Genome-wide identification and functional analysis of the cyclic nucleotide-gated channel gene family in Chinese cabbage

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Abstract

Cyclic nucleotide-gated channels (CNGCs) are a class of nonselective cationic channels that are widely found in animals and plants. Plant CNGCs participate in numerous biological functions that vary from development to stress tolerance. Most *CNGC* genes have been identified in plant genomes, but no such comprehensive study has yet been conducted on Chinese cabbage. In this study, thirty *BrCNGC* genes were identified, divided into five groups, and used for evolutionary analysis. We assigned names of all individual *CNGC* members on the basis of phylogenetic relationship with *A. thaliana* *CNGCs*. All *BrCNGC* genes were randomly distributed on chromosomes, and the A08 chromosome did not carry any *CNGC* gene. The *CNGC* genes of Chinese cabbage and *A. thaliana* from the same group displayed similar conserved motifs and gene structures. Especially the closer the homology, the higher the similarity. Quantitative expression analysis showed that most of the *CNGC* genes were expressed under four stresses, indicating that they play a key role in the stress response of Chinese cabbage. Expression patterns of 12 *BrCNGC* in the roots, stems, leaves, flowers, and siliques showed that *BrCNGC8* and *BrCNGC16* were specifically expressed only in flowers but not in other parts. This study lays a theoretical foundation for future research on the function of the CNGC gene family in Chinese cabbage.

Keywords Ca^{2+} · CNGC · Expression level · Phylogeny · QRT-PCR analysis

Abbreviations

AA	Amino acids
Ca^{2+}	Calcium
CaM	Calmodulin
CaMB	Calmodulin-binding domain
cGMP	Guanosine 3',5'-cyclic monophosphate
CNBD	Cyclic nucleotide-binding domain
CNGC	Cyclic nucleotide-gated channel
cNMPs	Cyclic nucleotide-monophosphate
GFP	The green fluorescence protein
MWs	Molecular weights
NCBI	National Centre for Biotechnology

PBC	Phosphate-binding cassette
PEG	Polyethylene glycol
Pi	Isoelectric point
QRT-PCR	Quantitative real-time polymerase chain reaction

Introduction

Calcium (Ca^{2+}) is an important ion that maintains the stability of plant cell structures (Hanson 1960). It usually functions as a cellular signal and second messenger in many pathways after internal or external stimulants such as plant hormones, pathogens, light signalling, temperature, salt, water or mechanical stress, among others (Dodd et al. 2010; Kudla et al. 2010). CaM is linked to the germination and growth of pollen tubes (Abbas and Chattopadhyay 2014). Pollen tube tip growth and fertilisation also depends on the participation of Ca^{2+} signals (Qin and Yang 2011; Hepler et al. 2012; Iwano et al. 2009; Zhou et al. 2009). Under elevated Ca^{2+} concentrations, interaction of this ion with CaM can lead to displacement of cyclic nucleotides and channel closure (Kaplan et al. 2007). When Ca^{2+} content is available

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at low levels in the external environment, plant development is affected as a result of Ca^{2+} deficiency (Choi et al. 2008).

Plant cyclic nucleotide-gated channels (CNGCs) were first identified in 1998 when screening CaM-conjugated transporters (*Hordeum vulgare* CaM-binding transporter, Hv CBT1) in barley (Mäser et al. 2001). These nonselective cationic channels are widely found in animals and plants (Yuen and Christopher 2013) and are universal Ca^{2+} sensors in eukaryotes. In the present study, it was confirmed that CNGCs occur in both monocotyledonous and dicotyledonous plants (Talke et al. 2003). Previous studies have shown that CNGCs are ligand-gated, Ca^{2+} -permeable ion transport channels that may be activated by cNMPs and inhibited by binding Ca^{2+} /CaM (Cukkemane et al. 2011; Ma and Berkowitz 2011; Spalding and Harper 2011). Structurally, CNGCs are composed of six transmembrane domains (S1–S6) and a pore region (P) between the fifth and sixth domains, as well as a C-terminal CaMB and a CNBD (Zelman et al. 2012). CNBD is the most conserved region of the CNGC, containing a PBC and a “hinge” region (adjacent to PBC); the PBC binds to the sugar and phosphate moieties of the cyclic nucleotide ligand (Cukkemane et al. 2011) and the “hinge” region contributes to ligand-binding efficacy and selectivity (Young and Krougliak 2004). It has been shown that the carboxyl tails of several plant CNGCs bind to CaM in a Ca^{2+} -dependent manner (Schuurink et al. 1998; Arazi et al. 2000a; Köhler et al. 2000; Reddy et al. 2002).

