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Genome-Wide Identification and Expression Analysis of the Barrel Medic (*Medicago truncatula*) and Alfalfa (*Medicago sativa* L.) Basic Helix-Loop-Helix Transcription Factor Family Under Salt and Drought Stresses

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Abstract

The basic helix loop helix (*bHLH)* transcription factor comprises one of the largest plant-specific transcriptional regulators in plant growth and development that response to biotic and abiotic stresses. Many members of *bHLH* play essential roles in the growth of root hair and response to drought, salt, and cold stresses. The family of *bHLH* genes has been found in many species; nevertheless, the barrel medic and alfalfa species still have a minute gap of *bHLH* new members thus far. This research aims to identify members of the *bHLH* family in barrel medic and alfalfa and elucidate their expression pattern level, network analysis, predictive 3D modeling and phylogenetic relationships. Here, we identified and characterized the *bHLH* gene family in both barrel medic and alfalfa plants and their genes expression response to drought, salinity, and cold stresses. A total of 159 *MtbHLH* and 133 *MsbHLH* genes were identified and characterized, divided into 18 subgroups and 17 subgroups, respectively. As a ubiquitous and popular method, neighbor-joining clustering was used. Based on the phylogenetic analyses, the VIII and IX subfamily and X subfamily were selected as the stress-related subfamily in these two species. The 154 *MtbHLH* genes were progressively distributed on the 8 chromosomes and 23 tandem duplicated genes, and 44 duplicated genes segments were detected in *MtbHLH* family. The analyses of gene ontology discovered the *bHLH* predominantly functions in protein and DNA binding in these two species. The results of Ka/Ks were < 1, which showed that the most orthologous of the *bHLH* gene values was found between *A. thaliana* and *M. truncatula* species. Remarkably, 7 *MtbHLH* and 10 *MsbHLH* genes were selected and validated with qRT-PCR after the treatment's samples sampled under stressed abiotic conditions. The similar expression patterns between *M. truncatula* and *M. sativa* L have demonstrated identical expression patterns level in the root, and contrasting patterns in the stems and leaves were diverse. It was highlighted that the gene expression analyses of 17 *bHLH* genes were up-regulated to stresses, respectively, apart from some genes that were timely trended down-regulated to control (0 h). This study provided a concise understanding of the tissue specific of *bHLH* gene functions in genome-wide levels under drought, salt, and cold stresses. Our analyses provide the first insights onto the *M. truncatula* and *M. sativa* L evolution that contributes to molecular breeding for improving plant yield and stress tolerance.

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Abbreviations

TFs	Transcription factors
bHLH	Basic helix-loop-helix
GRAVY	Grand average of hydropathicity
pI	Isoelectric point
CDS	Coding sequences
HMM	Hidden Markov Model
MW	Molecular weight
qRT-PCR	Quantitative real-time PCR
GO	Gene ontology
Mt	*Medicago truncatula*
Ms	*Medicago sativa* L
CDD	Conserved domain
HLH	Helix-loop-helix

Introduction

Abiotic stress is one of the significant environmental factors that negatively impact each plant aspect, leading to the momentous yield losses in agriculture around the world (Nakashima et al. 2009). Moreover, particular crops, including legumes, have been found to have a significant share of annual crop losses due to biotic and abiotic stresses. Legumes are the plants in family Fabaceae or Leguminosae, which are playing a crucial role in crop rotation due to their symbiotic nitrogen-fixing bacteria in structures (Complainville et al. 2003; Min et al. 2019; Staniak et al. 2018). In general, it includes several common legumes, including barrel medic, alfalfa, beans, lentils, common vetch, clover, peas, and peanuts (Complainville et al. 2003). Fabaceae is primarily grown for human consumption, for livestock forage and silage, and as soil-enhancing green manure (Min et al. 2019; Thomas 1995). The transcription factors play a crucial role in enhancing some legume species to adapt during biotic and abiotic stresses.

Nonetheless, the basic helix-loop-helix (bHLH) gene family has not been identified in *M. truncatula* and *M. sativa* L thus far. Here, we aimed to explore the analysis of the *bHLH* gene family to provide some clues to the functional research of its members in *M. truncatula* and *M. sativa* L, to a range of abiotic stresses and combinations at the genetics and transcriptional level. It is consequently overbearing to grow plants that are more tolerant to drought, salt, and cold stresses. So, combining the resources of multiple genetic engineering is necessary to improve plant tolerance, which will further upsurge plant yields under numerous stress conditions and accurate signaling.

The transcription factors (TFs) are the significant proteins that the different plants possess through regulating and activating the expressions of downstream genes (Agarwal et al. 2006; Pires and Dolan 2010; Rai et al. 2019). The TFs play a crucial role in the different molecular processes by prompting, converting, or transcribing DNA into mRNA that regulates the genes expression via RNA polymerase (Agarwal et al. 2010). It is a regulatory protein with DNA-binding domains, which mediates various aspects of cellular processes and collaborates with cis-element in the promoter regions of different downstream genes (Schwechheimer et al. 1998). A basic helix-loop-helix is one of the six essential and potential extensive TF families, which are known to mediate the signal transduction and stimulus–response pathway. It regulates the downstream of the target gene expression and leads to plant tolerance (Budak et al. 2013; Pires and Dolan 2010), including *MYB* (Du et al. 2012; Qiuling He 2016), *DREB* (Dehydration-responsive element binding) (Agarwal et al. 2006), *ERF* (Ethylene response binding factor), *bZIP* (basic-region leucine zipper motif), *NAC,* and *WRKY* members (Muthuramalingam 2018). The characterization of these six major gene families gives the needed understanding to manage the abiotic and biotic stresses in plants. The *bHLH* superfamily was classified as the second most significant transcription factor in plants (Hir et al. 2017). Through the discovery of the *bHLH* motif with DNA-binding and dimerization activities, members of the bHLH protein proved an increase of bio-physiological functions on animal muscle development. Besides that, the subordinating numbers were found in plants (Murre et al. 1989b; Quail 2000), and the finding has been characterized by three eukaryotic kingdoms (Murre et al. 1989a). The family defined by the *bHLH* domain, which comprises approximately 60 amino acids and contains two distinct regions, the basic regions and the *HLH* regions. The interactions of the two components can bind the specific part E-box [CANNTG] DNA sequence. The central DNA site recognized by *bHLH* TFs comprises a consensus core element known as the E-box (5′-CANNTG-3′), with the palindromic G-box (5′-CACGTG-3′) being one of the most common forms. The *HLH* region encompasses two amphipathic α helices with a linkage loop of variable lengths; the two helices combined with the basic region give the formation of homodimers or heterodimers (Ellenberger et al. 1994; Murre et al. 1989b). In addition, this typical region has six basic residuals and a highly conserved HER motif (His-5, Glu-9, and Arg-13) that is predicted to bind specific DNA sequences. The bHLH proteins are distinguished by specific signature domains consisting of about 60 amino acids. Every single domain has two distinct regions having

a different function. The first segment, which comprises about 15 amino acids with typical six basic residues that play a role in binding to DNA, is called a basic region, which settled in the N-terminus (Ellenberger et al. 1994). Increasing numbers of members of the *bHLH* gene family were deciphered in model plants to participate in a multitude of physiological processes that enable plants to withstand abiotic stress, including *Arabidopsis* and rice (Carretero-Paulet et al. 2010).

Among the various plant species, a growing number of members in the *bHLH* gene family were also found to cope with the abiotic stresses. For example, a total of 155 *bHLH* gene members were identified in common bean, and 21 subfamilies were categorized based on the phylogenetic tree as this number of subfamilies was also found in *A. thaliana* (Kavas et al. 2016). The *bHLH* gene family was identified in *B. rapa*, which contains 230 *bHLH* transcription factors and is clustered into 24 subfamilies (Song et al. 2014). 162 *bHLH A. thaliana* and 167 rice *bHLH* genes were also grouped into 25 subfamilies in *A. thaliana* (Li et al. 2006). Besides, different other studies on this *bHLH* family have been reported in plant species before phylogenetic tree comparison, as described by Miao et al. (2020). In legumes, studies have been showing that transcriptomic techniques are the most positive ways to elucidate abiotic stresses (Babar et al. 2014). Thus, leguminous (Fabaceae), including barrel medic and alfalfa, plays an essential part in the botanical sphere and are valuable sources of nutrients, drugs, and biofuels (Liancourt et al. 2013). The bHLH proteins were firstly identified and characterized in animals, plants, and fungi (Ledent and Vervoort 2001). Binding of heterodimers leads to the transcriptional activation of the target gene, and a large number of bHLH proteins are involved in plant developmental and cellular processes. The presence of preserved domains, such as the *bHLH* domain and MYC encoding *bHLH* domain association, is most likely the result of extensive investigations of *bHLH* genes in plants, which plays a crucial role in mitigating the harsh environmental conditions. Typically TFs are known as sequence-specific DNA-binding proteins capable of activating or repressing transcription (Bacsi and Hankinson 1996). Emerging evidence started to show that the stress signaling surges the large number of stress-responsive genes that can be used to identify the *bHLH* genes functions in legumes. Moreover**,** several genes of the *bHLH* family were involved in stress response, and their roles contribute much to unveil the new candidates in the different species. For example, *AtbHLH17* and *AtWRKY28* co-expressions granted *bHLH* binding to G-Box for salt tolerance (Chinnusamy et al. 2005; Mahajan and Tuteja 2005). Previous studies found that *OsRERJ1* is involved in wound and drought responses (Kiribuchi et al. 2005), *OsbHLH1* is involved in cold stress response (Wang et al. 2003), *AtbHLH92* is involved in osmotic stress (Jiang et al. 2009), and *bHLH106* is involved in cold, salt, and drought stress (Ahmad et al. 2015; Verslues et al. 2006). The *bHLH* family gene was identified in various species, including grape (Wang et al. 2018a), apple (Mao et al. 2017), common bean (Kavas et al. 2016), tomato (Sun et al. 2015), potato (Wang et al. 2018c), *Camellia sinensis* (Cui et al. 2018a), strawberry (Zhao et al. 2018), and *Fagopyrum tataricum* (Luo et al. 2016)

Last decade, various studies gave many contributions to the *bHLH* functions analysis; some genes were suggested to be responsive to regulating element deficiency and iron stress in *A. thaliana*, including *AtbHLH034* and *AtbHLH104*. The transgenic plants were performed and confirmed to be positively regulated in iron deficiency and iron stress. Even though these two genes were enhanced to respond to iron (Fe), but loss-of-function was demonstrated in the reduction mechanism of Fe content, disruption of Fe deficiency response, and uptake (Li et al. 2016). Similar roles were also accepted using multiple knockout mutants in *A. thaliana,* for example, *AtbHLH038*, *AtbHLH039*, *AtbHLH100*, and *AtbHLH101* genes played a gradually significantly increased role in iron deficiency responses and uptake under minimal iron conditions (Wang et al. 2013). At the same time, *AtbHLH112* showed a better ROS-scavenging potential, which also increased the level of Proline in *A. thaliana* and resulted in high resistance to several abiotic stresses, including drought and salt stress (Liu et al. 2015). The results from the research on over-expression also indicated that *AtbHLH122* be strappingly rooted by salt, drought, and osmotic stress (Liu et al. 2014). It is noticed that a stress responsive of *bHLH TF* plays a crucial role in the different plant species, for example, the functions in JA-mediated drought tolerance were shown at Rice (*OsbHLH148*). *Vitis vinifera* (*VvbHLH1*) has the potential to improve resistance to drought and salinity by controlling flavonoid aggregation and functions as an ABA signaling regulator (Wang et al. 2016). *RsICE1* from *Raphanus sativus* improves cold tolerance in rice by interacting with CBF/DREB1 (Man et al. 2017). *Pyrus ussuriensis* ICE1 plays a pivotal role in enhancing cold tolerance by increasing *PuDREB* transcriptional regulation via interaction with *PubHLH1* (Huang et al. 2015). However, based on the TFs information shortage as described in the previous study, for instance, less than 1% of transcription factor genes of the model legumes species was only characterized in *Lotus japonicus* and *Medicago truncatula* (Udvardi et al. 2007).

