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Insights into TtCIPK gene family and its roles in durum wheat in response to PEG and ABA treatments

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Drought is one of the significant abiotic stresses seriously affecting plant growth and productivity. In this regard, the identification and utilization of genetic factors improving mechanisms of drought tolerance should be of primary importance. Calcineurin B-like interacting protein kinases (CIPKs) are crucial regulators in calcium signaling pathways, mediating plant responses to abiotic stresses. The present study includes the first comprehensive analysis of the *CIPK* gene family in durum wheat. A total of 58 *TtCIPK*s were identified using bioinformatics prediction and then classified into six evolutionary groups by the phylogenetic analysis. Results from the structural analysis indicated variations in exon–intron organizations; members segregated into high- and low-exon-number subgroups. Predictions of subcellular localization indicated that most TtCIPKs are located in the chloroplast and cytosol. According to qPCR results, *TtCIPK* genes exhibited tissue-specific expression. Besides, it was stated that *TtCIPK* genes are induced in response to drought stress by both ABA-dependent and independent signaling pathways. Further, phosphorylation site predictions and conserved domain analyses showed functional diversity among TtCIPKs, supporting their roles in stress adaptation. These findings are important for understanding the molecular mechanism of drought tolerance in durum wheat and lay the foundation for developing stress-resilient wheat varieties. The findings provide new insights into ABA-mediated and independent pathways in durum wheat's drought response. These insights lay a foundation for leveraging *CIPK* genes in developing drought-tolerant wheat varieties, addressing a critical challenge in sustainable agriculture.

Keywords *CIPK* gene family, Calcium signaling, Drought-responsive gene, Gene Expression

Drought stress is a severe and rising constraint to worldwide agriculture and causes drastic impacts on plant development and physiological processes, and eventually reduces crop yield[1,2]. Stress caused by drought triggers metabolic imbalances, inhibition of photosynthesis, and retardation of plant growth and is a serious threat to global food security[3,4]. One of the most vital food crops in the world, wheat (*Triticum* spp.), is also especially vulnerable to water shortage. The enhanced occurrence and severity of drought associated with climate change also emphasize the importance of developing the molecular mechanisms of drought tolerance in wheat species[5,6].

Protein kinases are pivotal in plant stress signaling and are responsible for perceiving and transmitting environmental stimuli. Of importance among them is the Sucrose Non-Fermenting 1-related Kinase (SnRK) family and a subset of it—SnRK3 and the Calcineurin B-like protein-interacting Protein Kinases (CIPKs), also commonly referred to as the SnRK3 subfamily[7–9]. CIPKs and Calcineurin B-like proteins (CBLs) serve as calcium decoders of calcium fluctuations to direct targeted physiological responses[10,11]. These responses are the regulation of ion homeostasis, osmotic adjustment, stomatal movement, antioxidant defense, and activation of stress-responsive genes[12–14]. Because of their multifunctional capacities at transcriptional and post-translational levels, CIPKs are considered strategic targets to enhance crop drought tolerance[15,16].

Several studies have shown the functional significance of specific *CIPK* genes in crops. *OsCIPK23* and *AtCIPK23* play a fundamental role in potassium uptake and drought tolerance[17,18]; StCIPK18 promotes water holding and antioxidant activity in potato; and *TaCIPK2* enhances drought tolerance in Arabidopsis[19,20]. These are involved in ABA-mediated signaling, ROS detoxification, and regulation of stress-related gene expression. CIPKs share standard structural features such as an N-terminal kinase domain and a C-terminal regulatory

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domain with the NAF/FISL motif, a requirement for CBL binding and formation of a complex[21–24]. In addition, specific CIPKs function independently of CBLs as evidenced by CIPK6 from tomato, which regulates ROS production through alternative protein interactions[25].

In light of this progress in model plants and advanced crops, the functions and regulation mechanisms of the *CIPK* gene family in durum wheat (*Triticum turgidum* ssp. *durum*) are poorly understood. The lack of such information restrains the application of molecular breeding approaches to promote drought tolerance in the crop, which is of considerable importance to the economy and nutrition. Durum wheat is a good source of dietary fiber, vitamins (B, E), phenolic acids, carotenoids, and other bioactive molecules, and is predominantly utilized in the production of pasta and semolina[26–29]. Hence, deciphering the expression and regulatory patterns of *CIPKs* under drought and ABA stimuli in durum wheat has enormous implications in developing water-stress-compatible genotypes.

Here, we systemically identified and analyzed the *CIPK* gene family in durum wheat genomes. We compared their structural properties, expression patterns in response to drought and ABA treatment, and putative functions in ABA-dependent and ABA-independent pathways. By closing this knowledge gap, our research contributes to a better understanding of drought tolerance mechanisms and offers valuable targets for breeding climate-resilient durum wheat varieties.

Materials and methods

Identification of *CIPK* gene family members in *T. turgidum* genome

The *CIPK* genes of durum wheat (designated as *TtCIPK*) were investigated using the *Triticum turgidum* genome available at the Plant Ensembl database (https://plants.ensembl.org/Triticum_turgidum/Info/Index)[30]. To perform a homology search in the durum wheat genome, CIPK protein sequences from Arabidopsis and rice were used as reference queries in BLASTp tool with the e-value set to 1×10^{-5}. In CIPK protein sequences, the presence of protein kinase and NAF domains was confirmed by checking with the Conserved Domains Database (CDD)[31] and Pfam[32]. The physicochemical properties of TtCIPKs protein sequences, including molecular weight (MW), theoretical isoelectric point (pI), instability index (II), and grand average of hydropathicity (GRAVY) were assessed using the ProtParam prediction tool in the Expasy database (https,//web.expasy.org/protparam/)[33]. This research identified precise gene locations on chromosomes. It also mapped out exon–intron structure and conserved motifs, utilizing TBtools software (v0.6735)[34]. In addition, the prediction of TtCIPKs subcellular localization was conducted through the WoLF PSORT online tool (https://wolfpsort.hgc.jp/). Visualization of the *TtCIPK* gene properties was achieved through a graph created in GraphPad Prism 9.0.

TtCIPKs phylogenetic analysis

The amino acid sequences of CIPK in durum wheat, Arabidopsis, and rice (*Oryza sativa*) were obtained from the EnsemblPlants and NCBI databases. These sequences were then aligned by ClustalW[35]. The phylogenetic analysis, which studies the relationships among these plants, was carried out using the maximum likelihood (ML) method. This method was run 1000 times for bootstrap testing and was executed with the standard settings on the IQTREE web server[36]. The outcome file was transferred to the iTOL online tool to visualize the phylogeny tree[37]. The grouping of members in the phylogenetic tree was determined based on the location of clusters and the genetic distance between clusters.

Analysis of *TtCIPK*s duplication events

The duplicated *TtCIPK* genes were identified by comparing the coding sequences of pairs of *TtCIPK* genes and selecting those with an identity of 0.85 or higher. The Ka/Ks calculator in TBtools software was used to calculate the non-synonymous (Ka) and synonymous (Ks) values, which measure the purity selection pressure on duplicated genes[38]. To investigate the gene's conservation level, the Ka/Ks ratio was calculated[39]. Similar results regarding the presence of tandem and segmental duplications were obtained by gene duplication location analysis[40]. The approximate times of duplication events (in Mya) were estimated using the formula $T = Ks/2\lambda$; λ is equal to 6.5×10^{-9} substitutions per synonymous site per year and describes the average rate of synonymous substitution in durum wheat[41].

Promoter analysis of *TtCIPK* genes

The Plants Ensembl database was utilized to extract the 1500 bp promoter sequences preceding the ATG start codon for all *TtCIPK* genes. The analysis of *cis*-elements related to stresses, light, growth, and hormone responses was carried out through PlantCARE (http://bioinformatics.psb.-ugent.be/webtools/plantcare/html/)[42].

Post-translational phosphorylation site prediction in TtCIPK proteins

The reversible post-translation modification of phosphorylation, a significant in vivo event in the signaling pathway related to plant stress responses, was predicted using the NetPhos 3.1 site with a threshold probability of 0.8[43].

3D structure prediction and possible binding site identification in TtCIPK proteins

The 3D structure of TtCIPK proteins was predicted using the Phyre2 database[44]. This procedure employs a homology modeling methodology that compares the protein sequence to known structures, generating an optimal 3D conformation. The Phyre investigator tool from the Phyre2 server was used to predict the locations of pocket sites as ligand-binding regions in the three-dimensional structure of TtCIPK proteins.

RNA-seq data analysis

In the present study, the available RNA-seq datasets of durum wheat were investigated to illustrate the expression profile of *TtCIPK* genes. The raw data, including PRJNA780180 datasets, relating to increased temperature (+2 °C) stress, and PRJNA1089221 datasets related to 5 °C temperature[45], as cold stress, were investigated. Initially, low-quality regions and adapter sequences were recognized and removed using Trimmomatic, and then RNA-seq data were aligned using HISAT and HTSeq-count[46] with the reference genome of durum wheat, Svevo.v1. To show the expression profile, the fold change of *TtCIPKs* was calculated by the NOISeq package[47].

Durum wheat growth conditions

After surface sterilization, durum wheat seeds of the Dena cultivar were planted hydroponically and maintained at a temperature of 25 ± 3 °C, with relative humidity at 35 ± 5% and a 12-h light/dark photoperiod. At 10 days old, the seedlings were fed a half-strength Hoagland solution.