It has been shown that plant CNGCs participate in numerous biological functions that vary from plant development and stress tolerance (Kaplan et al. 2007) to disease resistance (Abdel-Hamid et al. 2011; Ma 2011), including ion transport, growth and development, pollen tube elongation, pathogen defense response, gravity, and heat resistance (Kaplan et al. 2007; Frietsch et al. 2007; Chin et al. 2009; Urquhar et al. 2011; Finka et al. 2012; Tunc-Ozdemir et al. 2013a, b). Previous experiments have shown that *CNGC18* plays an important role in the growth of pollen tubes (Frietsch et al. 2007). This is consistent with the pharmacological evidence that the cNMP signals in pollen can lead to growth-changing Ca^{2+} signals (Moutinho et al. 2011; Rato et al. 2004; Wu et al. 2011). According to report, *CNGC3*, *CNGC10*, *CNGC19*, and *CNGC20* are associated with the response to and tolerance of abiotic salt stress in *Arabidopsis thaliana* (Guo et al. 2008; Kugler et al. 2009). *AtCNGC1* participates in plumb (Pb^{2+}) (Sunkar et al. 2000) and Ca^{2+} uptake (Ma et al. 2006). Also, the *A. thaliana* orthologs (*AtCNGC2*) are essential for thermo tolerance (Finka et al. 2012). *CNGC5* and *CNGC6* encode Ca^{2+} channels that are activated by cGMP in cell membranes of *A. thaliana* stomata cells (Wang et al. 2013), and *CNGC7* and *CNGC8* are homologous genes that are expressed in pollen. Confocal laser scanning microscopy revealed that GFP-CNGC7 fusion

protein was located at the tip of the pollen tube. Individual *CNGC7* or *CNGC8* mutants were not significantly different from the wild-type in terms of pollen transport ability, but the co-mutation of the two genes resulted in a male sterility phenotype (Tunc-Ozdemir et al. 2013a, b).

Prior studies have also identified *AtCNGC2* and *AtCNGC4* from group IV-B as involved in disease resistance against various pathogens and in thermotolerance (Mäser et al. 2001; Maathuis 2006; Kugler et al. 2009; Yuen et al. 2010). In addition, *AtCNGC6* mediates heat-induced Ca^{2+} influx and the acquisition of thermotolerance (Saand et al. 2015), and *AtCNGC11* and *AtCNGC12* are involved in plant defense against pathogens (Kaplan et al. 2007). Nevertheless, further investigation is required to fully understand the signal components and molecular mechanisms underlying CNGCs' regulation of biological processes from plant development to stress tolerance.

Brassica is one of the most important cruciferous genera of the Brassicaceae family, and contains more than 40 species (Wang et al. 2011). Among them, Chinese cabbage (*Brassica rapa pekinensis*) is one of the most important vegetable crops in China with a long history of cultivation, various species, and distinct morphological features. Several *CNGC* genes have been identified in plant genomes, namely *A. thaliana* (Mäser et al. 2001), *Oryza sativa* (Bridges et al. 2005), *Populus trichocarpa* (Ward et al. 2009), *Pyrus bretschneideri* Rehd (Chen et al. 2015), a moss (*Physcomitrella patens*) and some algae (Verret et al. 2010; Zelman et al. 2013), but their phylogeny, evolution, and functions are still unclear and require further investigation. Until now, no comprehensive study has been conducted on Chinese cabbage. However, published genome sequence information on Chinese cabbage enables whole-genome analysis of *BrCNGCs*, for proper identification, characterization, and functional verification of this gene family, and to provide a reference for potential future research. In the present study, thirty *BrCNGC* genes were identified, divided into five groups, and used for evolutionary analysis. All *BrCNGC* genes were randomly distributed on nine chromosomes, and A08 chromosomes did not cover the genes. Quantitative expression analysis of twelve *BrCNGC* genes in the root, stem, leaf, flower, and siliques of Chinese cabbage showed that *BrCNGC8* and *BrCNGC16* were only expressed in flowers but not in other plant parts. This experiment lays a theoretical foundation for future research on the function and roles of the CNGC gene family in Chinese cabbage.

Materials and methods

Identification of CNGC family genes in Chinese cabbage

To identify all the CNGC gene family members in Chinese cabbage, the basic local alignment search tool using a protein query (blastp) was performed against the genomes deposited in *Brassica* database (<http://brassicadb.org/brad/>) using twenty *A. thaliana* CNGC full-length proteins obtained from the Arabidopsis Information Resource database (<http://arabidopsis.org/>) as queries. All retrieved non-redundant putative sequences were subject to domain analysis using Pfam (<http://pfam.xfam.org/>), SMART (<http://smart.embl-heidelberg.de/>), Conserved Domain Database (CDD) (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>), ExPASy-PROSITE, (<http://prosite.expasy.org/>) and SUPERFAMILY (<http://supfam.org/SUPERFAMILY/hmm.html>) to eliminate all sequences that did not contain the signature CNBD domain. The Chinese cabbage genome sequence is known, and we downloaded the gene and protein sequence of all members of the Chinese cabbage CNGC gene family from the *Brassica* database (*B. rapa* chromosome v1.5). Genes without CNGC-specific domains or that were <200 AA in size were excluded.

Phylogenetic analysis

To classify the *CNGC* genes identified in Chinese cabbage and investigate their phylogenetic relationships, their derived protein sequences were analysed. Phylogenetic trees were produced using the full-length sequences of CNGC proteins from Chinese cabbage and *A. thaliana*. Phylogenetic and molecular evolutionary genetics analyses were conducted using MEGA6 (<http://www.megasoftware.net/>) with the neighbour-joining method. The stability of branch nodes was assessed through 1000 bootstraps. To reflect the orthologous relationship between Chinese cabbage and (*A. thaliana* *CNGC* genes, the names of all individual *CNGC* members in (*B. rapa*) were assigned in ascending order in accordance with their phylogenetic relationships with 20 *A. thaliana* *CNGCs*, adding *Br* before gene name.