In this study, we obtained 159 *bHLH* genes in *M. truncatula* and 133 *bHLH* genes in *M. sativa* L members, respectively, based on the BLAST results and domain verification. The phylogenetic tree method has used to categorize and cluster the bHLH proteins into subfamilies. In general, *bHLH* genes were found to be assembled in the same families at a bootstrap value greater than 80,

which may be due to similar evolutionary history and their functions. Interestingly, gene families from various paths could be grouped in one large cluster. Those results could be the product of positions close to those *bHLH* gene subfamilies that narrowed down their divergence during their evolutionary history in these two plant species. Besides, the analysis of the bHLH protein motif organization indicated that each particular protein subfamily could be distinguished by at least one conserved motif. Moreover, *bHLH* family proteins, subfamilies, and group pathway clades shared mutual motifs with genes from both metabolic domain pathways. In this analysis, we also observed that the divergence time of the *bHLH* family gene between the chromosome positions of the *MtbHLH* genes increased at approximately 95% with the distance from the centromeric. Contrary, no chromosomes localization tested in *M. sativa* L due to the incompleteness of its genomes database. We also evaluated physiochemical properties to understand the amino acid reliability in *M. truncatula.* The findings showed that a GRAVY value < 0 means mainly hydrophilic proteins. GRAVY values were less than 0 for more than 90% of MtbHLH proteins, emphasizing low hydrophobicity. The results of Ka/Ks were < 1, which showed the most of the orthologous *bHLH* gene values between *A. thaliana* and *M. truncatula* species**.** Also, there was evidence of multiple individual intron loss events from a study of *bHLH* genes and protein features and their chromosome localization in *M. truncatula*. In supporting these data, we also employed network analysis and 3D modeling prediction to unveil the features of *bHLH* family genes in these two species. Finally, expression pattern analysis during the development of these two species and after specific stress treatments indicated diverse functions underlying abiotic stress. The genome-wide transcriptome obtainable through this study has advanced our knowledge of the physiological process by underlying a significantly altered *bHLH* gene that reveals the structural and functional stress related to abiotic stresses.

Materials and Methods

Plant Materials

The plant materials (*Medicago truncatula* Jemalong A17 and *Medicago sativa* L Zhongmu No.1) seeds were scarified and surface sterilized in 1.0% (v/v) sodium hypochlorite for approximately 5 min and then tap water washed three times and germinated at 20 °C in the dark. After 4 days, uniform seedlings were transplanted to ½ modified Murashige and Skoog solution in the sterilized aeroponic box as the regular nutrition, and the solution was changed once every 2 days. The pH of the culture solution was adjusted and maintained at 5.8 for the duration of the experiment. The seedlings were grown and irrigated in a room growth chamber conditions (27/23 °C light 14 h/10-h dark cycle and 70–80%, relative humidity) with aerated nutrition as described by Liu et al. (2017). The 30-day-old seedlings were treated with NaCl (180 mM and 250 mM), 15% polyethylene glycol (PEG-6000), or 4 °C for salt, drought, and low temperature stress conditions, respectively. After treatment, the leaves, stems, and roots were sampled in liquid nitrogen at 0 h (as control), 6 h, 24 h, and 48 h after stress for *M. truncatula*, and 0 h (as control), 3 h, 6 h, 12 h, and 24 h for *M. sativa*. To decrease the effect of circadian rhythm on plant growth and gene expression, samples from the control, salt, drought, and cold stress treatments were grown in parallel and harvested at the same time after 24 h or 48 h. All samples were immediately frozen in liquid nitrogen and stored in a refrigerator at − 80 °C until used for RNA isolation. All the experiments were performed in triplicate.

Transcriptome Analysis

For RNA isolation and cDNA synthesis, total RNA was extracted from previous plant materials of *M. truncatula* and *M. sativa* L using an RNA sangon product Kit, China, following the manufacturer's instructions. The extracted RNAs were computed on a Nanodrop 2000 spectrophotometer (USA) and examined on 1% agarose gels. The A260/280 ratio of each RNA sample ranged from 1.6 to 2.2 cDNA and was synthesized from the total RNA of each sample using the PrimeScript RT Perfect Real-Time reagent kit (Takara, sangon biotech, China). The cDNA was diluted 10 times with nuclease-free water. All of the procedures were conducted according to the manufacturers' protocols. The specific *MtbHLH/MsbHLH* gene primers for quantitative real-time PCR (qRT-PCR) were designed with Primer Premier 5. The qRT-PCR was conducted in a 10-µL reaction system with the following components: 5 µL KAPA SYBR FAST qPCR Kit Master Mix (2×) Universal, 0.5 µL forward primer, 0.5 µL reverse primer, 2µL diluted cDNA, and 2 µL ddH_2O (primers used in this study are detailed in Table S1). A 7500 Real-Time PCR System was used for the qRT-PCR. The procedure was 95 °C for 2 min and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. A melt curve was generated from 65 °C to 95 °C with increments of 0.5 °C every 5 s. Each gene was detected in three biological replicates and three technical replicates. The internal reference gene was *ef1α*. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Three biological replicates for each group were run, and each reaction was performed with three technical replicates.

Identification and Phylogenetic Analysis of the *bHLH* Gene Family in Both *M. truncatula* and *M. sativa* L

To further assess the incredible potential members of bHLH protein family, the *Arabidopsis* (133) (Bailey et al. 2003; Heim et al. 2003) and rice (155) bHLH protein sequences downloaded from Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html) were used as queries in Blastp (by BLAST 2.4.0 tool) searches against *Medicago truncatula* Genome Project v4.0 of JCVI database(https://www.jcvi.org/medicago/index.php). The *bHLH* domain (PF00010.25, Pfam; https://pfam.xfam.org/) was also employed as a query to perform blast search against the same genome database after retrieved CD-Hits to avoid the unnecessary alignments. The 159 bHLH proteins putative were retrieved. Occasionally, to identify and classify the *bHLH* family, the *Medicago sativa* protein sequences were searched and downloaded from the online databasehttps://www.alfalfatoolbox.org/doblast/?filterword=DOBLAST&function=function2by using 159 bHLH protein IDs of *Medicago truncatula* as query pursuits against the same genome database, CD-hits, and HMM profiles were used to retrieve the non-redundant, and then a total of 133 MsbHLH proteins putative were regained (Godzik and Li 2006). The identified 159 *MtbHLH* and 133 *MsbHLH* were renamed, respectively, according to bHLH proteins with the highest sequences similarity. To verify the reliability selection of the bHLH protein sequences, the structural and functional (HLH, domain) were predicted by the Conserved Domain Database (CDD) of NCBI and SMART analysis (Letunic et al. 2015). Meanwhile, the multiple sequence alignments of *bHLHs* of these two species were performed using the ClustalW program in MEGA 6 software. The unrooted phylogenetic tree was constructed by MEGA 6 based on the neighbor-joining method supported with 1000 bootstrap resampling with the pairwise deletion parameters.

Sequence Analysis, Chromosomal Localization, and Gene Structure Prediction of *bHLH* Gene Family

Gene structure was shown using the TBtools, a toolkit for biologists' software (Chen et al. 2018). The chromosomal localization was displayed using the MapChart 2.3 software for only *M. truncatula* bHLH gene sequences. Molecular weight, theoretical isoelectric point, and grand average of hydropathicity (GRAVY) of each *MtbHLH* (Table S2) were calculated by the ProtParam tool of ExPASy Server (https://web.expasy.org/protparam/). The genetic Ka/Ks analysis was performed by the Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and PAL2NAL (Suyama et al. 2006) programs using the pairwise sequences with more than 80% similarity. Moreover, due to the incompleteness of the *M. sativa* L database, we did not analyze chromosome localization, molecular weight, and theoretical isoelectric point of its amino acid sequences.

In Silico Analysis of Promoter Sequences, Conserved Amino Acid Residues, and Gene Expression Patterns

To further understand the in silico analysis of transcription factors, nucleotide sequences of 2000 bp upstream of genes transcription start site of *M. truncatula* were downloaded from the JCVI database. The PlantCARE analyzed the promoter sequences (Lescot et al. 2002). All transcriptome residues of the *MtbHLH* genes were carefully obtained from the comparison with the identified *bHLH* (Guenther et al. 2003; Hove and Bhave 2011; Tornroth-Horsefield et al. 2006; Zou et al. 2015). In addition, the multiple sequence alignments of the bHLH protein sequences and physical exertion in these two species were explored using the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/). To dissect the expression patterns of *MtbHLH* genes in different tissues, the *bHLH* gene chip expression data were retrieved from the *M. truncatula* Gene Expression Atlas (MtGEA) Project of NOBLE database (https://mtgea.noble.org/v3/) (Benedito et al. 2008). The BLAST program of this database was performed to search the probe IDs of corresponding genes, and further viewed the expressions of the corresponding probes. We processed the log2 and hierarchical cluster data and generated a heatmap. Furthermore, the searched contig IDs of all *MsbHLH* putative and the visualization transcript sets were well thought out and three organs/tissues data were downloaded. The HemI software was used to draw the gene expression profiles heatmap.

Gene Ontology Annotation and 3D Prediction Analysis of *bHLH* Genes in Both *M. truncatula* and *M. sativa* L

The functional categorization and annotation of *bHLH* sequences were performed by using Blast2GO software with default parameters, *M. truncatula,* and *M. sativa* L amino acid sequences were used as queries in BLASTP, a search was run against the non-redundant database at NCBI with an e-value of $1 \times E - 10$, and the top 20 alignments for each sequence were uniformly considered. The resulting Multi Blast data collection was then rehabilitated into a Blast2GO project. Go annotation was carried out an annotation score of each candidate Go term (Table S3), and visualization was approved by CELLO2GO online website (https://cello.life.nctu.edu.tw/cello2go/) (Table S4). To better understand the prediction of a 3D homology model of *bHLH* transcription factor proteins, the online website (https://www.sbg.bio.ic.ac.uk/phyre2) was used, Hidden Markov Models via HMM-HMM have aligned the proteins through the detection rate method, and the intensive mode was selected to increase

the accuracy of the alignments (Kelley and Sternberg 2009). The 3D protein modeling of the successfully predicted genes was carefully chosen above 90% of the confidence interval and the percentages of residue values varied from 80 to 100 for two plant species.

