Genome-wide characterization of MLO family genes in bread wheat shed light on their role in powdery mildew, rusts, blight, abiotic stresses, and development

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Abstract

Powdery mildew (PM), caused by the fungus, Blumeria graminis Bgt is a devastating and notorious disease that is prevalent across the globe and causes up to 62% of yield losses in wheat. The well-known 68 PM resistance loci are never long-lasting and break down when new races of pathogens interact with host plants. The editing of three known MLO genes through CRISPR/Cas9 has conferred resistance against PM. However, only 10 MLO genes were known in wheat to date. Taking advantage of IWGSC refseq v2.1 and using the gold-standard analysis pipeline that we have established, we expanded this number to 47 and reported 44 additional potential CRISPR targets that can be used to develop PM in wheat. We also performed phylogenetic, gene structure, Motif, and gene duplication, evolution, and comparative phylogenetic analysis with *Arabidopsis thaliana* and *brachypodium distachyon* to establish their evolutionary relationship; and finally, their expression under powdery mildew, rusts, head blight, abiotic stresses, and growth and development and predicted their potential roles in wheat under these conditions. We hope that the MLO genes identified in this study will be a great resource for the wheat community to develop PM resistance in wheat.

Key words: Wheat, *Arabidopsis thaliana*, *Brachypodium distachyon*, MLO, Powdery mildew, evolution, phylogenetic analysis, abiotic stresses

1. Introduction

The climate change not only alters temperature and rain patterns but it results in emergence of new races of pathogens. Several fungal pathogens lower wheat yield by causing diseases such as leaf rust, stripe rust, stem rust, smuts, septoria leaf blotch, fusarium head blight, and powdery mildew (Hussain et al., 2022). Powdery mildew (PM), caused by the fungus, *Blumeria graminis Bgt* is a devastating and notorious disease that is prevalent across the globe and causes up to 62% of yield losses in wheat (Singh et al., 2016). Therefore, efforts have been made to identify resistance genes against this pathogen. A total of 68 resistance genes (officially designated *Pm1* - *Pm68*) were mapped in wheat after the decades long efforts (McIntosh et al.; Hussain et al., 2022). However, resistance against ever evolving pathogens is never long-lasting and break down when new races of pathogens interact with host plants (Scholthof, 2007; Hussain, 2015).

Keeping in view the economic impact of this disease, ten genes were cloned in wheat to develop powdery mildew resistance till date i.e., (*Pm1a, Pm2, Pm3, Pm3b, Pm5e, Pm21, Pm24, Pm38/Lr34/Yr18/Sr57, Pm41, Pm46/Lr67/Yr46/Sr55, Pm60*) (Yahiaoui et al., 2004; Krattinger et al., 2009; Hurni et al., 2013; Moore et al., 2015; Sánchez-Martín et al., 2016; Xing et al., 2018; Zou et al., 2018; Li et al., 2020; Lu et al., 2020; Xie et al., 2020). However, cloning techniques such as map-based cloning are very difficult to perform, is tedious, time consuming, and need specialized lab facilities and skilled manpower, therefore, plants' race to adapt against evolving pathogens is unsuccessful in terms of slow progress in developing PM resistance varieties.

However, the onset of the CRISPR revolution in 2012 (Jinek et al., 2012) and its subsequent use for editing of powdery mildew susceptibility genes, *Mildew Locus O* (*MLO*), conferred resistance against PM as reported by two independent groups (Shan et al., 2014; Wang et al., 2014). The CRISPR/Cas9 based editing of *TaMLO-A1* allele resulted in increased resistance to PM (Wang et

al., 2014). Recently, another group used CRISPR technology to edit another MLO gene for the same purpose (Li et al., 2022). As CRISPR can be used to edit part of susceptibility genes that directly interacts with pathogens (Hussain et al., 2018; Hussain and Ahmad, 2022), so it provides durable resistance against diseases. However, only seven MLO genes were reported in 2010 (Li et al., 2022) and 15 MLO genes are well-known in *Arabidopsis thaliana* so we hypnotized that being a hexaploid, bread wheat should have around 45 MLO genes. In this study, we performed a genome-wide search to identify all MLO genes in bread wheat, studies their distribution in the genome, performed phylogenetic, gene structure, Motif and gene duplication analysis to establish their evolutionary relationship; and finally, their expression under powdery mildew, other diseases, abiotic stresses, and growth and development to predict their potential roles in wheat. We hope that additionally, MLO genes identified in this study will be a great resource for wheat community to develop PM resistance in wheat.

2. Methodology

2.1. Sequence Retrieval and Genome-wide identification of MLO Genes

The *T. aeastivum* genome assemblies, proteomes, transcriptomes along with general feature format files (GFF3) were downloaded from the International Wheat Genome Sequencing Consortium v2.1 (IWGSC RefSeq v2.1) (Zhu et al., 2021) website (https://urgi.versailles.inrae.fr/download/iwgsc/IWGSC_RefSeq_Assemblies/v2.1/). A total of 15 *Arabidopsis thaliana* MLO sequences were downloaded from The Arabidopsis Information Resource (TAIR).

Genome-wide identification of MLO gene family was performed for *Triticum aeastivum. Arabidopsis thaliana* MLO sequences were used as query sequences for the genome-wide search of MLO genes within the genomes of *Triticum aeastivum (Traes)* through BLAST. Additionally, an HMMER-based search was performed to predict the MLO proteins in wheat genomes (Potter et al., 2018). The presence of complete MLO superfamily domain in the obtained proteins was confirmed through the NCBI Conserved Domain Database (CDD) search tool v3.19

(https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Th proteins without complete MLO conserved domain were not considered for further analysis as previously described (Raza et al., 2022). Moreover, Expasy server's pI/Mw tool (https://web.expasy.org/compute_pi/) was used for the determination of biophysical characteristics like molecular weight and isoelectric point of the MLO peptides. The subcellular localization of proteins was predicted with a web-based server Bologna Unified Subcellular Component Annotator (BUSCA) (https://busca.biocomp.unibo.it/). For the purpose, the organ that proclaimed the highest value was taken as the location of the protein.

2.2. Chromosomal Mapping/Distribution across the genome

The identified genes were mapped on the chromosomes by using TB Tools software (Chen et al., 2020). The chromosomal location of genes extracted from GFF3 file.

2.3. Phylogenetic Analysis

The identified MLO sequences were aligned through multiple sequence alignment (MSA) tool i.e., Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al., 2011) with default settings. The resultant multiple sequence alignment was used to construct a phylogenetic tree with the maximum likelihood (ML) method using MEGA v11.0 (Kumar et al., 2018).