Location of CNGC genes on chromosomes, conserved motifs, and structural analysis

The position of each *CNGC* gene on the 10 Chinese cabbage chromosomes was determined from the *Brassica* database and a genetic linkage map was constructed using MapChart.

The conserved motifs of CNGCs proteins were detected using the MEME tool (<http://meme.sdsc.edu/meme/>). The

gene structure of *BrCNGC* was analysed online using the gene structure display server tool (<http://gsds.cbi.pku.edu.cn/>).

Plant materials, growth conditions, and abiotic stress treatments

Experiment materials for transcriptome sequencing were obtained from Chinese cabbages [doubled haploid (DH) line (FT)]. The artificial growth conditions were: photoperiod, 12 h/12 h, light/dark; temperature, 25/15 °C; and relative humidity, 50–60%. To better understand the response of each gene to different abiotic stresses, we divided germinated seedlings into four groups. Two groups were cultured in plastic pots in soil–vermiculite mixture and when seedlings reached the four-leaf stage, we treated each group with high or low temperature (38 °C or 4 °C, respectively) for 0, 3, and 12 h. After each period, fourth leaves were sampled for RNA extraction. The other two groups of seedlings were cultured in Hoagland's solution (hydroponic culture) and subject to osmotic or salt stress treatments at the four-leaf stage using 10% (w/v) PEG 6000 or 250 mM NaCl, respectively, for 0, 3, and 12 h. After each period, young roots were sampled for RNA extraction. All materials are cultured in a controlled-environment growth chamber. Three biological replicates were set up for each set of treatments.

Transcriptome analysis

Total RNAs were extracted from the root and leaf samples using TRIZOL reagent (TIANGEN, Beijing, China) (Chomczynski and Sacchi 1987) and RNA samples ($n = 12$) were sent to BGI (<http://www.bgitechsolutions.com>) for transcriptome sequencing using RNA-seq technology. An average of 23,594,868 raw sequencing reads were generated, and 23,521,984 clean reads were obtained after filtering out low-quality reads. After filtering, clean reads were mapped to the reference *B. rapa* genome sequence (version 1.5) using HISAT/Bowtie2 tool. The average mapping ratio was 76.09% for each gene and the average genome mapping ratio was 94.54%. For each sample, we conducted strict quality control from several aspects to evaluate if the sequencing data qualified. The RSEM tools were used for quantitative gene expression analysis based on the transcriptome sequences. To examine gene expression levels (to a certain degree) according to the fragments per kilobase of transcript per million mapped reads (FPKM) values (files with the gene.FPKM.xls suffix), we assessed the correlation between each two RNA samples and developed heat maps of relevance using the Gene Denovo online software (<http://www.omicshare.com/tools/Home/Soft/heatmap>).

RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from roots, stems, leaves, flowers, and siliques using the RNA extraction kit (TIANGEN, Beijing, China) according to the manufacturer's instruction. The RNA was reverse-transcribed into cDNA using the Fast Quant RT Kit (with gDNase, TIANGEN, Beijing, China), and purified cDNA samples were diluted (1:50) with ddH₂O before being used as templates in for qRT-PCR. The software Primer 5 was used to design specific primers for *BrCNGC* genes (Support Information Table 1), and gene *actin* was used as the internal control to normalise the expression level of target genes. A hybrid system that contained 29.4 µl ddH₂O, 1.4 µl of each of forward and reverse primer, and 2.8 µl diluted cDNA. After brief centrifugation, samples were protected from light and 35 µl Ultras SYBR Mixture (Low ROX, CWBIO, Beijing, China) were added. After removing the air bubbles, the liquid was dispensed into eight joint tubes (20 µl per tube). Three biological replicates were conducted for each experiment. The qRT-PCRs were performed using the Quantstudio™ 6 Flex Real-time PCR System (Applied Biosystems® by Life Technologies™, USA). The thermal cycling conditions were: pre-degeneration for 10 min at 95 °C; 40 cycles of denaturation for 15 s at 95 °C; annealing for 60 s at 60 °C; and final melting curve analysis for 15 s at 95 °C, 60 s at 60 °C, 15 s at 95 °C, and 15 s at 60 °C. We used the comparative – $\Delta\Delta C_t$ value method to analyse the relative gene expression (Heid et al. 1996). Finally, results were calculated using the $2^{-\Delta\Delta C_t}$ method according to previous reports (Livak et al. 2001).

Results

Identification of CNGC family members in the Chinese cabbage genome

The genome-wide identification of *CNGC* genes in Chinese cabbage was performed by blastp search using known *A. thaliana* CNGC proteins as the queries. After removal of the overlapping genes and alternative splice forms of the same gene, 46 candidate genes were identified as the most similar to the CNGC sequences used as the queries.

