

Genomic profiling and expression analysis of the diacylglycerol kinase gene family in heterologous hexaploid wheat

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Abstract

Background

The inositol phospholipid signaling system, which is based on the metabolism of phosphoinositide (PI), mediates plant growth, development, and responses to adversity. Diacylglycerol kinase (DGK) is one of the key enzymes in the PI-cycle, which catalyzes the phosphorylation of diacylglycerol (DAG) to form phosphatidic acid (PA). To date, comprehensive genomic and functional analyses of DGK genes have not been reported in wheat.

Results

In this study, 20 DGK gene family members from the heterologous hexaploid wheat genome (TaDGKs) were identified and analyzed. Each putative protein was found to consist of a DGK catalytic domain and an accessory domain. The analyses of phylogenetic and gene structure revealed that each TaDGK gene could be grouped to clusters I, II, or III. In each phylogenetic subgroup, the TaDGKs demonstrated high conservation in functional domains, for example gene structure and amino acid sequences. By cloning, four coding sequences were ascertained from Chinese spring wheat. Expression analysis of these four genes revealed that each had a unique spatial and developmental expression pattern, indicating their functional diversification in wheat growth and development processes. Additionally, *TaDGKs* were also prominently up-regulated under salt and drought stresses, suggesting their possible roles in dealing with adversity environment. Further cis-regulatory elements analysis elucidated transcriptional regulation and potential biological functions.

Conclusions

These results provide valuable information for understanding the putative functions of DGK genes in wheat, and conduce to ulterior functional analysis of this pivotal gene family. The 20 TaDGKs identified and analyzed in this study provide a strong foundation for further exploration of the biological function and regulatory mechanisms of *TaDGKs* in response to environmental stimuli.

Background

As the major components of biomembrane and the basis for lipid signaling, lipids are proposed that among the most important biomolecules found in plant tissues. The inositol phospholipid signaling system is based on the metabolism of phosphoinositide (PI), which is synthesized principally through the PI-cycle [PI → phosphatidylinositol 4-bisphosphate (PI4P) → phosphatidylinositol 4,5-bisphosphate (PI (4, 5) P₂) → diacylglycerol (DAG) → phosphatidic acid (PA) → cytidine diphosphate-diacylglycerol (CDP-DAG) → PI] [1–2]. These derivatives and catalytic enzymes play pivotal roles in the production of the lipid

messengers that mediate plant growth, development, and responses to biotic and abiotic cues [3–4]. Steps in the PI-cycle include conversion of DAG to PA catalyzed by diacylglycerol kinase (DGK) [1, 3, 5].

Plant DGKs have been cloned from many plant species, including *Arabidopsis thaliana* [6–7], tomato [8], maize (*Zea mays*) [9], rice (*Oryza sativa*) [10], *Malus prunifolia* [11], tobacco [12], and soybean (*Glycine max*) [13]. Based on their gene architectures, evolutionary relationships, and sequences, plants DGKs have been classified into three distinct phylogenetic clusters [5]. In the genome of *Arabidopsis thaliana*, seven AtDGK genes are present, of which AtDGK1/2 fall into cluster I, AtDGK3/4/7 into cluster II, and AtDGK5/6 into cluster III [5–6]. *AtDGK2/4/7* were expressed in *E. coli*, respectively, and the recombinant proteins demonstrated kinase activity *in vitro* [7, 14–15]. The expression patterns of various *AtDGK* genes reflect their different physiological roles in plant growth, development, and responses to stimuli. *AtDGK1* and *2* were expressed in the roots and leaves and shown to play a role in responding to cold stress [6, 14]. *AtDGK4* is highly expressed in the pollen and appears to regulate pollen tube growth by binding to nitric oxide [15]. In *Arabidopsis*, *AtDGK7* is expressed ubiquitously, especially in flowers and young seedlings [7]. *OsDGK1* is intensively expressed in roots system, and modulates the growth and development of roots, which is associated with lipid mediators that control rice root architecture [16].

Other plant DGK homologs have also been deposited in GenBank, including those in hybrid *Populus* (BU828590), grape (CB981130), apricot (*Prunus armeniaca*; CB821694), and wheat (BT009326) [14]. Wheat (*Triticum aestivum* L.) is an important crop that is limited in terms of both productivity and quality by drought, salinity and low temperatures. Several studies have shown that the phosphoinositide signaling pathway played a pivotal role at variety developmental stages and in abiotic stress-responsive in common wheat [17–18]. As early as 1992, the activity of DGK and phospholipase C (PLC), two key members in phosphoinositide signaling, were demonstrated in highly purified plasma membrane isolates from roots of wheat [19–21]. Additionally, overexpression of the wheat phospholipase D gene TaPLD α has been shown to enhance tolerance to drought and osmotic stress in transgenic *Arabidopsis thaliana* [3]. Previously, we had measured the TaPLC expression patterns at both the transcriptional and protein levels in response to drought and salinity stress, and our research indicated the possible roles of TaPLC1 in mediating seedling growth and responding to drought and salinity stress [22]. However, the molecular functions of lipid messengers remain unclear in wheat. Whole-genome sequencing has provided key insights into new methods for investigating wheat genes. In this study, we conducted a survey and analysis of TaDGK family members in genome-wide of wheat. We also analyzed TaDGKs expression patterns across various tissues, and under drought or salinity stress in order to provide information for the development of hardier varieties of wheat.

Results

Identification and chromosomal distribution of TaDGK family in wheat

To identify the members of TaDGK family, the DGK sequences from other plants, such as *Arabidopsis* and rice, were used to conduct local BLAST against wheat genome databases. Furthermore, the keyword and protein domain searchers were also executed. Ultimately, a total of 20 putative wheat diacylglycerol kinase (TaDGK) genes were identified (Table 1 and S1). All 20 genes were distributed almost evenly along the 14 wheat chromosomes (Chr), with the exception of Chr 4 (Fig. 1). In hexaploid wheat, homologous genes coming from the A, B, and D subgenomes respectively, were deemed as homoalleles of a single ancestral TaDGK gene that arose from a polyploidization event during genome evolution. Every TaDGK gene exhibited its own orthology among three diploid relatives, with the exception of TaDGK7A, which might have been lost throughout the course of asymmetric subgenome evolution. To determine when its homoalleles were lost during evolution, we blasted the genomes of ancestral species of modern wheat, including *Triticum urartu* (with an AA diploid genome), *Aegilops tauschii* (DD diploid genome), and *Triticum dicoccoides* (AABB tetraploid genome). Notably, we found orthologs in the A subgenomes of *Triticum urartu* (AA) and *Triticum dicoccoides* (AABB), which suggests TaDGK7A was lost after the allohexaploidy event.

Taking into account their chromosomal locations, 20 *TaDGK* genes were identified as *TaDGK1A/B/D*, *TaDGK2A/B/D*, *TaDGK3A/B/D*, *TaDGK4A/B/D*, *TaDGK5A/B/D*, *TaDGK6A/B/D*, and *TaDGK7B/D*, respectively (Fig. 1). The ORFs of these genes were 1,467–2,172 bp, encoding polypeptides of 488–723 amino acids, with predicted molecular weights of 54.25–80.33 kD (Table 1). Their theoretical isoelectric point (*P*) values ranged from 5.79 to 9.04 (Table 1).

The nucleotide and amino acid sequences of each gene are shown in supplementary Table S1.

Phylogenetic analysis of TaDGK genes

A phylogenetic analysis was conducted according to the protein sequences of *Arabidopsis*, rice, apple, soybean, and maize. The obtained unrooted phylogenetic tree confirmed that these DGK genes were grouped into clusters I, II, and III. TaDGKs were distributed as follows: TaDGK1A/B/D, TaDGK4A/B/D, and TaDGK5A/B/D were found in cluster I; TaDGK6A/B/D were found in cluster II; TaDGK2A/B/D, TaDGK3A/B/D, and TaDGK7B/D were found in cluster III (Fig. 2). The phylogenetic tree also revealed that the TaDGKs were more aptly classed with DGKs from the monocots rice and maize than those from the dicots *Arabidopsis* and soybean, and TaDGK2/TaDGK7 formed a clear paralogous pair.

Protein domains and sequence characterization of TaDGK genes

By utilizing IBS, a schematic diagram was developed for the protein domains in all TaDGKs (Fig. 3). This diagram demonstrated each TaDGK harbored a diacylglycerol kinase catalytic domain (DGKc) (PF00781) and one accessory domain (DGKa) (PF00609), and demonstrates that the domains of TaDGK have different distributions based on the conservation of the macro protein domains throughout the evolution of all three clusters and. Furthermore, all the TaDGK genes belonged to cluster I contained two C1 domains (PF00130), i.e., the trans-membrane domain and the DAG/phorbol ester (PE)-binding domain.

