Genome-wide identification and expression analysis of calmodulin and calmodulin-like genes in wheat (Triticum aestivum L.)

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ABSTRACT
Calmodulin (CaM) and calmodulin-like (CML) genes are widely involved in plant growth and development and mediating plant stress tolerance. However, the whole genome scale studies about CaM and CML gene families have not been done in wheat, and the possible functions of most wheat CaM/CML gene members are still unknown. In this study, a total of 18 TaCaM and 230 TaCML gene members were identified in wheat genome. Among these genes, 28 TaCaM/CML gene members have 74 duplicated copies, while 21 genes have 48 transcript variants, resulting in 321 putative TaCaM/CML transcripts totally. Phylogenetic tree analysis showed that they can be classified into 7 subfamilies. Similar gene structures and protein domains can be found in members of the same gene cluster. The TaCaM/CML genes were spread among all 21 chromosomes with unbalanced distributions, while most of the gene clusters contained 3 homoeologous genes located in the same homoeologous chromosome group. Synteny analysis showed that most of TaCaM/CMLs gene members can be found with 1–4 paralogous genes in T. turgidum and Ae. Tauschii. High numbers of cis-acting elements related to plant hormones and stress responses can be observed in the promoters of TaCaM/CMLs. The spatiotemporal expression patterns showed that most of the TaCaM/TaCML genes can be detected in at least one tissue. The expression levels of TaCML17, 21, 30, 50, 59 and 75 in the root or shoot can be up-regulated by abiotic stresses, suggesting that TaCML17, 21, 30, 50, 59 and 75 may be related to abiotic stresses in wheat. The spatiotemporal expression patterns of TaCaM/CML genes indicated they may be involved widely in wheat growth and development. Our results provide important clues for exploring functions of TaCaMs/CMLs in growth and development as well as responses to abiotic stresses in wheat in the future.

Introduction
As an important second messenger, calcium (Ca²⁺) plays pivotal roles in plant growth and development as well as stress signal transduction. Evidence shows that stimulations, such as plant hormones, gravity, light, cold, heat, drought, anoxia, salt, touch, wound and pathogen attack, can rapidly induce an increase of cytosolic-free Ca²⁺ concentrations ([Ca²⁺]cyt). These Ca²⁺ signatures can be decoded by effectors to generate specific responses. There are several Ca²⁺-sensing proteins found in plants, especially EF-hand domain containing proteins (i.e., a helix-loop-helix structure), playing principal roles in Ca²⁺ signal transduction. The major EF-hand containing proteins could be divided into three families, including calmodulins (CaMs) and calmodulin-like proteins (CMLs), calcineurin B-like (CBL), and Ca²⁺-dependent protein kinases (CDPKs/CPKs). As a conservative Ca²⁺-binding protein, the typical CaM is a soluble protein composed of 149 amino acids that has two pairs of EF-hand domains, while each EF-hand domain can be combined with one Ca²⁺ ion. CaMs can interact with the target proteins and affect their biological activity to regulate the growth and development of plants and respond to stresses. CMLs share 16–75% amino acid identity with typical CaM, which usually carry 1–6 EF-hands and no other known functional domains with variable amino acids length. Most of the CaM/CMLs usually have no enzymatic or biochemical functions, except for CaM7, which can act as a transcription factor to directly regulate the expression of the HY5 gene to regulate light morphogenesis. Different CaM/CMLs varied in the binding and regulation of target proteins, with slight differences in the structures of CaM/CML proteins that may result in considerable impacts on their binding to target proteins. CaM/CMLs are widely involved in plant growth and development and mediating plant stress tolerance.

