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# Decoding the CBL gene family in durum wheat: Insights into dual ABA signaling pathways and calcium-mediated drought tolerance

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## Abstract

Calcium signaling is an essential mechanism in plant responses to environmental stressors, facilitated by sensors like the calcineurin B-like (CBL) protein family. This study offers a comprehensive analysis of the *CBL* gene family in durum wheat (*Triticum turgidum* ssp. *durum*), emphasizing their expression patterns in reaction to drought stress and abscisic acid (ABA) treatment. Bioinformatics and gene expression analyses were conducted on the *CBL* gene family in durum wheat under drought and ABA application. A total of 23 *CBL* genes (*Triticum turgidum* CBLs [*TtCBL*s]) were identified and further grouped into four phylogenetic clusters. Further evolutionary analysis revealed that segmental duplication is the primary driving force in *CBL* family expansion, and strong purifying selection played a crucial role in their functional integrity. Promoter analysis showed ABA- and stress-responsive *cis*-elements, suggesting that the gene family has dual regulatory roles in both ABA-dependent and ABA-independent pathways. Expression profiling of *TtCBL*s demonstrated variable drought and ABA treatment patterns, with notable tissue-specific patterns. *TtCBL2* and *TtCBL10* emerged as promising candidates contributing to root-specific drought responses. In addition, *TtCBL19* emerged as a putative integrator of cross-tolerance mechanisms regulated by both ABA-mediated and non-ABA signaling. This work emphasizes the complex interplay of calcium signaling with ABA-mediated pathways and provides a platform for targeted genetic interventions enhancing drought resilience in cereal crops. Another important point would be to highlight how such findings open up perspectives regarding using in silico approaches to guide active molecular breeding strategies in agriculture.

## Plain Language Summary

Durum wheat, a major staple crop in arid and semiarid regions worldwide, faces considerable yield losses due to the effects of drought stress. Plants utilize calcium

**Abbreviations:** ABA, abscisic acid; ABREs, ABA-responsive elements; CBL, calcineurin B-like; CIPK, CBL-interacting protein kinases; CREs, *cis*-regulatory elements; GRAVY, grand average of hydropathy; MW, molecular weight; p*I*, isoelectric point; TtCBL, *Triticum turgidum* CBL.

signaling as a key pathway when adapting to environmental challenges. Due to its confirmed role in calcium-mediated signaling, the calcineurin B-like (CBL) gene family presents a strong potential for enhancing drought resilience. In this study, CBL genes were identified and analyzed in durum wheat. Our results found that durum wheat has 23 CBL genes with diverse sequence structure. In addition, it was stated that CBL genes of durum wheat involve both in ABA-dependent and -independent pathways in response to drought stress. Our findings provide a framework for the use of CBL genes in agricultural biotechnology breeding programs in durum wheat. The results of our study offer a foundation for integrating CBL genes into breeding programs to enhance drought tolerance of durum wheat.

## 1 | INTRODUCTION

Drought affects durum wheat (*Triticum turgidum* ssp. *durum*) by reducing the height of the plant, number of tillers, leaf area, and chlorophyll content, all of which are essential for optimal plant growth and photosynthesis. It also lowers grain yield and the number of grains per spike, ultimately compromising productivity. These physiological and yield-related reductions make durum wheat cultivation particularly challenging in regions with limited water availability. Notably, there are durum wheat genotypes that show higher drought tolerance, with improved water holding and photosynthesis under stress conditions—attributes widely targeted in breeding programs for the generation of drought-resistant cultivars (De Santis et al., 2021; Pour-Aboughadareh et al., 2020). Based on these findings, we focus on identifying and characterizing stress-resistance traits to support food security and sustainable agricultural production in drought-prone regions.

Plants respond to environmental stresses through complex signaling networks (Ghosh et al., 2022; Xu et al., 2022), wherein calcium signaling acts as a key second messenger in such processes (Kudla et al., 2010, 2018; Todaka et al., 2015). The EF-hand is a highly conserved calcium-binding domain characterized by a helix–loop–helix structure. It binds a calcium ion via its loop region by coordinating with oxygen atoms of the backbone's aspartate, glutamate, and carbonyl groups (Gifford et al., 2007). EF-hand proteins in plants have essential functions in decoding $Ca^{2+}$ signatures elicited by abiotic (e.g., drought, salinity, heat stress) and biotic (e.g., herbivores, pathogens) stress and, in turn, the modulation of downstream stress-adaptive responses (Naz et al., 2024). The calcineurin B-like (CBL) proteins represent key calcium sensors in deciphering calcium signals mediated by abiotic stresses like drought (Poovaiah & Du, 2018; W. Sun et al., 2021). Calcium binding through EF-hand motifs in CBL proteins results in the activation of specific responses. CBLs mediate this response by recruiting CBL-interacting protein kinases (CIPKs) to membrane sites that regulate ion channels and other proteins (Kolukisaoglu et al., 2004). As calcium sensors, CBLs interact with CIPKs to form signaling complexes that regulate ion homeostasis, hormonal signaling, and various physiological processes (Förster et al., 2019; Ketehouli et al., 2019; Xuan et al., 2019). Upon calcium binding, CBLs undergo conformational changes that enable them to interact with specific CIPKs via their N-terminal region. The interaction between them depends on the FISL/NAF domain found in CIPKs, which removes CIPK autoinhibition so that its target proteins in stress adaptation are phosphorylated (Pandey et al., 2014; Sanyal et al., 2015).

The CBL-CIPK complex mediates calcium signaling that cooperates with abscisic acid (ABA) pathways to regulate many plant stress responses (Bittner et al., 2022; Verma et al., 2022). Being a phytohormone, ABA mediates key regulatory roles such as stomatal closure, root architecture, and gene expression under water-deficit conditions (Hsu et al., 2021), while calcium acts as a second messenger to decode such stress-induced signals (Tian et al., 2020). For instance, the *Arabidopsis thaliana* CBL9-CIPK3 complex is known to be as directly involved in ABA biosynthesis and the process of seed germination, thereby linking calcium to the hormonal signaling pathway (Ma et al., 2020; Pandey et al., 2004, 2008). Indeed, evidence suggests that acting together could negatively regulate potassium ion channels such as guard cell outward rectifying K channel, maintaining ionic homeostasis under drought stress (Förster et al., 2019). Compared to model plants, such as *A. thaliana* (D'Angelo et al., 2006; Yadav et al., 2018), in which the CBL-CIPK module has been extensively investigated intensively, much less is known about its function in polyploid crops, including durum wheat.

Multiple *CBL* and *CIPK* genes have been identified, which perform diverse functions under drought stress. For instance, *AtCIPK1* regulates defense responses by interacting with *AtCBL1* and *AtCBL9*, and their malfunction may lead to

hypersensitivity to drought stress (D'Angelo et al., 2006). Overexpression of specific *CBL* genes, such as *CBL1*, in *Arabidopsis* confers tolerance to salt stress (Sharghi et al., 2016). Recently, a comprehensive study on the *CBL* and *CIPK* gene family in quinoa provided an overview of their roles in abiotic stresses, such as drought (Xiaolin et al., 2022). *GmCIPK29* and *GmCBL1* have been isolated from soybean (*Glycine max* L.) and are induced under drought stress. These proteins are involved in ABA signaling (C. Wang et al., 2023). Drought-induced ABA accumulation alters the expression of stress-related genes (Hussain et al., 2021; Mittler & Blumwald, 2015).

Recent advances in bioinformatics and transcriptomics have facilitated deeper insight into the stress-responsive gene families, particularly in polyploid crops. High-throughput RNA sequencing and promoter analyses have revealed not only spatial and temporal expression patterns but also the regulatory elements controlling gene function. In wheat, the *cis*-elements ABA-responsive elements (ABREs) and MYB motifs have emerged as key regulators of adaptation to abiotic and biotic stresses, linking genomic data to functional phenotypes (Hussain et al., 2021). Phylogenetic and duplication analyses have revealed how segmental duplications and selective pressures shaped functional diversity within gene families in complex genomes, such as that of durum wheat (Mohanta et al., 2015). This study employs bioinformatics tools to investigate the dual role of the *CBL* gene family in ABA and calcium signaling, enhancing our understanding of stress responses and tolerance mechanisms in durum wheat.

Durum wheat, a major staple crop in arid and semiarid regions, experiences significant yield losses due to drought stress (Giunta et al., 1993; Solomon et al., 2003). Although significant progress in genomics has been made, substantial gaps remain in understanding the molecular mechanisms underlying stress tolerance. The *CBL* gene family offers promising potential for enhancing drought resilience due to its established role in calcium-dependent signaling pathways. To our knowledge, this is the first comprehensive genome-wide analysis of the *CBL* gene family in durum wheat, investigating the responses of these genes to drought stress and ABA treatment. This study characterizes the *CBL* gene family in durum wheat, analyzing their phylogenetic relationships, gene duplications, and expression profiles in response to drought and ABA treatments. By combining bioinformatics analyses with transcriptomics data, we contribute hints regarding the dual role of *Triticum turgidum* CBL (*TtCBL*) genes in ABA-dependent and independent pathways, thus opening vistas for their application in molecular breeding and genetic engineering. These insights should guide breeding strategies to enhance drought resilience in polyploid crops.

**Core Ideas**

- Durum wheat included 23 calcineurin B-like (CBL) genes, arranged into four evolutionary groups.
- Segmental duplications drive CBL family expansion, under strong purifying selection.
- *Triticum turgidum* CBL (TtCBLs) operate both in abscisic acid (ABA)-dependent and -independent pathways.
- TtCBL2, TtCBL10, and TtCBL19 are proposed targets for engineering drought tolerance.