To make sure that the conserved sequences in TaDGKs, we performed multiple alignment of the domains of TaDGK for cluster I in three phases, the DGKc domain (Fig. 4A) and each of the two C1 domains (Fig. 4B, 4C, respectively). The alignment revealed that all TaDGKs, like AtDGKs, possessed a conserved DGKc domain containing a putative ATP-binding site with a GXGXXG consensus sequence (the red box in Fig. 4A) [23]. As in other studied plants, TaDGKs have the classical generalized structure as seen in other studied plants (Fig.5). Additionally, the two C1 domains harbor the sequences $HX_{14}CX_2CX_{16-22}CX_2CX_4HX_2CX_7C$ and $HX_{18}CX_2CX_{16}CX_2CX_4HX_2CX_{11}C$, respectively (Fig. 5) [11, 13, 14]. By sequence alignment, we found the upstream basic region and extCRD-like domain were substantially conserved, with only slight variation: in the basic region, conserved KA residues were replaced by K in TaDGK1 (A/B/D), and the flanking residue V of extCRD-like was replaced by L in TaDGK4A (Fig. 5).

Structures and protein motifs of TaDGK genes

Structural analysis was performed to obtain some valuable information about duplication events of gene families in the form of phylogenetic relationships. The exon-intron distributions of TaDGK genes were analyzed using Evolview, which showed that genes in the same Cluster were highly similar, especially the ones with closer evolutionary relationships. All of the TaDGKs in clusters II and III had 12 exons, while those in cluster I had 7 exons (Fig. 6A, B). The exons of genes within the same cluster showed extraordinary conservation in order and size. Among genes in the same cluster, not only homologs across different wheat chromosomes, but also rice *DGK* genes had very similar exon-intron structures (Fig. S1). This result showed the orthology of *DGK* genes across different plant species and suggested that *TaDGKs* have undergone gene duplications throughout their evolution.

However, we found the fifth introns of *TaDGK7B* and *TaDGK7D* were much longer than those of the others homologs, with lengths of 14,797 and 8,325 bp, respectively. The sequences of their second introns were blasted against the NCBI protein database. Interestingly, we found that the introns of *TaDGK7B* and *TaDGK7D* contained partial retrotransposon protein sequences (ABF96702.1 and XP_017609491.1, respectively), which suggests the formation of the second intron in TaDGK7 occurred through the insertion of transposons as potential controlling elements [24].

MEME analysis revealed 15 distinct motifs in the TaDGK family (Fig. 6A, C). Three copies of each TaDGK member presented the same motif compositions, and genes belonging to the identical cluster had similar motif compositions. Five motifs—namely 1, 8, 10, 12, and 14—were shared among all TaDGKs. Meanwhile, the motifs 4, 5, 11, and 15 were specific protein motifs to cluster I, and motif 13 was specific to cluster III. The motif 6 existed in clusters II and III. All the sequence logos for these motifs are showed in Fig. S2.

Cis-acting elements in the promoter of *TaDGKs*

Transcription factors regulate the target genes expression by binding to cis-regulatory elements [25]. *TaDGK* promoters, within 1500 bp upstream of the transcription start site, were analyzed to identify the putative cis-regulatory elements, using the PLACE and PlantCARE databases. CAAT-box and TATA-box

elements were overrepresented among all 20 *TaDGK* promoters (Fig. 7). Moreover, we selected some representative components for subsequent investigation of expression. Thus, the cis-acting elements could be classified into several groups according to abiotic stress responsiveness (water, dehydration, and temperature), biotic stress responsiveness (disease and pathogens), responses to plant hormones (ethylene, auxin, abscisic acid [ABA], gibberellic acid [GA], and salicylic acid [SA]), and metabolic processes (GA biosynthesis) (Fig. 7 and Table S2). In addition, almost all *TaDGKs* contain MYBCORE (water stress), MYB1AT (dehydration-responsive), and ASF1MOTIFCAMV (abiotic and biotic stress) elements (Fig. 7), which suggests that *TaDGKs* mediate stress responses in wheat.

Expression profiles of *TaDGK* in various tissues

We executed a microarray-based expression pattern analysis of *TaDGK* genes using public datasets from the wheat gene expression database hosted by the Triticeae Multi-omics Center. All *TaDGK* genes members were determined to have some level of tissue-specific expression, and none were constitutively expressed in all investigated tissues (Fig. 8A and Table S3). *TaDGK6A/B/D*, *TaDGK2A/B/D*, *TaDGK4A/B/D*, and *TaDGK5A/B/D* showed high expression levels in roots. *TaDGK7B/D* showed high expression in spikes. *TaDGK1A/B/D* and *TaDGK3A/B/D* showed high expression in grain. Almost all *TaDGK* genes showed low expression in leaves.

Real-time PCR was also carried out to investigate the expression patterns of some specific *TaDGKs*—namely *TaDGK2A*, *3A*, *4B*, and *5A*—in various organs. According to microarray data, *TaDGK2A* and *3A*, which belong to cluster II, were highly expressed in roots and granules, respectively. *TaDGK4B* and *5A*, which both belong to cluster I, were also highly expressed in roots. Our results showed that *TaDGK2A*, *4B*, and *5A* had strong expression levels in roots, while *TaDGK3A* was mostly highly expressed in stems and spikes. The expression of *TaDGK5* was much lower in most tissues/organs and indeed almost undetectable in tissues other than leaves (Fig. 8B). These results suggest that *TaDGK2/4/5/6* appear to be involved in root growth and development, while *TaDGK1/3/7* may be related to grain development.

***TaDGK* expression patterns under salinity and drought stress**

The promoters of almost all *TaDGKs* were enriched for abiotic stress responsive elements, strongly suggesting the potential functions of *TaDGK* genes in responses to salinity or drought stress. Accordingly, we tested the expression patterns of *TaDGKs* at the transcriptional level and found that expression of *TaDGK* genes was induced under stress. After only 10 min of salt treatment, the mRNA abundance of four tested *TaDGK* genes—*TaDGK2A/3A/4B/5A*—increased rapidly, with about 2-fold higher expression than controls (at 0 h). Three genes—*TaDGK2A/4B/5A*—that were highly expressed in the roots were significantly induced after 12 h, increasing by 25-, 18-, and 22-fold respectively, with subsequent gradual down-regulations of expression (Fig. 9A).

We also performed real-time PCR to obtain insights into expression patterns of *TaDGKs* under drought stress. *TaDGK2A/3A/4B/5A* were all also induced at 30 min, by approximately 2.5- and 4.5-fold, respectively, with the highest expression (8–32-fold increases) observed after 12 h of stress treatment. In

contrast to the expression of *TaDGK* induced by salt treatment, the transcript level of *TaDGk3*, which is higher in leaves, was increased most strongly under drought stress, by a factor of up to 30, while the other *TaDGKs* were relatively less induced (Fig. 9B). The control gene *TaDREB2* encoding a transcription factor, with major roles in dealing with abiotic stresses, has been demonstrated to be induced under drought stress and salt stress [26-27].

Subcellular localization of TaDGKs

Using the online prediction tool WoLF PSORT, subcellular localization of TaDGK expression was predicted. All cluster I TaDGKs have a trans-membrane region and were predicted to be distributed among multiple cellular organelles, though mainly within the nucleus and chloroplast. The cluster II TaDGKs TaDGK6A/B/D were mainly predicted to be localized to the chloroplast and cytoplasm. All TaDGK2/3/7 members were mainly predicted in the nucleus and cytoplasm (Table S4).

We selected TaDGK2A and TaDGK3A proteins for empirically assessing their predicted subcellular localization. Accordingly, TaDGK2A and TaDGK3A proteins fused a N-terminal GFP tag, were expressed in tobacco leaves (Fig. S3). Notably, the confocal microscopy results were consistent with the predicted subcellular localizations (Table S4). TaDGK2A was indeed expressed in the nucleus and cytoplasm. TaDGK3A, however, was mostly expressed in the cytoplasm based on confocal microscopy (Fig. 10), though its accumulation was predicted to be highest in the nucleus (Table S4).

Discussion

DGKs are involved in responses to osmotic stress in plants

Various lipids are related in controlling plant growth, development, and dealing with biotic and abiotic stresses, and their synthesis is modulated by lipid-signaling enzymes [28–29]. The PLC/DGK pathway is one of the most important signaling networks in response to biotic and abiotic stresses [1, 5, 18]. Our previous work revealed the function of TaPLC1 in controlling seedling growth and adapting to drought and salt stress [22], while the present study is focused on the role of DGK genes in wheat.