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strongly affect pollen germination and pollen tube growth,\textsuperscript{9,10} and AtCML24 can interact with AtGP4b to affect autophagy progression.\textsuperscript{11} AtCML39 can be involved in regulating seed development, germination, fruit development, and early seedling establishment in Arabidopsis.\textsuperscript{12,13} It has been found that CaM is involved in heat shock signal transduction in wheat.\textsuperscript{14} AtCaM3 can regulate the activity of CaM-binding protein phosphatase (AtPP7) or protein kinase (AtCBK3) to increase the activation of the heat shock transcription factor (HSF), resulting in improving heat tolerance in Arabidopsis.\textsuperscript{15,16} ShcML44, a CML gene isolated from cold tolerant wild tomato (Solana habrochaites), can enhance the tolerance to cold, drought, and salinity stresses in plants, and promote germination and seedling growth.\textsuperscript{17} A rice calmodulin-like gene, OsMSR2, can enhance drought and salt tolerance and increase ABA sensitivity.\textsuperscript{18,19} while OsCML4 can scavenge reactive oxygen species to improve drought tolerance in rice.\textsuperscript{20} The overexpression of GmCaM4 can promote the DNA binding activity of the MYB2 transcription factor to increase salt tolerance in plants.\textsuperscript{21} AtCaM1 and AtCaM4 can be directly bind with S-nitrosoglutathione reductase (GSNR), which can inhibit its activity and improve the internal level of nitric oxide (NO), resulting in increased salt resistance.\textsuperscript{22} In summary, itsuggests that CaM/CMLs play fundamental roles in Ca\textsuperscript{2+} signal transduction during development and stress adaptations in plants. However, the complex signal transduction of CaM/CMLs in regulating these pathways still needs to be explored.

Genes encoding CaM/CMLs have been identified at the whole genome scale in many plant species. For example, there are 7 CaMs and 50 CMLs in Arabidopsis;\textsuperscript{4} 5 CaMs and 32 CMLs in rice (Oryza sativa);\textsuperscript{23} 6 CaMs and 52 CMLs in tomato (Solanum lycopersicum);\textsuperscript{24,25} 7 CaMs and 19 CMLs in lotus (Lotus japonicas);\textsuperscript{26} 79 CMLs in Chinese cabbage (Brassica rapa L. ssp. pekinensis);\textsuperscript{27} and 4 CaMs and 58 CMLs in apple (Malus × domestica).\textsuperscript{28} In wheat, the cDNAs corresponding to 10 CaM genes have been isolated, which can be classified into 4 subfamilies. Using subfamily-specific DNA probes, the southern-blot analysis showed that there may be 10–20 copies of CaM genes located in the wheat genome.\textsuperscript{29} However, up to now, there are only 2 CML genes that have been reported in wheat. Overexpression of TaCML20 can enhance water soluble carbohydrate accumulation and yield in wheat,\textsuperscript{30} and TaCML36 can positively participate in an immune response to Rhizoctonia cerealis.\textsuperscript{28} Due to its large and complex genome, the whole wheat genome sequencing was relatively later than other model plant species.\textsuperscript{31} There is no study about CaM and CML gene families of wheat in whole genome scale, and the possible functions of most wheat CaM/CML gene members are still unknown.

In this study, 18 deduced CaM and 230 CML gene members were identified in the wheat genome using BlastP and HMMsearch methods, and their duplication copies, transcript variations, gene structure, protein motifs, isoelectric point, molecular weight, subcellular location, and subgroup classification were analyzed. We also analyzed the spatiotemporal expression patterns and expression profiles after abiotic treatments. This systematic analysis of the complete sets of CaM/CMLs in wheat will provide useful information for further gene cloning and functional exploration as well as the understanding of the Ca\textsuperscript{2+}-mediated signal transductions in wheat.

**Materials and methods**

**Identification and characterization of TaCaM/CML genes in wheat**

The CaM/CML proteins were identified and characterized following the method as described by Wang et al with some modifications.\textsuperscript{32} Protein sequences of wheat (Triticum aestivum, IWGSC 1.1), Triticum durndum (Svevo_v1), Aegilops tauschii (Aet_v4.0) were downloaded from the Ensemble database (http://plants.ensemble.org) to construct the local protein database. The database was then searched using known CaM and CML protein sequences collected from A. thaliana (7 CaMs and 50 CMLs), O. sativa (5 CaMs and 32 CMLs), and T. aestivum (10 CaMs) using the local BLASTP program with an e-value of 1e\textsuperscript{-5} and a threshold of 50% identity. Furthermore, a hidden Markov model (HMM) profile of EF-hand (PF00036) was downloaded from the Pfam database (http://pfam.xfam.org/) and used to search the wheat local proteins database using the HMM-search tool embedded in HMMER 3.2.\textsuperscript{33,34} BLAST and HMMER hits were compared and parsed by manual editing. Furthermore, a self blast of these sequences was performed to remove the redundancy, and the remaining sequences were considered as the putative TaCaM and TaCML proteins. These sequences were submitted to the NCBI Batch CD-search database (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) to confirm the presence and integrity of the EF-hand domain and without any other domains. The theoretical pI (isoelectric point) and Mw (molecular weight) of the putative TaCaM and TaCML proteins were calculated using the compute pI/ Mw tool online tool (http://web.expasy.org/compute_pI/).\textsuperscript{35} Subcellular localization of these genes was predicted using the CELLO v2.5 web server (http://cello.life.nctu.edu.tw/).\textsuperscript{36}