Because plant CNGCs are characterised by the presence of CNBD domains, TM or ITP regions, specifically PBCs and hinge region (Talke et al. 2003; Zelman et al. 2012; Ward et al. 2009; Zhorov and Tikhonov 2004), their presence in the AA sequence is a validation criterion for CNGCs. We used five different domain analysis programs (Pfam, SMART, CDD, ExPASy-PROSITE and SUPERFAMILY) to confirm the presence of a CNB domain (ITP domain in Pfam, cNMP domain in SMART, CAP_ED domain in CDD,

cNMP in ExPASy-PROSITE, and cAMP in SUPERFAMILY). Subsequently, the candidate sequences were examined for plant CNGC-specific motifs in the PBC and hinge region within CNBD. The blastp search also provided genes from potassium AKT/KAT channels as homologs of CNGCs due to the presence of CNBD and ITP domains, TM region, and additional ankyrin repeats (Su et al. 2001), but such unrelated genes were excluded from further analysis. Finally, 30 full-length *BrCNGC* genes were confirmed to contain the specific domains in their proteins sequences, and hence were identified as *CNGC* genes in Chinese cabbage (Table 1).

All members from group II contained IQ domains, which are consistent with the results reported by previous studies (Mumtaz et al. 2015). The IQ domain is conserved in plant CNGCs and it increases the variability of the Ca²⁺-dependent pathway control mechanism; emphasising the functional diversity within this multi-gene family. These group II genes are from the same domain, and it is noteworthy that genes of this group are relatively conservative in the evolutionary process.

Whole-genome information analysis of *BrCNGCs*

Members of the Chinese cabbage CNGC gene family and relevant information are listed in the summary table (Support Information Table 3). The length of *BrCNGCs* proteins ranged from 556 AA (Bra031515) to 760 AA (Bra021266), and their *Pis* were between 8.02 (Bra022702) and 9.83 (Bra022235) and distributed in the alkaline area. The MWs of these proteins were between 81342.2 Da (Bra008733) and 90343.81 Da (Bra022232). When blastp was used to identify CNGC members, a specific gene, Bra024083, with only 0.237 kb was located on chromosome A03. Its protein sequence consisted of only 78 AA, which was a tenth of the average length of the protein sequence of CNGC family members. The *Pi* was 4.54, and the MW was 8610.44 Da, which were much smaller than that of other gene family members. Chinese cabbage shares a common whole-genome triplication (paleohexaploidy) and diploid ancestor with the model species *A. thaliana*. Among the three subgenomes, we observed biased gene fractionation (gene loss). For almost every *AtCNGC* gene, there was a corresponding homologous *BrCNGC* gene. By searching each (*A*) *thaliana* orthologous gene (syntenic paralog in (*B*) *rapa*), we found a collinear relationship between Bra024083 and *AtCNGC17* (Support Information Table 5), which suggested that this gene might have been a member of the *CNGC* gene family, but a variable shear in the process of replication might have resulted in the absence of part of the gene fragment. Due to its short length, this gene is presumed to lack a specific motif. Thus, in the present study, Bra024083 was considered a member of the *non-CNGC* gene family and excluded from further analyses.

Table 1 Domain architecture of BrCNGC gene family

Group	Gene symbol	Gene locus	Domain architectures in different databases				
			PFAM	Smart	CDD	PROSITE	SUPERFAMILY
Group I	BrCNGC1	Bra000937	ITP	cNMP	CAP_ED	cNMP	cAMP
	BrCNGC2	Bra004537	ITP,CNMP	cNMP	CAP_ED,ITP	cNMP	cAMP
	BrCNGC3	Bra034281	ITP	cNMP	CAP_ED,ITP	cNMP	cAMP
	BrCNGC4	Bra031515	ITP	cNMP	CAP_ED,ITP	cNMP	cAMP
	BrCNGC5	Bra003323	ITP	cNMP	CAP_ED	cNMP	cAMP
	BrCNGC6	Bra022632	ITP,CNMP	cNMP	CAP_ED	cNMP	cAMP
	BrCNGC7	Bra003081	ITP,CNMP	cNMP	CAP_ED	cNMP	cAMP
Group II	BrCNGC8	Bra026086	ITP,CNMP	cNMP	CAP_ED	cNMP,IQ	cAMP
	BrCNGC9	Bra039221	ITP	cNMP	CAP_ED	cNMP,IQ	cAMP
	BrCNGC10	Bra032132	ITP	cNMP	CAP_ED	cNMP,IQ	cAMP
	BrCNGC11	Bra024067	ITP,CNMP	cNMP	CAP_ED	cNMP,IQ	cAMP
	BrCNGC12	Bra020402	ITP,CNMP	cNMP	CAP_ED	cNMP,IQ	cAMP
Group III	BrCNGC13	Bra007839	ITP,CNMP	cNMP	CAP_ED	cNMP	cAMP
	BrCNGC14	Bra032081	ITP,CNMP	cNMP	CAP_ED	cNMP	cAMP
	BrCNGC15	Bra018089	ITP,CNMP	cNMP	CAP_ED,ITP	cNMP	cAMP
	BrCNGC16	Bra008733	ITP,CNMP	cNMP	CAP_ED	cNMP	cAMP
	BrCNGC17	Bra011186	ITP,CNMP	cNMP	CAP_ED	cNMP	cAMP
	BrCNGC18	Bra011963	ITP,CNMP	cNMP	CAP_ED	cNMP	cAMP
	BrCNGC19	Bra021265	ITP	cNMP	CAP_ED,ITP	cNMP	cAMP
Group IV-A	BrCNGC20	Bra022232	ITP	cNMP	CAP_ED,ITP	cNMP	cAMP
	BrCNGC21	Bra022233	ITP	cNMP	CAP_ED	cNMP	cAMP
	BrCNGC22	Bra021266	ITP	cNMP	CAP_ED,ITP	cNMP	cAMP
	BrCNGC23	Bra022235	ITP		ITP		cAMP
	BrCNGC24	Bra031529	ITP	cNMP	CAP_ED	cNMP	cAMP
	BrCNGC25	Bra029958	ITP	cNMP	CAP_ED,ITP	cNMP	cAMP
	BrCNGC26	Bra001676	ITP	cNMP	CAP_ED	cNMP	cAMP
	BrCNGC27	Bra001678	ITP	cNMP	CAP_ED	cNMP	cAMP
	BrCNGC28	Bra008699	ITP,CNMP	cNMP	CAP_ED	cNMP	cAMP
Group IV-B	BrCNGC29	Bra003001	ITP	cNMP	CAP_ED	cNMP	cAMP
	BrCNGC30	Bra022702	ITP	cNMP	CAP_ED	cNMP	cAMP