DGKs are widely distributed in eukaryotes. In silico identification has been used for the functional prediction of DGKs family members in *Arabidopsis*, rice, maize, apple, and soybean [5, 9–11, 13]. Research assessing the response of DGKs to osmotic stress has mainly been performed through analysis of transcriptome and examination of mutants. *AtDGK1* was the first cloned plant DGK cDNA [6]. Both *AtDGK1* and *AtDGK2* genes were induced under low temperature (4 °C) [14, 30]. Later, *AtDGK1* and *AtDGK2* were determined to be cold-responsive genes using Affymetrix GeneChips [30]. In *Arabidopsis*, *AtDGK2*, *DGK3*, and *DGK5* also mediate the response to cold [7, 14, 31]. In line with this observation, *dgk2*, *dgk3*, and *dgk5* revealed improved tolerance and decreased PA synthesis under freezing temperatures [31]. Under optimal and high salinity conditions, double mutants *dgk3 dgk7*, *dgk5 dgk6*, and *dgk1 dgk2* exhibited lower germination rates, lower total respiration rates, use of an alternative respiratory pathway, and lower PA content in response to 24-epibrassinolide (EBL) treatment compared to wild-type plants [32].

In addition, all three ZmDGKs, six of the eight DGKs in the apple genome, and almost all GmDGKs had significantly induced expression under PEG or salt treatments [9–10, 13]. Although the functional identification of plant DGKs is still unthorough, these findings confirmed the role of DGK in osmotic stress, and it was benefit of researching onthe relationship between the paired PLC/DGK pathway and environmental stresses in plant responses [33].

Expansion of the DGK gene family in hexaploid wheat

In this study, we identified *TaDGKs* and analyzed their evolution and expression. We isolated 20 *TaDGKs* in hexaploid wheat (*T. aestivum*) using a genome-wide approach. The number of *DGKs* in wheat is approximately three-fold higher than that in *Arabidopsis* (i.e., seven *DGKs*) [7], rice (eight *DGKs*) [10], and other plant species [9, 11, 13]. This is because the ancestor of allohexaploid bread wheat (*T. aestivum*) underwent three polyploidizations [34]. Accordingly, three groups of genes corresponding to *TaDGKs* in the A, B, and D subgenomes formed clusters with bootstrap values of 100 (Fig. 2). These events, such as Intrachromosomal serial replication and gene-loss, may have occurred during the evolution process, whereas a gene loss event appears to have occurred among *TaDGKs* on wheat chromosome 7A. In addition, we identified the following two paralogous gene pairs among *TaDGKs*: *TaDGK1/4* and *2/7*. This pair corresponds to *AtDGK5/6* and *3/7*, *OsDGK1/2*, *MdDGK4/8*, *2/5*, and *3/7*, and *GmDGK1/3*, *8/9*, *5/6*, *7/10*, and *11/12* of other plant species (Fig. 2) [11]. It should be noted that segmental or tandem duplication events of *DGK* genes led to the expansion of gene families throughout plant genome evolution [35]. However, although *TaDGK1* occurs in a tandem array with *TaDGK5* on chromosome 5, it is more similar to *TaDGK4* in both sequence and structure (Figs. 1, 2 and 6).

DGK family members in mammals are grouped into five subtypes [36], whereas plants DGKs fall into three distinct clusters (I, II, and III). All plant DGK genes within Cluster I, which consist of the most intricate plant DGKs, most closely similar with the DGK genes in Type III, which are the most basic DGKs in mammalian cells [5]. Notably, plant phosphoinositide-dependent phospholipases C (PI-PLC) also closely resembles the most basic PLC ζ isoform of mammals [37]. This shows the difference in the DGK/PLC pathway between plant and animal cells, and it is unknown whether this structural simplification indicates functional simplification.

The results of phylogenetic analysis are further supported by the analyses of sequence and structural features of *TaDGK* genes. *TaDGK* homologs in the same clade, such as wheat and rice, own semblable exon-intron structures (Fig. 6). In addition, the structural organization of *TaDGKs* within clusters I and II comprised seven and twelve exons, respectively, consistent with other plant species, such as rice, apple, and soybean [11, 13, 38]. This conservation of gene structures across species shows the plant DGK family is also conserved in its genomic structure. It should be noted that the *TaDGK7B/D* genes have very long fifth introns. The fifth introns of *TaDGK7B* and *TaDGK7D* were 14,797 and 8,325 bp, respectively, and 10 and 5.5 times the length of each gene's ORF. This pattern has not yet been observed in other plants *DGKs* and might be related to the regulation of *TaDGK7* gene expression.

Expression and functional divergence of *TaDGK* genes

Usually, the gene expression patterns, in various tissues and organs, are detected to analyze corresponding biological functions. Based on the in-silico assessment of RNA-seq experiments and subsequent qRT-PCR confirmation, *TaDGKs* were observed to exhibit specific expression in the root, shoot, leaf, spike, and grain (Fig. 8, Table S3), implying that they may play a role in specific growth and development processes. Although the expression level of each *TaDGK* copy gene among the different chromosomes differed slightly from each other, the trend for each homolog was the same. Accordingly, the roles of *TaDGK2* and *6* in root development and *TaDGK1* in seed development merit further investigation. The expression level of *TaDGK1* was very low across all tissues, but 10 times higher in seeds than in any other organ. *TaDGK2* and *6* had higher transcript levels in all organ, especially in roots. The closest ortholog to *TaDGK2* is *OsDGK1* in rice, which is also highly expressed in roots and affects rice lateral root development and seminal root/ crown root growth [16]. The transcripts of *TaDGK7* were difficult to detect, both among tissues/organs and under hormone/stress treatment conditions, which might be owing to its exceptionally long fifth intron (Fig. 6). In wheat, the expression of all *TaDGKs* in leaves was significantly lower than that in roots. Unlike wheat, all *MdDGKs* in apple showed high expression in stems [11], and almost all *GmDGKs* in soybean showed noteworthy expression levels in leaves and roots, but no significant contrasts in expression levels between leaf and root tissues [13].

Cis-regulatory promoter elements of genes, which play key roles in regulating tissue-specific and stress-responsive expression of genes, can reveal transcriptional regulation and potential functions of *TaDGKs*. In the promoter regions of *TaDGKs*, we found many stress and hormone response elements (Fig. 7). We examined the expression profile of *TaDGKs* under osmotic stress and phytohormone treatments using publicly available microarray datasets. All *TaDGK1A/B/D* and *TaDGK2D* genes exhibited significantly increased transcript levels under 4 °C compared to 23 °C (Table S3). Similarly, *AtDGK1*, *2*, *3*, and *5* are induced by exposure to low temperature and contribute to the cold response in *Arabidopsis* [14, 30–31]. Low, non-freezing temperatures were found to trigger a very rapid PA increase and were primarily generated through DGK in *Arabidopsis* [39–40] suggesting that cold-induced membrane rigidification is upstream of DGK pathway activation. Cold treatment caused an increase in the expression of *ZmDGK2* and *3* in roots and leaves [9]. *TaDGK1* and *AtDGK1/2* belong to cluster I, while *TaDGK2D* is the closest ortholog to maize *ZmDGK2* and *3* in our phylogenetic analysis (Fig. 2). This suggests that sequence homology is related to functional similarity.

Among the several hormones explored through in-silico assessment of RNA-seq experiments, the most significant factor affecting *TaDGK* expression is ABA (Table S3), which is a major hormone that modulates the ability of plants to survive in harsh, changing environments. The transcript levels of *TaDGK2*, *3*, and *6* were severely suppressed by ABA (Table S3), although ABA-responsive elements are located in the promoters of *TaDGK1A/B/D* and *TaDGK4A/B/D* (Fig. 7, Table S2). Physiological analysis showed that PA triggers early signal transduction events that lead to responses to abscisic acid (ABA) during seed germination. In this reaction, it is the lipid phosphate phosphatase (LPP) *AtLPP2*, which catalyzes the conversion of PA to diacylglycerol (DAG), is a negative regulator of ABA signaling [41]. Recent research has shown that ABA can stimulate DGK activity independently of *AtLPP2* activity in *Arabidopsis* [42]. In short, DGK is involved in ABA signaling but the regulatory mechanism is unclear.

