Genome-Wide Identification and Characterization of the Wheat Remorin (*Ta*REM) Family during Cold Acclimation

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ABSTRACT Remorins (REMs) are plant-specific proteins that play an essential role in plant–microbe interactions. However, their roles in vernalization and abiotic stress responses remain speculative. Most remorins have a variable proline-rich *N*-half and a more conserved *C*-half that is predicted to form coils. A search of the wheat (*Triticum aestivum* L.) database revealed the existence of 20 different *REM* genes, which we classified into six groups on the basis of whether they shared a common phylogenetic and structural origin. Analysis of the physical genomic distributions demonstrated that *REM* genes are dispersed in the wheat genome and have one to seven introns. Promoter analysis of *TaREM* genes revealed the presence of putative *cis*-elements related to diverse functions like development, hormonal regulation, and biotic and abiotic stress responsiveness. Expression levels of *TaREM* genes were measured in plants grown under field and controlled conditions and in response to hormone treatment. Our analyses revealed that 12 members of the REM family are regulated during cold acclimation in wheat in four different tissues (roots, crowns, stems, and leaves), with the highest expression in roots. Differential gene expression was found between wheat cultivars with contrasting degrees of cold tolerance, suggesting the implication of *TaREM* genes in cold response and tolerance. Additionally, eight genes were induced in response to abscisic acid and methyl jasmonate treatment. This genome-wide analysis of *TaREM* genes provides valuable resources for functional analysis aimed at understanding their role in stress adaptation.

Abbreviations: ABA, abscisic acid; CBF, C-repeat binding factor; CRT, C-repeat; DRE, dehydration-responsive element; *GSD1*, *REM setting defect1*; IWGSC, International Wheat Genome Sequencing Consortium; MeJA, methyl jasmonate; MEME, Multiple Em for Motif Elucidation; NCBI, National Center for Biotechnology; REM, remorin; SA, salicylic acid

CORE IDEAS

- Twenty different wheat remorin (*TaREM*) genes were identified and classified into six phylogenetic groups.
- *TaREM* gene expression was measured in plants grown under both field and controlled cold conditions and in response to hormone treatment.
- Twelve *TaREM* were regulated during cold acclimation in four different tissues (roots, crowns, stems, and leaves).
- Several *TaREM* genes are implicated in cold response and tolerance.
- Eight genes were induced in response to abscisic acid and methyl jasmonate treatment.

Remorins are plasma membrane-associated proteins found explicitly in all embryophytes including angiosperms, gymnosperms, pteridophytes, and bryophytes (Checker and Khurana, 2013). Although REM proteins are specifically associated with the plasma membrane, they lack a transmembrane domain. They are characterized by a conserved *C*-terminal region with the signature coiled-coil structure, which is considered to be the family's signature (Marín and Ott, 2012), and a dynamic

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membrane-anchoring motif (Perraki et al., 2014). Experimental evidence has shown that the *C*-terminal region plays a determinant role in the mechanism by which most REM proteins bind specifically to the inner leaflet of membrane domains. This binding is mediated by (*S*)-acylation of cysteine residues in a *C*-terminal hydrophobic core (Konrad et al., 2014). The *N*-terminal region is highly variable and harbors many residues that can be phosphorylated under a wide range of biological conditions (Marín and Ott, 2012; Marín et al., 2012). Through a yeast two-hybrid interaction assay in *Arabidopsis thaliana* (L.) Heynh., Marín et al. (2012) demonstrated that the phosphorylation of a serine residue (Ser-66) in the intrinsically disordered *N*-terminal region of *AtREM1.3* mediates protein–protein interactions, and may constitute a regulatory domain stabilizing these interactions.

Remorins have diverse functions in plant–microbe interactions (Campo et al., 2008; Jarsch and Ott, 2011) and plant defense against some pathogens (Jacinto et al., 1993; Reymond et al., 1996; Raffaele et al., 2009), and have been found in membrane lipid rafts and plasmodesmata. It was reported that REMs impair cell-to-cell movement of *Potato virus X* by directly binding to the virus's movement protein, TGBp1 (Raffaele et al., 2009). Other REMs, identified as membrane-raft-associated proteins, interact with signaling proteins such as receptor-like kinases or act as scaffold proteins (Lefebvre et al., 2010).

Recent studies report that REMs are critical factors for plant signaling cascades, particularly during plant–microbe interactions (Lucau-Danila et al., 2010; Raffaele et al., 2009; Tóth et al., 2012; Demir et al., 2013), and play essential roles in signal transduction and plasma membrane trafficking (Reymond et al., 1996; Tóth et al., 2012; Marín et al., 2012; Marín and Ott, 2012). The REMs participate in plant hormone responses, as well as in cross-talk in several plant developmental processes (Gui et al., 2016). They are also associated with apical, vascular, and embryonic tissues (Bariola et al., 2004) and are involved in somatic embryogenesis, as found in chicory (*Cichorium intybus* L.) (Lucau-Danila et al., 2010) and in regulating stem development and phloem formation in *Populus deltoides* Marshall (Li et al., 2013). *Os*REM4.1 protein coordinates the antagonistic interaction between abscisic acid (ABA) and brassinosteroid signaling pathways to regulate plant growth and development (Gui et al., 2016). In rice (*Oryza sativa* L.), the *REM setting defect1* (*GSD1*) gene affects grain setting through regulating plasmodesmata conductance by interacting with actin. An overexpression of *GSD1* leads to a reduction in the grain setting rate, carbohydrate accumulation in leaves, and soluble sugar content in the phloem exudates (Gui et al., 2014, 2015). Recently, Gui et al. (2016) found that the *Os*REM4.1 plays an essential function in equilibrating plant growth with varying environments in rice. In addition to their role in plant defense and development, there is evidence that REMs may play a role in plants' adaptations to environmental conditions. The mulberry (*Morus indica* L.) remorin (*MiREM*) transcript was induced during salt and water stress in mature leaves (Checker and Khurana, 2013). In foxtail millet [*Setaria italica* (L.) P.Beauv.], the *SiREM6* transcript was induced by high salt and cold treatment but not by drought stress (Yue et al., 2014). Overexpression of these two *REM* genes improved salt (Checker and Khurana, 2013, Yue et al., 2014) and dehydration tolerance (Checker and Khurana, 2013) in transgenic *A. thaliana* during seed germination and seedling developmental stages. Although the *REM* gene family has been investigated in potato (*Solanum tuberosum* L.)(Jacinto et al., 1993), tobacco (*Nicotiana tabacum* L.) (Mongrand et al., 2004), tomato (*Solanum lycopersicum* L.) (Bariola et al., 2004), *A. thaliana* (Bhat et al., 2005), rice (Raffaele et al., 2007), and *Medicago trunculata* Gaertn. (Lefebvre et al., 2007), the structural features, phylogenetics, and functional properties of the *REM* gene family in common wheat (*T. aestivum)* have not been studied, especially in relation to cold tolerance and hormonal regulation.

In this study, 20 *TaREM* genes were identified from Ensembl wheat genome sequences and RNA-Seq data. Phylogenetic analysis, chromosomal localization, and expression profiling of these *REMs* were investigated during phenological development and cold acclimation and in response to hormonal treatment. The analyses revealed that *TaREMs* are expressed in roots, crowns, stems, and leaves. Several members of the wheat *REM* family were regulated during cold acclimation and are associated with cold tolerance, whereas others responded to ABA and methyl jasmonate (MeJA) treatment. This genome-wide analysis of *TaREM* genes provides valuable resources for functional analysis to determine their role in stress adaptations.

MATERIALS AND METHODS

Plant Material and Environmental Conditions

'Norstar' (Grant, 1980) winter wheat and 'Manitou' (Campbell, 1967) spring wheat were used for gene expression experiments, where plants were grown in environmentally controlled growth chambers as previously described (Badawi et al., 2007). Under the cold treatment (4°C), the sampling times were at 22 h and 7, 21, 35, and 56 d. Abscisic acid and MeJA treatments were performed as described previously (Danyluk et al., 1998; Diallo et al., 2014). Briefly, two groups of seedlings were sprayed with 150 μM MeJA or 100 μM ABA dissolved in 0.1% Tween 20 (Sigma-Aldrich, St Louis, MO) solutions. Each group of treated plants was watered with one of these treatment solutions. The untreated plants received a mock treatment of 0.1% Tween 20 solution and were used as a control. The samples were collected at 4 and 24 h for both ABA and MeJA. For the tissue-specific experiment, different tissues were collected at 22 h, 7, 21, 35, and 56 d for seedlings and at 1 and 8 d for roots, crowns, stems, and leaves. For all experiments, two biological replicates were collected for each sample for expression analysis.

For RNA-Seq analyses, developing crowns of Norstar and Manitou wheat grown under field conditions were

collected at five time points in 2010 as described by Li et al. (2018). Two biological replicates were collected for each sampling date and immediately frozen in liquid nitrogen and stored at −80°C for analysis.

Identification of the *REM* Genes in *T. aestivum*

To investigate the *REM* gene family in wheat, all *REM* sequences of *O. sativa* were used as queries for a BLAST search against the whole genome sequence of *T. aestivum* cv. Chinese Spring (release No. 42) from the Ensembl Plants database (http://plants.ensembl.org, accessed 21 Mar. 2019) (Kersey et al., 2014) with default parameters and from the International Wheat Genome Sequencing Consortium (IWGSC). All potential REM proteins were further screened to confirm the presence of the REM domain in the the National Center for Biotechnology (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome, accessed 8 Apr. 2019). The REM sequences were confirmed by the presence of a remorin domain, and the putative REM proteins were aligned to rice and *A. thaliana*. Remorin proteins were classified into different groups, as described by Raffaele et al. (2007). All putative annotations of the *REM* genes were retrieved from the *T. aestivum* genome website and the number and distribution of introns in *REM* genes were investigated through the use of *T. aestivum* genome annotation information.

Phylogenetic and Mapping Analyses

Rice and wheat REM protein sequences were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/protein/, accessed 21 Mar. 2019), Ensembl (http://plants.ensembl.org, accessed 21 Mar. 2019) (Kersey et al., 2014), and IWGSC (https://urgi.versailles.inra.fr/blast_iwgsc/, accessed 21 Mar. 2019) databases. These sequences were analyzed and 20 *TaREMs* and 20 *OsREM* genes were aligned with MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/, accessed 21 Mar. 2019). A maximum likelihood tree was derived from this alignment with the LG + Gamma model and a bootstrap value of 500 replicates in Molecular Evolutionary Genetics Analysis version 6 (Tamura et al., 2011). Graphical representations of *TaREMs*' positions on the chromosomes of *T. aestivum* were drawn with MapChart software (Voorrips, 2002).

Gene Structure and Conserved Motif Analyses

Genomic sequences and open reading frames of *TaREMs* were obtained from Ensembl (Kersey et al., 2014). The exon and intron structure was identified with Gene Structure Display Server version 2.0 (http://gsds.cbi.pku.edu.cn/, accessed 21 Mar. 2019) (Hu et al., 2015) by aligning the cDNA sequences to the corresponding genomic sequences. The *TaREMs*' coding sequences were translated to protein sequences with the TRANSLATE tool of the ExPASyserver (http://web.expasy.org/translate/, accessed 21 Mar. 2019). Conserved motifs of the genes were analyzed with the Multiple Em for Motif Elucidation (MEME) program (http://meme-suite.org/tools/meme, accessed 29 Mar. 2019) (Bailey and Elkan, 1994). The following parameters in the MEME tool were used for the distribution of motif occurrences: any number of repetitions, the maximum number of motifs was set to 20 motifs; optimum motif width was set to 6 to 100, and the ratio of minimum to maximum number of sites was 5:100. Identified MEME motifs other than the *C*-remorin domain was achieved with PROSITE and the Eukaryotic Linear Motif tools (http://elm.eu.org/, accessed 21 Mar. 2019) (Gould et al., 2010) for functional sites in proteins.

Database Searching and Identification of *Cis*-Regulatory Elements in the Promoter Region

Putative *cis*-acting regulatory DNA elements in *TaREM* genes were identified in the 1.5 kb upstream region preceeding the translation initiation site, as in previous studies (Badawi et al., 2008; Li et al., 2018). Promoter sequences were analyzed with PlantCARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed 21 Mar. 2019) as previously described (Lescot et al., 2002).

Protein subcellular localization was determined by CELLO (http://cello.life.nctu.edu.tw, accessed 21 Mar. 2019). The ExPASy tools (http://web.expasy.org/compute_pi, accessed 21 Mar. 2019) were used to predict some biochemical properties of REM proteins such as the molecular weight and the isoelectric point, which indicates the balance of positive and negative amino acid residues.

