that the average length of *M. crystallinum* bHLH genes is approximately 4,352 bp, with a maximum length of 24,507 bp, and a minimum length of 673 bp. The number of introns inserted ranges from 0 to 11. It was observed that genes such as *Mc04G00185* and *Mc03G02400* [subfamily III(d+e)], *Mc02G01577* (subfamily IIIf), and *Mc05G00505* [subfamily VII(a+b)] are relatively long, while genes in subfamilies like Ib(2) and VIIIc(2) are relatively short. Untranslated regions (UTRs) flank the gene sequence in most subfamilies; however, subfamilies such as VIIIb, VIIIc(1), and VIIIc(2) lack UTRs. Subfamilies like Ia and Ib(2) are intron-poor (≤ three introns per gene), whereas subfamilies IVb, IVc, and IVd are intron-rich (> three introns per gene). Furthermore, genes within subfamilies III(d+e), VIIIb, and Orphans lack introns entirely. This indicates functional diversity and complexity among these genes ([Fig. 1](#), [Supplementary Table S4](#)).

The domain composition of the *M. crystallinum* bHLH gene family was also analyzed, revealing diversified domain structures. The results showed 16 distinct types of bHLH domains with high diversity. Protein members within the same subfamily generally contain similar types of bHLH domains, with a few exceptions [e.g., Ib(2), IIIf] ([Supplementary Fig. S1](#)).

Regarding motif composition, 10 motifs were identified among the core *M. crystallinum* bHLH proteins. Motif 1 and Motif 2 are ubiquitous across all sequences. Subfamily XII contains Motif 5; additionally, protein *Mc06G01080* [subfamily VII(a+b)], *Mc04G00224* [subfamily III(d+e)], and *Mc01G00955* (subfamily Vb) also contain Motif 5, while others do not. Most proteins in subfamilies III(d+e) and Orphans contain Motif 6 and Motif 9, which can also be found in a few other subfamilies, such as protein *Mc06G00350* in subfamily XIV. Overall, while motif composition varies, proteins belonging to the same evolutionary clade tend to be similar. Almost all *M. crystallinum* bHLH proteins lack some motifs, and duplication events are relatively infrequent ([Fig. 1](#)).

Phylogenetic tree and gain/loss in the bHLH gene family

To achieve a detailed classification of the *M. crystallinum* bHLH gene family, a phylogenetic tree including bHLH family genes from *M. crystallinum* and the model species *A. thaliana* were constructed ([Fig. 2](#)). The maximum likelihood (ML) phylogenetic tree, constructed based on 91 *M. crystallinum* bHLH protein sequences and *A. thaliana* reference sequences, revealed that the family can be classified into 27 subfamilies: Ia, Ib(1-2), II, III(a+c), IIIb, III(d+e), IIIf, IVa-d, Va, Vb, VII(a+b), VIIIa-b, VIIIc(1), VIIIc(2), IX, X, XI, XII, XIII, XIV, XV, and Orphans. This classification was based on topological structure and the evolutionary relationships of homologous genes in *A. thaliana* ([Fig. 2](#)). The phylogenetic tree showed that most minor subfamilies belonging to the same major subfamily clustered together on the same branch and were closely related. Furthermore, *M. crystallinum* genes belonging to subfamilies such as Ib(2), XII, and III(d+e) were relatively more numerous.

By reconciling the phylogenetic tree with the species tree, Notung software was used to infer the history of gene gain and loss events in the bHLH gene family across the 13 study species,

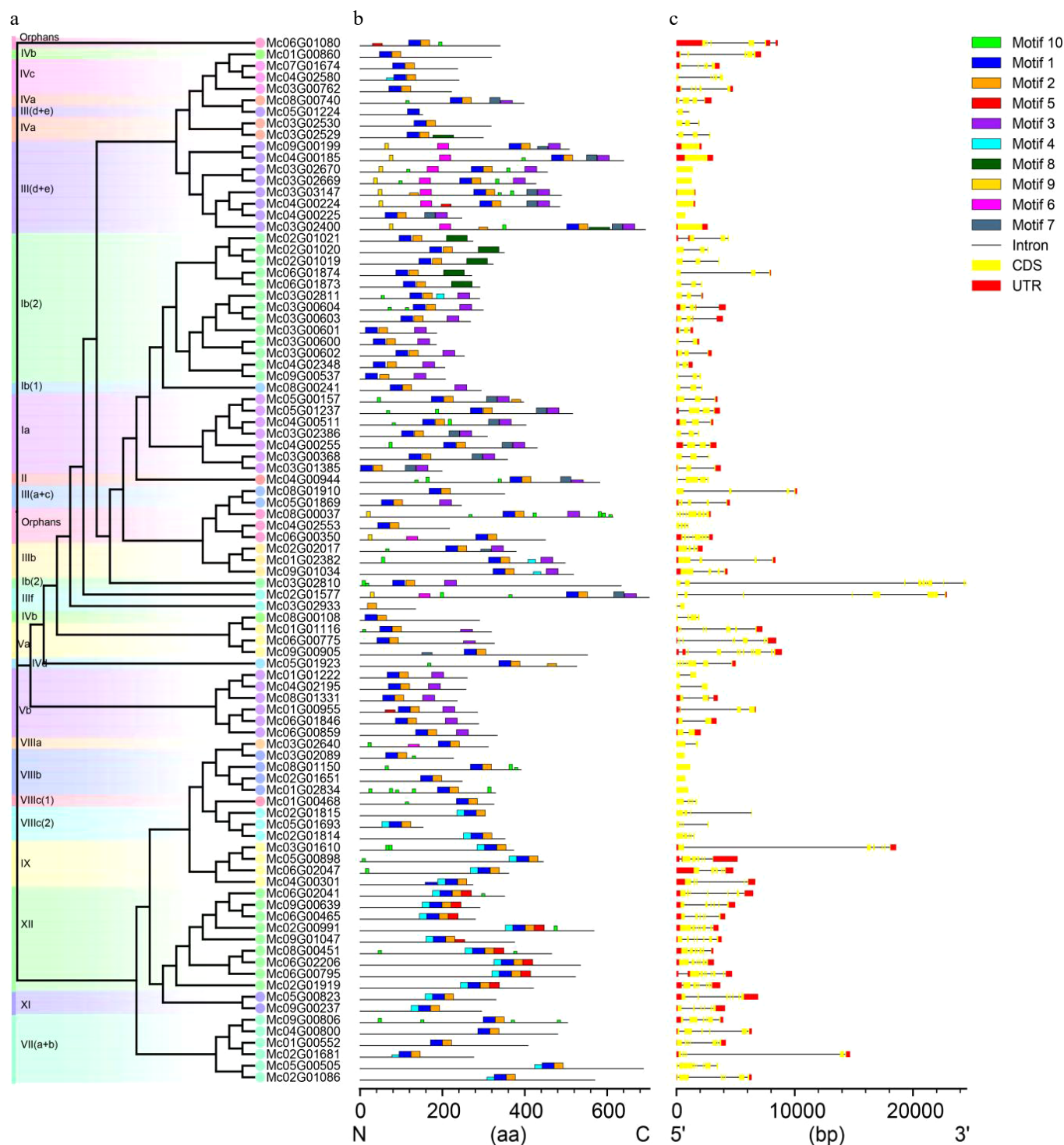


Fig. 1 Motif and gene structure analysis of the *M. crystallinum* bHLH gene family. (a) The phylogenetic tree of 91 *M. crystallinum* bHLH. (b) Conserved motif distribution of bHLH protein in *M. crystallinum*. (c) The genetic structure of the bHLH gene in *M. crystallinum*. The information of each gene structure is provided in [Supplementary Table S4](#). The red rectangle and the yellow rectangle represent the untranslated region (UTR), and the exon respectively. Introns are indicated by black lines. The scale is shown at the bottom of the chart.

