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Molecular characterisation and expression profiling of *calcineurin B-like* (*CBL*) genes in Chinese cabbage under abiotic stresses

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Abstract. Calcium signals act as a second messenger in plant responses to various abiotic stresses, which regulate a range of physiological processes. Calcium-binding proteins, like calcineurin B-like (CBL) proteins, belong to a unique group of calcium sensors that play a role in calcium signalling. However, their identities and functions are unknown in Chinese cabbage. In this study, 17 *CBL* genes were identified from the *Brassica rapa* L. (Chinese cabbage) database and Br135K microarray datasets. They were used to construct a phylogenetic tree with known CBL proteins of other species. Analysis of genomic distribution and evolution revealed different gene duplication in Chinese cabbage compared to *Arabidopsis*. The microarray expression analysis showed differential expression of *BrCBL* genes at various temperatures. Organ-specific expression was observed by RT–PCR, and qRT–PCR analyses revealed responsiveness of *BrCBL* genes to cold, drought and salt stresses. Our findings confirm that *CBL* genes are involved in calcium signalling and regulate responses to environmental stimuli, suggesting this family gene have crucial role to play in plant responses to abiotic stresses. The results facilitate selection of candidate genes for further functional characterisation. In addition, abiotic stress-responsive genes reported in this study might be exploited for marker-aided backcrossing of Chinese cabbage.

Additional keywords: calcium signalling, *CBL* family genes, expression analysis, gene evolution, microsynteny, protein interaction.

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Introduction

Abiotic stresses including cold, drought and salinity are significant environmental factors that cause crop losses worldwide. In plants, responses to environmental stresses depend upon the activation of signal transduction pathways to cope up with changes in their environment by controlling their metabolism, throughout their life cycle. Calcium (Ca^{2+}) is a primary signalling event that regulates important developmental process and adaptive responses of plants under environmental stress (Gilroy and Trewavas 2001; Sun *et al*. 2015). During stress induction, cytosolic Ca^{2+} concentration may be elevated by signals of environmental changes, like salinity, drought and cold (Knight and Knight 2001; Kader and Lindberg 2010; Abdula *et al*. 2016).

Sathyanarayanan and Poovaiah (2004) reported that Ca^{2+}elevation may be sensed by Ca^{2+} sensors or binding proteins that contain elongation factor (EF)-hand motifs and helix-loop-helix structures. Three major classes of EF-hand Ca^{2+} sensors have been characterised to date in plants, including calmodulin like proteins (CaMs), calcium-dependent protein kinases (CDPKs) and Calcineurin B-like protein (CBLs) (Snedden and Fromm 2001; Cheng *et al*. 2002). In Ca^{2+} sensors, most are known to be calmodulin and CaM related proteins, which are small proteins that contain multiple elongation factors for Ca^{2+} binding. Other known calcium sensors include calmodulin (CaM) and CaM-related proteins, which contain multiple EF-hand domains. CaMs interact with target proteins, which helps to mediate Ca^{2+} signalling to regulate gene activity.

Another important family of Ca^{2+} sensors, referred to as calcineurin B-like (CBLs) proteins, has been identified in *Arabidopsis* (Kudla *et al*. 1999). Except for EF-hand regions, there is no sequence similarity between *CBL*s and other Ca^{2+}

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sensors. CBL proteins interact with a single family of protein kinases and their targeted proteins. These kinases – referred as CIPKs (CBL-interacting protein kinases) – are specific serine-threonine protein kinases that are activated through interaction with CBLs (Ishitani *et al*. 2000), and they may represent a new subclass of protein kinases (Batistic and Kudla 2004). Activated CIPKs subsequently transduce calcium signals by phosphorylating downstream signalling components (Liu *et al*. 2000). Recent reports suggest that phosphorylation of CBL proteins via interaction with CIPKs is required for full activity of CBL-CIPK complexes towards their target proteins (Du *et al*. 2011; Hashimoto *et al*. 2012).The CBL-CIPK interaction network aids in ion transport at a cellular level. To date, 10 CBL and 26 CIPKs in *Arabidopsis*, and 10 CBLs (OsCBL) and 30 CIPKs in *Oryza sativa* L. have been identified to show distinct interplay of different CBL-CIPK combinations that, in turn, could decode the Ca^{2+} signals from different stimuli through spatiotemporal regulation of downstream signalling cascades. Several CBL-CIPK complexes are involved in mediated proteins via Ca^{2+} sensors. Two *CBL* genes of *Arabidopsis*, *AtCBL1* and *AtCBL9*, are closely related, and over 90% sequence identity is involved in stress responses. In plants, *AtCBL1* acts as a positive regulator of salt and drought stresses but as a negative regulator of cold stress (Cheong *et al*. 2003). In contrast, abscisic acid (ABA) signalling *AtCBL9* gene functions as a negative regulator and under stress conditions involved in ABA biosynthesis processes (Pandey *et al*. 2004). In the reactive oxygen species (ROS) signalling pathway, *AtCBL1* or *AtCBL9* interact with *AtCIPK26* and to form protein complexes to regulate respiratory burst oxidase homologue F (*AtRbohF*) (Drerup *et al*. 2013). In addition, *AtCBL1* and *AtCBL9* act as the regulator of pollen germination and growth of pollen tube through regulating K^+ homeostasis (Mähs *et al*. 2013). In *Brassica rapa* L., the *BnCBL1* and *BnCBL6* complex showed responses to high salinity and phosphorous deficiency as well as ABA signalling (Chen *et al*. 2012). In the Salt Overly Sensitive (SOS) pathway, CIPK24/SOS2 maintains ion homeostasis during salt stress by regulating Na^+/H^+ exchanger. In addition, *SOS1* interacts with *SOS3*/*CBL4* to promote transport of sodium ions out of the cell under salt stress (Liu *et al*. 2000; Xiong *et al*. 2002; Quintero *et al*. 2011). The SOS pathway is functionally conserved in rice (Martinez-Atienza *et al*. 2007), tomato (Olías *et al*. 2009) and *Populus trichocarpa* Torr. & A.Gray ex. Hook. (Tang *et al*. 2010). *SOS3* and *SOS2* were shown to interact in roots, whereas *CIPK24*/*SOS2* interacts with *CBL10*/*SCABP8* in shoots (Kim *et al*. 2007; Quan *et al*. 2007; Lin *et al*. 2009). In addition, the expression of maize *ZmCBL4* and pea *PsCBL* are differentially regulated by various abiotic stresses (Wang *et al*. 2007).

Chinese cabbage (*B. rapa* ssp. *pekinensis*) includes two inbred lines, Chiifu and Kenshin, adapted to cold and warm climates respectively. These two lines respond differently to temperature and vernalisation (Lee *et al*. 2010). Understanding the molecular mechanisms of *B. rapa* responses to abiotic stresses is a prerequisite for improving stress tolerance cultivars. One promising approach to improve stress tolerance of plants is through modulating the key tolerance genes via plant breeding. In the present study, we identified 17 *B. rapa CBL* (*BrCBL*) genes and analysed the phylogenetic relationship, exon-intron structure, genomic localisation, microsyntenic relationship, calculate synonymous and non-synonymous substitution rates, evolutionary divergence, gene duplication and interaction network of *BrCBL* genes. We also examined the microarray and organ-specific expression of all *BrCBL* genes and expression profiling by qPCR of the identified *BrCBL*s in response to abiotic stresses. Co-responsive expression of the genes against abiotic stresses revealed a role in stress tolerance. Therefore, extensive expression profiling of the identified genes will promote understanding of the roles of *BrCBL*-based networks in abiotic stress responses.