**Multiple sequence alignments and phylogenetic analysis**

Multiple sequence alignments were generated using the ClustalW tool.\textsuperscript{37} To investigate the evolutionary relationship among TaCaM/CML proteins, a maximum likelihood (ML) tree was constructed by MEGA X software based on the full-length of the TaCaM/CML protein sequences.\textsuperscript{38} Bootstrap test method was adopted, and the replicate was set to 1000.

**Gene structure and conserved motifs analysis**

Gene structures of the TaCaM/CML members were predicted using the gene structure display server 2.0 (http://gsds.gao-lab.org/) using the genomic and coding sequences.\textsuperscript{39} The conserved motifs of TaCaM/CML proteins were predicted using the MEME program (http://meme-
suite.org/tools/meme) with the following parameters: a maximum of 10 motifs and an optimum motif width between 10 and 50 amino acids.40

Chromosomal locations and synteny analysis of TaCaM/CML genes

The chromosomal locations were collected from the Ensembl plant database (http://plants.ensembl.org) and the location map was constructed using the TBtools v0.6654 software.41 The Multiple Collinearity Scan toolkit (MCScanX) was used to analyze the gene duplication events.42

Prediction of cis-acting elements in the promoters of TaCaM/CML genes

To investigate the cis-acting elements in the promoter sequences of the TaCaM/CML genes, 2-kb sequences upstream of the initiation codon (ATG) were collected from the Ensembl plant database (http://plants.ensembl.org) and subjected to the PLACE database (https://www.dna.affrc.go.jp/PLACE).43

The spatiotemporal expression patterns of TaCaM/CML genes

The spatiotemporal expression patterns of TaCaM/CML genes were analyzed following the method as described by Tyagi et al. with some modifications.44 High-throughput RNA sequence data with two biological replicates (PRJEB5314) were used for the expression analysis of the TaCaM/CML genes in three developmental stages of five different tissues, including the root, stem, leaf, spike, and grain.45 The transcripts per million mapped (TPM) values were used for the calculation of the expression data at a P-value of 0.001. The correlation between the replicates was determined by using log10TPM-transformed values for the tissue developmental stages. Heatmaps were constructed using the TBtools v0.6654 software.41

Plant Materials and Treatments

Plant growth and abiotic stress treatments were conducted as described previously.46 Jin he 9123, a wheat cultivar bred by ourselves and preserved in our lab, was used in this study. The seeds were surface-sterilized for 10 minutes in 1% NaOCl and repeatedly rinsed with tap water three times, then seeded in 1/2 Hoagland nutrient solution after immersion and imbibition for 12 h, and hydroponically cultivated in the incubator with a 16/8-h photoperiod at 25°C. Four 10-day-old homogeneous seedlings groups, each of which included 100 seedlings, were subjected to different treatments, including 16.1% PEG6000, 200 mM NaCl, cold (4°C), and heat (40°C). The seedlings without any treatment were used as control. The root and shoot tissues from ten seedlings were sampled at one time point for the experiment. Collected samples were immediately frozen in liquid nitrogen and stored at −80°C for RNA extraction.

RNA isolation and gene expression analysis

RNA isolation and gene expression analysis were carried out following the method as described by Yang et al. with some modifications.46 Total RNA of collected samples was isolated using the EasyPure® Plant RNA Kit (ER301-01, Transgen, China). For reverse transcription, the first-strand cDNA was synthesized using a PrimeScript™ RT reagent kit (RR047A, TaKaRa, Japan). Quantitative real-time PCR (qRT-PCR) for examination of the TaCMLs expression patterns were performed using the TB Green™ Premix Ex Taq™ II (RR820A, TaKaRa, Japan) with 7500 Real-Time PCR System (Applied Biosisystem, USA). Gene cluster-specific and internal reference gene TaActin primers were listed in additional file 4: Table S2. The qRT-PCR program was carried out as follows: pre-denaturation at 95°C for 30 s; denaturation at 95°C for 5 s, annealing at 58°C for 30 s, extension at 72°C for 34 s, 45 cycles. The 2−ΔΔCt method was used to analyze the data.47 All of the experiments were performed with three technical replicates and three biological replicates, and the data were represented by mean value ± standard error of three biological replicates.