Gene Expression Profile Analysis

The RNA-Seq data corresponding to the *TaREM* genes was downloaded from the Norstar and Manitou wheat crown transcriptome database and can be found in the Gene Expression Omnibus under the accession number GSE101118 (Li et al., 2018).

Illumina RNA-Seq data analyses were performed on crowns of plants grown under field conditions from early autumn to winter in 2010 (Li et al., 2018). The reads per million were obtained from the field condition RNA-Seq database subjected to surrogate variable R analysis (Leek, 2014) to correct variation in the transcriptome sequencing data. The expression cluster for each *TaREM* gene for each cultivar–time point combination represents the total reads of the three copies (A, B, and D) and the mean values of the two biological replicates (Li et al., 2018).

A BLASTN search of the gene sequence obtained from the Ensmbl wheat genome was performed against the *TaREM* genes identified in this study to find and confirm the corresponding Genevestigator REM identifiers (Supplemental Table S4). The RNA-Seq databases of *T. aestivum* from different tissues and developmental stages (Hruz et al., 2008) were analyzed with the Genevestigator tool (http://www.genevestigator.ethz.ch/, accessed 21 Mar. 2019). The expression patterns obtained are presented as heat maps in yellow and blue for different stresses and burgundy–white color-coding for tissues and developmental stages.

RNA Extraction and Expression Analyses of the *TaREM* Genes by Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from samples of all the experiments with the mirVana miRNA Isolation Kit (ThermoFisher-Scientific). The purity and quality of RNA were analyzed by NanoDrop 2000c (ThermoFisher-Scientific). A 0.9-μg aliquot of total RNA was treated with gDNA wipeout buffer in the QuantiTect Reverse Transcription Kit (Qiagen) and then reverse-transcribed. For the quantitative real-time polymerase chain reaction (qRT-PCR), 2 μL of cDNA template (equivalent to 90 ng of total RNA) were used with LightCycler 480 SYBR Green I Master (Roche Life Science). The qRT-PCR was performed with the FX96 Real-time Detection System (Bio-Rad) according to the manufacturer's instructions. The polymerase chain reaction conditions were as follows: 95°C for 15 min and 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. The experiments were repeated for the two biological replicates. Each reaction was conducted in duplicate to ensure the reproducibility of results. The $\Delta\Delta C_T$ method was used to calculate the relative expression levels of *TaREM* with using *18S* as the reference gene. The gene-specific primers were used to quantify the transcripts of *TaREM*. All the primers used for qRT-PCR are listed in Supplemental Table S5. Expression levels were calculated from the cycle threshold according to the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). The statistical analysis was done using Prism version 5.0 (GraphPad Software). Values are shown as means ± SD. Means were compared via one-way ANOVA followed by Tukey's test. Significance was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Analyses of gene families have become essential in the understanding of gene structure, protein function, and evolution. Here, we conducted a comprehensive analysis of the *TaREM* gene family to determine their potential functions in response to an abiotic stress (i.e., exceptionally cold temperatures), phenological development, and hormones such as MeJA and ABA.

Identification and Phylogenetic Analysis of the Wheat Remorin Gene Family

Twenty rice *REM* genes were used to search the Ensembl database (release 42) and then used to identify genes encoding *REMs* in the wheat genome. Twenty complete *TaREM* coding sequences were identified. Based on their genomic location, these genes were present as two or three homeoallelic coding sequences on the three genomes of hexaploid wheat (A, B, and D). On the basis of the phylogeny of their *C*-terminal domain, the identified *REM* genes were classified into six groups. This classification was based on nomenclature established by Raffaele et al. (2007) for the cereal species *O. sativa.* To be consistent with the method of Raffaele et al. (2007), we assigned identical gene numbers to orthologs of hexaploid wheat (Groups 0.1, 0.2, 1, 4, 5, and 6). In total, 58 *TaREM* copies were identified with their characteristic information such as the gene ID, position on the chromosomes, coding sequence size, amino acids, number of introns and exons, isoelectric point, and molecular weight of the predicted protein (Table 1). Most wheat *REM* gene loci have three copies distributed across the three wheat genomes except *TaREM0.12*, which has only two copies (Table 1). These analyses found that *TaREM* genes encode predicted polypeptides with a wide range of sizes (168 –622 amino acids) and isoelectric points (5.09–10.17). All identified *Ta*REM proteins had a typical remorin motif at the *C*-terminus (Fig. 1A, B). The *Ta*REM *C*-motif contained a coiled-coil domain, a signature of the remorin family. The multiple sequence alignment of the *C*-terminal region of all identified wheat REM proteins revealed a highly conserved coiled-coil domain (Fig. 1A). The number of REMs in rice, *A. thaliana*, and wheat were found to be much higher than in poplar (eight REMs) (Raffaele et al. (2007), and foxtail millet (11 REMs) (Yue et al., 2014), indicating that the REM family in the first three species has expanded over time. This expansion of the *TaREM* gene family is probably a result of whole-genome duplication or segmental duplication. Gene duplication played a crucial role in the expansion of gene families, creating the opportunity for changes in gene function, which, in turn, allowed for optimal adaptability to diverse environmental conditions (Xu et al., 2012).

Previously, Raffaele et al. (2007) showed that some monocot and eudicot *REM* sequences are separated on a phylogenetic tree, suggesting the existence of specific groups in monocots and eudicots. To study the phylogenetic relationships of the wheat *REM* family, we retrieved the REM proteins described by Raffaele et al. (2007), the 16 *A. thaliana* REM members, and the four *Populus* and *Medicago* group members associated with both genera as representatives of eudicot REMs, plus the 20 *O. sativa* REM members as additional monocot representatives. During our initial comparisons of REM proteins from different groups, it was difficult to align the entire protein of distant members with complete confidence. Therefore, we only aligned the amino acids encompassing the conserved *C*-motif from the 58 proteins. A maximum likelihood tree was derived from this alignment and is presented in Fig. 2. This analysis showed that REM proteins cluster into six monophyletic groups. Groups 0.2, 1, and 4 clearly show a separation of monocot and eudicot REM sequences, suggesting that only one representative per group may have existed during eudicot–monocot divergence. These groups now account for 10 REM proteins in each species. Groups 5 and 6 display two and four distinct branches that contain at least one REM member from each species, suggesting that the amplification of genes within these groups preceded eudicot–monocot divergence. These groups now contain six, nine, and eight REM proteins in *A. thaliana*, rice, and wheat, respectively. No *A. thaliana* REM proteins clustered with members from Group 0.1,

Table 1. Characteristics of remorin (*TaREM*) genes identified from the genome-wide search analysis.

Gene name	Gene ID†	Chromosome location†	ORF	Total deduced amino acid length	Intron no.	Exon no.	pI	Mol wt
			bp					Da
TaREM1.1	*TraesCS6A02G403500.1*	6AL	516	171‡	4	5	9.46	18,857.43
	TraesCS6B02G447400.1	6BL	522	173‡	4	5	9.52	19,092.80
	GFFI01045056.1	6DL	507	168‡	4	5	9.38	18,617.11
TaREM1.2	*TraesCS1A02G174700.1*	1AL	534	177‡	4	5	5.70	19,237.75
	GFFU01029238.1	1BL	534	177‡	4	5	5.50	19,248.68
	TraesCS1D02G164300.1	1DL	534	177‡	4	5	5.53	19,265.76
TaREM1.3	*TraesCS5A02G554600.1*	5AL	534	177‡	4	5	6.80	19,736.65
	TraesCS4B02G393000.1	4BL	528	175‡	4	5	7.79	19,554.47
	TraesCSU02G033600.1	U	528	175‡	4	5	7.79	19,554.47
TaREM1.4	*TraesCS6A02G237200.2*	6AL	582	193‡	4	5	9.57	20,963.35
	TraesCS6B02G265700.2	6BL	576	191‡	4	5	9.36	20,803.13
	TraesCS6D02G219800.1	6DL	576	191‡	4	5	9.49	20,834.19
TaREM1.5	*TraesCS2A02G369800.1*	2AL	633	210‡	4	5	5.29	22,953.06
	TraesCS2B02G386900.1	2BL	645	214‡	4	5	5.45	23,254.46
	TraesCS2D02G366300.2	2DL	645	214‡	4	5	5.36	23,343.51
TaREM0.1	*GFFI01031721.1*	6AL	1275	424‡	5	6	9.43	46,559.11
	GFFI01034102.1	6BL	1260	419‡	5	6	9.31	45,958.33
	TraesCS6D02G231600.1	6DL	1275	424‡	5	6	9.55	46,527.10
TaREM0.12	*GFFI01029091.1*	6BL§	1158	385	5	6	9.36	42,448.28
	GFFI01035312.1	6DL§						
TaREM0.2	*TraesCS1A02G057900.1*	1AS	1056	351‡	5	6	9.79	38,946.86
	TraesCS1B02G075600.1	1BS	1056	351‡	5	6	9.74	38,938.83
	TraesCS1D02G058700.1	1DS	1056	351‡	5	6	9.69	39,134.09
TaREM0.23	*TraesCS4A02G053800.2*	4AS	939	312‡	5	6	9.80	34,433.91
	TraesCS4B02G250900.1	4BS	942	313‡	5	6	9.88	34,656.20
	TraesCS4D02G250800.1	4DS	942	313‡	5	6	9.80	34,682.10
TaREM4.1	*TraesCS2A02G199300.1*	2AS	936	311‡	1	2	8.77	33,344.95
	TraesCS2B02G226600.1	2BS	927	308‡	1	2	6.90	33,229.94
	TraesCS2D02G206900.1	2DS	930	309‡	1	2	9.09	33,303.05
TaREM4.2	*TraesCS5A02G464900.1*	5AL	927	308‡	1	2	5.55	33,301.77
	TraesCS5B02G476700.1	5BL	921	306‡	1	2	5.61	33,121.61
	TraesCS5D02G477500.1	5DL	918	305‡	1	2	5.48	32,913.35
TaREM4.3	*TraesCS7A02G443500.1*	7AL	855	284‡	1	2	5.19	30,739.10
	TraesCS7B02G342300.1	7BL	837	278‡	1	2	5.09	29,975.17
	TraesCS7D02G432700.1	7DL	834	277‡	1	2	5.26	30,024.32
TaREM5.1	*TraesCS6A02G333200.1*	6AL	1722	573‡	6	7	9.56	61,521.94
	TraesCS6B02G363600.1	6BL	1764	587‡	6	7	9.56	63,001.60
	TraesCS6D02G312000.2	6DL	1755	584‡	6	7	9.48	62,847.65
TaREM5.3	*TraesCS5A02G237300.2*	5AL	1869	622‡	6	7	9.58	66,660.07
	TraesCS5B02G235500.2	5BL	1839	613‡	7	8	9.50	66,035.33
	TraesCS5D02G244300.2	5DL	1848	615‡	7	8	9.51	66,237.46
TaREM6.1	*TraesCS6A02G046400.1*	6AS	1527	508‡	5	6	6.23	55,660.06
	TraesCS6B02G062500.1	6BS	1527	508‡	5	6	6.14	55,640.13
	TraesCS6D02G053500.1	6DS	1530	509‡	5	6	6.29	55,759.28
TaREM6.2	*TraesCS5A02G517700.1*	5AL	1269	422‡	5	6	6.60	46,546.70
	TraesCS4B02G349100.1	4BL	1275	424‡	5	6	6.86	46,843.28
	TraesCS4D02G343800.1	4DL	1269	422‡	5	6	6.46	46,424.61
TaREM6.3	*TraesCS5A02G040500.2*	5AS	1314	437‡	3	4	10.17	47,821.99
	TraesCS5B02G041700.2	5BS	1329	442‡	3	4	10.05	48,211.22
	TraesCS5D02G047600.4	5DS	1314	437‡	3	4	10.07	47,620.63
TaREM6.4	*GEDT01053800.1 TSA*	2AL	1242	413‡	3	4	8.85	45,404.38
	TraesCS2B02G363600.1	2BL	1242	413‡	3	4	8.85	45,376.32
	TraesCS2D02G343900.1	2DL	1248	415‡	3	4	8.85	45,534.48

(cont'd.)

Table 1. Continued.

Gene name	Gene ID†	Chromosome location†	ORF	Total deduced amino acid length	Intron no.	Exon no.	pI	Mol wt
TaREM6.5	*TraesCS4A02G238400.1*	4AL	1050	349‡	3	4	9.35	37,536.95
	TraesCS4B02G076500.1	4BS	1065	354‡	3	4	9.51	37,959.39
	TraesCS4D02G075000.1	4DS	1062	353‡	3	4	9.47	37,866.32
TaREM6.6	*TraesCS2A02G464000.1*	2AL	1581	526‡	5	6	8.77	57,219.99
	TraesCS2B02G485700.1	2BL	1587	528‡	5	6	8.86	57,391.01
	TraesCS2D02G464900.1	2DL	1590	529‡	5	6	8.94	57,591.43

† Accession numbers (Gene ID) and chromosome locations have been taken from from Ensembl or NCBI.