Results and Discussions

Identification of bHLH Proteins of *M. truncatula* and *M. sativa* L and Comparative Analyses

To better understand the transcription factors in both barrel medic and alfalfa, our search for *bHLH* domain enfolding proteins identified that a total of 159 distinct *bHLH* in *M. truncatula* and 133*bHLH* distinct in *M. sativa* L via the transcriptome analysis described above were renamed, respectively, as shown in Supplementary Figure S1. These proteins characterize the major evolutionary lineages of the two species for their analysis of the *bHLH* transcription factors. Previous research has reported the various analyses, which have proved the coincidence and coexistence of the gene's subfamilies compared by *Arabidopsis thaliana* through the gene structure and motif prediction probes. A set of 159 MtbHLH protein sequences was between 81 and 721 in length (with an average of 333), while the 133 MsbHLH protein sequences were between 70 and 1038 in length (average of 381). The alignments were observed in the two major types, with more conservation found in helices than the basic region, as illustrated in Supplementary Figure S1. The identification of the residues across all members was estimated based on the lowest and highest percentages of the consensus in *M. truncatula* and *A. thaliana* species, 51.5% and 52.5%, respectively, as demonstrated in Table 1. The motifs were at least presented at every single amino acid whereby the motif was from 1 to 9 in barrel medic, as shown in Supplementary Figure S2, while the MsbHLH protein sequences are ranged between 1 to 13 motifs, as shown in Supplementary Figure S3. Based on the rule created by Toledo-Ortiz et al. (2003), we identified 156 DNA binding *bHLHs* and 3 non-DNA binding *bHLHs* in *M. truncatula*, while 118 DNA binding *bHLHs* and 15 non-DNA binding *bHLHs* in *M. sativa* L. The DNA binding *bHLHs* were further divided into 146 E-box binders (containing Glu-9 and Arg-12) and 23 non-E-box binders (lacking Glu-9 or Arg-12) based on the presence or absence of Glu-13 and Arg-16. Most of the non-DNA binding and non-E-box *bHLHs* were distributed in subfamilies 4, 6, 9, 10, 12, 19, and 21. Among 146 E-box *bHLHs*, 97 proteins (containing His/Lys-5, Glu-9, and Arg-13) were predicted to bind the G-box motif (CAC GTG), while 49 proteins were predicted to recognize other types of E-boxes (CANNTG) and were defined as non-G-box binders (Supplementary Figure S4) in *M. truncatula*. On contrary, 97 E-box binders (containing Glu-9 and Arg-12)

Table 1 Comparison of the representative bHLH proteins, components of the bHLH domain in *Arabidopsis* and barrel medic plants

Position in the alignment by Atchley et al. (1999)	Position in the alignment in *Medicago truncatula*	Region	Consensus Motif Amino Acid Frequency within the bHLH Domain (Atchley et al. 1999)	Amino Acid Frequency within the *Medicago truncatula* bHLH Domain
1	1	Basic	K (27%), R (61%)	K (6%), R (4%)
2	2	Basic	K (16%), R (77%)	K (16%), R (77%)
9	9	Basic	E (93%)	E (86%), A (9%)
10	10	Basic	R (81%), K (14%)	R (86%), K (11%)
12	12	Helix1	R (91%)	R (95%)
16	16	Helix1	I (35%), L (33%), V (23%)	I (42%), L (41%), V (16%)
17	17	Helix1	N (74%)	N (43%), S (27%)
20	20	Helix1	F (72%), I (9%), L (14%)	F (34%), I (11%), L (26%), M (17%)
23	23	Helix1	L (98%)	L (99%)
24	24	Loop	K (35%), R (44%)	K (2%), R (34%), Q (30%), G (4%)
47	47	Helix2	K (58%), R (24%)	K (59%), R (4%)
50	49	Helix2	K (93%)	T (10%)
53	50	Helix2	I (74%), T (15%), V (7%)	I (35%), T (1%), V (18%), M (27%)
54	51	Helix2	L (98%)	L (66%), V (7%)
57	54	Helix2	A (76%)	A (59%), I (17%), V (11%), T (10%)
58	55	Helix2	I (31%), T (23%), V (27%)	I (56%), T (2%), V (21%)
60	57	Helix2	Y (77%)	Y (63%)
61	61	Helix2	I (69%), L (16%), V (8%)	I (8%), L (86%)
64	68	Helix2	L (80%), M (7%)	L (74%), M (7%)

Table 2 Comparative of *bHLH* gene family identified unto three species

Subfamily	*A. thaliana*	*M. truncatula*	*M. sativa* L	*Ratios*		
				Mt/A. thaliana	*Ms/A. thaliana*	*Ms/M. truncatula*
I	6	3	1	0.50	0.17	0.33
II	5	4	2	0.80	0.40	0.50
III	10	10	8	1.00	0.80	0.80
IV	4	3	0	0.75	0.00	0.00
V	4	31	20	7.75	5.00	0.65
VI	6	5	5	0.83	0.83	1.00
VII	3	2	3	0.67	1.00	1.50
VIII	8	7	7	0.88	0.88	1.00
IX	10	15	14	1.50	1.40	0.93
X	6	9	8	1.50	1.33	0.89
XII	6	11	3	1.83	0.50	0.27
XV + a	15	12	12	0.80	0.80	1.00
XVI	7	9	13	1.29	1.86	1.44
XVII	5	3	3	0.60	0.60	1.00
XVIII	17	14	15	0.82	0.88	1.07
XIX + a	13	13	13	1.00	1.00	1.00
XX	4	3	1	0.75	0.25	0.33
XXI + a	4	5	5	1.25	1.25	1.00
Total	133	159	133	1.20	1.00	0.84

Number of *bHLH* genes subfamily members categorized into various organisms

and 11 non-E-box binders (lacking Glu-9 or Arg-12) were divided based on the presence or absence of Glu-9 and Arg-12. Most of the non-DNA binding and non-E-box *bHLHs* were distributed in subfamilies 1, 2, 4, 6, 12, 19, and 21. Among 97 E-box *bHLHs*, 80 proteins (containing His/Lys-5, Glu-9, and Arg-13) were predicted to bind the G-box motif (CACGTG), while 17 proteins were predicted to identify other types of E-boxes (CANNTG) and were defined as non-G-box binders (Supplementary Figure S5) in *M. sativa* L.

Phylogenetic Relationships and Multiple Sequence Alignments of the Both *M. truncatula* and *M. sativa* L bHLH Proteins

To obtain more insights into the bHLH protein family, the phylogenetic relationships of the *bHLH* genes family were classified into subfamilies (Table 2). To investigate the classification and evolution of both plant species, we referred to the barrel medic pseudomolecules with other plant sequencing that have been shown the contiguous relationship, for example, synteny and genome duplication history of *Arabidopsis thaliana* displayed the closest similarity (Young et al. 2011). This genomic comparison indicates that there is the same strong ancestors' alteration of the subclades between those plant species. The evolution of 159 full-length *bHLH* of *M. truncatula* (Fig. 1) and 133 full-length *bHLH* of *M. sativa* L protein sequences was rebuilt by using 133 full-length bHLH protein sequences in *Arabidopsis thaliana* as a reference via NJ method, as described in Supplementary Figure S6.

All protein sequences were aligned and the phylogenetic tree analysis showed that MtbHLH proteins could be divided into 18 subfamilies and 17 subfamilies in *MsbHLH* based on the 21 subfamilies that were studied in *Arabidopsis thaliana* (Toledo-Ortiz et al. 2003). The categorizations of these subfamilies were newly renamed based on their members as the clades were represented. Compared to their neighbor clade relationship, each clade was suggested that an individual clade was specific to *M. truncatula* and *M. sativa* L. This indicated that they were segregated to the ancestors from *Arabidopsis thaliana*, but that the 11, 13, and 14 subfamilies of *Arabidopsis thaliana* were not presented because no members of both *M. truncatula* and *M. sativa* L were identified. Remarkably, subfamily 4 in *M. sativa* L did not coincide to the ancestors of *Arabidopsis thaliana*. However, no monophyletic clades were observed in both species. By distinction, among each subfamily, the amino acid sequence was conserved from the short branch length at the information of the tree, which implicates the robust biological process relationships among taxonomic group members. Our understanding of the analysis of the *bHLH* gene family at genome-wide level in both *M. truncatula* and *M. sativa*