## 2 | MATERIALS AND METHODS

### 2.1 | Identifying *CBL* family genes in durum wheat

The *CBL* gene family members were identified in durum wheat using the Svevo.v1 genome assembly available at Ensembl Plants (https://plants.ensembl.org/Triticum_turgidum/Info/Index) (Bolser et al., 2017). BLASTP and TBLASTN searches used CBL protein sequences from *A. thaliana* (TAIR database; http://www.arabidopsis.org) and *Oryza sativa* (TIGR database). CBL protein sequences from *Triticum urartu*, *Triticum aestivum*, *Hordeum vulgare*, and *Zea mays* were extracted from Ensembl Plants and NCBI databases. To confirm the discovered proteins, EF-hand domains were analyzed utilizing Pfam (https://www.ebi.ac.uk/interpro/) (Paysan-Lafosse et al., 2023), the Conserved Domain Database (Marchler-Bauer et al., 2015), and PANTHER (https://www.pantherdb.org/). Proteins devoid of EF-hand motifs or possessing incomplete sequences were filtered out. The designated genes were assigned names according to their chromosomal positions. The physicochemical parameters of each CBL protein, such as molecular weight (MW), isoelectric point (p*I*), grand average of hydropathy (GRAVY), and instability index, were calculated using the ProtParam tool in the ExPASy database (https://web.expasy.org/protparam/) (Gasteiger et al., 2005). Subcellular localization predictions were determined using WoLF PSORT (https://www.genscript.com/wolf-psort.html).

### 2.2 | Phylogenetic analysis

The evolutionary relationships of TtCBL proteins were analyzed using multiple sequence alignments with their homologs in *A. thaliana*, *T. urartu*, *T. aestivum*, *H. vulgare*, and *Z. mays*. Alignments were performed using Clustal

Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers & Higgins, 2018). A phylogenetic tree was constructed using the maximum likelihood method with 1000 bootstrap replications, and the tree was displayed using the iTOL tool (https://itol.embl.de/) (Letunic & Bork, 2019).

## 2.3 | Gene duplication events analysis in *TtCBLs*

Gene duplication occurrences in *TtCBL*s were found by analyzing gene pairs with coding sequence similarity exceeding 85%. The rates of Ka (non-synonymous substitution) and Ks (synonymous substitution), together with their ratio (Ka/Ks), were computed utilizing TBtools (https://github.com/CJ-Chen/TBtools) (Yaghobi & Heidari, 2023). The divergence period for duplicated genes was estimated using the method $T = (\mathrm{Ks}/2\lambda) \times 10^{-6,}$ where ($\lambda = 6.5 \times 10^{-9}$) substitutions per site per year (Yang et al., 2008).

## 2.4 | Upstream regions analysis

The upstream regions (1500 bp) of *TtCBL* genes were obtained from the Ensembl Plants database. The PlantCARE tool (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) analyzed these regions for *cis*-regulatory elements (CREs) (Lescot et al., 2002). The identified elements were categorized based on their roles, and their frequencies were measured.

## 2.5 | Prediction of three-dimensional (3D) protein structures and ligand-binding sites of TtCBLs

The 3D conformations of TtCBL proteins were forecasted utilizing the Phyre2 platform (http://www.sbg.bio.ic.ac.uk/phyre2/) (Kelley et al., 2016). Binding sites for ligands were identified using the Phyre Investigator tool, which also determined amino acid composition in the pocket regions. The relative abundances of amino acids in these regions were visualized as bar graphs.

## 2.6 | Prediction of phosphorylation sites in TtCBLs

Potential phosphorylation sites in TtCBL proteins were discerned based on serine, threonine, and tyrosine residues. Sequences were examined utilizing the NetPhos 3.1 server (http://www.cbs.dtu.dk/services/NetPhos/) (Blom et al., 2004). Sites with values surpassing 0.80 were deemed phosphorylation candidates.

## 2.7 | Interaction network of TtCBLs

An interaction network was constructed for the TtCBL proteins to identify the interacting partners responsible for influencing their activity. The protein sequences of TtCBLs were submitted to the online database STRING (https://string-db.org/) (Von Mering et al., 2003) against the reference monocot genome, *O. sativa* L. ssp. *japonica*. The minimal required interaction score was set to be 0.7 or higher for high confidence, and up to 50 first-layer interactors and up to five second-layer interactors can be used for network visualization. Enrichment analysis was performed for significant gene ontology (GO) terms related to biological processes, molecular function, and cellular components, with a threshold FDR of $< 0.05$.

## 2.8 | Expression profile of *TtCBL* genes

Subsequently, RNA-seqence (RNA-seq) datasets were analyzed to investigate the expression profiles of *TtCBLs* under abiotic stress. Two publicly available datasets (Díaz et al., 2019), including PRJNA1089221 (cold stress at 5°C) and PRJNA780180 (increasing temperature [+2°C]). The reads in FASTQ format were downloaded and then quality controlled using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Low-quality reads were removed, and adaptors were eliminated using Trimmomatic. Then, cleaned reads were mapped against the Svevo.v1 reference for durum wheat using HISAT2, and quantifications at the gene level were estimated with HTSeq-count software (Anders et al., 2015). *TtCBL*s expression was then normalized and analyzed for differential expression by the R package NOISeq. Results were reported as $\log_2$ fold changes between stress and control conditions (Tarazona et al., 2012).

## 2.9 | Plant growth and drought stress treatment

The factorial experiment was conducted as a completely randomized design in the research greenhouse of Ferdowsi University of Mashhad. The factors included drought stress at two levels of zero and PEG 15% (w/v), ABA treatment at two levels of zero and 100 μM, and the third factor included the time of sampling after drought stress, including 0, 1 (D1), 24 (D24), and 72 h (D72). The durum wheat Dena cultivar, which is relatively tolerant to drought and obtained from the Seed and Plant Certification and Registration Institute in Iran, was used in this study. To cultivate the plant, seeds were placed in 5% (w/v) sodium hypochlorite for 15 min and then were washed five times with sterile distilled water. The sterilized seeds were soaked in deionized water for 5 h. Then

we cultivated the seeds hydroponically in the autoclaved sand bed in greenhouse conditions at a temperature of 25 ± 2°C, with a relative humidity of 35 ± 5% and a photoperiod of 12 h of light and 12 h of darkness. Ten-day-old seedlings were nourished with a half-strength Hoagland nutrient solution.

For the ABA treatment, shoots of the plants were treated with 100 μM ABA in the presence of 0.02% (v/v) Triton X-100 as a surfactant in the evening, 3 days before the application of drought stress. Drought stress was imposed by adding PEG6000 to the nutrient solution at a 15% w/v concentration, thereby increasing osmotic potential and reducing water availability for the roots. At each of the 0, D1, D24, and D72 time points, samples of root and shoot tissues were collected, immediately frozen in liquid nitrogen, and then stored at −70°C until use.

### 2.10 | RNA extraction and quantitative polymerase chain reaction (qPCR) analysis

From these seedlings, total RNA was extracted from the roots and shoots using a Sambio RNA Extraction Kit (Gene Azma). Three biological replicates were isolated for each treatment; quality and quantity were checked accordingly via the NanoDrop spectrophotometer (Implen). Total RNA was subsequently transformed to a complementary first-strand DNA using a reverse transcriptase kit (Yekta Tajhiz Azma) according to the producers' instructions. Primer design for one representative *TtCBL* gene from each subfamily was done using Primer-BLAST (Table S1), and *TtActin7* was used as a reference gene for normalization. qRT-PCR was performed in the ABI StepOne Plus system using Ampliqon 2X qPCR Master Mix Green High Rox (Ampliqon). The relative expression levels were calculated by the delta-delta Ct method (Livak & Schmittgen, 2001). Hence, the control plants (0% PEG 6000 and 0 μM ABA treatments) served as a point of reference. Comparisons of treatments with their respective controls have been analyzed using a two-tailed *t*-test, $p < 0.05$, whenever the differences in expression have to be gauged between a treatment against its control condition.

## 3 | RESULTS

### 3.1 | Identification of *TtCBL* genes in durum wheat

Using BLASTP and TBLASTN, 23 putative *CBL* genes were identified in the durum wheat genome. These genes were named according to the order of their placement on the chromosomes, *TtCBL1* to *TtCBL23* (Table 1; Table S2). Physicochemical analysis indicated that all the TtCBLs were acidic in nature, with p*I* less than 6. The MW ranged from 21.1 kDa in the case of TtCBL3 to 38.8 kDa in TtCBL13. The amino acid sequences had lengths varying from 183 to 346 amino acid residues. Furthermore, all TtCBLs, except TtCBL18, showed a negative GRAVY, which indicates that most of these proteins are hydrophilic.

### 3.2 | Investigating the evolutionary relationship of *TtCBL* genes

A phylogenetic analysis of the amino acid sequences of TtCBLs with their homologous CBL proteins from related species like *A. thaliana*, *O. sativa*, *T. aestivum*, *Z. mays*, *H. vulgare*, and *T. urartu* gives an idea of the evolutionary path that the *TtCBL* gene family has taken. The phylogenetic tree clustered all TtCBLs into four groups (Figure 1), among which Group 3 possesses the maximum number of genes, while Group 2 is the smallest. Further, each group exhibited some features that are unique from structural and functional aspects, therefore indicating functional diversification under evolution. The closest evolutionary relationships were detected between TtCBLs and their monocot orthologs, especially *T. aestivum* and *T. urartu*. This closeness shows that important CBL functions have been kept similar among monocots and emphasizes how TtCBL has changed after dicots and monocots evolved separately. Based on the number of exons, members of Group 2 all had seven exons, and members of Groups 3 and 4 had seven to nine exons (Figure 2a). Furthermore, for p*I*, all members of phylogeny Group 2 had a range close to each other, while more diversity was observed between members of Groups 3 and 4 (Figure 2b). For the GRAVY index, TtCBL groups had differences with each other, with the highest range in Group 3 and the lowest range in Group 1 (Figure 2c). Furthermore, the level of stability for TtCBL groups was variable, so all members of Group 2 and most members of Group 1 were predicted as unstable proteins, but 50% of members of Groups 3 and 4 were unstable (Figure 2d). Evolutionary analysis, therefore, established that *TtCBL* genes not only maintained a structural entity but also underwent functional diversification for the adaptation of durum wheat to take place under various abiotic stresses. This understanding of how calcium signaling works in polyploid crops provides a solid foundation and can help guide the creation of specific genetic changes to enhance plant resilience to stress.