including *M. crystallinum* (Fig. 3). The results indicated that the current number of bHLH genes in *M. crystallinum* (91) was formed from its ancestral genes through the loss of 66 genes and the duplication of seven genes. The evolutionary paths for other species were as follows. The common ancestor of the 13 study species contained 135 bHLH genes. It experienced the gain of 57 genes and the loss of 67 genes, forming the extant bHLH family in the monocot model species *O. sativa*. This ancestor experienced the gain of 68 genes

and the loss of 12 genes, resulting in an ancestral pool of 191 genes for the remaining 12 species. This ancestral pool then underwent the gain of 64 genes and the loss of 97 genes, forming the extant bHLH family size in the dicot model species *A. thaliana*. The ancestral pool of 191 genes for the 12 species experienced the gain of 21 genes and the loss of 28 genes, forming an ancestral pool of 184 genes for *V. vinifera*, and the other 10 species. This pool subsequently experienced the gain of 24 genes and the loss of 93 genes,

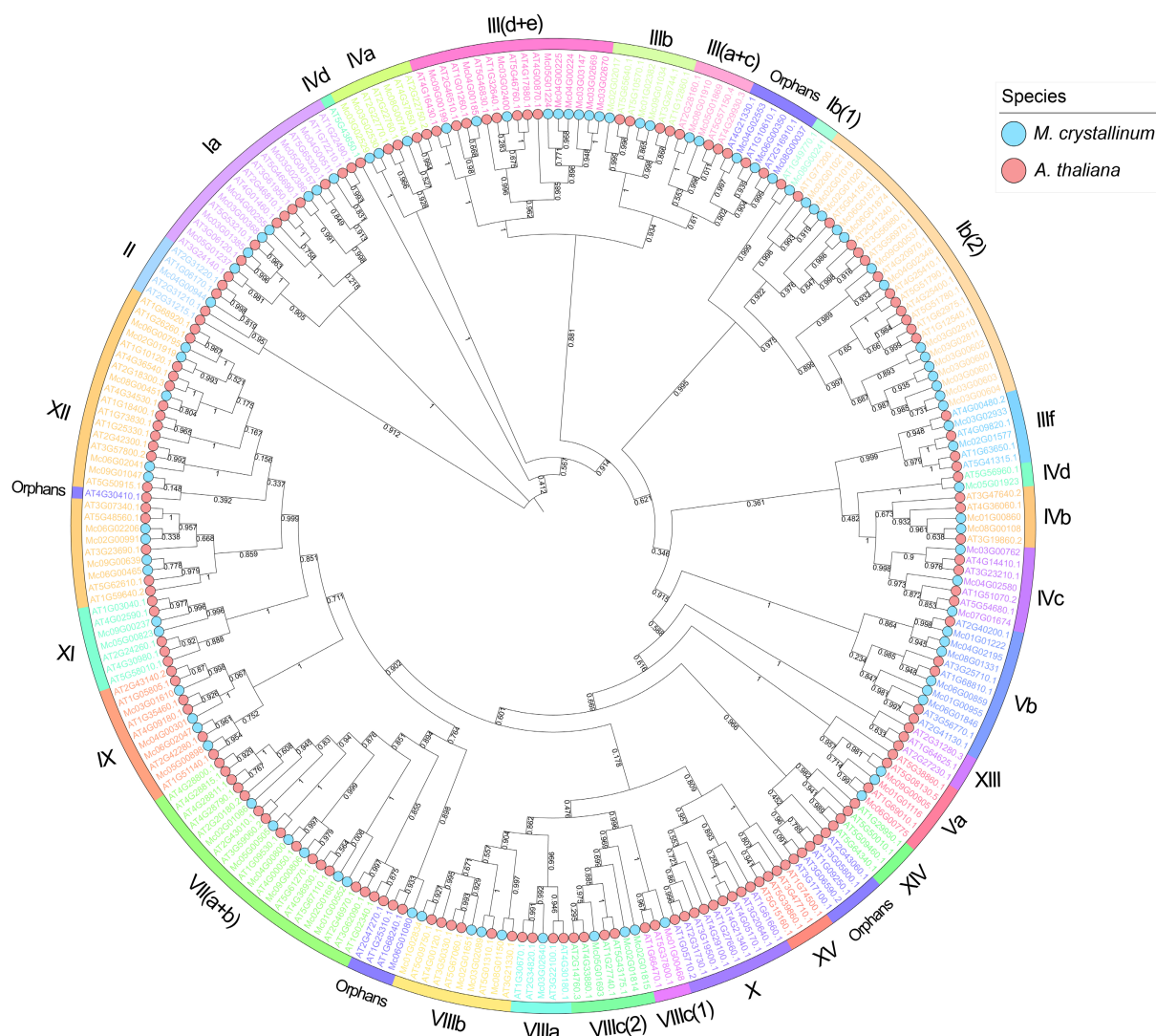


Fig. 2 Phylogenetic analysis of the *bHLH* gene family from *M. crystallinum* and *A. thaliana*. Phylogenetic trees were constructed using the *bHLH* protein sequences and different colors indicate different clades.

forming the extant *bHLH* family size in the genomic model species *V. vinifera*. Similarly, the extant *bHLH* family sizes in the related species of *M. crystallinum* were formed through gene loss and duplication events.

Through comparison, it was found that *A. thaliana* experienced the highest number of *bHLH* gene losses (97), while the related species *C. quinoa* experienced the fewest losses (10). Notably, among these 13 species, only *C. quinoa* had more gene duplications (23) than losses (10). In all other species, gene duplication was less frequent than gene loss. Overall, during the evolution of the *bHLH* family in the 13 study species, gene loss (1,046 events) significantly outnumbered gene duplication (733 events) (Fig. 3).

Synteny analysis of *bHLH* genes in *M. crystallinum* and related species

To investigate the chromosomal distribution characteristics of the *M. crystallinum* *bHLH* gene family, chromosomal localization of all 91 *bHLH* genes in *M. crystallinum* and their orthologs in three closely related species were performed (Fig. 4). The results showed that *M. crystallinum* *bHLH* genes were distributed across all nine chromosomes, with significant non-uniform distribution patterns observed in both *M. crystallinum* and the three related species. In *M. crystallinum*, chromosomes 2, 3, and 4 exhibited dense gene clustering,

while chromosome 7 contained only a single gene (subfamily IVc). However, none of the three related species showed such extreme cases of chromosomes containing very few genes. Overall, most *M. crystallinum* *bHLH* genes were located near chromosome termini, a distribution pattern consistent with their orthologs in related species.

Expansion of the *M. crystallinum* *bHLH* gene family primarily occurred through dispersed duplication and segmental duplication (Supplementary Table S5). Intrasppecific synteny analysis using MCScanX identified eight syntenic gene pairs distributed across chromosomes 3–9 (Fig. 5). Exon-intron structures of most syntenic pairs were highly conserved (only *Mc03G00602*–*Mc03G02810* differed in intron category), indicating functional conservation post-duplication.