Materials and methods

Plant materials

Chinese cabbage (*Brassica rapa* L. 'SUN-3061') plants were grown in the Department of Horticulture, Sunchon National University, Korea. Fresh roots, stems, leaves and flower buds were harvested, frozen immediately in liquid nitrogen then stored at –80°C for RNA isolation.

Abiotic stress treatments

For abiotic stress treatments, two contrasting *B. rapa* inbred lines 'Chiifu' and 'Kenshin' were used. Among them 'Chiifu' is cold tolerant and 'Kenshin' is cold sensitive because of their origin; Chiifu originated in temperate regions, whereas Kenshin in subtropical and tropical regions. Plants were cultivated under aseptic conditions in semi-solid medium for 10 days, afterward plants were transferred into liquid medium to minimise stress during the treatment. Stress treatments (cold, drought and salt) were applied to 4-week-old plants at the vegetative stage for continuous time courses (0, 1/2, 1, 4, 8, 12, 24 and 48 h). Plants were transferred to the incubator at 4°C to induce cold stress. Drought/desiccation stress was simulated by drying the plants on Whatman 3 mm filter papers, and salt stress was induced by transferring plant samples to rectangular Petri-dishes (72 × 72 × 100 mm) containing 200 mM NaCl. Fresh roots and leaves (third and fourth leaves) from five plants were harvested as biological replicates, then immediately frozen in liquid nitrogen and stored at −80°C for RNA extraction.

RNA extraction

Total RNA was extracted from roots, stems, leaves and flower buds of frozen samples using an RNeasy mini kit (Qiagen). RNA was treated with RNase-free DNase (Promega) to remove genomic DNA contaminants. The cDNA was synthesised using the Superscript III First-Strand synthesis kit (Invitrogen) according to manufacturer's instructions.

Database search and sequence analysis

The *B. rapa* genomic database (BRAD: http://brassicadb.org/brad/, accessed 10 August 2016) was searched to identify *BrCBL* genes using tBLASTN with the entire *Arabidopsis* CBL amino acid sequences (48). We also investigated microarray annotated database for two cold-treated *B. rapa* inbred lines, Chiifu and Kenshin, using the keyword 'CBL'. To confirm the presence of the CBL domain, we used the web tool from EMBL (http://smart.embl.de/smart/set_mode.cgi?GENOMIC=1, accessed 10 August 2016) and conducted protein homology searches using the

Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/, accessed 14 August 2016) using the candidate *CBL* genes in *B. rapa*. The primary structure of genes was analysed using ProtParam (http://expasy.org/tools/protparam.html, accessed 25 August 2016). The number of introns and exons was determined by comparing predicted coding sequences (CDS) with the corresponding genomic sequences using the GSDS 2.0 software (http://gsds.cbi.pku.edu.cn, accessed 28 August 2016) (Guo *et al*. 2007). The conserved motifs in thr BrCBL proteins were analysed using MEME software (http://meme.sdsc.edu/meme/intro.html, accessed 8 September 2016). This software was executed with the following parameters: (1) optimum motif width $\geq$6 and $\leq$50; (2) maximum number of motifs = 15. Multiple sequence alignment was performed using the ClustalW program (Thompson *et al*. 1997) and GeneDoc. The phylogenetic trees of CBL proteins were generated using MEGA (V6.0) (http://www.megasoftware.net/, accessed 15 September 2016) (Tamura *et al*. 2011) with the neighbour-joining (NJ) method. Specific protein interaction networks were constructed with the STRING software (Search Tool for the Retrieval of Interacting Genes/Proteins, http://string-db.org/, accessed 20 September 2016) (Liu *et al*. 2013).

Chromosome localisation, gene duplications and divergence time

The positions of *BrCBL*s were mapped to 10 *B. rapa* chromosomes by Mapchart software. The physical locations of *CBL* genes were obtained from the BRAD database. To identify the duplicated *BrCBL* genes, BrCBL protein sequences were searched against themselves using BLASTP with an E-value cut-off of 1×10^{-10} and identity >80%. The synonymous rate (*Ks*), non-synonymous rate (*Ka*), and evolutionary constraint (*Ka*/*Ks*) was calculated between the duplications pairs of *BrCBLs* using the method by Nei and Gojobori (1986) as implemented in *Ka*/*Ks* calculator. The divergence time was calculated with the formula $t = Ks/2r$. (*Ks* being the synonymous substitutions per site and r is taken to be 1.5×10^{-8} substitutions per site year^{-1} for dicotyledonous plants) (Zhang *et al*. 2006).

Microsynteny analysis

The microsyntenic relationship of *CBL* genes among *B. rapa*, *B. oleracea* L., and *Arabidopsis thaliana* (L.) Heynh. were detected using Blast against whole genome of such crop species. *CBL* genes positions on chromosome were collected from database and the relationship among the three crop species were plotted using Circos software (http://circos.ca/, accessed 30 September 2016) (Krzywinski *et al*. 2009).

Microarray expression analysis

Temperature-treated microarray data for *CBL* genes were collected from the data by Jung *et al*. (2014). For microarray data, two inbred lines of *B. rapa* ssp. *pekinensis*, namely cold-tolerant Chiifu and cold-sensitive Kenshin, were treated with different temperatures *viz. 22*, 4, 0, −2, and −4°C for 2 h. To generate a heat map based on transcript abundance value of *CBL* genes using Cluster 3.0 and tree view software (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm, accessed 9 October 2016).

Expression analysis

RT–PCR was performed using an AMV one step RT–PCR kit (Takara). Specific primers for all genes were used for RT–PCR and primers *Actin* of *B. rapa* were used as a control (see Table S1, available as Supplementary Material to this paper). The PCR reactions were performed using 50 ng cDNA from the roots, leaves, stems, and flower buds as templates. Briefly, 10 pmol each primer, 150 µM each dNTP, 1.2 U Taq polymerase, 1 × Taq polymerase buffer, and double-distilled H_2O to a final volume of 20 µL were added to 0.5 mL PCR tubes and mixed. The samples were subjected to initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. Real-time PCR (qPCR) was performed using 1 µL cDNA in a 25 µL reaction volume with iTaq SYBR Green Super-mix with ROX. Specific primers for genes were used to conduct real-time PCR (Table S1). The thermal cycler conditions were as follows: 10 min at 95°C, followed by 40 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s. The fluorescent products were detected in the last step of each cycle. Amplification, detection, and data analysis were carried out using qPCR value of 3 replicates following a Rotor-Gene 6000 real-time rotary analyser (Corbett Life Science).