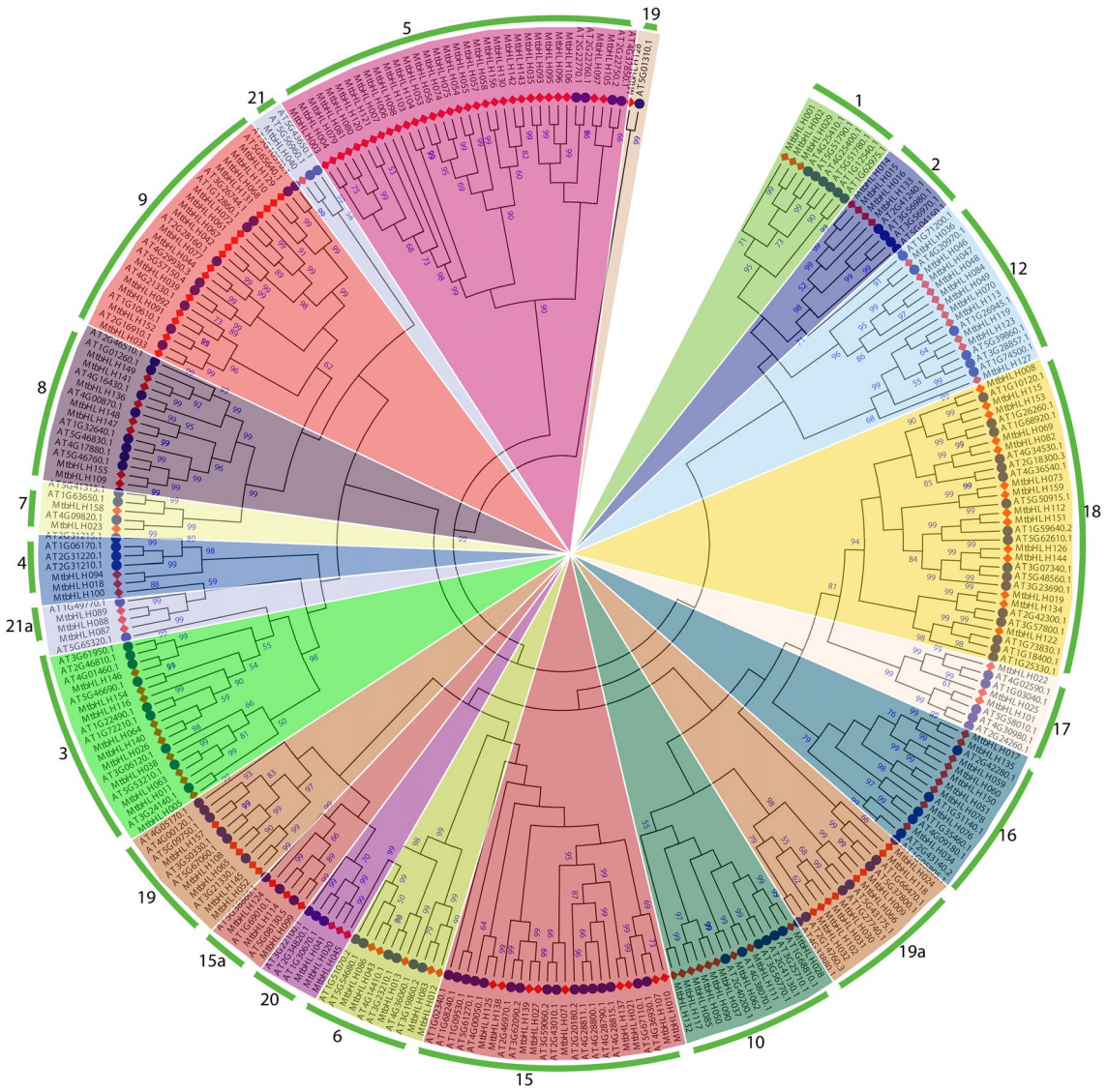

Fig. 1 Phylogenetic relationships of *bHLH* genes from *Medicago truncatula*. A Neighbor-Joining (NJ) phylogenetic tree of all detected bHLH genes was constructed, using MEGA6 program with a bootstrap test of 1000 replicates and Poisson correction distance. Arabic numerals correspond to the *bHLH* subfamily. The colored branch indicates the various subfamilies

L was to make comprehensive predictions of *bHLH* gene function. It is to be noted that the location of *bHLH* gene in the same branch of the phylogenetic tree gives a rough suggestion of their gene function.

Chromosome Location, Tandem, and Gene Structure Prediction of the *bHLH* Genes

Based on the position of each gene, a distinctive pattern of the *bHLH* gene appears in the various certain way; the chromosome was stained to the centromeric arm, respectively. 154 out of 159 *MtbHLH* genes were distributed to all eight chromosomes of the *M. truncatula*. The numbers of genes were increased with the distance from the centromeric and, except the 5 scaffolds genes which were not presented to any chromosome arm, the chromosome 4 was highly found with the largest number of 28 (17.61%) genes which followed by chromosome 1 with 27 (16.98%) genes. By contrast, chromosome 6 was the lowest in number with only 4 (2.5%) genes. The tandem and segmental duplication of *MtbHLH* members were characterized; 23 tandems duplicate *MtbHLH* genes were identified based on the phylogenetic tree's clades information (Fig. 1), where the tandem considered was above 80% of the clades and 44/159 (27.76%) genes were segmented among all the *bHLHs* in *M. truncatula* (Supplementary Figure S7). In order to better understand the divergence and duplication of *MtbHLH* gene family, we

also examined the synonymous substitution rate (Ks) and non-synonymous substitution rates (Ka); the ratio of Ks/Ka for the tandem duplication was ranged from 0.001 to 0.86 (Table S5). The results of gene structure revealed 5.66% (*MtbHLH071*, *-137*, *-024*, *-113*, *-071*, *-119*, *-123*,*-127*, and *-040*) of *MtbHLH* genes without intron in *M. truncatula,* as shown in Supplementary Figure S2.

Network Analysis of *bHLH* Genes and 3D Modeling Prediction

The above results, which are quite significant, asserted that the 7 *MtbHLH* and 10 *MsbHLH* genes which were selected, respectively, are involved in the abiotic stress responses in these two species as the result of interaction evidence with other plants, including *Arabidopsis thaliana*. A computation tool, string, was used to compute the protein-proteins interactive network as a ubiquitous and well-thought-out method (Kovács et al. 2019). We endeavored to construct a comprehensive regulatory network of both species by analyzing protein–protein interactions and target predictions of *MtbHLH* and *MsbHLH* relationships to abiotic stresses. Based on the analysis shown above, the regulatory network established that the 159 MtbHLH proteins contain the bitscore ranged between 68.9 and 547.7, only five proteins (*MtbHLH018*, *MtbHLH049*, *MtbHLH087*, and *MtbHLH100*) did not share the interaction network, and 133 MsbHLH proteins shared the internodes interactions with the bitscore ranging between 68.2 and 555.4; not including the five proteins (*MsbHLH087*, *MsbHLH090*, *MsbHLH094*, *MsbHLH123*, and *MsbHLH129*), the comparable mechanism in both plants species was observed at the transcription factors levels. The *MsbHLH* gene family has the closest common interaction network with the *MtbHLH* genes between them, and strongly resembles the gene's functions of *Arabidopsis thaliana* that were used as a reference. This indicates a high degree of likelihood that the unmatched genes could have the same structure and function. Within this network, multiple interaction patterns were recognized, such as one-to-one, one-to-many, and many-to-many. We extracted the different sub-network from the above-mentioned regulatory network to further investigate *bHLH* a-dependent regulatory relationships under abiotic stresses. The essentials core elements were analyzed (ICE1 mutants are defensive in cold-regulated gene expression *MsbHLH056,-055,-56,-57* and *MtbHLH061*; *MtbHLH131* shared interactive network; MYC2 with the cooperation of MYB2 is involved in the regulation of ABA-inducible genes under drought stress conditions which shared the interactive network with *MsbHLH043* and *MsbHLH042*; and *MtbHLH109* shared the network with *AtbHLH093* (Heim 2003), which were well known in those sub-networks, which were integrated between them. It was noted that these associations confirmed a clear selectivity and affinity of the positive proteins that are more resilient to plant stress and supported the phylogenetic relationships (Supplementary Figure S8; Supplementary Figure S9). The 3D modeling studies shown that the stability and the vibrant form of these proteins may play key roles in response to abiotic stresses. Homology 3D protein structures represent the most important problems in computational structural biology (Zhang 2008). Our results showed that 12 genes of *MtbHLH* were able to predict a 3D proteins homology, the confidence was > 90%, and the percentage residues ranged from 80 to 100, including *MtbHLH001, -036, -038, -047, -084, -092, -100, -111, -113, -119,-123, and -127* in *Medicago truncatula* (Fig. 2), while a total of 25 MsbHLH proteins were predicted to the 100% of confidence and the percentage residues were varied from 70 to 100. The structure encloses the conserved hour-glass model with a pore-forming integral membrane protein, including the formation of six transmembrane helices (TM, S1 to S6) that were downloaded, as shown in Supplementary Figure S10. After identification of the *bHLH* genes in these two plants, we then inquired whether all genes shared the response to abiotic stresses. To assess this, we analyzed GO terms (see methods); our results showed that the 159 *MtbHLH* and 133 *MsbHLH* genes were assigned terms under the cellular component, biological process, and molecular function, respectively. It was observed that the biological process was quite high than others in both plants, including cellular process, biological regulation, and metabolic process (see Supplementary Figure S11).

Expression Patterns of *bHLH* Genes in Various Organs and at Different Abiotic Stresses

To unveil the *MtbHLH* and *MsbHLH* genes' characteristics that regulate the barrel medic and alfalfa species development in the three different tissues/organs, root, stem, and leaf, the expression profile were conducted under standard growth conditions. We clarified the profound understanding of the transcripts, that is, sensing of stress, organs/tissues response, and adaptation of the *M. truncatula* and *M. sativa* L genes. The expression profiles of each gene were downloaded at the *Medicago truncatula* Gene Expression Atlas (MtGEA) Web Server (https://mtgea.noble.org/v3/) and https://plantgrn.noble.org/AGED/index.jsp to assess the biological processes in roots, stems, and leaves. The analysis of every single gene was revealed and 159 *MtbHLH* genes in the three organ/tissue samples of *M. truncatula* (Fig. 3), 20 *MtbHLH* genes were not identified in any organs, and 139 genes were presented in at least one of the three organ/tissues. Even though these genes were found in every single organ, the roots were not only highly expressed but also highly sensitive to abiotic stress, which implicated the alterability of the expression patterns in the remaining organ/tissues. Nevertheless, the heatmap

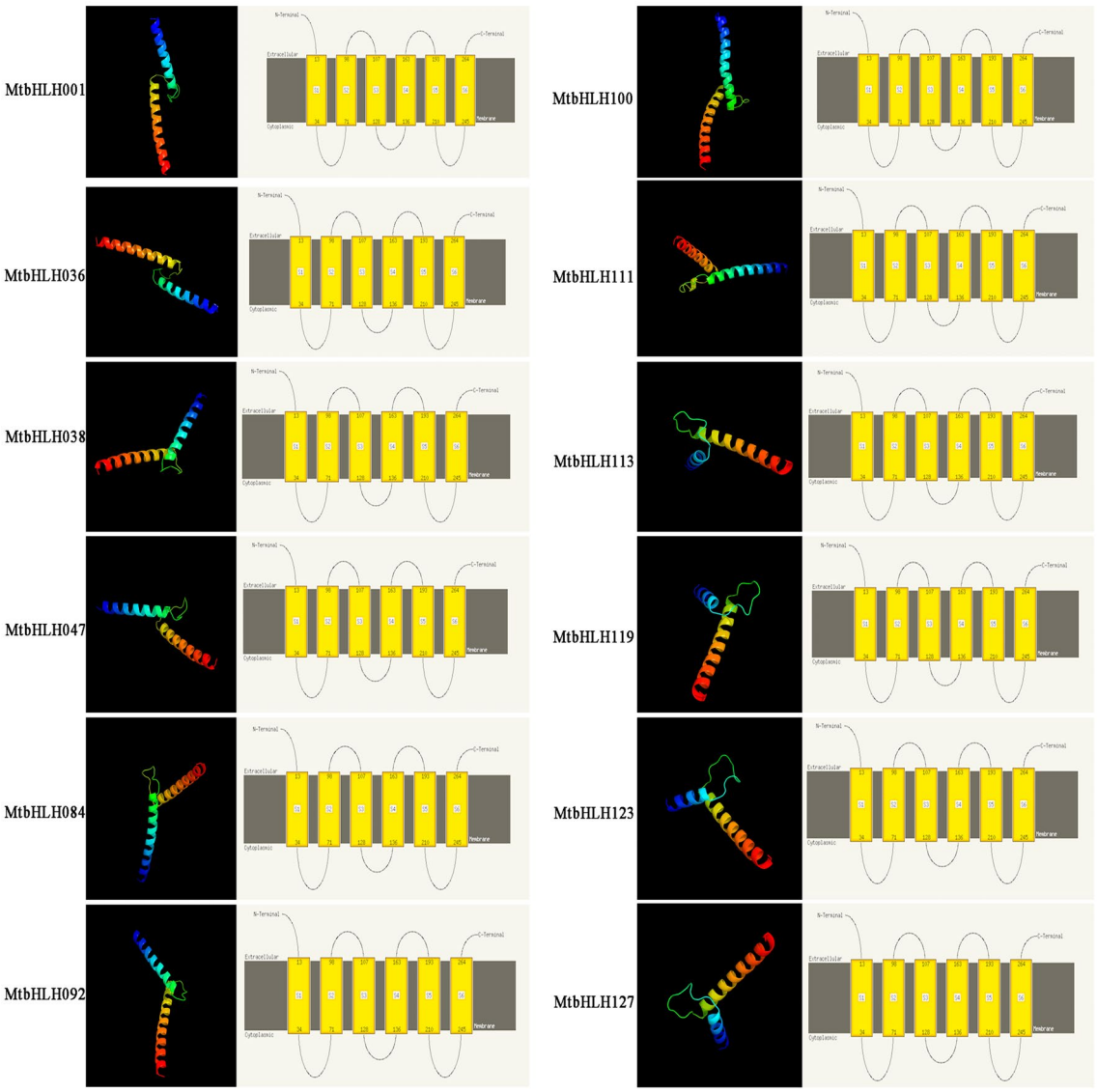

Fig. 2 Predicted 3D Modeling structure and transmembrane helix of 12 MtbHLH proteins. The 3D structure and TM Helix of *MtbHLH* are representing each subfamily. Modeled at > 90% confidence level by using Phyre2 server. The extracellular and cytoplasmic sides of the membrane are labeled and the beginning of each transmembrane helix established with a number representing the residue index.TM, Transmembrane