ITP ion transport protein domain, cNMP cyclic nucleotide-monophosphate binding domain or cyclic nucleotide-binding domain (CNBD), CAP_ED cap family effector domain, which binds cAMP

By comparing the genomic information of Chinese cabbage (Support Information Table 3) and *A. thaliana* (Support Information Table 4), we found that the number of exons in gene group IV-A, the length of genes and proteins, Pis and MWs were almost indistinguishable between the two genomes. In the gene block column (Support Information Table 3), all group IV-A genes seem to be from the F region, while the other groups of genes apparently show no regularity.

Phylogenetic relationship between Chinese cabbage and *A. thaliana* CNGCs

To determine the phylogenetic relationships between Chinese cabbage and *A. thaliana* CNGCs, a maximum

likelihood phylogenetic tree was constructed using full-length AA sequences. The previously defined clade IV comprised two sub-clades: A and B (Mäser et al. 2001). In total, the 20 AtCNGCs and 30 BrCNGCs were classified into five groups: I, II, III, IV-A, and IV-B (Fig. 1), containing different numbers of members.

The sizes of the groups were unequal. Group I constituted the largest clade with thirteen members: seven members from Chinese cabbage (BrCNGC1–BrCNGC7) and six from *A. thaliana* (AtCNGC1, 3, 10, 11, 12, and 13). Group IV-B was the smallest with five members: three Chinese cabbage CNGCs (BrCNGC28–BrCNGC30) and two *A. thaliana* CNGCs (AtCNGC2 and AtCNGC4). Although Chinese cabbage and *A. thaliana* CNGC proteins also formed a cluster in group I, they showed large genetic distances,

Fig. 1 Phylogenetic tree of BrCNGC and AtCNGC proteins. Thirty of the BrCNGC proteins (marked with red squares) and twenty of the AtCNGC proteins (marked with purple triangles) were used to construct the neighbour-joining tree. The bootstrap values from 1000 resampling are given at each node

which suggested they might be evolving in different direction. The number of members in group II was similar for both Chinese cabbage and *A. thaliana*, i.e., five Chinese cabbage CNGCs (BrCNGC8–BrCNGC12) and five *A. thaliana* CNGCs (AtCNGC5–AtCNGC9). Both group III and group IV-A contained 11 members: group III had six Chinese cabbage CNGCs (BrCNGC13–BrCNGC18) and five *A. thaliana* CNGCs (AtCNGC14–AtCNGC18), while group IV-A had nine CNGCs from Chinese cabbage (BrCNGC19–BrCNGC27) and two CNGCs from *A. thaliana* (AtCNGC19 and AtCNGC20).

Location of CNGC genes on chromosomes

Chromosome localisation analysis demonstrated that the *BrCNGCs* are located on nine chromosomes (A01–A07, A09, and A10) (Fig. 2). The *BrCNGC* genes were unevenly distributed: chromosome A01 carried six *CNGC* genes; chromosomes A02 and A04 each carried three *CNGC* genes; chromosomes A03, A05, and A10 each carried four *CNGC* genes, while A06, A07, and A09 carried two genes. No *CNGC* gene was found on chromosome A08.

In the present study, both segmental and tandem duplication events were investigated to elucidate the underlying mechanism of evolution of the *BrCNGC* gene family.

Chromosomal localisation analysis showed that most *BrCNGC* genes were randomly scattered throughout the genome, except for four pairs of *CNGC* genes. As evidenced in Fig. 2, genes *BrCNGC22* and *BrCNGC19* were located at adjacent positions on chromosome A01. This distance was particularly short and the distribution of these two genes in the chromosome appeared to almost overlap. The length, starting position, and AA number of these genes was also very similar (Support Information Table 3), suggesting that these closely co-located genes were probably generated from a tandem duplication event (Chen et al. 2015). Similarly, another three groups of genes, *BrCNGC26* and *BrCNGC27*, *BrCNGC23*, *BrCNGC21*, and *BrCNGC20*, *BrCNGC28* and *BrCNGC16*, that were co-located at adjacent positions on chromosomes A03, A05, and A10, respectively, probably underwent tandem duplication events. Therefore, we hypothesised that these gene duplications will not only expand the number of *CNGC* family members in Chinese cabbage but will also increase their functional diversity.