Results

Identification and characterization of TaCaM/CML genes in wheat

Using BlastP and HMM-search methods, a total of 18 TaCaM and 230 TaCML gene members were identified in wheat genome. It can be found that 28 TaCaM/CML gene members have 74 duplicated copies, which located adjacent on the chromosome and from the same cluster in phylogenetic tree; as while 21 genes have 48 transcript variants, resulting in 321 putative TaCaM/CML proteins totally (Additional file 1: Table S1). To obtain the subfamily classification of the 321 TaCaM/CML proteins, multiple sequence alignment was performed using the amino acid sequences, and a maximum likelihood (ML) tree was constructed. The TaCaM/CML proteins were clustered into 7 groups according to their similarities and relationships with each other, and each group contained 18, 57, 65, 34, 44, 12, and 18 gene members, while the group I was CaM family. We also found that 18 TaCaM members were grouped into 6 gene clusters, and 230 TaCML gene members were grouped into 82 gene clusters (Figure 1). The predicted wheat CaM/CML genes were designated as TaCaM1 to TaCaM6 and TaCML1 to TaCML82, plus the suffix corresponding to the specific wheat sub-genome identifier (A, B, or D). The genes in the same cluster were considered as the homoeologous genes of one wheat CaM/CML cluster. It showed that 77 TaCaM/CML gene clusters each contained 3 homoeologous gene members, while 6 other gene clusters (TaCML4-A/D, 8-A/B, 11-B/D, 23-A/D, 30-B/D, and 47-A/D) harbored 2, and 5 remaining gene clusters (TaCML22-A, 53-D, 56-B, 67-A, and 68-A) had 1 (Figure 1).

The genes located adjacent on the chromosome and from the same cluster in phylogenetic tree were defined as duplicated genes, such as TraesCS5A02G057100.1 and TraesCSSA02G057200.1 from TaCML2-A gene cluster. The duplicated genes were named as gene member plus
duplication number and wheat sub-genome identifier. For example, duplicated genes of TaCML2 member are named as TaCML2-1-A (TaCC5A02G57100.1) and TaCML2-2-A (TaCC5A02G57200.1). It showed that 28 gene members have 74 duplicated copies, including 16 genes (TaCML2-A/D, 14-B/D, 17-D, 32-B, 40-A/B/D, 43-A/B, 44-A/B/D, 45-B, and 49-A) have 2 duplicated copies, 8 genes (TaCML32-A, 45-D, 51-A/B/D, and 73-A/B/D) have 3 duplicated copies, 2 genes (TaCML32-D, and 46-A) have 4 duplicated copies, and 2 genes (TaCML46-B/D) have 5 duplicated copies (Additional file 1: Table S1).

The transcript variations are named as gene member plus transcript variation number and wheat sub-genome identifier. For example, transcript variations of TaCaM2-A cluster are named as TaCaM2-1-A and TaCaM2-2-A. It can be found that 21 genes have 48 transcript variants, including 16 gene members (TaCaM2-A, 3-B, and 5-B; TaCML1-A, 37-D, 38-A/D, 61-B, 71-B/D, 76-A/B, 77-B, 81-D, and 82-A) have 2 types of transcript variants, and 5 genes (TaCaM2-B, 6-B; and TaCML37-A, 38-B, 66-B, and 81-B) have 3 types of transcript variants (Additional file 1: Table S1).

The TaCaM proteins ranged in length from 128 (TaCaM5.2-B) to 183 (TaCaM6.1-B), with molecular weights ranging from 14.50 kDa (TaCaM5.2-B) to 20.48 kDa (TaCaM6.1-B) and isoelectric points ranging from 3.83 (TaCaM1-B) to 4.25 (TaCaM6.2-B). These TaCaM proteins ranged in length from 53 (TaCML46-3-B) to 1299 (TaCML81.3-B) amino acids, with molecular weights ranging from 5.65 kDa (TaCML46-3-B) to 139.62 kDa (TaCML81.3-B) and isoelectric points ranging from 3.70 (TaCML57-D) to 10.28 (TaCML33-B/D). Additionally, 289 TaCaM/CML proteins are acidic proteins, while only 32 TaCMLs (TaCML18-A/B/D, 32-1-A/B/D, 32-2-A/B/D, 32-3-A/D, 32-4-D, 33-A/B/D, 34-A/B/D, 36-A/B/D, 55-B, 61-A/D, 61.1/2-B, 66-A/D, 66.1/3-B, 67-A, and 68-A) were basic proteins with isoelectric points higher than 7. Subcellular localization prediction indicated that 253 TaCaM/CML proteins are localized in the cytoplasm, 20 in the nucleus, 13 in the chloroplast, 5 in the mitochondria, 15 in the extracellular matrix, 8 in the periplasm, 6 in the outer membrane, and 1 protein in the plasma membrane (Additional file 1: Table S1).

