Identification and expression analysis of BnaCNGC family gene in the response to phytohormones, abiotic and biotic stresses in Brassica napus

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ABSTRACT

The cyclic nucleotide-gated ion channel (CNGC) family affects the uptake of Ca\textsuperscript{2+}, regulates the growth and development, pathogen defense, and abiotic stress tolerance in plants. However, the systematic identification, origin, and function of the CNGC gene family has not been performed in Brassica napus. In the present study, we identified 61 putative BnaCNGC genes in the B. napus genome, which are non-randomly localized on 18 chromosomes, and could be classified into five major groups: Group I, II, III, IV-a, and IV-b. Gene structure, multiple sequence alignment, and MEME analysis showed that all the CNGC genes are intron rich and conserved. The expression analysis showed that the BnaCNGC genes have different expression patterns in B. napus, under different phytohormones, abiotic stresses (cold, hot, waterlogging), and infection of Sclerotinia sclerotiorum. Among them, four genes (BnaC03g31050D, BnaC03g31720D, BnaA05g01380D and BnaC09g01250D) from Group I and two genes (BnaCng45430D and BnaA03g34680D) from Group IV-a, all were strongly induced by SA and infection of S. sclerotiorum, and reduced by cold and heat stresses, suggesting their importance in the abiotic and biotic stress responses in rapeseed. Our comprehensive genome-wide analysis represents a rich data resource for studying the CNGC gene family in B. napus.

1. Introduction

Ca\textsuperscript{2+}, an important second messenger, has been shown to be involved in plant response to environmental changes including abiotic and biotic stresses and developmental cues (Tuteja and Mahajan, 2007). However, the calcium-permeable channels in plants have remained largely unknown. One of the potential pathways for the uptake of Ca\textsuperscript{2+} ions in signal transduction is via cyclic nucleotide-gated ion channels (CNGCs) (Chin et al., 2009; Jammes et al., 2011).

CNGCs, a kind of calcium-permeable cation channels, have been demonstrated to play a vital role in multiple molecular functions involved in plant development and environmental stress tolerance (Kaplan et al., 2007). In plants, CNGCs localized in the plasma membrane (Borsics et al., 2007), vacuole membrane (Yuen and Christopher, 2013), or nuclear envelope (Charpentier et al., 2016), are composed of six transmembrane (TM) domains with one pore region (P loop) between the fifth and sixth TM domains, one C-terminal cyclic nucleotide-binding domain (CNBD), one C-terminal CaM-binding domain (CaMBD), or isoleucine-glutamine-glycine domain (IQD) (Chen et al., 2015; Hao and Qiao, 2018). CNGC activity can be regulated by cNMPs such as cAMP and CaM binding to the highly conserved CNBD (Wang et al., 2020). CNBD has a phosphate-binding cassette (PBC) and a hinge region which are plant CNGC-specific motifs (Saand et al., 2015). In Arabidopsis, AtCNGC1 and AtCNGC2 are permeable to both K\textsuperscript{+} and Ca\textsuperscript{2+}, and AtCNGC5, 6, 7, 8, 9, 10, 14, 16, and 18 are Ca\textsuperscript{2+}-permeable cation-selective channels (Wang et al., 2019). CNGCs as Ca\textsuperscript{2+}-permeable channels interacted with the ubiquitous Ca\textsuperscript{2+}/calmodulin (CaM) via diverse CaMBDs, including IQD (Fischer et al., 2017). A latest study showed that CNGC12 gene channel functionality depends on the conserved IQ-motif in the C-terminus of the channel (DeFalco et al., 2016).

Previous studies have shown that CNGCs play an important role in plant growth and development. In Arabidopsis, 20 CNGC genes are differentially expressed in all tissues and their functions critically depend on the presence of cAMP and/or CaM that function as signaling molecules (Nawaz et al., 2014). AtCNGC18 is expressed primarily in pollen, and the loss of AtCNGC18 causes abnormalities in pollen tube growth (Frietsch et al., 2007). AtCNGC7/8 and AtCNGC18 together reported as pollen-tube-specific CNGC proteins, interacting with calmodulin 2 (CaM2) constitute a molecular switch that either opens or closes the calcium channel depending on cellular calcium levels (Pan et al., 2019). OsCNGC13 is preferentially expressed in the pistils and facilitates the penetration of the pollen tube in the style for successful double fertilization and seed-setting in rice (Xu et al., 2017). AtCNGC11/10 has been shown to play a role in the roots of Arabidopsis (Ma et al., 2006; Borsics et al., 2007). AtCNGC14-dependent Ca\textsuperscript{2+} signaling is essential for regulating the early posttranscriptional phase of auxin growth responses in Arabidopsis roots (Shih et al., 2015).
AtCNGC5 and AtCNGC6 genes encode unique cGMP-activated nonselective Ca\(^{2+}\)-permeable cation channels in the plasma membrane of Arabidopsis guard cells (Wang et al., 2013).