‡ Complete protein.

§For *TaREM0.12*, neither the 6D nor 6B copies are annotated in ENSEMBL; the 6B and 6D copies may be pseudogenes.

pI, isoelectric point; U, unknown chromosome; L, long arm; S, short arm; Mol wt, molecular weight.

suggesting its specificity to the monocot group. The REM phylogenetic tree showed mostly the same clustering patterns in the two members of the grass family, wheat and rice. The phylogenetic similarity between *T. aestivum* and *O. sativa* REM proteins suggests that they might have evolved conservatively. In total, 19 REM proteins out of 20 were clustered as pairs, revealing their orthologous relationship. Furthermore, *TaREM0.12* and *OsREM5.2* represent additional genes that may have explicitly duplicated in wheat and rice, respectively. Yue et al. (2014) showed that the 11 *SiREMs* (foxtail millet) were classified into four phylogenetic groups. In *A. thaliana*, all 16 *AtREM* genes were divided into five groups (1, 3, 4, 5, and 6). These results indicate that *TaREM*, *OsREM*, and *AtREM* genes may have undergone different duplication events. As shown in Supplemental Fig. S1, the orthologs from the A, B, and D genomes tended to form an orthologous pair at the branch end, indicating that the orthologs from A, B, and D had a close relationship.

Chromosomal Locations of Wheat Remorin Members

TaREM genes were located on all the homeologous groups of wheat chromosomes except Group 3 (Fig. 3), whereas no *REM* genes were found in rice chromosomes 1, 5, 6, and 7. *REM* genes in *A. thaliana* were distributed among all five chromosomes (Raffaele et al., 2007). *TaREM* genes were over-represented on Group 6 and 4 chromosomes, which contain six and four *TaREM* genes respectively (Fig. 3). Interestingly, the Group 6 chromosomes had the greatest number of *COR* genes (Li et al., 2018). *TaREM4.2*, *TaREM5.3*, and *TaREM6.3* were located in Group 5 chromosomes, which are known to harbor most of the genes that regulate cold tolerance and development. It is known that chromosome 5A has the freezing tolerance locus (*FR2*) and contains a cluster of C-repeat binding factor (*CBF*) genes (Miller et al., 2006).

Gene Structure of Wheat Remorins

The exon–intron structure of the *TaREMs* was analyzed (Fig. 4A and B) to gain further insight into the evolution of *TaREM*. This analysis showed a conserved intron number ranging from one to six per gene between wheat and rice (Fig. 4B). The confined number of introns suggests that there is similar gene structure diversity for the *REM* genes in monocots. All genes from phylogenetic Groups 0.1 and 0.2 and three from Group 6 (*TaREM6.1*, *TaREM6.2*, and *TaREM6.6*) contained five introns (Table 1, Fig. 4A, B), whereas all members of Group 1 had four introns. *TaREM5.3* was the only member that had six to seven introns, whereas Group 4 contained only one intron. *TaREM6.3*, *TaREM6.4*, and *TaREM6.5* have three introns, whereas *TaREM5.1* has six introns. The most important differences in the structure of *TaREMs* were in the intron length (Fig. 4A). The presence of several introns in these genes could cause alternative splicing and different splice variants of *REMs*. Sequence analysis shows that all *REMs* from the six groups produced different transcripts from each corresponding gene, indicating that *TaREMs* are subjected to alternative splicing. The number of splicing sites varied from two to seven sites (Supplementary Table S1). In general, most of the genes in the same group had similar sequence identities and shared the same number of intron structures, indicating a close evolutionary relationship and the related gene structure diversity of *REM* genes. The intron lengths within genes showed similar patterns within groups. The intron–exon organization and intron numbers of *REM* genes in the wheat genome were similar to those of rice, except for the genes *TaREM5.1*, *TaREM6.3*, and *TaREM6.5* (Fig. 4B, Supplemental Table S1), suggesting an evolutionary conservation of *REM* genes in monocots. Similar numbers of introns were also observed in *A. thaliana* (Supplemental Table S1, Fig. 4B), which may suggest the evolutionary conservation of *REM* genes in plants. The *TaREM* genes were strictly related to recent progenitors. *TaREM1.2* to *TaREM1.5* were more closely related to *TuREM*, which is the A-genome donor relative from *Triticum urartu* Thumanjan ex Gandilyan (Ling et al., 2013). Sequence analysis showed that these four *REM* genes have 99, 99, 96, and 98% identity with *TuREM*, respectively. The *TaREM* genes on the A and B genomes also had a closer identity to the allelic genes on the D genome. For example, *TaREM5.1* on chromosome 6B was similar to the *TaREM5.1* on chromosome 6D, whereas the *TaREM6.3* on chromosome 5A was similar to *TaREM6.3* on chromosome 5D (Supplemental Fig. S1). Marcussen et al. (2014) proposed that several rounds of hybridization may have produced *T. aestivum*. Additionally, phylogenomic analyses showed

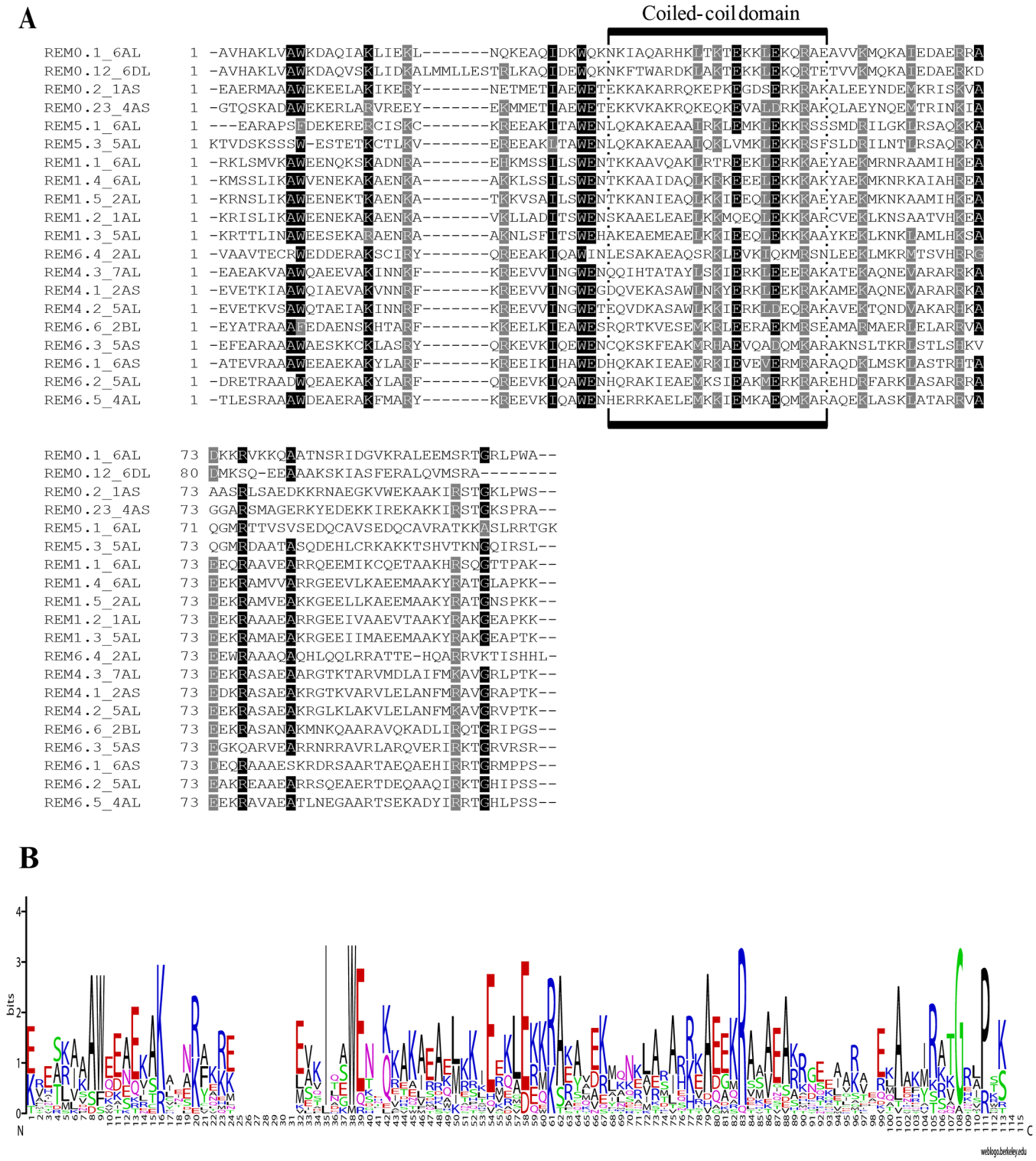


Fig. 1. Conserved motif analysis of the remorin (REM) *C*-terminal sequences in wheat. (A) Multiple sequence alignment of wheat REM motif is shown. The homeologous A copy from all REM genes were used in the analysis, except for *TaREM0.12*, for which the D copy was used. The coiled-coil domain in the REM motif is boxed. The conserved amino acid and blocks of similar amino acid residues are shaded in black and gray, respectively. (B), Sequence representation LOGO derived from the multiple sequence alignment of the remorin *C*-motif. The *x*-axis represents the conserved sequence. The *y*-axis is a scale that reflects the conservation rate of each amino acid across all proteins.

that the D genome may be a product of homoploid hybrid speciation between the A and B genome ancestors 5 to 6 million yr ago. The retention of all *REM* genes in the three genomes of *T. aestivum* may have enhanced the function of each *REM* group by adding extra copies.

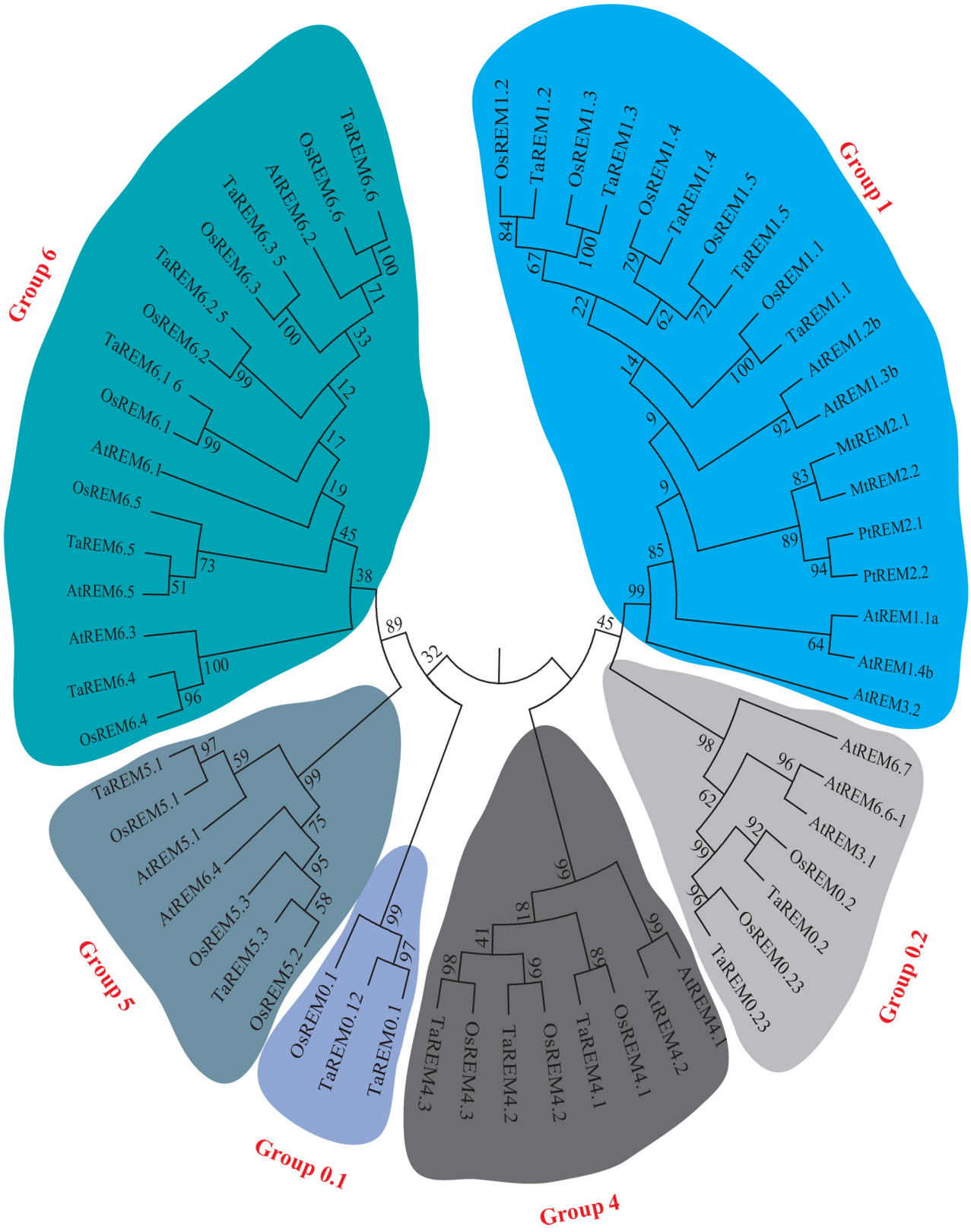


Fig. 2. Phylogenetic relationships between all remorin proteins from *T. aestivum*, *O. sativa*, and *A.thaliana*. The amino acids corresponding to the conserved *C*-motif from the three species were aligned by MUSCLE in Molecular Evolutionary Genetics Analysis version 6. A maximum likelihood tree (ML) was derived from this alignment via the LG + Gamma model and a bootstrap value of 500 replicates. In addition, four proteins from the *Populus* + *Medicago* [Group 2-specific remorins in Raffaele et al. (2007)] were included for comparison. The wheat remorins were grouped into six distinct groups and annotated with different colors. Genes belonging to the different groups are listed in Table 1.