Results and discussion

Identification and characterisation of CBL genes in B. rapa *(*BrCBL*)*

Searches of the *B. rapa* genome database (BRAD) using *Arabidopsis* CBL proteins using as query probes returned 17 genes from *B. rapa*. We also searched the microarray Br135K annotated database using the keyword 'CBL'. Based on such annotation and sequence analysis, these genes were designated as *B. rapa Calcineurin B-like* (*BrCBL*) genes. Results of BLAST searches against previous published of *OsCBL* genes were similar to those orthologous genes as annotated (Kolukisaoglu *et al*. 2004; Hwang *et al*. 2005).

The 17 *BrCBL* genes encode putative proteins ranging from 110 to 449 amino acids with predicted pI values ranging 4.70 to 6.02. The predicted molecular masses were ranged from 12.76 to 50.53 kDa, and all BrCBL proteins contained EF-hand domains (Table 1). Notably, BrCBL2–2 and BrCBL7 have *N*-terminal extensions, with predicted molecular masses of 25.15 and 28.00 kDa respectively. In addition, BrCBL3–2 has a *C*-terminal extension, with a predicted molecular mass of 50.53 kDa (Table 1; see Fig. S1, available as Supplementary Material to this paper). The amino acid sequence identity of different BrCBLs ranged from 28 to 98%, with a highly conserved domain in the *C*-terminal regions that flank the EF-hand domains (Table S2). As in *AtCBL* genes, four EF-hand structures were found in *BrCBL*s. EF-hand motifs form the structural basis for calcium binding site, and each EF hand consists of a 12-aa loop flanked by two helices (Fig. 1*a*, *b*). However, some of the EF hands in the BrCBLs differed from the canonical EF-hand domain. In particular, EF1 loop contained

Table 1. List of 17 *CBL* genes identified in *Brassica rapa* and their sequence characteristics
Abbreviations: aa, amino acids; bp, base pair; kDa, kilo dalton; ORF, open reading frame; pI, isoelectric point

Serial no.	Gene name	Accession no.	Chromosome no.	ORF (bp)	Protein Length (aa)	Protein Molecular weight (kDa)	pI	Number of exons	*Arabidopsis thaliana* accession no.
1	*BrCBL1–1*	Bra040169	A01	642	213	24.58	4.82	8	At4g17615
2	*BrCBL1–2*	Bra012655	A03	642	213	24.60	4.77	8	
3	*BrCBL2–1*	Bra035598	A02	681	226	25.86	5.04	8	At5g55990
4	*BrCBL2–2*	Bra028949	A03	663	220	25.15	5.11	7	
5	*BrCBL3–1*	Bra026421	A01	657	218	24.88	4.96	8	At4g26570
6	*BrCBL3–2*	Bra019099	A03	1350	449	50.53	6.02	16	
7	*BrCBL4–1*	Bra009743	A06	666	221	25.58	5.14	8	At5g24270
8	*BrCBL4–2*	Bra026462	A01	666	221	25.48	5.00	8	
9	*BrCBL4–3*	Bra029396	A02	666	221	25.36	4.87	8	
10	*BrCBL5*	Bra002301	A10	333	110	12.76	5.31	4	At4g01420
11	*BrCBL7*	Bra026422	A01	753	250	28.00	5.41	7	At4g26570
12	*BrCBL8*	Bra027703	A09	645	214	24.75	5.37	8	At1g64480
13	*BrCBL9–1*	Bra022104	A02	642	213	24.35	4.75	8	At5g47100
14	*BrCBL9–2*	Bra017504	A09	642	213	24.35	4.75	8	
15	*BrCBL10–1*	Bra034543	A08	741	246	28.46	4.95	9	At4g33000
16	*BrCBL10–2*	Bra011404	A01	636	211	24.33	4.77	8	
17	*BrCBL10–3*	Bra037030	A03	777	258	29.87	4.70	9	

an insertion of two amino residues S/A and V/I between positions 1 and 3 respectively. Furthermore, BrCBL5 lacked the EF1 and EF2 motifs entirely. Therefore, a motif scanning program was used for searching other motif that could be functionally important in the BrCBL proteins. We found that 11 BrCBL proteins started with a conserved *N*-myristoylation motif (GXXXS/T) (Towler *et al*. 1988) that might be functional in membrane targeting of the CBL-CIPK complex, whereas the other six CBL proteins did not have this sequence motif (Fig. S1).

Chromosomal distribution and evolution of BrCBL *genes*

Genomic distribution and evolution of 17 *BrCBL* genes showed their location on chromosomes A01, A02, A03, A06, A08, A09 and A10. Most of the *BrCBL* genes were concentrated on chromosomes A01 and A03 (Fig. 2). Evolutionary history of *BrCBL* families were analysed. Regarding this, we retrieved *CBL* genes from *A. thaliana* and *O. sativa* using similarity based searches. *BrCBL* orthologs genes in *A. thaliana* were identified using BLASTP. Most of the *CBL* orthologs in *Arabidopsis* obtained two or three copies that were connected to their related *CBL* of *B. rapa* (Table 1). A series of genetic changes were evolved during evolution of *BrCBL* genes at the time of genome triplication in Brassica. Segmental and tandem duplication influenced the distribution of the *CBL* gene in a family (Lynch and Conery 2000). In addition, *B. rapa* genome triplication events might also have played an important role in the extension of *CBL* gene family. We found nine pairs of segmental duplication genes in *BrCBL* family. In addition, to determine the divergence times and selection pressures of these duplicated *BrCBL* genes, we calculated the substitution ratio of non-synonymous (*Ka*) to synonymous (*Ks*) per site between duplicated pairs (Table 2). We considered, if value of *Ka*/*Ks* < 1, the duplicated gene pairs may evolve from purifying selection (also called as negative selection); *Ka*/*Ks* $= 1$ means neutral selection; whereas *Ka*/*Ks* > 1 means positive selection. Five duplicated pairs (i.e. *BrCBL1-1* vs *BrCBL1-2*, *BrCBL2-1* vs *BrCBL2-2*, *BrCBL4-1* vs *BrCBL4-3*, *BrCBL10-1* vs *BrCBL10-2* and *BrCBL10-2* vs *BrCBL10-3*), had *Ka*/*Ks* ratios > 1, representing accelerated evolution with positive selection on these duplicated pairs (Table 2). Four duplicated gene pairs (i.e. *BrCBL3-1* vs *BrCBL3-2*, *BrCBL4-1* vs *BrCBL4-2*, *BrCBL4-2* vs *BrCBL4-3* and *BrCBL9-1* vs *BrCBL9-2*,) had *Ka*/*Ks* ratios < 1, those are evolved under strong purifying selection pressure in *B. rapa*. Our results indicated that positive and purifying selection played key role for functional divergence of *BrCBL* genes. Koch *et al*. (2000) predicted the evolutionary timescale of Brassicaceae on the basis of synonymous substitution rate. We calculated the divergence times of duplicated *BrCBL* genes (Table 2) indicating divergence of *BrCBL* family members took place ~1.39–3.99 million years ago (MYA) after the triplication events of *B. rapa* (Cheng *et al*. 2011).