ABA treatment and drought stress

For drought treatment, 45-day-old durum wheat plants were exposed to 15% (w/v) polyethylene glycol (PEG6000)[48,49]. The pre-treatment with ABA occurred three days before drought stress, administered by external spraying of 100 µM ABA[50,51]. All treatments were conducted in a greenhouse with three biological replicates. Samples from both shoot and root tissues were collected at 0 (control), 1 (D1), 24 (D24), and 72 h (D72) after the drought treatment, frozen in liquid nitrogen, and stored in a − 70 °C freezer.

Isolation of RNA and production of cDNA

Total RNA was isolated from shoot and root tissues using the Sambio™ RNA Extraction Kit. Complementary DNA (cDNA) was synthesized according to the provided protocol of the Sambio™ cDNA Synthesis Kit, utilizing the supplied components for converting total RNA or mRNA into single-stranded cDNA.

Designing primers for the selected *TtCIPK* genes

Based on evolutionary analysis, a selection of six *TtCIPK* genes, which include *TtCIPK33*, *TtCIPK42*, *TtCIPK12*, *TtCIPK43*, *TtCIPK27*, and *TtCIPK29*, were investigated for gene expression. Suitable primers for the selected genes were designed (Supplementary Table 1) using the CDS sequences with the help of the online software Primer3Plus (http://www.bioinformatics.nl/primer3plus)[52,53]. Subsequently, the specificity of the primers was verified using Primer-BLAST in NCBI.

Real-time PCR analysis

The Sambio™ qPCR Master (EVA) with ROX was employed to assess relative expression levels according to the manufacturer's instructions. The length of the fragments amplified by the specific primers ranges from 80 to 250 base pairs. The amplification conditions for PCR were 95 °C for 5 min, followed by 25–40 cycles at 95 °C for 15 s, 63 °C for 20 s, and 72 °C for 35 s. In this analysis, relative expression levels of each target gene were determined using the $2^{-\Delta\Delta Ct}$ method and normalized to the housekeeping gene Actin.

Results

Characterization of TtCIPK family members

In the first phase, the homology search using Arabidopsis and rice sequences revealed 58 potential CIPK proteins in durum wheat. Subsequently, the presence of NAF and kinase domains in all members of the TtCIPK family was confirmed by Pfam and NCBI-CDD databases. Their nomenclature was updated to represent their chromosomal locations, from TtCIPK1 to TtCIPK58 (Table 1). Based on physicochemical properties, all the TtCIPK proteins ranged between 1035 to 1608 base pairs in CDS sequence length. The number of exons in *TtCIPK* genes varied from 1 to 15. TtCIPK10, the smallest protein, has 344 amino acids and a molecular weight of 37.30 kDa, while TtCIPK26, the largest protein, has 535 amino acids and weighs 58.69 kDa. GRAVY values ranged from -0.545 to -0.078, indicating hydrophilicity. Isoelectric points (pI) ranged from 5.95 for TtCIPK1 to 9.51 for TtCIPK28 and TtCIPK48 (Table 1).

Phylogenetic analysis of *TtCIPK* gene family

Phylogenetic analysis using the maximum likelihood method, based on protein sequences from durum wheat, rice, and Arabidopsis, clustered the 58 TtCIPK proteins into six evolutionary groups (Fig. 1). The TtCIPK proteins were unevenly distributed across these six groups. Group 1 comprised six proteins: TtCIPK14, TtCIPK9, TtCIPK33, TtCIPK41, TtCIPK40, and TtCIPK35. Group 2 consisted of eight TtCIPK proteins (including TtCIPK47, TtCIPK42, TtCIPK18, TtCIPK13, TtCIPK57, TtCIPK55, TtCIPK30, and TtCIPK21), and Group 3 consisted of seven TtCIPK proteins (including TtCIPK53, TtCIPK17, TtCIPK12, TtCIPK6, TtCIPK2, TtCIPK5, and TtCIPK1). Group 4 consisted of ten TtCIPK proteins (including TtCIPK48, TtCIPK43, TtCIPK32, TtCIPK25, TtCIPK50, TtCIPK46, TtCIPK16, TtCIPK10, TtCIPK26, and TtCIPK19), and Group 5 consisted of six TtCIPK proteins (including TtCIPK56, TtCIPK54, TtCIPK8, TtCIPK4, TtCIPK29, and TtCIPK23). Group 6 stood out as the biggest, housing the other 21 proteins. This hints that group 6 members may have followed a different evolutionary path or have special functional traits; CIPK proteins from rice and Arabidopsis were spread across all groups. This points to a complicated evolutionary background and wide-ranging functional roles.

The phylogenetic groups of TtCIPKs were compared based on their physicochemical characteristics. The exon number in groups 1, 2, and 3 falls between 12 and 15, with 14 exons being the most common. This points to structural likeness and, as a result, more functional similarity among these three phylogenetic groups. Groups 4, 5, and 6 have one exon, with some cases showing two. This suggests that these groups are related in evolution

Gene ID	Gene name	Exon number	Protein(aa)	MW(KD)	GRAVY	PI
TRITD1Av1G028970	*TtCIPK1*	13	494	55.15	−0.313	5.95
TRITD1Av1G029130	*TtCIPK2*	12	471	52.05	−0.263	7.68
TRITD1Av1G182120	*TtCIPK3*	1	472	53.61	−0.488	9.35
TRITD1Av1G194670	*TtCIPK4*	1	521	57.99	−0.429	8.39
TRITD1Bv1G038160	*TtCIPK5*	14	482	54.05	−0.343	6.39
TRITD1Bv1G038220	*TtCIPK6*	12	466	51.68	−0.244	6.63
TRITD1Bv1G169190	*TtCIPK7*	1	472	53.80	−0.483	9.4
TRITD1Bv1G185360	*TtCIPK8*	1	519	57.91	−0.423	8.39
TRITD2Av1G025290	*TtCIPK9*	14	447	50.44	−0.371	8.69
TRITD2Av1G028700	*TtCIPK10*	2	344	37.30	−0.117	6.14
TRITD2Av1G028770	*TtCIPK11*	1	452	51.06	−0.396	9.07
TRITD2Av1G044340	*TtCIPK12*	13	451	51.02	−0.412	7.13
TRITD2Av1G137450	*TtCIPK13*	14	461	51.74	−0.429	9.15
TRITD2Bv1G032930	*TtCIPK14*	14	447	50.46	−0.392	8.7
TRITD2Bv1G037460	*TtCIPK15*	1	452	51.01	−0.391	9.13
TRITD2Bv1G037620	*TtCIPK16*	1	436	47.36	−0.171	6.48
TRITD2Bv1G058000	*TtCIPK17*	13	451	51.02	−0.415	7.15
TRITD2Bv1G122190	*TtCIPK18*	14	462	51.80	−0.429	9.15
TRITD3Av1G052120	*TtCIPK19*	1	522	57.21	−0.316	8.11
TRITD3Av1G052250	*TtCIPK20*	1	466	52.44	−0.445	9.25
TRITD3Av1G121900	*TtCIPK21*	14	449	50.51	−0.33	7.67
TRITD3Av1G191580	*TtCIPK22*	2	477	53.34	−0.364	9.46
TRITD3Av1G191600	*TtCIPK23*	1	521	58.02	−0.293	8.65
TRITD3Av1G212750	*TtCIPK24*	1	507	57.58	−0.545	8.75
TRITD3Av1G251490	*TtCIPK25*	1	432	47.70	−0.204	9.13
TRITD3Bv1G056130	*TtCIPK26*	1	535	58.64	−0.317	8.85
TRITD3Bv1G063790	*TtCIPK27*	1	464	52.24	−0.476	9.34
TRITD3Bv1G174030	*TtCIPK28*	2	470	52.28	−0.337	9.51
TRITD3Bv1G174140	*TtCIPK29*	2	530	59.15	−0.279	8.87
TRITD3Bv1G190960	*TtCIPK30*	14	449	50.55	−0.337	6.59
TRITD3Bv1G195320	*TtCIPK31*	1	507	57.47	−0.541	8.75
TRITD3Bv1G244100	*TtCIPK32*	1	379	42.08	−0.21	8.21
TRITD4Av1G064230	*TtCIPK33*	14	451	51.16	−0.369	8.27
TRITD4Av1G072180	*TtCIPK34*	2	441	50.34	−0.347	9.15
TRITD4Av1G146770	*TtCIPK35*	14	456	52.09	−0.39	8.07
TRITD4Av1G148390	*TtCIPK36*	2	435	47.48	−0.187	8.94
TRITD4Av1G152750	*TtCIPK37*	1	449	51.04	−0.416	9.19
TRITD4Bv1G046280	*TtCIPK38*	2	435	47.52	−0.181	9.08
TRITD4Bv1G049240	*TtCIPK39*	1	444	50.28	−0.429	9.03
TRITD4Bv1G057520	*TtCIPK40*	14	456	52.08	−0.381	7.64
TRITD4Bv1G111600	*TtCIPK41*	14	451	51.17	−0.365	8.58
TRITD4Bv1G182520	*TtCIPK42*	14	422	47.65	−0.233	6.14
TRITD5Av1G026500	*TtCIPK43*	1	433	47.62	−0.187	9.19
TRITD5Av1G112430	*TtCIPK44*	1	438	49.16	−0.378	9.29
TRITD5Av1G143220	*TtCIPK45*	1	446	47.92	−0.142	9.18
TRITD5Av1G200030	*TtCIPK46*	1	431	47.17	−0.078	8.66
TRITD5Av1G237590	*TtCIPK47*	15	492	55.34	−0.308	6.77
TRITD5Bv1G024270	*TtCIPK48*	1	432	47.42	−0.185	9.51
TRITD5Bv1G133150	*TtCIPK49*	1	447	48.06	−0.153	9.08
TRITD5Bv1G194940	*TtCIPK50*	1	431	47.13	−0.08	8.82
TRITD6Av1G045160	*TtCIPK51*	1	481	54.57	−0.518	9.12
TRITD6Bv1G058210	*TtCIPK52*	1	491	55.74	−0.486	9.22
TRITD6Bv1G066770	*TtCIPK53*	14	439	48.84	−0.181	8.17
TRITD7Av1G189080	*TtCIPK54*	2	456	50.80	−0.311	8.61
TRITD7Av1G204030	*TtCIPK55*	13	421	47.19	−0.238	8.97
TRITD7Bv1G155170	*TtCIPK56*	2	455	50.81	−0.312	8.95