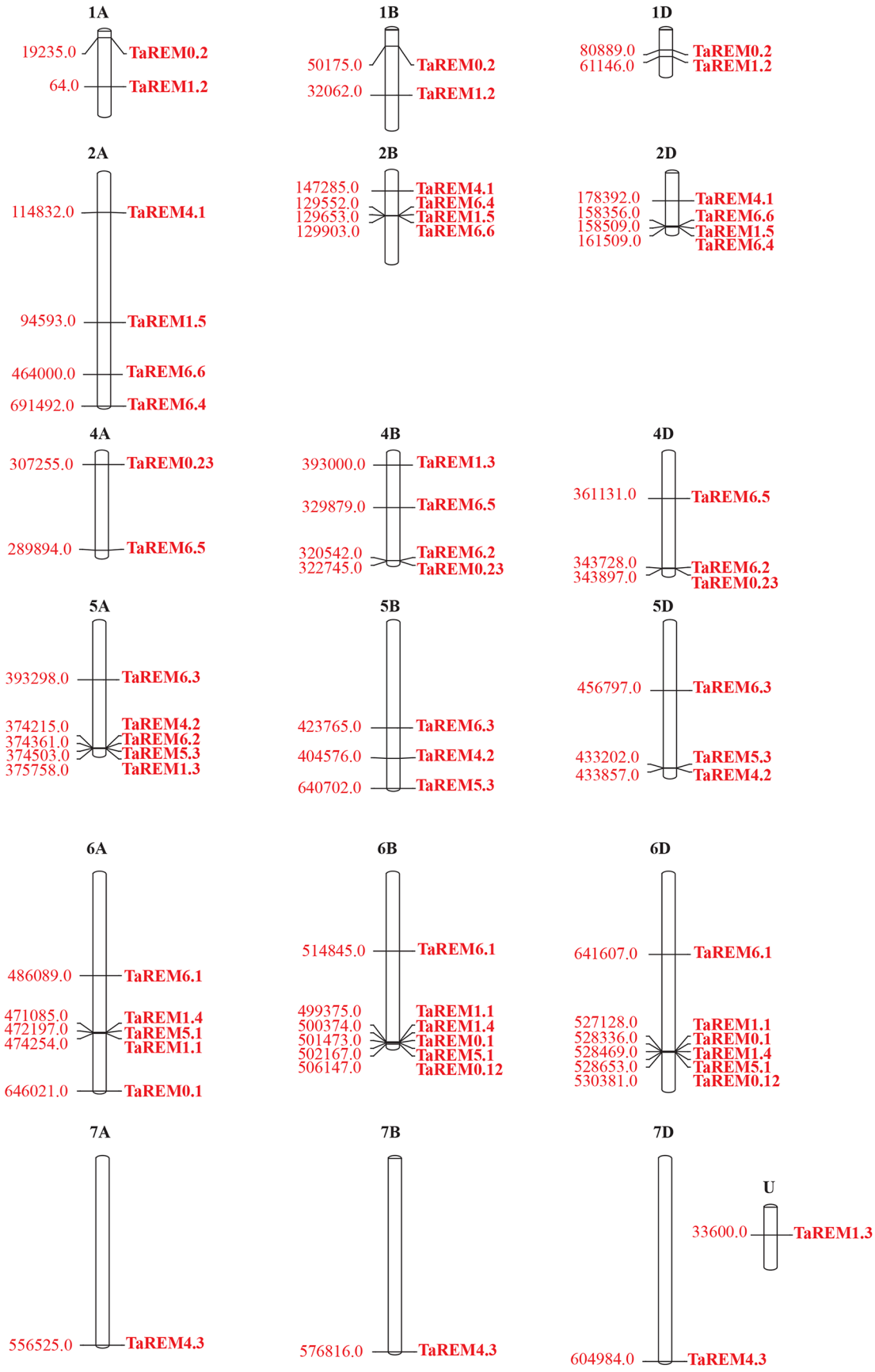
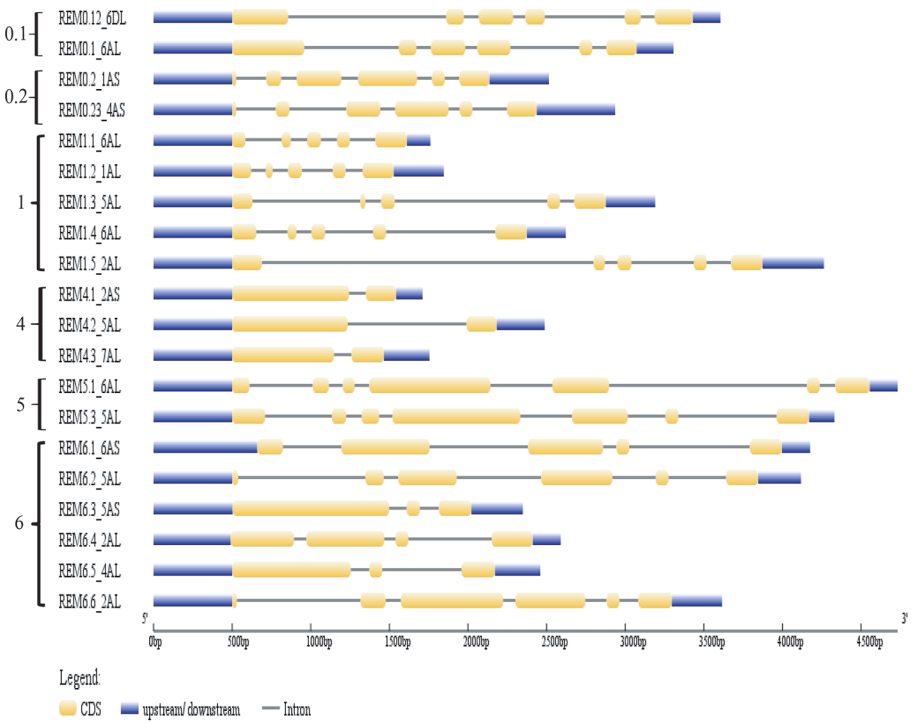


Fig. 3. Chromosomal locations of remorin (*TaREM*) genes in the wheat genome. The distributions of the 58 remorin genes were determined according to the scaffold number and are shown in red. Chromosome 3 does not have any remorins and was excluded in this figure. The numbers on the top indicate each chromosome number and the genome (A, B, or D). Positions are indicated in kb on chromosomes and bases on scaffolds. U, unknown chromosome.

A



B

Phylogenetic group	Gene name	Exons numbers		
		Wheat	Rice	*Arabidopsis*
1	REM1.1	5	5	5 (AtREM1.1)
	REM1.2	5	5	5 (AtREM1.2)
	REM1.3	5	5	4 (AtREM1.3)
	REM1.4	5	5	5 (AtREM1.4)
	REM1.5	5	5	4 (AtREM3.2)
0.1	REM0.1	6	6	-
	REM0.12	6	-	-
0.2	REM0.2	6	6	3 (AtREM3.1) 7 (AtREM6.6) 6 (AtREM6.7)
	REM0.23	6	-	-
4	REM4.1	2	2	2 (AtREM4.1)
	REM4.2	2	2	2 (AtREM4.2)
	REM4.3	2	2	-
5	REM5.1	6	7	7 (AtREM5.1)
	REM5.2	-	7	-
	REM5.3	7	7	4 (AtREM6.4)
6	REM6.1	6	6	6 (AtREM6.1)
	REM6.2	6	6	-
	REM6.3	4	5	-
	REM6.4	4	5	6 (AtREM6.3)
	REM6.5	4	3	3 (AtREM6.5)
	REM6.6	6	6	6 (AtREM6.2)

Fig. 4. Gene structure of the remorin (REM) gene family in wheat. (A) The exon–intron structures were determined by comparing the coding sequences of *TaREM* genes and their corresponding genomic sequences. Exons, introns, and upstream or downstream untranslated regions are represented, respectively, by yellow rectangles, black lines, and blue rectangles. The scale bar on the bottom line indicates genes size in bp. The number at the left side represents the phylogenetic groups and their corresponding REM genes. (B) Comparison between the remorin exon numbers in wheat, rice, and *A. thaliana*. The analyses of exon–intron structures were performed with Gene Structure Display Server software (http://gsds.cbi.pku.edu.cn, accessed 22 Mar. 2019). –, no equivalent gene was present in the species.

Motifs and Subcellular Localization Analysis

Motifs were identified with the MEME program (http://meme-suite.org/tools/meme, accessed 22 Mar. 2019) to study the diversity and conservation among the *TaREM*s. Twenty distinct motifs were identified among the 20 *Ta*REM proteins. The distribution of motifs in the different remorin groups, the protein motifs, and their consensus are shown in Fig. 5 and Supplemental Table S2. The size of the identified motifs ranged from 10 to 107 amino acids (Supplemental Table S2). The closely related genes within each group in the phylogenetic tree shared common motif sequences and positions (Fig. 2 and Fig. 5). In general, remorins have a conserved *C*-terminal domain, considered to be a signature of the protein; a predicted coiled-coil; and a variable *N*-terminal region. Motif analyses showed that the remorin *C*-domain (Motif 1) is uniformly present in all wheat remorin proteins, suggesting that they are evolutionarily conserved in plants. The remorin *N*-domain is about 64 amino acids (Motif 2) and was found only in Group 1 remorins. This group has both remorin *N*- and remorin *C*-domains, except for *Ta*REM1.1, which is missing the remorin *N*-domain but still contains high proline content, like other members of Group 1. Exclusively, Group 4 members have similar numbers of proline in the *N*-terminal region. It has been reported that Group 1 remorins from dicots have a higher percentage of prolines in this region but the biological significance of this remains unclear (Yue et al., 2014; Raffaele et al., 2007). We found that Group 1 remorins have several motifs (3–10) in the *N*-terminal region. Group 4 was characterized by the presence of a serine rich sequence in the *N*-terminal region that contains one specific motif (Motif 11) corresponding to the putative glycosylation site. Group 5 contains only one conserved motif in the *N*-terminal region and has a putative phosphorylation site (Motif 14). Group 6 has four conserved motifs (Motifs 15–18, corresponding to Collagen IV, a carboxyl-terminal non-collagenous (NC1) domain profile collagen; LDL-receptor class B repeat profile; a protein kinase C phosphorylation site; and LIG_LIR_Gen_1, a canonical LIR motif that binds to Atg8 protein family members to mediate processes involved in autophagy) at the *N*-terminus, except *Ta*REM6.4 and *Ta*REM6.5, which contained only two motifs (15 and 16). Most of the closely related members in the phylogenetic tree had identical motif compositions, suggesting functional similarities among the REM proteins within the same group (Fig. 2, Fig 5, and Supplemental Table S2). The motif distribution and organization showed that remorin proteins were conserved during evolution and the motif distribution in different groups of proteins could be the source of the functional divergence in remorin genes over time. The presence of variable *N*-terminal regions in remorin genes suggests different structures and functions (Marín and Ott, 2012).