Furthermore, we examined the transcript levels of *TaDGK* genes under salt and drought stress treatments. According to RNA-seq data in public databases, *TaDGK2* and *3* were up-regulated under drought conditions, especially *TaDGK3*, which was strongly induced (Table S3). Using qRT-PCR, we examined *TaDGK2A*, *3A*, *4B*, and *5A*, expression patterns under drought simulated by PEG and high salt, specifically. Our results confirmed that *TaDGK3* was most strongly induced by drought. In addition, the transcriptional expression of almost all the tested genes was induced by drought and salt (Fig. 9). Although the expression of all *TaDGKs* in leaves was significantly lower in wheat, the variation in *DGK* expression patterns in response to drought or salt stress reflects its metabolic activities in leaves. Multiple members of the *DGK* family responded to salt or drought in wheat, as occurs in many plant species, such as maize [9], apple [11], and soybean [13]. PA, considered to be a significant second messenger, has been demonstrated to be induced in response to ethylene [43–44], abscisic acid [45], wounding and Nod factors [43], osmotic pressure [46–47], cold [48], salinity [49], temperature changes [39], pathogens [50–51], and drought [11]. Overall, analyses based on data from the expression database supported that *DGK/PA* messengers participate in similar physiological processes in wheat.

Conclusion

A total of 20 *TaDGKs* genes were identified from wheat genome and its close relatives. Based on comparative analyses, we identified putative *TaDGKs* and inferred their phylogenetic relationships, sequence characteristics, cis-regulatory promoter elements, and subcellular localization patterns. From these results, we obtained insights into the putative functions of *TaDGKs*, which has the potential to contribute to their further functional dissection in future research. Expression profiles of *TaDGKs* at a transcriptional level showed each member of this family had specific spatial and developmental expression patterns. In addition, our results indicated that some *TaDGKs* were significantly induced by salinity or drought stress, suggesting their possible function in responses to environmental stimuli.

Methods

Identification and chromosomal location of *TaDGKs*

To study the *TaDGK*-coding genes in common hexaploid wheat, several methods were employed. We first performed a local BLASTN against the wheat genome database by using identified *DGK* sequences from *Arabidopsis* and rice as queries (<http://plants.ensembl.org/index.html>) to find their homologous genes. Additionally, all sequences that were acquired by a search of the gene name “diacylglycerol kinase,” were submitted to WheatExp (<https://wheat.pw.usda.gov/WheatExp/>) to confirm the presence of putative *DGK* genes in wheat. Protein motifs were queried using the NCBI BlastP program (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome), and those that lacked the conserved *DGK* catalytic domain were ignored. Chromosomal localization of genes was obtained from Ensemblplant. The online ExPASy Molecular Biology Server (<http://web.expasy.org/protparam/>) and TMHMMServer2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>) tools were used for sequence analyses.

Multiple sequence alignments and phylogenetic analysis

Multiple sequence alignments of DGK sequences were created using DNAMAN and MEGA7.0 programs with default parameters. Again using MEGA7.0, all DGK sequences acquired from the NCBI protein database, including those from *Arabidopsis*, rice, soybean, apple, maize, and wheat, were aligned to analyze their evolutionary relationships, again using the default parameters, and a phylogenetics tree was produced using the neighbor-joining (NJ) method with 1000 replicates to determine bootstrap support.

Sequence analysis methods

The exon-intron structures and motifs of *DGK* genes were generated online with Evolview. Protein domains analyses were conducted using SMART (<http://smart.embl-heidelberg.de/>) and Pfam (<http://pfam.sanger.ac.uk/>) databases, and IBS software was used to diagram the domain structures.

Promoters (within 1500 bp upstream of the transcription start site) of *DGK* genes were surveyed to identify putative cis-regulatory elements using the Plant Cis-acting Regulatory DNA Elements (PLACE) (<http://www.dna.affrc.go.jp/PLACE/>) and PlantCARE databases (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). The subcellular localization of DGK was predicted using WoLF PSORT (<https://www.genscript.com/wolf-psort.html>).

TaDGK expression profiles based on RNA-Seq data

To investigate the expression profiles of *TaDGKs* among various tissues, organs, and stress treatments, three sets of transcriptome data were obtained from the wheat expression database hosted by the Triticeae Multi-omics Center (<http://202.194.139.32/expression/index.html>).

Plant materials and stress treatments

The wheat seeds (Chinese Spring) were provided by professor Yanyun Pan (Key Laboratory of Hebei Province for Plant Physiology and Molecular Pathology) [22]. Seeds were briefly surface-sterilized with ethanol (70%), then that were immersed in bleach solution (30%) for 10 min. After being washed with sterilized water three times, they were germinated and cultured with a hydroponic system under 16-h days at 24°C in a growth chamber.

For the salinity and drought treatments, 2-week-old wheat seedlings were treated with 200 mM NaCl and 20% PEG6000, respectively. Then, those plants along with control plants were sampled at 0, 1/6, 1, 2, 6, 12, and 24 h post-treatment. Additionally, at different developmental stages, including the two-node stage, various tissues, including roots, stems, spikes, and leaves, were sampled and rapidly frozen in liquid nitrogen prior to storage at -80°C.

RNA extraction and gene expression analysis

Total RNA was extracted using UNIQ-10 Trizol reagent (Sangon Biotech, Shanghai, China). Then, gDNA was extracted, and first-strand cDNA was synthesized with PrimeScript™ RT reagent Kit (TaKaRa,

Dalian, China). In triplicate, quantitative real-time PCR was performed using the Bio-Rad Chromo 4 real-time PCR system (Bio-Rad, Hercules, CA, USA) with SYBR Green PCR master mix (TransGen Biotech, Beijing, China). Three biological replicates with prepared wheat materials were conducted using the specific primers listed in Table S5.

Plasmid vector construction and plant transformation.

To express TaDGK2A-GFP and TaDGK3A-GFP in *Nicotiana benthamiana* driven by the 35S promoter, the *TaDGK2A* and *TaDGK3A* coding sequences without stop codons were cloned into pSUPER1300, a binary vector with a C-terminal fusion with the GFP tag, by cloning it into the *Spe*I/*Kpn*I and *Xba*I/*Kpn*I restriction enzyme sites. DNA sequences for all cloning primers used are listed in Table S5. The *35S:TaDGK2A-GFP* and *35S:TaDGK3A-GFP* vectors were introduced into *N. benthamiana* leaves by *Agrobacterium*-mediated transformation.

Subcellular localization

The *35S:TaDGK2A-GFP* and *35S:TaDGK3A-GFP* vectors were delivered into the epidermis of *N. benthamiana* leaves by *Agrobacterium tumefaciens* (EHA105). Three days after infection, images were captured under fluorescence with a Leica TCS SP8 confocal microscope (Leica, Wetzlar, Germany). To capture GFP fluorescence, the excitation light wavelength was set to 488 nm and detected between 510 and 550 nm.

Abbreviations

DGK: diacylglycerol kinase; DAG: diacylglycerol; PA: phosphatidic acid; PI: phosphatidylinositol; PI-cycle: phosphatidylinositol cycle; PLC: phospholipases C; ORF: open reading frame; aa: amino acid; DREB: dehydration-responsive element-binding protein 2; GFP: green fluorescent protein; qRT-PCR: quantitative real-time PCR.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data generated or analyzed during this study are included with this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YP and KZ conceived the original research project and analyzed the data. XJ, XS and YJ performed the research with the help of YJ and HZ. YJ and ST performed the confocal microscopy. YP and XS wrote the manuscript. All Authors read and approved the manuscript.