### 3.3 | Prediction of duplication events

The genomic distribution of the 23 identified *TtCBL* genes showed that segmental duplications were the main factor in *TtCBL* gene family expansion in durum wheat. Of the seven chromosomes, chromosome 1B harbored the highest number with seven members (*TtCBL5*, *TtCBL6*, *TtCBL7*, *TtCBL8*, *TtCBL9*, *TtCBL10*, and *TtCBL11*), while fewer

**TABLE 1** The physicochemical parameters of *Triticum turgidum* CBL (TtCBL) proteins. More details are provided in Table S2.

| Gene ID | Gene name | Exon number | Protein(aa) | MW (kDa) | GRAVY | p*I* |
|---|---|---|---|---|---|---|
| TRITD1Av1G138820 | *TtCBL1* | 8 | 215 | 24.67 | −0.248 | 4.82 |
| TRITD1Av1G165570 | *TtCBL2* | 8 | 224 | 25.89 | −0.309 | 4.98 |
| TRITD1Av1G199350 | *TtCBL3* | 7 | 183 | 21.11 | −0.315 | 4.93 |
| TRITD1Av1G202220 | *TtCBL4* | 7 | 222 | 25.28 | −0.376 | 4.9 |
| TRITD1Bv1G132830 | *TtCBL5* | 8 | 215 | 24.67 | −0.248 | 4.82 |
| TRITD1Bv1G151110 | *TtCBL6* | 8 | 224 | 25.78 | −0.289 | 4.91 |
| TRITD1Bv1G189370 | *TtCBL7* | 8 | 215 | 24.84 | −0.285 | 4.76 |
| TRITD1Bv1G189690 | *TtCBL8* | 8 | 215 | 24.88 | −0.28 | 4.66 |
| TRITD1Bv1G193960 | *TtCBL9* | 7 | 222 | 25.26 | −0.4 | 4.78 |
| TRITD1Bv1G194200 | *TtCBL10* | 7 | 261 | 29.38 | −0.317 | 4.97 |
| TRITD1Bv1G194230 | *TtCBL11* | 7 | 222 | 25.17 | −0.359 | 4.91 |
| TRITD3Av1G013810 | *TtCBL12* | 9 | 292 | 32.95 | −0.031 | 5.00 |
| TRITD3Av1G132180 | *TtCBL13* | 9 | 346 | 38.78 | −0.159 | 5.18 |
| TRITD3Bv1G017060 | *TtCBL14* | 9 | 296 | 33.53 | −0.078 | 5.65 |
| TRITD3Bv1G121400 | *TtCBL15* | 9 | 288 | 32.73 | −0.128 | 4.96 |
| TRITD4Av1G181930 | *TtCBL16* | 9 | 258 | 29.13 | −0.222 | 5.53 |
| TRITD4Bv1G023460 | *TtCBL17* | 9 | 260 | 29.50 | −0.226 | 5.61 |
| TRITD4Bv1G203360 | *TtCBL18* | 7 | 248 | 28.09 | 0.056 | 4.87 |
| TRITD5Av1G029330 | *TtCBL19* | 8 | 226 | 25.91 | −0.175 | 4.91 |
| TRITD5Av1G093590 | *TtCBL20* | 7 | 261 | 30.13 | −0.182 | 4.61 |
| TRITD5Av1G254480 | *TtCBL21* | 8 | 215 | 24.66 | −0.153 | 4.76 |
| TRITD5Bv1G029010 | *TtCBL22* | 8 | 244 | 28.22 | −0.21 | 5.75 |
| TRITD5Bv1G076480 | *TtCBL23* | 8 | 226 | 26.03 | −0.292 | 4.79 |

Abbreviations: aa, amino acid; GRAVY, grand average of hydropathy; kDa, kilodalton; MW, molecular weight; pI, isoelectric point.

genes were located on chromosomes such as 3A and 4A (Figure 3a). Among these, only one tandem duplication event was detected: *TtCBL10* and *TtCBL11* on chromosome 1B (Figure 3a; Table S3), while all the other duplications were considered segmental. The evolutionary analysis of these duplications indicated that the Ka/Ks ratios of all the duplicated gene pairs were below 0.4, indicating that purifying selection played a major role in maintaining the functional integrity of *TtCBL* genes. Estimations based on this provide evidence that these duplications might have occurred around 28–31 million years ago (Table S3), thus coinciding with the evolutionary expansion of the monocot species. These results have indicated the evolutionary stability of the *TtCBL* gene family and supported the maintenance of selective pressures on their critical functions in calcium signaling and stress responses.

## 3.4 | Subcellular location of TtCBLs

This suggested that the subcellular localizations of TtCBL proteins are diverse among the cellular compartments, supporting their functional diversity. Most of the TtCBL proteins were predicted to be localized in the cytoplasm and chloroplasts, although several predictions have shown them present in the mitochondria and nucleus. Certain proteins were associated with the endoplasmic reticulum, vacuole, plasma membrane, and apoplastic spaces and thus were involved in intercompartmental signaling (Figure 3b).

Comparing the subcellular localization patterns among phylogenetic groups, distinct preferences were observed. For example, Group 1 proteins were mainly targeted to mitochondria and chloroplasts, while Group 2 members were mostly nuclear or chloroplastic. Group 3 and Group 4 proteins showed dual localizations, including in the cytoplasm and nucleus (Table S2), suggesting their possible role in integrating intracellular and extracellular calcium signals during abiotic stress responses. These results support that subcellular localization has been a major determinant of functional diversification within the *TtCBL* gene family, hence allowing these proteins to mediate calcium-dependent signaling across several cellular contexts. This functional plasticity may form the very basis of durum wheat's responsive capabilities to a wide

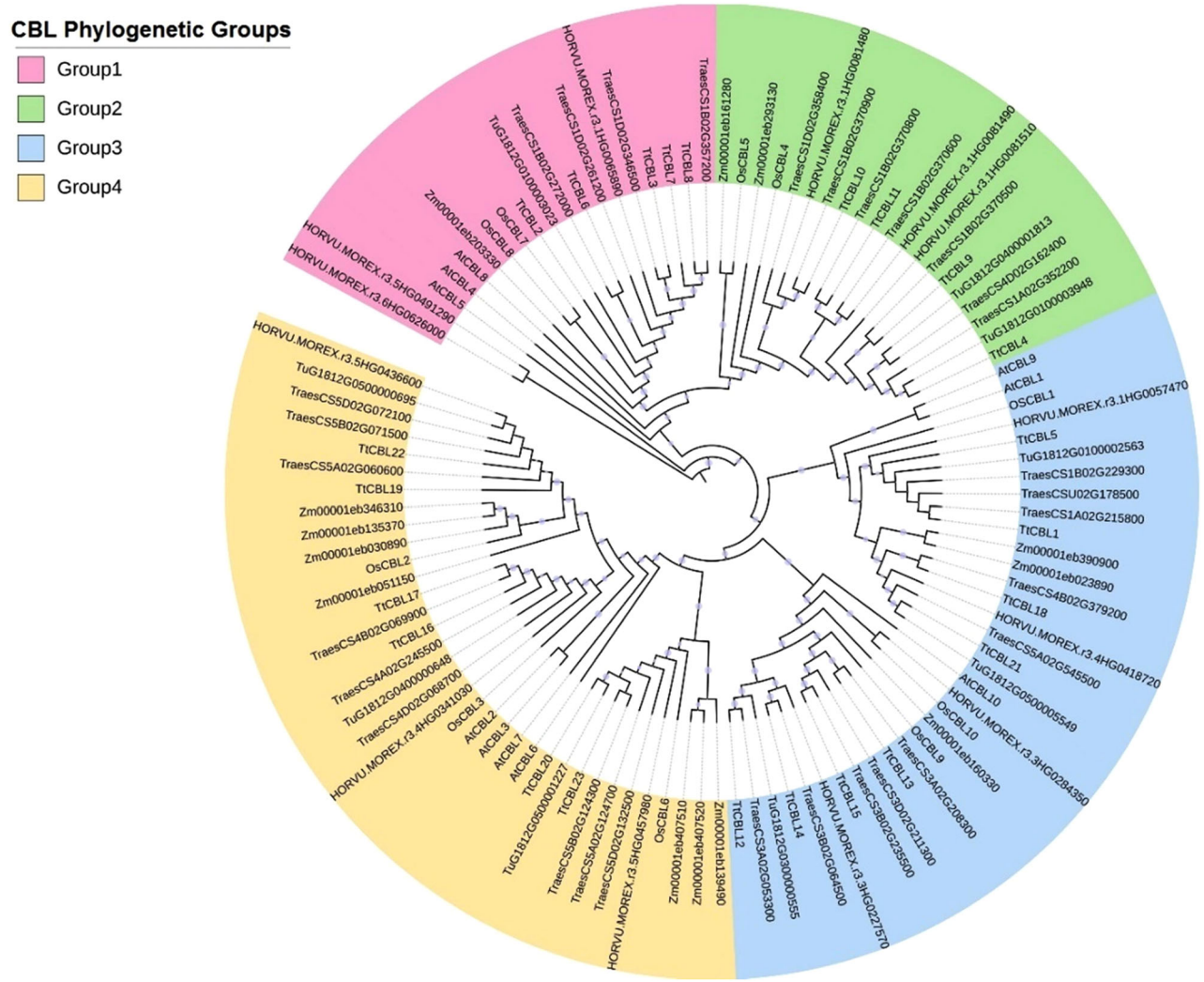


**FIGURE 1** Phylogenetic tree of durum wheat, bread wheat, barley, corn, red wild einkorn wheat, rice, and *Arabidopsis* calcineurin B-like (CBL) proteins.

array of environmental challenges and strengthens the role of *TtCBL*s in stress tolerance.

