



Cloning and characterization of nutrient deficiency and salinity stress responsive *TaCBL4* gene from bread wheat (*Triticum aestivum* L.)

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Abstract

Environmental stresses, such as drought, salinity, extreme temperatures and nutrient deficiencies affect plant productivity adversely. Calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases (CIPKs) are components of calcium mediated stress response pathways in many plants. In the present study, *TaCBL4* gene homologous to salt stress responsive *Arabidopsis CBL4* (*AtSOS3*) was cloned from wheat and sequence characterized. The predicted protein of *TaCBL4* showed 95, 83, 70 and 61% similarity with amino acid sequences of barley, rice, maize and *Arabidopsis CBL4*, respectively, demonstrating that *CBL4* genes are conserved between dicots and monocots. *TaCBL4* protein consists of three EF-hand calcium binding motifs, a characteristic feature of calcium sensor proteins. The modelled image of *TaCBL4* protein was highly identical to the already resolved structure of the *AtCBL4* protein. In addition to salinity stress, the expression of *TaCBL4* gene was also up-regulated by deficiency of major nutrients, namely, nitrogen, phosphorus and potassium. This suggests that *TaCBL4* may serve as node for signaling crosstalk between salinity and nutrient deficiency stress signaling pathways and nutrient homeostasis under salinity stress. The present study lays foundation for functional studies on role of *TaCBL4* gene in nutrient use efficiency and for its further utilization in breeding new wheat varieties with enhanced nutrient use efficiency and salinity tolerance.

Key words: Wheat, CBL, SOS, salinity, nutrient stress

Introduction

Worldwide wheat production is impacted by various abiotic stresses, like water deficit, salt, high temperature and nutrient deficiencies. Genetic improvement for abiotic stress tolerance in wheat can be greatly aided by knowledge of abiotic stress signaling. Calcium ions (Ca^{2+}) act as one of the second

messenger of signal transduction pathway by inducing specific cytosolic Ca^{2+} signatures in response to environmental cues (Steinhorst and Kudla 2013). These temporal and spatial attributes of specific calcium signatures are sensed by Ca^{2+} sensor protein families (Luan et al. 2002). There are three major Ca^{2+} sensor proteins in plants viz., calmodulins (CaM), calcium-dependent protein kinases (CDPK) and calcineurin B-like (CBL) proteins (Pandey et al. 2004). CBLs are similar to neuronal calcium sensors in animals; they interact with a group of serine/threonine kinases designated as CBL-interacting protein kinases (CIPKs) (Luan et al. 2002). Both CBL and CIPK are encoded by multiple genes and the formation of different CBL/CIPK complexes constitutes a specific regulatory network of Ca^{2+} signaling in plant cells (Luan 2009). Genome-wide analysis has identified 26 CIPKs in *Arabidopsis*, 33 CIPKs in rice, 27 CIPKs in poplar and 43 CIPKs in maize (Kudla et al. 1999; Batistic and Kudla 2004; Kolukisaoglu et al. 2004) The CBL/CIPK network intervenes diverse signaling pathways and first well defined CBL/CIPK pathway was identified by screening *Arabidopsis* mutants for a salt overly sensitive (SOS) phenotype (Dodd et al. 2010). This pathway starts with *SOS3* (*CBL4*), which acts as Ca^{2+} sensor and further activates and recruits *SOS2* (*CIPK24*) to plasma membrane where they phosphorylates and activates *SOS1*, a plasma membrane bound sodium (Na^+)/proton (H^+) antiporter (Liu et al. 2000; Yang et al. 2009). Xu et al. (2006) demonstrated that the *Arabidopsis CBL1/CBL9/CIPK23* complex phosphorylates the potassium (K^+) transporter AKT1 and enhance high affinity K^+ uptake. Recently Liu et al. (2013) also reported that CIPK9

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and *CBL3* work together and function in potassium homeostasis under low- K^+ stress in *Arabidopsis*. Further study revealed that *CBL10* directly interacts with K^+ channel (AKT1) and modulates its activity in regulating K^+ homeostasis in *Arabidopsis* under ion stress conditions in a CIPK-independent manner (Liu et al. 2013). Similarly, *CBL2* and *CBL3* were also found to regulate the activity of Vacuolar-ATPase required for ion homeostasis and maintenance of turgor (Ren et al. 2013). Studies on CBL/CIPK pathways are limited in plants except for *Arabidopsis*. Gu et al. (2008) studied the expression pattern of rice CBL genes (*OsCBLs*) in response to environmental stresses and concluded that *OsCBL8* showed a salt stress responsive mechanism. Over expression of a maize *ZmCBL4* conferred salt tolerance to wild type and *sos3 Arabidopsis* mutant (Wang et al. 2007). In addition, over expression of *GmCBL1* from soybean also imparted drought and salinity stress tolerance and promoted hypocotyl elongation in *Arabidopsis* (Zhao et al. 2009). Zhang et al. (2014) identified and characterized seven CBL and 23 CIPK genes from *Brassica napus*. Chen et al. (2012) found that *Brassica CBL1/CIPK6* component regulate the plant response to abiotic stress and ABA signaling.