Phylogenetic analysis of the BrCBL *gene family*

For phylogenetic relationships 17 *B. rapa CBL* genes, 10 rice CBL genes, 10 *Arabidopsis CBL* genes, together with five calmodulins (CaMs) or calcium-dependent protein kinases (CDPKs) were used for comparative analysis (Fig. 3). The phylogenetic analysis indicated that the *BrCBL* family genes were distributed in four clades and CaMs and CDPKs were in separate clade. In group II and III, each group contained five CBL members of *B. rapa*. In contrast, groups I and IV contained four and three members of *BrCBL* genes respectively. All *CBL* genes clearly formed a separate group from other types of calcium sensor proteins (Fig. 3). The number of genes were increased through tandem and segmental duplications during evolution of the gene families (Bancroft 2001). These might be the reasons to identify nine segmental duplicated gene pairs in *BrCBL* family members (Table 2) and segmental duplication was the main

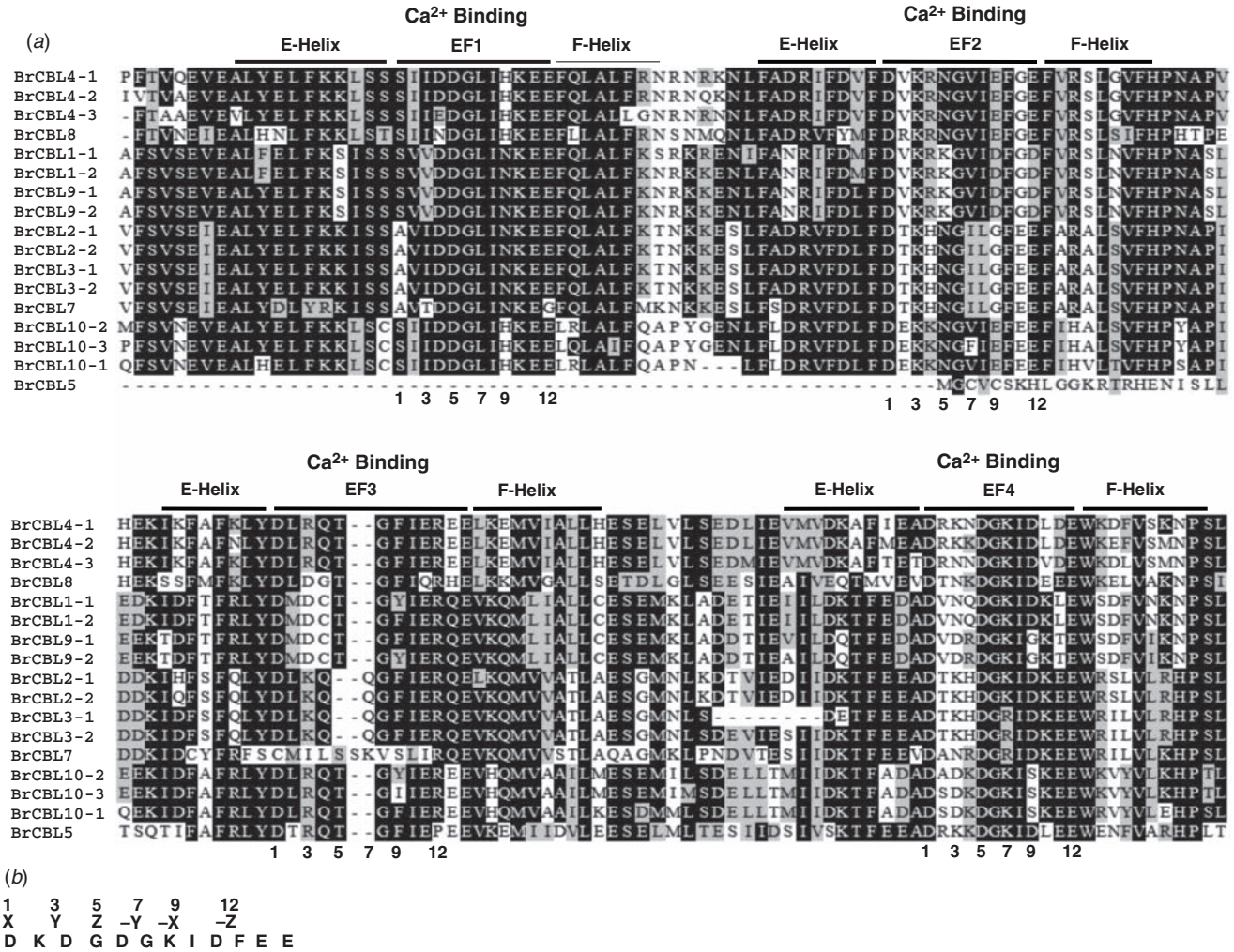

Fig. 1. (*a*) Analysis of amino acid sequences in *Brassica rapa* CBLs. All BrCBLs contain four EF-hand calcium-binding motifs (dark lines over the sequences). The calcium-binding loops, flanked by E and F helices, are marked. Numerical values denote amino acid residues important for calcium-binding and EF-hand structure. (*b*) Canonical EF-hand consensus sequence and coordinates for interaction with Ca^{2+}.

provider to the expansion of this gene family. These results suggest that *BrCBL*s may be extended rapidly after speciation from *A. thaliana* by genome triplication.

Exon-intron distribution and motif analysis

We determined the exon-intron structure of the *BrCBL* genes based on the genomics DNA and CDS sequence information. Mount (1982) suggested that the sequence of all exon-intron junctions confirmed the GT-AG intron splicing donor-acceptor sequence rule. As shown in Fig. S2, the coding regions of *BrCBL* genes consist of 4–16 exons separated by various lengths of introns. The length of introns was varied, but rather conserved in phase and position among all *BrCBL* genes. The similarity among BrAL protein sequences was very high; therefore, five motifs (1, 2, 3, 4 and 5) were common among all groups (Fig. S3). In most cases, *BrCBL* family members in the same group shared common motif mixtures. The unique motifs (motif numbers 7, 13, 14 and 15) were present in group II. Motif 9 and motif 10 were only found in Group IV. Motif 8 was present in group I and III, whereas motif 11 was found only in group III (Fig. S3).

Microsynteny relationships

A microsynteny map was constructed using orthologous gene pairs of *CBL* genes among *B. rapa*, *B. oleracea* and *A. thaliana* to investigate the evolutionary history and relationships of *CBL* genes among the plant species (Fig. 4). We identified 17 orthologous gene pairs between *B. rapa* and *A. thaliana*, whereas 19 orthologous gene pairs were found between *B. rapa* and *B. oleracea* (Fig. 4). Results suggested that *BrCBL* genes are more closely related to *B. oleracea* and *A. thaliana CBL* genes. Among the *BrCBL* genes, nine pairs of genes were segmental duplicated, as represented as black line in Fig. 4. For simplicity, we have also depicted the *BrCBL* duplicated gene pairs in chromosome map (Fig. 2).