Interspecific synteny analysis revealed evolutionary relationships between *M. crystallinum* and other species. Synteny gene pairs with *A. thaliana* and *O. sativa* were predominantly concentrated in terminal chromosomal regions (Fig. 5c), whereas those with the closely related species *H. undatus* covered broader segments, implying shared ancestral genomic architecture. *Ka/Ks* analysis showed all homologous gene pairs had *Ka/Ks* ratios < 1 (Table 1), indicating purifying selection during evolution.

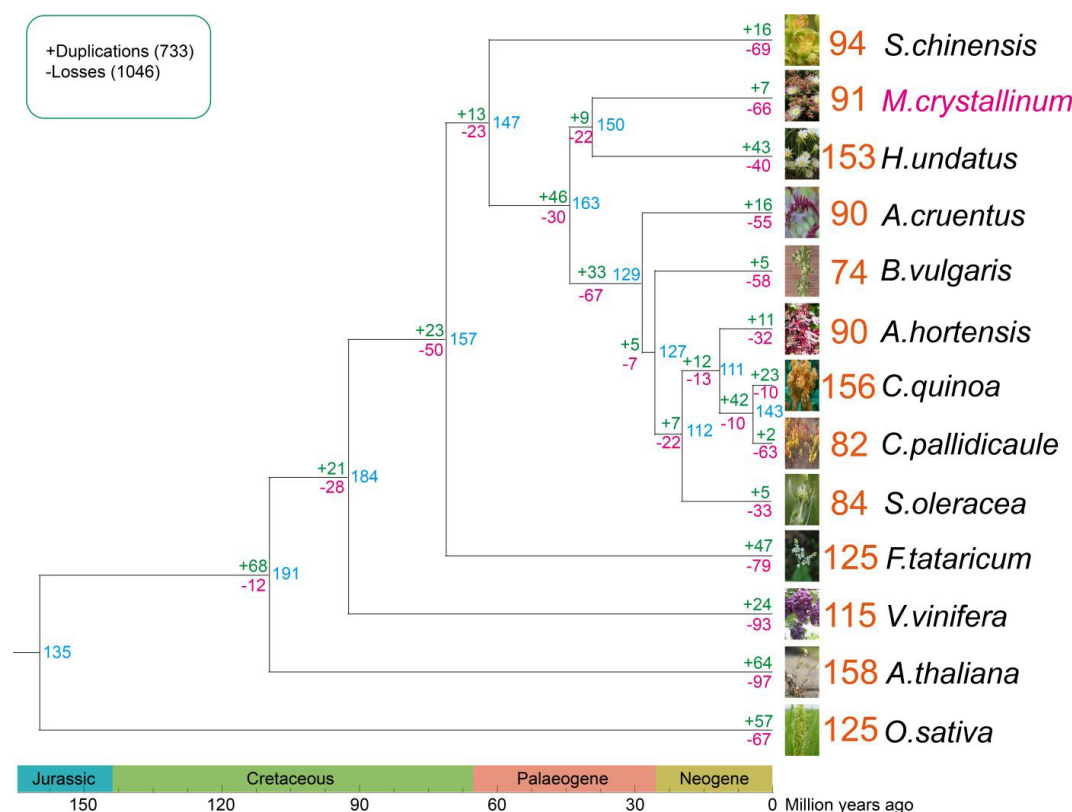


Fig. 3 Analysis of the gain and loss of the *bHLH* gene and evolutionary time in 13 species. Schematic diagram of the gain and loss of the *bHLH* gene in *M. crystallinum* and their closely related species. The green rectangle and the numbers in yellow font on the right side of the species picture represent the quantity of *bHLH* genes in ancestors and existing species. The + sign and the - sign respectively indicate the gain and loss of genes.

Co-expression, protein network, and cis-acting element of *M. crystallinum* *bHLH* genes

Co-expression analysis of transcriptome data extracted gene pairs with $PCC > 0.95$, revealing exclusively positive correlations (all $PCC > 0.95$; none < -0.95 ; Fig. 6). Network analysis demonstrated that *bHLH* genes correlated highly with diverse genes, highlighting their central transcriptional roles and synergy with other families. *Mc03G00604* co-expressed significantly with MYB family gene *Mc01G01299* (MYB20, lignin metabolism), glycosyltransferase gene *Mc01G00687* (mucilage biosynthesis), 2OG-Fe(II) oxygenase gene *Mc09G00173* (disease defense), and *bHLH* gene *Mc03G00603* (*bHLH126*). *Mc05G01224* co-expressed with WRKY family gene *Mc04G02119* (WRKY65).

In the intraspecific synteny pair *Mc03G00604*-*Mc03G00603* ($PCC = 0.992$), *Mc03G00603* was annotated as transcription factor (TF) *bHLH120* (AT5G51790), with GO enrichment for 'DNA-binding TF activity (GO:0003700)' and 'RNA polymerase II transcription regulation (GO:0006357)', suggesting a core role in transcriptional cascades. Co-expression of *bHLH* genes with ABC transporter (*Mc02G02356*), GST (*Mc03G01764*), and copper transporter (*Mc03G00444*) genes further implicated roles in transport and detoxification.

Notably, *bHLH* members showed significant co-expression with key ABA/JA pathway genes: *Mc01G00468* and *Mc02G01019* co-expressed with the ABA signaling gene *Mc06G01384*. *Mc03G00601* co-expressed with the ABA receptor gene *Mc08G01193*. *Mc03G00601*, *Mc03G00603*, and *Mc03G00604* co-expressed with the JA metabolic gene *Mc02G00882*. *Mc03G00600*, *Mc03G00603*, and *Mc08G01910* co-expressed with the JA-responsive TF gene *Mc06G02065*. *Mc03G00601* co-expressed with both ABA receptor and JA metabolic enzyme genes, indicating potential cross-talk

mediation. Additionally, *Mc03G01764* is strongly associated ($PCC = 0.989$) with glutathione S-transferase GSTU8 (detoxification). Genes linked to *Mc02G01019* (*Mc05G01832*, *Mc06G01713*) showed synergy with auxin transporter PIN2 and ethylene response factor WIN1 (Supplementary Table S6).

Annotation analysis of the *M. crystallinum* *bHLH* TF family identified key regulatory members: *Mc09G00905* (PIF4) involved in light signaling, *Mc02G01681* (PIL5/PIF1) associated with circadian regulation and cold response, and *Mc04G00224* (SCRM2/ICE2) functioning in negative gene expression regulation. GO analysis revealed enriched molecular functions including DNA binding (GO:0003677), TF activity (GO:0003700), and protein dimerization (GO:0046983), with significant involvement in biological processes such as light response (GO:0009416), seed germination regulation (GO:0010114), and iron ion homeostasis (GO:0055072). *Mc06G01874* was annotated as a core regulator of cellular iron starvation response (GO:0010106). Domain analysis confirmed the universal presence of the conserved HLH domain (PF00010.27) in all members, with additional *bHLH*-MYC_N domains (PF14215.7) detected in subsets (e.g., *Mc02G01577*), implying MYC-like regulatory networks. Functional complexity was noted: *Mc03G02400* (MYC2-like) contains both HLH domain and metabolic enzyme annotation (2-alkenal reductase), implying dual regulatory-metabolic roles (Supplementary Table S7).

