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# New Insights into Structure and Function Predictions of *TIFY* Genes in Barley: A Genome-Wide Comprehensive Analysis

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**Abstract:** Barley (*Hordeum vulgare* L.) is the fourth-largest cereal crop widely grown for livestock feed, brewing malts and human food. The *TIFY* family is a plant-specific protein family with diverse functions in plant growth, development and stress responses. However, a genome-wide comprehensive analysis of the *TIFY* gene family has not yet been characterized in *Hordeum vulgare*. In the present study, 21 and 22 *TIFY* family members were identified in the genomes of *Hv_Morex* and *Hv_Barke*, respectively. The *HvTIFY* proteins could be divided into the TIFY, ZIM/ZML and JAZ groups, and the JAZ group could be further clustered into six subgroups. *HvTIFY* genes were conserved in the two genotypes, and all of the duplicated gene pairs in the barley *TIFY* family were dominated by intense purifying selection. Tandem duplication was the main driving force for the expansion of the *HvTIFY* gene family. In silico gene expression profiling revealed most members of the *Hv_Morex JAZ* group were predominantly expressed in reproductive organs and root tissues and were also more involved in the responses to cold treatment and spot blotch infection than other groups. Quite a few *JAZ* genes (*Hv_MoJAZ1*, *Hv_MoJAZ4*, *Hv_MoJAZ6*, *Hv_MoJAZ9*, *Hv_MoJAZ11*, *Hv_MoJAZ12* and *Hv_MoJAZ14*) were found to be tightly associated with the growth of barley and the responses to cold and spot blotch infection stresses. The genome-wide comprehensive analysis of the structure and function of the *HvTIFY* genes will contribute further to our understanding of the functions of these genes in response to abiotic and biotic stresses in *Hordeum vulgare*.

**Keywords:** *TIFY* family; *JAZ* gene; *Hordeum vulgare*; phylogenetic analysis; expression profile

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**Citation:** Li, J.; Xu, X.; Wang, H.; Zhang, Y. New Insights into Structure and Function Predictions of *TIFY* Genes in Barley: A Genome-Wide Comprehensive Analysis. *Agronomy* **2024**, *14*, 1663. https://doi.org/10.3390/agronomy14081663

Academic Editor: Fangbin Cao

Received: 27 May 2024
Revised: 21 July 2024
Accepted: 24 July 2024
Published: 29 July 2024

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## 1. Introduction

The *TIFY* gene family is plant-specific gene family coding for transcription factors (TFs) involved in the regulation of plant growth and development and a variety of stress responses. All *TIFY* factors feature a conserved TIFY motif (TIF[Y/F]XG) that is composed of approximately 36 conserved amino acids (AA) [1,2]. *AT4G24470*, the first characterized *TIFY* gene, was reported to be involved in inflorescence and flower development [1,3]. It was previously annotated as *ZIM* for its structure of a C2C2-GATA zinc-finger structure and later renamed as the *TIFY* family gene to avoid confusion with the *ZIM* nomenclature [1,4]. In general, *TIFY* genes can be classified into the four different subfamilies of *JAZ* (jasmonate ZIM-domain), *ZIM/ZML* (Zinc-finger Inflorescence Meristem: ZIM and ZIM-like), *TIFY* and *PPD* (PEAPOD) in dicots and the three distinct subfamilies of *JAZ*, *ZIM/ZML* and *TIFY* in monocots. For these subfamilies, the *TIFY* subfamily contains only a TIFY domain, while the others have at least one other structural domain besides the TIFY domain, with the *JAZ* subfamily containing a JA-associated (Jas, CCT2) domain for the inhibition of the jasmonate acid (JA) signaling pathway by interaction with the MYC2 proteins [5]; the

*ZIM/ZML* subfamily holding a CCT (CONSTANS, CO-like and TOC1) domain and a GATA zinc-finger domain; and the *PPD* subfamily involving a PPD domain and a partial Jas domain that lack the conserved PY (Proline -Tyrosine) at their C-terminus region [6–9].

To date, quite a few *TIFY* genes have been identified to be involved in many biological processes of plant growth and development, as well as phytohormone responses. In addition to participating in the development of inflorescence and flowering, *AtTIFY1* (*ZIM*) could promote the extension of petioles and hypocotyls by regulating cell elongation [3,5]. *AtTIFY4a* (*PPD1*) and its closest homolog *AtTIFY4b* (*PPD2*) participated in the synchronized growth process of leaves and could promote leaf growth by adjusting the lamina size and curvature [10,11], and the homolog *AtTIFY4b* (*PPD2*) could also regulate leaf flatness and lateral organ development [11,12]. *OsTIFY3*/*OsJAZ1* was found to be involved in the development of spikelets through interaction with *OsCOI1b* and *OsMYC2* during the rice reproductive stage [13]. *OsTIFY11b*/*OsJAZ10* was related to increased grain-size through enhancing the accumulation and translocation of carbohydrates in the stems and leaf sheaths [14]. *SlJAZ2* was involved in regulating vegetative growth and early flowering in tomatoes. Furthermore, several studies reported that the overexpression of truncated JAZ proteins, including AtTIFY6b/JAZ3 and AtTIFY9/JAZ10, could result in the occurrence of a jasmonate-insensitive phenotype [15–18].

Apart from their critical roles in plant development and phytohormone responses, the *TIFY* family genes also respond to various stresses through the regulation of defense responses in plants. On the one hand, many studies have shown that the *TIFY* genes are broadly involved in the response to abiotic stresses such as drought, low temperature, salt, alkaline conditions, etc. *AtJAZ7*-overexpressing plants exhibited a significant drought tolerance phenotype [19], and the overexpression of *OsTIFY11a* conferred enhanced tolerance to dehydration and salt stress in rice [20]. *OsJAZ1* could also regulate drought tolerance through interaction with *Osb-HLH148* in a jasmonate signaling pathway [21]. Overexpressed *JAZ1* or *JAZ4* caused negative responses to freezing stress in *Arabidopsis thaliana* [22]. *GhJAZ2*-overpressing cotton plants showed increased sensitivity to salt stress [23]. *AtTIFY10a* and *AtTIFY10b*, *GsTIFY10a* and *OsJAZ8* were found to be involved in the response to alkaline and salt stresses, respectively [24,25]. On the other hand, *TIFY* genes also play crucial roles in regulating plants' responses to biotic stresses. *JAZ* genes in *Arabidopsis* were reported to be involved in modulating the response to wounding and herbivory [26]. The overexpression of *TaJAZ1* increased powdery mildew resistance in transgenic bread wheat by a reactive oxygen species-regulating strategy [27]. In addition, *Arabidopsis AtJAZ10* and rice *OsJAZ8* were reported to participate in responses to *Pseudomonas syringae* DC3000 and bacterial blight resistance, respectively [28,29]. Given the multiple roles of *TIFY* family genes in regulating plant growth and development, as well as stress responses, it is necessary to conduct a genome-wide survey of the *TIFY* family genes in crop plants.

Barley (*Hordeum vulgare* L.) is the fourth-largest cereal crop grown worldwide after maize, rice and wheat and is mainly used for livestock feed, brewing malts and human food. Barley is adaptable to a greater range of climate than its close relative wheat, with quite a few varieties suited to temperate, subarctic or subtropical areas [30]. However, with the degradation of global climate, barley is constantly facing various abiotic environmental stresses, such as cold damage and drought stress, during the growing season. Moreover, its productivity is limited due to the prevalence of pests and diseases in cultivated plant species. As a plant-specific protein family, the crucial roles of TIFY family in plant growth and development, stress responses and signal transduction have been attracting much attention. *TIFY* genes have been identified and functionally characterized in many cereal species, including wheat [31,32], rice [20], maize [33,34] and sorghum [35,36]. Yet, there are few reports on a comprehensive genome-wide survey and characterization of the *TIFY* gene family in barley and its involvement in abiotic and biotic stresses to date. In this study, we carried out a genome-wide identification and characterization of the *TIFY* family from two assembled barley genomes, including *Hv_Morex* (v3.0) and *Hv_Barke*. All identified barley

*TIFY* genes were subjected to phylogenetic, gene structure, conserved domain or motif, cis-regulatory element, chromosome distribution and gene duplication analysis. Further, their synteny and evolutionary relationships with one dicotyledon (*A. thaliana*) and four monocotyledons (*Oryza sativa*, *Brachypodium distachyon*, *Sorghum biocolor* and *Setaria italic*) were also attempted. Moreover, the expression profiling of the barley *TIFY* genes in various tissues, and the responses to cold stress and spot blotch infection, were also analyzed. Our analysis results provide a foundation for further functional study of *TIFYs* in barley and also contribute to elucidating their roles in abiotic and biotic stress responses.

## 2. Materials and Methods

### *2.1. Genome-Wide Identification of TIFY Gene Family Members*

We downloaded the whole-genome data of two barley genotypes *Morex* (version 3, a reference cultivar) and *Barke* (a successful German variety) from the Barley Genome Database (https://barley-pangenome.ipk-gatersleben.de, accessed on 10 August 2023) [30]. We performed a genome-wide identification of barley *TIFY* genes via two basic local alignment search tool (BLAST) methods. First, we aligned the whole barley genome with the *TIFY* genes of *Arabidopsis thaliana* [1,18] and rice (*O. sativa*) [20] to obtain candidate *HvTIFY* genes. We downloaded the TIFY protein sequences of *A. thaliana* and *O. sativa* from the European Nucleotide Archive (https://plants.ensembl.org/info/data/ftp/index.html, accessed on 15 August 2023). Second, we searched for the potential members of *HvTIFY* gene family through a hidden Markov model (HMM) profile of the conservative functional domain of TIFY (PF06200) using HMMER 3.0 software (http://HMMER.org/, accessed on 15 August 2023). Finally, using a Prosite search (https://prosite.expasy.org/, accessed on 15 August 2023) and the InterPro tool (https://www.ebi.ac.uk/interpro/, accessed on 15 August 2023), we further confirmed the conserved domain architectures of all candidate genes. We excluded those sequences without the typical functional domain of TIFY and used the remaining protein sequences containing the conservative TIFY domain for subsequent analysis. The basic physicochemical properties of each barley TIFY protein, including molecular weight (MW) and isoelectric point (pI), were analyzed by Expasy's ProtParam server (https://web.expasy.org/protparam/, accessed on 15 August 2023). The subcellular localization of the barley TIFY proteins was predicted by the web-based software of Cell_PLoc 2.0 package (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/, accessed on 15 August 2023).