Conserved motifs and phylogenetic analysis

To better understand the evolution of the *CNGC* gene family in Chinese cabbage and *A. thaliana*, we constructed phylogenetic trees for the conserved motifs obtained using MEME

Fig. 2 Distribution of *BrCNGC* genes on the chromosomes. The chromosomal positions of the *BrCNGC* genes were mapped according to the information available in the Chinese cabbage genome database. The chromosome numbers are indicated at the top of each chromosome. The scale is in mega bases (Mb)

(Fig. 3). According to E-values (E-value < e-100 criterion), the top 20 specific and conserved motifs were retained and downloaded (see details of motifs on Support Information Table 2). The comparative analysis between the phylogenetic trees and conserved motifs revealed that conserved motifs from members of the same group were similar, and that the conserved motifs of homologous genes were highly consistent. The closer the homology, the higher the similarity of the motifs. In addition, the number of conserved motifs in groups I, II, and III was approximately the same, with an average of 17, while group IV-B contained the least number of conserved motifs, with an average of 13. Each of the 30 CNGC proteins contained conserved motif 10 (Fig. 3), and motifs 1, 2, 3, 5, 6, 7, 8, 9, 11, 13, 14, and 15 were common to almost all CNGCs. Conserved motifs 16, 18, and 20 were endemic to group IV-A and, therefore, some of the functions of the CNGCs within group IV-A were not available in the other groups. Hence, we hypothesised that the differences in the types and quantities of conserved motifs in each group might lead to changes in their evolutionary rate and degree.

Genetic structure analysis

Gene structure analyses can provide valuable information concerning duplication events when interpreting phylogenetic relationships within gene families. To better understand the relationship between the genetic structure of Chinese cabbage and *A. thaliana* CNGCs, we used online analysis software to obtain intron and exon structures (Fig. 4). It is noteworthy that the number of introns was different, but the genes that clustered together had very similar distributions of exons and introns. Overall, the number of exons determined for CNGCs in Chinese cabbage ranged from 5 to 11

(Support Information Table 3), as in *A. thaliana* (Support Information Table 4). The number of exons in the *BrCNGCs* from group I were between 6 and 9, while the number of exons of *AtCNGCs* within the same group ranged from 7 to 9. The number of exons in *BrCNGC* and *AtCNGC* from group II were between 5 and 7, the CNGCs from group III had 6 or 7 exons and group IV-A CNGCs had 10 or 11 exons in both Chinese cabbage and *A. thaliana*. In group IV-B, the *BrCNGCs* had eight exons and the *AtCNGC* gene had nine exons. Thus, the numbers of exons in CNGCs from the same group were strikingly similar between *A. thaliana* and Chinese cabbage, although usually higher in *AtCNGCs* than in *BrCNGC*. Because the CNGCs in group IV-A have the highest number of exons, they are more likely to undergo alternative cleavage, generate new genes, and increase the functional diversity of the CNGC gene family.

Quantitative expression analysis of genes under different stresses

In the transcriptome analysis, genes *BrCNGC4*, *BrCNGC16*, and *BrCNGC21* were oddly found to be unresponsive to the four abiotic stresses tested here. Although *BrCNGC8* had no changes in expression under salt and osmotic stresses, it was significantly upregulated after 12 h at a high temperature. We hypothesised that these four genes were not differentially expressed in response to abiotic stresses, but might respond to other specific spatiotemporal conditions.

The heat map constructed for temperature stress (Fig. 5a) showed that almost all genes were downregulated at the initial stage of high temperature (HT0). With the extension of stress time, gene expression gradually increased, and eventually was upregulated. In addition, the specific expression of

Fig. 3 MEME domain analysis and schematic diagram for main motif structures of CNGC genes in Chinese cabbage and *A. thaliana*. Different motifs are indicated by different colors numbered 1–20. For details of motifs, refer to Support Information Table 2

BrCNGC1, *BrCNGC3*, *BrCNGC8*, *BrCNGC9*, *BrCNGC12*, *BrCNGC13*, *BrCNGC23*, *BrCNGC24*, and *BrCNGC30*, was particularly pronounced. Only five genes: *BrCNGC7*, *BrCNGC11*, *BrCNGC18*, *BrCNGC27*, and *BrCNGC28*, showed weak downregulated expression. Therefore, the highly expressed genes under high-temperature stress might be responsible for heat tolerance. Under low-temperature stress, the expression of *BrCNGC1*, *BrCNGC2*, *BrCNGC3*, *BrCNGC10*, *BrCNGC17*, *BrCNGC22*, *BrCNGC23*, *BrCNGC27*, and *BrCNGC29* was upregulated during stages LT0–LT3 and LT3–LT12, while *BrCNGC7*, *BrCNGC12*, and *BrCNGC30* were always down-regulated during the same stages. In conclusion, five of the 30 genes, *BrCNGC1*, *BrCNGC3*, *BrCNGC10*, *BrCNGC17*, and *BrCNGC29*, were upregulated during the entire temperature stress experiment.