Analysis of 1.5 kb promoter sequences upstream of start codons identified enriched cis-acting elements (Fig. 7): (1) Stress-responsive elements: LTR (low-temperature), MBS (drought), TC-rich repeats (defense), with salt-specific elements ARE and GC-motif enriched in *Mc05G01869* and *Mc06G01874*; (2) Hormone-responsive elements: ABRE (ABA), CGTCA/TGACG-motif (MeJA), GARE/P-box (GA); (3) Light-responsive elements: G-box (CACGTG), I-box; (4) Other

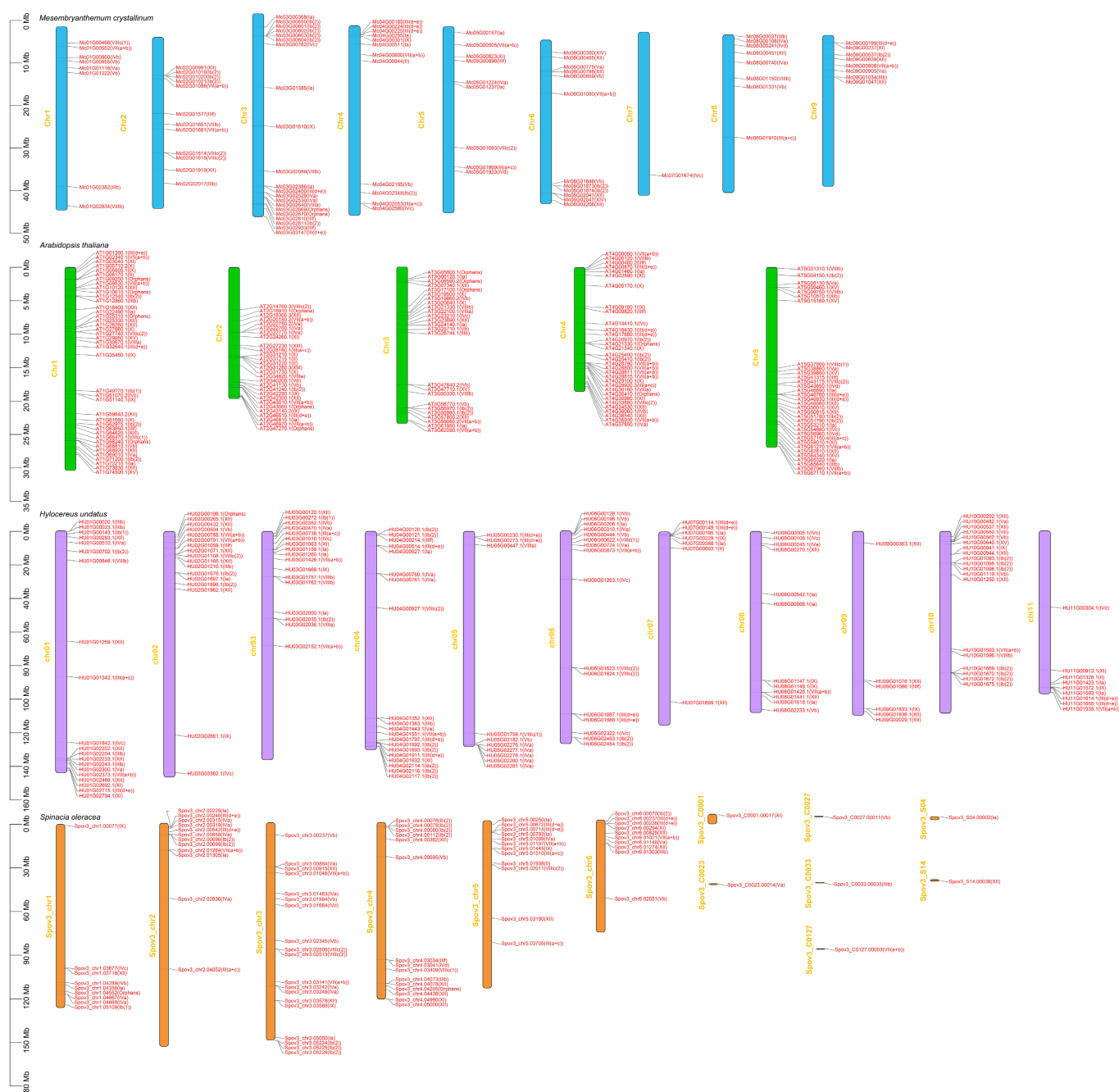


Fig. 4 Chromosome localization of the *bHLH* gene family in *M. crystallinum*, *A. thaliana*, *H. undatus*, and *S. oleracea*. The left scale indicates the chromosome length (Mb), with *bHLH* gene markers on the right side of each chromosome. Among them, blue represents *M. crystallinum*, green represents *A. thaliana*, purple represents *H. undatus*, and orange represents *S. oleracea*.

functional elements: circadian (diurnal rhythm), MSA-like (cell cycle). Stress-responsive elements predominated, with notable clustering (e.g., LTR in *Mc03G03147*) (Fig. 7). These results indicate *M. crystallinum* *bHLH* genes integrate light, hormone, and stress signals through complex promoter *cis*-element networks (Supplementary Tables S8–S10).

To understand the biological functions of *bHLH* proteins, the STRING database was used to analyze the protein interaction networks of their expressed proteins. The results showed that 89 candidate proteins of *M. crystallinum* successfully match the homologous proteins of *A. thaliana*, with sequence similarities ranging from 25.9% to 91.1%. The core functional modules include: (1) MYC2 can bind to *cis*-elements such as G-box and Z-box, and participate in

the signaling pathways of light, ABA, and jasmonic acid (JA). PIF1 activates genes such as SOM by binding to the G-box *cis*-element, and cooperates with *bHLH122* to enhance the functions mediated by JA/ABA signals. (2) The stomatal dynamic regulation module: SPCH and FAMA interact with SCRM to inhibit excessive stomatal closure. *BHLH85* and *GL3* are involved in the life processes of epidermal cells and negatively regulate stomatal formation. (3) The metabolic reprogramming module: *BIM1* interacts with *PIF4* and *PIF3* to activate photoperiod response genes. *TT8* and *BHLH93* enhance cell membrane stability by regulating flavonoid synthesis genes. Members such as *BHLH160* form a specific interaction network with *BHLH162* in *M. crystallinum*, possibly optimizing the expression of osmotic adjustment genes through variations in the