## 3.5 | Structure of *TtCBL* genes and distribution of conserved domains

Structural analysis of the 23 *TtCBL* genes showed that their exon–intron structure is highly conserved, with several exons ranging from seven to nine (Figure 4a). These structural uniformities suggest that during evolution, *TtCBLs* may be conserved and imply functional stability over time. In Group 2, most of the seven exon genes remained constant, while Groups 3 and 4 varied widely, even including some with nine exons. These structural variations might contribute to the functional diversification among the *TtCBLs*, particularly for stress-specific signaling roles.

Bioinformatics analysis of the protein sequences demonstrated that all TtCBLs have the common calcium-binding domains of EF-hand motifs (Figure 4b), one of the main characteristic calcium-binding domains that determines their role in signal events. Most of the members have two EF-hand structures. However, a few gene members, including the *TtCBL1*, *TtCBL4*, *TtCBL5*, *TtCBL18*, and *TtCBL21*, were predicted to hold only a single EF-hand calcium-binding structure. The expression of various numbers of single EF-hand-containing *TtCBL* members suggested variation in affinity to calcium binding through this gene family and perhaps modification of its current regulatory role. Notably, all *TtCBL*s shared high conservation of the EF-hand motifs, underlining their role in specificity related to calcium signaling. Further, the spatial distribution of these motifs within the different groups showed partial support for subcellular localization patterns and a role for specific stress.

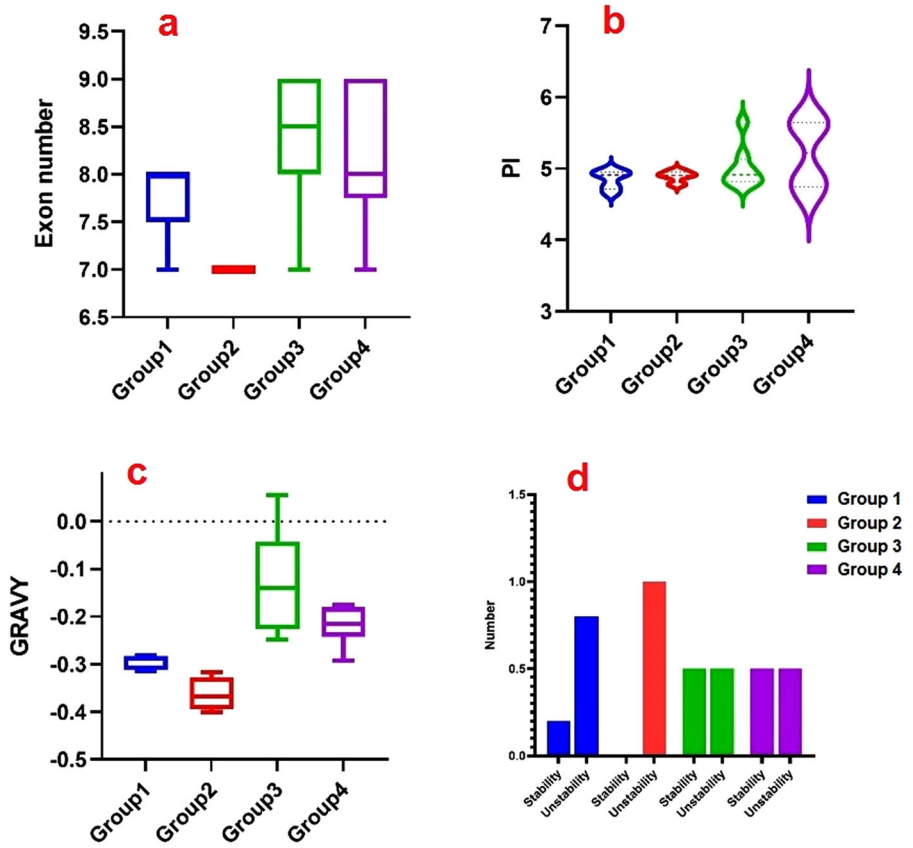


**FIGURE 2** General information of *Triticum turgidum* CBL (TtCBL) phylogenetic groups.

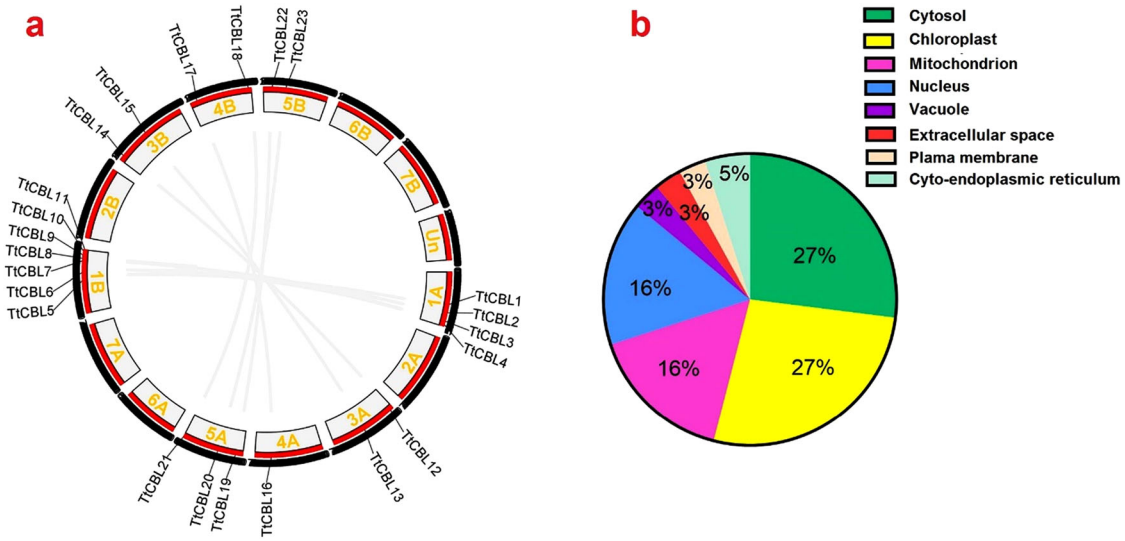


**FIGURE 3** Identification of *Triticum turgidum* CBL (TtCBL) gene locations on chromosomes and assessment of gene duplication events. Full details of duplication events are provided in Table S3. Chromosome numbers are labeled within each bar. Frequency of TtCBLs in different subcellular locations.

For instance, some features in EF-hand arrangement were noticed for proteins from Group 3, with both cytoplasmic and nuclear localizations, which might be responsible for the cross-compartmental signal transduction.

## 3.6 | 3D structure of TtCBLs

Homology modeling of the 3D structures of the 23 TtCBL proteins indicated group-specific changes in their structural

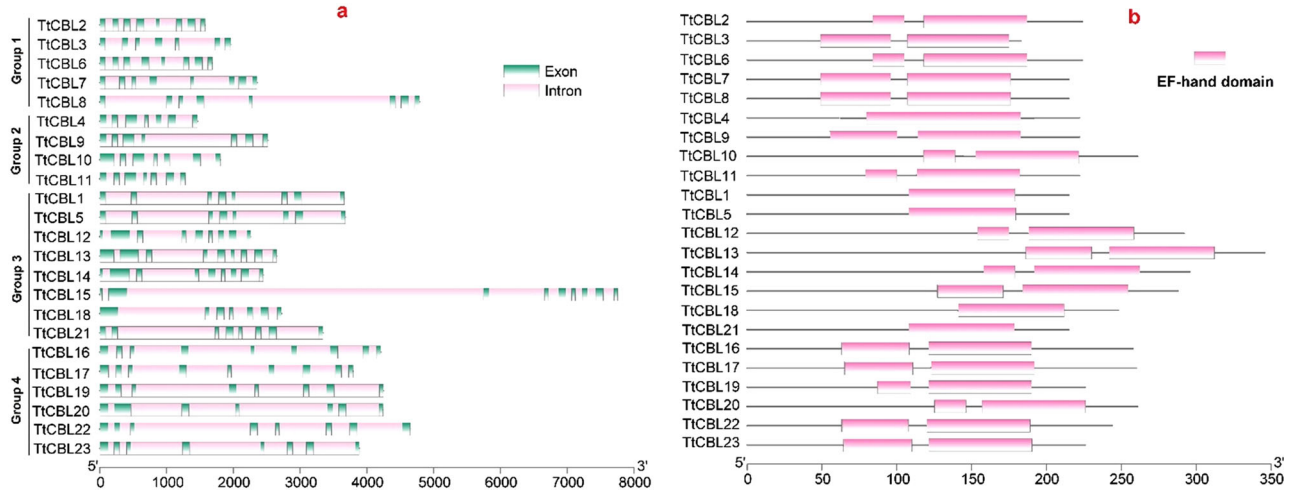


**FIGURE 4** The arrangement of exons and introns in the *Triticum turgidum* CBL (TtCBL) gene family (a), conserved EF-hand domains in TtCBL amino acid sequences (b).