### *2.2. Multiple Sequence Alignment and Phylogenetic Tree Construction*

We performed multiple sequence alignments (MSAs) using MAFFT v7.487 [37] and manually edited them using Geneious Prime v2021.1.1. Phylogenetic analysis was carried out for the resultant MSAs using IQtree v2.2.0, and then the tree topology support was assessed based on bootstrap analysis with 1000 replicates. Furthermore, the phylogenetic tree was annotated and visualized with the iTOL v6 webserver (https://itol.embl.de/, accessed on 15 August 2023). Finally, the sequence logos of TIFY domain and Jas domain were generated by using WebLogo v3.7.4 (http://weblogo.threeplusone.com/, accessed on 15 August 2023) [38].

### *2.3. Analysis of Gene Structure, Conserved Motif and Cis-Acting Element*

We identified the exon/intron organization of *HvTIFY* genes based on barley genome annotation data retrieved from the Barley Genome Database and also analyzed the domain composition of HvTIFY proteins using the Pfam database. The conserved motif patterns of HvTIFY proteins were further detected by using the MEME suite v5.5.0 (https://meme-suite.org/meme/tools/meme, accessed on 15 August 2023), with the maximum number of conserved motifs set to 10 [39]. Then, the gene structures and conserved motifs in the *HvTIFY* family were visualized by TBtools [40]. We extracted 2 kb upstream sequences of *HvTIFY* genes from the barley genome sequence as promoter sequences, and we performed a comprehensive analysis of the promoter region for cis-element identification us-

ing PlantCARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 15 August 2023) [41].

### *2.4. Analysis of Chromosomal Location, Gene Duplication and Gene Synteny*

We obtained the position information of the *HvTIFY* genes along chromosomes based on barley genome assembly and annotation. MCScanX analysis was performed for detecting duplication types and the intraspecific/interspecific collinearity of *TIFY* family members. We constructed chromosomal location maps and analyzed the collinearity relationship of the *TIFY* genes in each genotype by selecting the Advanced Circles function in the TBtools software (version 2.003). The synteny relationship of the orthologous *TIFY* genes from *Hv_Morex* or *Hv_Barke* was determined and exhibited using the Multiple Synteny Plot function in TBtools. We also conducted the homology analysis of the *TIFY* genes between barley and model plants or closely related cereal species including *A. thaliana*, *B. distachyon*, *O. sativa*, *S. biocolor* and *S. italica* using the Dual Synteny Plot in TBtools. All the genome sequence information of the five selected plants has been downloaded from the European Nucleotide Archive. In addition, we calculated the nonsynonymous rate (Ka), synonymous rate (Ks) and the evolutionary constriction (Ka/Ks) using the Simple Ka/Ks Calculator (NG) of TBtools for duplicated and syntenic *TIFY* gene pairs [42].

### *2.5. In Silico Expression Analysis of Barley TIFY Genes*

We performed expression pattern analysis of the *TIFY* genes in various tissues based on *Hv_Morex* tissue RNA-seq data downloaded from EoRNA (ERR1457163, ERR1457188 to ERR1457235, ERR1457259), a barley gene and transcript abundance database [43]. To determine the expression profiles of *TIFY* family genes in response to stress factors, the RNA-seq data of *Hv_Morex* after cold stress (4 °C for 2 h and 24 h) and a normal growth temperature (18 °C for 2 h and 24 h) were further mined from EoRNA (ERS3631845 to ERS3631859) and analyzed. To explore the expression patterns of *HvTIFYs* in response to biotic stress, we carried out a transcriptome analysis of *Hv_Morex* leaves infected by the spot blotch pathogen based on retrieved data from EoRNA (SRR3290245 to SRR3290262). Heat maps of the expression profile of *TIFY* genes have been generated through the Heatmap Illustrator in TBtools.

## 3. Results

### *3.1. Identification of TIFY Genes in Barley*

We identified a total of 21 and 22 *TIFY* genes in the whole genome of *Morex* and *Barke*, respectively (Supplementary Table S1), and renamed these genes as *Hv_MoTIFY*, *Hv_MoZML1* to *Hv_MoZML4*, *Hv_MoJAZ1* to *Hv_MoJAZ17* (absent for *Hv_MoJAZ10*) or *Hv_BaTIFY*, *Hv_BaZML1* to *Hv_BaZML4* and *Hv_BaJAZ1* to *Hv_BaJAZ17* on the basis of their homologous genes in *Arabidopsis* and rice and the corresponding chromosomes which they were being assigned. Extensive variation was noticed for the length of these identified *TIFY* genes. The amino acid sequence lengths of *Hv_Morex* TIFYs and *Hv_Barke* TIFYs varied from 136 aa (Hv_MoJAZ6 and Hv_MoJAZ13) to 380 aa (Hv_MoJAZ4) and from 138 aa (Hv_BaJAZ13 and Hv_BaJAZ14) to 417 aa (Hv_BaJAZ4), respectively (Supplementary Table S1). Meanwhile, the molecular weights of *Hv_Morex* TIFYs and *Hv_Barke* TIFYs ranged from 14.49 (Hv_MoJAZ13) to 40.20 (Hv_MoJAZ4) kDa and from 14.49 (Hv_BaJAZ14) to 44.06 (Hv_BaJAZ4) kDa, respectively. The isoelectric points (pI) of 17 out of 21 TIFYs in *Hv_Morex* and 17 out of 22 TIFYs in *Hv_Barke* were higher than 7.0, suggesting that most of the barley TIFYs were characterized by an enrichment of basic amino acids. Furthermore, all barley *TIFYs* were located in the nucleus based on subcellular localization prediction.

### *3.2. Multiple Sequence Alignment, Phylogenetic Analysis and Classification of the Barley TIFY Genes*

To explore the classification and evolutionary relationship of the barley *TIFY* gene family, we constructed a phylogenetic tree based on the multiple sequence alignment of

81 TIFY protein sequences, including 21 TIFYs in *Hv_Morex*, 22 TIFYs in *Hv_Barke*, 18 TIFYs in *Arabidopsis* and 20 TIFYs in rice (Figure 1). According to the phylogenetic relationship reflected from the tree and the classification method of TIFY proteins previously reported in *A. thaliana* [1] and rice [20], 21 *Hv_Morex* TIFYs and 22 *Hv_Barke* TIFYs could be classified into three major phylogenetic groups: JAZ, ZIM/ZML, and TIFY. The group JAZ contained the highest number of members with 16 *Hv_Morex* JAZs and 17 *Hv_Barke* JAZs, and could be further clustered into six subgroups, i.e., JAZ I to JAZ VI (Figure 1 and Supplementary Table S1). Of the six JAZ subgroups, JAZ VI was the largest subgroup with eight *Hv_Morer* JAZ and nine *Hv_Barke* JAZ members, followed by JAZ II with three members and JAZ V with two members for both *Hv_Morer* and *Hv_Barke*, while each of the JAZ I, JAZ III and JAZ IV subgroups contained only one member for both *Hv_Morex* and *Hv_Barke*. The group ZIM/ZML was the second-largest group that included eight barley ZIM/ZML proteins, with each of the four members being from *Hv_Morex* and *Hv_Barke*, respectively. The TIFY group contained one *Hv_Morex* TIFY and one *Hv_Barke* TIFY (Figure 1 and Supplementary Table S1). In addition, we also analyzed the phylogenetic relationships among the members of the barley TIFY family based on phylogenetic tree construction using the alignment of the 43 protein sequences of *Hv_Morex* and *Hv_Barke* TIFYs characterized herein, and we found that the TIFY proteins derived from the same group were clustered in a common clade (Figure 2A), which was in accordance with that of the above constructed phylogenetic tree from barley, rice and *Arabidopsis* TIFY sequences (Figure 1).