results confirmed the clusters through the subfamily, one hundred and thirty-nine *MtbHLHs* belonging to 18 subfamilies were differentially high in both stem and leaf. Among these, *MtbHLH* genes, 33 were highly expressed in root organ. In this regard, 7 *MtbHLH* genes (*MtbHLH012*, *-141*, *-061*, *-083*, *-109*, *-131*, and *-149*) were selected as the best highly expressed at the transcription levels that might respond to abiotic stress, including drought and salt stresses. Despite that, the expression profile of *Medicago sativa* L data was accessed and visualized at the online web server, three biological processes in tissues/organs (root, elongation stem, and leaf). 133 *MsbHLH* genes, the identified *M. sativa* L sequences, were used to retrieve the contig numbers (Table S6) but some genes were not found, including *MsbHLH071*, *-137*, *-024*, *-113*, *-070*, *-123*, *-127*, and *-040*, as shown in Fig. 4. One hundred and twenty-three *MsbHLHs* were constructed heatmap. Interestingly, we also observed that *MsbHLH* proteins of subgroup VIII and IX displayed crucial expression profiles in various organs and in response to abiotic stresses, which were similar to the results reported in previous studies (Bailey et al. 2003; Carretero-Paulet et al. 2010; Toledo-Ortiz et al. 2003). Hence, we considered 10 *MsbHLH* genes (*MsbHLH056*, *MsbHLH041*, *MsbHLH042*,

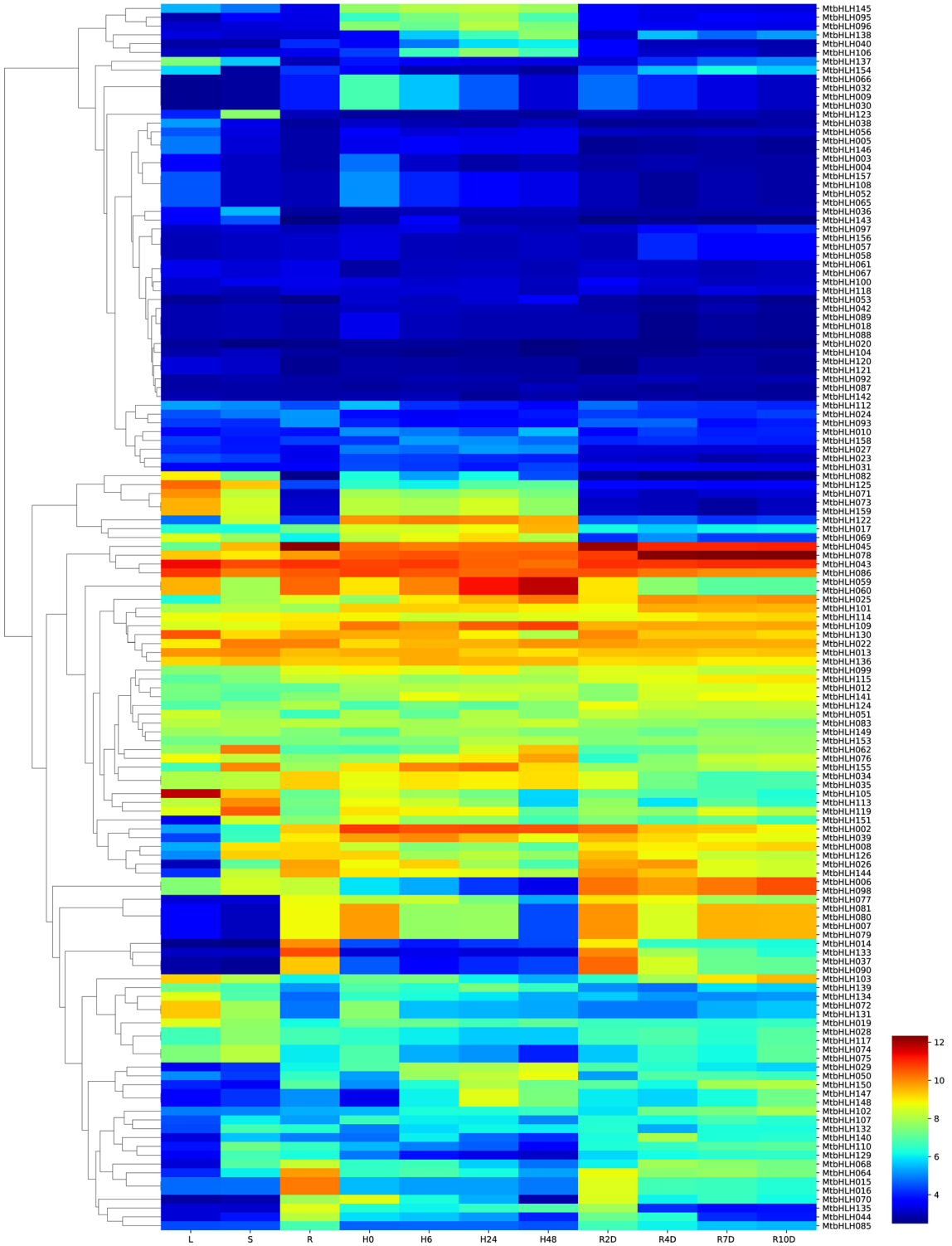

Fig. 3 Expression profile of *bHLH* genes in various organs of *Medicago truncatula* at different abiotic stresses. Clustering expression analysis of the 139 *bHLH* genes was illustrated in various organs. Fragments per kilobase of transcript per million mapped reads (FPKM) values of *MtbHLH* genes were transformed by log2, and the heatmap was constructed with HemI. The clustering tree was constructed by hierarchical clustering using the average linkage method. The color represented the relative expression

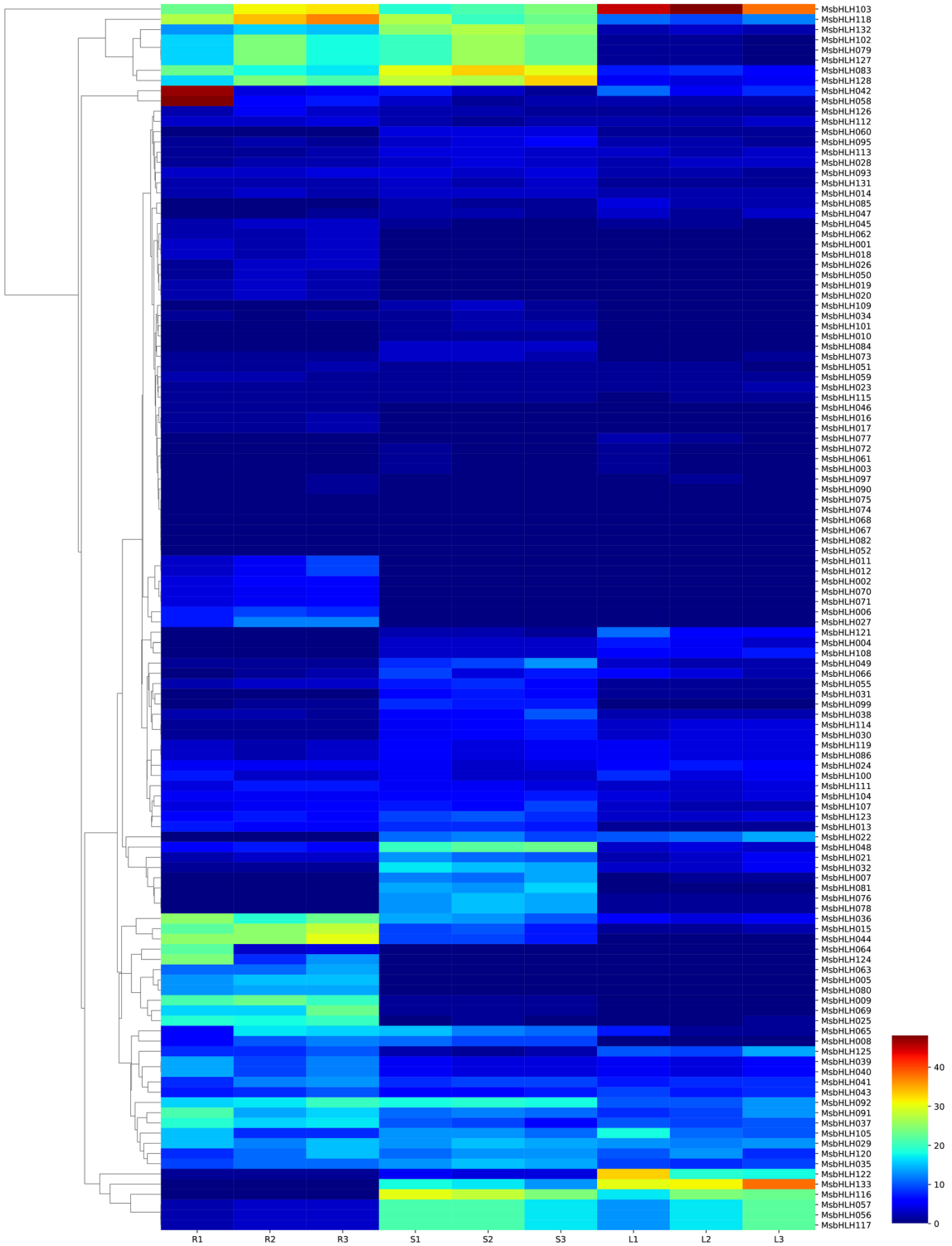

Fig. 4 Expression profile of *bHLH* genes in various organs of *Medicago sativa* L at different abiotic stresses. Clustering expression analysis of 123 *bHLH* genes illustrated in various organs. *MtbHLH* genes were transformed by log2, and the heatmap was constructed with HemI. The clustering tree was constructed by hierarchical clustering using the average linkage method. The color represented the relative expression

-043 (VIII), *MsbHLH055*, *-057*, *-058*, *-102*, *-117* (IX), and *MsbHLH063* (X)) were chosen to scrutinize the expression profile levels in the three tissues/organs (leaf, stem and root) for both drought, salt, and cold stress conditions, as shown in Supplementary Figure S9. We found that all the genes were expressed at least in one tissue/organ of alfalfa, except the five genes were not expressed in any organs, including *MsbHLH082*, *MsbHLH052*, *MsbHLH067*, *MsbHLH068,* and *MsbHLH074* as they are appearing in light green color, as shown in Fig. 4.