Gene structure and conserved motifs of TaCaM/CMLs

The exon and intron structures of TaCaM/CML genes were analyzed using the GSDS database. The numbers of introns in all the TaCaM/CML gene transcripts varied from 0 to 15, in which 184 TaCaM/CML transcripts has no intron, as well as the transcripts of 26, 13, 17, 20, 19, 6, 8, 3, 4, 5, 3, 7, 4, 1 and 1 have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 introns, respectively (Additional file 1: Table S1; Additional file 2: Figure S1).

The conserved motifs of the TaCaM/CML proteins were predicted using the MEME program. A total of 10 motifs were identified among the 321 TaCaM/TaCML proteins. Motif 1 – the predicted EF-hand domain – was observed in all of the TaCaM/CML proteins, while motif 10 only...
appeared in 13 TaCML proteins of TaCML37/38 clusters (TaCML37.1/2/3-A, 37-B, 37.1/2-D, 38.1/2-A, 38.1/2/3-B, and 38.1/2-D). It showed that 37, 90, 79, 109, and 6 TaCML/CML proteins contain 1, 2, 3, 4, and 5 EF-hands, respectively. (Additional file 1: Table S1; Additional file 3: Figure S2). In general, genes and proteins belonging to the same cluster contained similar structures and motifs. Among 83 gene clusters harboring multiple-gene members, similar gene structures with the same numbers of exons and coding exons can be observed in 59 gene clusters, and similar distributions of protein motifs can be found in 56 gene clusters (Additional file 1: Table S1; Additional file 2: Figure S1; Additional file 3: Figure S2).

Chromosome locations and syntenic analysis of TaCaM/CML genes

To determine the distribution of TaCaM/CML genes in 21 chromosomes of wheat, we analyzed their chromosomal locations. Among 248 wheat CaM/CML genes, 83, 82, and 82 gene members were mapped in each of the sub-genomes A, B, and D, respectively; while the TaCML60-U gene was located on the unanchored scaffolds. The TaCaMs/CMLs genes were spread among all 21 chromosomes with unbalanced distributions. 33, 34, 42, 28, 49, 23, and 38 TaCaM/CML genes were located in homoeologous-group 1, 2, 3, 4, 5, 6, and 7 chromosomes, respectively. Additionally, chromosome 5A had the highest numbers of 17 TaCaM/CML gene members, and chromosome 6D contained the lowest numbers of 7 gene members (Figure 2).

To understand the evolution of TaCaM/CML, the CaM/CML gene members were identified in ancestral species, T. turgidum (AABB) and Ae. tauschii (DD) genome. It showed that there were 152 and 88 TaCaM/CML gene members identified in the genome of T. turgidum and Ae. tauschii, respectively (Additional file 5: TableS3). Collinearity diagrams among TaCaM/CML gene members were further analyzed using MCscanX software. Among 248 TaCaM/CML gene members in wheat, 230 and 222 gene members were found with 1–4 paralogous genes in T. turgidum and Ae. Tauschii, respectively (Figure 3; Additional file 6: TableS4).

Cis-acting elements in the promoters of the TaCaM/CML genes

Phytohormone and stress responses cis-acting elements in the promoter regions of the 321 TaCaM/CMLs transcripts were predicted: auxin responsive element (AuxRR-core: GGTCCAT, TGA-element: AAGCAG), JA-responsive element (CGTCA-motif: CGTCA), abscisic acid (ABA)-responsive element (ABRE: ACCTGGA/T), dehydration-responsive element/C-repeat element (DRE/CRT: A/GCCGAC), low-temperature responsive element (LTR: CCGAA), WRKY binding site (W-box: T/CTGACC/T), and sulfur-responsive element (SURE: GAGAC).