2.4. Comparative Phylogenetic Analysis

The identified MLO sequences along with 15 *AtMLO* sequences were aligned through Clustal Omega and the rest of procedure was the same as described above.

2.5. Gene Structure and Motif Analysis

The coding sequences (CDS) along with respective genomic sequences for all *TraesMLO* genes were pasted in the Gene Structure Display Server (GSDS) tool (http://gsds.gao-lab.org/) to visualize the exons, introns, and down and upstream regions of the genes.

Conserved motifs/domain analysis was performed by online tool MEME 5.1 (http://meme-suite.org/tools/meme). We tried different settings for the proper identification of conserved motifs and found a suitable setting of 16 as maximum number of motifs and 50 as maximum length of motifs. The motif data was downloaded as an XML file to be used by TB tools software (Chen et al., 2020) to visualize and synchronize the gene structure.

2.6. Divergence (Gene Duplication) and Evolution Analysis

We followed the methodology described by Raza and colleagues (Raza et al., 2022) for the purpose. The Coding sequences (CDS) of all *TraesMLO* genes were subjected to MSA using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al., 2011) and aligned sequences were MAFFT aligned (pairwise) in all possible combinations using Sequence Demarcation Tool v1.2 **(Muhire et al., 2014)** to calculate the sequence identities. Gene pairs with 90% identity (E value < $1e^{-10}$) were classified as duplicated. The duplicated gene pairs that were mapped on the same chromosome were considered as tandem duplication and the ones located on different chromosomes were defined as segmental duplication.

The CDS of duplicated genes were MAFFT aligned before being subjected to the Synonymous Non-Synonymous Analysis Program v2.1.1 (www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html) to compute the synonymous (Ks) and non-synonymous (Ka) substitutions. The Ka/Ks was also calculated to predict the codon selection during evolution. The calculation for an approximate divergence time for the duplicated genes was done by using formulae T = Ks/2r × 10−6 assuming a substitution rate (r) of **6.5 × 10−9** wheat genome paper substitutions/synonymous site/year **(El Baidouri et al., 2017).**

2.7. Expression Analysis

RNA-seq based expression patterns of all identified MLO genes during the whole growth and development period, as well as under different abiotic (cold, drought, heat and phosphate starvation) and biotic (fusarium head blight, powdery mildew and stripe rust) stresses were retrieved from the expVIP Wheat Expression Browser as Log2 TPM (processed expression value in transcripts per million (Borrill et al., 2016; Ramírez-González et al., 2018). Expression heatmaps were generated with TBtools (Chen et al., 2020).

3. Results and Discussion

3.1. Discovery of MLOs in bread wheat

Using the bread wheat genome Refseq v2.1, we initially identified 61 genes encoding MLO superfamily proteins using MLO protein sequences from *Arabidopsis thaliana* as queries. Two duplicate genes were deleted by sorting the genes on the basis of their names. Multiple sequence alignment identified four pseudogenes with incomplete domain and probably lost function of the

proteins (Panchy et al., 2016). And phylogenetic tree helped to identify 8 isomers thus leaving behind 47 non-redundant MLO genes. NCBI CDD search was used to confirm the presence of MLO super family principal domain in the protein sequence of these genes. Following the confirmation, renaming of these TraesMLO genes was done in ascending order according to their positions on the chromosomes (TraesMLO1 to TraesMLO47) following the renaming in other species (Deshmukh et al., 2014; Kusch et al., 2016; Win et al., 2018). The peptide length of TraesMLO proteins ranged from 409 to 612 amino acids and average length was 514 amino acids (**Table 1**). The isoelectric point values varied from 7.36 to 9.87, while the molecular weight of the proteins was in range of 46.98 to 68.50 kDa, with a mean kDa value of 58.34 (**Table 1**).

Several other groups reported a varying number of MLO superfamily members in different plant species. For example, 15 MLO genes were identified in Arabidopsis (Devoto et al., 2003), whereas 20 in pumpkin (Win et al., 2018), 15 in lentil (Polanco et al., 2018), seven in bread wheat (Konishi et al., 2010), 20 in strawberry (Tapia et al., 2020), 68 in octaploid strawberry (Tapia et al., 2020), 39 in soybean (Deshmukh et al., 2014) etc. As several species have 15 MLO genes therefore, wheat has 47 MLO genes being a hexaploid.

Table 3. Detailed properties of identified MLO genes in *Triticum aestivum* (AABBDD genome).