In rice, OsCNGC genes were highly responsive to multiple stimuli including hormonal, biotic, and abiotic stress (Nawaz et al., 2014). For example, the function of two closely related CNGC genes, OsCNGC14 and OsCNGC16, are investigated for increasing temperature-stress tolerance in rice (Oryza sativa). A rice plasma membrane-localized Ca\(^{2+}\)-permeable nonspecific cation channel OsCNGC9 enhances chilling tolerance in rice, through regulating cold-induced calcium influx and cytoplasmic calcium elevation (Wang et al., 2021). Arabidopsis AtCNGC2 and CNCGb gene from Physcomitrella patens act as the primary thermosensors of land plant cells. CNCGb loss-of-function causes a hyper-thermo-responsive Ca\(^{2+}\) influx and altered Ca\(^{2+}\) signaling (Fink et al., 2012). Arabidopsis plants may contain a cyclic nucleotide-based signaling pathway that directly affects Na\(^+\) transport and improves Arabidopsis salinity tolerance (Maathuis and Sanders, 2001). The AtCNGC10 channel is involved in Na\(^+\) and K\(^+\) transport during cation uptake in roots and in long-distance transport, and the AtCNGC10 antisense lines were more sensitive to salt stress compared with the wild-type (Guo et al., 2008). AtCNGC19 and AtCNGC20 are also involved in salt stress responses (Kugler et al., 2009; Yuen and Christopher, 2013).

CNGCs also play important roles in the resistance to biotic stress. Loss-of-function AtCNGC2 exhibited increased resistance to pathogen infection after infection with some avirulent pathogens (Clough et al., 2000; Chin et al., 2013). AtCNGC4 gene expression is induced by pathogen infection and some pathogen-related signals (Balagué et al., 2003). AtCNGC11 and AtCNGC12 are involved in R gene-mediated resistance and exhibits increased resistance to pathogen infection (Yoshioka et al., 2006; Moeder et al., 2011). The mutation of AtCNGC20 partially restores disease resistance in eds1 (ENHANCED DISEASE SUSCEPTIBILITY1) (Zhao et al., 2021). In rice, OsCNGC9 positively regulates the resistance to rice blast disease (Wang et al., 2019). SlCNGC genes of Group IV-b regulate different types of resistance against diverse pathogens in tomato (Saand et al., 2015).

Brassica napus (2n = 4x = 38, AACC) is an important allopolyploid oil crop derived from interspecific crosses between Brassica rapa (2n = 2x = 20, AA) and Brassica oleracea (2n = 2x = 18, CC) (Challoub et al., 2014). The production of B. napus is highly influenced by environmental stress conditions. Although the CNGC gene family members have been identified in Arabidopsis (Bridges et al., 2005), rice (Nawaz et al., 2014), tomato (Saand et al., 2015), pear (Chen et al., 2015), maize (Hao and Qiao, 2018), Brassica oleracea (Kakar et al., 2017), Brassica rapa (Li et al., 2019), and Chinese jujube (Wang et al., 2020), the CNGC gene family has not been studied in B. napus.

In this study, 61 putative CNGC genes were identified in B. napus and classified into five groups (Group I, II, III, IV-a, IV-b). The analysis of evolutionary and structure implied that the CNGC genes were conserved and there was an expansion of Group IV-a members in Brassicaceae. The expression analyses showed that BnaCNGC genes were particularly sensitive multiple stimuli including hormonal, abiotic, and biotic stress in B. napus. Our objective elucidated the expansion and functional diversification of the CNGC gene family and identified some novel genes potentially useful for breeding in B. napus.

2. Materials and methods

2.1 Identification of CNGC in B. napus

To identify the CNGC in B. napus, 20 Arabidopsis CNGC protein sequences were used as queries to perform BLASTP for the B. napus genome in the Ensembl Plants database (http://plants.ensembl.org/index.html). All non-redundant protein sequences were retrieved, and the conserved domains were analyzed with Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). Protein sequences containing membrane-spanning regions, pore region, CNBD, CaMBD, or IQD were recognized as members of CNGCs. Amino acid residues properties, including molecular weight (kDa) and isoelectric points (pI), were determined using the ProtParam tool (http://web.expasy.org/prot-param/). Multiple sequence alignments of the putative CNGC proteins were aligned by the CLUSTALW program (https://www.genome.jp/tools-bin/clustalw). A phylogenetic tree was constructed using the EvolView (https://evolgenius.info/evolview-v2/).

2.2 Chromosomal locations and gene duplication events

Details regarding the chromosomal locations of the BnaCNGC genes were obtained from the Ensembl Plants database. TBTools (Chen et al., 2020) was employed to analyze chromosomal localization.

2.3 Analysis of gene structure, motif composition

Gene exon/intron structures were predicted with the TBTools (Chen et al., 2020), with genomic and coding sequences from the Ensembl Plants database (http://plants.ensembl.org/index.html). The conserved motifs in the CNGC sequences were identified using the MEME (https://meme-suite.org/meme/tools/meme) with the following parameters: optimal motif width: 6–50; maximum number of different motifs: 10.