In comparison to other species, little is known about CBL-CIPK pathway in wheat. One of the CIPK components characterized in wheat is *WP4*, involved in light, nutrient deprivation and cytokinin signaling (Ikeda et al. 1999). Another wheat CIPK gene *TaCIPK29* conferred salt tolerance to transgenic *Arabidopsis* by improving the K^+/Na^+ ratios, Ca^{2+} content and by decreasing H_2O_2 accumulation and membrane damage (Deng et al. 2014). Also, ectopic expression of *TaCIPK14* conferred cold and salinity tolerance in tobacco (Deng et al. 2013). Although few evidences are reported in wheat for involvement of CBL-CIPK in response to various environmental stimuli and only limited number of *CBL* genes are characterised. Therefore, in the present study, an attempt was made to clone a putative *CBL4* homologue from bread wheat and analysed its expression pattern in response to salinity and different nutrient stresses.

Materials and methods

Plant growth conditions and stress treatments

For cloning complete cDNA sequence (CDS) of *TaCBL4* gene, bread wheat (*Triticum aestivum* L.) genotype Kharchia 65, was planted in 30 cm earthen pots filled with soil and FYM mixture. Plants were grown for 30

days with recommended practices. Salinity stress was imposed by irrigating the pots using 100mM NaCl solution. Uppermost expanded leaves were sampled after 24 hrs of treatment and immediately frozen and stored at $-80^\circ C$ until further use. To study the quantitative real time PCR (qRT-PCR) expression of *TaCBL4* gene under various nutrient and salinity stress, kernels Kharchia 65 were sterilized and germinated on sterile filter paper. After removing the endosperm, 5 day old seedlings were shifted to glass tubes containing 50 ml of Hoagland solution. After 3 days of growth in Hoagland solution, seedlings were subjected to nutrient stress by exposing to Hoagland solution without nitrogen (-N), phosphorus (-P), without potassium (-K), excess (25mM) magnesium (+Mg) ions and 100mM NaCl for 24 hours. Root tissue collected from each treatment and control (C) plants were flash frozen and stored at $-80^\circ C$ until further use.

RNA extraction and RT-PCR

Total RNA was extracted using RNeasy plant minikit (Qiagen Inc., Chatsworth CA 91311, USA, Cat No: 749040), followed by on-column DNA digestion with DNase I (Qiagen Science, Maryland, USA) to remove DNA contamination. For cloning of *TaCBL4*, wheat homologues were identified based on the sequence similarity to partial CDS of *TaCBL4* and *Oriza sativa CBL4* genes. These input sequences were used to identify complete gene sequences from wheat genome database (<http://wheat-urgi.versailles.inra.fr/Seq-Repository/BLAST>) and confirmed by comparing with expressed sequence tags (ESTs) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and tentative consensus sequences available with DFCI gene index (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=wheat>). Primers for amplifying complete *TaCBL4* CDS were designed and one-step RT-PCR was performed to amplify the CDS using Qiagen one-step Reverse Transcription-PCR (RT-PCR) kit on QN 96 Thermal cycler (Quanta biotech, England). To estimate the size, RT-PCR product was visualised on agarose gels stained with ethidium bromide using Alphamager® imaging system (Protien simple, USA).

Sequence analysis and Phylogenetic tree construction

RT-PCR products were purified following agarose gel electrophoresis and sequenced (Chromous Biotech Pvt. Ltd., Bengaluru, India). To study the sequence conservation and evolutionary relationship, phylogenetic tree of the candidate gene sequence and related sequences from other genera was generated

based on maximum likelihood method using Mega 6.0 software (Tamura et al. 2013).