S#	Gene Name/ID	New Gene Names	LINKAGE Group	Gene Start	Gene End	Gene Length (bp)	Strand	pI	Mw (kDa)	Protei Lengt (AA)
1	TraesCS1A03G0539600.1	TraesMLO1	Chr1A	364505363	364509878	4515	-	9.36	63.32	56
2	TraesCS1A03G0668200.1	TraesMLO2	Chr1A	457568166	457572624	4458	-	9.08	56.52	49
3	TraesCS2A03G0163000.1	TraesMLO3	Chr2A	42897580	42900950	3370	-	9.45	55.86	49
4	TraesCS2A03G0163100.1	TraesMLO4	Chr2A	42905631	42908828	3197	-	9.63	55.33	48
5	TraesCS2A03G0775400.1	TraesMLO5	Chr2A	539693546	539696928	3382	-	9.03	55.33	49
6	TraesCS2A03G1299000.1	TraesMLO6	Chr2A	768633437	768638338	4901	-	9.22	68.50	60
7	TraesCS3A03G0013800.1	TraesMLO7	Chr3A	6965742	6973314	7572	-	8.51	61.25	54
8	TraesCS3A03G0944700.1	TraesMLO8	Chr3A	648479170	648482130	2960	-	9.33	54.81	48
9	TraesCS4A03G0580300.1	TraesMLO9	Chr4A	518674297	518679167	4870	+	8.62	58.39	50
10	TraesCS5A03G0963900.1	TraesMLO10	Chr5A	599592378	599596257	3879	+	9.12	62.37	56
11	TraesCS5A03G1150400.1	TraesMLO11	Chr5A	661126976	661129967	2991	-	9.87	61.05	54
12	TraesCS5A03G1158600.1	TraesMLO12	Chr5A	664743006	664756436	**13430**	+	7.36	64.06	58
13	TraesCS5A03G1159400.1	TraesMLO13	Chr5A	664797864	664801136	3272	-	9.53	60.29	53
14	TraesCS6A03G0387200.1	TraesMLO14	Chr6A	154203217	154207538	4321	+	8.70	56.92	49
15	TraesCS6A03G0805500.1	TraesMLO15	Chr6A	545643614	545648358	4744	-	9.28	54.72	48
16	TraesCS7A03G0928000.1	TraesMLO16	Chr7A	561794230	561801544	7314	-	9.51	54.31	47
17	TraesCS1B03G0628500.1	TraesMLO17	Chr1B	398073082	398077619	4537	-	9.41	63.28	56
18	TraesCS1B03G0759600.2	TraesMLO18	Chr1B	484447774	484452526	4752	-	9.15	56.25	49
19	TraesCS2B03G0227100.1	TraesMLO19	Chr2B	65216529	65219835	3306	-	9.52	55.85	49
20	TraesCS2B03G0227200.1	TraesMLO20	Chr2B	65315357	65318657	3300	-	9.59	55.36	48
21	TraesCS2B03G0856800.1	TraesMLO21	Chr2B	479483631	479486919	3288	+	8.99	55.06	49
22	TraesCS2B03G1489500.1	TraesMLO22	Chr2B	788988904	788994031	5127	-	9.38	68.44	61
23	TraesCS3B03G1076900.1	TraesMLO23	Chr3B	689913560	689916559	2999	+	9.34	55.05	48
24	TraesCS4B03G0222000.1	TraesMLO24	Chr4B	104220694	104226387	5693	+	7.97	59.19	51
25	TraesCS4B03G0835400.1	TraesMLO25	Chr4B	611711110	611728680	**17570**	+	9.72	60.87	54
26	TraesCS4B03G0836900.1	TraesMLO26	Chr4B	612012622	612014655	2033	-	9.49	60.39	53
27	TraesCS5B03G1014800.1	TraesMLO27	Chr5B	590155469	590160172	4703	+	9.00	59.26	53
28	TraesCS6B03G0494800.1	TraesMLO28	Chr6B	232292016	232296365	4349	-	8.55	56.56	49
29	TraesCS6B03G0955000.1	TraesMLO29	Chr6B	601764897	601769827	4930	-	9.14	56.54	49
30	TraesCS7B03G0775700.1	TraesMLO30	Chr7B	524484720	524491473	6753	-	9.14	57.36	50
31	TraesCS1D03G0519600.1	TraesMLO31	Chr1D	293427154	293431832	4678	-	9.42	63.60	56
32	TraesCS1D03G0630400.1	TraesMLO32	Chr1D	357744682	357749428	4746	-	9.09	56.56	49
33	TraesCS2D03G0160400.1	TraesMLO33	Chr2D	37249636	37253058	3422	-	9.53	55.81	49
34	TraesCS2D03G0160500.1	TraesMLO34	Chr2D	37272260	37275524	3264	-	9.76	54.30	49
35	TraesCS2D03G0715400.1	TraesMLO35	Chr2D	400923755	400926943	3188	-	8.60	55.34	49
36	TraesCS2D03G1262400.1	TraesMLO36	Chr2D	638659281	638665452	6171	-	9.28	68.46	61
37	TraesCS3D03G0010400.1	TraesMLO37	Chr3D	1805922	1810130	4208	-	9.06	50.84	44
38	TraesCS3D03G0877800.1	TraesMLO38	Chr3D	513351507	513355165	3658	-	9.41	55.19	48
39	TraesCS4D03G0182500.1	TraesMLO39	Chr4D	69109133	69114152	5019	+	8.93	57.68	50
40	TraesCS4D03G0743900.1	TraesMLO40	Chr4D	483173859	483176970	3111	+	9.77	60.55	54
41	TraesCS4D03G0744500.1	TraesMLO41	Chr4D	483205801	483208560	2759	-	9.52	60.45	53
42	TraesCS5D03G0918700.1	TraesMLO42	Chr5D	481711884	481717428	5544	+	9.07	60.50	54
43	TraesCS6D03G0340700.1	TraesMLO43	Chr6D	148637851	148642268	4417	-	8.80	56.91	49

44	TraesCS6D03G0681600.1	TraesMLO44	Chr6D	418198229	418205340	7111	-	9.35	46.98	40
45	TraesCS7D03G0041500.1	TraesMLO45	Chr7D	8641641	8683479	**41838**	-	9.83	53.97	46
46	TraesCS7D03G0062000.1	TraesMLO46	Chr7D	14007248	14014362	7114	+	9.69	54.81	48
47	TraesCS7D03G0891400.1	TraesMLO47	Chr7D	493179947	493187259	7312	-	9.15	57.52	50

3.2. Distribution of MLOs in bread wheat genome/s

Total 16, 14, and 17 MLO genes were mapped on A, B, and D Genomes of Traes, respectively in non-random fashion (**Figure 1**). Such non-random chromosomal distribution of genes has been reported in other species as well (Ahmed et al., 2021). Nonrandom distribution of plant gene families originates from long terminal repeat retrotransposons through evolution (Gao et al., 2004). Chromosomes 2A, 2B, and 2D harbored maximum four TraesMLO genes each while chromosome 1A, 1B, 1D, 6A, 6B, and 6D contained two genes each. Interestingly, chromosome 5A harbored four TraesMLO genes while 5B and 5D contained only one gene. Similarly, chromosome 4A harbored only one gene in contrast to three TraesMLO genes on both 4B and 4D. Additionally, the number of mapped genes on 7D were three in comparison to one gene on 7A and 7B while two, one, and two genes were mapped on chromosome 3 in three genomes, respectively (**Figure 1**).

Previously, seven MLO gene members were identified in wheat and none of these genes showed change in their expression under abiotic stresses in 2010 (Konishi et al., 2010). This could be linked with non-availability of reliable wheat reference genome till that date. However, the availability of whole genome refence sequence of bread (IWGSC, 2014; Appels et al., 2018) has provided us with an opportunity to scan whole genome and were able to identify 47 MLO genes. Additionally, these seven MLO gene members were not assigned to chromosomes due to non-availability of annotated genome (Konishi et al., 2010) that we have shown here.