Continued

Gene ID	Gene name	Exon number	Protein(aa)	MW(KD)	GRAVY	PI
TRITD7Bv1G157470	*TtCIPK57*	14	446	49.95	−0.18	8.62
TRITD0Uv1G054740	*TtCIPK58*	2	402	45.70	−0.336	9.13

Table 1. Physicochemical features of TtCIPK proteins. More details are illustrated in Supplementary Table 2.

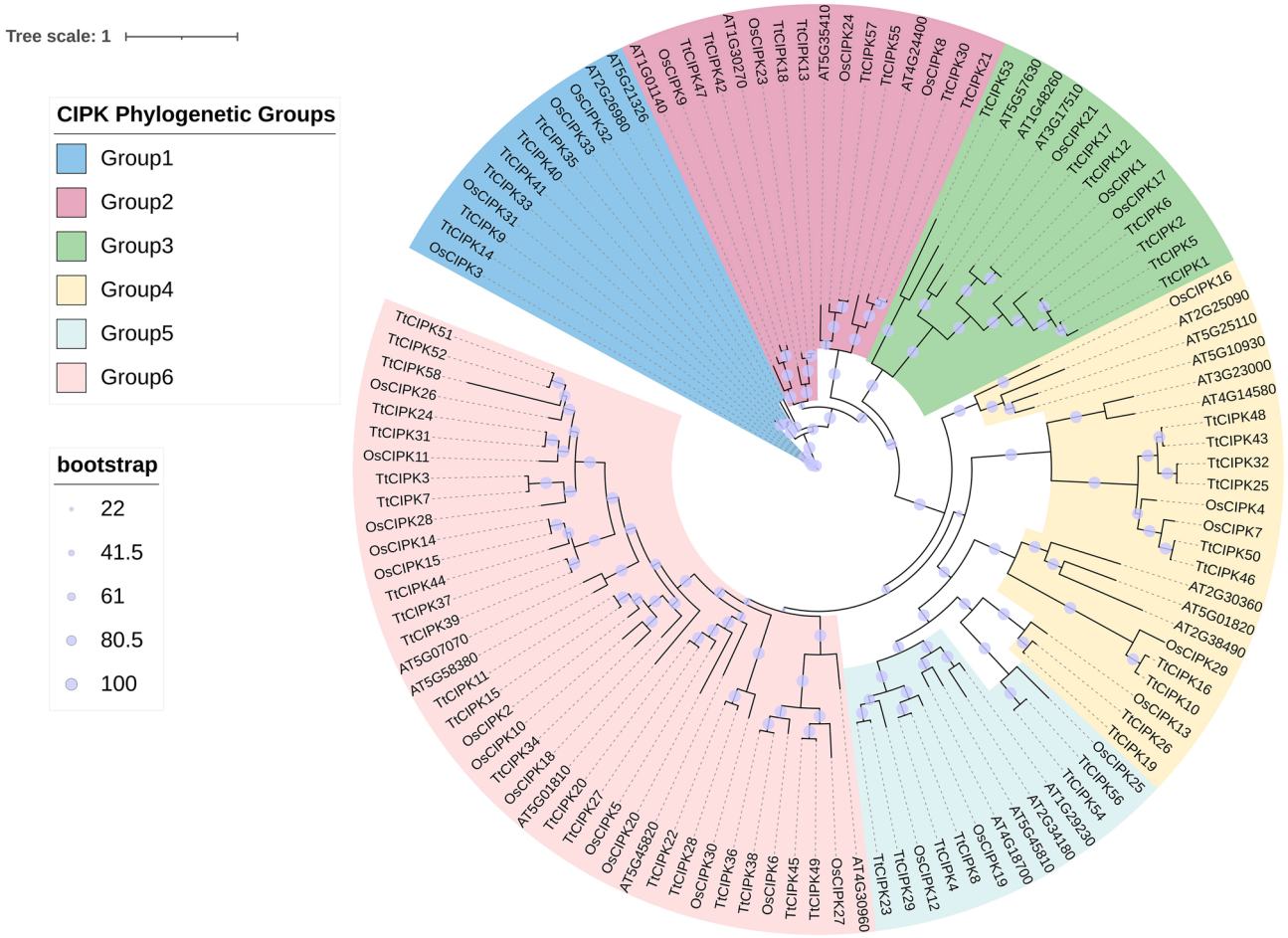

Fig. 1. Phylogeny tree of CIPK gene family in durum wheat (TtCIPK), Arabidopsis (started with AT), rice (started with Os).

(Fig. 2a). Based on these results, the members of this gene family can be divided into two groups: low exon and high exon number. The isoelectric point (pI) of proteins in evolutionary groups 2 and 4 covers a broader range and hovers around 9. Groups 5 and 6 have a more limited isoelectric point close to 9 (Fig. 2b). The GRAVY index is negative for all groups, which means they are hydrophilic. Except for group 1, where members mostly share a similar GRAVY index, the other groups display a diverse range of GRAVY values (Fig. 2c). The stability index was measured across different groups, revealing that all members of group 5 are unstable, while all members of group 3 are stable. In the other groups, both stable and unstable members are present. Groups 1, 2, and 6 have more stable than unstable members, while group 4 has more unstable members (Fig. 2d).

Structure analysis of TtCIPK family members

All proteins within this gene family possess a conserved kinase domain, which is directly related to their function. It is located in a nearly identical position in all members, shown in blue in Fig. 3a. The NAF domain is a crucial conserved domain in TtCIPKs, playing a regulatory role in the function of these proteins, and is found in a relatively similar position in all proteins, depicted in pink in Fig. 3a. According to gene structure analysis, the genes *TtCIPK21* and *TtCIPK30* have long introns, which may be associated with functional diversity, gene expression regulation, or their role in evolution (Fig. 3b).