2.4 Plant materials and treatments, heat map analysis of the BnaCNGC transcriptome data

For different abiotic stress and phytohormones treatments, the ZS11 (B. napus L. cv. Zhonghuang 11) seeds were germinated and transplanted to pots containing soil or vermiculite. The growth conditions, phytohormones treatments, and abiotic stress conditions were as described previously (He et al., 2019).

For waterlogging treatment, the 15-day-old seedlings of winter-type rapeseed variety Avatar and semi-winter rapeseed variety ZS9 were submerged for 24 h, and the expression data of BnaCNGC genes were from the GEO database (GEO: GSE140828) (Wittig et al., 2021).

For infection with S. sclerotiorum, healthy leaves of susceptible (Westar) and resistant (ZY821: Zhongyou821) genotypes of rapeseed were inoculated at the bloom stage, and the expression data of BnaCNGC genes were from the
3. Results

3.1 Identification of CNGC genes in B. napus

Using the 20 AtCNGC proteins as queries in BLASTp search for the Ensembl Plants database, 61 putative CNGC proteins were identified in the B. napus genome. Among them, 51 members contain all the essential CNGC-specific domains (TM, CNBD, and CaMBD/IQD), and 10 members were truncated proteins lacking one or two of the CNGC-specific domains.

The physiological and biochemical properties of the putative 61 BnaCNGC proteins were determined by computing different parameters, and are tabulated in Table S1. These 51 typical BnaCNGC proteins varied in length from 600 to 801 amino acid residues in molecular weights from 70.24 to 90.74 kDa, and in pI from 8.35 to 9.88 (Table S1). The predicted subcellular localization showed that most of CNGC proteins were localized in the plasma membrane, while AtCNGC20 and its 11 orthologous BnaCNGC proteins were localized in the chloroplast membrane (Table S1).

3.2 Phylogenetic analysis of CNGC proteins in B. napus, B. rapa, and B. oleracea

Multiple sequence alignments and a maximum likelihood phylogenetic tree between BnaCNGCs (61), AtCNGCs (20), BrCNGCs (30) (Li et al., 2019), and BoCNGCs (26) (Kakar et al., 2017) were constructed. Multiple sequence alignments and a maximum likelihood phylogenetic tree were built using MEGA X software. The phylogenetic tree revealed that the CNGC family genes could be divided into five major groups: Group I (homologous to AtCNGC1/3/10/11/12/13), Group II (homologous to AtCNGC5/6/7/8/9), Group III (homologous to AtCNGC14/15/16/17/18), Group IV-a (homologous to AtCNGC19/20), and Group IV-b (homologous to AtCNGC2/4) (Figure 1). As expected, each AtCNGC gene has orthologous genes in B. napus, B. rapa, and B. oleracea. Notably, there was an expansion of CNGC genes in Group IV-a, 18, 8, and 9 genes orthologous with AtCNGC19/20 in B. napus, B. rapa, and B. oleracea, respectively (Figure 1 and Table S2).

According to the orthologous gene sets among the Ar (B. rapa), Co (B. oleracea), An-, and Cn-subgenomes of B. napus, 25 Ar-Co-An-Cn pairs were identified (Table S2). Fifty of 61 BnaCNGC genes with a subgenome assigned were found in the corresponding An- and Cn homoeologous chromosomes, and they had homoeologous genes both in Ar (B. rapa) and Co (B. oleracea). Three members (BnaC06g16330D, BnaC02g14560D, and BnaC09g29350D) from Group I had no orthologous genes in B. oleracea, though they had orthologous genes in both B. rapa and An-subgenomes of B. napus. BnaC01g24610D (homologous to AtCNGC19) had no orthologous genes in the corresponding An homoeologous chromosome, though it was found to have an orthologous gene in both B. rapa and B. oleracea (Table S2). Additionally, there were four members (BnaC03g31050D, BnaC06g16240D, Bna031515.1 and BnaAnng17920D) from Group I that had no orthologous genes.

3.3 Chromosomal distribution and diversification of BnaCNGC genes

The 61 BnaCNGC genes were mapped onto B. napus chromosomes and the position of each locus was determined (Figure 2(B)). The BnaCNGC genes were unevenly distributed (1–5 genes on one chromosome) on 18 chromosomes (i.e. BnaA01-07 and BnaC01-C09). Additionally, there was a tandem duplication (a three-gene cluster) in the orthologous regions between BnaA05 and BnaC05 chromosomes (Figure 2(B)). As expected, the distribution of CNGC genes in B. rapa and B. oleracea was similar to the distribution of the orthologous BnaCNGC genes in the B. napus An-subgenome and Cn-subgenome, respectively (Figure 2).

3.4 Gene structure and motif composition analysis

To characterize the structural diversity of the CNGC family members, we analyzed the exon–intron organization of individual CNGC genes. Closely clustered CNGC genes in the same clades were similar in the number of exons and intron. The majority of the CNGC genes from phylogenetic Groups I, II, III, IV-b contained 6–9 exons, while the Group IV-a members had 10–12 exons (Figure 3). A comparison between the exon–intron organizations of BnaCNGC and AtCNGC genes clustered in the same phylogenetic groups revealed that there was one more or less intron in BnaCNGC genes than that in AtCNGCs.