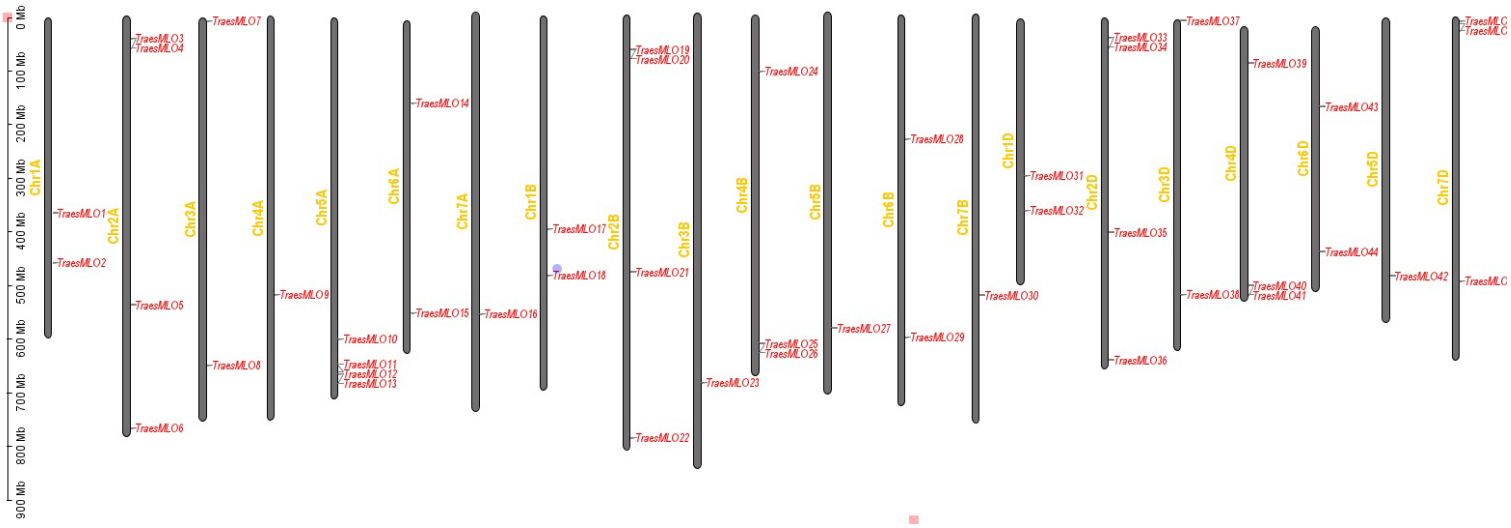

Figure 1: Chromosomal mapping of 47 bread wheat MLO genes on all 21 chromosomes of wheat

3.3. Phylogenetic Classification of wheat MLO family

We constructed an unrooted phylogenetic tree to demonstrate the evolutionary relationship of the MLO members in ancestral and modern wheat. The phylogenetic tree showed the distribution of MLO proteins into three distinct groups (Fig. 2). The distribution of MLO superfamily members into three groups is because group-specific motifs are conserved among the proteins. Group I represent the largest group with 28 members out of 47 MLO superfamily proteins identified in bread wheat, but physiological roles of most of these proteins are not known yet (Fig. 2). Group two and three were relatively small. Interestingly, the group I members shared some unique motifs among MSA e.g., VPLLSLEAJHQLHIFIFVLAVTHVLFSAITMLLGGAKIRQWKKWE, KRHKKALYEALLKVKEELMLLGFISLLLTVTQGPISKICIP, and ARRLLAGGAASDDHC (Fig. 2). Total thirteen TraesMLO superfamily proteins were present Group II with variable forms of conserved motifs e.g., GDLVVKPSDDHFWFGRP, ARRLLAGGAASDDHC and LRGNPKFBFHK (Fig. 2). Group III had only six TraesMLO superfamily proteins with variable motifs i.e., ESAMSHMLPCKPPPHSVAAEG and LRGNPKFBFHK (Fig. 2).

3.5. Conserved Domain and Gene Structure Analysis

3.5.1. Sequences and characteristics of identified MOTIFs

The MEME online server was used to perform conserved domain analysis to further figure on the diversity in the structure of TraesMLO genes. A total number of 16 conserved domains were

identified among 47 TraesMLO genes (**Table 2; Fig 2**). The width of the conserved domains was found to be ranging from 11 (Motif 12) to 50 (Motif 5) amino acids (**Table 2**), while the different motifs are illustrated with different colors in Figure 3. Within the groups, the nature, number, and patterns of motifs were observed almost same but variations among the groups and subgroups can easily be seen. For example, in group III, there are 9-10 motifs that are highly conserved, while in group I, there are 11-14 motifs that are conserved. Furthermore, group I also shows differentiation within its subgroups, namely IA, IIB, and IC based on conserved motifs (**Fig 2**). Furthermore, some motifs clearly differentiate the groups and subgroups based on their presence and absence. For example, motif 11 with 21 amino acids (ESAMSHMLPCKPPPHSVAAEG) was absent in group III but present in group II (**Fig 2**).

Similarly, if we see the distinctness within the group then motif 16 with 32 amino acids (AHPVHRVPPCDGWRSASSPALDAHIPGADFCF) was differentiating between the subgroups IIA and IIB as it is absent in subgroup IIA but present in all the member of subgroup IIB (**Fig 2**). The four motifs, Motif 1 with 32 amino acids (LIHFILFQNAFEIAFFFWILVTYGFKSCIMGN), motif 2 with 33 amino acids (YMIRALEDDFKKVVGISWYLWAFVVIFLLLNVN), motif 3 with 45 amino acids (VPLLSLEAJHQLHIFIFVLAVTHVLFSAITMLLGGAKIRQWKKWE) and motif 4 with 41 amino acids (KRHKKALYEALLKVKEELMLLGFISLLLTVTQGPISKICIP) are conserved in all the 47 TraesMLO genes that demonstrate the basic structural and functional resemblances among all the genes. It is reported that MLO gene belongs to a group of plant disease susceptible genes with leucine rich characteristics (Singh et al., 2012). We found that all the putative 47 TraesMLO genes are leucine rich. Furthermore, it was also observed that each member of the TraesMLO genes adheres to the seven-transmembrane structure, a significant hallmark of the MLO gene family (Devoto et al., 2003). While not all MLO genes across various species follow this specific structure, they are still classified as MLO genes. For example, in soybean, among the 39 identified MLO genes, only 18 possess the precise configuration of seven transmembrane domains; others depict variations with more or fewer transmembrane domains (Deshmukh et al., 2014).

Table 2: Multi-level consensus sequences for the MEME defined domains observed among different TaMLO proteins.