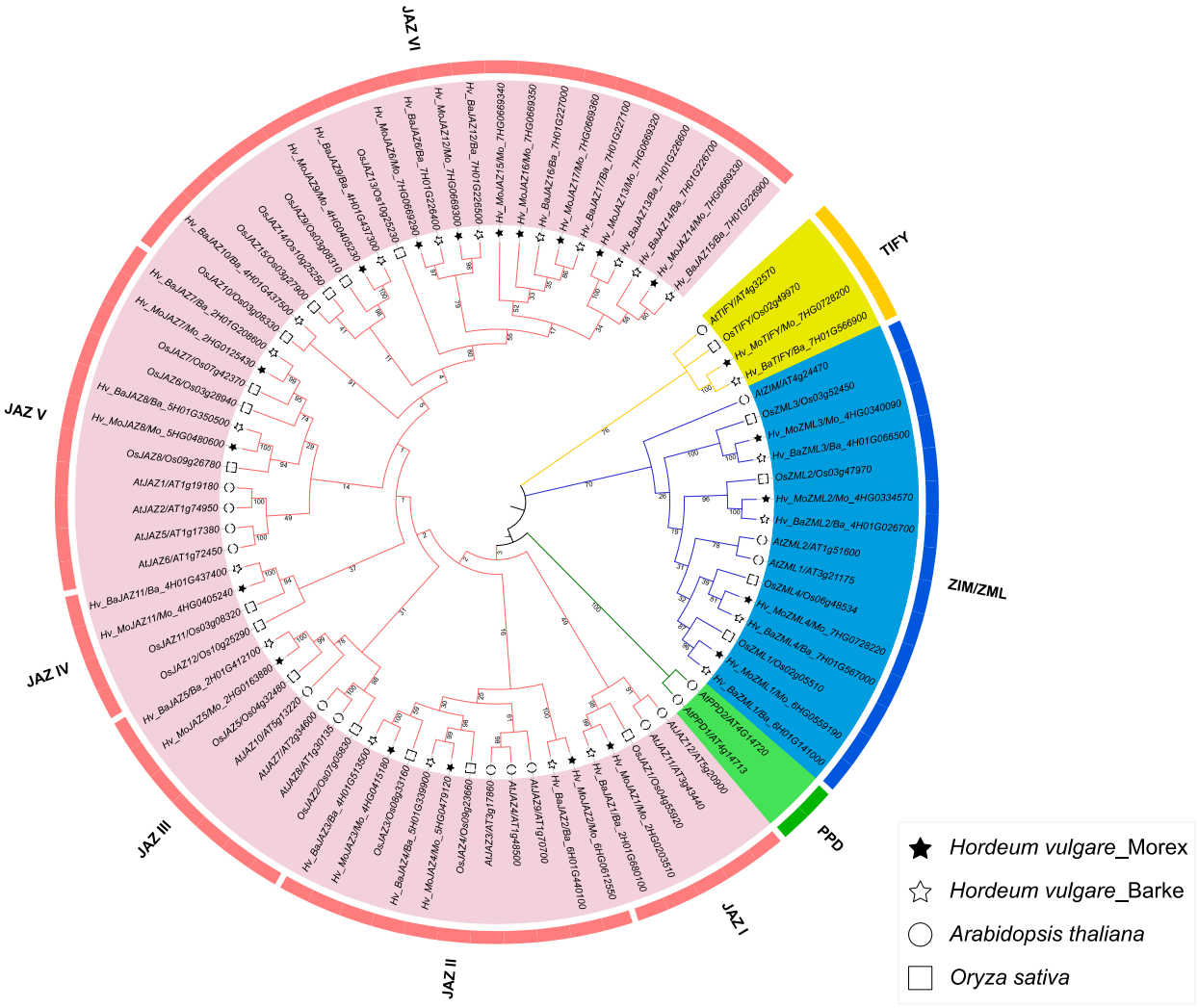



**Figure 1.** Maximum likelihood phylogenetic relationships of the TIFY proteins from barley, *A. thaliana* and *O. sativa*.

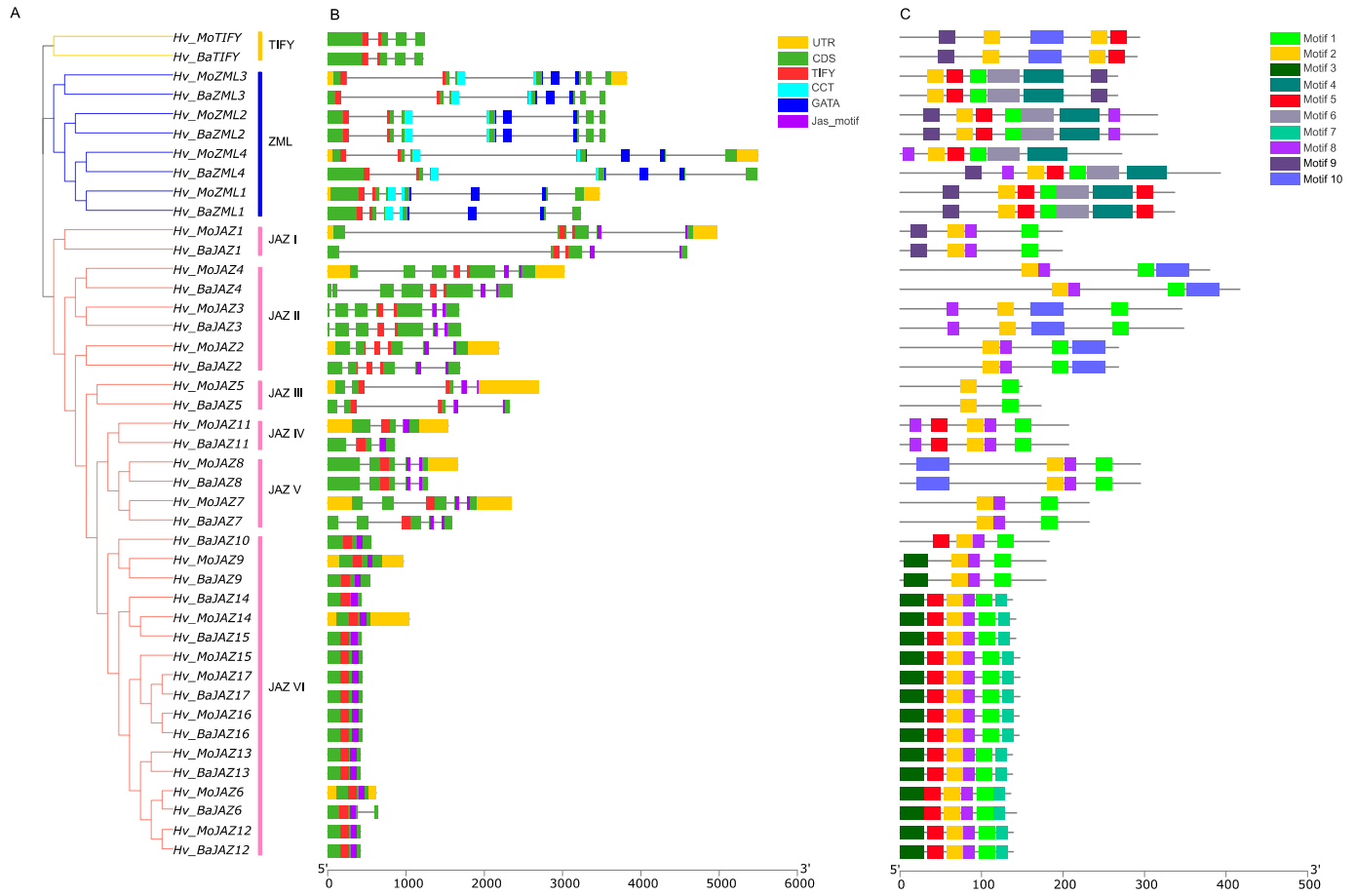



**Figure 2.** Phylogenetic relationships, gene structure and conserved protein motifs in barley TIFY family. (**A**) The phylogenetic tree of barley TIFY proteins. (**B**) Exon–intron structure and the characteristic domain distribution of barley *TIFY* genes. (**C**) The motif composition of barley TIFY proteins.

To reveal the domain profiles of TIFYs in different groups, we conducted a comparative analysis focusing on the amino acid sequences of the domains of 43 HvTIFYs (21 in *Hv_Morex* and 22 in *Hv_Barke*). All 43 HvTIFY proteins within the three groups contained one TIFY domain with a conserved TX4G motif, 33 members of the group JAZ contained a Jas domain with a conserved RX2SLX2FX3RX2/4R motif in addition to the TIFY domain, and 8 proteins of the group ZIM/ZML had an additional CCT and GATA domain, whereas 2 members of the group TIFY were characterized by only the TIFY domain but lacking any other domain (Figure 2B and Supplementary Table S1).

### *3.3. Gene Structure and Protein Motif Analyses of HvTIFY*

Barley *TIFY* genes from different groups and subgroups showed great differences in the number and distribution of exons, with the number of exons ranging from one to eight unevenly (Figure 2B and Supplementary Table S1). The genes in the *ZIM/ZML* group held the largest number of exons, with an average number greater than seven, of which all genes contained eight exons except for *Hv_MoZML1*, *Hv_MoZML4* and *Hv_BaZML4*. The group *TIFY* contained four exons for both *Hv_MoTIFY* and *Hv_BaTIFY*, while the genes in the group *JAZ* exhibited a large difference in their exon number—with only one exon for most members of the subgroup *JAZ VI* but three to eight exons for the members of the other *JAZ* subgroups.

MEME motif analysis was performed for the conserved structural motifs in barley *TIFY* family members. We detected a total of 10 conserved motifs, and named them Motif 1 to Motif 10 (Figure 2C and Supplementary Table S2). No member of the barley *TIFY* family has been found to hold a complete set of 10 conserved motifs, and the motif number

of the barley *TIFYs* ranged from 2 to 7. In addition, except for Motif 2 that was widely distributed in every member of the barley *TIFY* family, unique motifs were identified in the TIFY proteins of some groups or subgroups. Motif 4 and Motif 6 were unique to the group *ZIM/ZML*; Motif 3 and Motif 7 were unique to the subgroup *JAZ VI* except for the members of JAZ 9 (Motif 7 missing) and JAZ 10 (both motifs missing). Moreover, the *TIFY* genes from the same group or subgroup had the similar number and type of motifs, and the similar distribution pattern of motifs as well. For example, the two members of the *TIFY* group possessed four conserved motifs with the same structural distribution, the members of the *ZIM/ZML* group mainly included five motifs and most members of the subgroup *JAZ VI* had six similarly distributed motifs (Figure 2C).

To further understand the structural conservation of the barley TIFY and Jas domains in the different groups and subgroups, we conducted sequence alignment analysis for both domains in all barley *TIFY* family members and obtained the corresponding logos (Figure 3). The domain logos revealed that not all the TIFY domains of barley *TIFY* members were completely conserved, but most of the TIFY domain sequences shared common motifs, such as TIFYXG, TXFYNG and TLX2QG. For these conserved motifs, the motif TIFYXG was the most dominant motif, and 14 out of 21 *Hv_Morex* and 15 out of 22 *Hv_Barke* TIFYs contained this motif; the motif TIFYXG was shared by the members of the subgroups JAZ I and JAZ IV to VI and the two members of JAZ II; the motif TXFYNG was co-owned by one JAZ II member and one JAZ III member; while the motif TLX2QG was co-possessed by the ZIM/ZML members (Supplementary Table S1). Owing to its existence only in the Jas group, the Jas domain was more conservative among all members of the subgroups I to VI and the shared residue motif of PX2RX2SLX2FX2KRX2R (Figure 3A).