The protein sequences were analyzed using MEME, for analyzing the conservation of motifs in CNGC, 10 motifs were detected and arranged in all typical CNGC proteins as follows: motif10-9-4-7-1-2-3-8-6-5 (Figure 3). The typical domain including TM domain, CNBD (the C-terminus of motif 6) and IQ domain (motif 5) were found to be the most conserved and were present in most of BnaCNGC members (Figure 3 and Figure S2). Additionally, the IQ domain (motif 5) was found to be absent in six BnaCNGC members (BnaC03g31050D, BnaCnng45430D, BnaA03g34680D, BnaC03g40070D, BnaA03g34700D, and BnaA03g34940D). Three members (BnaC03g31050D, BnaC02g44680D and BnaAnng17920D) lack the CNBD (Figure 3).
3.5 Expression patterns of BnaCNGC in different rapeseed tissues

We investigated the expression patterns of BnaCNGC in 12 tissues (i.e. root, stem, leaf, bud, sepal, stamen, new pistil, blossom pistil, wilting pistil, ovule, pericarp, and silique) using RNA-sequencing data. It was found that BnaCNGC genes were differentially expressed in the 12 tissues at different developmental stages (Figure 4). Eighteen BnaCNGC genes from Group I (10/15), II (3/10) and IV-b (5/6) members were constitutively expressed (FPKM>10) in all 12 rapeseed tissues. In contrast, the majority of BnaCNGC genes from Group III and IV-a were lowly or not expressed (FPKM<3) in any of the tested 12 rapeseed tissues.

From Group I, BnaA04g15220D and BnaC04g38170D (a pair of genes homologous to AtCNGC11/12) were predominantly expressed in root. BnaA05g01380D and BnaC04g01250D (a pair of genes homologous to AtCNGC3) were predominantly expressed in pericarp and sepal. BnaC03g31720D and BnaA03g26780D (a pair of genes homologous to ATCNGC13) were predominantly expressed in leaf and sepal. BnaA02g10440D and BnaC02g14560D (a pair of genes homologous to ATCNGC1) had high transcription levels in almost all tissues. In particular, BnaA10g06480D and BnaC09g29350D (another pair of genes homologous to ATCNGC1) were expressed specifically in the stamen.

From Group II, BnaA04g15220D and BnaC04g38170D (a pair of genes homologous to AtCNGC5) was relatively highly expressed in root, sepal, and blossom pistil, and its expression level is higher than BnaA02g07990D (another homologous to AtCNGC5) in all rapeseed tissues. BnaA03g50250D (homologous to AtCNGC9) was relatively highly expressed in newpistil and blossompistil. BnaC04g36890D (homologous to AtCNGC6) was expressed specifically in root, sepal and ovule.

From Group III, BnaA10g19030D and BnaC09g42720D (a pair of genes homologous to AtCNGC18) were predominantly expressed in bud and newpistil tissues. BnaA01g06670D and BnaC01g08020D (a pair of genes homologous to ATCNGC17) were relatively highly expressed in root.

From Group IV-a, BnaA05g22550D, BnaCnng48280D, and BnaC05g35840D were predominantly expressed in root, and BnaCnng45430D was predominantly expressed in leaf, sepal, and pericarp. BnaC05g35840D was relatively highly expressed in root and leaf and BnaCnng48270D was relatively highly expressed in leaf, stem, pericarp, and silique. These genes in pairs had no similar expression patterns.

3.6 Expression patterns of BnaCNGC in response to abiotic stresses and phytohormones

We also examined the BnaCNGC expression levels under abiotic stresses (NaCl, PEG, cold, heat, waterlogging) and phytohormones treatments (ABA, MeJA, Ethylene, and SA).
As shown in Figure 5, the majority of members from Group II and III were slightly or not responsive to all the tested stresses and photohormone treatments, while the majority expressed members from Group I, IV-a, and IV-b were regulated by temperature stresses and SA treatment. For example, the expressions of BnaA05g01380D/BnaC04g01250D (a pair of genes homologous to AtCNGC3) and BnaA03g26780D/BnaC03g31720D (homologous to AtCNGC13) from Group I all were significantly up-regulated with SA treatment and down-regulated with ABA treatment and cold/heat/NaCl stresses. BnaC03g31050D (from Group I) and BnaCnng45430D/BnaA03g34680D (from Group IV-a) were strongly induced by NaCl and PEG treatments. From Group IV-b, BnaA02g09790D and BnaC02g44680D (a pair of genes homologous to AtCNGC4) were up-regulated with cold treatment for (at 3 h treatment point) and heat treatment (at 6 h treatment point).