Protein domain and structure analysis

To study the conservation of protein across genera, ClustalW2.1 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) multiple sequence alignment of deduced protein sequences from bread wheat and corresponding sequences from model organisms was prepared, the final alignment was visualized on Jalview (Clamp et al. 2004). Deduced protein sequences were analyzed for the presence of conserved domains using protein blast (<http://blast.ncbi.nlm.nih.gov/>) with default parameters. The protein structure of *A. thaliana* (Sanchez-Barrena et al. 2004) CBL4 protein sequence was used as template to model TaCBL4 protein structure. Protein model template sourced from Protein Database (PDB) server (<http://pdbeta.rcsb.org/pdb/Welcome.do>) was used to perform homology modeling in swissmodel (<http://swissmodel.expasy.org/>).

Expression analysis using qRT-PCR

Total RNA isolated from wheat root tissue subjected to various stresses were used for qRT-PCR expression analysis. RNA was quantified on agarose gel (1% w/v) and by thermo nanodrop 2000c spectrophotometer. DNase treated RNA was reverse transcribed using superscript III Reverse transcriptase kit (Invitrogen Life Technologies, USA). Gene specific primers were used in qRT-PCR using Power SYBR[®] Green Master Mix (Applied Biosystems, USA) on iQ[™] 5 real time PCR detection system (Bio Rad, USA). Melt curve data collection and analysis was enabled. Normalization of the data for each transcript was carried out using wheat *TaActin* as an internal control and level of expression were analyzed using 2^{-DDCt} method (Livak and Schmittgen 2001).

Results and discussion

Cloning and phylogenetic analysis of TaCBL4 gene

Recently, we have established that the differential transcript abundance of SOS pathway genes including *TaSOS1*, *TaSOS2* and *TaSOS3* (*TaCBL4*) is associated with salinity tolerance of bread wheat (Lekshmy et al. 2015). In the current study the partial CDS sequence of *TaCBL4* (Lekshmy et al. 2015) and *OsCBL4* (Martínez-Atienza et al. 2007) gene sequences were used as queries to identify wheat homologues in wheat genome database by tBLASTn (wheat-urgi.versailles.inra.fr/Seq-Repository/BLAST), and primers were

designed. Complete coding sequence (703 bp) of *TaCBL4* gene was cloned from salt tolerant wheat genotype Kharchia 65 by utilizing RT-PCR technique (Fig. 1). The chromosomal localization and physical

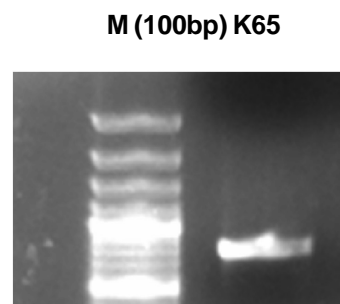


Fig. 1. RT-PCR amplification of complete cDNA of SOS3 (703bp) from bread wheat cv Kharchia 65 (M: 1kb marker)

position in bacterial artificial chromosome (BAC) were searched in wheat high throughput genomic sequences (<http://urgi.versailles.inra.fr/SeqRepository/BLAST>), which indicates presence of three copies of *TaCBL4* gene in wheat genome, one copy each on long arm of chromosomes 1A, 1B and 1D, respectively. The gene under present study showed close homology with *HvCBL4*, the candidate gene in the barley salt tolerance QTL *HvNAX4* (Rivandi et al. 2011). *HvNax4* was fine-mapped on the long arm of barley chromosome 1H in the QTL marker interval ABC152-7SGlob. Further, the RFLP marker ABC152 of barley mapped to long arm of wheat chromosome 1 homeologues (Rivandi et al. 2011), which supports our observation on *TaCBL4* localization on chromosomes 1A, 1B and 1D. The cloned sequence showed maximum similarity to the D genome homologue. The expression of *TaCBL4* homologues belonging to A, B and D genomes also showed wide variation (wheat.pw.usda.gov/Wheat Exp/) in response to developmental stages, heat and drought stresses (Data not shown). Genome specific expression profiling and gene mining of *TaCBL4* might help in understanding nutrient deficiency response of bread wheat.

To understand the evolutionary relationship of *TaCBL4* gene with sequences from other genera, phylogenetic analysis was carried using Mega 6.0 (Fig. 2). *CBL4* sequences of rice, maize, barley, *Arabidopsis* and other plants identified from NCBI database were used in phylogenetic tree construction (Fig. 2). *TaCBL4* sequence belonged to a clade containing sequences of *Hordeum* sp. Other four clades of the tree had



Fig. 2. Phylogenetic analysis of *TaCBL4* cDNA sequence with *CBL4* cDNA sequences of other plant species. The phylogenetic tree was constructed following maximum likelihood method in MEGA 6 software

sequences from rice, maize and *Arabidopsis* and *Brassica*, respectively, demonstrating that *CBL4* genes are conserved between dicots and monocots. Kushwaha et al. (2011) observed huge variation in sequence identity between *Brassica juncea* *CBL4* sequence with that of *Arabidopsis* and other species and thus concluded that *CBL4* gene might have undergone species specific evolution in *Brassicaceae*. Similarly, the distinct position of *TaCBL4* sequence also point out the species and genera specific evolution of *CBL4* sequence in plants.