Motif	Multilevel Consensus Sequence	E-value	No. of Sites	*Width
1	LIHFILFQNAFEIAFFFWILVTYGFKSCIMGN	7.1e-1067	47	32
2	YMIRALEDDFKKVVGISWYLWAFVVIFLLLNVN	3.5e-1016	47	33
3	VPLLSLEAJHQLHIFIFVLAVTHVLFSAITMLLGGAKIRQWKKWE	3.0e-1360	47	45
4	KRHKKALYEALLKVKEELMLLGFISLLLTVTQGPISKICIP	4.4e-1192	47	41
5	YVIARLVJGVIVQVLCSYSTLPLYAJVTQMGSSFKKSIFDEQVQEGLVNW	4.7e-1362	45	50
6	ILSWMRSFFKQFYGSVTKEDYTTLRLGFIMKH	5.0e-905	46	32
7	GWHTYFWISFVPLILLLVVGTKLEHIITELAYEVAZKHAAI	2.2e-1070	46	41
8	GGGEARTLELTPTWIVASVCSVIVIISLLFERLLHRLGKWL	4.8e-949	42	41
9	GDLVVKPSDDHFWFGRP	7.9e-427	44	17
10	ENAGNGPSMIRHVHZFKFIRDHFKGHGKR	2.4e-416	37	29
11	ESAMSHMLPCKPPPHSVAAEG	2.10E-233	41	21
12	LRGNPKFBFHK	4.10E-153	44	11
13	ARRLLAGGAASDDHC	7.60E-127	27	15
14	EPIDEANGGAVQMTNTRANSSVEQGTARL	2.10E-102	6	29
15	QPSRGSSPVHLLHKAGARSDDPQSAPPSP	1.60E-102	10	29
16	AHPVHRVPPCDGWRSASSPALDAHIPGADFCF	4.60E-101	7	32

3.5.2. Comparison of Gene Structure and MOTIFs with Phylogenetic Tree

Different exon and intron structures are of great importance for plant biodiversity and variation among gene families. These distinct structures play an important role in the fate of genes (Xu et al., 2012). Our Gene Structure analysis revealed that *TraesMLO* genes have introns ranging from 9 to 15 (**Fig 2**). Similar exon/intron patterns have been outlined in bitter gourd (*Momordica charantia*) with 8 to 17 introns (Chen et al., 2021) and bottle gourd (*Lagenaria siceraria*) with 5 to 15 introns (Wang et al., 2023). On the other hand, the gene structure analysis in melon (*Cucumis melo*) showed the range of introns from 1 to 16 (Zhang et al., 2023). Total 21 Out of 47 *TraesMLO* genes shared the same 13 number of introns. Furthermore, results revealed that only one gene TraesMLO40 contains 9 introns, *TraesMLO11*, *TraesMLO12* and *TraesMLO25* contains 10 introns, *TraesMLO13*, *TraesMLO26*, *TraesMLO41* and *TraesMLO45* contains 11 introns, *TraesMLO44* contains 12 introns, 8 genes contain 14 introns, and 9 genes contains 15 introns (**Fig 2**). It was also noticed that 6 genes (*TraesMLO11*, *TraesMLO12*, *TraesMLO15*, *TraesMLO23*, *TraesMLO25*, *TraesMLO29*) have no upstream and downstream regions, 3 genes (*TraesMLO10*, *TraesMLO27* and *TraesMLO38*) without upstream and 2 genes (*TraesMLO16* and *TraesMLO45*) without downstream region. In comparison with the phylogenetic tree, we found that all the

members of group III in phylogenetic tree have same intron/exon distribution patterns that confirmed the evolutionary phylogenetics in our studies. All the members of phylogenetic subgroups IIA and IIIB also follow the same pattern of intron/ exons patterns (**Fig 2**). Overall, our gene structure analysis strongly supports evolutionary phylogenetic relationships.

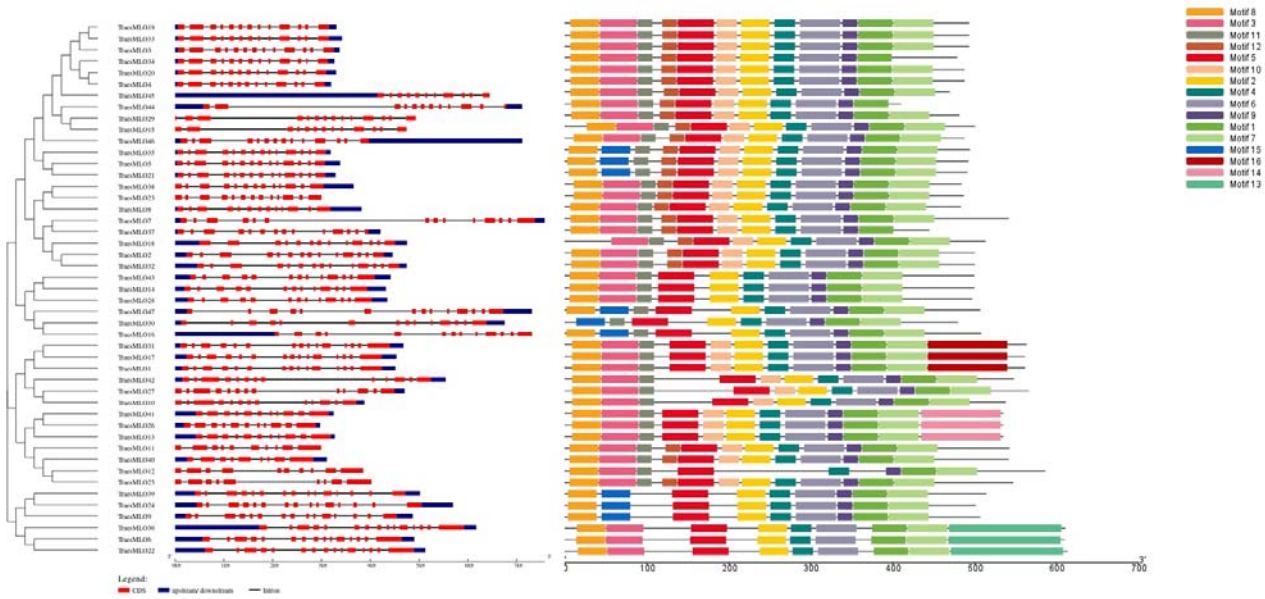

Figure 2: Comparative phylogenetic tree of 47 TraesMLO genes with *Arabidopsis thaliana* genes

3.4. Comparative Phylogeny of wheat MLOs with model and cereal plants

Comparative phylogenetic analysis was performed by using the 15 reported proteins of *Arabidpsis thaliana*, 12 proteins of *Brachypodium distachyon* (expanded from previous reported 11) and newly identified 47 MLO proteins of *Triticum asestivum* to deep dive into the evolutionary patterns and functional confirmation of MLO proteins. As a result, we found that all the proteins are distributed into six clades (**Fig 3**). Similarly, six clade pattern has been reported in Milberry (Ramesha et al., 2020), Melon (*Cucumis melo*) (Zhang et al., 2023), *Fragaria vesca* (Jambagi et al., 2017). We classified the clades according to the pattern of Arabidopsis reported in Devoto et al., 2003. In the Clade I, AtMLO04, AtMLO11, AtMLO14 were distributed with 2 BradiMLO and 6 TraesMLO genes and , Clade II contained AtMLO13, AtMLO01, AtMLO15 genes with 4 BradiMLO and 14 TraesMLO genes, Clade III contained AtMLO09, AtMLO05, AtMLO08,