As shown in Figure 5, 16 members were up-regulated by submergence treatment. BnaA10g18740D and BnaC09g42460D (a pair of genes homologous to AtCNGC4) from Group IV-b were up-regulated in both two B. napus cultivars ZS9 and SAV with submergence treatment. Meanwhile, BnaC02g44680D and BnaA02g09790D (a pair of genes homologous to AtCNGC4), from Group IV-b, were down-regulated after submergence stress.
3.7 Expression patterns of BnaCNGC in response to the infection of Sclerotinia sclerotiorum

As shown in Figure 5, all 20 SA-responsive BnaCNGC genes were regulated with the infection of S. sclerotiorum. For example, four genes (BnaC03g31050D, BnaC03g31720D, BnaA05g01380D and BnaC04g01250D) from Group I and three genes (BnaCnng45430D, BnaA03g34680D, and BnaC03g40070D) from Group IV-a, all were strongly induced by SA and the infection of S. sclerotiorum, but the change-fold (24 hpi/control) in a susceptible rapeseed cultivar (Westar) was higher than that in a tolerant one (Zhongyou 821). Moreover, all the members from Group IV-b were reduced by SA, and were reduced in the susceptible cultivar Westar but induced in the tolerant cultivar Zhongyou 821 after the infection of S. sclerotiorum.
3.8 Clustering analysis of expression with BnaCNGC and BnaCaM

Since a large number of studies showed that the interaction between CNGC and CaM plays an important role in plant growth and development (Fischer et al., 2017), the cluster analysis of gene expression patterns of BnaCNGC and BnaCaM was performed as shown in Figure 6. A pair of BnaCNGC11/12 (BnaA04g15220D/BnaC04g38170D) and BnaCaM7 (BnaA06g20230D) had the same expression pattern and were predominantly expressed in the root. BnaCNGC9 (BnaA03g50250D) and BnaCaM7 (BnaA06g19660D) were both predominantly expressed in newpistils. A pair of BnaCaM2/3/5 (BnaA03g19320D/BnaC03g23130D) and a pair of BnaCNGC19...
(BnaA05g22570D/BnaC05g35860D) were expressed in roots and strongly reduced by salt and PEG treatments.

4. Discussion

As an important calcium channel protein, CNGCs participate in plant immunity and the tolerance to salt and temperature stress in many crops (Saand et al., 2015; Hao and Qiao, 2018; Meena et al., 2019; Wang et al., 2019; Wang et al., 2021). However, a functional identification of CNGC genes has not been reported in B. napus. In this study, we identified 61 putative B. napus CNGC genes, and determined that the BnaCNGC gene family is larger than the CNGC families of most of the reported crops, such as 18 in tomato (Hao and Qiao, 2018), 21 in pear (Chen et al., 2015), and 28 in rice (Nawaz et al., 2014). The results of synteny analysis indicated that the significantly higher number of BnaCNGC genes may be attributed to the expansion of members in Group IV-a (CNGC19/20) in B. napus. Meantime, the results showed that all the BnaCNGC members (18) from Group IV-a in B. napus had corresponding orthologous genes in B. rapa (9) and B. oleracea (9), which implied that CNGCs in Group IV-a were expanded both in B. rapa and B. oleracea, and all were retained after the whole-genome duplication (WGD) event in B. napus (allopolyploidy rape-seed). Like AtCNGC19/20, most of the Br/Bo/BnaCNGCs from Group IV-a were tandem genes (Figure 3). Gene duplications during evolution increase the genomic content and expand gene functions to optimize the adaptability of plants (Zelman et al., 2012). In Arabidopsis, AtCNGC19/20 have been found to regulate cell death and respond to various biotic and abiotic stresses, such as salinity, Pb²⁺ and Cd²⁺ stress, herbivory insect, plant pathogenic fungi and bacteria, and endophytes. We presume that the expansion of CNGC19/20 in Brassicaceae could greatly increase the adaptation of plants to stress conditions.

Homologous genes within the same taxonomic group are assumed to exhibit similar structural, functional, and evolutionary properties (Zelman et al., 2012), which may help clarify the roles of BnaCNGC genes. Similar to the orthologous CNGC proteins in Arabidopsis, the 51 typical BnaCNGCs proteins had conserved gene structures and
conserved domains including six membrane-spanning regions, pore region, CNBD, CaMBD, and/or IQD (Figure 3 and Figure S1). Since CaMBD and/or IQD were the CaM-binding sites, the proteins lacking these two domains may lose the function in regulating Ca^{2+} signaling (Fischer et al., 2013; Fischer et al., 2017). Therefore, it is reasonable

Figure 6. Expression of BnaCNGC and BnaCaM under plant hormone treatments, abiotic stress and infection of Sclerotinia sclerotiorum. Leaf: untreated leaves; Cold: leaves treated with 4 °C; Hot: leaves treated with 40 °C; ABA: leaves treated with 100 μM abscisic acid; MeJA: leaves treated with 100 μM methyl jasmonate; ET: leaves treated with 10 μg/mL ethephon; SA: leaves treated with 1.0 mM salicylic acid. Root: untreated roots; NaCl: roots treated with 200 mM NaCl; PEG: roots treated with 20 % polyethylene glycol 6000. Colored rectangles indicate normalized expression levels of BnCNGC genes (FPKM). Red means high expression; Blue means low expression. Following parameters was used in TBtools program: Cluster Rows, Show ori value; Row Scale, ZeroToOne; Title Shape: Circle, Scale Size By Area; Dist Method: PearsonDist; Cluster Method: Complete; Branch Form: Cladogram.
to presume that the absence of CaMBD and IQ domain in six BnaCNGC members (BnaCnng45430D, BnaA03g34680D, BnaC03g40070D, BnaA03g34700D, BnaC03g31050D, and BnaC02g44680D) raised the possibility that they were function abnormal CNGC proteins.