Discussion

The myriad problems that affect plants today are the revolutionized environmental and abiotic stresses that limit plant growth and yield (Yadav and Sharma 2016). A various genomics approach has suggested and revealed a clear overlap in the gene expression in the different stresses. A basic helix-loop-helix transcription factor, as mentioned as the second largest potential transcriptional factor gene family that contributed to control the expression of multiple target genes concerned in abiotic stress, plays a pivotal role in response to signal transduction and environment stimuli, which enhances stress tolerance (Yadav et al. 2013). Nonetheless, this *bHLH TF* family has not been reported in barrel medic and alfalfa. To elucidate this problem, we tended to identify *bHLH* members in *M. truncatula* and *M. sativa* L species underlying abiotic stress. A compendious analysis of 159 and 133 *bHLH* was used to obtain a global view of gene function under various abiotic stresses in both species, respectively. Most *bHLH* functional studies currently focus on the response to abiotic stresses, such as drought, salt, and cold stresses. In this analysis, quantitative PCR in real-time was used to examine the patterns of expression of the 17 *bHLH* genes in *M. truncatula* and *M. sativa* L species. Ascertain many *bHLH* members in *A. thaliana* and rice have been investigated in detail, and their homologous genes could be used to predict the functions of *bHLH* TF in *M. truncatula* and *M. sativa* L based on the biological roles of *Arabidopsis* and Rice. To investigate the *bHLH* genes family, we compared the phylogenetic tree relationship between *MsbHLH* (17) and *MtbHLH* (18) proteins, respectively. The subfamily showed the strong similarity of the proteins in both species. Among these *bHLH* genes, VI, VIII, and IX subgroups in *M. truncatula* showed high similarity to *AtbHLH* genes (III and IV) (Bailey et al. 2003; Zhao et al. 2013), which could positively be the interesting key role that is proving the higher transcriptional activity of genes response to drought, salt, and cold stress conditions (Gao et al. 2019). It is noted that seven genes in *MtbHLH* (*MtbHLH012*, *-061*, *-083*, *-109*, *-131*, *-141*, and *-149*) and ten genes in *MsbHLH* (*MsbHLH056*, *-041*, *-042*, *-043*, *-055*, *-057*, *-058*, *-062*, *-063*, *-102*, and *-117*) could be deciphered by the fact that these genes develop different regulatory mechanism to respond to multiple abiotic stresses.

Studies have accepted that abiotic stresses, such as salt stress, drought stress, and cold stress, influence the development of alfalfa and barrel medic in most research on the stress tolerance of legumes (Jin et al. 2019). Meanwhile, a specific nodulation inhibition and resulting nitrogen deficiency may depress yields in such saline soils. In *A. thaliana*, functions of many bHLH proteins have been characterized. In subgroup Ia, MUTE (MacAlister and Bergmann 2011; Pillitteri et al. 2008), SPCH (MacAlister and Bergmann 2011), and bHLH071 were associated with stomatal development, and sepal fusion and organ size related to HWS (Lang et al. 2018). Meanwhile, in *M. truncatula* and *M. sativa* L, MUTE (*MsbHLH110* and *MtbHLH038*) and SPCH (*MsbHLH109*, *MtbHLH011,* and *MtbHLH063*) were also suggested to play a role in the differentiation and growth of stomatal cells, and they are suggesting that members of subgroup Ia could be mainly involved in cell division and organ differentiation in both plant species. In subgroup Ib, ORG2, ORG3 (Omidbakhshfard et al. 2018), and bHLH100 (Sivitz et al. 2012) play a crucial role in the iron deficiency response. Furthermore, FRU (*MtbHLH044*, *MtbHLH077*, *MsbHLH070*, *MsbHLH069*, and *MsbHLH071* in this study) is also suggested to be an essential regulator for Fe uptake and iron use in Alfalfa (Cui et al. 2018b; Fourcroy et al. 2014).

In *A. thaliana*, the three bHLH010, bHLH089, and bHLH091 members of subgroup II were reported to interact with downstream *bHLH* Factors Promotes DYT1 Nuclear Localization and participate redundantly in the anther development (Cui et al. 2016). Similarly, *M. truncatula* and *M. sativa* L DYT1 (*MsbHLH089*, *MtbHLH091*, and *MtbHLH092*) were suggested to play a key role in early-stage anther development. AMS (*MsbHLH053* and *MtbHLH033*) and DYT1 (*MsbHLH089*) were identified as master regulators of pollen production in subgroup IIIa (Sorensen et al. 2003). AtICE1, AtICE2, *MsbHLH055*, *MtbHLH072*, *MtbHLH067*, *MtbHLH061*, *MtbHLH042*, *MsbHLH056*, *MsbHLH057,* and *MsbHLH117* were shown to be involved in the response to deep freezing in IIIb (Budhagatapalli et al. 2016; Huang et al. 2015). For *A. thaliana*, mutual of ICE1 and *AtbHLH093* were related for deacclimation (Oono et al. 2006), which was investigated in cold stress, and AT1G01260 suggested transcription regulation activity (Heim et al. 2003; Riechmann et al. 2000). In *A. thaliana*, functionally characterized only one member of Subgroup IIIc, At4g29930, played a crucial role in hypocotyls control and root to abiotic stress (Dinneny et al. 2008). In contrast, three *M. sativa* L members shared a relationship with *MsbHLH058*, *MsbHLH070*, and *MsbHLH071,* which were suggested to

be in transcriptional induction activation during wound and drought stresses. In *A. thaliana*, IIId members (JAM1-JAM3 and bHLH014) and IIIe bHLHs (MYC2–MYC4) were proposed to take part in JA-mediated plant development. In cooperation with MYB2, ABA-inducing genes are regulated under conditions of drought stress (Sasaki-Sekimoto et al. 2013). Similarly, in *M. truncatula* and *M. sativa* L, some *bHLH* genes (*MsbHLH041*, *MsbHLH042*, *MsbHLH043*, *MtbHLH155*, *MtbHLH149*, *MtbHLH148*, *MtbHLH147*, and *MtbHLH109*) shared a relationship with MYC2, suggested to be played an important role in regulation of drought conditions. Subgroup III Protein TT8 participates in anthocyanin and PA pathways, close to IIIf members in *A. thaliana*, suggesting participation in the biosynthesis of anthocyanidin. Similar functions were observed in *M. truncatula* and *M. sativa* L and two members (*MsbHLH115* and *MtbHLH023*) of *bHLH* family gene were suggested to participate in the biosynthesis of anthocyanidin. Besides, three other III proteins were found to be involved in the formation of epidermal cells in *A. thaliana* (GL3, EGL3, and MYC1) (Bernhardt et al. 2003). Proteins IVb (PYE) and IVc (bHLH034, bHLH104, bHLH115, and ILR3) have been shown to modulate metal homeostasis (Gao et al. 2020). Similarly, *M. truncatula* and *M. sativa* L GL3 (*MsbHLH040*), EGL3 (*MtbHLH158* and *MsbHLH039*) in subgroup VII and subgroup II proteins bHLH034 (*MsbHLH026* and *MsbHLH002*), subgroup VI bHLH104 (*MtbHLH013*), bHLH115, and ILR3 (*MsbHLH025*, *MtbHLH043*, *MtbHLH086*, *MtbHLH045*, and *MsbHLH024*) were also suggested to control iron homeostasis. Members of subgroup VIIIc (RHD6, RSL1, RSL2, RSL4, and At2g14760) were suggested as necessary for root hair growth (Bernhardt et al. 2003; Lin et al. 2015; Rymen et al. 2017), while two proteins studied (bHLH068 and bHLH112) were able to respond to abiotic stress (Wang et al. 2018b). Similarly, members of subgroup XIX were suggested to play a crucial for root hair growth in *M. truncatula* and *M. sativa* L, including RHD6 (*MtbHLH118*), RSL4 (*MtbHLH066*), RSL1, RSL2 (*MtbHLH009*, *MtbHLH030*, and *MtbHLH031*), and At2g14760 (*MtbHLH102*, *MtbHLH032*, *MsbHLH017,* and *MsbHLH018*). The present study reveals that stress tolerance of leaf, stem, and root is enhanced by abiotic stresses on these plant parts in *M. truncatula* (drought and salinity stresses) and *M. sativa* L (drought, salinity, and cold stresses) species.

To increase the evidence, we verified 7 *MtbHLH* genes to the various types of stress responsiveness, including *MtbHLH012*, *-061*, *-083*, *-109*, *-131*, *-141*, and *-149* (MBS*), MtbHLH149* (TC-rich repeats); *MtbHLH141*, *MtbHLH012*, *MtbHLH061,* and *MtbHLH149* (ARE); *MtbHLH109* and *MtbHLH131* (ABRE); *MtbHLH012* and *MtbHLH061* (ERE); *MtbHLH012*, *-061*, *-083*, *-109*, *-131*, *-141*, and *-149* (HSE); and *MtbHLH012* and *MtbHLH061* (LTR) for the selected *bHLH* genes in *M. truncatula* under abiotic stresses. The observations above are supporting the interaction regulatory network of barrel medic gene expression. Furthermore, the analyses of gene ontology enhance the significant