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Table

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Figures

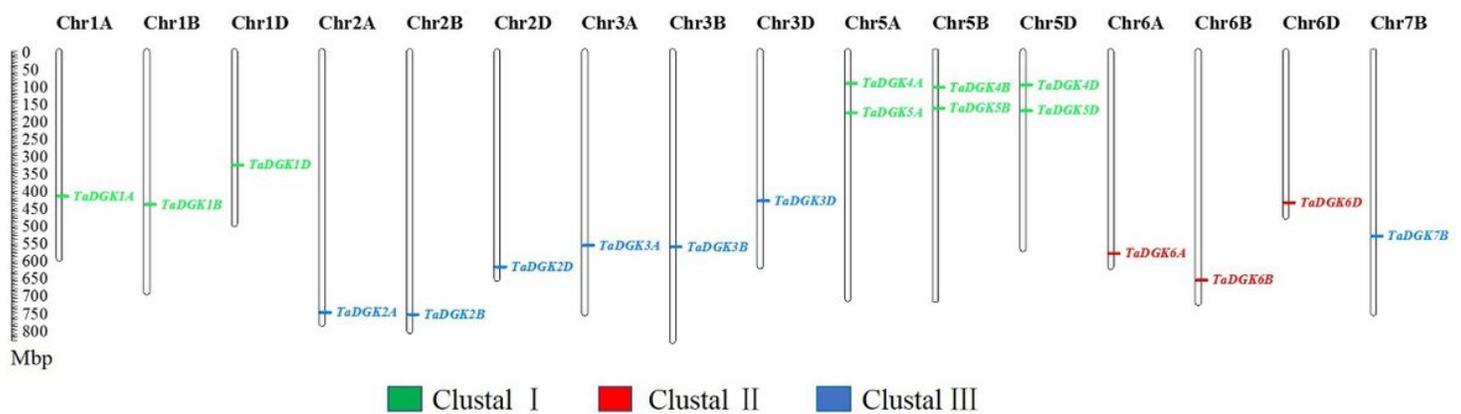


Figure 1

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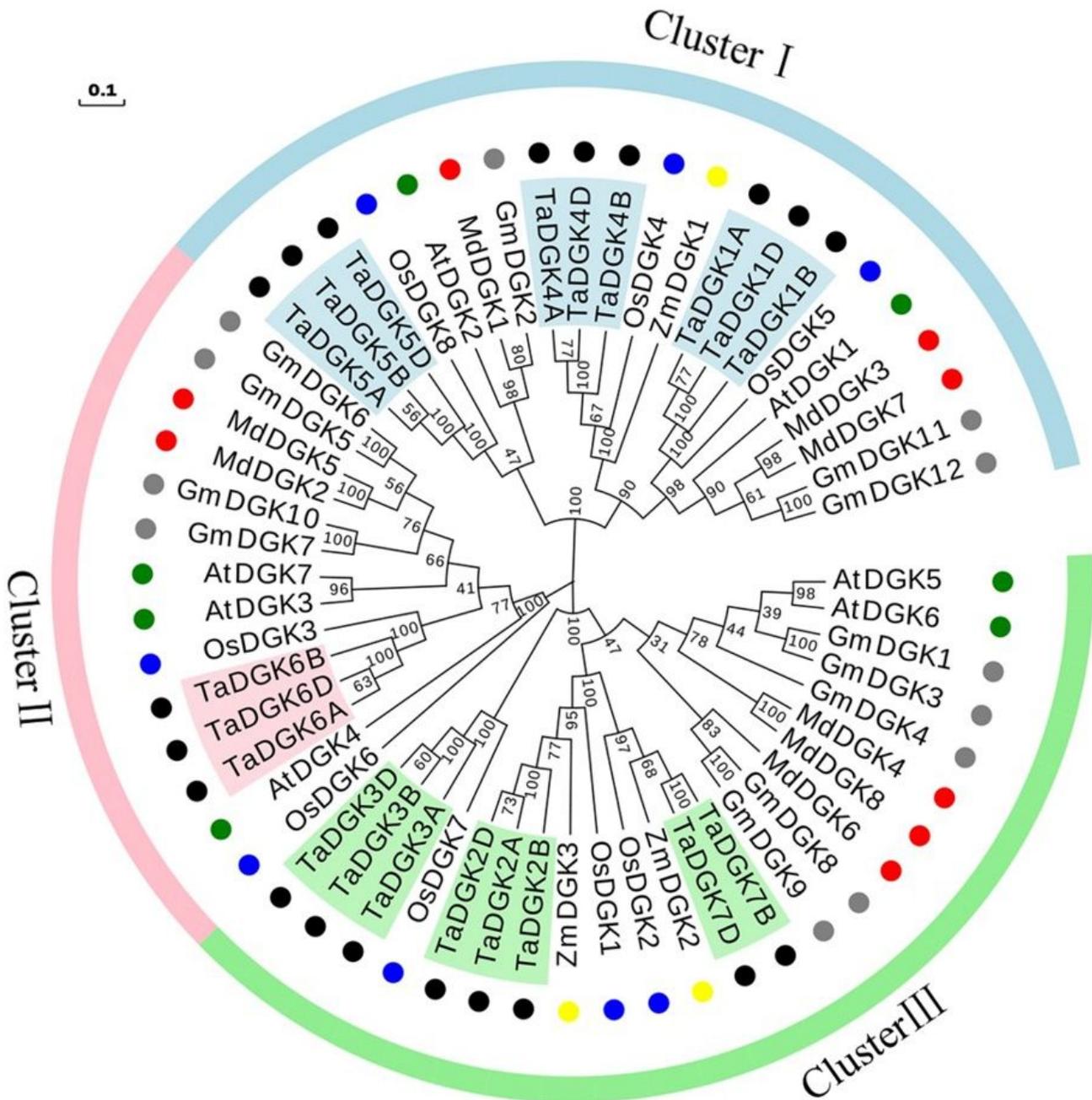


Figure 2

Phylogenetic analyses of DGK genes in *Triticum aestivum* (Ta), *Glycine max* (Gm), *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Zea mays* (Zm), and *Malus domestica* (Md). Clusters are indicated by colors. The following genes are shown with the following corresponding symbols: TaDGKs, black circles; GmDGKs, gray circles; AtDGKs, green circles; OsDGKs, blue circles; ZmDGKs, yellow circles; MdDGKs, red circles.

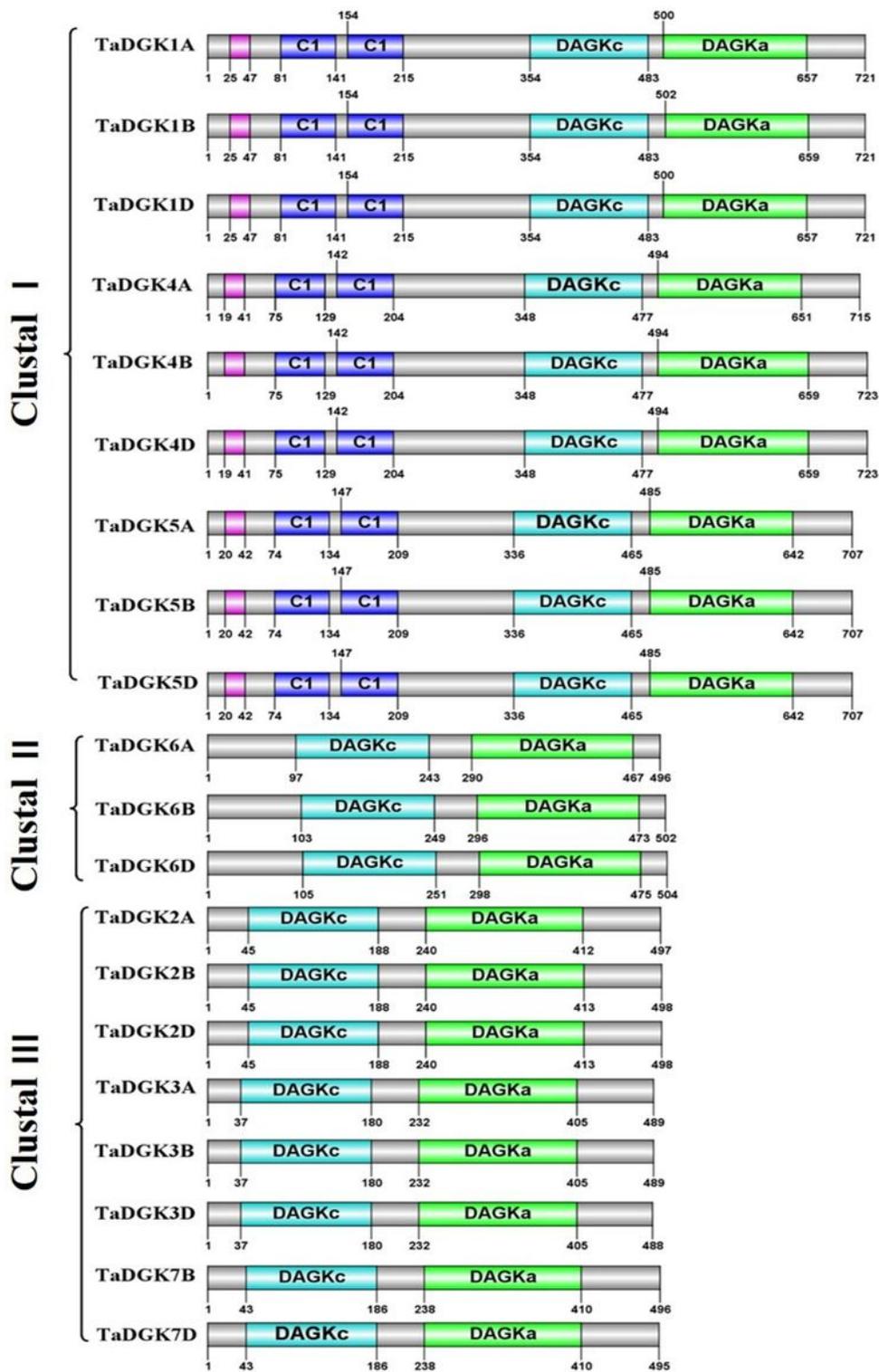


Figure 3

Functional domain analysis of wheat diacylglycerol kinase (DGK) proteins. The numbers shown indicate the position of each amino acid in the protein.