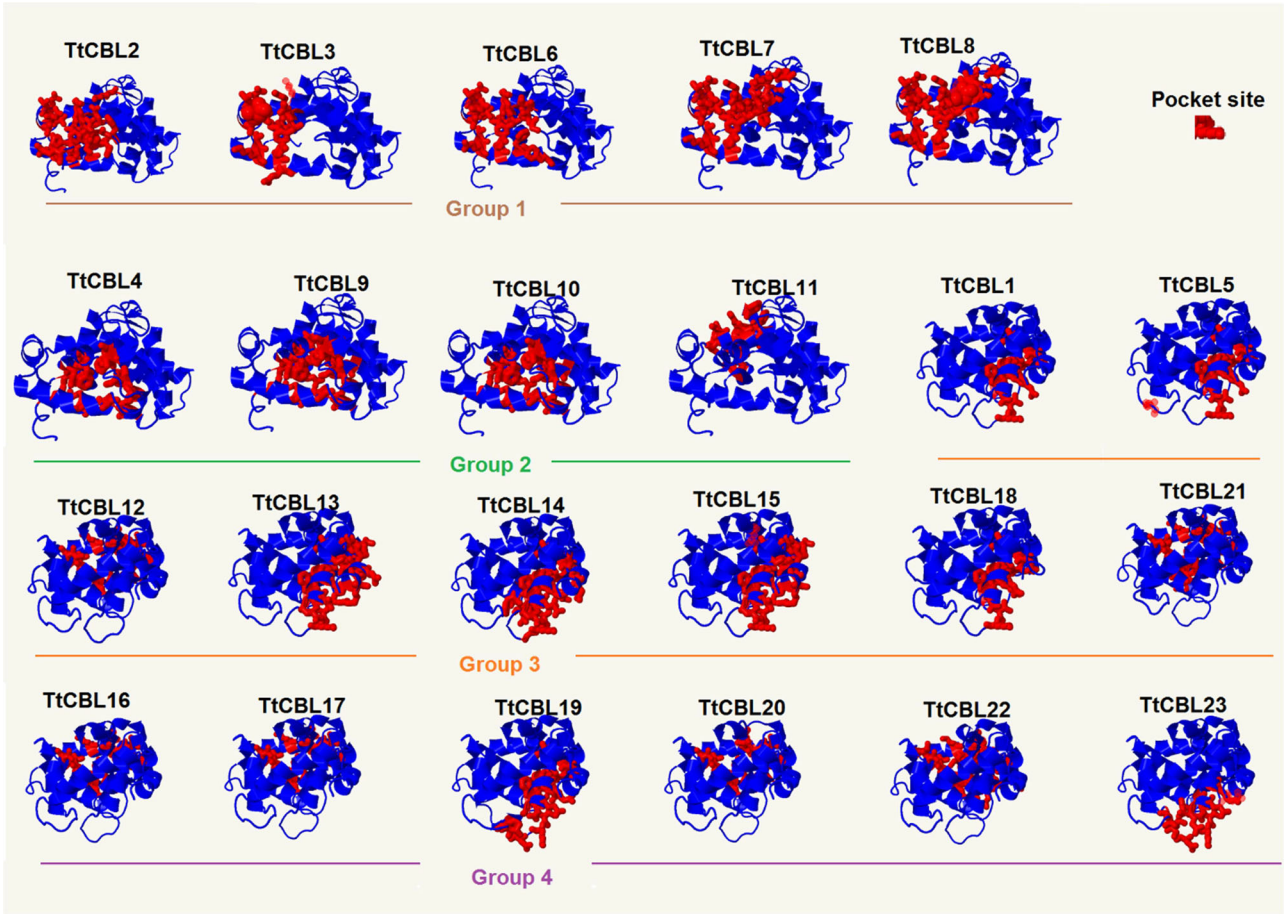


**FIGURE 5** Three-dimensional (3D) structure of *Triticum turgidum* CBL (TtCBL) proteins. Ligand-binding regions (pocket sites) are marked in red on the structure. The grouping of proteins is based on the results of phylogeny.

features (Figure 5). All TtCBL proteins shared a common architecture corresponding to their established role as calcium sensors, characterized by a core structure that contains conserved EF-hand calcium-binding motifs. These motifs were located at strategic positions for effective coordination with calcium ions, necessary in signal transduction.

Phylogenetic groups were structurally diverse, mainly in EF-hand motif arrangements and overall binding pocket

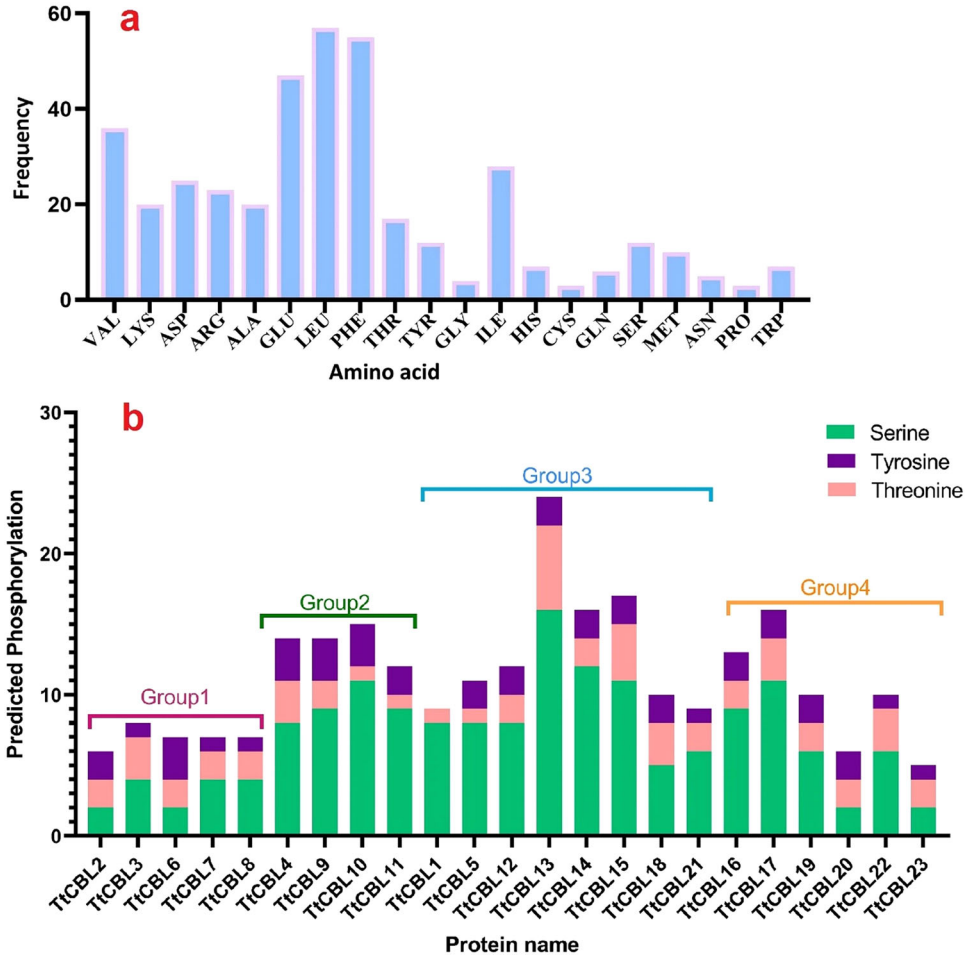


**FIGURE 6** Occurrence of amino acids located within the binding sites of *Triticum turgidum* CBL (TtCBL) proteins (a). The phosphorylation regions in TtCBLs for serine, tyrosine, and threonine residues across phylogenetic groups (b).

configurations. Overall, proteins within Groups 3 and 4 shared closely related structural features, which implied similar functional properties. However, TtCBLs from Groups 1 and 2 displayed individual conformations that might refer to their specialized functions during calcium-mediated signaling pathways. A detailed analysis of the ligand-binding pockets across the TtCBLs indicated differences in their size, shape, and composition, which are crucial for interaction specificity. Among such amino acids, leucine, phenylalanine, glutamic acid, valine, and isoleucine are the most abundant (Figure 6a). Such amino acids often participate in important interactions that stabilize the interaction with ligands and are thus important for signal fidelity during abiotic stress responses.

The spatial arrangements of the ligand-binding pockets also vary between groups. For instance, proteins of Group 3, associated with cytoplasmic and nuclear localization, usually contain more expanded binding pockets that can potentially accommodate a wider spectrum of interaction partners. At the same time, members of Group 1 contain more compact pockets and fit well with the predicted mitochondrial and chloroplastic localization while carrying specialized signaling functions.

## 3.7 | Prediction of phosphorylation sites

Phosphorylation, a critical posttranslational modification, was predicted in all 23 TtCBL proteins to understand their regulatory roles in signal transduction pathways. Using NetPhos 3.1, potential phosphorylation sites at serine, threonine, and tyrosine residues were identified, which have been reported to be pivotal in protein activation and signaling under abiotic stresses. Most of the targeted sites were found to belong to serine residues; the others corresponded to threonine and tyrosine in that order. Most interestingly, TtCBL13 showed a profoundly greater number of phosphorylation events, as indicated on serine and threonine residues that were distributed along its sequence. TtCBL1 in the third phylogenetic group is only phosphorylated at serine and threonine. This might have therefore shown that it possesses some rigid regulatory role and has not much flexibility in its functioning

compared with the other members of its group. In other TtCBLs, phosphorylation is predicted in all three amino acids (Figure 6b).

The distribution of phosphorylation sites differed among the phylogenetic groups. Proteins in Groups 3 and 4 had the highest density of phosphorylation sites, consistent with their established roles in dynamic and stress-responsive signaling pathways. A more specialized signaling function was represented by a smaller number of phosphorylation sites within Group 1 proteins, the majority of which are localized in organelles such as chloroplasts and mitochondria.

## 3.8 | Upstream analysis of *TtCBL* genes

The promoter of *TtCBL* genes was examined to identify the known regulatory elements, and based on their role, they were divided into three categories related to hormones, stress, and light and growth (Figure 7). In stress-related elements, MYB motifs that respond to stress are the most abundant (Figure 7a). Among the stress response elements, two CREs, W-box, and MBS, which are related to drought stress, were identified. W-box *cis*-element in *TtCBL4* in the second group and MBS *cis*-element in *TtCBL13* in the third group are more frequent. Overall, CREs related to phytohormones were more abundantly distributed in the promoter of *TtCBL* genes (Figure 7b). It was found that the most CREs were related to hormones, among which the elements responsive to ABA and methyl jasmonate (MeJA) were the most frequent (Figure 7c). The high abundance of ABA- and drought-related motifs, along with the presence of stress-specific elements, underlines further the importance of *TtCBL* genes in mediating abiotic stress responses. Furthermore, the multitude of CREs related to hormonal and environmental signaling pathways suggested that *TtCBL*s function as integrators of multiple signals, which allow durum wheat to adapt to complex conditions of stress.