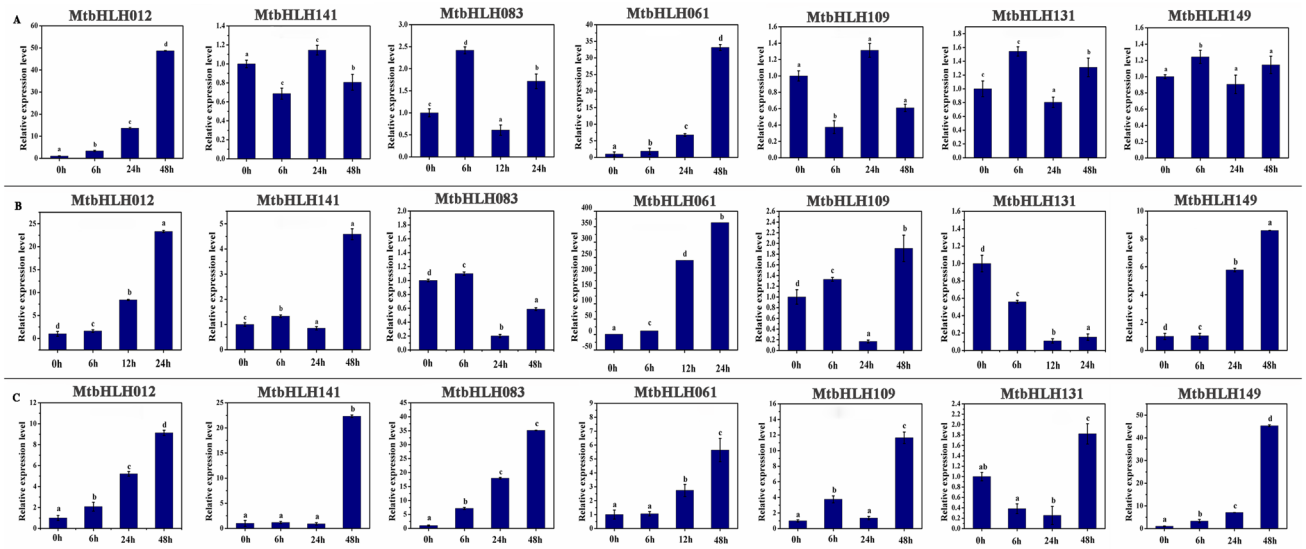

Fig. 5 qRT-PCR validation of *MtbHLH* genes in the response to salt stress. The relative expression levels of the *bHLH* genes in (**a**) the leaf, (**b**) stem, and (**c**) root of *M. truncatula* seedlings treated with 180 mM NaCl were determined by qPCR. The Errors bars were obtained from three measurements. Small letter(s) above the bars indicate significant differences ($\alpha = 0.05$, LSD) among the treatments. Duncan's test was used and the results are represented by means ± standard deviations; relative expression level is compared with that of the control (0 h). The relative expression level was calculated by $2^{-\Delta CT}$ method comparing with that of *ef1a.* LSD, least significant difference; qPCR; qRT-PCR, quantitative real-time PCR

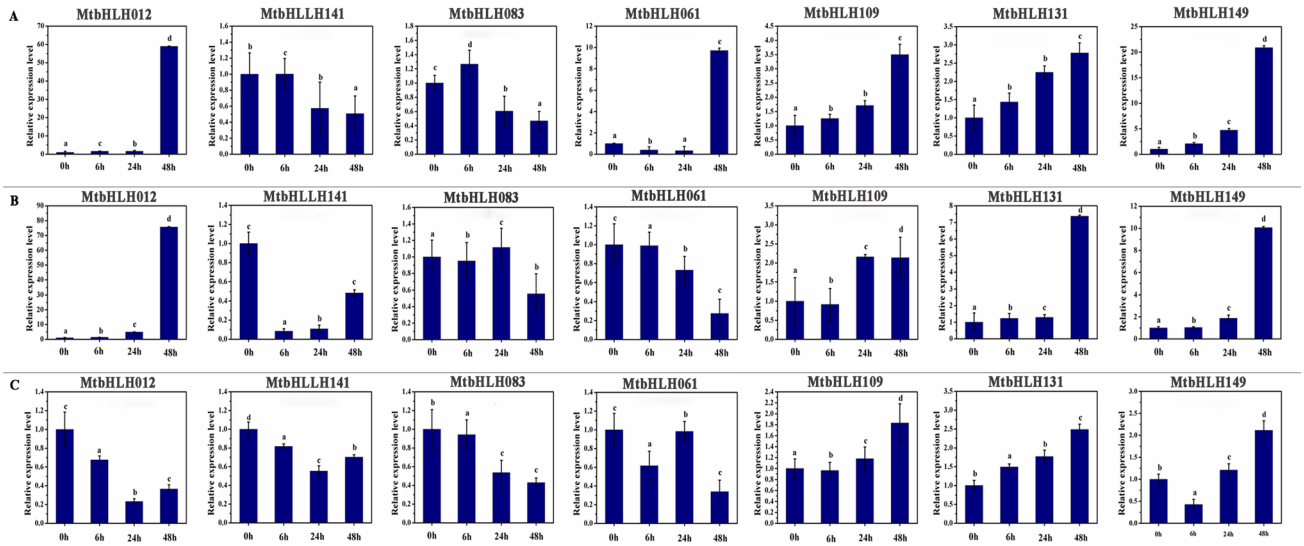

Fig. 6 qRT-PCR validation of *MtbHLH* genes in the response to drought stress. The relative expression levels of the *bHLH* genes in (**a**) the leaf, (**b**) stem, and (**c**) root of *M. truncatula* seedlings treated with (15% PEG-6000) were determined by qPCR. The Errors bars were obtained from three measurements. Small letter(s) above the bars indicate significant differences ($\alpha = 0.05$, LSD) among the treatments. Duncan's test was used Duncan's test was used and the results are represented by means ± standard deviations; relative expression level is compared with that of the control (0 h). The relative expression level was calculated by $2^{-\Delta CT}$ method comparing with that of *ef1a.* LSD, least significant difference; qPCR; qRT-PCR, quantitative real-time PCR

functions of those genes related to abiotic stresses. Gene ontology analyses discovered that most of the *bHLH* genes members in both barrel medic and alfalfa were localized in the nucleus, as described in Supplementary Figure S11. Also, some biological processes were overrepresented in all *bHLH* genes identified in these two species, including metabolic process (GO: 0008152), cellular process (GO: 0009987), and biological regulation (GO: 0065007). These phytohormones play a crucial role in plant growth and development, especially MBS had confirmed the drought stress in all seven *MtbHLH* genes that were validated by quantitative real-time PCR analyses.

A remarkable conclusion in these *bHLH TF* analyses is that not only root tissues, which are the most severe stress-related, but also the other plant's tissues/organs, especially in *M. truncatula* (Fig. 5). The expression profile showed that the three sampled organs were expressed in different ways; it is important to note that the transcription factor in either up- or down-regulation genes of *bHLH TF* was revealed to be the positive biological pathway mechanisms (Figs. 5, 6, 7, and 8). It directed us to understand the most severely stressed part of each plant species (Figs. 3 and 4) deeply. Different reports showed the highest expression in two tissues/organs (leaf and root) to salt stress and others to drought and cold stresses (Amirbakhtiar et al. 2019; Chinnusamy et al. 2003; Gao et al. 2017; Golldack et al. 2014). The quantitative real-time PCR results showed that *MtbHLH012*, *MtbHLH109*, *MtbHLH131,* and *MtbHLH149* were up-regulated in all three plant tissues (leaf, stem, and root), while *MtbHLH061*, *MtbHLH083*, and *MtbHLH141* were down-regulated, for drought stress-related, as shown in Fig. 6. The qRT-PCR also proved that *MtbHLH012* and *MtbHLH061* were up-regulated in leaf, stem, and root tissues; *MtbHLH083*, *MtbHLH141*, and *MtbHLH149* were up-regulated in only stem and root tissues, while *MtbHLH109* and *MtbHLH131* were down-regulated in leaf, but *MtbHLH109* was seemed to be up-regulated in stem and root for salt-related stress after stress treatment, and the results were analyzed based on the control (regularized to 1). Based on the highest expressed genes in transcriptome data, *MtbHLH149* was up-regulated to qRT-PCR results for salt and drought stresses (Figs. 5 and 6). Interestingly, some *bHLH* genes, including *MtbHLH149* and *MtbHLH109* was suggested to stake the same function to the mutant of *MtbHLH-658* underlying salt tolerance (Aftab et al. 2015; Zahaf et al. 2012).

Meanwhile, the validation of quantitative real-time PCR of the 10 genes selected and conducted in *M. sativa* L revealed that except *MsbHLH063* was down-regulated in stem tissues and *MsbHLH042*, *MsbHLH102*, and *MsbHLH058* were down-regulated in root tissues, others were all up-regulated (*MsbHLH056*, *MsbHLH041*, *MsbHLH055*, *MsbHLH057*, *MsbHLH043*, *MsbHLH102*, and *MsbHLH117*) in leaf, stem, and root tissues after salt treatment (Fig. 7). The *MsbHLH063* was down-regulated in stem tissue; *MsbHLH042*, *MsbHLH058*, *MsbHLH043*, and *MsbHLH102* were down-regulated in roots after salt