AtMLO07, AtMLO10 genes with 3 BradiMLO and 6 TraesMLO genes, and Clade V contained AtMLO02, AtMLO06, AtMLO12 with BradiMLO02 and 7 TraesMLO genes. Interestingly, there was not a single gene that clustered in the clade with AtMLO03 but we kept this as clade VI keeping in mind the previous studies that supported six clades pattern in monocots especially as reported in cereal model species- *Brachypodium distachyon* (Ablazov and Tombuloglu, 2016). It is possible that the absence of genes exhibiting a similar phylogeny to AtMLO03 in wheat and *Brachypodium distachyon* might be attributed to difference between Arabidopsis and wheat with respect to monocot and dicot point of view. Furthermore, the intriguing identification of Clade IV exclusively containing 13 TraesMLO genes with 02 BradiMLO genes, devoid of any AtMLO genes, provides a valuable insight into the monocot-specific nature of this clade. This observation may potentially indicate distinct functional roles and evolutionary paths that are specific to monocotyledonous species, separate from their dicotyledonous counterparts. In addition, we used *Brachypodium distachyon*, a model species in cereals, solely for the purpose of verifying the absence of AtMLO genes within this clade. It is thinkable that this clade could be specific to cereals, thus offering valuable insights to researchers in discerning the evolutionary history and phylogenetics of cereals. In conclusion, our comparative phylogenetic analysis of MLO proteins in *Arabidopsis thaliana*, *Brachypodium distachyon* and *Triticum aestivum* has yielded valuable insights into the evolutionary patterns and functional implications of these proteins. The consistent six-clade distribution pattern across diverse plant species suggests a deep-rooted and robust evolutionary framework. The alignment with well-established Arabidopsis patterns, as well as the observed monocot-specific clades, adds further layers of complexity and specificity to the understanding of MLO protein evolution. This study contributes to the broader field of plant molecular evolution and lays the foundation for future investigations into the functional significance of these identified clades in the context of disease resistance and plant development.

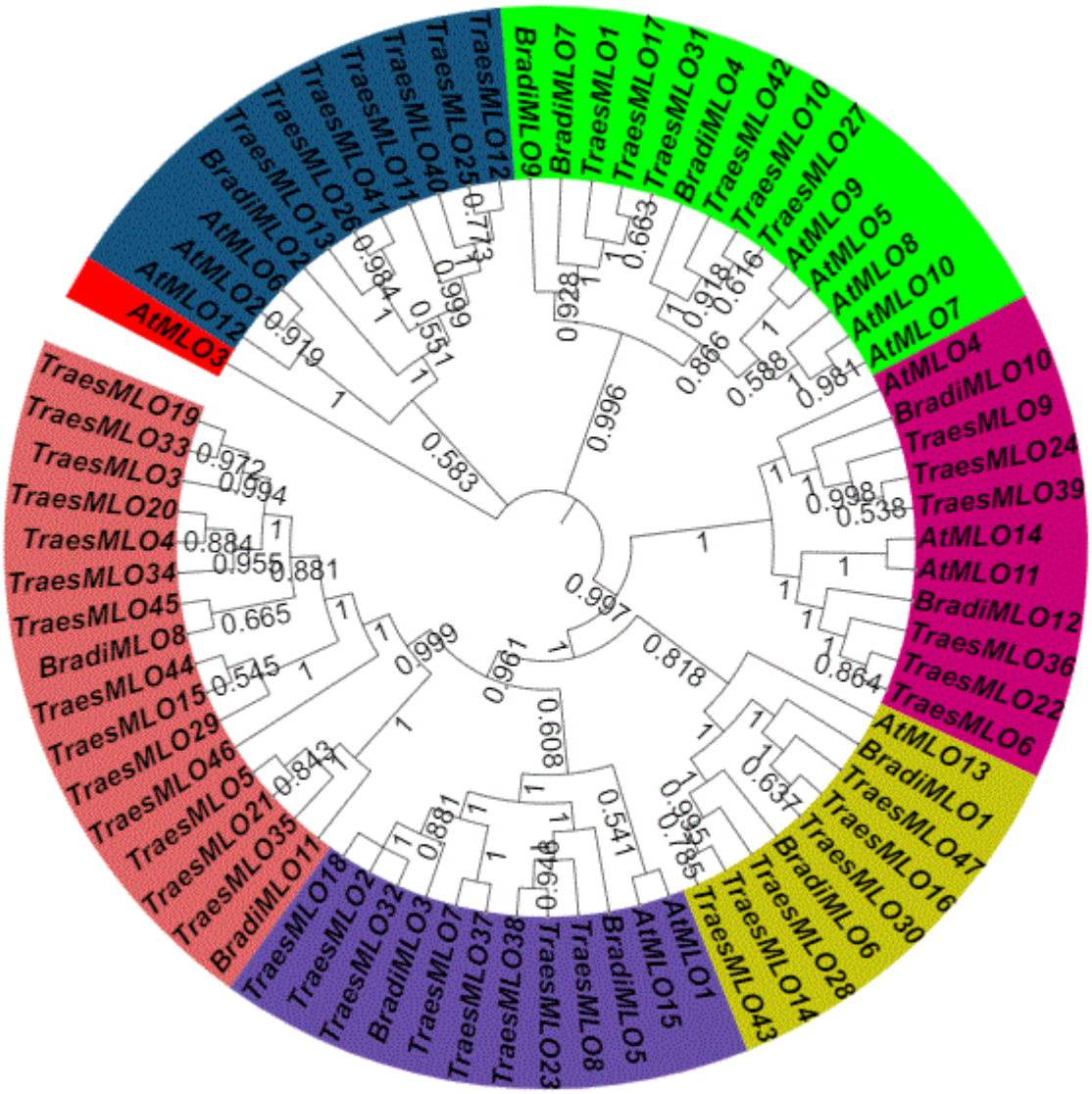

Figure 3: Comparative phylogenetic tree of 47 *TraesMLO* genes with *Arabidopsis thaliana* and brachypodium proteins

3.6. Gene Duplication Analysis

All duplications are segmenal.