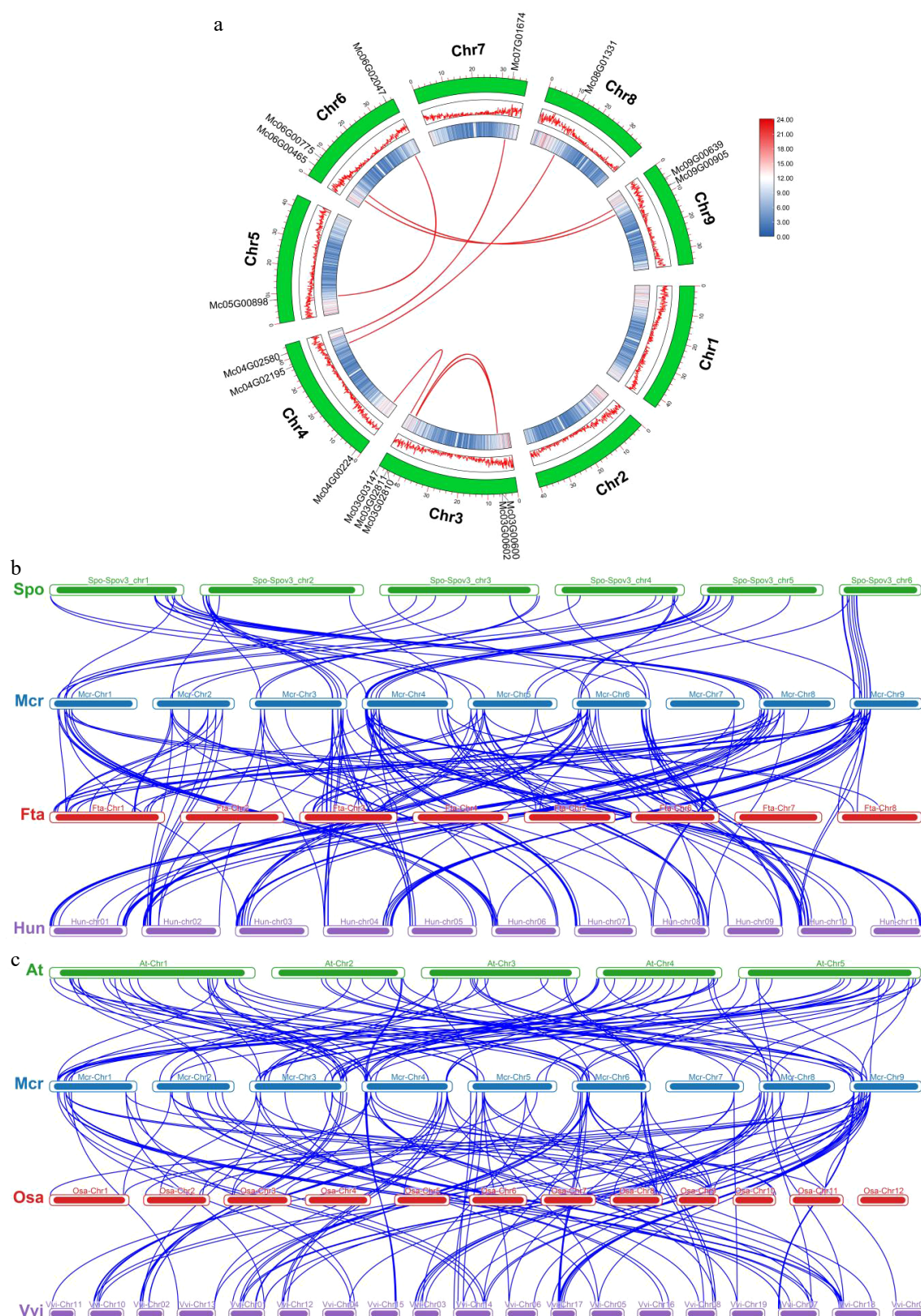


Fig. 5 Synteny analysis of the bHLH gene family. (a) Synteny within the bHLH gene family in *M. crystallinum*. (b) Interspecific synteny of the bHLH gene family in *M. crystallinum* and model species. (c) Interspecific synteny of the bHLH gene family between *M. crystallinum* and its closely related species.

bHLH domain (Fig. 8). This network reveals that the bHLH family supports the adaptive evolution of *M. crystallinum* in high-salt environments through both conserved stress signal transduction and species-specific metabolic integration.

Expression analysis profiling of bHLH under salt stress in *M. crystallinum*

Based on the expression data analysis of *M. crystallinum*, the gene expression of *M. crystallinum* shows significant tissue preference

Table 1. *Ka/Ks* calculation of the *bHLH* gene family in *M. crystallinum*.

Seq_1	Seq_2	Ka	Ks	Ka/Ks
Mc03G00600	Mc03G02811	0.5342569753824004	1.9079257380987593	0.28001979569434676
Mc03G00602	Mc03G02810	0.672448903885827	3.866700798259868	0.17390766417418418
Mc03G03147	Mc04G00224	0.6099490421022348	NaN	NaN
Mc04G02580	Mc07G01674	0.40884308246042134	3.5979201086646384	0.1136331741985685
Mc04G02195	Mc08G01331	0.4931212289557203	1.576299173683655	0.3128347950619963
Mc05G00898	Mc06G02047	0.5463903540215208	2.0177864227266507	0.270787010888486
Mc06G00465	Mc09G00639	0.3443717448863296	2.745644206336303	0.12542475244665727
Mc06G00775	Mc09G00905	0.4561463021811314	NaN	NaN

NaN indicates that the result cannot be calculated.

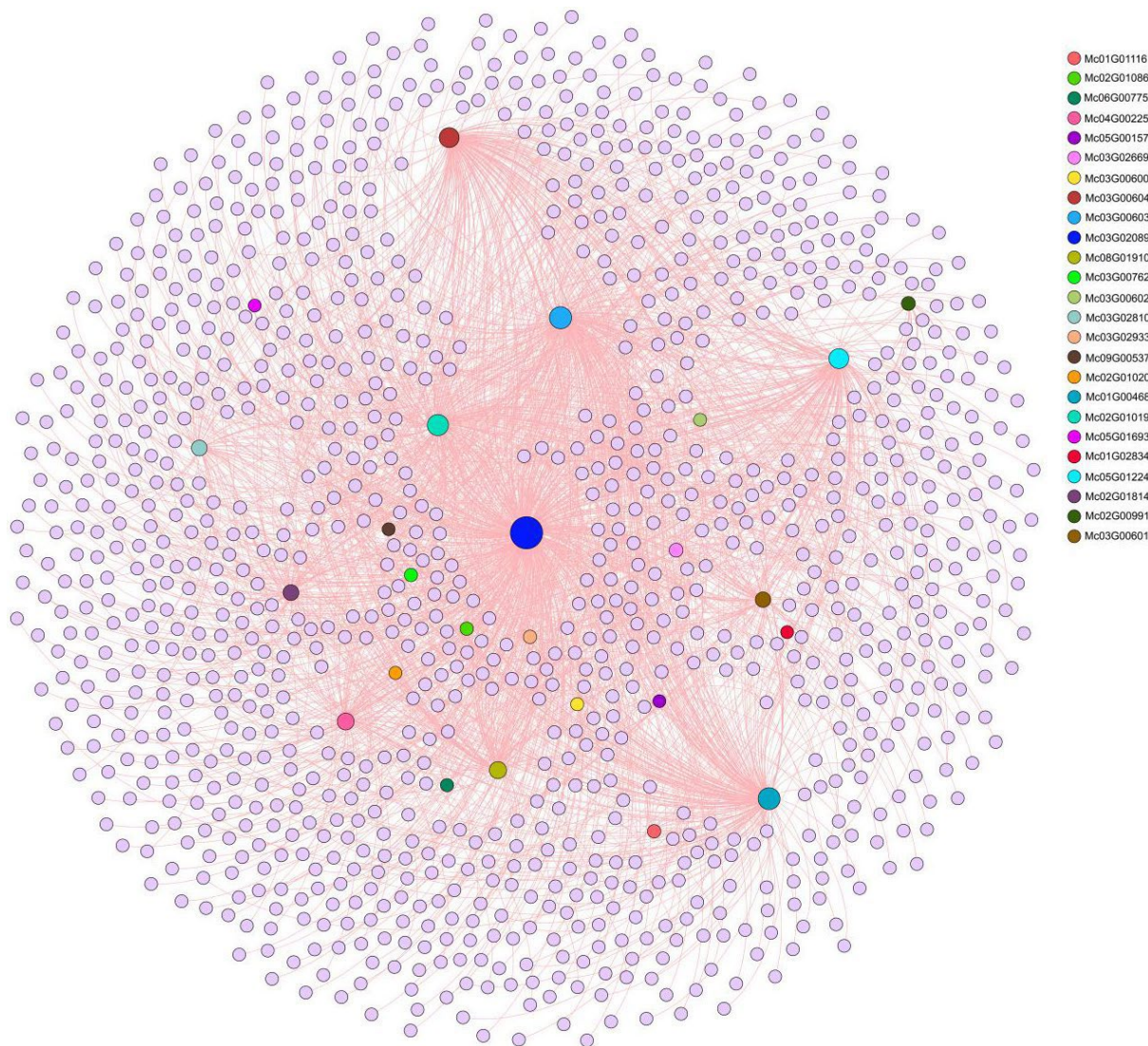
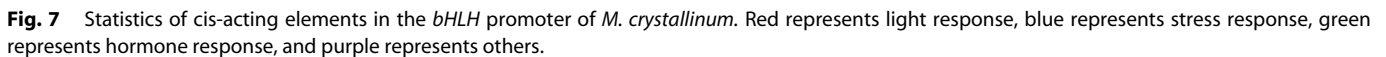