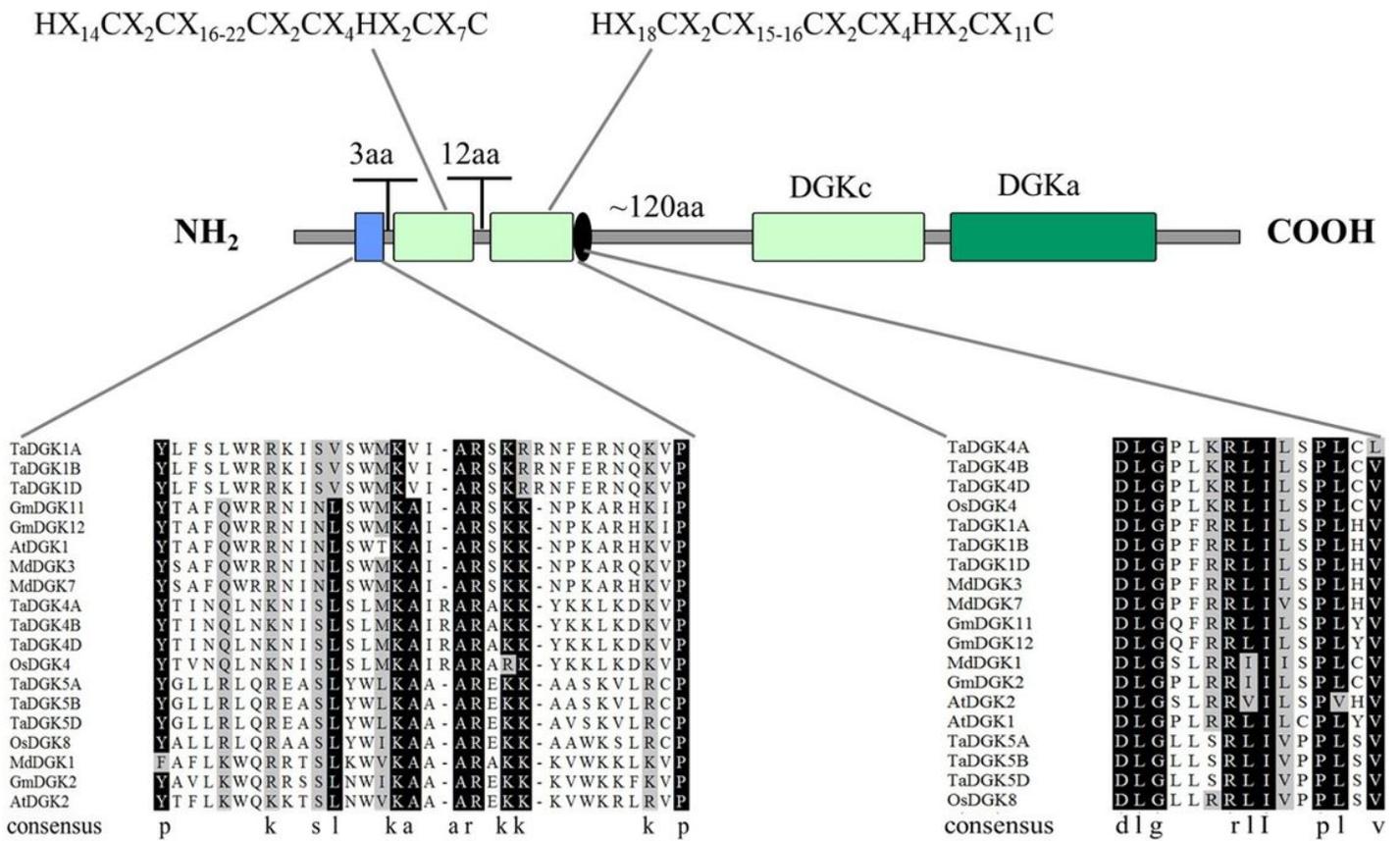


Figure 5

A detailed view of domains of diacylglycerol kinase (DGK) genes in cluster I. The domains of DGKs in cluster I with the predicted location and the sequences of conserved C6/H2 cores; the extended cysteine-rich (extCRD)-like domain and the upstream basic regions are also shown.

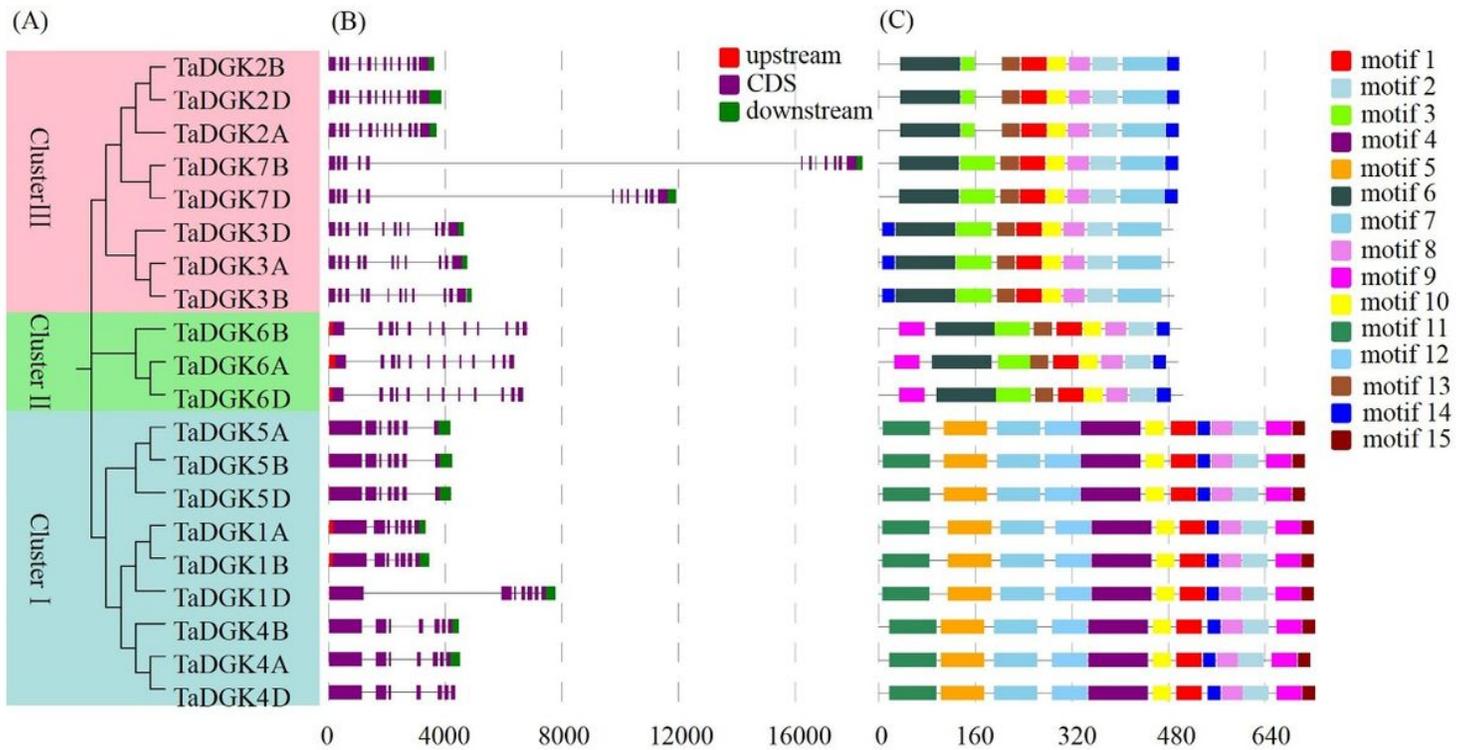


Figure 6

Phylogenetic relationship, gene structure, and motifs of the *Triticum aestivum* diacylglycerol kinases (TaDGKs). (A) Phylogenetic analysis, with different colors indicating genes in individual clusters. (B) Schematic diagram of exon/intron structures of DGK genes in rice and wheat. Red, purple, and green boxes represent upstream regions, CDSs, and downstream regions, respectively. (C). Schematic diagram of the DGK gene motifs. Different colored boxes are assigned to each motif.