Though the BnaCNGCs were similar with AtCNGCs in the gene sequences and structures, their spatiotemporal and stress-response expression patterns were quite different. There are many reports showed that most of AtCNGCs from Group II and Group III in Arabidopsis are mainly involved in the growth of pollen tube (Pan et al., 2019), guard cells (Wang et al., 2013), root hair (Tan et al., 2020), auxin signaling, salt, and \( \text{P}_{2+}/\text{Ca}^{2+} \) tolerance (Moon et al., 2019). Transcriptional data showed that the majority of BnaCNGCs from Group III were low- or no-expression genes, members from Group II and III all were slightly or not responding to all the tested stresses and photohormone treatments. Only BnaC09g42720D and BnaA10g19030D (a pair of genes homologous to AtCNGC18) from Group III were expressed predominantly in bud and new pistil, while a pair of genes homologous to AtCNGC1 (BnaA10g06480D and BnaC09g29350D) from Group I were expressed predominantly in stamen (Figure 4). Unlike AtCNGCs of Group II and III, the members from Group I, IV-a, and IV-b are mainly involved in plant immunity and cell death (Kugler et al., 2009; Chin et al., 2013; Yu et al., 2019).

During the process of plant immune response, there is a complex genetic regulatory network that affects SA-mediated signaling (An and Mou, 2011). The application of SA enhanced the resistance to S. sclerotiorum infection in B. napus (Wang et al., 2012). In our study, most of expressed BnaCNGCs from Group I, IV-a, and IV-b were induced and/ or reduced strongly by SA treatment and infection of S. sclerotiorum indicating that those BnaCNGCs play important roles in the SA-mediated response to S. sclerotiorum in B. napus. Additionally, studies showed that the members from Group IV-b are involved in thermotolerance in Arabidopsis (Finka et al., 2012) and rice (Wang et al., 2021). Our study showed that only two \((\text{BnaC02g44680D/ BnaA02g09790D})\) of six members from Group IV-b were slightly induced with cold and heat stresses (about twofold change). However, 16 BnaCNGCs from the other four groups were induced and/or reduced by cold and/or heat stresses.

Meanwhile, a large number of studies have shown that the interaction between CNGC and CaM plays an important role in plant growth and development (Fischer et al., 2017). For example, in Arabidopsis, AtCNGC7/8 and AtCNGC18 together interacting with calmodulin 2 (CaM2) constitute a molecular switch that controls the calcium channel during the pollen tube growth (Pan et al., 2019). The activity of AtCNGC12 was significantly enhanced when CaM1 was co-expressed in oocytes (Zhang et al., 2019). Cluster analysis results showed that some BnaCNGC and BnaCaM gene had the same and specific expression patterns and were clustered together. For example, a pair of BnaCNGC11/12 (BnaA04g15220D/BnaC04g38170D), and BnaCaM7 (BnaA06g20230D) had the same expression pattern and were predominantly expressed in the root. BnaCNGC9 (BnaA03g50250D) and BnaCaM7 (BnaA06g19660D) were both predominantly expressed in new pistils. A pair of BnaCaM2/3/5 (BnaA03g19320D/BnaC03g23130D) and a pair of BnaCNGC19 (BnaA05g22570D/BnaC05g35860D) were expressed in roots and strongly reduced by salt and PEG treatments. The generated data may be useful for constructing the CNGC-CaM interaction networks. Further characterization of the interactions between BnaCNGCs and BnaCaMs will expand our understanding of the functions of calcium oscillations in the development and adaptation of B. napus.

This work is the first comprehensive and systematic analysis of CNGC gene family in B. napus. There are 61 BnaCNGC genes that are identified in B. napus, and are classified into five major groups (i.e. Groups I, II, III, IV-a, IV-b), and Group IV-a appears to have expanded through WGT. Our results display that BnaCNGC have a very conservative domain, exon–intron structure, and this study assists to elucidate the functional diversity of BnaCNGC genes in the regulation of plant development and stress response in B. napus, and provides valuable information for the genetic and breeding improvement of rapeseed.

**Abbreviations**

CNGC cyclic nucleotide-gated channel
cAMP cyclic Adenosine MonoPhosphate
cGMP cyclic Guanosine MonoPhosphate
cBD cyclic nucleotide binding domain
IQD isoelucine-glutamine domain
CaM \( \text{Ca}^{2+} \)-activated calmodulin
CaMBD CaM-binding domain
TM transmembrane

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DeFalco TA, Marshall CB, Munro K, Kang HG, Moeder W, Ikura M, Snedden WA, Yoshikawa K. 2016. Multiple calmodulin-binding sites positively and negatively regulate Arabidopsis CYCLIC NUCLEOTIDE-GATED CHANNEL12. Plant Cell. 28:1738–1751.