Protein structure analysis

ClustalW2.0 multiple sequence alignment analysis depicted that *TaCBL4* has 95% homology with barley *CBL4*. There was 83, 70 and 61% similarity with amino acid sequences of rice, maize and *Arabidopsis*, respectively (Fig. 3a). To mark the conserved domains and motifs, the predicted amino acid sequence of *TaCBL4* gene was analysed using Blastp. *TaCBL4*

predicted protein contained three EF-hand calcium binding motifs, a characteristic feature of calcium sensor proteins (Fig. 3b). The presence of conserved EF hand motifs are characteristic feature of *CBL4* protein sequences in plants (Steinhorst and Kudla 2013). The X-ray structure of the *AtSOS3* protein has already been solved by Sanchez-Barrena et al. (2004) which shows that the overall fold of an *SOS3* protein is almost identical with that found for our study (Fig. 3c).

Quantitative real time – PCR expression analysis

We have identified *TaCBL4* gene showing similarity to *AtCBL4* and *OsCBL4* genes, component of SOS pathway for salinity tolerance (Liu et al. 2000; Martínez-Atienza et al. 2007). To study the salinity stress regulated

transcriptional changes of *TaCBL4* gene, qRT-PCR analysis was carried out using RNA, isolated from control and salinity stressed root tissues of tolerant wheat genotype Kharchia 65. Degenerate primers designed based on *TaCBL4* cDNA sequence was used for qRT-PCR analysis. Here we observed upregulated expression of *TaCBL4* gene by salinity (100mM NaCl) stress (Fig. 4).

We further analysed the effect of nutrient stresses apart from salinity on the expression of *TaCBL4* gene. *TaCBL4* gene expression was also found to be up-regulated by –N, –P and –K treatments. There was significantly higher expression in response to excessive +Mg treatment as well (Fig. 4). However the highest level of expression was observed in plants facing salinity stress. In the similar lines, Kushwaha et al. (2011) observed that expression of *BjSOS3* gene in *B. juncea* was responsive to a wide range of abiotic stresses, but the salinity stress induced the gene expression most.