Table 3: Gene duplication analysis of

S#	Gene 1	Gene 2	Chr.*(Gene 1; Gene 2)	% Identity	Duplication Type
1	*TraesMLO40*	*TraesMLO25*	4D; 4B	96.30	Segmental
2	*TraesMLO13*	*TraesMLO26*	5A; 4B	95.00	Segmental
3	*TraesMLO13*	*TraesMLO41*	5A; 4D	95.64	Segmental
4	*TraesMLO26*	*TraesMLO41*	4B; 4D	97.13	Segmental

5	*TraesMLO1*	*TraesMLO31*	1A; 1B	97.56	Segmental
6	*TraesMLO1*	*TraesMLO17*	1A; 1D	97.27	Segmental
7	*TraesMLO31*	*TraesMLO17*	1B; 1D	98.40	Segmental
8	*TraesMLO27*	*TraesMLO42*	5B; 5D	98.00	Segmental
9	*TraesMLO27*	*TraesMLO10*	5B; 5A	97.83	Segmental
10	*TraesMLO42*	*TraesMLO10*	5D; 5A	96.78	Segmental
11	*TraesMLO43*	*TraesMLO14*	6D; 6A	96.99	Segmental
12	*TraesMLO43*	*TraesMLO28*	6D; 6B	97.31	Segmental
13	*TraesMLO14*	*TraesMLO28*	6A; 6B	97.79	Segmental
14	*TraesMLO30*	*TraesMLO16*	7B; 7A	96.36	Segmental
15	*TraesMLO30*	*TraesMLO47*	7B; 7D	95.18	Segmental

3.7. Expression Analysis of MLO genes

3.7.1. TaMLOs expression at different stages of developments

MLO genes expression patterns have been extensively investigated in crop plants during growth and development, as well as under biotic and abiotic stresses. In this study, we analyzed *TaMLO*s expression perturbations in several tissues at different time points to get insights into functional roles under prevalent conditions. Out of total 47 genes, 28 (~60%) were expressed in at least two different tissues during whole growth and development period (**Figure 4A**). Among these expressed genes, at least 9 (*TaMLO2, TaMLO3, TaMLO13, TaMLO14, TaMLO26, TaMLO28, TaMLO41, TaMLO43* and *TaMLO45*) were ubiquitously expressed in nearly all investigated tissues. Interestingly, all these genes exhibited highly significant expression patterns in flag leaf sheath and blade during reproductive phase (30% spike to grain ripening stages) (**Figure 4A**). However, several other genes exhibited tissue specific and growth stage specific expression perturbations. Collectively, these expression patterns indicate diverse functions of MLO genes in wheat growth and development.

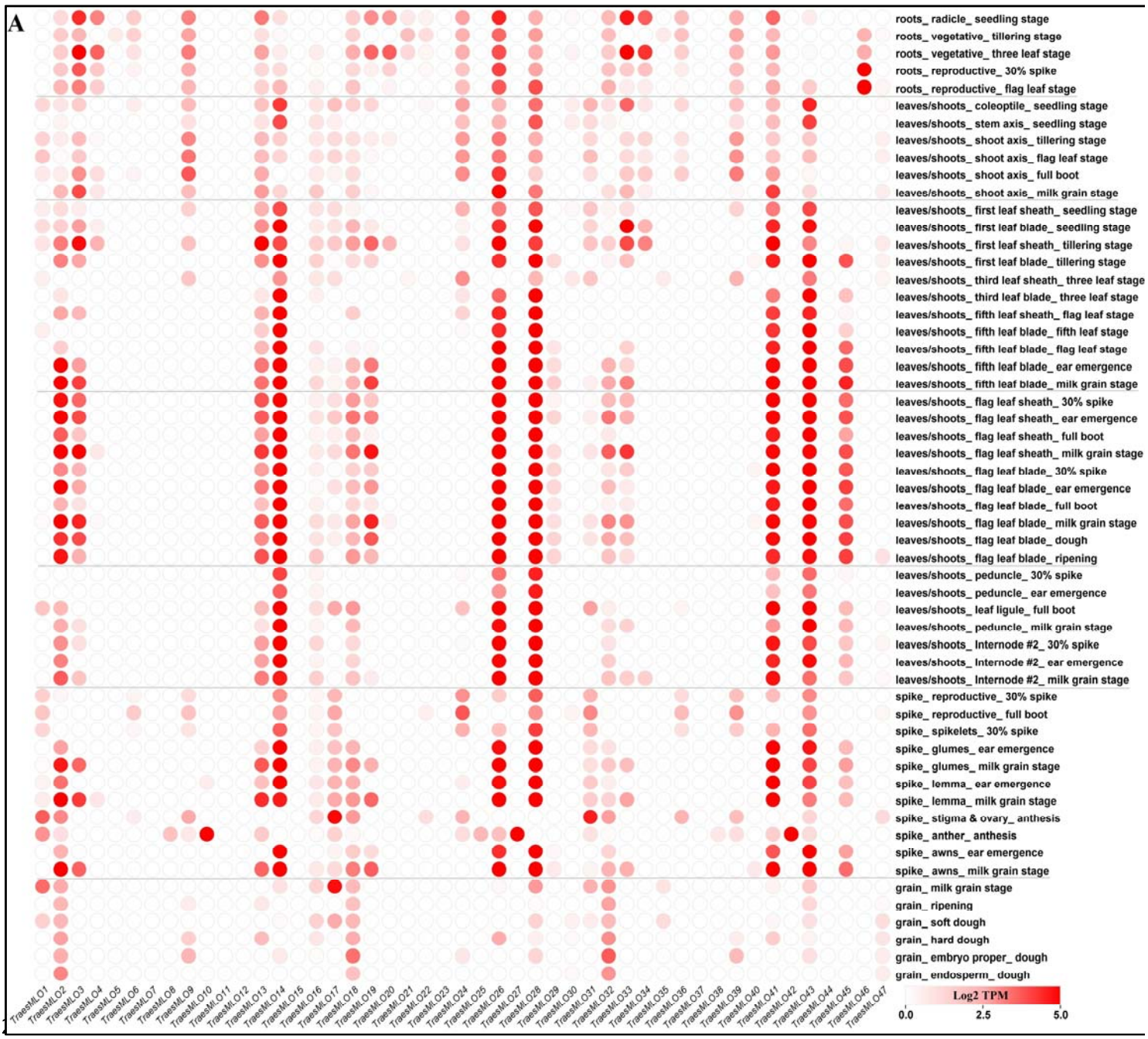

Figure 4A： Expression patterns of TaMLOs during different stages of growth and development.

3.7.2. TaMLOs expression at different biotic and abiotic stresses

We also studied the transcriptional changes in *TaMLO*s were under four different abiotic stresses including cold, drought, heat and phosphate starvation (**Figure 4B**), as well as under three different biotic stresses including fusarium head blight (FHB), powdery mildew (PM) and stripe rust (SR) inoculations (**Figure 4C**). Likewise, expression patterns during growth and development,

almost ≥60% genes were expressed after at least one abiotic and/or biotic stress application (**Figure 4B** & **4C**).