Finka A, Cuendet AF, Maathuis FJ, Saidi Y, Goloubinoff P. 2012. Plasma membrane cyclic nucleotide gated calcium channels control land plant thermal sensing and acquired thermotolerance. Plant Cell. 24:3333–3348.

Fischer C, DeFalco TA, Karia P, Snedden WA, Moeder W, Yoshikawa K, Dietrich P. 2017. Calmodulin as a Ca2+-sensing Subunit of Arabidopsis cyclic nucleotide-gated channel complexes. Plant Cell Physiol. 58:1208–1221.

Fischer C, Kugler A, Hoth S, Dietrich P. 2013. An IQ domain mediates the interaction with calmodulin in a plant cyclic nucleotide-gated channel. Plant Cell Physiol. 54:573–584.

Fritschi S, Wang YF, Sladek C, Poulsen LR, Romanowsky SM, Schroeder JJ, Harper JF. 2007. A cyclic nucleotide-gated channel is essential for polarized tip growth of pollen. P Natl Acad Sci USA. 104:14531–14536.

Girard I, Tong C, Becker MG, Mao X, Huang J, de Kievit T, Fernando WGD, Liu S, Belmonte MF. 2017. RNA sequencing of Brassica napus reveals cellular redox control of Sclerotinia infection. J Exp Bot. 68:5079–5091.

Guo KM, Babourina O, Christopher DA, Borsics T, Rengel Z. 2008. The cyclic nucleotide-gated channel, AtCNGC10, influences salt tolerance in arabidopsis. Physiol Plant. 134:499–507.

Hao LD, Qiao XL. 2018. Genome-wide identification and analysis of the CNGC gene family in maize. PeerJ. 6.

He X, Liu W, Li WQ, Liu Y, Wang WP, Xie P, Kang Y, Liao L, Qian LW, Liu ZS, et al. 2020. Genome-wide identification and expression analysis of CaM/CML genes in Brassica napus under abiotic stress. J Plant Physiol. 255.

He X, Xie S, Xie P, Yao M, Liu W, Qin LW, Liu ZS, Zheng M, Liu HF, Guan M, Hua W. 2019. Genome-wide identification of stress-associated proteins (SAP) with A20/AN1 zinc finger domains associated with abiotic stresses responses in Brassica napus. Environ Exp Bot. 165:108–119.

Jammes F, Hu HC, Villiers F, Routen R, Kwak JM. 2011. Calcium-permeable channels in plant cells. FEBS J. 278:4262–4276.

Kakar KU, Nawaz Z, Kakar K, Ali E, Almonafay AA, Ullah R, Ren XL, Shu QY. 2017. Comprehensive genomic analysis of the CNGC gene family in Brassica oleracea: novel insights into synteny, structures, and transcript profiles. BMC Genomics. 18.

Kaplan B, Sherman T, Fromm H. 2007. Cyclic nucleotide-gated channels in plants. FEBS Lett. 585:2237–2246.

Kugler A, Kohler B, Palme K, Wolf P, Dietrich P. 2009. Salt-dependent regulation of a CNG channel subfamily in arabidopsis. BMC Plant Biol. 9:140.

Li QQ, Yang SQ, Ren J, Ye XL, Jiang X, Liu ZY. 2019. Genome-wide identification and functional analysis of the cyclic nucleotide-gated channel gene family in Chinese cabbage. Biotech. 3:9.

Ma W, Ali R, Berkowitz GA. 2006. Characterization of plant phenotypes associated with loss-of-function of AtCNGCl1, a plant cyclic nucleotide-gated cation channel. Plant Physiol Biochem. 44:494–505.

Maathuis FJM, Sanders D. 2001. Sodium uptake in Arabidopsis roots Is regulated by cyclic nucleotides. Plant Physiol. 127:1617–1625.

Meena MK, Priyapati R, Krishna D, Divakaran K, Pandey Y, Reichelt M, Mathew MK, Boland W, Mithofer A, Yadassy J. 2019. The Ca2+ channel CNGC19 regulates Arabidopsis Defense against spodoptera herbivory. Plant Cell. 31:1539–1562.

Moeder W, Urquhart W, Ung H, Yoshikawa K. 2011. The role of the cyclic nucleotide-gated ion channels in plant immunity. Mol Plant. 4:442–452.

Moon JY, Belloeil C, Jania ML, Shin R. 2019. Arabidopsis CNGC family members contribute to heavy metal Ion uptake in plants. Int J Mol Sci. 20.

Nawaz Z, Kakar KU, Saand MA, Shu Q-Y. 2014. Cyclic nucleotide-gated ion channel gene family in rice, identification, characterization and experimental analysis of expression response to plant hormones, biotic and abiotic stresses. BMC Genomics. 15:853.