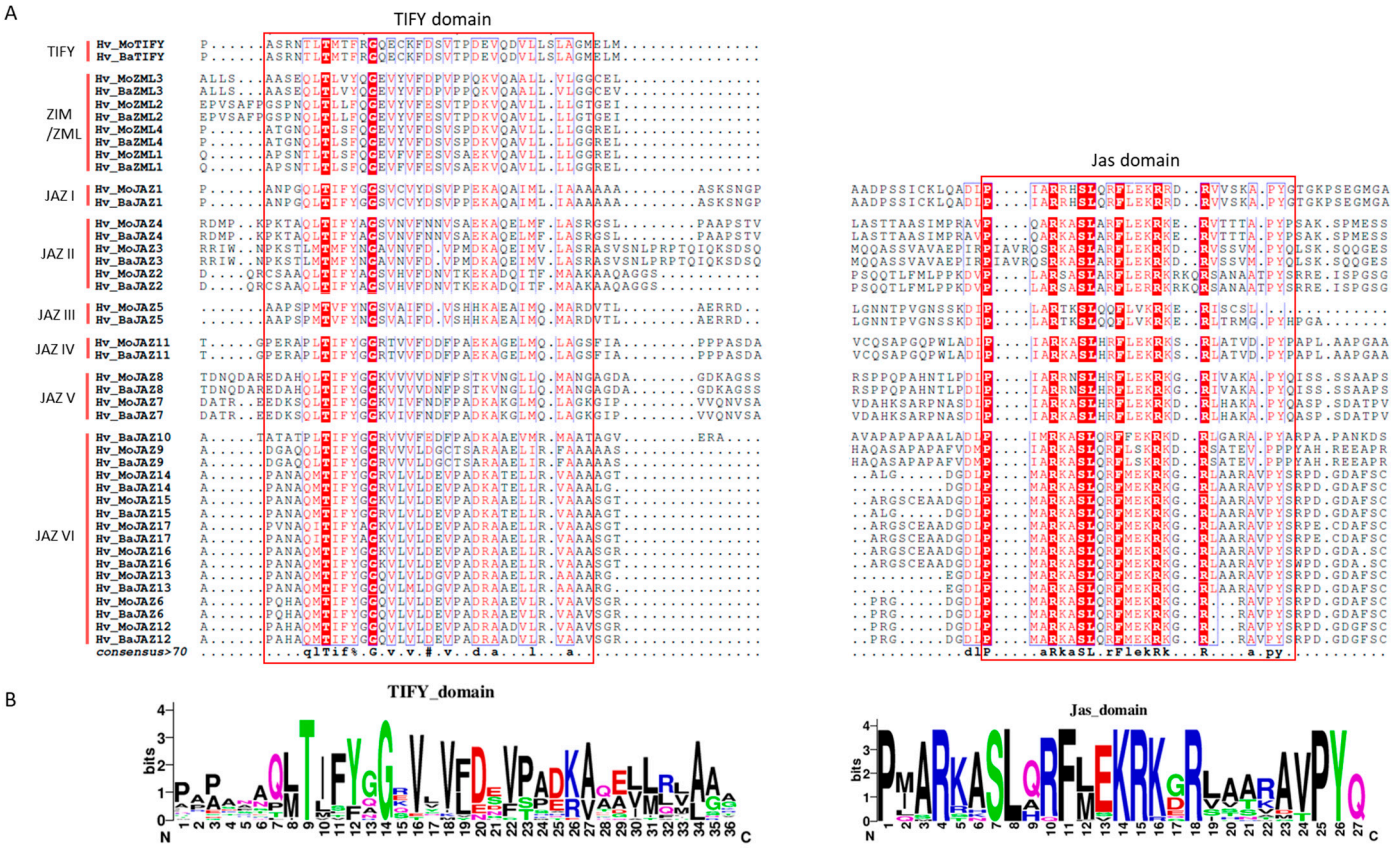



**Figure 3.** Sequence alignment of the barley TIFY and Jas domain and the conserved sequence logos of the barley *TIFY* family member. (**A**) Multiple sequence alignments of barley *TIFY* family members. (**B**) Consensus sequence logos of TIFY and Jas domain sequences. The red box indicates the conserved domain of *HvTIFY* family members, and the highlighted blocks in red show a 100% amino acid identity to each other for *HvTIFY* members.

### *3.4. Cis-Acting Element Analysis of TIFY Gene Family Members in Barley*

In order to explore the underlying expression patterns of the barley *TIFY* genes, we detected the cis-elements in the promoter regions of these *HvTIFY* genes. The results revealed that the promoter regions of the *HvTIFY* family members contained abundant putative cis-elements, falling into three main categories: stress response elements, phytohormone elements and plant growth and development elements (Supplementary Table S3). We detected 12 types of stress response-related cis-elements in the promoter regions of the *HvTIFY* gene family, mainly including light-responsive elements (G-box, Sp1 and GT1-motif), anaerobic induction elements (AREs), low-temperature-responsive elements (LTRs), the MYB binding site involved in drought-inducibility (MBS) and defense and responsive elements (TC-rich repeats), etc. Among them, except that the promoters of all barley *TIFY* genes carried the G-box element, the second most distributed cis-element was the ARE in the *Hv_Morex TIFY* family members, while the MBS was in the *Hv_Barke TIFYs.* Ten types of phytohormone-related cis-acting elements were found in the promoters of *HvTIFY* family members, primarily involving abscisic acid responsiveness (ABRE), MeJA-responsiveness (TGACG-motif, CGTCA-motif), auxin responsiveness (AuxRR-core, TGA-element), salicylic acid responsiveness (TCA-element) and gibberellin-responsiveness (P-box, GARE-motif, TATC-box). Of these, the cis-elements of ABRE and MeJA-responsiveness were detected in the promoter regions of most *HvTIFY* family genes. There were 21, 20 and 19 members in the *Hv_Morex TIFY* family, and 22, 20 and 21 members in the *Hv_Barke TIFY* family containing the three elements, respectively. In addition, some *HvTIFY* gene promoters were found to contain cis-acting elements related to plant growth and development, such as zein metabolism regulation (O2-site), meristem expression (CAT-box), circadian control (circadian), endosperm expression (GCN4-motif) and seed-specific regulation (RY-element). In general, the type and the average number of the cis-elements relating to plant growth and development were less than those relating to stress response or phytohormone. For the most identified elements on plant growth and development, an O2-site and CAT-box were identified in the promoters of 10 and 12 *Hv_Morex TIFY* genes, respectively, and each of the two elements was detected in 12 *Hv_Barke* TIFY members. In brief, our cis-elements analysis implies that a high proportion of barley *TIFY* genes are likely to respond to various environmental stresses.

### *3.5. Chromosomal Location and Duplication Analyses of TIFY Gene Family Members in Barley*

The analysis results demonstrated that the *HvTIFY* genes were distributed irregularly on the barley chromosomes. Both genotypes of *Hv_Morex* and *Hv_Barke* had a similar chromosomal distribution for *TIFY* genes, except for *Hv_BaJAZ10* mapping to Chr4H in *Hv_Barke* but the corresponding gene being missing in *Hv_Morex* (Figure 4A,B). All *HvTIFY* family members were unevenly mapped to the same five of the seven chromosomes of *Hv_Morex* or *Hv_Barke* (Figure 4A). Among them, Chr7H anchored the greatest number of genes with nine *HvTIFY* genes in both *Hv_Morex* and *Hv_Barke*, followed by Chr4H with five *HvTIFY* members in *Hv_Morex* and six members in *Hv_Barke*, Chr2H with three *HvTIFY* members in both *Hv_Morex* and *Hv_Barke* and Chr5H and Chr6H each with two *HvTIFY* members in either *Hv_Morex* or *Hv_Barke*.

MCScanX analysis revealed a couple of gene duplication events occurred throughout the barley *TIFY* family's history. Although five gene pairs in the *HvTIFY* family were identified as tandem duplications in both *Hv_Morex* and *Hv_Barke*, some gene pairs displayed differences between the two genotypes. For example, except for four pairs of similar duplication genes in two genotypes, one tandem duplication gene pair was *Hv_MoJAZ14*/*Hv_MoJAZ15* in *Hv_Morex*, but that was *Hv_BaTIFY*/*Hv_BaZML4* in *Hv_Barke*. In addition to tandem duplications, one and three segmental duplication events were also detected in the *Hv_Morex* and *Hv_BarkeTIFY* families, respectively (Figure 5 and Supplementary Table S4). Hence, tandem duplication was the main form of gene duplication in the barley *TIFY* family.