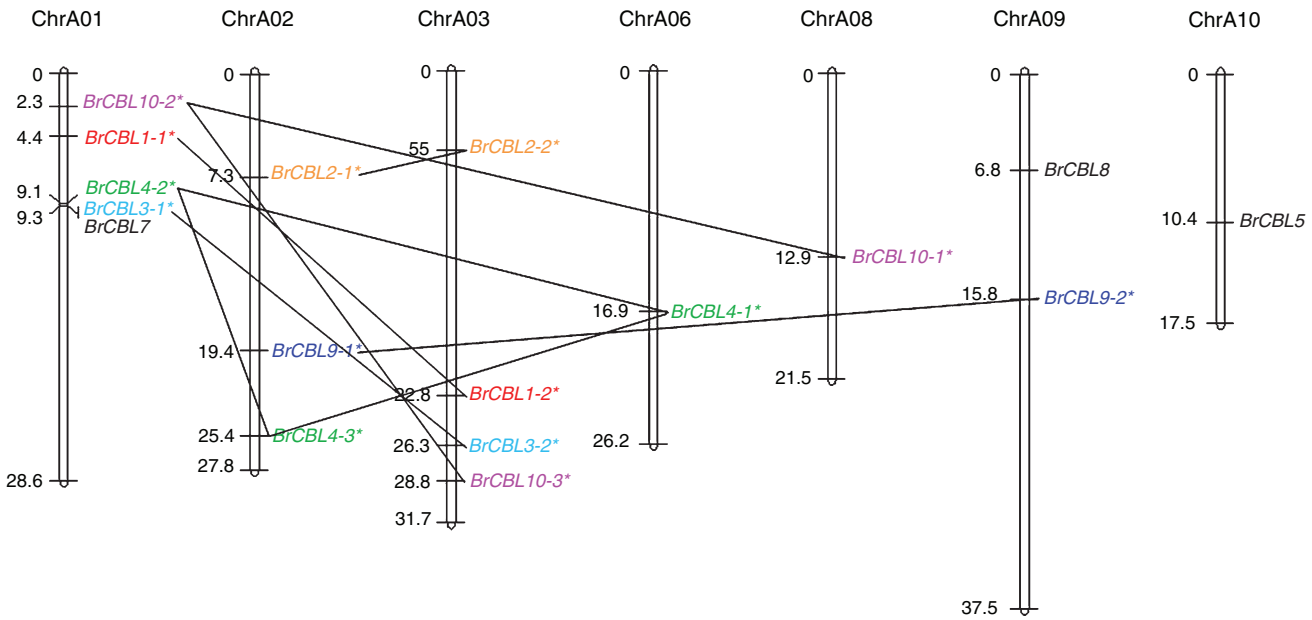

Fig. 2. Chromosomal distribution of *BrCBL* gene family members in the *Brassica rapa* genome. Physical location of the *BrCBL* genes are represented in megabase pairs (Mb). Thin line connected the segmental duplicated gene pairs.

Table 2. Estimated *Ka*/*Ks* ratios of the duplicated *BrCBL* genes with their divergence time in *B. rapa*

Note: *Ks* is the number of synonymous substitutions per synonymous site; Ka is the number of nonsynonymous substitutions per nonsynonymous site; MYA is millions of years ago

Duplicated gene pairs		*Ks*	*Ka*	*Ka*/*Ks*	Duplication type	Types of selection	Time (MYA)
BrCBL1–1	BrCBL1–2	0.0709	0.0723	1.0197	Segmental	Positive	2.32
BrCBL2–1	BrCBL2–2	0.0642	0.0673	1.0483	Segmental	Positive	2.14
BrCBL3–1	BrCBL3–2	0.0612	0.0542	0.8856	Segmental	Purifying	2.04
BrCBL4–1	BrCBL4–2	0.0942	0.0727	0.7718	Segmental	Purifying	3.14
BrCBL4–2	BrCBL4–3	0.1198	0.0589	0.4917	Segmental	Purifying	3.99
BrCBL4–1	BrCBL4–3	0.0924	0.1088	1.1775	Segmental	Positive	3.80
BrCBL9–1	BrCBL9–2	0.0731	0.0717	0.9808	Segmental	Purifying	2.43
BrCBL10–1	BrCBL10–2	0.0630	0.0749	1.1889	Segmental	Positive	2.10
BrCBL10–2	BrCBL10–3	0.0417	0.1038	2.4892	Segmental	Positive	1.39

Microarray expression

Microarray expression of 17 CBL genes of *B. rapa* was observed using previously published microarray data, in which two contrasting inbred lines of *B. rapa* 'Chiifu' and 'Kenshin' were exposed to cold and freezing temperature (4, 0, –2 and –4°C) (Jung *et al.* 2014). These lines were responded differently in microarray expression due to their origin (see 'Materials and methods'). We developed a heat map based on microarray expression of *BrCBL* genes (Fig. 5).

In the cold map, we identified three clusters based on the differential expression patterns of *BrCBL* genes between 'Chiifu' and 'Kenshin' lines in response to cold and freezing stress. Cluster I (five genes) showed a higher transcript abundance in response to cold and freezing temperature in 'Chiifu' compared with 'Kenshin'. These genes might be responsive to cold and freezing tolerance in 'Chiifu'. In the cold stress conditions, *BrCBL5*, *BrCBL7* and *BrCBL8* genes were included in cluster II, showing higher expression in 'Kenshin' than 'Chiifu'. In cluster III, most of the *BrCBL* genes were up-regulated in response to cold or freezing condition in 'Kenshin', whereas these genes were down-regulated in 'Chiifu' (Fig. 5), so these may play roles in cold or freezing susceptibility in 'Kenshin'.

Organ-specific expression analysis

We examined the expression level of *BrCBL* genes in different organs of *B. rapa* using RT–PCR, with cDNA templates prepared from isolated mRNA of roots, stems, leaves, and flower buds. *BrCBL1-1*, *2-1*, *2-2*, *3-1*, *3-2*, *4-1*, *4-3*, *7*, *9-1*, *9-2*, *10-1* and *10-2* were highly expressed in all tested organs but *BrCBL4-1* was slightly expressed in stem. However, all *BrCBL* genes were abundantly expressed in roots except *BrCBL5* and *BrCBL10-3*, whereas *BrCBL1-2* was slightly expressed in roots. Among the 17 *BrCBL* genes, only three genes (*BrCBL1-2*, *BrCBL4-2* and *BrCBL8*) were absent in stem and leaf, whereas *BrCBL4-1*, *BrCBL5* and *BrCBL10-3*