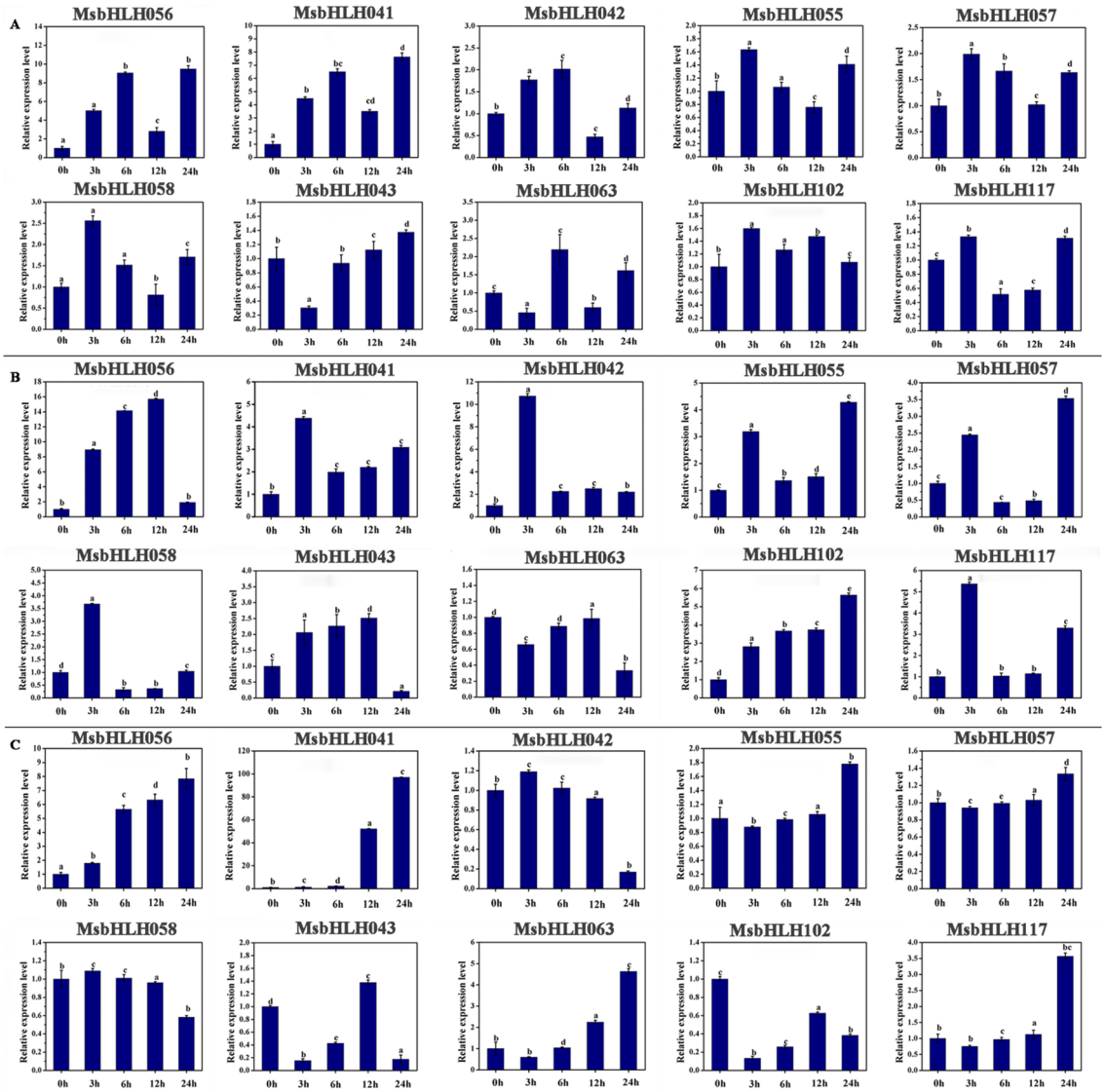

Fig. 7 qRT-PCR validation of *MsbHLH* genes in the response to salt stress. The relative expression levels of the *bHLH* genes in (**a**) the leaf, (**b**) stem, and (**c**) root of *M. sativa.* L seedlings treated with 180 mM NaCl were determined by qPCR. The Errors bars were obtained from three measurements. Small letter(s) above the bars indicate significant differences ($\alpha = 0.05$, LSD) among the treatments. Duncan's test was used and the results are represented by means ± standard deviations; relative expression level is compared with that of the control (0 h). The relative expression level was calculated by $2^{-\Delta CT}$ method comparing with that of *ef1a.*LSD, least significant difference; qPCR; qRT-PCR, quantitative real-time PCR

treatment, while *MsbHLH056*, *MsbHLH041*, *MsbHLH055*, *MsbHLH057*, and *MsbHLH117* were up-regulated in three tissues/organs (leaf, stem, and root) after drought stress treatment, as shown in Fig. 8. In addition, the *MsbHLH102* was down-regulated in leaf, while other genes were up-regulated for the 6 h after cold stress treatment. The *MsbHLH042* and *MsbHLH055* were down-regulated in root tissue, whereas the *MsbHLH056*, *MsbHLH041*, *MsbHLH117*, *MsbHLH057*, *MsbHLH058*, *MsbHLH043*, *MsbHLH102*, and *MsbHLH063* were up-regulated in stem and root tissues after cold stress treatment, as shown in Supplementary Figure S12. In brief, the expression pattern and qRT-PCR validation under abiotic stresses are reliable according to the results proved by the protein–protein interaction network analyses in *M. truncatula* and *M. sativa* L species. Howbeit, the entire selected new candidates consequently showed mutually exclusive of its expression pattern to play a pivotal role in plant regulation and developmental processes. Accordingly,

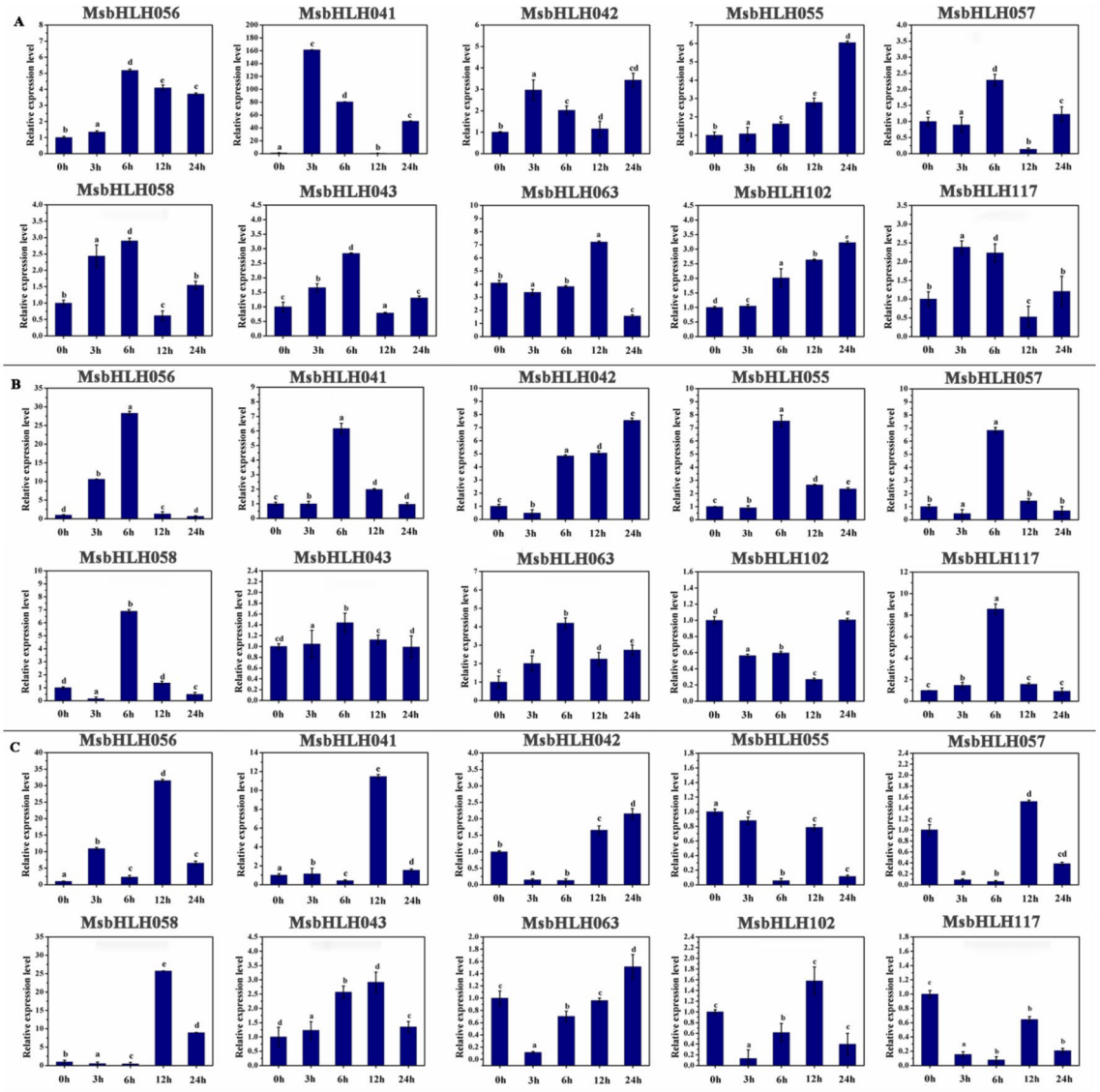

Fig. 8 qRT-PCR validation of *MsbHLH* genes in the response to drought stress. The relative expression levels of the *bHLH* genes in (**a**) the leaf, (**b**) stem, and (**c**) root of *M. sativa* L seedlings treated with (15% PEG-6000) were determined by qPCR. The Errors bars were obtained from three measurements. Small letter(s) above the bars indicate significant differences ($\alpha = 0.05$, LSD) among the treatments. Duncan's test was used Duncan's test was used and the results are represented by means ± standard deviations; relative expression level is compared with that of the control (0 h). The relative expression level was calculated by $2^{-\Delta CT}$ method comparing with that of *ef1a.* LSD, least significant difference; qPCR; qRT-PCR, quantitative real-time PCR

MtbHLH012&MtbHLH083, *MtbHLH149,* and *MsbHLH057* (Heim 2003) suggested a resilient fitness characteristic against salt stress; salt and drought stresses; and cold stress, respectively. For instance, *MtbHLH012* and *MtbHLH083* have shared the ancestor in the same clade with *AtbHLH034,* which is involved in response to salinity stress, modulation glucose (Glc), and ABA (Min et al. 2017). Besides, it is found that MYC2 was highly expressed in various treatments, including, MeJA, ABA, Salinity, and drought stresses as well as pathogen infection. This shows the *bHLH* genes functionally evolvement (Fengli Zhao 2018); some *bHLH* genes in *M. truncatula* and *M. sativa* L were interacted with MYC2, including, *MsbHLH041*, *MsbHLH042*, *MsbHLH043*, *MtbHLH109*, *MtbHLH147*, *MtbHLH148*, and *MtbHLH155* (Supplementary Figures S8 and S9). In these two species, results of *M. truncatula* show that the expression pattern levels were compared with the *bHLH* mutants at *Medicago truncatula* mutant resource: https://medicago-mutant.noble.org/mutant/index.php and we found that *bHLH* mutants are corresponded to the gene expression at rate percentage range of 95 to 100, apart from *MtbHLH061* was 69% as

described in Table S7. In conclusion, the *bHLH* gene family suggested the functional enhancement of the two plant tissues expressed stress-induced shreds of evidence in barrel medic and alfalfa. This study will provide an insight into a further understanding of *bHLH* TFs member's functions in *M. truncatula* and *M. sativa* L.

Conclusion

The presented results in this study highlight and provide an outline of systematic molecular response to abiotic stress in both barrel medic and alfalfa. We describe and perform a compendious genome wide of the *bHLH* gene family in these two species as the notable legumes species around the globe. A total of 159 *bHLH* genes in *M. truncatula* and 133 *bHLH* genes in *M. sativa* L were identified and characterized, divided them into 18 subgroups and 17 subgroups, respectively. The *MtbHLH* and *MsbHLH* family are likely to be phylogenetic and highly complex and might include a range of key processes to cope with the abiotic stresses in these two plant species. Both hierarchical clusters and screening of genes ontology predictions, which supported by the protein–protein network interaction analyses, established that there is a major distinction time progression from the drought-stress-related, salt-stress-related, and cold-stress-related sensitively. The expression profile of the *bHLH* genes denotes that the different organs/tissues evolved in the acclivity of this gene family in these plant species. For instance, the expression of *MtbHLH012*, *MtbHLH109*, *MtbHLH131,* and *MtbHLH141; MsbHLH056*, *MsbHLH041*, *MsbHLH117*, *MsbHLH057*, *MsbHLH058*, *MsbHLH043*, *MsbHLH102*, and *MsbHLH063* was significantly induced after stresses conditions. Based on these analyses, we suggested that this transcription factor (TF) plays a pivotal role in regulating genes that respond to abiotic stresses. Further, the transcriptome storage data that we have generated here will provide valuable perceptions about the molecular mechanism of the metabolic pathways for improving tolerance in plants. In addition, the predominance of genes induction under the salt, drought, and cold stresses evaluated in both *M. truncatula* and *M. sativa* L indicates a continuous adaptation over an extended period of time. However, further molecular analysis is needed to study the overexpression of these up-regulated genes, endeavoring at enhancing the abiotic stresses tolerance in barrel medic and alfalfa species.

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Author Contributions WL and BN conceived and designed research. BN, XJ, XM, XS, and XF performed the experiments. NB, XM, and WL draw figures and tables, and analyzed the data. BN and WL wrote the manuscript. All the authors read and approved the manuscript.

Compliance with Ethical Standards

Conflict of interest No competing financial interests exist.

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