Fig. 6 Co-expression network of the *bHLH* gene family in *M. crystallinum*. Based on the correlation of expression levels among the genes of *M. crystallinum*, a co-expression network is formed. The light purple circle indicates that the genes *M. crystallinum* do not belong to the *bHLH* family, and the genes belonging to the *bHLH* family are marked with different colors. The correlations in the figure are all greater than 0.95.

(Supplementary Table S11): *Mc08G00451* is highly expressed in leaves (40) and stems (47), but is relatively low in roots (6), while *Mc03G02400* is highly expressed in roots (138) and stems (105), but is relatively low in leaves (47). *Mc09G00237* maintains high expression in roots (26.72), stems (22.67), and leaves (18.27), possibly participating in the multi-tissue coordinated regulation of basic metabolic pathways. *Mc04G00255* has a significantly higher expression in leaves (18) compared to roots (2), and stems (0)

(Fig. 9a), suggesting that it may be preferentially involved in stress signal transduction in leaves. The expression of *Mc06G00859* gene increases first, then slightly decreases, and then stabilizes as the salt concentration continuously rises, indicating that it may be expressed under salt conditions. The expression of multiple genes in *M. crystallinum* shows significant dynamic responses with the increase of salt concentration. *Mc05G01869* increases to 33 under T75mM treatment, approximately



To validate the response of *M. crystallinum* *bHLH* transcription factors to salt stress, eight significantly differentially expressed *bHLH*

Mounting evidence indicates that plant *bHLH* genes participate in diverse physiological and biological processes, including development and stress responses^[41]. However, research on the facultative CAM plant *M. crystallinum*, renowned for its extreme salt tolerance, remains limited. The release of a high-quality *M. crystallinum* genome^[6] enables in-depth investigation of its *bHLH* family. Based on *A. thaliana* phylogenetic classification, *bHLH* genes from *M. crystallinum*, *A. thaliana*, and closely related species, were categorized into 27 subfamilies, with *M. crystallinum* distributed across 23 subfamilies. Thus, a comprehensive classification and systematic analysis of the *M. crystallinum* *bHLH* family was conducted.

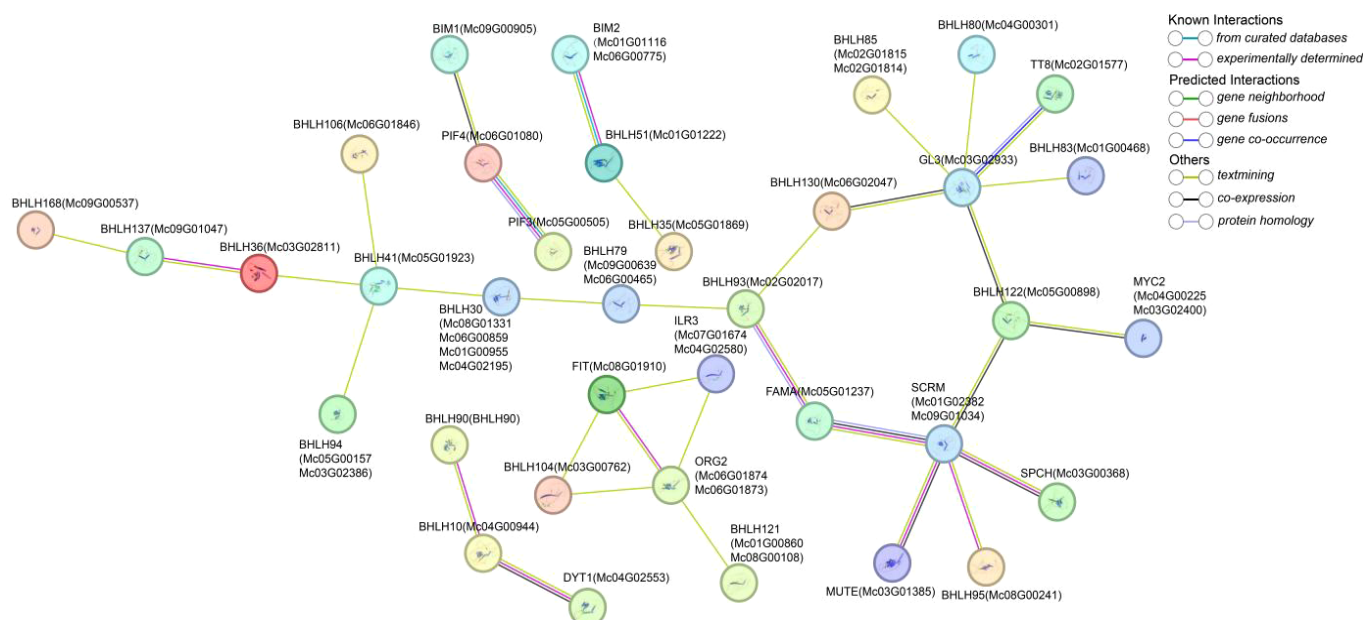


Fig. 8 Protein interaction network of the *bHLH* gene family in *M. crystallinum*. PPI network based on the *bHLH* homolog in *A. thaliana*.

Evolution of the *M. crystallinum* *bHLH* gene family

bHLH genes emerged in land plants 440 million years ago^[15] and expanded dramatically in higher plants^[42]. In this study, expansion of the *M. crystallinum* *bHLH* family primarily occurred through dispersed duplication (56 genes), followed by whole-genome/segmental duplication (16 genes), tandem duplication (TD) (15 genes), and singleton (four genes). This indicates dispersed duplication as the dominant expansion mechanism, consistent with findings by Yu et al.^[43]. Polyploidization events significantly drive family expansion^[44], which was validated in *M. crystallinum*. Notably, TD contributed to *bHLH* family expansion at a level comparable to genome doubling. Previous studies demonstrated that TDs facilitated functional innovation in disease resistance genes^[45], C4 photosynthesis genes^[44], and aspartate protease genes^[46]. The present findings revealed that *M. crystallinum* *bHLH* members evolved under multiple duplication mechanisms, providing insights for future studies on evolution-function relationships.

The *Ka/Ks* ratios of all synteny duplicated gene pairs in *M. crystallinum* were less than 1 (e.g., *Mc04G02580*–*Mc07G01674* pair, *Ka/Ks* = 0.11), indicating that the *bHLH* genes in *M. crystallinum* have undergone strong purifying selection during evolution (Table 1). By exploring the synteny of *bHLH* genes with other species, a significant number of synteny results were identified, suggesting the evolutionary conservation of the *bHLH* gene family in *M. crystallinum* and other higher plants. *M. crystallinum* formed 61, 50, and 120 synteny gene pairs with its close relatives *S. oleracea*, *F. tataricum*, and *A. cruentus*, respectively, with the highest number of synteny gene pairs observed with *A. cruentus*, implying a closer evolutionary relationship between the *M. crystallinum* *bHLH* family and *A. cruentus*.