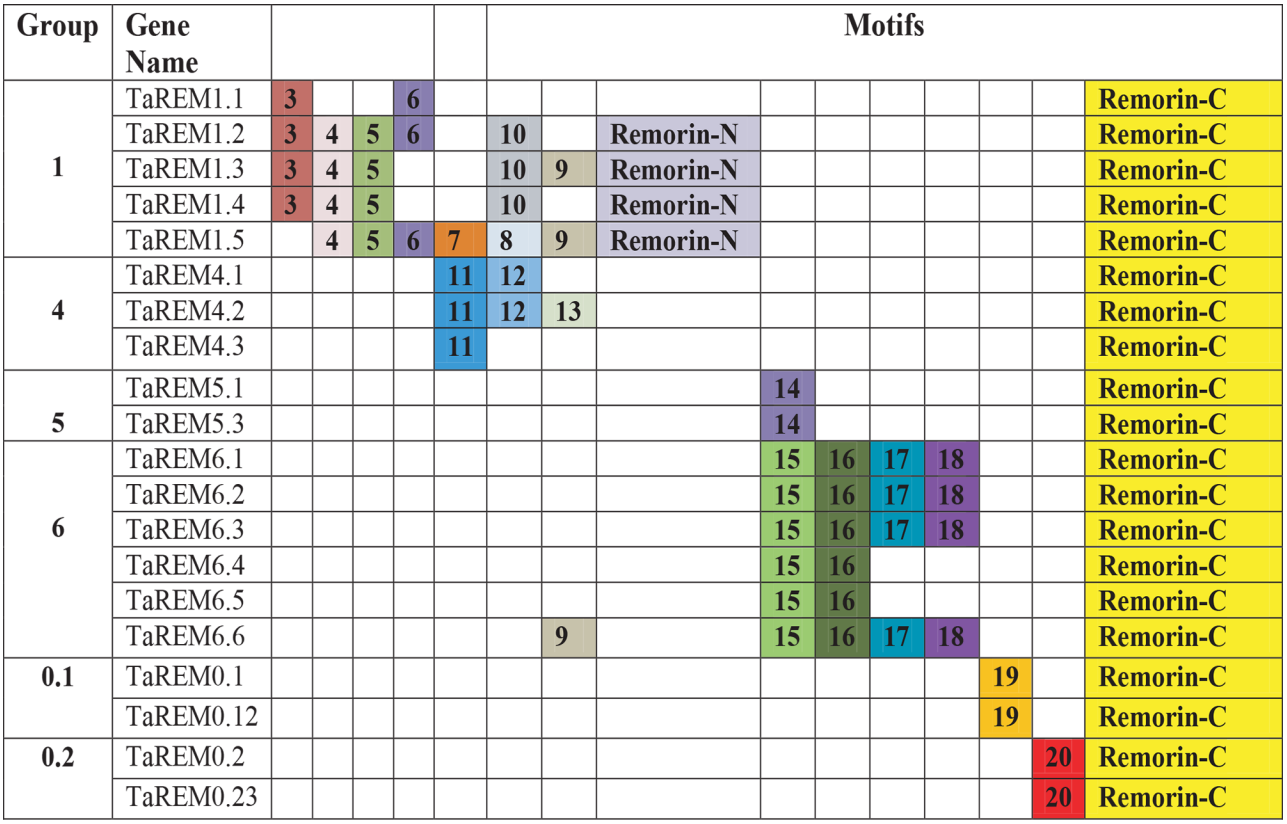


Fig. 5. Schematic diagram of the conserved motifs in 20 wheat remorin (*REM*) genes. The motifs were identified by Multiple Em for Motif Elucidation (MEME) software. Each colored number represents a motif. Yellow box: the remorin *C*-domain represents Motif number 1 and the remorin *N*-domain represents Motif 2. The other motifs are shown in different colors. The consensus sequences of the motifs identified by MEME and their function identified by the PROSITE and Eukaryotic Linear Motif resource tools are presented in Supplemental Table S2.

Subcellular localization of the *Ta*REM proteins was predicted with the CELLO program. The putative *Ta*REM protein subcellular localizations showed that most *Ta*REMs were concentrated in the nucleus. However, a few REMs proteins (TaREM1.2, TaREM1.3, TaREM1.5D, and TaREM4.1B) were localized in the cytoplasm (Table 2). These differences may be related to the motif sequence and suggest that the subcellular localization of *Ta*REMs is diverse and complex.

Bioinformatics Analysis of Putative *TaREM* Promoters

To elucidate the regulatory mechanisms of 20 *TaREMs*, the transcriptional response elements of their promoters were identified from the regions upstream of the start site in the *T. aestivum* genome through use of the PlantCARE promoter databases. All the putative *TaREM* promoters possessed typical TATA and CAAT boxes (Table 3, Supplemental Table S3), which are the core elements in promoter and enhancer regions. The predicted regulatory *cis*-elements include transcription binding sites and numerous elements related to stress, phytohormone, and light responses (Table 3). Light-responsive elements, which contain different core elements (Supplemental Table S3), represent most of the predicted elements. Every promoter possessed 10 to 20 types of light core elements, indicating that light might differentially regulate *TaREMs*. The presence of a light-responsive element was reported in a soybean [*Glycine max* (L.) Merr.] remorin gene regulated under drought stress (Marcolino-Gomes et al., 2014). The 13 predicted hormone-responsive regulatory elements are associated with ABA (ABA-responsive element, CE1, CE3, and Motif IIb), ethylene (ERE), MeJA (a *cis*-acting regulatory element involved in MeJA-responsiveness; CGTCA and TGACG motifs), gibberellic acid (GARE motif, TATC-box, and P-box), auxin (AuxR-core, TGA-box, and TGA element), and salicylic acid (SA) (TCA-element) responses. The ABA-responsive *cis*-elements in *REM* gene promoters were also found in foxtail millet (Yue et al., 2014). Regulation of *REM* gene expression in response to hormone treatment including ABA, MeJA, auxin, and/or salicylic acid has been reported in different species (Checker and Khurana, 2013; Yue et al., 2014; Kong et al., 2016). The induction of *MiREM* and *SiREM6* in response to exogenous ABA treatment in mulberry and foxtail millet, respectively, suggest that these two genes could be involved in abiotic stress tolerance through the ABA-dependent signal pathway (Checker and Khurana, 2013; Yue et al., 2014). The predicted *TaREM* promoters had many *cis*-elements related to endosperm expression, such as Skn-1 and GCN4; RY, which is a *cis*-acting regulatory element involved in seed-specific regulation; and the CCGTCC-box, which is a *cis*-acting regulatory element related to meristem specific activation, as shown in Supplemental Table S3. The tissue specificity of *REM* genes has

Table 2. Prediction of cell localization of wheat remorin proteins in comparison with rice and *A. thaliana* predictions and to the experimental location in *A. thaliana*.

	Prediction†			
Gene name	Wheat	Rice	*A. thaliana*	Experimental in A. *thaliana*‡
TaREM1.1_6AL	Nuclear	*OsREM1.1* Nuclear	*AtREM1.1* Nuclear	Cytosol
TaREM1.1_6BL	Nuclear			
TaREM1.1_6DL	Nuclear			
TaREM1.2_1AL	Cytoplasmic	*OsREM1.2* Cytoplasmic	*AtREM1.2* Nuclear	Cytosol
TaREM1.2 _1BL	Cytoplasmic			
TaREM1.2_1DL	Cytoplasmic			
TaREM1.3_5AL	Cytoplasmic	*OsREM1.3* Nuclear	*AtREM1.3* Cytoplasmic	Cytosol
TaREM1.3_4BL	Nuclear			
TaREM1.3_U	Cytoplasmic			
TaREM1.4_6AL	Nuclear	*OsREM1.4* Nuclear	*AtREM1.4* Nuclear	Cytosol
TaREM1.4_6BL	Nuclear			
TaREM1.4_6DL	Nuclear			
TaREM1.5_2AL	Nuclear	*OsREM1.5* Nuclear	*AtREM3.2* Nuclear	Cytosol
TaREM1.5_2BL	Nuclear			
TaREM1.5_2DL	Cytoplasmic			
TaREM0.1_6AL	Nuclear	*OsREM0.1* Nuclear	–	–
TaREM0.1_6BL	Nuclear			
TaREM0.1_6DL	Nuclear			
TaREM0.12_6BL	Nuclear	–	–	–
TaREM0.12_6DL	Nuclear			
TaREM0.2_1AS	Nuclear	*OsREM0.2* Nuclear	*AtREM3.1* Nuclear	Cytosol
TaREM0.2_1BS	Nuclear		*AtREM6.6* Nuclear	Cytosol
TaREM0.2_1DS	Nuclear		*AtREM6.7* Nuclear	Plasma membrane
TaREM0.23_4AS	Nuclear	–	–	–
TaREM0.23_4BL	Nuclear			
TaREM0.23_4DS	Nuclear			
TaREM4.1_2AS	Nuclear	*OsREM4.1* Nuclear	*AtREM4.1* Nuclear	Cytosol
TaREM4.1_2BS	Cytoplasmic			
TaREM4.1_2DS	Nuclear			
TaREM4.2_5AL	Nuclear	*OsREM4.2* Nuclear	*AtREM4.2* Nuclear	Cytosol
TaREM4.2_5BL	Nuclear			
TaREM4.2_5DL	Nuclear			
TaREM4.3_7AL	Nuclear	*OsREM4.3* Nuclear	–	–
TaREM4.3_7BL	Nuclear			
TaREM4.3_7DL	Nuclear			
TaREM5.1_6AL	Nuclear	*OsREM5.1* Nuclear	*AtREM5.1* Nuclear	Cytosol
TaREM5.1_6BL	Nuclear	*OsREM5.2* Nuclear		
TaREM5.1_6DL	Nuclear			
TaREM5.3_5AL	Nuclear	*OsREM5.3* Nuclear	*AtREM6.4* Nuclear	Plasma membrane
TaREM5.3_ 5BL	Nuclear			
TaREM5.3_5DL	Nuclear			
TaREM6.1_6AS	Nuclear	*OsREM6.1* Nuclear	*AtREM6.1* Nuclear	Plasma membrane
TaREM6.1_6BS	Nuclear			
TaREM6.1_6DS	Nuclear			
TaREM6.2_5AL	Nuclear	*OsREM6.2* Nuclear	–	–
TaREM6.2_4BL	Nuclear			
TaREM6.2_4DL	Nuclear			
TaREM6.3_5AS	Nuclear	*OsREM6.3* Nuclear	–	–
TaREM6.3_5BS	Nuclear			
TaREM6.3_5DS	Nuclear			

(cont'd.)

Table 2. Continued.

Gene name	Prediction†			Experimental in A. *thaliana*‡
	Wheat	Rice	*A. thaliana*	
TaREM6.4_2AL	Nuclear	*OsREM6.4* Nuclear	*AtREM6.3* Nuclear	Cytosol
TaREM6.4_2BL	Nuclear			
TaREM6.4_2DL	Nuclear			
TaREM6.5_4AL	Nuclear	*OsREM6.5* Nuclear	*AtREM6.5* Nuclear	Cytosol
TaREM6.5_4BS	Nuclear			
TaREM6.5_4DS	Nuclear			
TaREM6.6_2AL	Nuclear	*OsREM6.6* Nuclear	*AtREM6.2* Nuclear	Plasma membrane
TaREM6.6_2BL	Nuclear			
TaREM6.6_2DL	Nuclear			

† CELLO was used for the localization predictions (http://cello.life.nctu.edu.tw/, accessed 25 Mar. 2019).

‡ Experimental localization of the *Arabidopsis* remorin *C*-terminal anchor was done by Konrad et al. (2014).

§ U, gene is localized in an unknown chromosome; –, gene does not exist in the species.

Table 3. In silico promoter analysis of remorin (*TaREM*) genes from the PlantCARE database program, showing numbers of stress-related *cis*-elements and regulatory and development elements predicted in the regions 1500 bp upstream of *TaREM* genes. The *cis*-motifs identified in *TaREM* candidate genes are given in relation to the transcription start site.

Gene name	DRE	ABRE	MeJA	Other hormones‡	LRE§	Regulatory¶	Development#	Promoter size in different genomes
								bp
TaREM1.1	2	22	14	7	87	60	17	1500 (A, B, D)
TaREM1.2	9	17	11	6	73	39	20	1500 (A, B, D)
TaREM1.3	1	7	8	3	44	32	16	1500 (A, B, U)
TaREM1.4	4	4	20	6	57	45	18	1500 (A, B, D)
TaREM1.5	3	13	18	–	64	61	42	1500 (A, B, D)
TaREM0.1	–	1	10	12	36	72	14	1500 (A, B, D)
TaREM0.12	1	2	2	8	38	61	12	1500 (D)
TaREM0.2	2	3	12	9	36	54	30	1500 (A, B, D)
TaREM0.23	6	7	10	13	74	77	21	1500 (A, B, D)
TaREM4.1	6	4	12	12	49	46	15	1500 (A, B, D)
TaREM4.2†	6	15	2	5	63	35	25	950 (A) 1500 (B) 1500 (D)
TaREM4.3	4	6	24	12	46	49	23	1500 (A, B, D)
TaREM5.1	16	13	8	2	75	33	14	1500 (A, B, D)
TaREM5.3	12	16	24	11	53	61	19	1500 (A, U, D) 1500 (A, B,)
TaREM6.1†	8	1	18	7	43	67	17	750 (D)
TaREM6.2	1	6	4	5	45	48	9	1500 (A, B, D)
TaREM6.3	2	16	2	4	100	85	22	1500 (A, B, D)
TaREM6.4	5	2	4	2	24	14	2	1500 (A, B, D)
TaREM6.5	1	1	14	4	48	55	15	1500 (A, B, D)
TaREM6.6†	–	3	6	10	46	18	8	1500 (A) 1100 (B) 1500 (D)

† Sequences less than 1500 bp were used for Copy A of *TaREM4.2*, Copy D of *TaREM6.1*, and Copy B for *TaREM6.6* promoter sequences because these were less than 1500 bp.