These genes are likely to function on specific occasions to help Chinese cabbage adapt to harsh environments and continue to grow.

Under salt stress (Fig. 5b), most genes were upregulated, while under PEG stress almost all genes were downregulated. Among the most upregulated genes, the differential expression of *BrCNGC7*, *BrCNGC10*, *BrCNGC12*, *BrCNGC13*, *BrCNGC19*, *BrCNGC22*, and *BrCNGC23* was significant in stages NC0–NC3 and NC3–NC12. Among the downregulated genes, *BrCNGC6*, *BrCNGC29*, and *BrCNGC30* had significantly lower expression levels in the same stages. Under PEG stress, the expressions of *BrCNGC3*, *BrCNGC7*, *BrCNGC14*, and *BrCNGC17* gradually increased in stages PEG0–PEG3 and PEG3–PEG12 and the differences in the expressions of *BrCNGC3* and *BrCNGC14* were very obvious. In contrast,

Fig. 4 Gene structure of 30 members of Chinese cabbage CNGC gene family. The exon/intron structure of *BrCNGC* gene was analysed online by the tool gene structure display server. The green bar repre-

sents the exon, while the black line represents the intron. Gene models are drawn to scale as indicated on the bottom, the direction is from 5' to 3'

BrCNGC5, *BrCNGC13*, *BrCNGC19*, *BrCNGC20*, *BrCNGC22*, *BrCNGC25*, and *BrCNGC28* were downregulated in all experiments. Moreover, the expression of *BrCNGC5* at the early stage of PEG stress was particularly high and gradually decreased as the stress period was prolonged. The upregulation of these genes under stress suggests they might be responsible for plant stress resistance, and that these versatile genes enable Chinese cabbage to survive even in harsh climatic conditions.

Expression patterns in different organs of Chinese cabbage

To further explore the roles of *CNGCs* in the growth and development of Chinese cabbage, we selected 12 genes

for qRT-PCR analysis in roots, stems, leaves, flowers, and siliques: three genes from groups I and II and two genes from each of the remaining three groups. Results of fluorescence quantitation showed that most of these genes were mainly expressed in flowers, followed by roots and siliques, while the number of genes expressed in stems and leaves was the lowest (Fig. 6). No expression was detected for *BrCNGC20* at any organ, probably because this gene is not expressed in the selected tissues. Two genes, *BrCNGC8* and *BrCNGC16*, were only expressed in flowers. Therefore, we propose that these two genes are closely related to the process of flowering or pollen tube formation in Chinese cabbage. Whether or not a male sterile transgenic plant can be obtained by individually

Fig. 5 Expression pattern of Chinese cabbage *CNGC* gene in different environments. Hierarchical clustering of *BrCNGC* genes that were significantly differentially expressed under high- and low-temperature stress (a) or salt and polyethylene glycol stress (b). The amount of gene specific-expression is indicated by the color scale of the transition from green to red. The green color indicates that the gene is downregulated in expression; the red color indicates that the gene is upregulated in expression. The expression levels at 0, 3, and 12 h

at high temperature were expressed as HT0_FPKM, HT3_FPKM, and HT12_FPKM, respectively; at 0, 3, and 12 h of cold stress, the expression levels were expressed as LT0_FPKM, LT3_FPKM, and LT12_FPKM, respectively; at 0, 3, and 12 h under salt stress, they were expressed as NC0_FPKM, NC3_FPKM, and NC12_FPKM, respectively; the expression levels of genes at 0, 3, and 12 h under polyethylene glycol stress were, respectively, expressed by PEG0_FPKM, PEG3_FPKM, and PEG12_FPKM

silencing or knocking out these two genes requires further investigation.

The expression of *BrCNGC1* was used as control in the histogram of the expression of different genes in the same organ (Fig. 6). Although *BrCNGC20* was not expressed at any organ, the remaining 11 genes were all expressed in flowers, and the relative expression of *BrCNGC6* was the highest while that of *BrCNGC16* was the lowest. In the siliques, the relative expression level of *BrCNGC28* was the highest, and the relative expression level of *BrCNGC22* was the lowest. However, in the root, stem, and leaf, the highest relative expressions were found for *BrCNGC22*, *BrCNGC12*, and *BrCNGC28*, respectively, and the lowest relative expression was found for *BrCNGC3*.

Considering the expression of the same gene in different organs, using the root as control (Fig. 7), most genes showed relatively higher expression levels in stems and leaves than in other organs. The relative expression levels of *BrCNGC1*, *BrCNGC3*, *BrCNGC10*, *BrCNGC17*, and *BrCNGC20* were relatively high in the leaves; the relative expression levels of *BrCNGC1* and *BrCNGC3* were lowest in the siliques, and the relative expression levels of *BrCNGC10*, *BrCNGC17*, and *BrCNGC20* were relatively low in the roots.

Previous studies showed that homologous genes may have similar functions. In our current research, *BrCNGC8*

was only expressed in flowers. Similarly, its homologue in *A. thaliana*, *AtCNGC7*, was also only expressed in flowers, and mutations in genes *AtCNGC7* and *AtCNGC8* cause *A. thaliana* to develop a male sterile phenotype. In addition, *BrCNGC16* was only detected in flowers and its homologue *AtCNGC18* in *A. thaliana* is associated with pollen tube elongation.