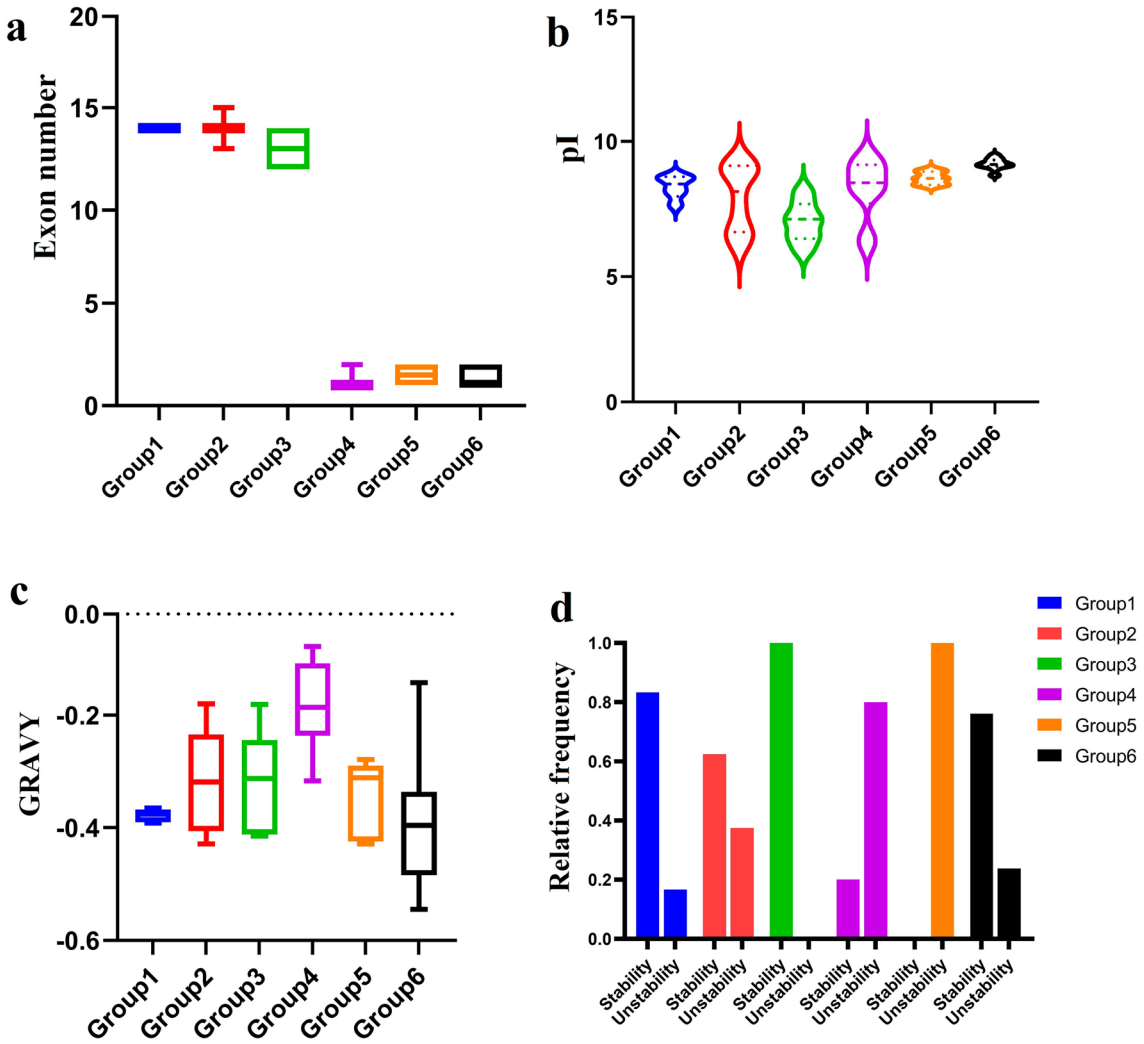

Fig. 2. Physiochemical properties of phylogeny tree groups of TtCIPK family in terms of exon number (**a**), pI values (**b**), GRAVY index (**c**), and instability index (**d**).

Duplication events and subcellular localization of TtCIPKs

TtCIPK gene family in durum wheat is distributed across all chromosomes with at least one *TtCIPK* gene in each chromosome. The genomic positions of the *CIPK* gene family members in durum wheat are well illustrated in Fig. 4a. An analysis of duplication events within this gene family revealed that some members have undergone segmental duplication. We could not detect any tandem duplications in *TtCIPKs*. The earliest duplication event in these genes occurred approximately 16 million years ago between *TtCIPK25* and *TtCIPK49*. A Ka/Ks ratio less than 0.4 in duplicated genes means Ks is much higher than Ka, which means strong negative selection on these genes (Supplementary Table 3). This implies these genes are functional and have retained their function over time.

Subcellular localization of TtCIPK proteins based on their amino acid sequences indicated that these proteins are present in different cellular compartments. Most CIPK proteins are likely to be localized in chloroplast and cytosol respectively. The presence of these proteins in other cellular compartments is possible, with the lowest probabilities being in vacuoles and mitochondria (Fig. 4b). It is predicted that TtCIPK11 and TtCIPK15, belonging to group 6, are more likely to be found in the cell nucleus (Supplementary Table 2).

Pocket site of CIPK proteins

Three-dimensional structure analysis revealed that TtCIPK proteins have a conserved structure and share minimal structural variation (Supplementary Fig. 1). However, there were differences in the binding and interaction sites (pocket site). The nature of amino acids within the active site of proteins determines the chemical properties of this region, thus affecting the ability of the protein to interact with a variety of other ligands and molecules. An analysis of the amino acids in the pocket site of the TtCIPK proteins shows that the most abundant are leucine, valine, aspartic acid, and lysine, while tryptophan and proline are rare in this region (Fig. 5).

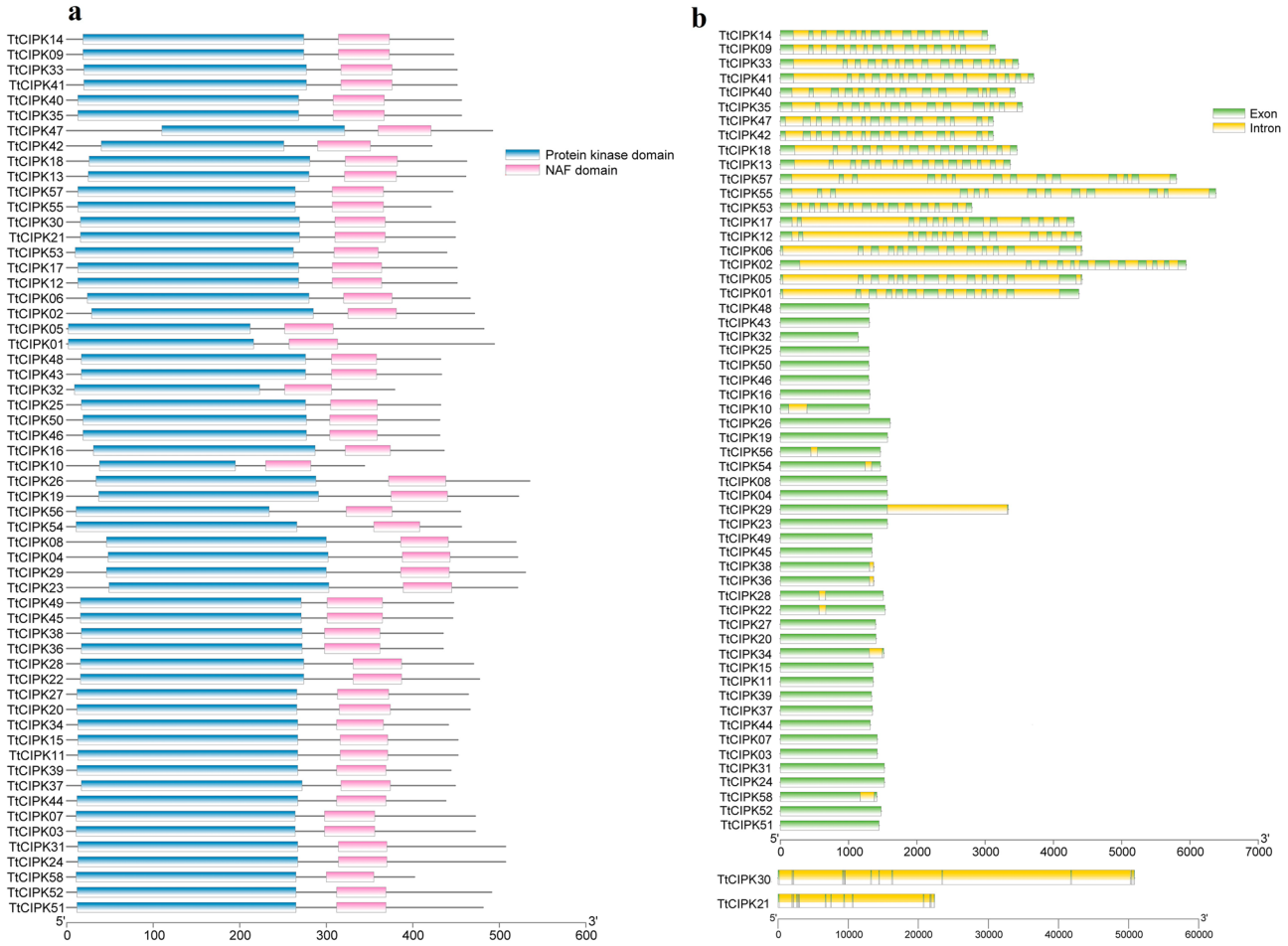

Fig. 3. Sequence analysis of TtCIPK family members based on conserved motifs distribution (**a**), and gene structure (**b**).

Post-translational modifications in CIPKs

Phosphorylation-induced conformational modifications are considered an effective mechanism that regulates the activity of CIPKs[54]. In line with this, we predicted amino acids within TtCIPK structures, which most likely will be phosphorylated. The prediction results indicate that all three major amino acids involved in phosphorylation, serine, threonine, and tyrosine, are likely to be phosphorylated with a probability of 0.8 in TtCIPK proteins. Among these proteins, serine is most likely to be the common amino acid receiving a phosphate group in all the phylogeny groups (Fig. 6). Additionally, the results revealed that there is a great diversity between the TtCIPK proteins in the potential phosphorylation sites, which can affect their function and lifespan.

Expression patterns of *TtCIPK* genes based on RNA-seq data

The expression profile based on RNA-seq data disclosed that the transcription of *TtCIPK* genes under temperature changes exposed the powerful and versatile role of these genes in signaling pathways related to abiotic stress (Fig. 7). Especially, the *TtCIPK49* gene was upregulated under cold stress, which might indicate its functional role in cold tolerance. On the other hand, low expression levels of *TtCIPK13* and *TtCIPK26* under cold stress may indicate that these genes were downregulated by such a type of stress. Generally, 58 *TtCIPK* genes showed a decreasing trend in the expression analysis except *TtCIPK38*, *TtCIPK36*, and *TtCIPK34*. Interestingly, both *TtCIPK38* and *TtCIPK26* were highly upregulated under heat stress; therefore, they are good candidates to be overexpressed to develop improved heat stress tolerance. Further, detailed studies of the expression of such genes are required for a crystal-clear understanding of the underlying molecular mechanisms of abiotic stress tolerance in plants.