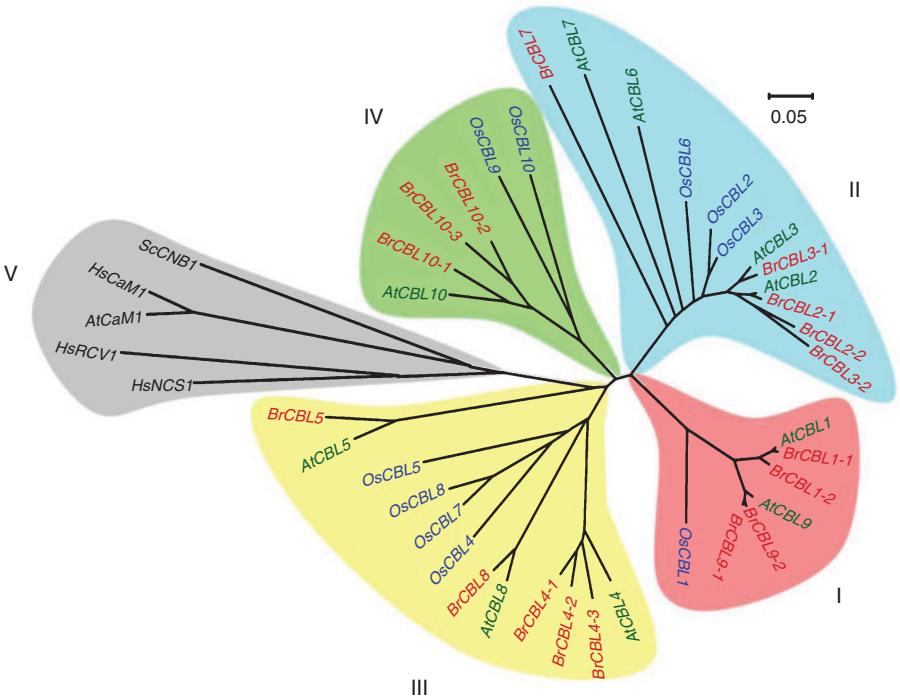

Fig. 3. Phylogenetic relationships of *Brassica rapa*, *Arabidopsis* and rice CBLs with related calcium-binding proteins. Gene names are coloured according to species.

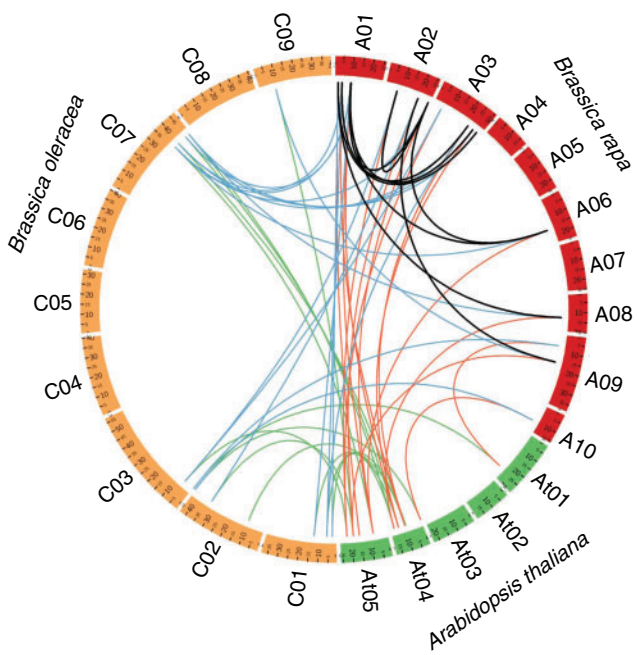

Fig. 4. Microsynteny analysis of *CBL* genes among *Brassica rapa*, *Brassica oleracea* and *Arabidopsis thaliana*. The chromosomes from the three species are indicated in different colours, red, green and yellow colours represent *B. rapa*, *A. thaliana* and *B. oleracea* chromosome respectively. Black lines depict duplicated *BrCBL* genes on 10 *B. rapa* chromosomes.

showed very low expression in stem and leaf. Moreover, all *BrCBL* genes were differentially expressed in flower buds (Fig. 6). Thapa *et al*. (2011) reported that *OsCBL* genes have tissue specificity or stimulus responsiveness of expression. The expression pattern reflects the functions of the *CBL* genes in plant development and signalling. Hence, Kolukisaoglu *et al*. (2004) suggested that *CBL* genes share characteristic features that make them particularly responsive to salt, ABA, and drought. Here, we found that *BrCBL* genes were predominantly expressed in all organs, suggesting the possible roles of *BrCBL* genes might mediate through primary signalling network in all environmental stress conditions.

Expression analysis in response to abiotic stresses

We analysed relative expression of *BrCBL* genes, using real-time PCR to know the responsive of the genes against drought, salt, and cold stresses focusing various time points in two contrasting inbreed lines 'Chiifu' and 'Kenshin' of *B. rapa*. In a comparison between the two lines, most of the *BrCBL* genes were differentially expressed under various stress situations (Fig. 7). In case of drought stress, expression of only few genes showed a significant effect in the line 'Chiifu', among them *BrCBL4-2* showed the highest relative expression with ~27-fold up-regulation at the 8 h time point. The same gene also showed up to 15-fold higher expression at 48 h time point. These results indicate that gene *BrCBL4-2* might be the candidate to overcome drought at early as well as at later stages of seedling growth. Whereas, *BrCBL1-2*, *9-1*, *10-2* and *10-3* genes showed relatively higher expression in 'Kenshin' against drought stress (Fig. 7*a*), this result was expected in this genotype because of its tropical origin.

Tai *et al*. (2016) reported Plant CBL-interacting protein kinases (CIPKs) play an important role in stress signalling transduction and enhancing plant stress tolerance, and

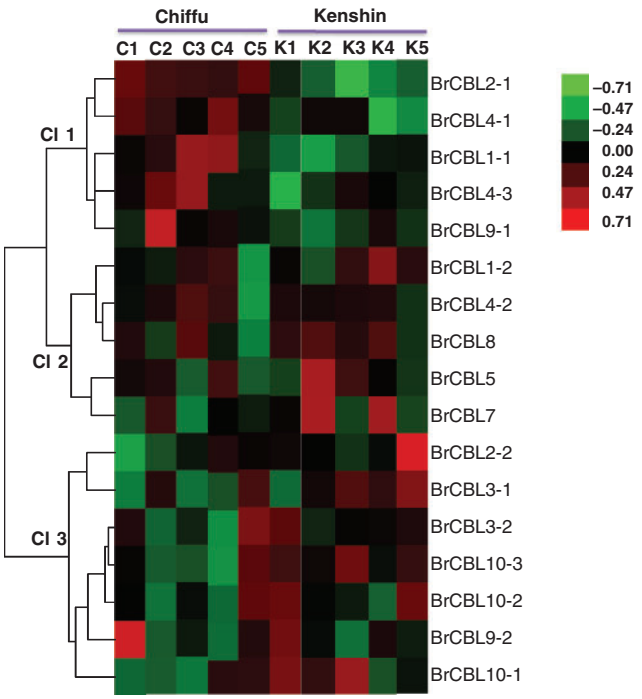

Fig. 5. Differential expression profiles of *BrCBL* genes in different temperatures. C and K, indicating Chiifu and Kenshin, respectively, were treated under five temperatures as control (C1 and K1), 4°C (C2 and K2), 0°C (C3 and K3), −2°C (C4 and K4), and −4°C (C5 and K5). Expression clusters are shown in the left (Cl1–Cl3) and gene name against each expression is mentioned on the right side. Colour bars with values at right represent differential expression in microarray.

qRT–PCR analysis revealed the mRNA accumulation of ZmCIPK8 in maize leaves and roots promoted by drought stress. In salt stress situation the genes *BrCBL1-1*, *1-2*, *2-2*, *4-1*, *4-2*, *7*, *8*, *9-1*, *9-2* and *10-2* showed differential expression. However, *BrCBL9–1* showed striking expression effect with 4- to 14-fold upregulation at 1 and 48 h time points for both 'Chiifu' and 'Kenshin' (Fig. 7*b*), indicating this gene might be the candidate in Chinese cabbage for elucidating salt stress.