The results showed that, among 321 TaCaM/CML transcripts, 68, 156, 300, 224, 242, 92, 121, 302, and 293 of TaCaM/CML transcripts have AuxRR-core, TGA-element, CGTCA-motif,

Figure 2. Chromosome locations of wheat CaM/CML genes. The chromosome numbers are shown at the left of each bar. The scale is represented in megabases (Mb).
ABRE, DRE/CRT, G-box, LTR, W-box, and SURE in their promoters, respectively (Figure 4), while 1, 18, 59, 95, 42, 15, and 2 transcripts have 9, 8, 7, 6, 5, 4, 3, and 2 types of cis-acting elements in their promoters, respectively. (Additional file 7: Table S5).

The spatiotemporal expression patterns of TaCaM/CML genes

To detect the spatiotemporal expression patterns of the TaCaM/TaCML genes, the gene expression TPM values of 321 TaCaM/CML transcripts in three developmental stages of five different tissues (including the root, stem, leaf, spike, and grain) were calculated by exploiting the previously reported RNA-Seq data. The heatmap of the expression patterns of the TaCaM/TaCML transcripts were prepared using the TPM values (Figure 5). Most of the TaCaM/TaCML genes were detected in at least one tissue, except for TaCML29-A, 32-3-D, 38.1-A, 45-1-D, 73-2-D, and 81.2-B, which can not be detected in any tissue. Several genes are expressed at almost one time point in specific tissue; for example, the expressions of TaCML12-A/B/D, TaCML22-A, TaCML23-A/D, TaCML26-A/B/D, and TaCML27-B/D can be detected mainly in the late stage (Z65) during spike development, implying their specific functions. Generally, most gene members from the same cluster showed similar but not exactly the same expression patterns, implying their redundant and partially differentiated functions. However, there are some transcript variations or gene duplications showed different expression levels; for example, TaCaM2.2-A/2.2-B was highly expressed in all detected tissues, while the expressions of TaCaM2.1-A/2.1-B/2.3-B was lower (Figure 5).
Expression patterns of TaCML genes under abiotic stress

In order to study whether wheat CaMs/CMLs are involved in the plant responses to abiotic stresses, 6 CML gene clusters – TaCML17, 21, 30, 50, 59, and 75 – were selected to analyze their expression patterns in the root and shoot of 10-day seedlings after NaCl, polyethylene glycol (PEG), cold, and heat treatment using quantitative real-time PCR (qRT-PCR) methods. In control seedlings, the expression levels of 6 CML genes showed no significant change in root or shoot (Figure 6a). Under NaCl stress, the expressions of TaCML50 and 75 in root, and TaCML30, 50 and 75 in shoot increased significantly; while the expressions of TaCML17, 21 and 59 in root, and TaCML17 and 59 in shoot decreased (Figure 6b). In response to PEG treatment, the expressions of TaCML30, 50 and 75 in root, and TaCML17, 30, 50 and 75 in shoot were markedly enhanced; and the expressions of TaCML17 and 59 in root were inhibited; while the expressions of TaCML21 in root, and TaCML21 and 59 in shoot were inhibited at early stage and up-regulated afterward (Figure 6c). In the cold treatment assay, the expressions of TaCML30 and 50 in root, and TaCML17, 21, 30, 50 and 59 in shoot were significantly up-regulated; while the expressions of TaCML17, 59 and 75 in root were down-regulated (Figure 6d). In the heat treatment group, the expressions of TaCML17, 30, 50, 59 and 75 in root, and TaCML21 in shoot increased; while the expressions of TaCML17, 30, 50 and 59 in shoot decreased (Figure 6e).

Discussion

In this study, 18 TaCaM members from 6 gene clusters and 230 TaCML members from 82 gene clusters were identified in the wheat genome, which can be classified into 7 subfamilies. It can be found that the size of CaM/CML family in wheat is larger than most plant species, including Arabidopsis (7 CaMs and 50 CMLs),^4^ rice (5 CaMs and 32 CMLs),^23^ tomato (6 CaMs and 52 CML genes),^24^ and lotus (7 CaMs and 19 CMLs).^26^ Considering that wheat is heterohexaploid, the size of CaM/CML family in wheat is similar to cabbage (79 CML genes),^27^ apple (7 CaMs and 83 CMLs),^49^ T. turgidum (AABB genome, 152 CaM/CMLs), and A. Tauschii (DD genome, 88 CaM/CMLs) (Additional file 5: TableS3). It can be found that the ancestral species contained a large numbers of CaM/CML genes, and most of TaCaM/CML gene members were found with orthologous genes in T. turgidum and Ae. Tauschii, as well as a few numbers of TaCaM/CMLs’ orthologous genes were not found (Figure 3; Additional file 6: TableS4). It indicated that the high numbers of TaCaM/CML genes may be due to the whole-genome duplications during chromosome polyploidization, and gene duplication after polyploidization event.