Upstream analysis of *TtCIPK* genes

The analysis of the upstream regions revealed a high diversity among the members of this family based on the frequency and type of *cis*-element in the promoter of *TtCIPK* genes. Our finding indicated that, overall, MYB and MYC *cis*-regulatory elements are the most frequent in the promoters of *TtCIPK* genes (Fig. 8a). Besides, the results show that most *cis*-regulatory elements are located upstream of *TtCIPK* genes associated with stress

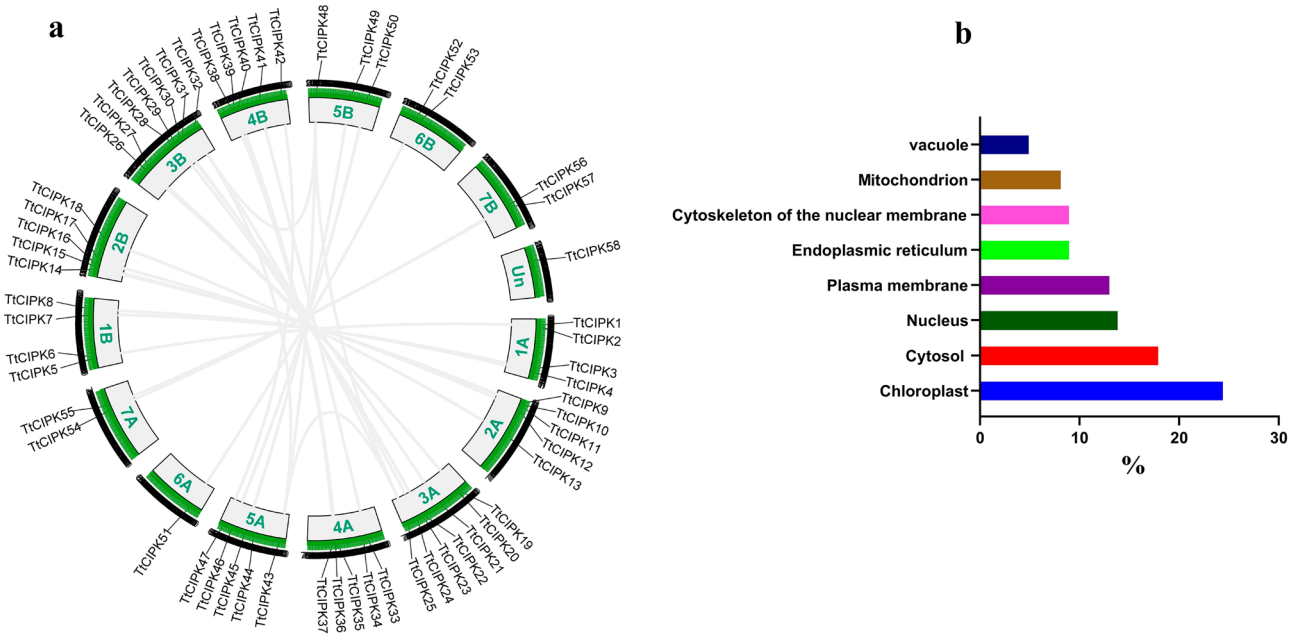

Fig. 4. Location of *TtCIPK* genes on the genome of *T. turgidum* (**a**). Duplicated genes are linked by gray lines. Subcellular localization of TtCIPK proteins (**b**).

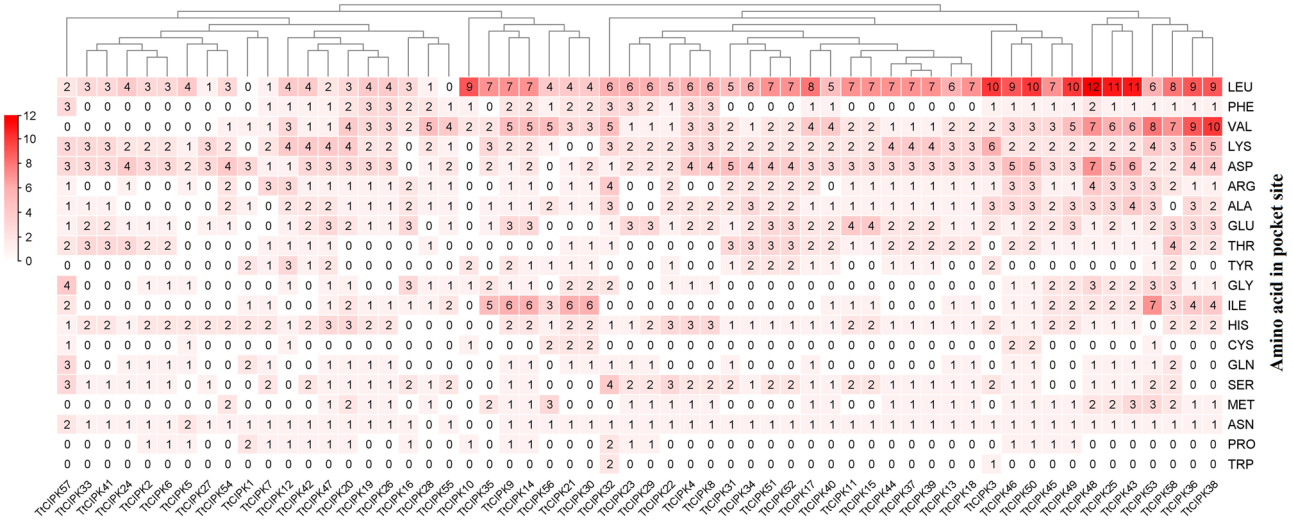

Fig. 5. Frequency of residues located in the pocket site of TtCIPK proteins.

responses (Fig. 8b). Among the hormonal *cis*-elements, which are the second most abundant after stress-related *cis*-elements, those responsive to ABA and methyl jasmonate are more prevalent (Fig. 8c).

Expression levels of *TtCIPK* genes in shoot tissues in response to drought stress

In the shoot tissues, the relative expression levels of a selected number of *TtCIPK* genes from each phylogenetic group were tested under drought stress conditions at 1, 24, and 72 h after treatment (indicated as D1, D24, and D72, respectively) with or without the application of ABA (Fig. 9). Upregulation of *TtCIPK12*, *TtCIPK27*, *TtCIPK29*, and *TtCIPK33* expression was induced in shoot tissue upon drought treatment; this upregulation was significantly suppressed with the application of ABA at D24 for *TtCIPK12*, *TtCIPK27*, and *TtCIPK29*. For *TtCIPK33*, significant upregulation was observed upon drought stress at D1 and D24, and with the application of ABA, the transcription of this gene is intensified. This includes expression upregulation in *TtCIPK43* following drought exposure at D1 and D24, but downregulated with ABA application at these time points, then upregulated at D72. Taken together, the upregulation of these genes by drought in shoots indicates that they have a vital role in responding to such stress.

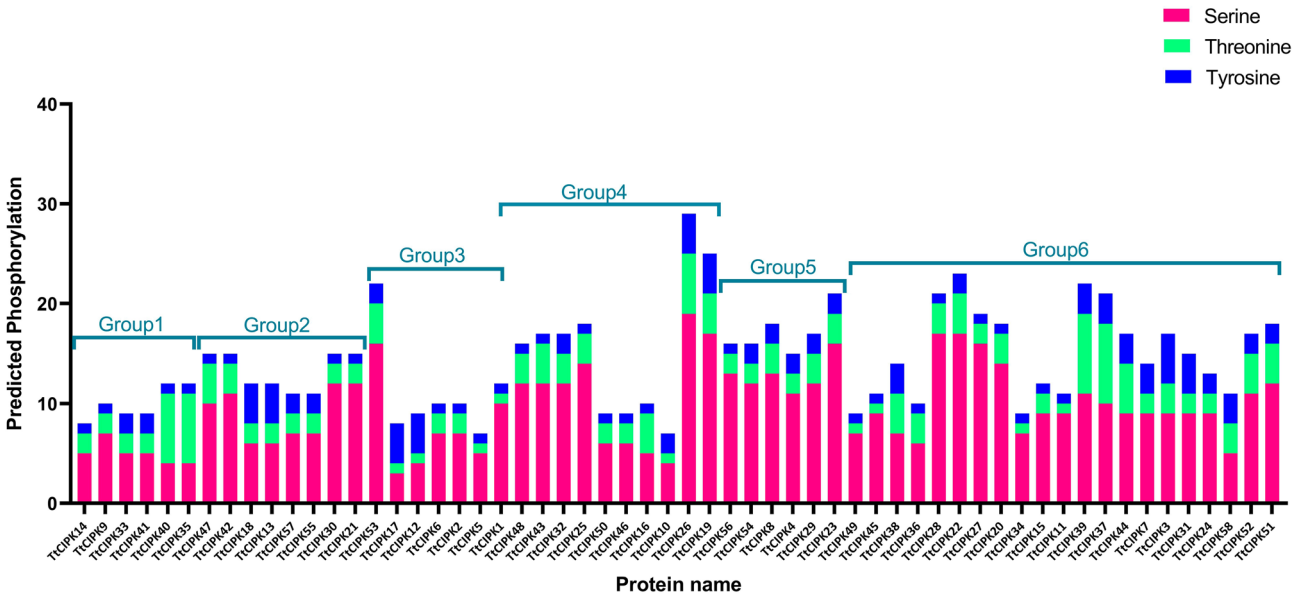

Fig. 6. Prediction of the phosphorylation sites in TtCIPK proteins.

Expression levels of *TtCIPK* genes in root tissues in response to drought stress

In root tissues, *TtCIPK12* was upregulated after 1 (D1) and 24 h (D24) under drought stress (Fig. 10). With ABA treatment, it was up-regulated in control and down-regulated at D1 and D24. The *TtCIPK27* gene showed increased expression at D24, which was reduced upon ABA application (Fig. 10). The *TtCIPK33* gene demonstrated a significant increase in expression in the control plants treated with ABA. For *TtCIPK42*, ABA application resulted in elevated gene expression in both control plants and at D24. The *TtCIPK43* gene exhibited increased expression at D1 under drought stress, and ABA had a notable effect on its expression in control plants, reducing expression at D1 and increasing it at D72. Roots exhibit a lesser tendency for *TtCIPK* gene upregulation than shoots under drought stress. Moreover, the effect of ABA in inducing gene upregulation in roots mainly occurred in control plants.