‡ Other hormone-responsive elements include salicylic acid, gibberellin, ethylene, and auxin.

§ This includes 45 different light-responsive elements (LREs) like G-box, ACE, GAG, Box 4, Sp1, BoxI, Box II, Box III, the CAG motif, etc. For more information on LRE regulatory and development elements, see Supplemental Table S3.

¶ Regulatory elements include the AT-rich element, CAAT box, 3-AF3 binding site, 5UT Py-rich stretch, A-box and OBP-site.

Development elements include AC-I, AC-II, and H-Box.

†† DRE, dehydration-responsive element; ABRE, ABA-responsive element; MeJA, methyl jasmonate-responsive element; –, element does not exist in the promoter.

been reported in many tissue localization studies (Li et al., 2013, Bariola et al., 2004; Yue et al., 2014; Kong et al., 2016). The presence of the tissue-specific element in *TaREM* gene promoters reveals their potential role in plant development. Furthermore, putative *TaREM* promoters have different types of stress-responsive regulatory elements (abiotic and biotic), including LTRs, which is a *cis*-acting element involved in low-temperature responsiveness; ARE, which is a *cis*-acting regulatory element essential for anaerobic induction; HSE, which is a *cis*-acting element involved in heat stress responsiveness; MBS, which is a MYB binding site involved in drought-inducibility; TC-rich repeats, which are *cis*-acting elements involved in defense and stress responsiveness; and Box-W1, a fungal elicitor responsive element (Supplemental Table S3). Tissue-specific and stress-related *cis*-elements in the promoters may be responsible for multiple functions of *TaREMs* through complex regulatory mechanisms.

In Silico Expression Profile of *TaREM* Genes in the Crowns of Two Wheat Genotypes Grown Under Autumn Field Conditions

To understand the function of *TaREM* genes in response to cold acclimation and vernalization, we analyzed the transcriptome of developing crowns from five time points for two genotypes grown under field conditions from early autumn to winter in 2010 at Saskatoon, SK, Canada. The winter (Norstar) and spring (Manitou) wheat genotypes differed in vernalization requirements and freezing tolerance potential (Limin and Fowler, 2002; Li et al., 2018). The transcript abundance of 20 *TaREM* genes was determined from the RNA-Seq data as reads per million (Fig. 6A, B). Digital gene expression analysis revealed that wheat *REM* genes varied in their expression depending on the genotype and on the exposure time to cold. *TaREM* genes were divided into three clusters on the basis of their regulation in response to cold: upregulated, downregulated, or constitutively expressed. The upregulated *REM* genes included the highly induced *TaREM1.2* (30-fold) and the moderately induced *TaREM1.3* (4-fold) and *TaREM1.1* (2.5-fold). These genes have the putative C-repeat (CRT)/dehydration-responsive element (DRE) or low-temperature-responsive elements in their promoter regions (Table 3). Interestingly, their expression was higher in the cold-tolerant Norstar than the less tolerant Manitou, indicating their possible association with freezing tolerance and vernalization in the crown tissue. *TaREM0.12* showed an inverse association in the less tolerant spring wheat Manitou. The downregulated *REM* genes included 10-fold repression of *TaREM0.23* and twofold repression of *TaREM4.1* and *TaREM4.3*. The other *REM* genes did not reveal any up- or down regulation of more than twofold and were considered to be constitutively expressed. Another interesting observation was that some genes were expressed in the cold-tolerant Norstar at a higher level across all time points analyzed. Norstar had higher expression of *TaREM1.1*, *TaREM1.3*, *TaREM4.3*, *TaREM6.1*, *TaREM6.5*, and *TaREM6.6*, whereas the less tolerant Manitou showed higher expression of *TaREM1.4*. The expression of these genes reflects the genetic background and could be considered positively or negatively associated with freezing tolerance and vernalization in the crown tissue. The expression levels of all the *TaREM* genes were also examined in the publicly available data of spring wheat transcriptomics from multiple RNA-Seq experiments with different tissues and abiotic or biotic stress conditions (Hruz et al., 2008). Unfortunately, there are no public data available for winter wheat for comparison. The Genevestigator ID corresponding to the *TaREM* genes is shown in Supplemental Table S4. The RNA-Seq data in Supplemental Fig. S2 showed that some *TaREM* genes (*TaREM4.1, TaREM 4.2*, and *TaREM 4.3*) are responsive to cold treatment (Hruz et al., 2008), but others were not. As reported in many species, the *REMs* identified in this study are also responsive to drought and heat treatment (Supplemental Fig.S2). They were also expressed in different tissues and developmental stages (Supplemental Fig. S3A, B). This suggests that *REM* genes have diverse functions during abiotic stresses.

Expression Profiles of *TaREM* Genes During Cold Acclimation in a Controlled Environment

Quantitative Real-Time PCR was used to determine the expression levels of 20 *TaREM* genes in the aerial tissues of Norstar and Manitou and to compare the RNA-Seq data from the field experiment with those of the controlled environment. The *REM* genes were classified into three groups according to their expression patterns: upregulated, downregulated, or constitutively expressed (Fig. 7A, B). The first group represents the genes that were upregulated in response to cold acclimation and correspond to *TaREM1.1, TaREM1.2*, *TaREM4.1*, *TaREM4.2*, *TaREM4.3*, *TaREM6.3*, *TaREM6.4*, *TaREM0.1*, *TaREM0.12*, and *TaREM0.23*. Interestingly, all of the Group 4 remorins were strongly induced by cold. The highest increase in expression (sevenfold) was found in *TaREM4.3*, followed by *TaREM4.2* (4.5-fold), after 56 d of cold treatment, suggesting an association with late cold response. In contrast, expression of the other eight *TaREMs* peaked after 7 d of cold treatment and then decreased toward the end of the treatment, suggesting an association with early responses to cold. The second group represents the downregulated genes and contained two genes from Group 1 (*TaREM1.3* and *TaREM 1.4*) that were also associated with cold response. The third group contained eight constitutively expressed genes (*TaREM0.2*, *TaREM1.5*, *TaREM5.1*, *TaREM5.3*, *TaREM6.1*, *TaREM6.2*, *TaREM6.5*, and *TaREM6.6*) that were not significantly responsive to cold (Fig. 7A, B).

Regulation of *REM* genes under abiotic stress has been reported in several species. In mulberry, *MiREM* was the first reported remorin gene involved in abiotic stress. The heterologous expression of *MiREM* in *Arabidopsis* improved drought and salinity tolerance during the germination and seedling stages (Checker and Khurana, 2013). In another study, *SiREM6* from

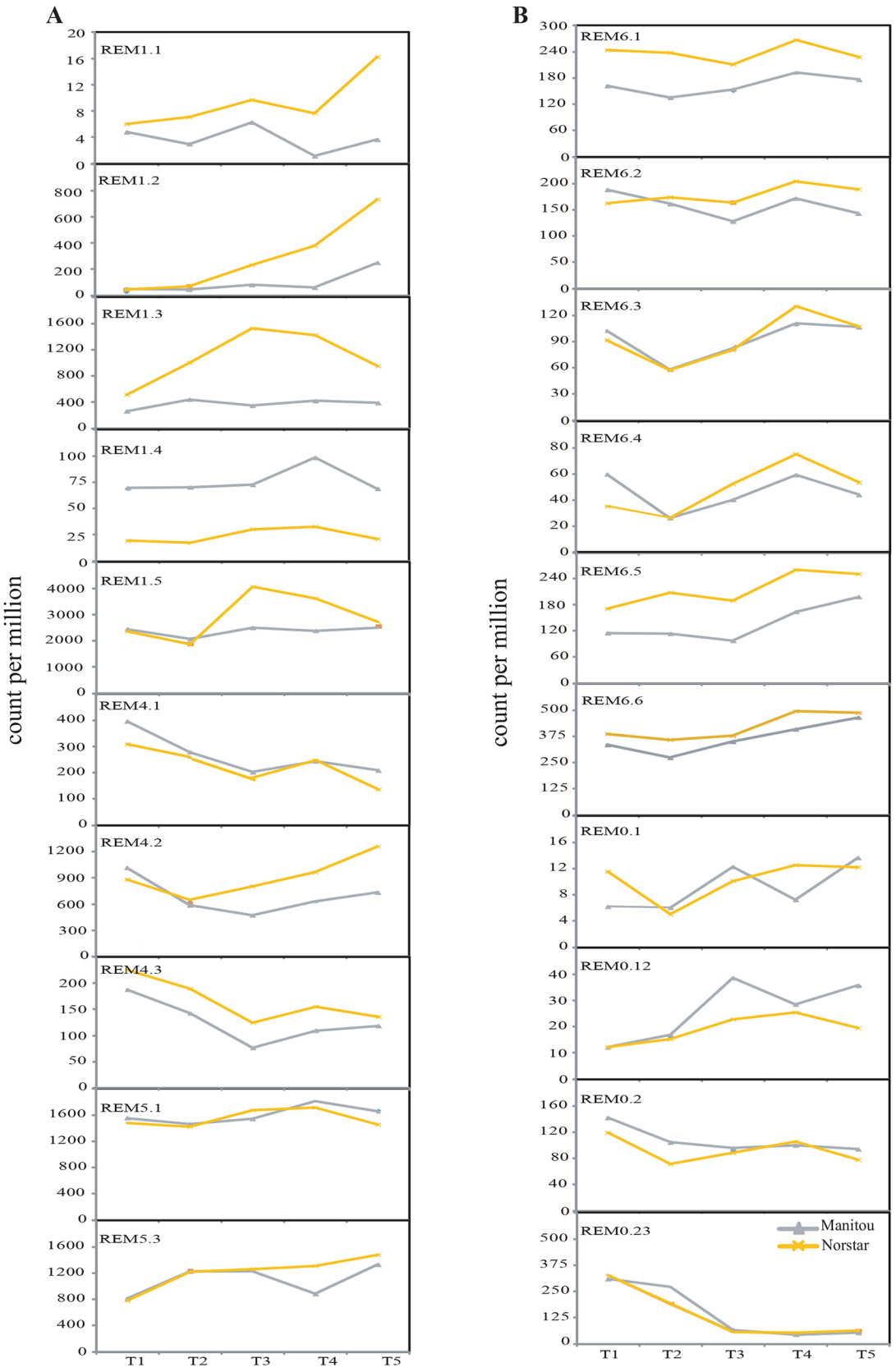


Fig. 6. Digital gene expression profiles of remorin (*TaREM*) genes based on the transcriptome data from the field study of 2010. The expression profiles of 20 REMs, representing the combined counts of the three homeologous copies, were deduced from the Illumina RNA-Seq data of the winter wheat genotype Norstar and the spring wheat genotype Manitou sampled from early autumn to winter. (A) Genes in phylogenetic Groups 1, 4, and 5; (B) genes in phylogenetic Groups 6, 0.1, and 0.2. The *y*-axis represents counts per million *REM* genes. The gene expression in this experiment represents the three copies combined; the means between the two biological replicates are presented. T1, T2, T3, T4, and T5 represent the five time points during autumn cold acclimation of crowns.