Structural analysis of the *M. crystallinum* *bHLH* family indicates that genes within the same phylogenetic tree classification exhibited overall structural similarity, consistent with findings from most gene family studies^[43]. Additionally, previous studies have demonstrated that *AtbHLH106* (*At2g41130*) responds to salt stress^[47], its ortholog in *M. crystallinum* is *Mc06G01846*, which clusters within the same phylogenetic clade, and exhibits significant upregulation under salt stress. However, divergent responses were observed for certain *M. crystallinum* genes (*Mc01G00860*, *Mc08G00108*,

Mc05G00898) phylogenetically close to the salt-responsive *A. thaliana* genes *AtbHLH121* (*At3g19860*) and *AtbHLH122* (*At1g51140*), as these ice plant homologs showed minimal salt induction. Furthermore, salt-tolerant *A. thaliana* genes such as *AtbHLH092* (*At5g43650*) and *AtbHLH112* (*At1g61660*) are evolutionarily distant from *M. crystallinum* *bHLH* members, reflecting functional divergence between species.

The number of introns in *M. crystallinum* *bHLH* genes varied significantly (0–11), with subfamilies III(d + e) and VIIIb being intronless, while subfamilies IVb, IVc, and XII were intron-rich (Supplemental Table S4)^[48]. This structural divergence may enhance functional diversity through alternative splicing mechanisms. Focused on the structural differences in synteny gene pairs, it was found that most synteny gene pairs within *M. crystallinum* belonged to the same structural category (e.g., synteny gene pairs *Mc03G00600*–*Mc03G02811* belonged to the intron-poor category, *Mc05G00898*–*Mc06G02047* belonged to the intron-rich category, while *Mc03G03147*–*Mc04G00224* had no intron insertions). However, there was one exception: the synteny gene pair *Mc03G00602*–*Mc03G02810*, where the former was intron-poor (two introns) and the latter was intron-rich (nine introns).

More deeply, the evolutionary trajectory of *bHLH* family size changes in *M. crystallinum* and its close relatives was inferred. In the context of the *bHLH* family across 13 examined species, it was deduced that the ancestral *bHLH* family size was at least 135 genes. Overall, the number of *bHLH* gene losses far exceeds the number of gene duplications. Previous studies have shown that whole-genome duplication (WGD) events are often accompanied by substantial gene loss^[49], and the *bHLH* family is no exception. In fact, similar phenomena have been observed in studies of other gene families. Intriguingly, during the duplication and loss events at ancestral nodes, some nodes showed a higher number of duplications than losses. For example, the ancestral *bHLH* family of *M. crystallinum*, *H. undatus*, *A. cruentus*, *B. vulgaris*, *S. oleracea*, *A. hortensis*, *C. quinoa*, and *C. pallidicaule* experienced more duplications than losses (46:30). This reflected the distinct evolutionary histories of *bHLH* families in various plants. Therefore, the present study elucidated the evolutionary history of the *bHLH* family in *M. crystallinum* and

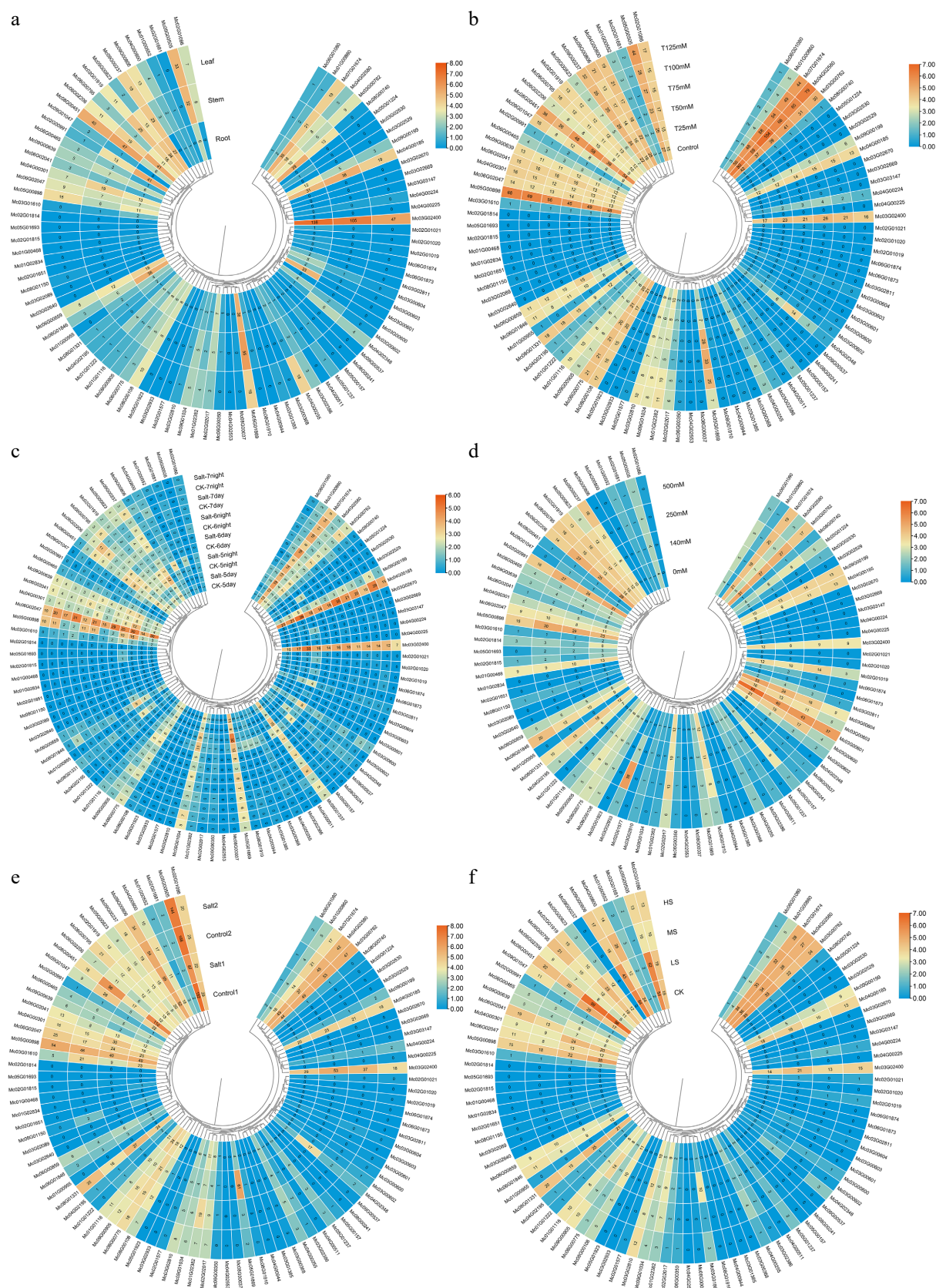


Fig. 9 Expression pattern analysis of *bHLH* genes in *M. crystallinum*. Expression levels of the *bHLH* gene in the (a) roots, stems and leaves. (b) *M. crystallinum* grown under different salt concentrations (0, 25, 50, 75, 100, and 125 mM NaCl). (c) *M. crystallinum* with control and salt treatment (500 mM NaCl) collected at 8:00 and 20:00 from 5 to 7 d after treatment. (d) *M. crystallinum* under different salt treatments (0, 140, 250, and 500 mM NaCl). (e) *M. crystallinum* treated with 500 mM NaCl salt concentration and the control group on the first and second days. (f) *M. crystallinum* treated with CK, LS (100 mM), MS (300 mM), and HS (500 mM) NaCl. The expression levels increase from blue to white and then to red.