Discussion

Given the role of CIPKs in plant physiological processes, including stress responses, hormonal signaling, and growth and development, their study in crop plants seems essential[55–57]. CIPKs interact with CBLs to form a network that establishes a physiological link between plant growth and stress tolerance[58]. Durum wheat is essential in regions affected by water stress limitations[59]. The *CIPK* gene family has been extensively studied in various plant types like Arabidopsis, wheat, tomato, and soybean[10,60–62]. However, so far, the members of this gene family have not been identified and investigated in durum wheat. In this study, 58 members of the TtCIPK family were identified, showing differences in structure and some physicochemical properties. The large number of members of this gene family in durum wheat can be related to the complete doubling of its genome, and considering the role of CIPK in response to environmental stresses, increasing the number of members has been effective in increasing the tolerance of this plant. The study of evolutionary relationships showed that TtCIPKs are separated into six groups, and a high genetic distance was observed between them. This genetic distance and the placement of orthologs of TtCIPKs in monocot and dicot model plants in the phylogeny tree convey the message that the diversity in the members of this gene family occurred both before and after the derivation of dicots and monocots. Domain analysis indicates that TtCIPKs have their kinase domains located always near the N-terminus, while NAF domains are uniformly found close to the C-terminus. The structural analysis of TtCIPK evolutionary groups indicates that groups 1, 2, and 3 are intron-rich, whereas those belonging to groups 4, 5, and 6 are known for their lack of apparent introns. This scenario has also been noted during other plants' CIPK gene family investigations, such as banana[11,63]. Two genes, *TtCIPK30* and *TtCIPK21*, belonging to the second evolutionary group, contain long introns. Long introns may indicate the complexity of gene expression regulation[64].

Introns harbor regulatory elements that influence transcription, splicing, and mRNA stability. These elements help to precisely regulate gene expression in response to different stimuli across tissues. Additionally, since introns are not translated, they tolerate mutations and contribute to genetic diversity over evolutionary time. Although CIPKs phosphorylate downstream targets in stress response pathways, they are also subject to phosphorylation. For example, phosphorylation of *MdCIPK22* has been reported to be induced by ABA and occurs at threonine 411[56]. We analyzed post-translational modifications in the TtCIPK family and predicted that all TtCIPK proteins are likely phosphorylated at serine residues, with less likelihood at threonine and tyrosine residues. A conserved structure was observed based on the three-dimensional structure prediction in TtCIPK proteins. However, based on the position and type of amino acid, TtCIPK family members differed from each

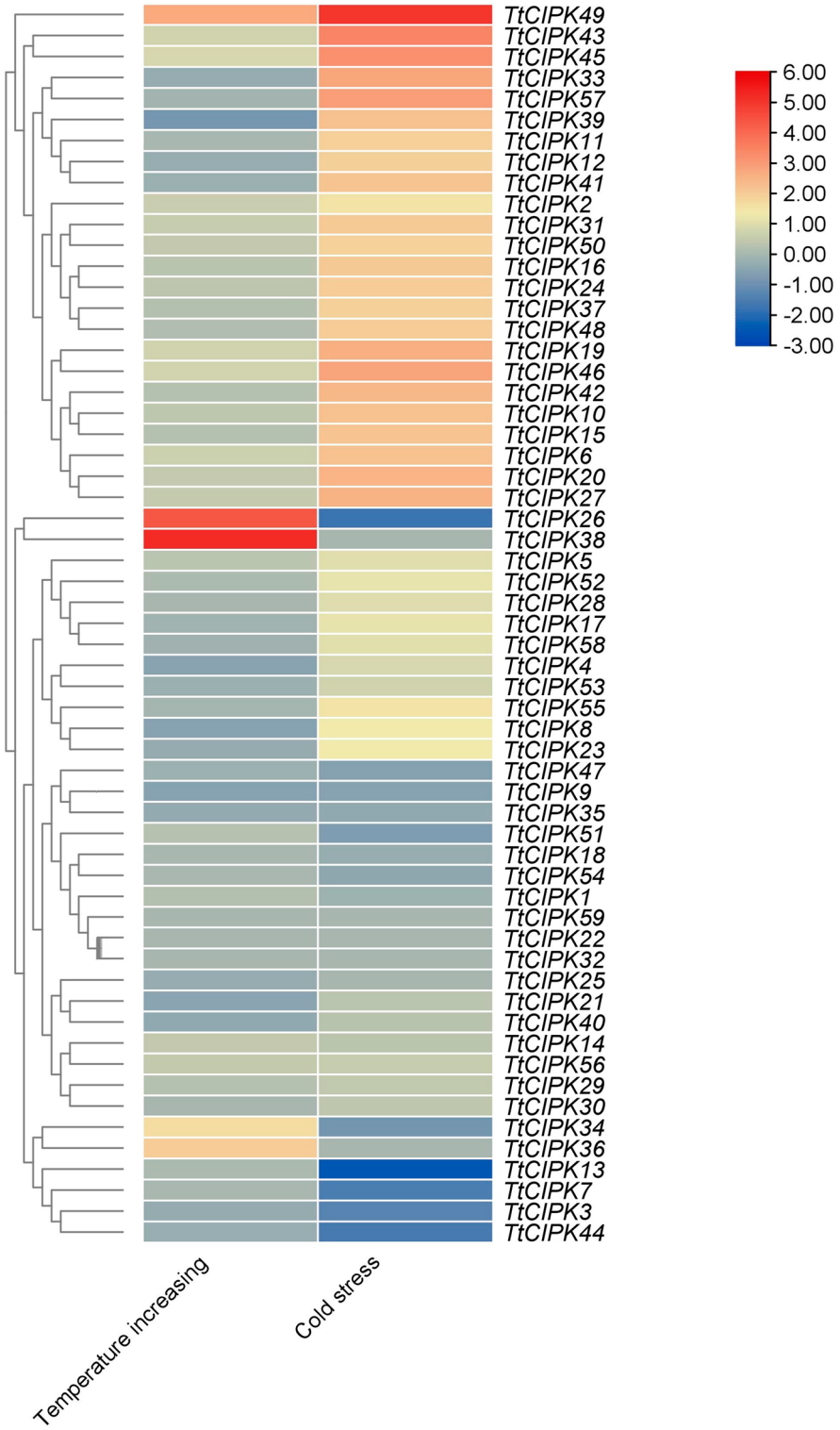

Fig. 7. Expression profile of TtCIPK genes in response to temperature increasing and cold stress.

other. The origin of this difference can be related to the mutations that have been created and stabilized during evolution, resulting in functional diversity. In addition, it seems that the amino acids leucine, valine, aspartic acid, and lysine play a key role in the function and interaction of TtCIPKs in the associated signal transmission networks, although further studies are needed.

Of the 58 identified *TtCIPK* genes, 50 contained the ABRE (Abscisic Acid Responsive Element), a crucial *cis*-element in stress responses[65]. ABA has shown a significant role in enhancing tolerance to environmental challenges[66,67]. Many *CIPK* genes are regulated by ABA under stress conditions, and some participate in the