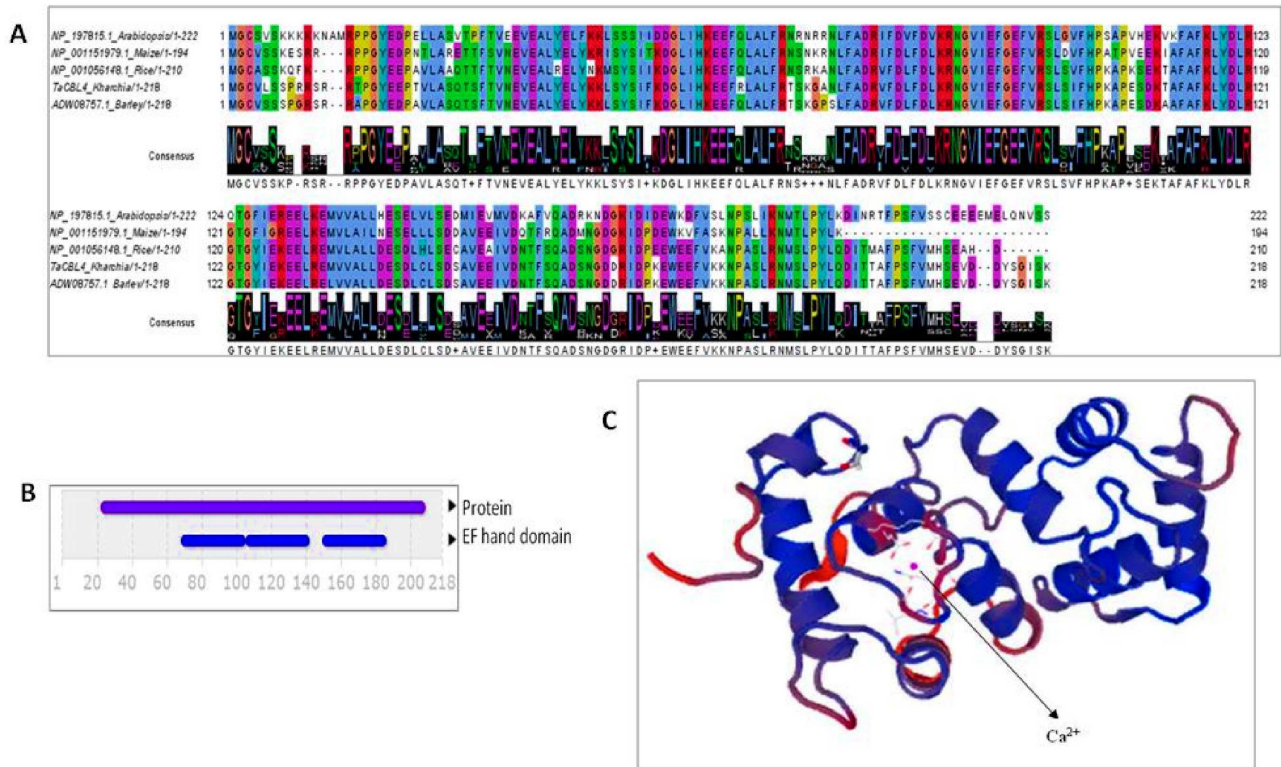


Fig. 3. A: Multiple sequence alignment and comparison of deduced CBL4 protein sequences of bread wheat, *Arabidopsis*, maize, rice and barley. Final alignment was made using multiple alignment editor Jalview; **B:** Interpro analysis of *TaCBL4* deduced protein sequence showing three conserved calcium ion (Ca²⁺) binding EF-hand motifs and **C:** Modelled image of CBL4 in *Triticum aestivum* showing the conserved domain structure of the protein and the calcium ion ligand in contact with amino acids

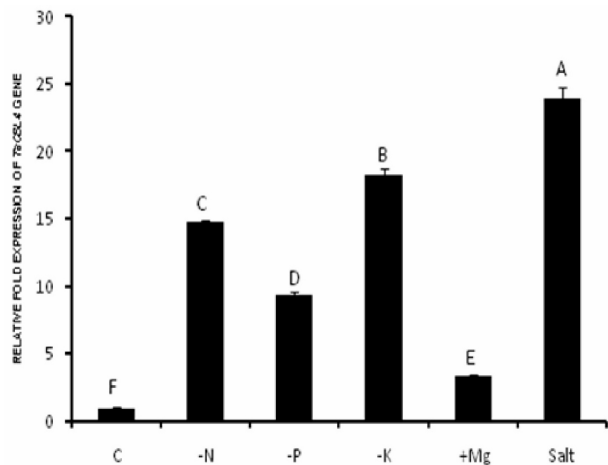


Fig. 4. qRT-PCR Analysis of *Triticum aestivum* Calcineurin B Like 4 (*TaCBL4*) gene in wheat root tissue in response to different nutrient stresses (C = control; -N = without nitrogen; -P = without phosphorus; -K = without potassium; +Mg = excess (25mM) magnesium and salt (100mM NaCl) stress

Previous studies enumerate that CBL proteins regulate diverse physiological and developmental functions including stress responses and there may be crosstalk between these functions (Kolukisaoglu et al. 2004). Recently, Wang et al. (2014) also reported that maize *CBL* family genes were differentially regulated by low potassium conditions. Dong et al. (2015) found that over expression of *Nicotiana sylvestris* calcineurin B-like protein NsYICBL10 conferred salinity tolerance in *Arabidopsis*. In our study it is found that *TaCBL4* gene expression is up-regulated by deficiency of N, P and K and also by excessive Mg²⁺ and Na⁺ ions. Transgenic manipulation of *TaCBL4* might help in further understanding the crosstalk between salinity and nutrient deficiency stress signaling of plants.

A salt stress responsive gene (*TaCBL4*) from bread wheat has been cloned, encoding calcineurin B like calcium signaling protein. Phylogenetic analysis of *TaCBL4* indicates conserved evolutionary relationship with other plant species. In addition to

salinity stress, the expression of *TaCBL4* gene was also up-regulated by deficiency of major nutrients namely nitrogen, phosphorus and potassium. This suggests that *TaCBL4* may serve as node for signaling crosstalk between salinity and nutrient deficiency stress signaling pathways and nutrient homeostasis under salinity stress. This study lays foundation for functional studies on role of *TaCBL4* gene in nutrient use efficiency and for its further utilization in breeding new wheat varieties with enhanced nutrient use efficiency and salinity tolerance.

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