Majority of these genes expressed under phosphate starvation and FHB infections. Interestingly, all those genes which were highly ubiquitously expressed during different growth and development stages were also highly expressed under abiotic and biotic stresses, except for *TaMLO45*. Under abiotic stresses, seven genes after two weeks of cold stress (*TaMLO1, TaMLO13, TaMLO18, TaMLO31, TaMLO32, TaMLO33* and *TaMLO45*), six genes after 1–12 hours of drought stress (*TaMLO4, TaMLO19, TaMLO20, TaMLO32, TaMLO33* and *TaMLO34*), three genes after 1–6 hours of heat stress (*TaMLO29, TaMLO33* and *TaMLO44*) and three genes under phosphate starvation at vegetative stage (*TaMLO16, TaMLO33* and *TaMLO39*) exhibited substantial transcriptional perturbations compared with their respective control (CK) treatments (**Figure 4B**).

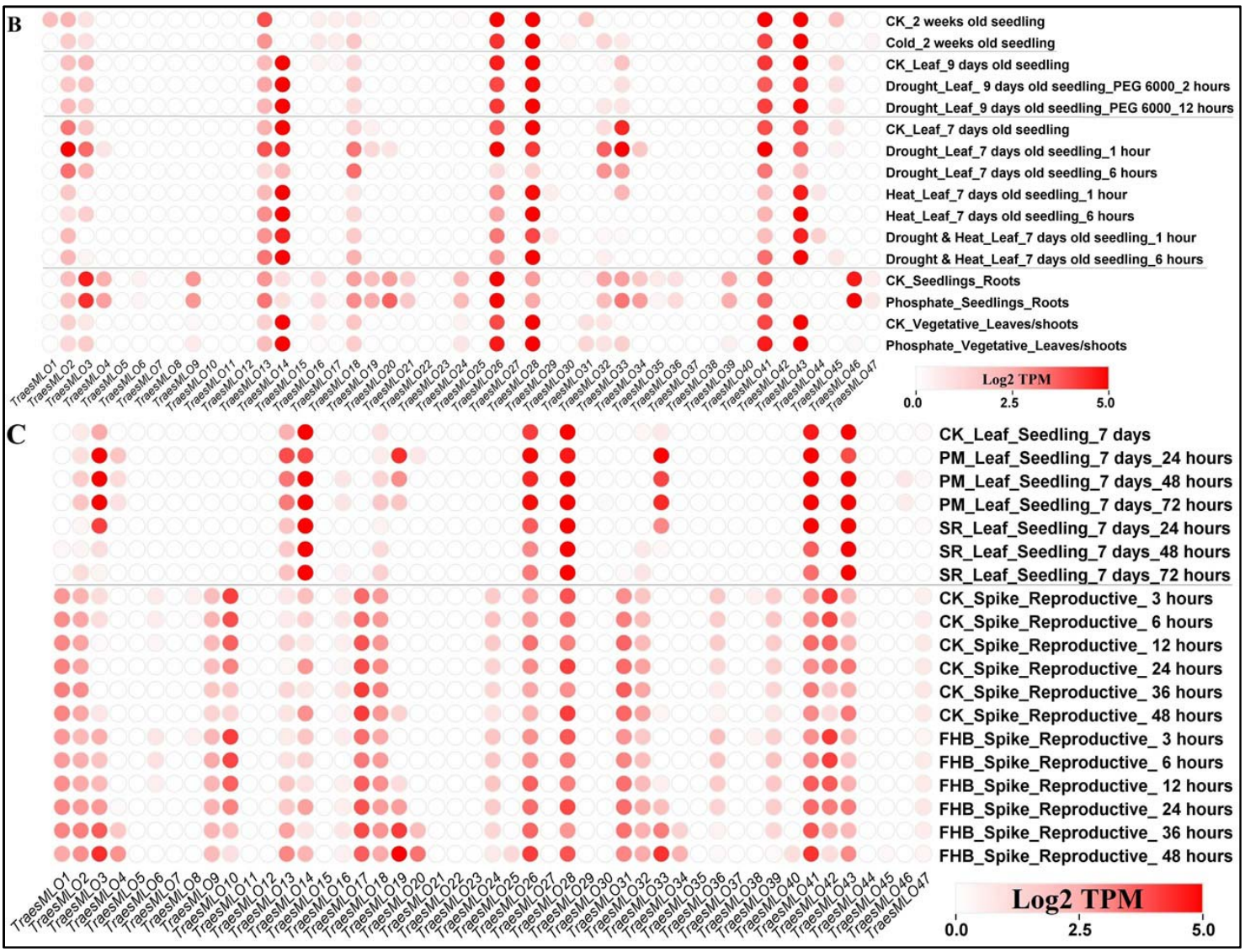

Figure 4: Expression patterns of TaMLOs under different (B) abiotic and (C) biotic stresses.

Under biotic stresses, at least four genes (*TaMLO4, TaMLO13, TaMLO16 and TaMLO46*) exhibited powdery mildew specific transcriptional perturbation when compared with their respective control treatment. Whereas, only *TaMLO32* showed stripe rust specific expression changes. Likewise, at least five genes (*TaMLO4, TaMLO6, TaMLO19, TaMLO20, TaMLO33* and *TaMLO34*) exhibited FHB specific expression patterns when compared with respective control treatments (**Figure 4C)**. Overall, these transcriptional perturbations during growth and development and under different abiotic and biotic stresses indicate that *TaMLO*s regulate multifarious processes to successfully complete bread wheat life cycle under continuously changing environmental conditions.

MLO family genes are unique to plants, governing resistance against powdery mildew infections through a negative regulatory pathway However, apart from conferring plant immunity, previous studies have also reported their diverse roles in growth and development and stress responses (Bindzinski et al., 2014; Tian et al., 2022), just like multifarious roles of MADS-box family genes in bread wheat (Raza et al., 2022). For example, a systematic study of rice MLO genes indicated their roles in morphological development, photoperiod sensitivity, abiotic stress tolerance and resistance against different biotic stresses (Nguyen et al., 2016). In this study, a substantial number of MLO genes also showed ubiquitous expression patterns during growth and development, abiotic and biotic stresses (**Figure 4A**, **4B** & **4C**). These results support previous findings about functional roles of MLO genes and reveal that these plant specific genes contribute paramount roles during successful completion of wheat plant life cycle and survival under unfavorable conditions. Detailed functional characterization of significantly perturbed MLO genes using modern molecular and biotechnological techniques such as CRISPR-Cas could yield strong candidates for development of climate-resilient and super-immune wheat cultivars.

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