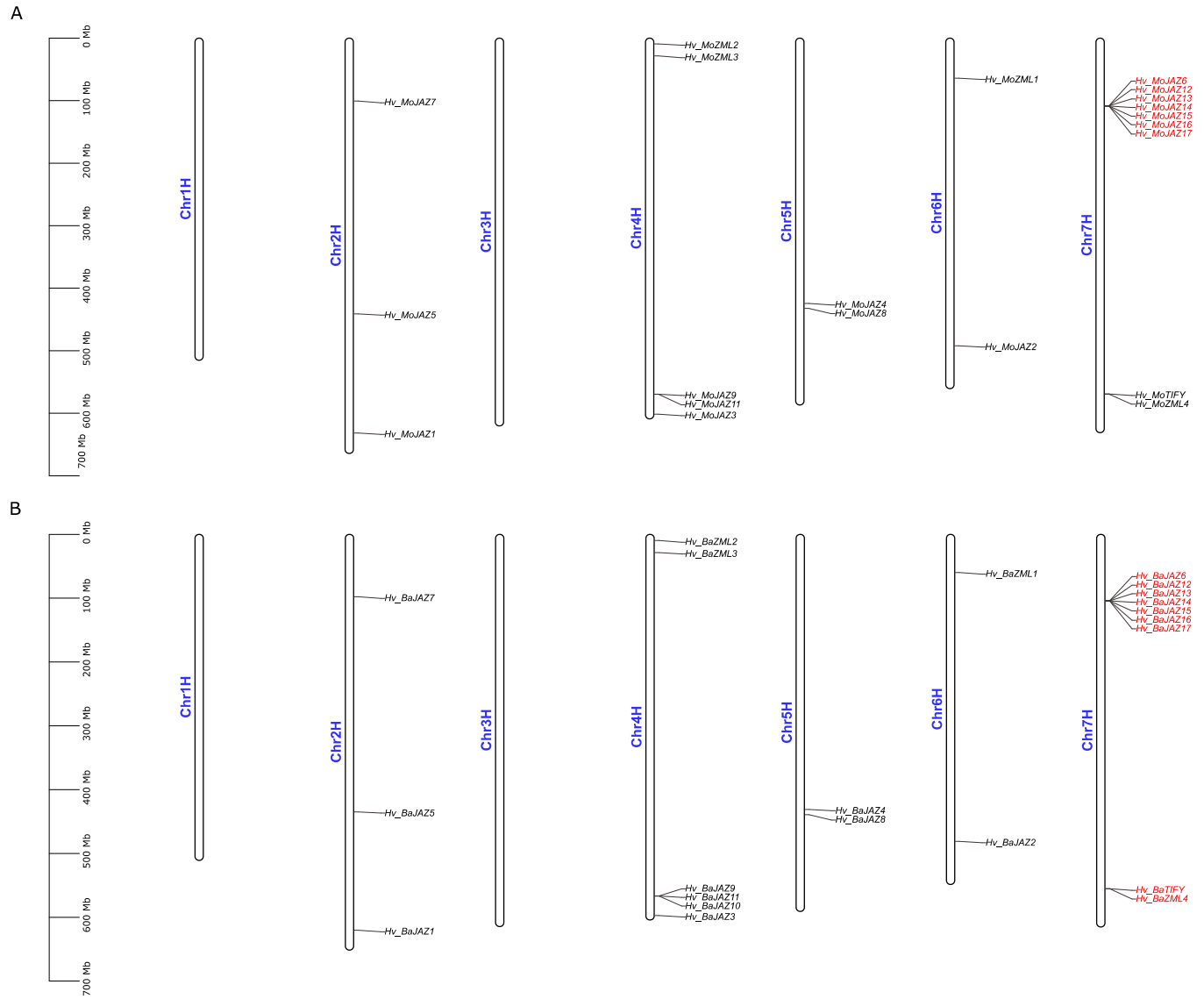



**Figure 4.** Distribution of the *HvTIFY* genes on barley chromosomes. (**A**) The gene distribution on *Hv_Morex* chromosomes. (**B**) The gene distribution on *Hv_Barke* chromosomes. The tandem gene duplication is shown by the red font. The chromosome numbers are indicated at the left side of each chromosome image. The scale on the left is in million bases (Mb).

### *3.6. Synteny and Evolutionary Analyses of Barley TIFY Genes and Other Plant TIFYs*

To analyze barley *TIFY* family evolution, we constructed a series of collinear maps of barley associated with other five plant species, i.e., *A. thaliana*, *O. sativa*, *B. distachyon*, *S. biocolor* and *S. italica* (Figure 6). The comparative maps revealed that a total of 11 *Hv_Morex TIFY* genes showed syntenic relationships with those in each species of *B. distachyon*, *S. biocolor* and *O. sativa*, followed by 9 and 1 *Hv_Morex TIFY* genes syntenic with those in *S. italica* and *A. thaliana*, respectively. The number of *Hv_Morex TIFY* orthologous genes in *B. distachyon*, *O. sativa*, *S. biocolor*, *S. italica* and *A. thaliana* was 12, 12, 10, 9 and 1, respectively (Figure 6 and Supplementary Table S5). Among the orthologous gene pairs, the *Hv_Morex TIFY* genes of *Hv_MoJAZ4*, *Hv_MoZML1* and *Hv_MoZML4* in the syntenic analysis of *Hv_Morex* and *B. distachyon*, of *Hv_MoJAZ7*, *Hv_MoJAZ4*, *Hv_MoZML1* and *Hv_MoZML4* in the syntenic analysis of *Hv_Morex* and *O. sativa*, of *Hv_MoJAZ4* in the syntenic analysis of *Hv_Morex* and *S. biocolor* and also in the syntenic analysis of *Hv_Morex* and *S. italica* were detected to be associated with two syntenic gene pairs. More homologous gene pairs were found between *Hv_Morex* and monocots than those between *Hv_Morex* and *A. thaliana*.

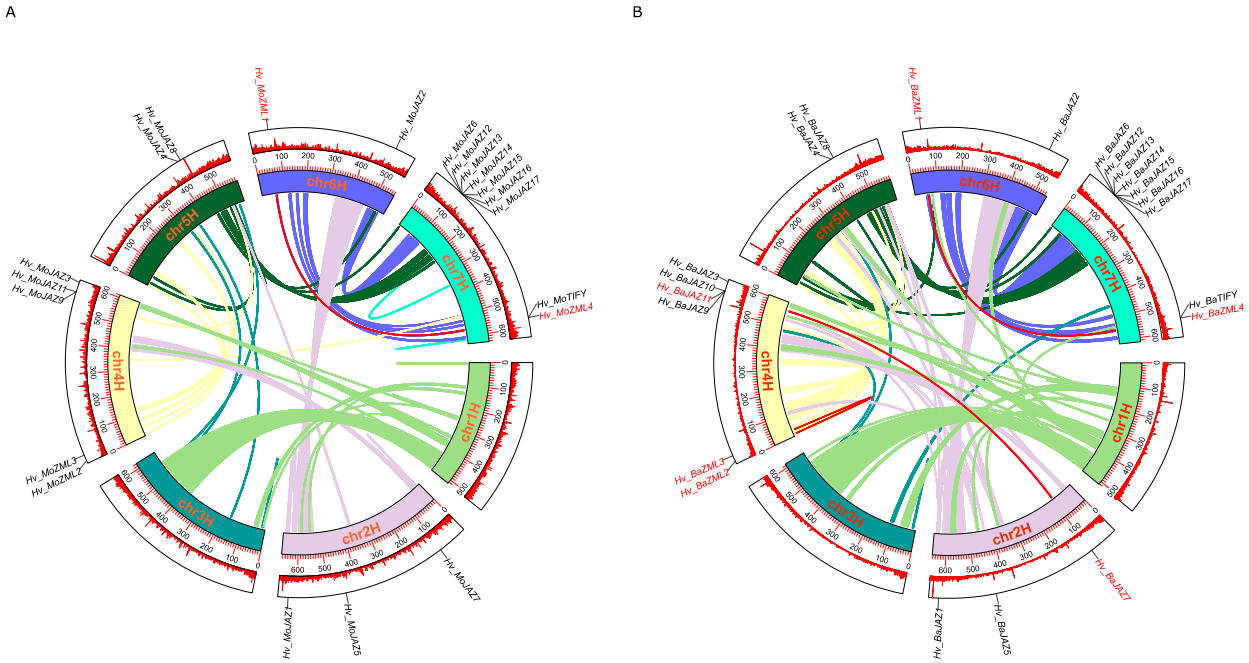



**Figure 5.** Collinear region of the barley *TIFY* genes. (**A**) The collinear result of the *Hv_Morex TIFY* genes. (**B**) The collinear result of the *Hv_Barke TIFY* genes. All collinear blocks are shown with colored lines in the *Hv_Morex* or *Hv_Barke* genome, and segmental duplications are represented with red lines. Chromosome numbers are indicated at the center of each chromosome.

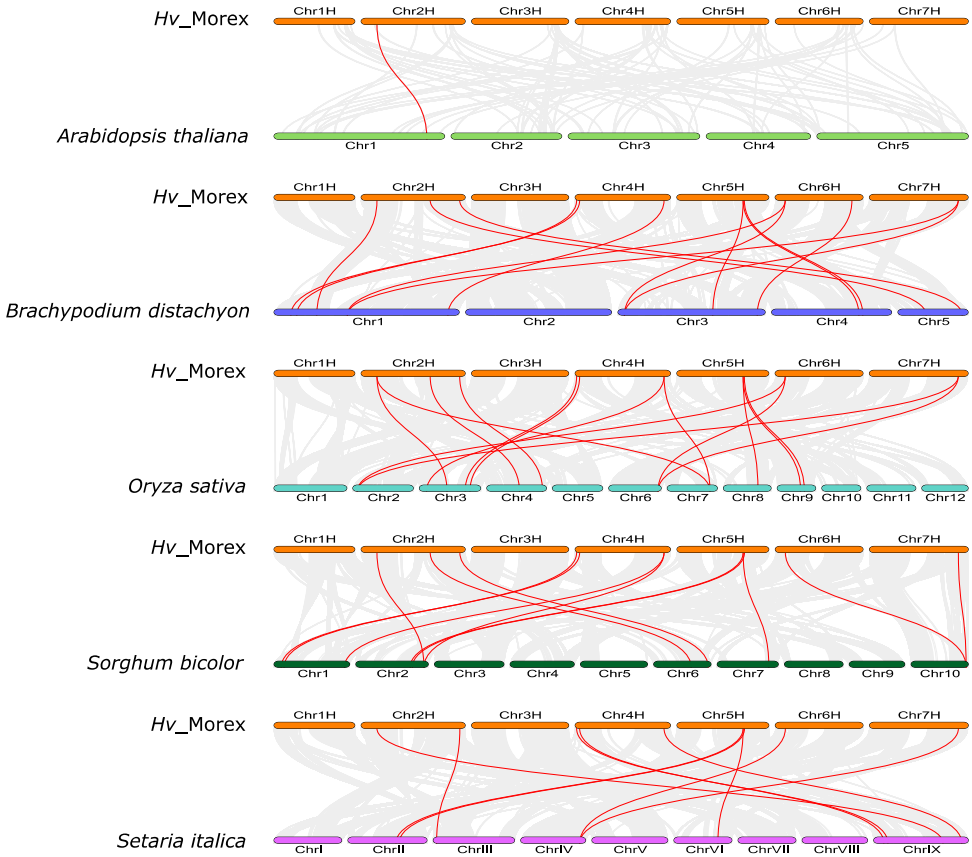



**Figure 6.** Synteny relationships between *Hv_Morex* and *A. thaliana*, *B. distachyon*, *O. sativa*, *S. bicolor* and *S. italica TIFY* family genes. The collinear blocks within *Hv_Morex* and other plant genomes are shown in gray lines in the background, while the syntenic *TIFY* gene pairs are highlighted in red lines.