Most TaCaM/CML genes from the same cluster had similar gene structures and protein motifs, as well as located in the same homoeologous chromosome group, except for TaCML77 and TaCML82, in which TaCML77-A and TaCML82-A were located in 5A, while TaCML77-B and TaCML82-B were located in 4B, and TaCML77-D and TaCML82-D were located in 4D (Figure 2). This may be due to the 4AL/5AL reciprocal translocation during the structural evolution of wheat chromosomes.\(^{50}\)

In previous work, a southern-blot analysis used a probe derived from the 3’-UTR of TaCaM1-3, suggested CaM genes from cluster 1 were located only on 3AS and 3BS, but not on 3DS.\(^{29}\) However, they were located in all three homoeologous-group 3 chromosomes (3A, 3B, and 3D) in our analysis, which may be because the probe in the southern-blot analysis was not effective enough to detect TaCaM1 gene member located in chromosome 3D.
The differential spatiotemporal expression patterns of TaCaMs/CMLs may provide important clues for exploring their functions in growth and development of wheat in the future. In this study, most of the TaCaM/CML genes can be detected in at least one tissue (Figure 5), implying wide involvement of TaCaM/CML genes in wheat growth and development. However, TaCML29-A, 32-3-D, 38.1-A, 45.1-D, 73-2-D, and 8L2-B were not expressed in any tissue (Figure 5), suggesting these genes may be related to other biological processes, such as abiotic or biotic tolerance, or due to homologous gene silencing.

During growth and development, wheat is often affected by abiotic stresses, which cause great losses to wheat production. It suggested that the losses in wheat yields caused by abiotic stresses such as salinity, drought, and heat more than biotic influences. Therefore, it is indispensable to understand the complex network of wheat responses to abiotic stresses for improving wheat yields. Increasing evidence shows that CaMs/CMLs in other plant species are involved in responses to abiotic stresses, including heat, cold, drought, and salinity. In Arabidopsis, it has been shown that AtCaM1, 3, and 4, as well as AtCML9, 18, 24, 37, and 42 were involved in resistance to abiotic stresses. Additionally, OsMSR2, OsCML4, and OsCaM1-1 in rice were related to tolerance to abiotic stresses in plants. In this study, high numbers of cis-acting elements related to plant hormones and stress responses can be observed in the promoters of TaCaM/CMLs (Figure 4; Additional file 7: Table S5), implying the potential roles in plant growth and resistance to abiotic stresses. It also showed that TaCML17, 21, 30, 50, 59, and 75 can be up-regulated significantly in at least one stress treatment (Figure 6), implying wide involvement of TaCaM/CML genes in resistance to abiotic stresses, providing important clues for future function studies of TaCaMs/CMLs in wheat.
Abbreviations

Ca\(^{2+}\): Calcium; [Ca\(^{2+}\)]\(_{cyt}\): Ca\(^{2+}\) concentrations; CaM: Calmodulin; CBK3: CaM-binding protein kinase 3; CBL: Calcineurin B-like; CDPKs/CPKs: Ca\(^{2+}\)-dependent protein kinases; CML: Calmodulin-like; GSNOR: S-nitrosoglutathione reductase; HSF: Heat shock transcription factor; MI: Maximum likelihood; MW: Molecular weight; NO: Nitric oxide; qRT-PCR: qPCR: Isoelectric point; PP7: Protein phosphatase 7; Quantitative real-time PCR; TPM: Transcripts per million.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Authors’ contributions

LYW, YF, and ZH performed the bioinformatics analysis, and generated and analyzed the data. CWY, DFS, WJ, and CJF performed the experiments. SYH, LLB, JWZ contributed to the wheat seedling treatments and samples collection. JB, LMY and LY wrote the manuscript. ZS and LYY designed the study and edited the manuscript. All of the authors read and approved the final manuscript.

Availability of Data and Materials

All data generated or analysed during this study were included in this published article and the additional files.

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