In case of cold stress *BrCBL1-1* gene showed very high expression with ~30-fold- up-regulation at the 4 h time point, thereafter its expression gradually decreased with advancement of time in 'Chiifu', indicating this gene may be responsive for the early stage of cold stress. In contrast, none of the genes in 'Kenshin' displayed significant expression like in 'Chiifu' (Fig. 7*c*). This result was expected owing to the origin of the genotypes 'Chiifu' and 'Kenshin'. *CBL* genes have distinct differential expression patterns and function in different pathways. Previous reports suggest that the expression of *AtCBL1*is induced by stresses such as salt, drought and cold, whereas that of other *AtCBL* genes is not (Batistic and Kudla 2004; Kim *et al*. 2007). Wang *et al*. (2007) reported that *ZmCBL4* and *PsCBL* are differentially regulated by various abiotic stresses. These findings agree with the observation by Zhang *et al*. (2014) in canola, where *CBL* and *CIPK* exhibited differential responses to multiple stress treatments and these

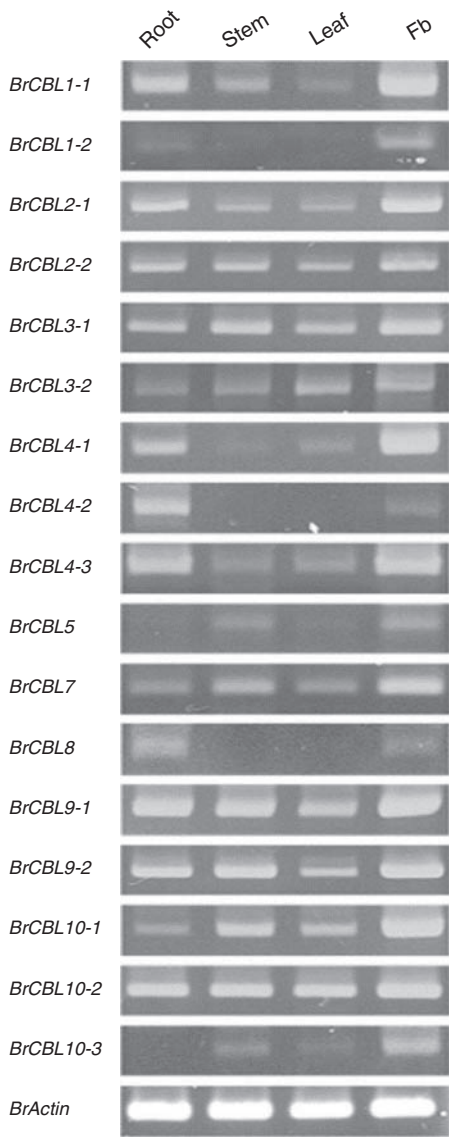

Fig. 6. Analysis of organ-specific expression patterns of 17 *BrCBL* genes by RT–PCR. The cDNA was prepared from mRNA isolated from root (R), stem (S), leaves (L) and flower buds (Fb).

authors concluded multiple CIPKs seemed to be necessary to co-ordinate with one specific stress stimulus.

Analysis of BrCBL *cis-acting elements and protein interactions*

Regulatory gene networks in stress response cascades involve various *cis*-elements, ABREs, DREs and LTREs, which have been well characterised for their roles in activation of gene expression under abiotic stress conditions (Narusaka *et al*. 2003). We analysed 1000-bp sequences upstream of 5′ end of full-length cDNAs for stress-inducible *BrCBL* genes to identify putative stress-responsive *cis*-elements. In all, 10 *BrCBL* genes contained a putative ABRE, DRE or LTRE in their promoter regions, with the exceptions of *BrCBL3-1*, *3-2*, *4-2*, *5*, *7*, *9-2* and *10-1* (Fig. S4). The ABRE (*BrCBL1-1*, *1-2*, *4-1*, *4-3*, *8* and *10-3*),