## 3.9 | RNA-seq data of *TtCBL* genes

We further analyzed the RNA-seq data to explore the expression patterns of *TtCBL* genes under abiotic stresses, including cold stress at 5°C and increased temperature at +2°C (Figure 8). This clearly showed that the transcriptional activities of *TtCBL* genes have changed significantly under abiotic stresses, indicating their stress-responsive roles. Among the upregulated genes in cold stress response, *TtCBL10* demonstrated very high induction due to cold stress, which could indicate that this gene has a potential role in the calcium-mediated mechanism for cold adaptation. A very high decrease in the level of the *TtCBL6* transcript suggested that it was little involved or negatively controlled in the cold stress condition. Among upregulated genes in increased temperature response, *TtCBL13* showed the highest expression under high-temperature treatment and thus may play a significant role in heat stress tolerance. The downregulated genes include *TtCBL2*, which was highly downregulated, indicating a contrasting regulatory role in the case of heat stress compared to cold conditions. These expression patterns indicate functional diversity among the *TtCBL* genes and suggest that some members may contribute more to the temperature stress response.

## 3.10 | Expression of *TtCBL* genes in response to drought stress and ABA treatment

### 3.10.1 | Expression profiles in root tissues

Expression analysis of *TtCBLs* in root tissues subjected to drought stress and ABA treatment showed differential temporal regulation (Figure 9). Expression of *TtCBL2* and *TtCBL10* was significantly induced within 1 h of stress at $p < 0.05$, which continued to be upregulated until 72 h. Application of ABA increased their expression at all tested points, which shows the involvement of ABA in the enhancement of drought responsiveness. *TtCBL12* transcript was significantly enhanced 24 h after the drought stress treatment. Application of ABA further enhanced its transcription at 24 and 72 h after drought stress ($p < 0.05$), suggesting it may contribute to ABA-mediated stress adaptation. The expression of *TtCBL19* gene was induced at 24 and 72 h after drought stress. This gene further showed increased expression with ABA treatment under all conditions, indicating that it may be involved in long-term stress responses.

### 3.10.2 | Expression profiles in shoot tissues

The expression of *TtCBL* genes in shoot tissues was dynamically regulated, as opposed to root tissues (Figure 9). *TtCBL2* gene was induced by short-term drought treatment at 1 and 24 h and was strongly downregulated after 72 h ($p < 0.05$); its expression was induced by the ABA treatment under a control condition but then reduced at 24 and 72 h of drought stress, suggesting a tissue-specific control. *TtCBL12* expression was significantly enhanced only after 72 h of drought stress and did not respond effectively to ABA application, indicating its late response in the shoot tissues. *TtCBL19* gene demonstrated complex expression patterns, with highly transcribed conditions at 1 and 24 h after drought stress; by 72 h it decreased. ABA application highly enhanced its expressions after drought stress, with massive accumulations 1 and 72 h after drought stress ($p < 0.05$).

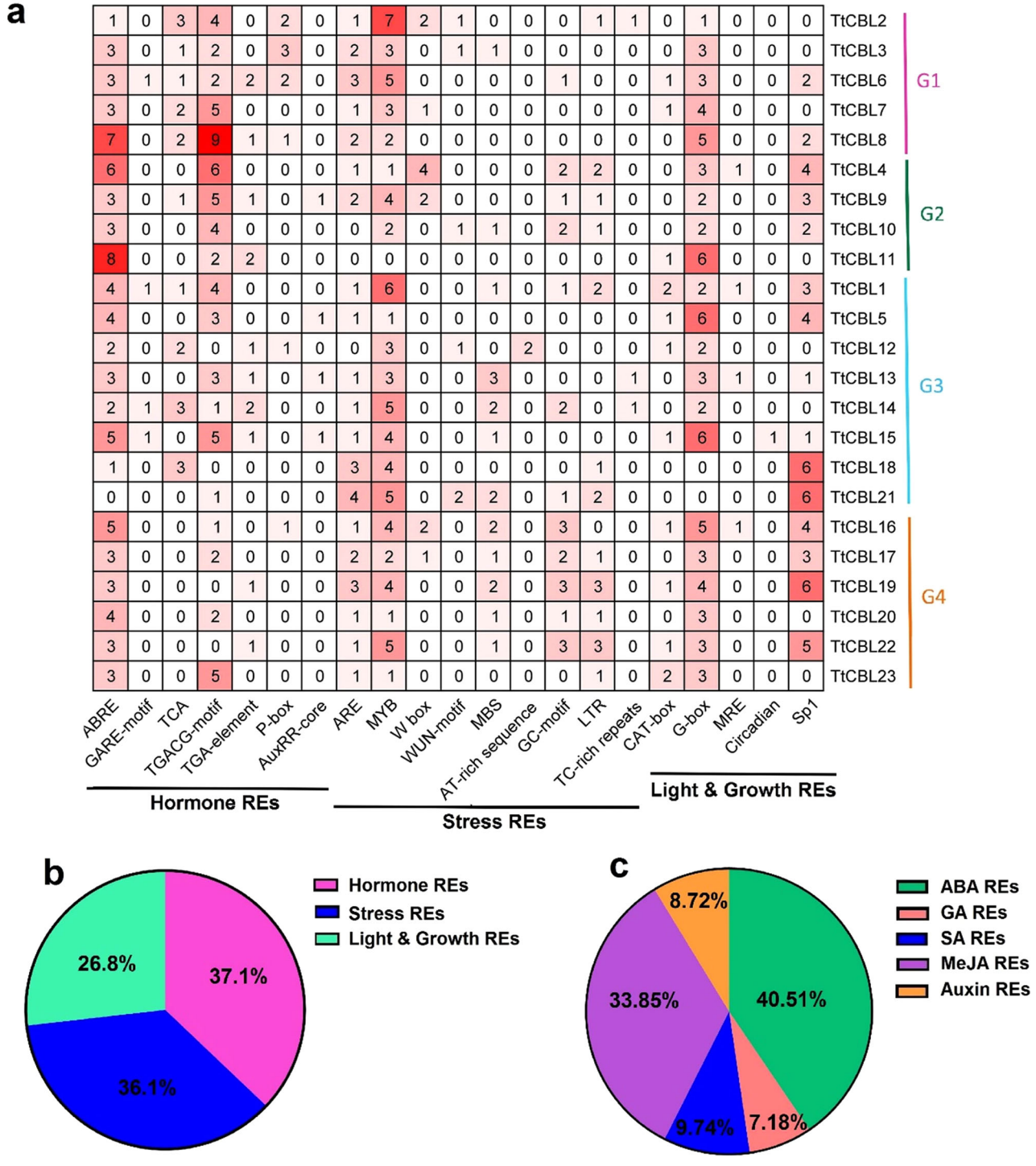


**FIGURE 7** Promoter region *cis*-elements of *Triticum turgidum* CBL (TtCBL) gene family members (a). Pie chart representing the percentage of *cis*-regulatory elements responsive to physiological events in the promoters of *TtCBL* genes (b). Pie chart for identifying the *cis*-regulatory elements associated with different plant hormones in *TtCBLs* (c).

## 3.11 | TtCBL interaction network

The interaction network showed that there is a strong connection between signal transduction and phosphorylation pathways and TtCBL (Figure 10). For example, CIPKs involved in signal transduction pathways dependent on cytosolic calcium changes showed the highest number of interactions with TtCBLs. In addition, a strong interaction was observed between TtCBLs and potassium transporters such as HAK5, SOS1, and AKT1. These results show that TtCBLs are involved in the response to salinity and drought stress. Besides, GO enrichment analysis was performed to determine

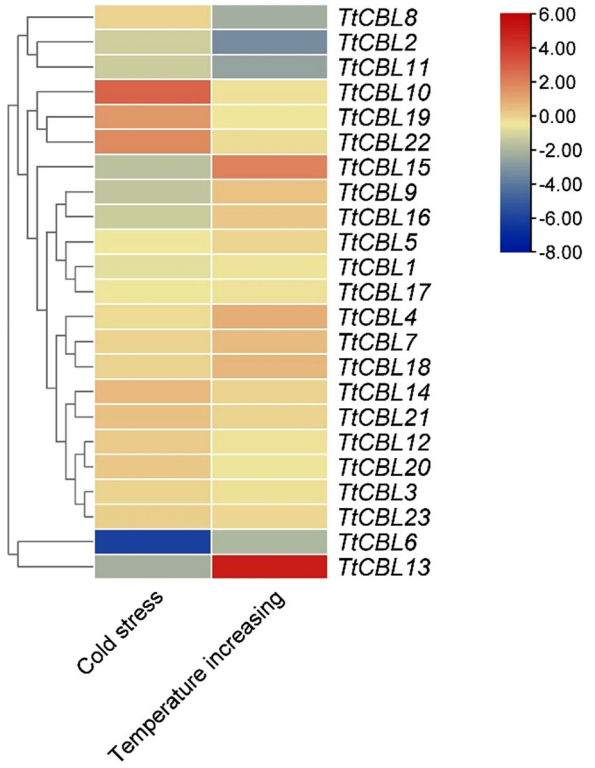


**FIGURE 8** Expression profile of *Triticum turgidum* CBL (*TtCBL*) genes in response to cold stress and face to increased temperature (+ 2°C) based on RNA-seqence (RNA-seq) data.

the biological processes, molecular functions, and cellular component terms significantly linked with the TtCBL network nodes (Table S4). The molecular function terms, including protein serine/threonine kinase activity, ion/calcium/ATP binding, and catalytic activity, were significantly linked with the TtCBL network. The cellular component terms, such as vacuole, cytoplasm, and cellular anatomical entity, were significantly enriched. In addition, several biological process terms, including signal transduction, protein phosphorylation, calcium-mediated signaling, and potassium ion transmembrane transporter, were strongly linked with the TtCBL network. The interaction network revealed that TtCBLs cooperate in ion binding and signal transduction with other key stress response factors.