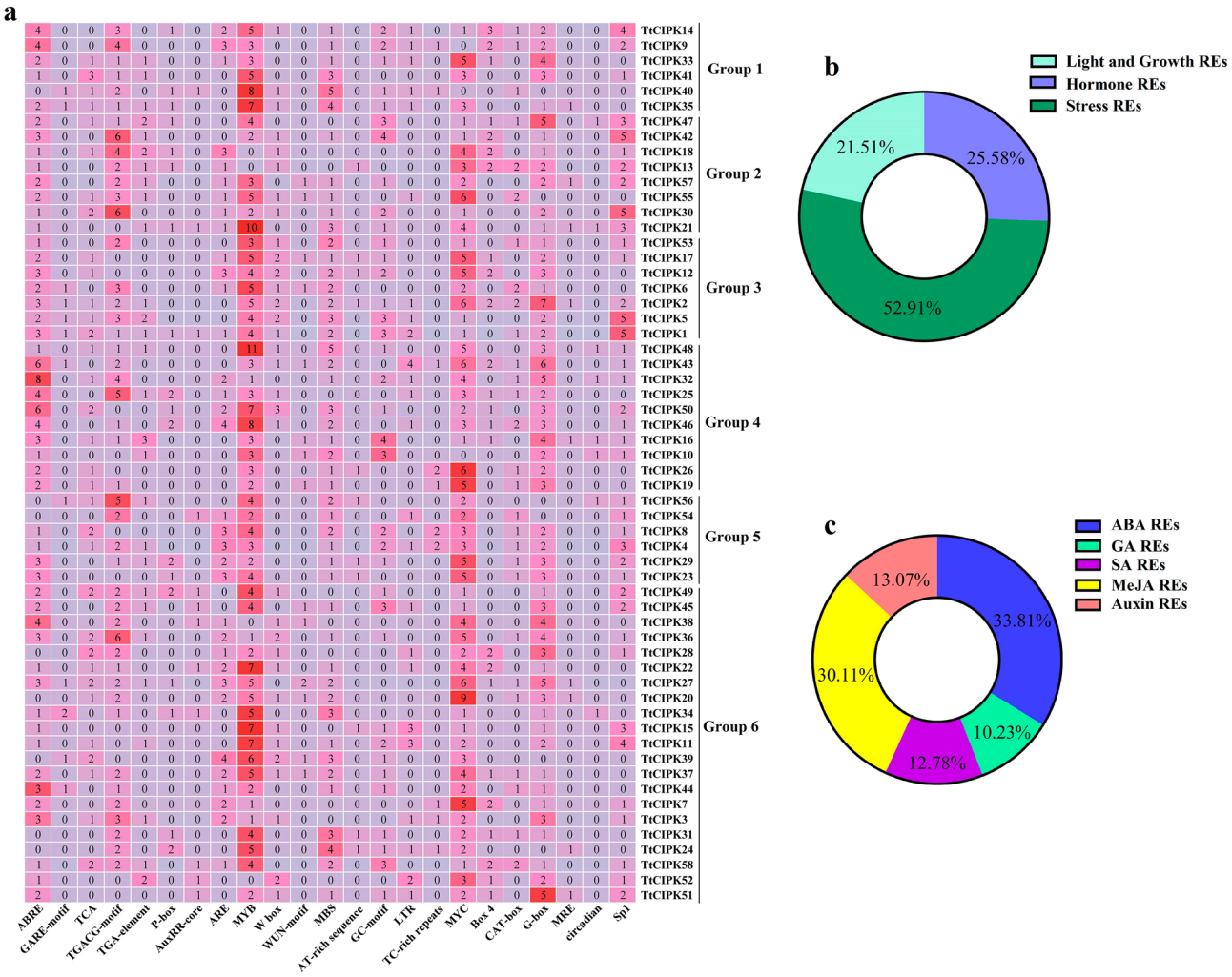

Fig. 8. Promoter analysis of *TtCIPK* genes. Distribution of *cis*-regulatory elements in the promoter region of *TtCIPK* genes (**a**). Frequency of *cis*-regulatory elements based on their function (**b**) and frequency of *cis*-regulatory elements related to hormones (**c**).

ABA signaling pathway[12,68]. The results from the expression analysis of *TtCIPK*s indicated that these genes were dynamically and differentially expressed in the root and shoot tissues under drought stress. Their regulations were often differentially influenced by ABA treatments. Of these, *TtCIPK12* and *TtCIPK27* were identified as two major ABA-independent genes playing key roles during the early stages of drought responses (Fig. 11). Both genes were significantly upregulated during the early stages of drought stress, both in root and shoot tissues, suggesting that their participation in rapid stress sensing and mitigation is relevant. Results are in agreement with literature data pointing out the involvement of both factors in early stages of stress responses using calcium ions as second messengers within the stress signal transduction pathways[69]. On the contrary, *TtCIPK33* was strongly induced in roots and shoots, whereas *TtCIPK42* was only strongly induced in roots upon ABA treatment; hence, they may take part in the ABA-mediated signaling pathways (Fig. 11). Notably, *TtCIPK33* presented a constitutive expression pattern in several tissues and at different time points, which agrees with a dual regulatory role and a candidate function in sustained adaptation responses. These observations are consistent with similar calcium signaling previously reported in other cereals[21].

These results highlight that the spatial and temporal expression patterns of *TtCIPK* genes under drought stress in shoot and root tissues reflect differential functional aspects. For instance, specific genes like *TtCIPK27* and *TtCIPK29* responded promptly to the early drought signal in shoot tissues, whereas others, such as *TtCIPK33*, continued their expression in longer-term stress. Significantly, these responses were modulated by ABA treatments in either upregulation or downregulation depending on specific genes and tissues, respectively, in agreement with previous studies that have emphasized the vital role of ABA in inter-tissue coordination of various stress responses. The integration of *TtCIPK* genes into ABA-dependent and ABA-independent pathways points out their varied functions in durum wheat's mechanisms of drought tolerance. The present work is one of the few pioneering studies to clearly outline the dual regulatory roles of certain CIPKs in durum wheat and has highlighted the space and time patterns of its expression. In addition, a detailed study on *TtCIPK12*, *TtCIPK33*, and *TtCIPK43* builds a good foundation for future studies on their involvement in cross-talk with stress signaling.

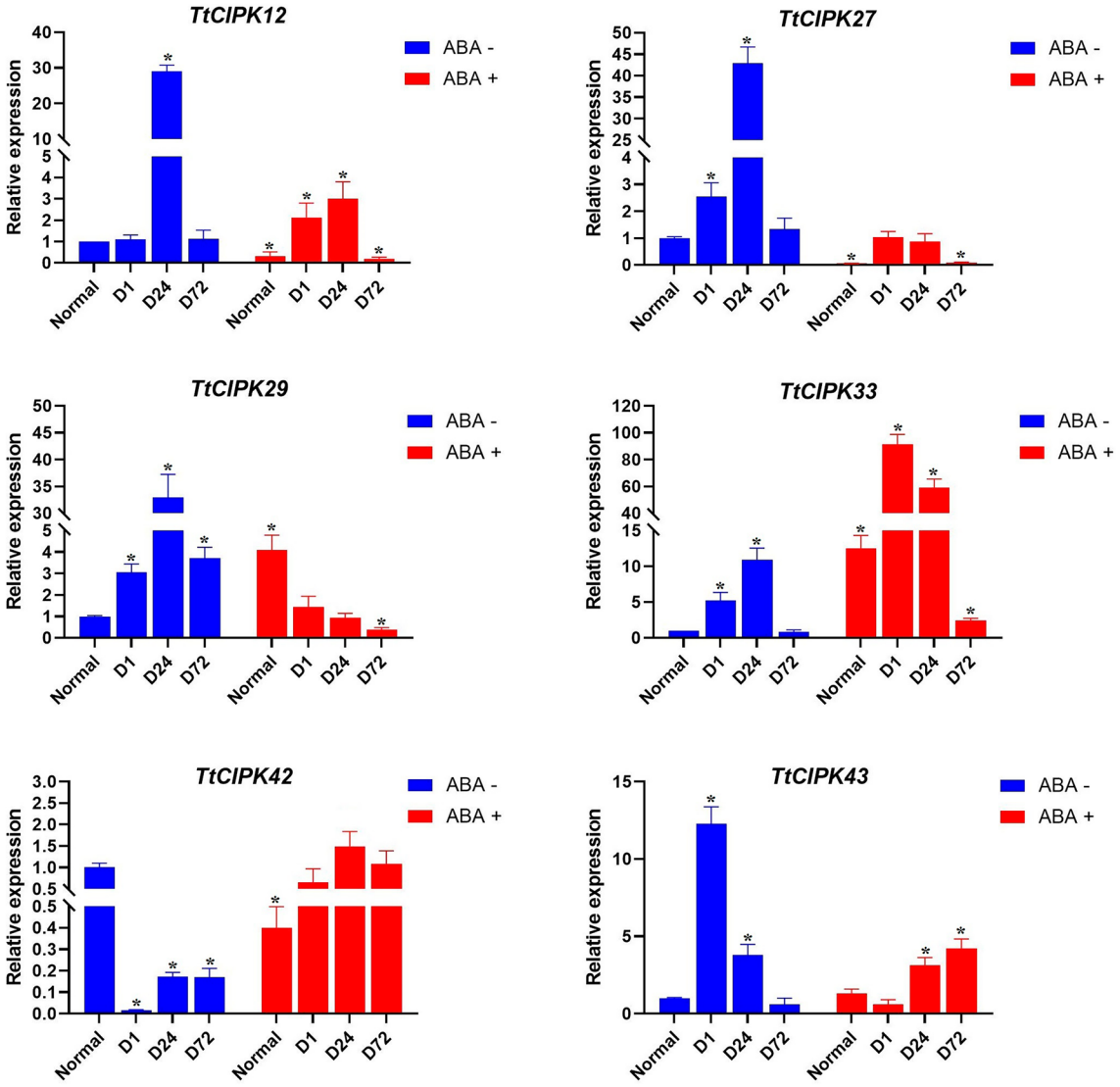

Fig. 9. Relative expression of candidate *TtCIPK* genes in shoot tissues in response to drought stress and ABA application. Each column is marked with an asterisk to show a significant variance (p-value < 0.05) from the control group, based on the t-test.

Although the gene expression level alone cannot provide the accurate function of *TtCIPK*s, *TtCIPK33* was considered the most promising candidate for improving drought tolerance since it was always involved in drought stress responses. Other similar members of this CIPK family have already been used in other plant species to enhance drought and abiotic stress tolerance[19,55]. Significant abiotic stresses were checked along with the expression of other genes in *TtCIPK* genes; some included being treated with ABA, polyethylene glycol, high temperature, or cold stress. The presented study contributes to a basic understanding of evolutionary development expressed within the *CIPK* gene family of durum wheat and thus provides the real grounds for functional future investigations among such genes.

The basis of this study is the previous studies that, for the first time, compare ABA-dependent and ABA-independent CIPK pathways directly in durum wheat, integrating promoter element analyses with tissue-specific expression profiling. Unlike the previously reported Arabidopsis and rice studies that mainly focused on a single pathway under specified stress, this research takes a broad approach to unraveling the regulatory mechanisms underlying the activity of CIPK. Furthermore, a few unique phosphorylation sites in durum wheat CIPKs have been identified. They are adding new dimensions to functional diversity and regulatory mechanisms, thus complementing the findings from potato and quinoa studies[19,70].