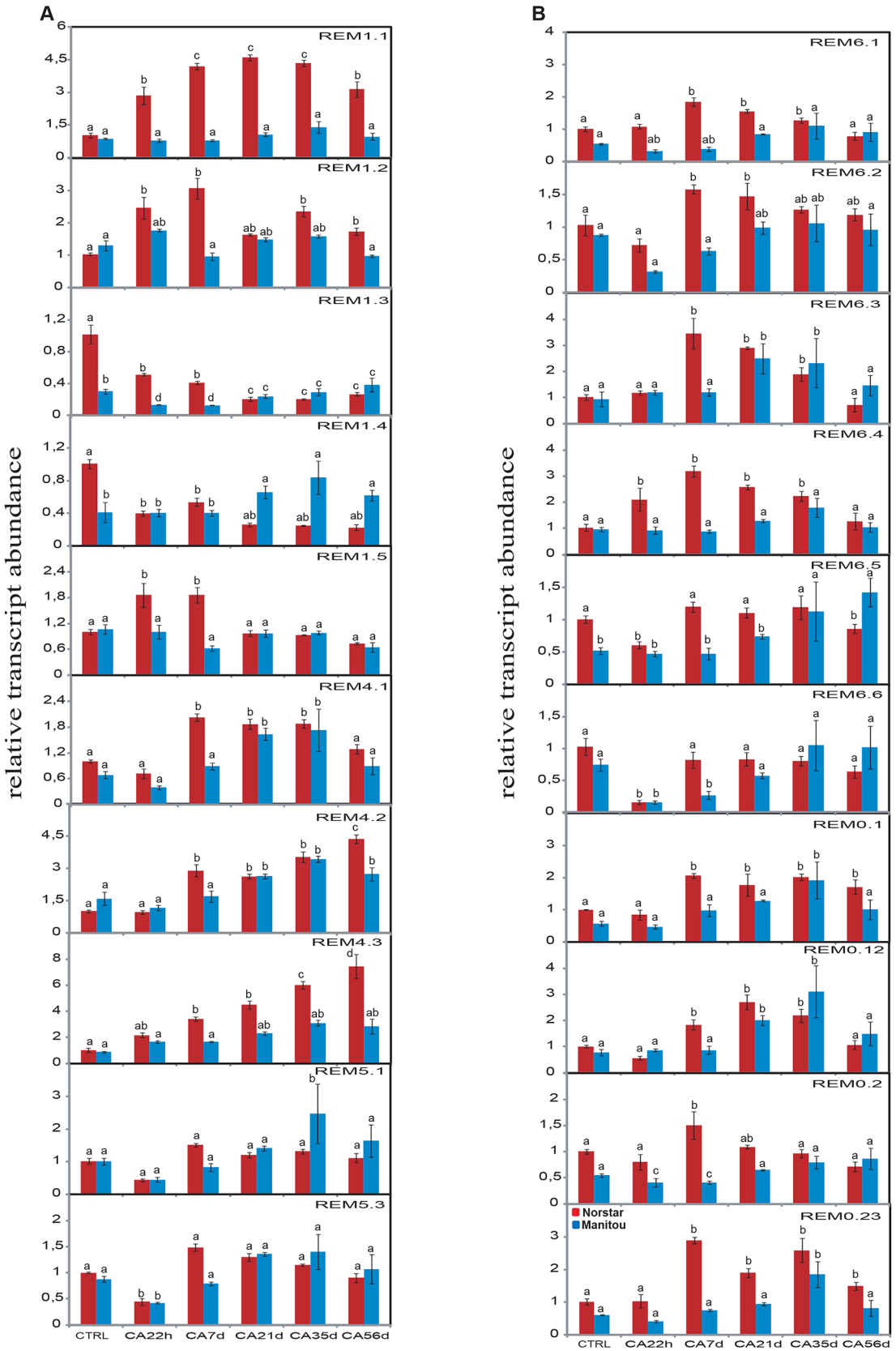


Fig. 7. Expression profiles of remorin (*TaREM*) genes in aerial tissues of winter (Norstar) and spring (Manitou) wheat during cold acclimation under experimental conditions via quantitative polymerase chain reaction (qPCR). Expression of *REM* genes was compared via qPCR between the control (CTR) and cold-acclimated (CA) plants after 22 h and 7, 21, 35, and 56 d, respectively. (A) Genes in phylogenetic Groups 1, 4, and 5; (B) genes in phylogenetic Groups 6, 0.1, and 0.2. The *y*-axis represents the relative expression levels of *REM* genes compared with *18S*. Bars represent the mean values of two biological and technical replicates ± SD. The different lower-case letters represent statistically significant differences between samples ($P < 0.05$ by Tukey's test).

foxtail millet increased by 5.2-fold under high salinity, fourfold in response to low temperatures, and 9.1-fold in resonse to ABA treatment. Overexpression of *SiREM6* in *A. thaliana* enhanced tolerance to high salt stress during seed germination and seedling development stages (Yue et al., 2014). This gene has homology with *TaREM1.5*, which increased in expression by almost twofold after 7 d of cold treatment (Fig. 7A). In a previous study, it was shown that several *REMs* in Group 1 respond to abiotic stress and ABA treatment (Checker and Khurana, 2013). Interestingly, most *TaREMs* contain the CRT/DRE element in their promoter (Table 2), suggesting that *TaREMs* can be regulated by DRE-binding or CBF transcription factors in wheat. Byun et al. (2015) showed that *Da*CBF7 binds to the upstream region of an endogenous *REM*, which has a putative CRT/DRE. The overexpression of *DaCBF7* in rice enhanced tolerance to cold stress through the upregulation of dehydrin, remorin, and several unknown or hypothetical genes (*Os03g63870*, *Os11g34790*, and *Os10g22630*). Moreover, all the induced *TaREM* genes were expressed to a significantly higher level in the winter cultivar in at least one time point compared with the spring cultivar suggesting their potential implication in freezing tolerance and a possible association with phenological development. However, additional work is needed to confirm their role. The pattern of gene expression revealed by quantitative PCR in aerial wheat tissues was like that detected in the crowns with the RNA-Seq data for some *REM* genes such as *TaREM0.12*, *TaREM1.1*, and *TaREM1.2* (upregulated) and *TaREM1.5*, *TaREM5.1*, *TaREM5.3*, *TaREM6.1*, *TaREM6.2*, *TaREM6.5*, and *TaREM6.6* (constitutively expressed). Interestingly, *TaREM0.12* and *TaREM1.1* are located on chromosome 6, which is known to harbor the most *COR* genes (Li et al., 2018). These results support the possible role of *TaREM0.12*, *TaREM1.1*, and *TaREM1.2* in a more generalized cold response. The differences observed in other *REM* expression profiles between both studies could result from the type of tissues used (crowns vs. aerial tissues) and the different experimental conditions (field conditions vs. controlled conditions). Several transcriptional studies of cold acclimation in wheat have shown that changes observed under controlled environments can be different from those in field studies (Campoli et al., 2009; Greenup et al., 2011; Laudencia-Chingcuanco et al., 2011; Wang et al., 2014), as plants grown under field conditions are exposed to more variable factors than those grown under controlled conditions.

Remorin Regulation under Hormone Treatment

Plant hormones, such as ABA and MeJA, are involved in regulating several biotic and abiotic processes (Mittler and Blumwald, 2015; Wasternack and Parthier, 1997). Most *TaREM* genes considered in this study were downregulated in response to ABA treatment (Fig. 8A, B). Thirteen out of the 20 *TaREM* genes were repressed to different degrees, whereas the remaining genes showed no change (*TaREM4.3* and *TaREM6.4*) or were upregulated (*TaREM1.1*, *TaREM1.2*, *TaREM1.5*, *TaREM4.2*, and *TaREM0.23*) under ABA treatment (Fig. 8A, B). The upregulated genes *TaREM1.2* and *TaREM1.5* exhibited a high level of transcript abundance, with 15- and 3.5-fold increases, respectively. The *REM* genes from Groups 5 and 6 (*TaREM5.1*, *TaREM5.3*, *TaREM6.1*, *TaREM 6.5*, and *TaREM6.6*) were highly repressed by 50- to 400-fold. The other genes (*TaREM0.1*, *TaREM0.12*, *TaREM0.2*, *TaREM1.3*, *TaREM1.4*, *TaREM4.1*, *TaREM6.2*, and *TaREM6.3*) were moderately repressed (2- to 21-fold changes) after ABA treatment (Fig. 8A, B). These results indicate that 16 *TaREM* genes responded in an ABA-dependent manner and may play a role in ABA signaling. *REM* was also upregulated in response to exogenous ABA in rice, suggesting that *REM* is involved in the ABA signal transduction pathway (Lin et al., 2002). In *A. thaliana*, *REM*s are induced through the binding of transcription factors to specific *cis*-elements in both the ABA-dependent and ABA-independent pathways (Raffaele et al., 2007). These results demonstrate that *TaREM1.1* and *TaREM1.2* are responsive to cold as well as to exogenous ABA treatment. In contrast, *TaREM0.23* and *TaREM4.3* are regulated in response to cold exposure but not to ABA treatment, suggesting that ABA-dependent and independent pathways may regulate *REM* expression in response to cold acclimation.

In addition to ABA, *REM* genes are regulated in response to several hormones including SA, MeJA, and brassinosteroid (Gui et al., 2016; Kong et al., 2016). Recently, Kong et al. (2016) showed that *StREMa4* expression levels were regulated by SA, MeJA, and ABA, indicating that *StREMa4*, and perhaps other *REMs*, are part of a complex regulatory network affecting plant host interactions with pathogens. This gene has the strongest homology with wheat *REM* genes from Group 1. The *OsREM4.1* gene was induced by an ABA signal through the transcriptional activator OsbZIP23 and plays a role in modulating brassinosteroid signaling (Gui et al., 2016). Jasmonate positively regulates plant responses to freezing stress through a critical upstream signal of the inducer of CBF expression-CBF/ DRE-binding pathway to regulate freezing tolerance in *A. thaliana* (Hu et al., 2013). In our study, MeJA treatment induced the expression of *TaREM1.2*, *TaREM1.5*, *TaREM4.2*, and *TaREM0.23* genes (Fig. 8A, B). The expression of *TaREM1.1*, *TaREM1.4*, *TaREM4.3*, and *TaREM6.4* showed no significant changes after MeJA treatment (Fig. 8A, B). As in the ABA treatment, the expression of *TaREM5.1*, *TaREM5.3*, *TaREM6.1*, *TaREM6.5*, and *TaREM6.6* was severely repressed (55- to 111-fold changes). *TaREM0.1*, *TaREM0.12*, *TaREM0.2*, *TaREM1.3*, *TaREM4.1*, *TaREM6.2*, and *TaREM6.3* were moderately repressed in response to MeJA treatment (Fig. 8A, B). These results suggest that the 16 differentially expressed genes may play a role in jasmonic acid signaling. All *REM* genes identified in *A. thaliana* were mostly involved in hormone and biotic or abiotic stress responses (Raffaele et al., 2007). Accumulation of *REM* in plants has often been associated with defense signaling molecules (Wu et