Discussion

The CNGC gene family has been reported in many agricultural crops (Almoneafy et al. 2014; Chen et al. 2015). However, Chinese cabbage *CNGCs* had not yet been subject to genome-wide identification and annotation. There are 20 members of the CNGC gene family in *A. thaliana*, 18 members in tomato (Mumtaz et al. 2015), 21 members in pear, and 16 *CNGC* genes in rice (Nawaz et al. 2014). Here, we identified 30 Chinese cabbage *CNGC* genes, which is much higher than the number reported for other plant species, suggesting that the CNGC gene family has expanded in Chinese cabbage. There are many reasons for an increase in the number of gene family members. Research has shown that tandem duplication, segmental duplication, and transposition events are the main reasons for such expansion (Kong

Fig. 6 Expression analysis of different genes in the same organ of Chinese cabbage. The horizontal axis indicates different genes, and the vertical axis indicates relative expression levels

et al. 2007). In Chinese cabbage, whole-genome triplication also contributed to an increase in the number of *CNGCs* and likely produced a diversity of functions. We propose that the enlargement of this gene family is of great benefit to the growth, survival, and adaptability of Chinese cabbage, as the increase in the number of members enriches the functions of this gene family and creates a suitable internal environment for the growth and development of the plant under several environmental conditions. Thus, the increase in the number of *CNGC* members was a necessary event that occurred during the plant gene evolutionary process, conferring plants the ability to adapt to changing environmental conditions.

To elucidate the role of Chinese cabbage *CNGCs*, we hypothesised that the same group of homologous genes

would have similar structures, functions and evolutionary properties. This gene family is known to have a specific cNBD domain, and we used five methods to identify all *BrCNGCs* based on the expected specific domains. All members from group II contained IQ domains (Table 1), which is consistent with the findings of previous studies (Mumtaz et al. 2015). The IQ domain is conserved in plant *CNGCs* and increases the variability of the Ca^{2+} -dependent pathway control mechanism, which emphasises the functional diversity within this multi-gene family. Although they are present in the IQ domain family and CaM-binding transcriptional activator, the function of IQ domains in plant proteins has not been studied in detail in *CNGCs* (Baler and Rhoads 2002; Reddy et al. 2002; Abel et al. 2005). Although *BrCNGC23*

Fig. 7 Expression analysis of the same gene in five different organs of Chinese cabbage. The horizontal axis indicates different organs, and the vertical axis indicates relative expression levels

from group IV-A lacked a cNMP domain according to the analyses performed in PROSITE and SMART, it comprised a cAMP-binding site, according to SUPERFAMILY analysis. These results might indicate that *BrCNGC23* lost the IQ domain in the evolutionary process, but gained functional diversity; however, further investigation is required to verify this hypothesis.

During the evolution of gene families, gene duplication plays an important role in generating new members and creating novel gene functions, thereby increasing the number and diversity of genes in the gene family (Cannon et al. 2004; Chauve et al. 2008). In our study, the phylogenetic analysis between Chinese cabbage and *A. thaliana* followed a previously described model (Bowers et al. 2003) and demonstrated the evolutionary trend of gene duplication in the two taxa.

In addition, we have proposed important research topics for further investigation. The increase or decrease in the expression level of some genes in the leaves or young roots of Chinese cabbage grown under stress might be due to the up- or downregulation of certain genes, whose functions are related to plants' adaption to environmental changes. However, the regulatory mechanisms underlying such functions are still unclear. For instance, the expression of *BrCNGC7*, a member of group I of the Chinese cabbage CNGC gene family was downregulated under temperature stress but upregulated under salt and osmotic stress, thereby differing from other genes within the same group. We therefore recommend that future assessments on the stress resistance of Chinese cabbage consider the role of *BrCNGC7*.

It is also noteworthy that two genes, *BrCNGC8* and *BrCNGC16*, are specifically expressed in flowers. Thus, future

studies should investigate whether these genes are involved in important flower-related regulation processes, to obtain a network mechanism for gene-regulation of the flowering process in Chinese cabbage.

Overall, our findings suggest that *BrCNGC* genes from the same group might play different roles in Chinese cabbage, while genes from different groups may function similarly under both biotic and abiotic stress responses. Although it remains unclear if different genes interact, and how these types of interactions function to regulate networks, and further investigations are required, the results of the present study provide a theoretical basis for further research on the CNGC gene family in Chinese cabbage, and in other crops.

Conclusion

In our study, genome-wide characterization and functional analysis of the CNGC gene family were conducted in Chinese cabbage. As a result, thirty *BrCNGC* genes were identified. We conducted a more comprehensive analysis of their phylogenetic relationships, distribution on chromosomes, conserved domains, and gene structures. The specific expression of CNGCs under four abiotic stresses as well as in different tissues was investigated. The results obtained in this study provided some valuable information about the CNGCs gene family, and laid a theoretical foundation for future research on the function of the CNGC gene to regulate growth, development and abiotic stress response of Chinese cabbage.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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