As shown in the homologous gene maps of *Hv_Barke* and the five representative plant species (Figure 7), a total of 11 *TIFY* family members of *Hv_Barke* were found to be homologous to the genes in each species of *B. distachyon*, *O. sativa* and *S. biocolor*, followed by 9 and 3 *Hv_Barke TIFY* genes showing syntenic relation to the genes of *S. italica* and *A. thaliana*, respectively. The number of *Hv_Barke TIFY* orthologous genes was 12, 13, 11, 10 and 1 in *B. distachyon*, *O. sativa*, *S. biocolor*, *S. italica* and *A. thaliana*, respectively (Figure 7 and Supplementary Table S6). In the identified homologous gene pairs, one *Hv_Barke TIFY* gene (*Hv_BaJAZ7*) showed association with three corresponding genes in *O. sativa* (*Os03t0402800-01*, *Os03t0180900-01* and *Os07t0615200-01*) and three genes in *S. biocolor* (*EER95398*, *EER92157* and *EER97533*). Two *Hv_Barke TIFY* genes (*Hv_BaJAZ7* and *Hv_BaJAZ8*) were found to have syntenic genes in both monocot plants and *Arabidopsis*. However, the *Hv_BaJAZ11* gene had its corresponding syntenic genes in *Arabidopsis* and the three monocot plants (*O. sativa*, *S. biocolor*, *S. italica*), but not in *B. distachyon* (Supplementary Table S6).

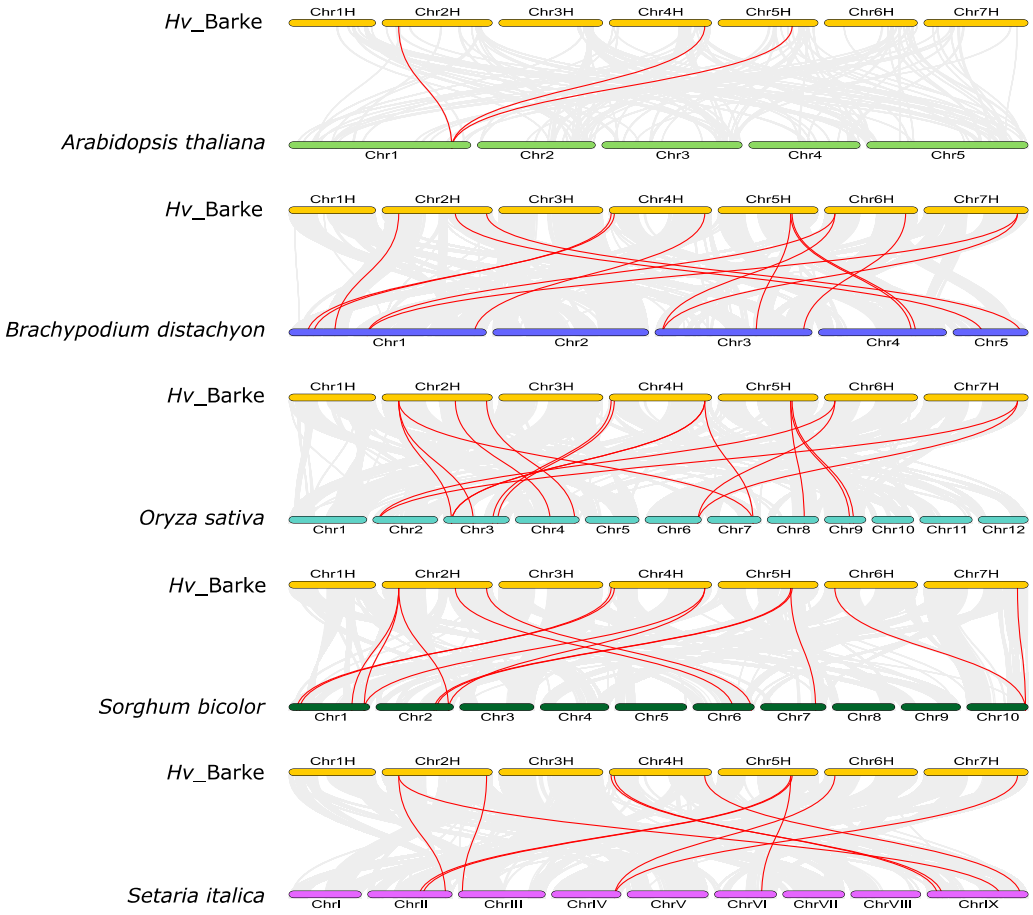



**Figure 7.** Synteny relationships between *Hv_Barke* and *A. thaliana*, *B. distachyon*, *O. sativa*, *S. bicolor* and *S. italica TIFY* family genes. The collinear blocks within *Hv_Barke* and other plant genomes are shown in gray lines in the background, while the syntenic *TIFY* gene pairs are highlighted in red lines.

To further understand the syntenic relationships among the barley *TIFY* gene family, homologous analysis was performed for the *TIFY* genes between the two genomes of *Hv_Morex* and *Hv_Barke* (Figure 8). The results indicated that a total of 16 *TIFY* genes from each of the two genotypes displayed collinearity, and 19 collinear gene pairs were found between *Hv_Morex* and *Hv_Barke* (Figure 8 and Supplementary Table S7).

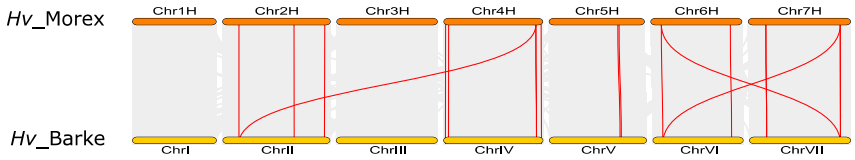



**Figure 8.** Synteny relationships of *TIFY* genes between two different barley genotypes. The collinear blocks within *Hv_Morex* and *Hv_Barke* genomes are shown in gray lines in the background, while the syntenic *TIFY* gene pairs are highlighted in red lines.

In addition, the Ka/Ks ratios were calculated to estimate the selective pressure on *TIFY* genes. The measurement showed that except for 11 collinear *TIFY* gene pairs between *Hv_Morex* and *Hv_Barke* (the Ks value being 0, or >1 for Ka/Ks), all other collinear gene pairs showed Ka/Ks < 1, implying that the *HvTIFY* family might have experienced changes in purifying selection during barley genome evolution (Supplementary Tables S5–S7).

### *3.7. Expression Pattern Analysis of Barley TIFY Genes*

In the present study, the expression patterns of the *Hv_Morex TIFY* genes in twelve tissues or tissue parts (grain, embryo, epidermis, inflorescence, shoot, lemma, lodicules, tiller, palea, rachis, root and leaf) were analyzed based on published transcriptome data (REF). As shown in Figure 9, the *TIFY* genes except for *Hv_MoJAZ13* were expressed in at least one of these tissues, and quite a few *TIFY* genes were expressed in a tissue-specific manner. Compared with other *TIFY* family members, *Hv_MoZML1*, *Hv_MoJAZ1*, *Hv_MoJAZ4*, *Hv_MoJAZ5*, *Hv_MoJAZ6*, *Hv_MoJAZ7*, *Hv_MoJAZ8*, *Hv_MoJAZ9*, *Hv_MoJAZ11*, *Hv_MoJAZ12* and *Hv_MoJAZ14* had relatively higher expression levels in some tissues or tissue parts. Among them, *Hv_MoJAZ1*, *Hv_MoJAZ4* and *Hv_MoJAZ7* were highly expressed in all twelve tissues, and the expression levels of *Hv_MoJAZ1* in inflorescence, lemma and palea, while the expression levels of *Hv_MoJAZ4* and *Hv_MoJAZ7* in epidermis, lemma, lodicules and palea were particularly prominent, implying that these genes may be important for the regulation of normal development of the tissues. *Hv_MoJAZ5*, *Hv_MoJAZ6*, *Hv_MoJAZ8*, *Hv_MoJAZ9*, *Hv_MoJAZ11*, *Hv_MoJAZ12* and *Hv_MoJAZ14* were highly expressed in inflorescence (lemma, lodicule, palea, rachis) or root tissues, suggesting they might be involved in inflorescence and root system adjustments in barley. Although *Hv_MoZML1* was clearly expressed in all tissues except for grain, no obvious tissue-specific expression pattern was observed.