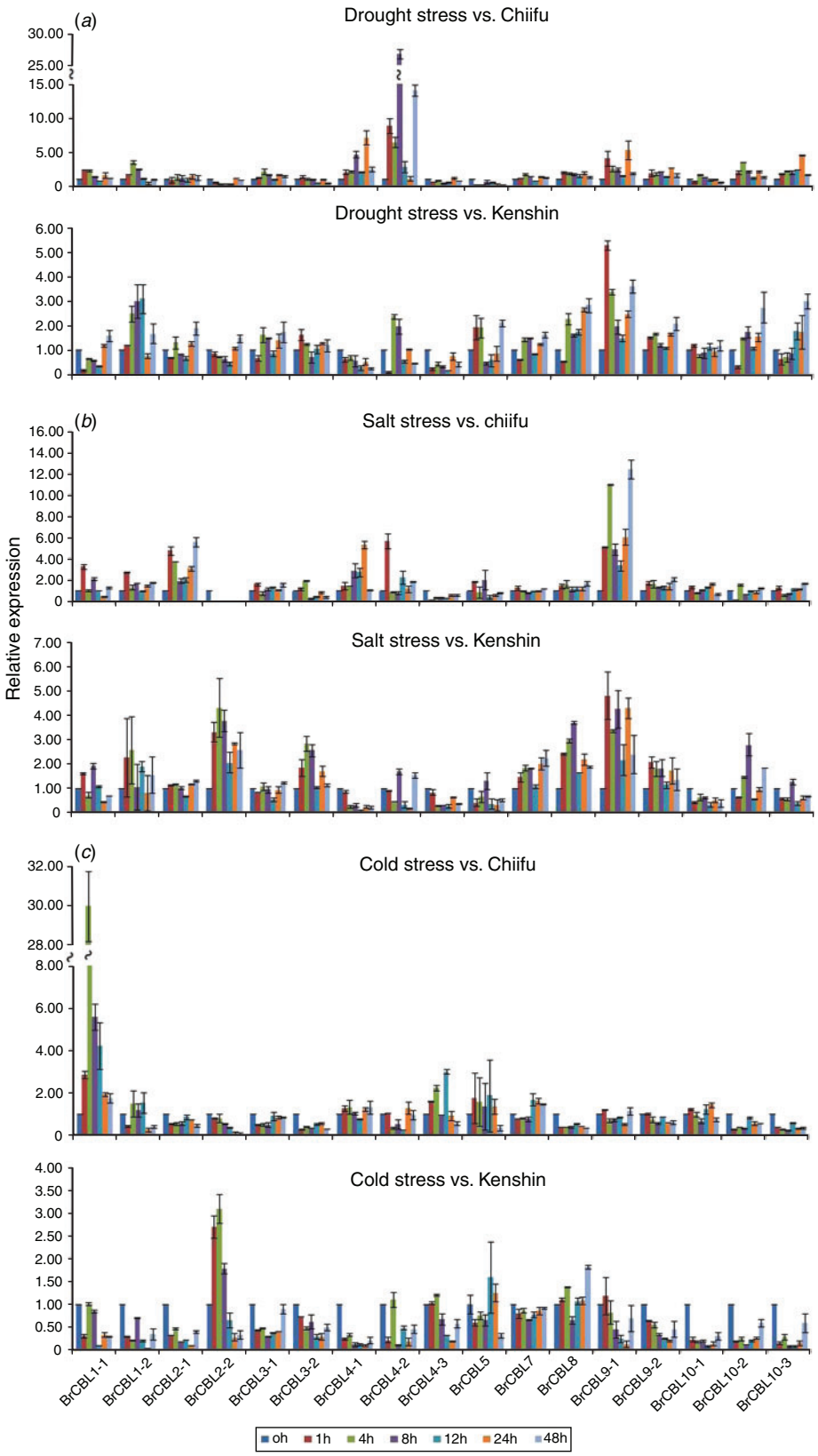

Fig. 7. Real-time PCR expression analysis of *BrCBL* genes after treatment with (*a*) drought (*b*) salinity and (*c*) cold. Error bars represent the s.e. from three replications.

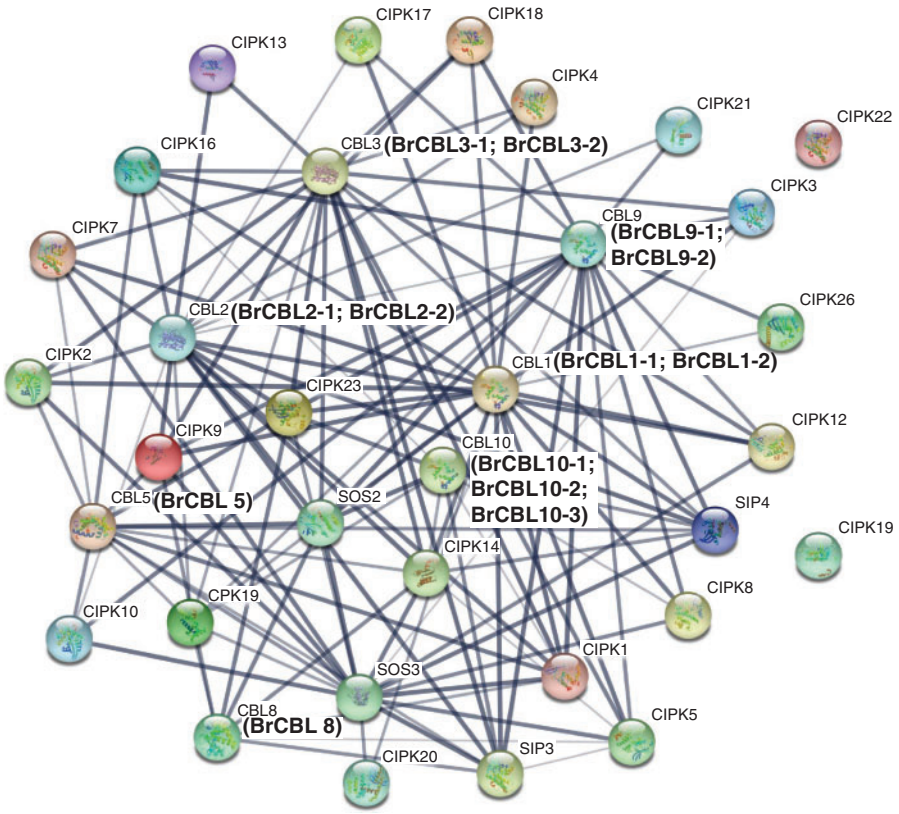

Fig. 8. Interaction network of 17 BrCBL proteins identified in *Brassica rapa* and related *Arabidopsis* CBL and CIPK proteins. Stronger associations are represented by thicker lines.

DRE (*BrCBL1-1*, 2-*1*, *8* and *9-1*), and LTRE (*BrCBL2-1*, *2-2*, *8*, *9-1*, *10-2* and *10-3*) elements are found in various promoter regions generally induced by drought, salt, and cold stress respectively (Brown *et al*. 2001; Dubouzet *et al*. 2003; Narusaka *et al*. 2003). Although none of these *cis*-elements were identified in the promoter regions of *BrCBL3-1*, *3-2*, *4-2*, *5*, *7*, *9-2* and *10-1*, we found that these genes are responsive to salt, drought and cold stress conditions. It is possible that novel stress-responsive *cis*-elements so far unidentified are absent in the promoters of these stress-inducible genes.

Additionally, *B. rapa* and *A. thaliana* protein interactions, including functional and physical interactions were examined using STRING software (Fig. 8). BrCBL1-1, BrCBL1-2; BrCBL2-1, BrCBL2-2; BrCBL3-1, BrCBL3-2; BrCBL9-1, BrCBL9-2 and BrCBL5 proteins that exhibited relatively high similarity to CBL1, 2, 3, 5 and 9 proteins of *Arabidopsis*, respectively, are involved in stronger (thicker lines) interaction networks, those proteins are involved in calcium signals triggered by environmental stresses (Fig. 8). In our analyses, CBLs proteins showed strong interaction with CIPKs and SOS3. *N*-myristoylation motifs function in membrane targeting of CBL-CIPK complex, and they are required for the function of SOS3 pathways (Hwang *et al.* 2005; Wang *et al*. 2007). CBL-CIPK plays a key regulatory role in plant response to different abiotic stresses like cold, salt and drought (Cheong *et al*. 2007; Piao *et al.* 2010). BrCBL1-1; 1-2 showed strong interaction with CIPK7, 8, 9, 12, 14, 17, 18, 4, 3 and 23. Huang *et al*. (2011) showed that CBL1 interacting with CIPK7 in *A. thaliana* is involved in cold responses. Moreover, CBL-CIPK also plays important roles in potassium (K) uptake and modulates plant growth. CBL1 and CBL9 are involved in the regulation of K uptake in plant body and in stomatal movements (Cheong *et al*. 2010). Taken together, our data suggest that functional analyses of the *BrCBL* genes identified in this work can provide an important foundation for further functional dissection of these important plant-specific signalling pathways.

Conclusion

Comprehensive and systemic analysis of CBL genes in *B. rapa* were conducted; here we studied gene structure, classification and expression pattern in different organs as well as responses to various abiotic stresses. The CBL genes of *B. rapa* were differentially expressed in different organs, indicating these genes have important role in morphogenetic and development processes. Several *BrCBL* genes showed significant effects and up-regulation induced by drought, salt and cold stresses; these may have functions in responses to multiple stresses. Furthermore, the highly expressed *CBL* genes against various abiotic stresses might be exploited for molecular breeding of *B. rapa*. Our data also facilitate selection of suitable candidate genes for further functional characterisation.

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