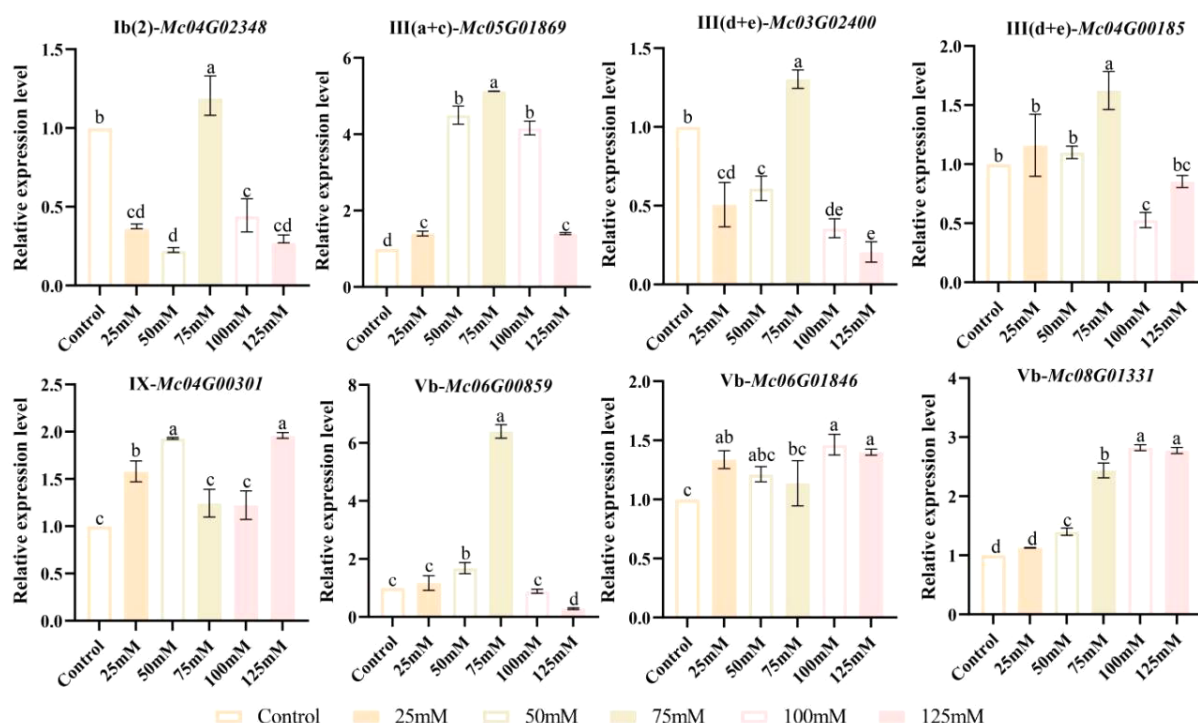


Fig. 10 qRT-PCR analysis of eight differential expression *bHLH* gene in leaf under different concentrations of salt irrigation. Different letter designations for the same gene indicate significant differences ($p < 0.05$).

comparative species, providing a valuable reference for studying gene duplication, loss, and retention in other species.

Function of *bHLH* in *M. crystallinum* and exploration under salt stress

Expression profiling was conducted and validated through qRT-PCR experiments showing that specific *M. crystallinum* genes exhibit significant differential expression under salt stress. The involvement of *bHLH* TFs in *M. crystallinum* salt stress response aligns with the findings of Takahashi et al.^[24], who reported that the *bHLH* TF *Mc010456.002* (*At2g42280.1*) in *M. crystallinum* participates in stomatal movement by activating genes encoding inward-rectifying potassium channels. Guan et al.^[50] also found that *M. crystallinum* *bHLH* TFs may be involved in mechanisms of osmotic regulation and stomatal regulation. It is noteworthy that *Mc03G02400*, a MYC2-like transcription factor, likely increases salt sensitivity by regulating proline biosynthesis and the JA signaling pathway^[51]. PIF (Phytochrome-Interacting Factors), belonging to the *bHLH* transcription factor superfamily, serves as a central hub for light signal transduction and modulating ABA signaling pathways^[52]. *Mc09G00905* is involved in light signaling, and *Mc02G01681* is associated with circadian regulation, which may be related to the function of regulating photosynthetic pathways and enhancing salt tolerance.

Co-expression network analysis provided corroborating evidence: *Mc05G01224* showed significant co-expression ($PCC = 0.989$) with glutathione transferase *GSTU8* (*Mc03G01764*), and *Mc04G00225* displayed strong co-expression ($PCC = 0.999$) with calcium exchanger *CAX9* (*Mc04G01942*) (Fig. 8), suggesting that *bHLH* genes may enhance plant stress tolerance by coordinating ion homeostasis (e.g., Na^+ efflux) and antioxidant defense^[53]. Additionally, *Mc03G02810*, the longest *bHLH* gene in *M. crystallinum* (Supplementary Table S4), exhibits high correlation with multiple genes. Among those co-expression genes, *Mc01G00538* and *Mc04G01204* are involved not only in gene silencing regulation but also in responses to salt stress, suggesting related functions for *Mc03G02810*. The

qRT-PCR verification of the *bHLH* gene (Fig. 10) also confirmed our correlation analysis: the expression dynamics of *Mc06G01846* showed upregulation when salinity increased, which was consistent with the effect of *A. thaliana* genes. In summary, the present work reveals the response pattern of ice plant *bHLH*s under salt stress. However, more in-depth research is needed to elucidate its specific salt-stress response mechanisms.

Conclusions

This study systematically analyzed the molecular characteristics and functional mechanisms of the *bHLH* gene family in the facultative CAM plant *M. crystallinum* through genome-wide analysis. A total of 91 *bHLH* genes were identified in its genome, revealing that gene loss dominates the family's evolution, with dispersed duplication as the primary driving force for expansion, and synteny gene pairs within the species generally undergoing purifying selection. The promoter regions are enriched with stress-responsive elements and hormone-responsive elements, suggesting that *bHLH* genes regulate salt adaptation by integrating light signals, ABA/JA pathways, and redox balance. Co-expression network and protein interaction analyses revealed that *M. crystallinum* *bHLH* genes collaborate with *MYB*, *GST*, and other gene families to form a multidimensional regulatory network, with gene expression under salt stress exhibiting tissue specificity. This study provides important theoretical insights into elucidating the functions and salt tolerance mechanisms of the *M. crystallinum* *bHLH* gene family.

Author contributions

The authors confirm their contributions to the paper as follows: study conception: Zhang Y, Song X; *bHLH* gene family, data collection, and bioinformatics analysis: Quan J, Chen H; experimental implementation: Dong Y, Han B, Zhu H, Nan Z; writing of the manuscript: Quan J, Zhang Y, Chen H; proofreading of the

manuscript: Guo D; revision of the manuscript: Zhang Y, Song X, Quan J, Chen H, Dong Y, Han B, Zhu H, Nan Z, Guo D. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data generated or analyzed in this study are included in this published article and the its supplemental files.

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Conflict of interest

The authors declare that they have no conflict of interest.

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