The expression patterns of *Hv_Morex TIFY* family members under cold stress (4 °C for 2 h and 24 h) as well as normal growth temperature (18 °C for 2 h and 24 h) were further analyzed on the basis of EoRNA data. As shown in the results in Figure 10, the expression levels of *Hv_MoZML1*, *Hv_MoZML4* and *Hv_MoJAZ5* decreased obviously under cold stress and normal temperature, with the exception of the increased expression of *Hv_MoJAZ5* under normal growth temperature for 2 h. Conversely, the genes *Hv_MoZML2*, *Hv_MoJAZ7*, *Hv_MoJAZ8* and *Hv_MoJAZ12* showed an upregulation of the expression level during the cold stress, and the increase trend of *Hv_MoZML2* and *Hv_MoJAZ12* was more obvious. In addition, the genes of *Hv_MoJAZ1*, *Hv_MoJAZ4*, *Hv_MoJAZ6*, *Hv_MoJAZ9*, *Hv_MoJAZ11* and *Hv_MoJAZ14* exhibited a higher expression level only at a certain stage of the cold stress. For example, the expression of *Hv_MoJAZ1* was lower under cold stress for 2 h, but higher under the stress for 24 h. *Hv_MoJAZ4* was highly expressed under cold stress for 2 h, while *Hv_MoJAZ6* showed increased expression under the stress for 24 h. The diversity of *Hv_Morex TIFYs* expression patterns implies that these *TIFY* genes might participate in the regulation of plant responses to cold stress in a complex manner.

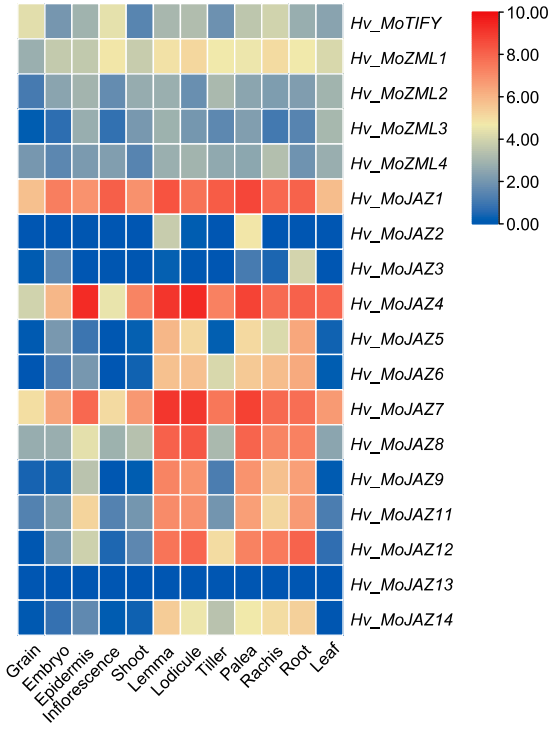



**Figure 9.** Expression patterns of *Hv_Morex TIFY* genes in twelve different tissues.

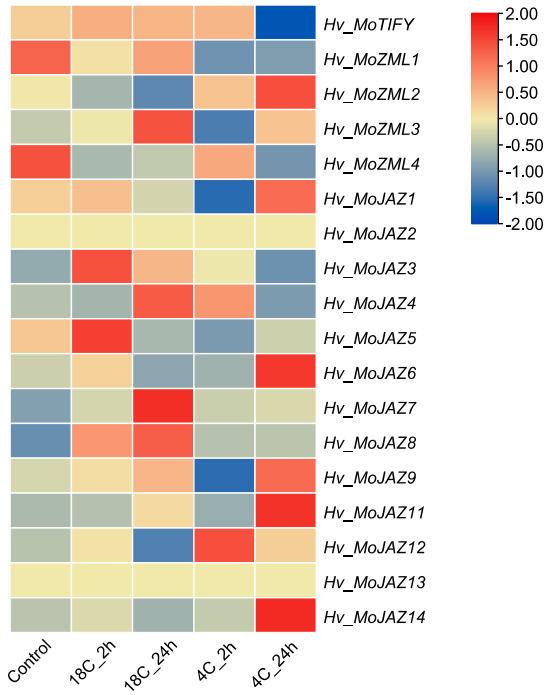



**Figure 10.** Expression patterns of *Hv_Morex TIFY* genes under cold stress. Control represents samples before normal temperature or cold temperature treatment. The labels 18C_2h, 18C_24h and 4C_2h, 4C_24h represent hours after normal temperature (18 °C) or cold temperature (4 °C) treatment.

The expression profiles of *Hv_Morex TIFY* family genes participating in spot blotch infection were also analyzed using EoRNA data. The gene expression results are shown in Figure 11. Most of the *TIFY* genes showed changes in expression level after spot blotch pathogen invasion. *Hv_MoTIFY*, *Hv_MoJAZ1*, *Hv_MoJAZ4*, *Hv_MoJAZ5*, *Hv_MoJAZ6*, *Hv_MoJAZ7*, *Hv_MoJAZ8*, *Hv_MoJAZ9*, *Hv_MoJAZ11*, *Hv_MoJAZ12* and *Hv_MoJAZ14* were expressed with similar patterns. Different from the control, these genes showed a

higher expression level in the early stage of spot blotch infection and then displayed a gradual or rapid decrease trend with the extension of infection time. On the contrary, the expression of *Hv_MoZML1*, *Hv_MoZML2*, *Hv_MoZML3* and *Hv_MoZML4* after spot blotch infection was lower than that without spot blotch inoculation. The three genes *Hv_MoJAZ2*, *Hv_MoJAZ3* and *Hv_MoJAZ13* showed no changes in expression level for both control and spot blotch invasion. These results suggested that quite a few *Hv_Morex TIFY* family members might play certain roles in responding to spot blotch infection.

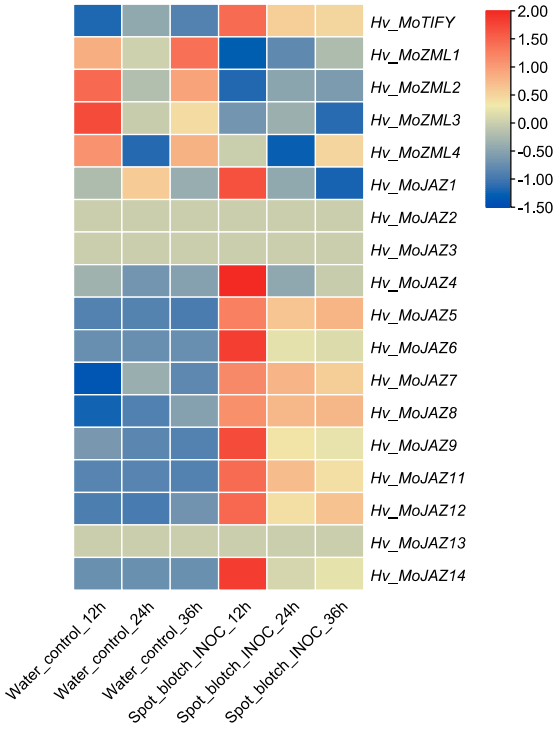



**Figure 11.** Expression patterns of *Hv_Morex TIFY* genes in the process of spot blotch invasion. Water_control_12h, Water_control_24h and Water_control_36h represent hours post spraying with water, and Spot_blotch_INOC_12h, Spot_blotch_INOC_24h and Spot_blotch_INOC_36h indicate hours post inoculation with spot blotch.

## 4. Discussion

The *TIFY* gene family is a transcription factor family unique to plants, and it has been proven to take pivotal roles in regulating plant development, physiological processes and biotic and abiotic stress responses. Thus far, the *TIFY* family has been reported in a large number of plant species. There are 18 *TIFY* family members in *Arabidopsis* [1], 20 in rice [20], 48 in maize [34,44], 63 in wheat [31,32,45], 19 in sorghum [35,36] and 21 in *Brachypodium* [33] according to genome-wide analyses. In the current study, there were at least 21 and 22 *TIFY* genes identified in the genomes of *Hv_Morex* and *Hv_Barke*, respectively, which could be classified into three groups showing clear differences in their gene structures. The difference in the *TIFY* gene number between the two barley genotypes was due to the different numbers of *JAZ* group members. The *JAZ* group in *Hv_Morex* embraced 13 members, while the group in *Hv_Barke* had 14 members. Since gene duplication and loss are the major forces of genome expansion or contraction, quite a few gene redundancies are believed to be a result of gene duplication [46,47]. In view of this, the difference in the number of *TIFY* family members between *Hv_Morex* and *Hv_Barke* might be due to gene duplication or loss in the two genotypes during the evolution of their genomes.

The *TIFY* genes display diverse characteristics in their structure. In many plant species, such as rice [20], wheat [32], kiwifruit [47], walnut [48], cucumber [49] and *Brassica rapa* [50],

the *TIFY* family members displayed variations in the number of exons and introns, in their protein sequence length and in conserved domains and motifs. In this study, a high variation in protein sequence structure was identified among the barley *TIFY* family members. On the one hand, the variation range of the *Hv_Morex* TIFY protein length was 136 aa to 380 aa, while that of *Hv_Barke* TIFYs was 138 aa to 417 aa. On the other hand, the variation range of the number of *HvTIFY* gene exons was one to eight. The conserved motif numbers of *HvTIFY* genes ranged from two to seven, and the conserved domains in HvTIFY proteins were also different between different groups. In addition, some conserved motifs or domains were unique to a certain group or subgroup. These structural divergences of *HvTIFY* genes could cause differences in their function to a considerable extent.