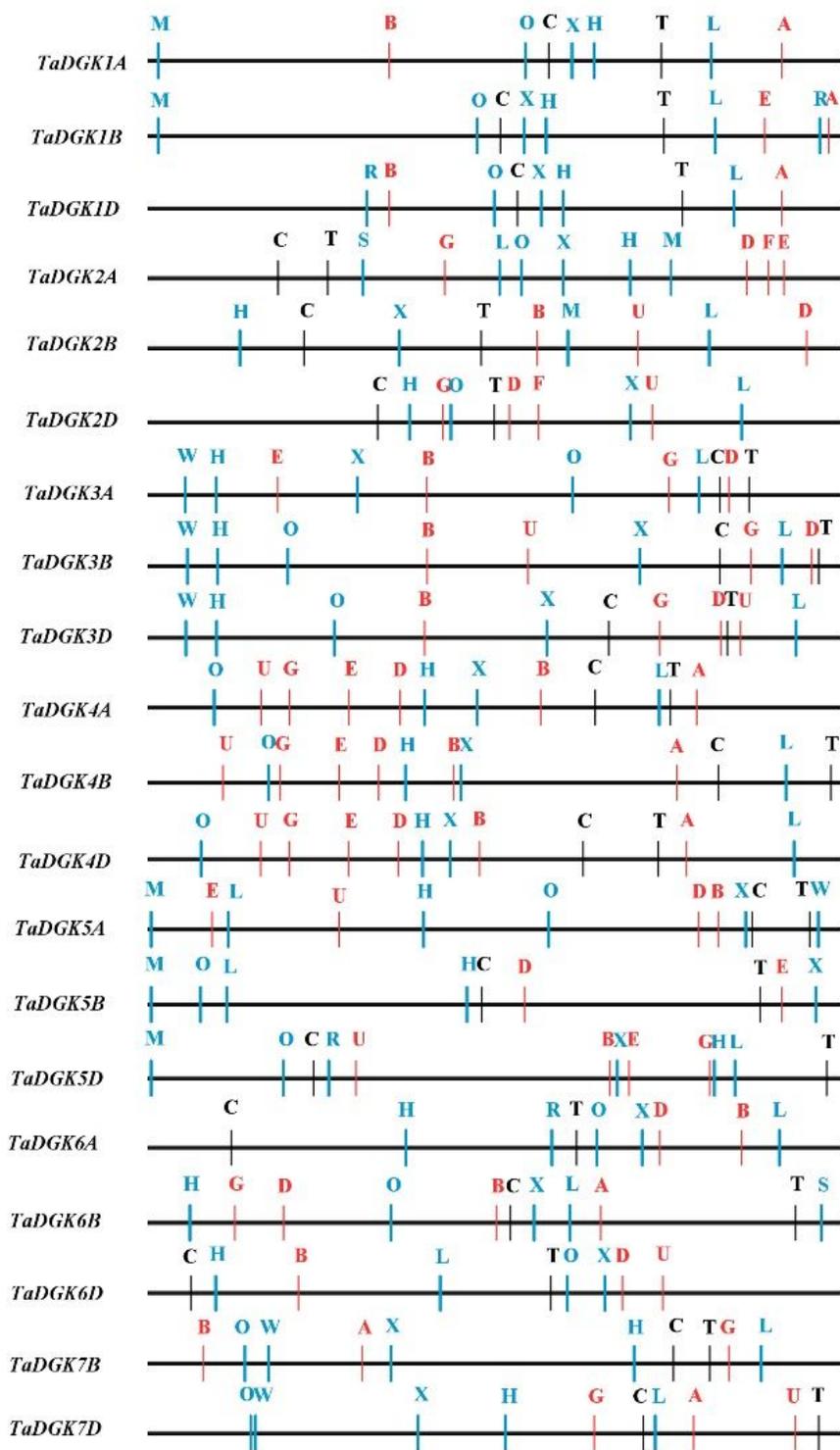


Figure 7

Putative regulatory cis-elements in the diacylglycerol kinase (DGK) gene promoters of wheat. The hormone and stress responsive cis-elements are in red and blue, respectively. The relative positions of elements are labeled with capital letters here and are denoted in Table S4.

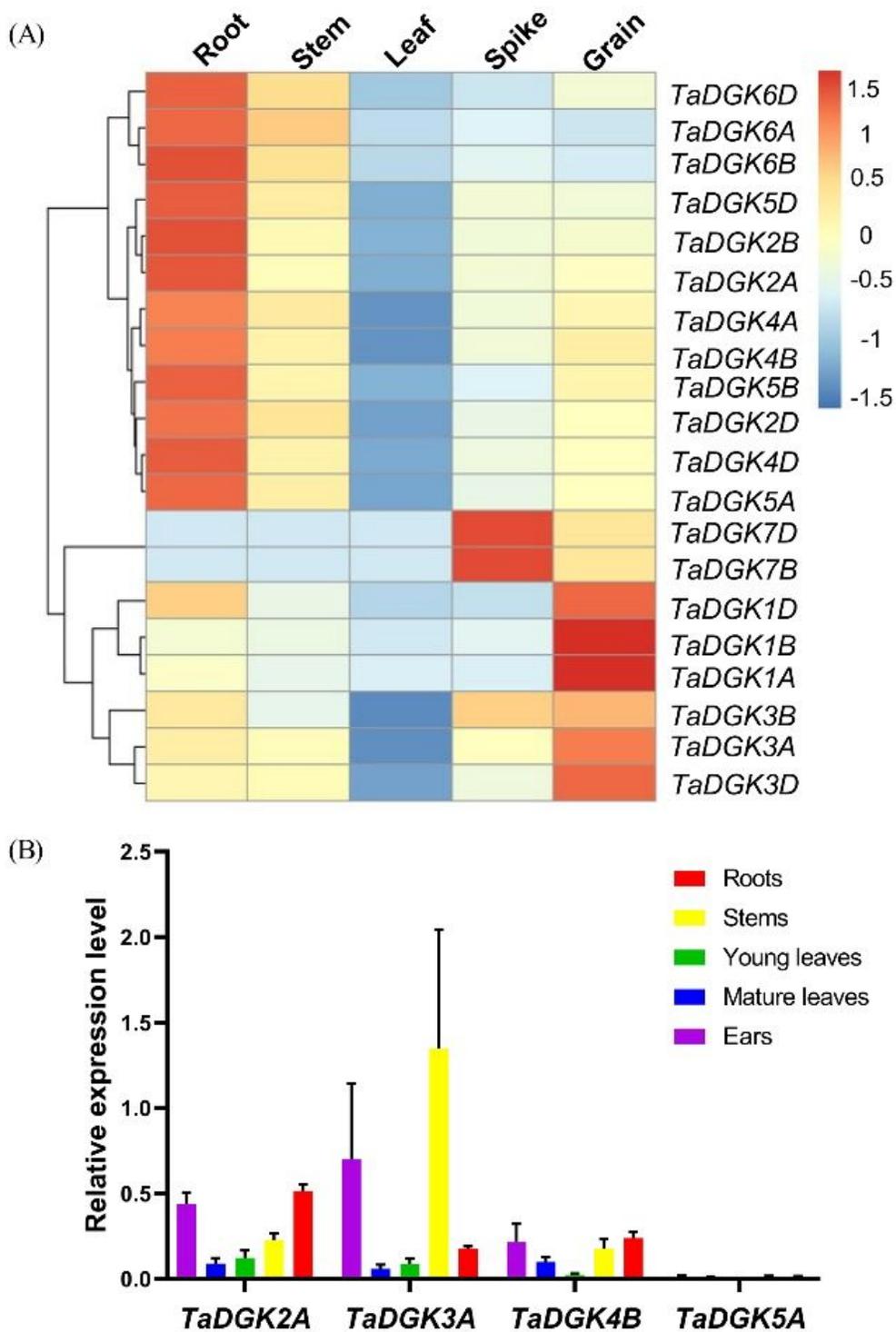


Figure 8

Tissue expression analysis of *Triticum aestivum* diacylglycerol kinase (*TaDGK*) genes. (A). RNA-seq data analysis results. The data for the analysis of gene expression in roots, stems, leaves, spikes, and grains were retrieved from the Triticeae Multi-omics Center. The color scale at the right of the heat map indicates the relative expression levels, where light blue and red indicate low and high expression, respectively. (B). Tissue-specific expression in roots, stems, young leaves, mature leaves, and spikes from wheat. Actin

was used as the reference gene. Mean values were obtained from three replicates. Vertical bars indicate standard deviations. Lowercase letters above each bar indicate the significance level ($\alpha = 0.05$) of each gene in different tissues.