## 4 | DISCUSSION

The CBLs are pivotal calcium sensors that decode calcium signals into adaptive physiological and developmental responses in plants (Jiang et al., 2020; Sanyal et al., 2020; Tang et al., 2020; Tong et al., 2021). In this context, durum wheat has emerged as a nutritionally rich, stress-resilient crop of global significance (Maccaferri et al., 2019). Drought severely affects durum wheat by reducing the height of the plant, number of tillers, leaf area, and chlorophyll content, all of which are essential factors in growth and photosynthesis. It also lowers grain yield and the number of grains per spike, ultimately compromising productivity. These factors make it difficult to cultivate wheat in regions with low water availability. Some durum genotypes are less affected by increased water content and have better photosynthetic efficiency during stress. This difference is helpful in breeding programs (De Santis et al., 2021; Pour-Aboughadareh et al., 2020). These observations emphasize the power of choosing a set of characteristics to breed drought-resistant varieties in support of food security and sustainable agriculture in areas prone to drought. A total of 23 *TtCBL* genes identified in durum wheat underscore both the evolutionary conservation and functional complexity of this gene family. In contrast to the 10 *CBL* genes reported in diploid species such as *A. thaliana* and *O. sativa* (Kanwar et al., 2014; Kolukisaoglu et al., 2004), polyploid crops like durum and bread wheat possess larger *CBL* gene families, suggesting expansion associated with increased functional demand for stress adaptation. For instance, bread wheat contains 24 *CBL*s across its A, B, and D sub-genomes (T. Sun et al., 2015), while diploid barley (*H. vulgare*) and rice harbor only 10 *CBL*s each (Jiang et al., 2020). Interestingly, comparative evolutionary analysis suggests that the number of *CBL* genes is not directly correlated with genome size across species (Mohanta et al., 2015). Comprehensive in silico analyses were executed on the members of the *TtCBL* gene family. The analysis of their phylogeny indicated that these TtCBLs were to fall into four phylogenetic clusters. Gene duplication has generally been considered one of the major sources of genetic novelty that may give rise to new genes, which subsequently evolve to fulfill new functions useful for crop improvement (Du et al., 2023). Segmental duplication events have been considered one of the major driving forces for evolutionary expansions of the *TtCBL* gene family in durum wheat. However, analysis of the Ka/Ks ratio indicates that duplicated *TtCBLs* have undergone negative selection.

It has previously been demonstrated that proteins of the *CBL* family are localized in diverse cellular regions such as cytoplasm, nucleus, plasma membrane, and vacuole (Sanyal et al., 2015). However, for durum wheat, our predictions indicate that TtCBL proteins localize mainly to the cytosol, chloroplast, nucleus, and mitochondria. This difference in localization could be due to species-specific functional diversification or adaptation toward different signaling environments in plant cells. Localization is regulated by several factors like p*I*, structural motifs, and signaling peptides (Kiraga et al., 2007; Tokmakov et al., 2021). For example, it has been found that cytoplasmic proteins showed distinct clustering at p*I* 5.0–6.0 (Schwartz et al., 2001). Several studies suggested that discrete p*K*a values for various amino acids may underlie p*I* multimodality in various proteomes (Garcia-Moreno, 2009; Kozlowski, 2021; Wu et al.,

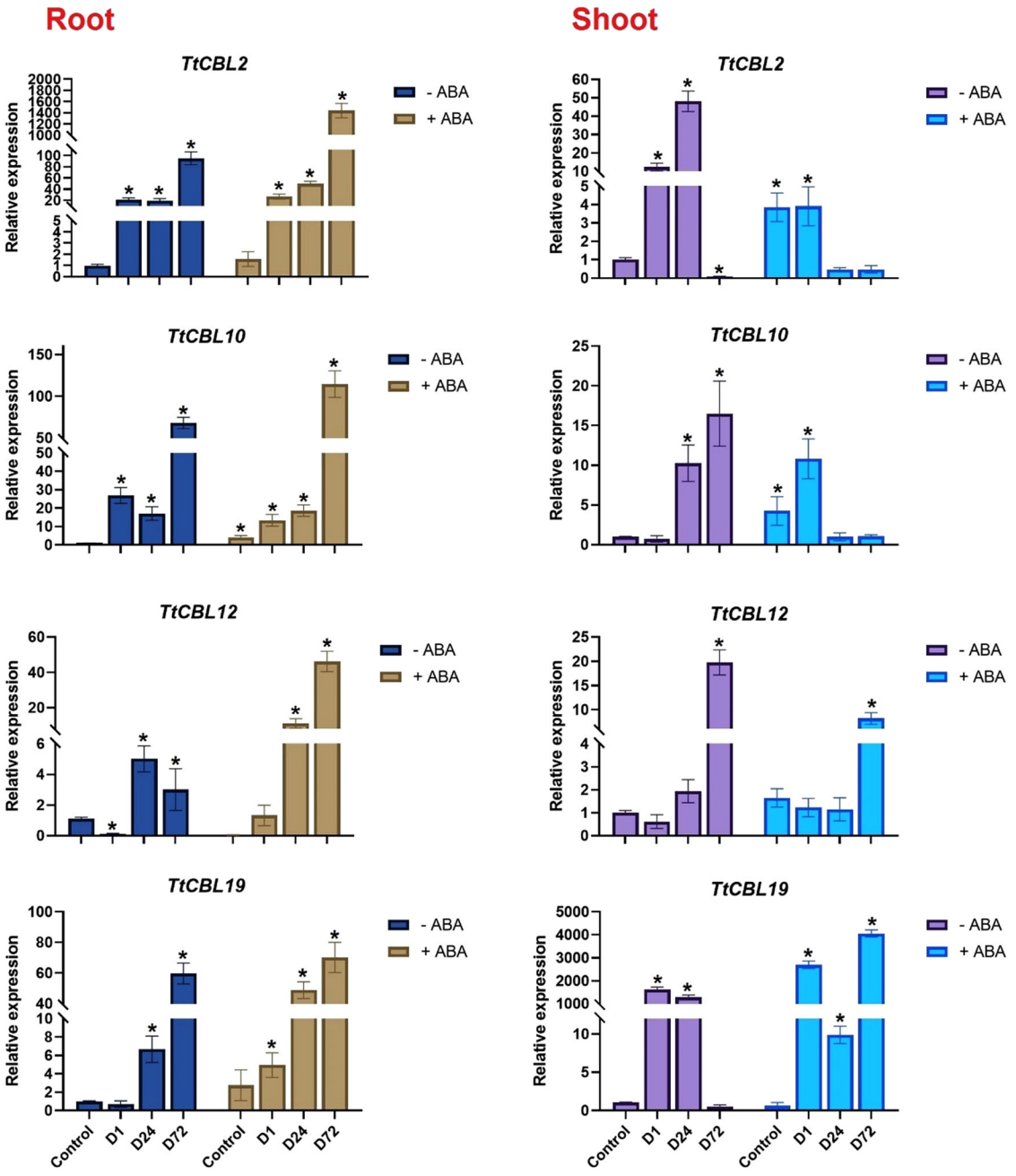


**FIGURE 9** Expression profile of *Triticum turgidum* CBL (*TtCBL*) genes in response to drought stress (D) and application of abscisic acid (ABA) (+ABA). D1, D24, and D72 are shown the drought stress after 1, 24 and 72 h, respectively. The asterisk above each column indicates a significant difference ($p$-value < 0.05) with the control.

2006). Future studies can study p*I* dynamics under different cellular states, for example, under stressed conditions, to further elucidate these interactions.

To study the *TtCBL* genes, we figured out where the exons and introns are based on the genetic information from durum wheat. *TtCBL* genes have exons that range from seven to nine. Among *TtCBL* genes, the most common structure has eight exons. Furthermore, in 15 different grass species, the majority of *CBL* genes contain seven introns (Jiang et al., 2020). A unique feature of CBLs is that their first EF-hand domain contains 14 amino acids, whereas in other $Ca^{2+}$ sensors, this domain contains only 12 amino acids (Beckmann et al., 2016). The proteins exhibited a fluctuation in the number of EF-hand domains, with counts ranging from one to six (Day et al., 2002). Investigation of TtCBL peptide sequences showed that most of the CBL proteins in *T. turgidum* contained two EF-hand domains. The conserved structural features and domain organization of the TtCBLs point toward their evolutionary importance concerning calcium-mediated stress signaling. Differences in the arrangements of EF-hand motifs,