Both segmental duplication and tandem duplication are important to functional differences of family genes during genome evolution in organisms. In the present study, five tandem duplication events were found in both the *Hv_Morex* and *Hv_Barke TIFY* gene families, while only one segmental duplication was found in the former and three in the latter. The higher frequency of tandem duplication in the *HvTIFY* family is consistent with the previous findings that tandem or local duplication was the key mechanism for gene family expansion [51]. Although many studies have revealed that tandem or duplicated genes usually have similar functions and expression patterns, a considerable portion of duplicated genes have displayed different expression patterns during the growth and development of organisms or when the organisms have been subjected to environmental stress [52]. In this study, the tandem duplicated gene pair of *HvJAZ6* and *HvJAZ12* had a basically consistent expression pattern in the same tissue; however, the duplicated gene pair of *HvJAZ13* and *HvJAZ14* showed completely different expression patterns in the tissues of the lemma, lodicule, tiller, palea, rachis and root, and the pair of *HvZML1* and *HvZML4* displayed distinct expression patterns in most of the 12 examined tissues. These duplicated gene members of *TIFY* family showing different expression patterns might play different functions in barley tissue development [53].

Generally, the function of transcription factors can be predicted according to the cis-elements identified from their promoter. Similar to the trait of *TIFY* gene expression in many species that the *JAZ* subfamily genes are usually expressed at a high level [9,20,33], here we also found that *JAZ* genes (except for *Hv_MoJAZ2*, *Hv_MoJAZ3* and *Hv_MoJAZ13*) displayed a relatively high level of expression in most examined tissues. In particular, *Hv_MoJAZ1*, *Hv_MoJAZ4* and *Hv_MoJAZ7* were highly expressed in all examined tissues, and the promoter of these genes had stress-response elements: LTRs and G-box elements involved in temperature and light responsiveness, hormone-response elements of the GTCA-motif and the ABRE element involved in MeJA and abscisic acid responsiveness. Meanwhile, *Hv_MoJAZ5*, *Hv_MoJAZ6*, *Hv_MoJAZ8*, *Hv_MoJAZ9*, *Hv_MoJAZ11*, *Hv_MoJAZ12* and *Hv_MoJAZ14* exhibited high expression levels in the reproductive organs and root tissues, and multiple cis-elements, mainly related to the stress response, phytohormones and plant growth and development, were detected from the promoter regions of these genes. Based on these identified cis-regulatory elements, we could infer that these barley *JAZ* genes may be involved in the response to varieties of stresses, multiple hormones response processes and the growth and development of reproductive organs and root system in barley.

Barley inevitably encounters diverse stresses during its growth and development, especially the threats of spring cold stress and spot blotch disease during the growth process. Cold stress generally causes positive feedback which generates, together with gene expression, fluctuations in plants. In response to low temperatures, diverse plant species increase their freezing tolerance to a varying degree via a vast reprogramming of gene expression [54]. As transcription factors, *TIFYs* play crucial roles in the regulation of cold tolerance in plants. Studies have shown that JAZ proteins are generally negative regulators of freezing stress tolerance [55]. JAZ1 and JAZ4 physically interacted with ICE1/2, a core component of the cold-induced ICE-CBF-COR pathway, inhibiting the activation of ICE1/2 and its downstream genes, thereby negatively altering cold tolerance

in *Arabidopsis* [22]. In rice, the interaction between OsMYB30 and OsJAZ9 could suppress the expression of *BMY* genes, thus negatively regulating cold tolerance [56]. In apples, both JAZ and MIEL1 proteins interacted with MdBBX37 to impair the activation of MdBBX37, thus destroying the BBX37–ICE1 complex and reducing the cold stress tolerance [57]. In addition, MdJAZ1 and MdJAZ2 negatively modulated JA-mediated cold tolerance in apples through the JAZ-ABI4-ICE1-CBF regulatory cascade [58]. In this study, we found that the expressions of *Hv_MoZML2* and *Hv_MoJAZ12* were upregulated and maintained at a higher level during the whole freezing treatment process compared with the normal growth temperature, and six *JAZ* subfamily genes (*Hv_MoJAZ1*, *Hv_MoJAZ4*, *Hv_MoJAZ6*, *Hv_MoJAZ9*, *Hv_MoJAZ11* and *Hv_MoJAZ14*) showed upregulated expression induced by cold stress only at a specific stage. These genes are valuable candidate genes for genetic improvement of barley freezing tolerance. In view of the fact that the Jas domain is the essential factor in the degradation of JAZ subfamily proteins [15,16,18,59,60], further work is needed to verify the functions of Jas domain proteins in regulating freezing tolerance in barley.

Previous studies have shown that *JAZ* genes are involved in the process of plant responses to a variety of pathogen infections [9,15,16,61–63]. Spot blotch disease incited by *Cochliobolus sativus* is a serious foliar disease limiting grain yield and malting quality in barley [64,65]. To overcome the existing threat of spot blotch to barley production, exploring resistance genes to spot blotch and breeding resistant varieties should be an effective way to control the disease. In the current study, we analyzed the expression patterns of *TIFY* genes in barley after spot blotch infection. For 11 upregulated *Hv_Morex* genes (*Hv_MoTIFY*, *Hv_MoJAZ1*, *Hv_MoJAZ4*, *Hv_MoJAZ5*, *Hv_MoJAZ6*, *Hv_MoJAZ7*, *Hv_MoJAZ8*, *Hv_MoJAZ9*, *Hv_MoJAZ11*, *Hv_MoJAZ12* and *Hv_MoJAZ14*), except for *Hv_MoJAZ1* and *Hv_MoJAZ4*, all of the other nine genes could maintain a relatively higher expression level during the whole process of spot blotch infection compared to the control. It is worth noting that the expression levels of *Hv_MoTIFY*, *Hv_MoJAZ1*, *Hv_MoJAZ4*, *Hv_MoJAZ6*, *Hv_MoJAZ9*, *Hv_MoJAZ11*, *Hv_MoJAZ12* and *Hv_MoJAZ14* were clearly higher in the initial stage of infection (12 h after inoculation) than in other stages of infection, and the genes of *Hv_MoJAZ5*, *Hv_MoJAZ7* and *Hv_MoJAZ8* were expressed at stable levels across the whole infection. These results imply that these 11 *Hv_Morex TIFY* genes, especially the three stably expressed genes, may be candidate genes in response to spot blotch infection and play specific roles in the process of spot blotch invasion.

## 5. Conclusions

The barley *TIFY* family genes were comprehensively and systematically characterized in this study. A total of 21 and 22 nonredundant *TIFY* family members were genome-widely identified in *Hv_Morex* and *Hv_Barke* genomes, respectively. The identified barley *TIFY* genes could be sorted into three main groups, i.e., *TIFY*, *ZML* and *JAZ*, and the group *JAZ* could be further divided into six subgroups, with members of the same group or subgroup having a similar gene structure and motif composition. A relatively high frequency of tandem duplication events was detected in barley *TIFY* family. The molecular evolution analysis revealed that all of the duplicated barley *TIFY* gene pairs were dominated by intense purifying selection. Cis-elements analysis showed that stress response-related and phytohormone-related elements were present in the promoters of all or most *HvTIFY* family genes. In silico expression analysis revealed most members of *Hv_MorexJAZ* group were predominantly expressed in reproductive organs and root tissues, and they were also more involved in the responses to freezing treatment and spot blotch infection than other groups; quite a few *HvJAZ* genes (*Hv_MoJAZ1*, *Hv_MoJAZ4*, *Hv_MoJAZ6*, *Hv_MoJAZ9*, *Hv_MoJAZ11*, *Hv_MoJAZ12* and *Hv_MoJAZ14*) might be associated with barley growth and development and the responses to cold and spot blotch invasion stresses. Therefore, the results of this study lay a foundation for future research related to the functional genomics of barley *TIFY* family genes.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy14081663/s1, Table S1: List of the identified *TIFY* family genes in barley; Table S2: The information of conserved motifs in barley TIFY proteins; Table S3: Analysis of cis-elements in the promoter regions of barley *TIFY* genes; Table S4: Tandemly and segmentally duplicated barley *TIFY* gene pairs; Table S5: One-to-one orthologous relationships of *TIFY* genes between *Hv_Morex* and other five representative plant species; Table S6: One-to-one orthologous relationships of *TIFY* genes between *Hv_Barke* and other five representative plant species; Table S7: The homologous relationships between *Hv_Morex* and *Hv_Barke TIFY* gene pairs.

**Author Contributions:** Conceptualization, J.L.; methodology, J.L. and X.X.; software, J.L. and H.W.; formal analysis, J.L. and Y.Z.; investigation, J.L. and X.X.; resources, J.L.; data curation, J.L., Y.Z. and X.X.; writing—original draft preparation, J.L.; writing—review and editing, J.L. and H.W.; visualization, J.L. and H.W.; supervision, J.L.; funding acquisition, J.L. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the National Natural Science Foundation of China (Grant number 32072608), the Jiangsu Agricultural Science and Technology Independent Innovation Fund (Grant number CX (22) 3175) and the China Scholarship Council (CSC) with File No. 201908320053.

**Data Availability Statement:** The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

**Acknowledgments:** The authors thank Nils Stein, M. Timothy Rabanus-Wallace and Awais Muhammad of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) group of the Genomics of Genetic Resources (GGR), Germany, for their guidance in the process of data analysis.

**Conflicts of Interest:** The authors declare no conflicts of interest.

## Abbreviations

| | |
|---|---|
| TF | Transcription factor |
| AA | Amino acids |
| MW | Molecular weight |
| pI | Isoelectric point |
| CDS | Coding sequences |
| MSA | Multiple sequence alignments |
| ARE | Anaerobic induction element |
| LTR | Low-temperature responsiveness element |
| MBS | MYB binding site |
| ABRE | Abscisic acid responsiveness |
| chr | Chromosome |
| kDa | Kilodaltons |
| Ka | Nonsynonymous |
| Ks | Synonymous |
| JA | Jasmonic acid |
| JAZ | Jasmonate ZIM-Domain |
| ZIM/ZML | Zinc-finger inflorescence meristem/ZIM-like |
| PPD | PEAPOD |
| CCT | CONSTANS, CO-like and TOC1 |
| BLAST | Basic local alignment search tool |
| HMM | Hidden Markov model |

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