Pan Y, Chai X, Gao Q, Zhou L, Zhang S, Li L, Luan S. 2019. Dynamic interactions of plant CNGC subunits and calmodulins drive oscillatory Ca2+ channel activities. Dev Cell. 48.

Saand MA, Xu YP, Li W, Wang JP, Cai XZ. 2015. Cyclic nucleotide gated channel gene family in tomato: genome-wide
identification and functional analyses in disease resistance. Front Plant Sci. 6:303.

Shih HW, DePew CL, Miller ND, Monshausen GB. 2015. The cyclic nucleotide-gated channel CNGC14 regulates root gravitropism in Arabidopsis thaliana. Curr Biol. 25:3119–3125.

Tan YQ, Yang Y, Zhang A, Fei CF, Gu LL, Sun SJ, Xu W, Wang L, Liu H, Wang YF. 2020. Three CNGC family members, CNGC5, CNGC6, and CNGC9, Are required for constitutive growth of Arabidopsis Root hairs as Ca2+-permeable channels. Plant Commun. 1:100001.

Tuteja N, Mahajan S. 2007. Calcium signaling network in plants: an overview. Plant Signal Behav. 2:79–85.

Wang J, Liu X, Zhang A, Ren Y, Wu F, Wang G, Xu Y, Lei C, Zhu S, Pan T, et al. 2019. A cyclic nucleotide-gated channel mediates cytoplasmic calcium elevation and disease resistance in rice. Cell Res. 29:820–831.

Wang J, Ren Y, Liu X, Luo S, Zhang X, Liu X, Lin Q, Zhu S, Wan H, Yang Y, et al. 2021. Transcriptional activation and phosphorylation of OsCNGC9 confer enhanced chilling tolerance in rice. Mol Plant. 14:315–329.

Wang LX, Li M, Liu ZG, Dai L, Zhang ML, Wang LL, Zhao J, Liu MJ. 2020. Genome-wide identification of CNGC genes in Chinese jujube (ziziphus jujuba mill.) and ZjCNGC2 mediated signalling cascades in response to cold stress. Bmc Genomics. 21.

Wang YF, Munemasa S, Nishimura N, Ren HM, Robert N, Han M, Puzzorjova I, Kollist H, Lee S, Mori I, Schroeder JI. 2013. Identification of cyclic GMP-activated nonselective Ca2+-permeable cation channels and associated CNGC5 and CNGC6 genes in Arabidopsis guard cells. Plant Physiol. 163:578–590.

Wang Z, Tan X, Zhang Z, Gu S, Li G, Shi H. 2012. Defense to Sclerotinia sclerotiorum in oilseed rape is associated with the sequential activations of salicylic acid signaling and jasmonic acid signaling. Plant Sci. 184:75–82.

Wittig PR, Ambros S, Muller JT, Bammer B, Alvarez-Cansino L, Konnerup D, Pedersen O, Mustroph A. 2021. Two Brassica napus cultivars differ in gene expression, but not in their response to submergence. Physiol Plant. 171:400–415.

Xu Y, Yang J, Wang Y, Wang J, Yu Y, Long Y, Wang Y, Zhang H, Ren Y, Chen J, et al. 2017. OsCNGC13 promotes seed-setting rate by facilitating pollen tube growth in stilar tissues. PLoS Genet. 13: e1006906.

Yoshioka K, Moeder W, Kang HG, Kachroo P, Masmoudi K, Berkowitz G, Klessig DF. 2006. The chimeric Arabidopsis CYCLIC NUCLEOTIDE-GATED ION CHANNEL11/12 activates multiple pathogen resistance responses. Plant Cell. 18:747–763.

Yu X, Xu G, Li B, de Souza Vespoli L, Liu H, Moeder W, Chen S, de Oliveira MVV, Ariadina de Souza S, Shao W, et al. 2019. The receptor kinases BAK1/SERK4 regulate Ca2+ channel-mediated cellular homeostasis for cell death containment. Curr Biol. 29:3778–3790 e3778.

Yuen CCY, Christopher DA. 2013. The group IV-A cyclic nucleotide-gated channels, CNGC19 and CNGC20, localize to the vacuole membrane in Arabidopsis thaliana. Aob Plants. 5.

Zelman AK, Dawe AS, Gehring CA, Berkowitz GA. 2012. Evolutionary and structural perspectives of plant cyclic nucleotide-gated cation channels. Front. Plant Sci. 3:95–95.

Zhang Z, Hou C, Tian W, Li L, Zhu H. 2019. Electrophysiological studies revealed CaM1-mediated regulation of the Arabidopsis calcium channel CNGC12. Front. Plant Sci. 10.

Zhao CH, Tang YH, Wang JL, Zeng YH, Sun HQ, Zheng ZC, Su R, Schneeberger K, Parker JE, Cui HT. 2021. A mis-regulated cyclic nucleotide-gated channel mediates cytosolic calcium elevation and activates immunity in arabidopsis. New Phyto. 230:1078–1094.