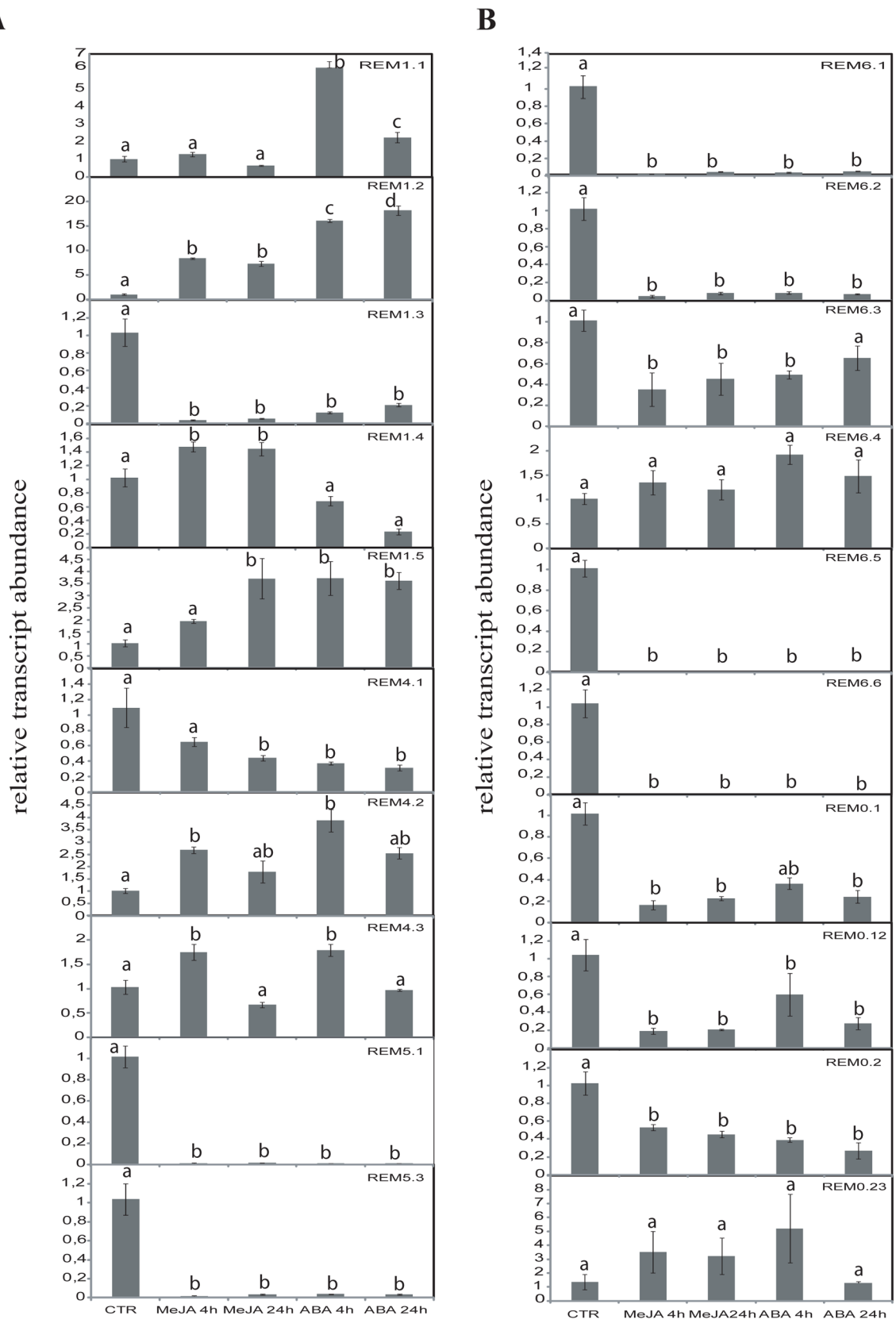


Fig. 8. Expression profiles of remorin (*TaREM*) genes in winter wheat (Norstar) in response to methyl jasmonate (MeJA) and ABA treatment via quantitative polymerase chain reaction. Expression of *REM* genes was compared between control (CTR) and plants treated with MeJA and ABA after 4 and 24 h. (A) Genes in phylogenetic Groups 1, 4, and 5; (B) genes in phylogenetic Groups 6, 0.1, and 0.2. The *y*-axis represents the relative expression levels of remorin genes compared with *18S*. Bars represent the mean values of two biological and technical replicates ± SD. The different lower-case letters represent statistically significant differences between samples ($P < 0.05$ by Tukey's test).

al., 2006; Anderson et al., 2004; Chen and Charles-An, 2006). Interestingly, in our study, the quantitative PCR expression analyses revealed that the transcripts of *TaREM1.2* and *TaREM4.2* accumulated on exposure to low temperature, ABA, and MeJA. This suggests that both ABA and MeJA may mediate their expression during cold acclimation.

TaREM Gene Expression in Different Tissues

Expression of *REM* genes is known to be tissue-dependent (Raffaele et al., 2007). Therefore, to gain insight into the tissue preference of *TaREMs* during cold acclimation in wheat, we examined the expression profiles of all *REM* genes in four organs (leaves, stems, crowns, and roots) by qRT-PCR. All 20 *TaREM* genes were expressed in the tested tissues; the expression patterns revealed spatial variation in the expression of *TaREM* genes in different organs (Fig. 9A, B). Some *TaREM*s are expressed preferentially in a specific tissue and this includes *TaREM1.2* in leaves, *TaREM6.3* in stems, and *TaREM1.1*, *TaREM1.3*, *TaREM1.4*, *TaREM4.2*, *TaREM4.3*, and *TaREM0.23* in roots. *TaREM* gene expression was markedly less in leaves during cold acclimation. Seven *TaREM* genes (*TaREM5.1*, *TaREM5.3*, *TaREM6.1*, *TaREM6.2*, *TaREM6.5*, *TaREM6.6*, and *TaREM0.1*) displayed significantly lower expression in leaves; otherwise, they did not express a pronounced preference in any other tissue. *TaREM* genes showed differential expression in various tissues, as reported in other plants (Raffaele et al., 2007; Yue et al., 2014). The expression of six *TaREMs* was positively associated with preferential root expression but seven *TaREMs* showed a substantial loss of expression in leaves during cold acclimation. The higher expression level in roots indicates a possible role in root development during cold stress or may reflect the higher susceptibility of underground tissues to biotic stress under these conditions. The lower expression of *TaREMs* in leaves may stem from the reduced growth or developmental activity of the mature leaf tissue compared with stems or crowns in cereals. In tomato, foxtail millet, and *A. thaliana* (Group 1b genes), several members of the REM family were expressed in the vascular system (Bariola et al., 2004; Yue et al., 2014). In foxtail millet seedlings, *SiREM6* was expressed in different tissues (roots, stems, leaves, and inflorescences) in the early stage of vascular development (Yue et al., 2014). In *P. deltoides*, *PdREM* was expressed in different tissues like leaf buds, and immature and mature phloem, indicating the possible function of *PdREM* in stem development and phloem formation (Li et al., 2013). In rice, the *REM* gene *GSD1* was localized at the plasma membrane and plasmodesmata of phloem companion cells and affected grain set by regulating the transport of photoassimilates (Gui et al., 2015). According to the analysis of putative *TaREM* promoters, the *TaREM* gene family harbors different numbers and types of *cis*-elements involved in responses to abiotic and biotic stresses, low temperatures, ABA, and MeJA. Additionally, *TaREM* genes showed tissue-specific responses during cold acclimation. Together, these observations help to explain the complex response of *TaREMs* to abiotic stresses and hormonal treatments. These results indicate that *TaREM* genes have several functional roles in response to hormone and cold treatment.

CONCLUSIONS

In this study, we evaluated the potential functional importance of *REM* gene family members in wheat. Twenty nonredundant *REM* genes were identified and phylogenetically clustered into six distinct subfamilies. Phylogenetic analysis showed that *TaREMs* and other monocot *REMs* (rice) are homologous, suggesting a probable functional similarity among them. Analysis of intron length, position, and splicing suggested that introns were highly conserved within the same subfamily. The response of wheat *REM* genes to low temperatures, ABA, and MeJA indicated that the action of several genes was specific to low temperatures, whereas others responded to low temperatures, ABA, and MeJA. This suggests that some genes are regulated by cold through hormonal signaling pathways. *Cis*-element analyses of putative *REM* promoters revealed the presence of *cis*-motifs specific to cold response, other abiotic stresses, hormone regulation, tissue specificity, and development, indicating that the expression of the *REM* genes was modulated, in part, by the regulatory elements in their promoters. The expression profiles of the 20 wheat *REM* genes in leaves, stems, crowns, and roots showed that they were expressed in all tissues, with higher expression in roots. Comparisons between autumn field and controlled environments showed common expression for some *TaREM* genes but not in others. The contradictions between the two experiments may be caused by the type of tissues used and the different experimental conditions. The expression of *REM* genes at different wheat developmental stages and in different tissues and their association with cold acclimation and hormonal responses suggest a potential role during wheat development. Understanding the function of each gene during the wheat life cycle may help us to select for varieties that tolerate higher biotic and abiotic stresses.

Supplemental Information

Supplemental File S1. The remorin sequences used in the present study.

Supplemental Table S1. Wheat, rice, and *A. thaliana* intron–exon number.

Supplemental Table S2. Identificaton of consensus sequence of *TaREM* by MEME and their function by Prosite and Eukaryotic Linear Motif resource software.

Supplemental Table S3. The *cis*-acting regulatory DNA elements of 20 *TaREM* promoters.

Supplemental Table S4. Remorin Genevestigator ID.

Supplemental Table S5. The primers sequences used in the present study for qRT-PCR.

Supplemental Fig. S1. Phylogenetic relationships among *T. aestivum* remorin proteins (from three genomes: A, B, and D).

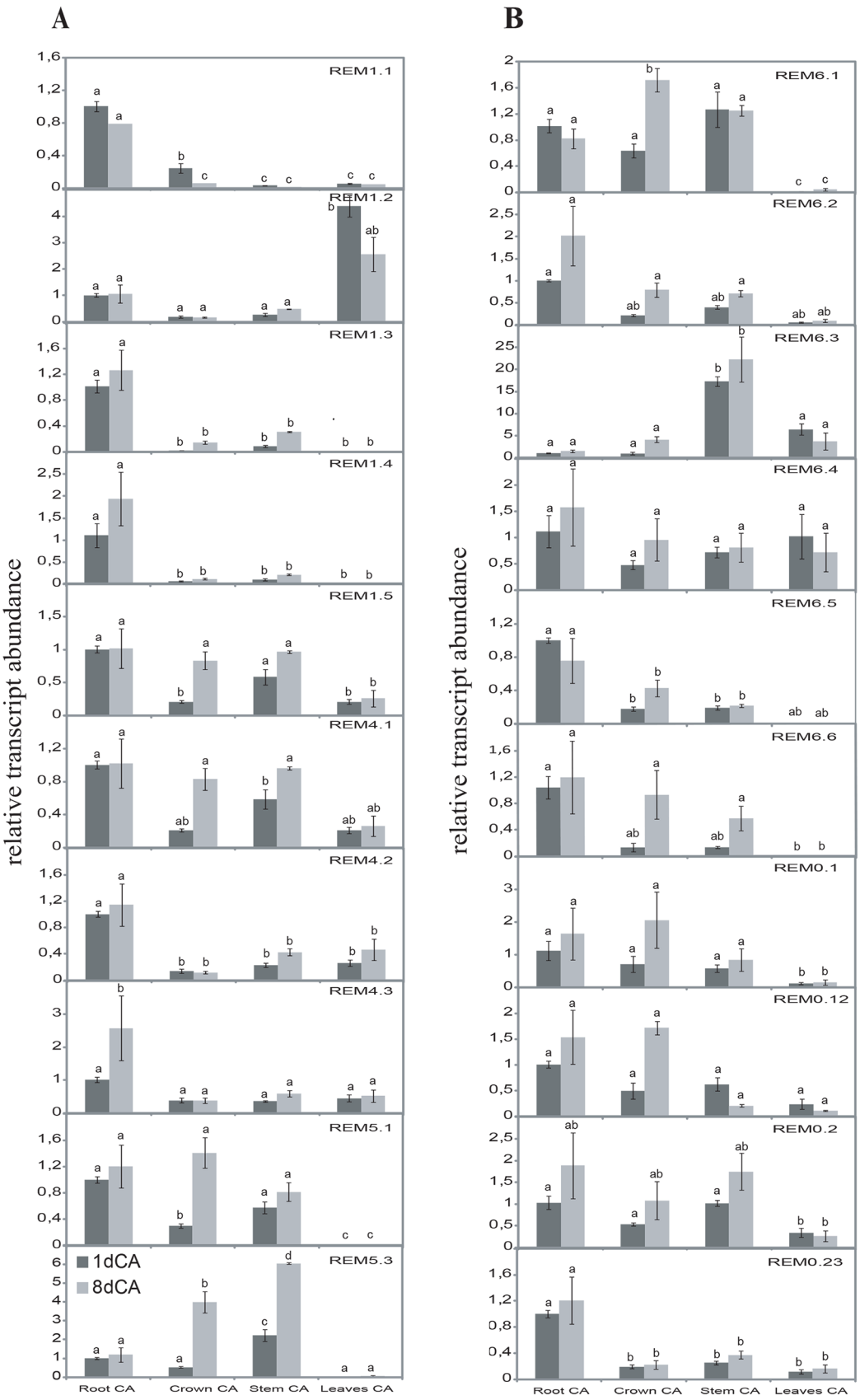


Fig. 9. Expression profiles analysis of remorin (*TaREM*) genes in different tissues of winter wheat (Norstar) during cold acclimation using quantitative polymerase chain reaction. The relative expression of *REM* genes was compared among roots, crowns, stems, and leaves from plants after 1 and 8 d of cold acclimation (1dCA, 8dCA). (A) Genes in phylogenetic Groups 1, 4, and 5; (B) genes in phylogenetic groups 6, 0.1, and 0.2. The *y*-axis represents the relative expression levels of *REM* genes compared with *18S*. Bars represent the mean values of two biological and technical replicates ± SD. The different lower-case letters represent statistically significant differences between samples ($P < 0.05$ by Tukey's test).

Supplemental Fig. S2. Gene expression analysis of 20 *TaREM* genes from published RNA-Seq data.

Supplemental Fig. S3. A gene expression analysis of 20 *TaREM* genes from published RNASeq data from different anatomical parts and developmental stages.

Author Contributions

MAB, JD, and FS designed the overall study. MAB and JD performed the biological experiments under controlled conditions. ZA and MZ performed the biological experiments for ABA and MeJA. DBF, JZ, and QL performed the biological experiments under field conditions and provided the RNA-Seq data. MAB, ZA, and MZ performed the preselection of *REM* genes. MZ performed the Genevestigator analysis. MAB performed the quantitative PCR experiment and the bioinformatics analyses. MAB, ZA, and FS wrote the article. All authors read, edited, and approved the manuscript.

Conflict of Interest Disclosure

The authors declare that there is no conflict of interest.

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