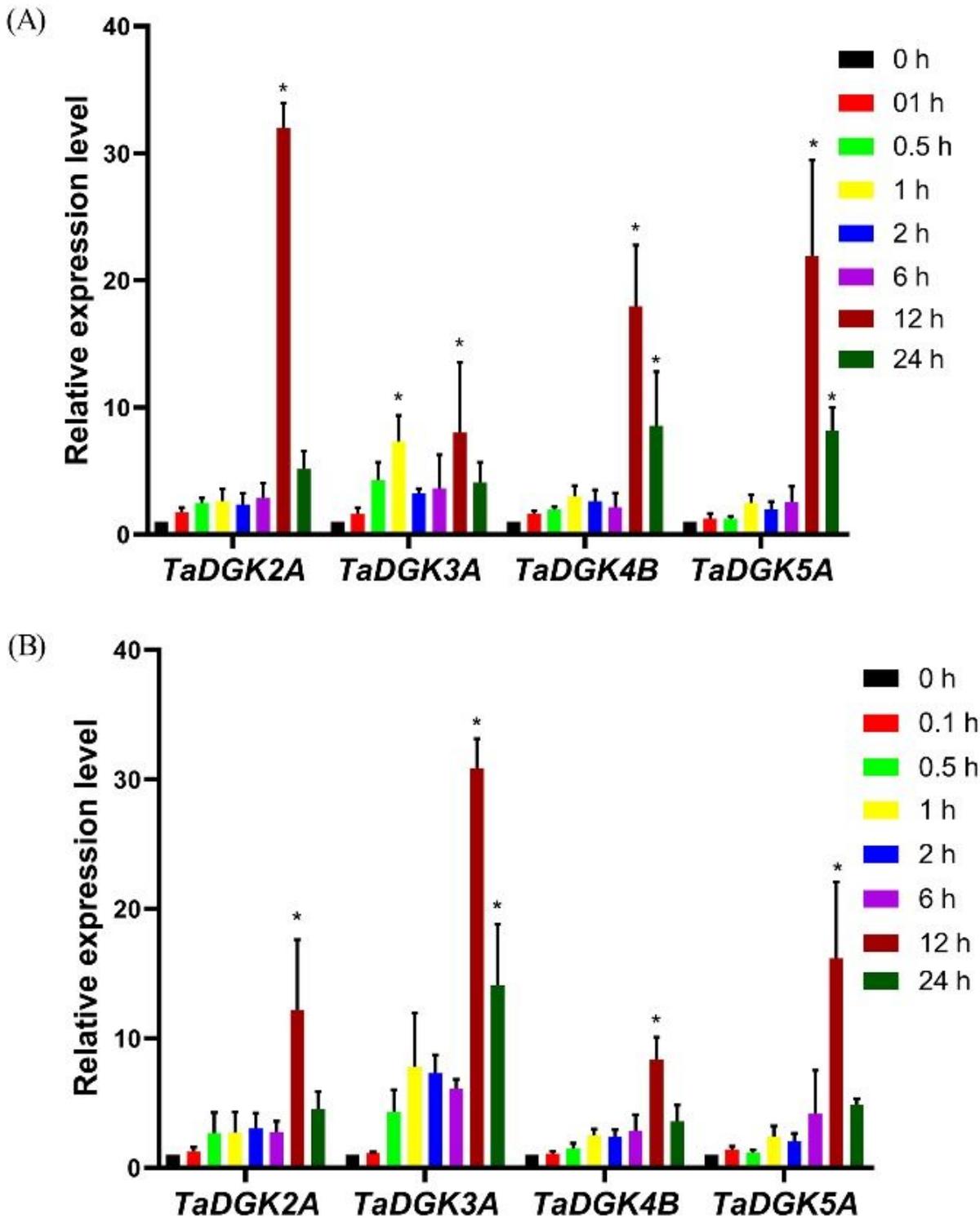


Figure 9

Analysis of *Triticum aestivum* diacylglycerol kinase (TaDGK) gene expression under salt and drought stress conditions. (A). qRT-PCR results of TaDGK under salt stress conditions. Wheat leaves were

sampled after 0, 0.5, 1, 2, 6, 12, 24, and 48 h of treatment with 200 mM NaCl. (B). qRT-PCR results for TaDGK genes under drought conditions. Wheat leaves were sampled after 0, 0.5, 1, 2, 6, 12, 24, and 48 h of treatment with 20% PEG. Actin served as the reference gene. Mean values were obtained from three replicates. Vertical bars indicate standard deviations. Asterisks above error bars indicate significant differences ($\alpha = 0.05$) compared with the control (expression at 0 h).

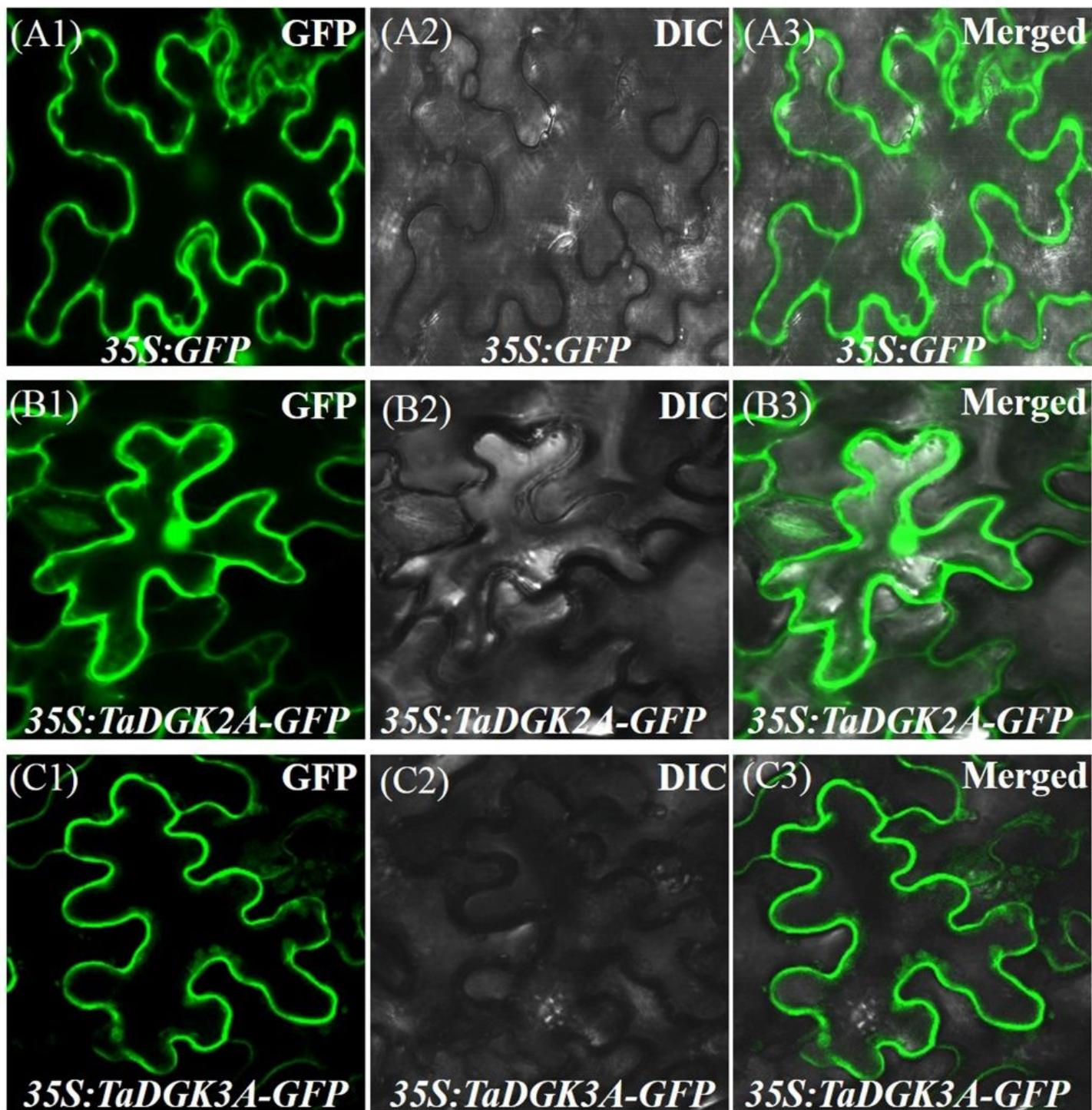


Figure 10

The subcellular localization of TaDGK2A and TaDGK3A proteins in tobacco leaves. (A). GFP under the control of the CaMV35S promoter. (B). TaDGK2A-GFP under the control of the CaMV35S promoter. (C). TaDGK3A-GFP under the control of the CaMV35S promoter. Localization of GFP signals from TaDGK proteins fused with GFP. Bright field, epifluorescence, and merged images of tobacco leaves transfected with constructs are shown expressing different fusion proteins. Scale bars are 50,000 nm in length.

Supplementary Files

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