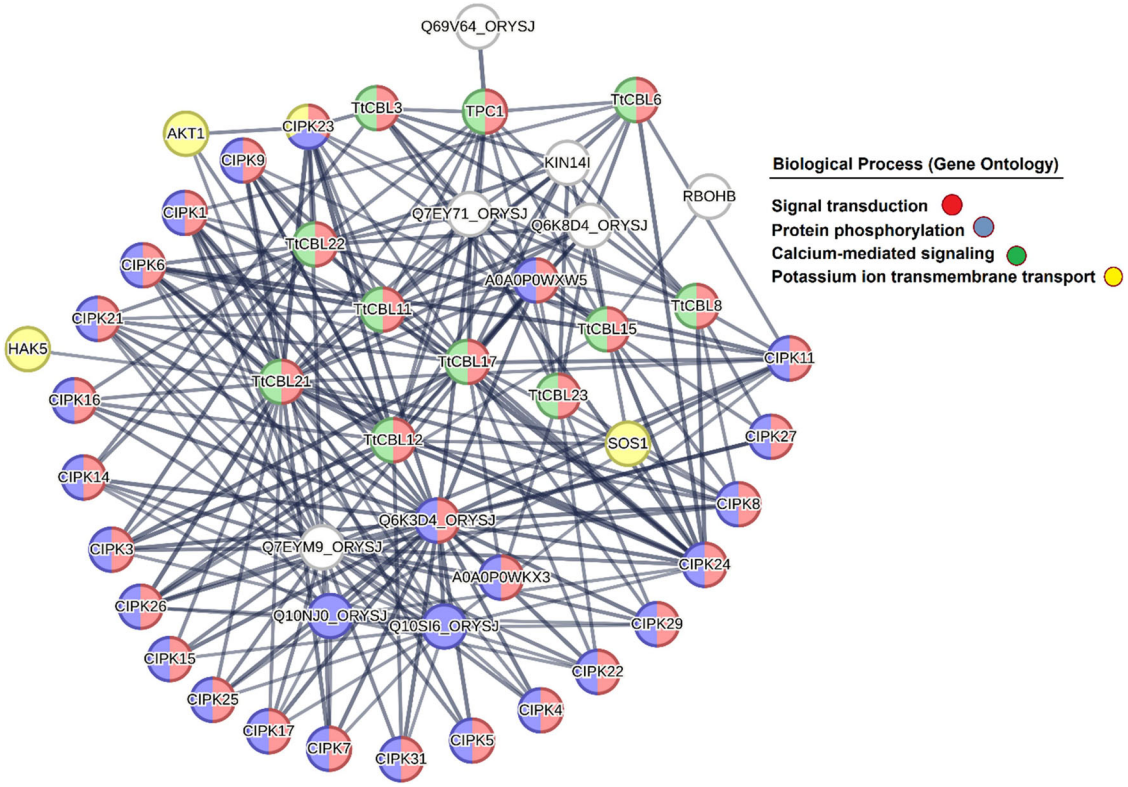


**FIGURE 10** Interaction network of *Triticum turgidum* CBLs (TtCBLs) based on available data from rice genome. The significant gene ontology (GO) terms are provided in Table S4.

exon–intron structures, and the structural features of the ligand-binding pocket suggest that the TtCBLs are fine-tuned toward the mediation of stress-specific responses, including drought and salinity tolerance.

The GRAVY index quantifies protein hydrophobicity and hydrophilicity based on amino acid sequences, taking into account protein charge and size, with scores ranging from negative to positive (Kyte & Doolittle, 1982; Rafique & Nasrullah, 2020). The positive score indicates hydrophobicity, and the most hydrophobic proteins are intrinsic plasma membrane proteins (Rafique & Nasrullah, 2020; Rustagi et al., 2023). Proteins with negative values of GRAVY are highly hydrophilic, and this renders them more soluble in aqueous solutions. Such hydrophilicity is crucial to their biological activity and interactions (Dunker et al., 2001; Jault et al., 2013; Polańska et al., 2024; H. Wang et al., 2021). The GRAVY index for protein hydrophobicity revealed that the majority of TtCBLs are hydrophilic, and such a property could contribute toward increasing their aqueous cellular environment solubility and interaction propensity (Kyte & Doolittle, 1982; Rafique & Nasrullah, 2020). This resonates with LEA proteins, which have a strong hydrophilic tendency and are responsible for drought stress tolerance due to their protective roles (Li et al., 2018). Phosphorylation, one of the key posttranslational modifications, mediates plant stress responses by regulating signaling pathways, such as those of the CBL-CIPK module (Song et al., 2022; Zhang et al., 2025). Differential phosphorylation capacities among TtCBL proteins are revealed by our analysis, with TtCBL13 holding the top position, indicating that they have a key function in stress signal transmission. The patterns indicate a complex mechanism for stress adaptation in durum wheat based on calcium.

*TtCBL* promoters contain *cis*-regulatory factors such as ABA-responsive motifs (ABRE), MeJA-response motifs (TGACG-motif), salicylic acid, auxin, and gibberellin (GA). What deserves particular mention are the MYB and W-box abundant motifs, which point to their possible contribution toward abiotic stress responses. For example, the promoters of *TtCBL2* and *TtCBL10* showed a lot of elements that respond to stress and hormones, suggesting they are involved in how plants deal with drought and ABA signaling. Under drought and high-salinity stress conditions, plants have two pathways: ABA-independent and ABA-dependent. ABA-dependent pathways induce the expression of stress-response genes by activating the AREB/ABF transcription factors. In contrast, the ABA-independent pathways involved in stress-related transcription factors, including MYB, MYC, NAC, and WRKY, bind to *cis*-elements associated with stress response genes and ABA, leading to stress-related protein expression. (Singh & Laxmi, 2015). These proteins include various functional classes, including osmoprotectants (e.g., LEA proteins), antioxidant enzymes (e.g., SOD, APX, CAT), and other stress-responsive factors like chaperones, dehy-

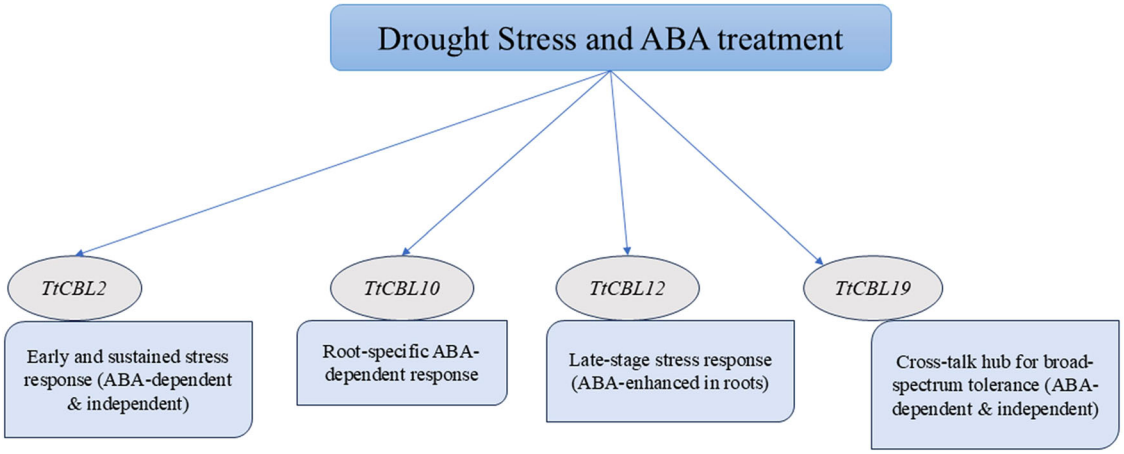


**FIGURE 11** Predicted roles of *Triticum turgidum* CBL (*TtCBL*) genes in abscisic acid (ABA) signaling pathways based on tissue-specific expression profiles under drought and ABA treatments.

drins, and ion transporters (Kaur et al., 2014; Szlachtowska & Rurek, 2023; Vaseva et al., 2012). CBLs regulate ABA-dependent and ABA-independent ABA-signaling pathways during drought stress by interacting with CIPKs (Kaya et al., 2024; Ma et al., 2020). For instance, the CBL1/9-CIPK1 module in *Arabidopsis* downregulates drought stress through phosphorylation of the PYLs ABA receptor (You et al., 2023).

The combined effects of drought stress and ABA-induced expression patterns of *TtCBL*s show that *TtCBL*s may play different roles at different times, in different tissues, and through different pathways to help plants adapt to stress. These results also reflect the functional diversities of *TtCBL*s in drought signaling and their potential to engineer enhanced drought tolerance in plants.

Both root and shoot tissues consistently documented the induction of *TtCBL2* under drought stress. The *TtCBL2* gene's induction, synergistically brought about by ABA in the roots, underlines its important role in the ABA-dependent signaling network responsible for drought responses (Figure 11). Strong *TtCBL2* activation shortly after drought stress starts indicates that it plays an early role in how shoots respond to stress. The gene may function in ABA-dependent and ABA-independent pathways; such dual regulation underlines its versatility in responding to stress. It is therefore considered a good candidate for genetic engineering in the improvement of drought tolerance, as its activity in early responses to stress and sustained signaling has been confirmed.

*TtCBL10* is mainly expressed in the roots and is significantly induced under drought stress. Moreover, ABA treatment further enhanced this drought-induced induction, firmly positioning it as a contributor to root-specific ABA-dependent pathways. The evidence suggests that the strong influence of ABA on transcription in the present study assigns the gene to the group of ABA-dependent genes and underlines its critical importance for root-mediated drought responses.

*TtCBL12* shows late but prominent upregulation, especially in roots, and increases even more when ABA is applied to them (Figure 11). *TtCBL19* is expressed in a complicated way, showing higher levels of transcription in both roots and shoots, particularly during extended drought stress. *TtCBL19* is complexly expressed, showing a transcriptional increase in roots and shoots, especially under prolonged drought stress. *TtCBL19* is likely an integrator of signals emanating from ABA-dependent and ABA-independent pathways; hence, it serves as a cross-talk hub in calcium signaling. Its responsiveness to both ABA and drought stress in different tissues raises its potential for broad-spectrum stress tolerance.

The patterns of *TtCBL2*, *TtCBL10*, *TtCBL12*, and *TtCBL19* in different tissues and over time during drought and ABA treatment show that they work together in a system that includes both ABA-dependent and independent pathways, suggesting they could be specifically improved for better drought tolerance. Despite these findings, the present work heavily depends on in silico predictions and transcriptional profiling. To confirm how *TtCBL*s help wheat adapt to salt and heat stress, future studies will need to include gene knockout/overexpression and interaction tests with the CIPKs. Future studies on salt and heat stress would help confirm how *TtCBL*s can be used to create wheat varieties that can handle multiple stresses.

## AUTHOR CONTRIBUTIONS

**Hadiseh Sadat Hosseini Pouya**: Conceptualization; data curation; formal analysis; investigation; methodology; writing—original draft. **Monireh Cheniany**: Conceptualization; investigation; validation; writing—review and editing. **Parviz Heidari**: Conceptualization; data curation; formal analysis; investigation; methodology; validation; visualization; writing—original draft; writing—review and editing.

## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

Data are available upon request with corresponding author.

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## SUPPORTING INFORMATION

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