This also suggests that TtCIPKs play a core role in calcium signaling, mediating stress responses that are potential means of improving crop resilience. By mediating stomatal closure, osmotic adjustment, and ROS detoxification, TtCIPKs contribute to water-use efficiency and drought tolerance[20,71]. Further diversity in *cis*-regulatory elements is involved in the response of plants to other abiotic stresses, including salinity and cold, positioning these genes as candidates for multi-stress tolerance breeding in crops. Progress in genome-editing

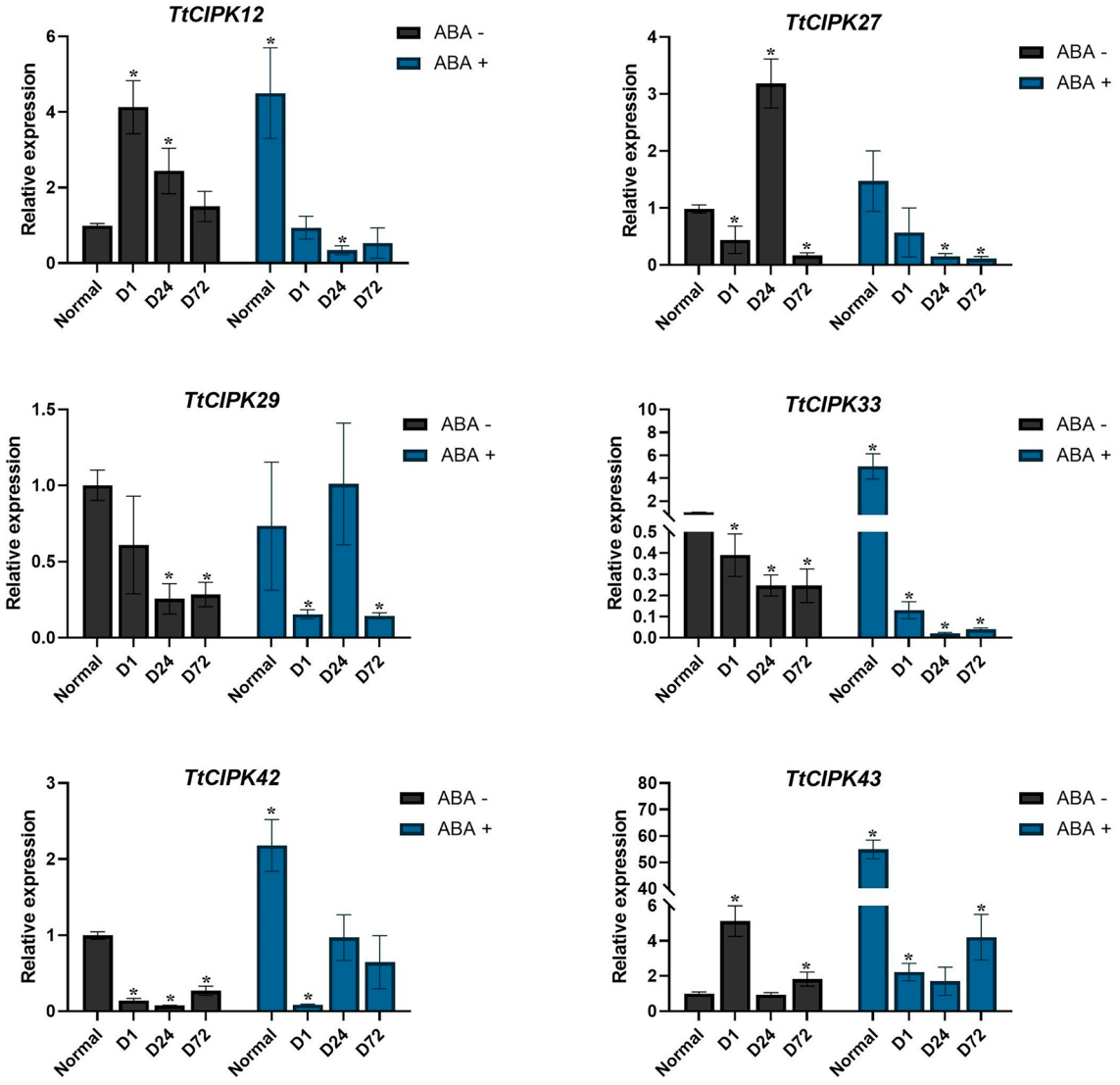

Fig. 10. Relative expression of candidate *TtCIPK* genes in root tissues in response to drought stress and ABA application. Each column is marked with an asterisk to show a significant variance (p-value < 0.05) from the control group, based on the t-test.

technologies has allowed or will allow exact modification of promoter regions, including the recently developed CRISPR-Cas9 system, which might lead to new ways of activating stress-responsive genes and new strategies for crop improvement. This work also presents not only the complex and multi-dimensional nature of the drought and ABA stress response mechanism but also points toward the *TtCIPK* gene family as a potential key player in enhancing stress tolerance and resilience in durum wheat.

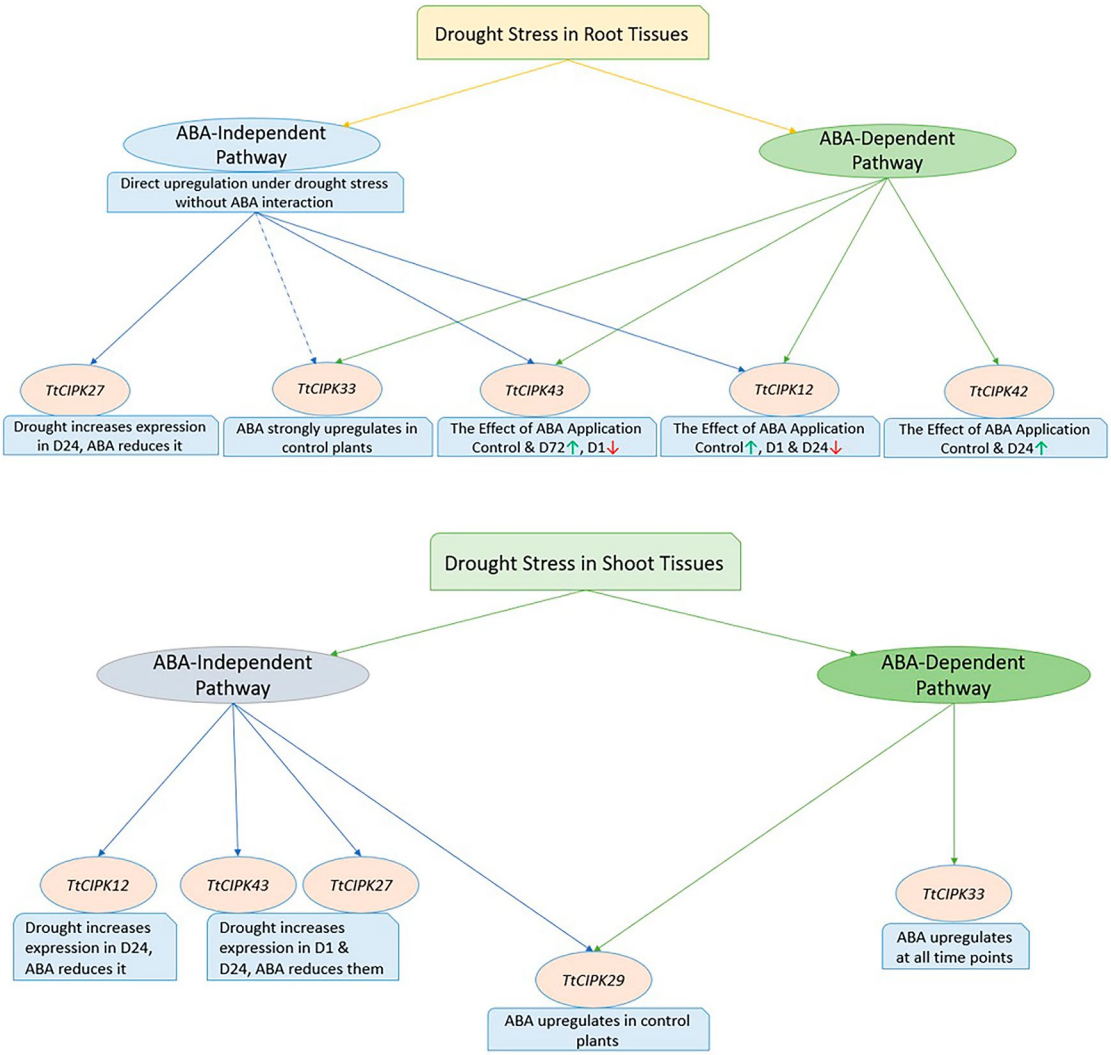

Fig. 11. Schematic representation of ABA-dependent and independent pathways and candidate *TtCIPK* genes involved in them during drought.

Data availability

The data generated or analyzed in this study are included in this article. Other materials that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

M. C. and P. H. conceived and design the study. H. S. H. P., M. C., and P. H. organized and performed the experiments. H. S. H. P., M. C., and P. H. were involved in data interpretation. H. S. H. P. wrote the manuscript. M. C. and P. H. planned and supervised the study and edited the final version of the manuscript. All authors read and approved the final version of the manuscript.

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Declarations

Competing interests

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Additional information

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