Genome-wide Identification and Analysis of the CNGC Gene Family in *Zea mays*

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Abstract

As one of the non-selective cation channel gene family, the cyclic nucleotide-gated channel (CNGC) gene plays vital roles in plant physiological processes which are related to signal pathways, plant development, and environment stresses. However, genome-wide identification and analysis of the *CNGC* gene family in maize have not yet been conducted. In this study, 12 *ZmCNGC* genes are detected in the maize genome, which are unevenly distributed on chromosomes 1, 2, 4, 5, 6, 7 and 8. They are classified into four major groups, including group I, II, III, and IV. Prediction of cis-acting regulatory elements show that 137 putative cis-elements which are related to hormones-response, abiotic stress-related and organ development-related. Gene ontology (GO) analysis demonstrated that most of the ZmCNGCs are involved in various biological processes including cellular processes, establishment of localization, and transmembrane transport. Furthermore, the co-expression network analysis of *ZmCNGC* genes may establish the importance of better understanding ZmCNGC transduction pathways in maize. AndAdditionally, expression profiles of *ZmCNGC* genes are shown to express in a tissue-specific expressedpattern. Our results provide valuable information to increase our understanding of the *CNGC* gene family in maize.

Subjects: Bioinformatics, Genomics, Plant Science

Keywords: CNGC, *Zea mays*, Gene family, Expression profiles

INTRODUCTION

In the process of organism evolution, itthe forms aformation of complex nutrient absorption and transport system thats includes ion channels, ion pumps and carriers, and it has been previously showedn that those system ares respond to endogenous and abiotic stimuli (Saand et al. 2015b). CThe cyclic nucleotide-gated channel (CNGC) is a Ca^{2+}-permeable cation transport channel, which is suggested to behave one of the fundamental mechanism ins for organismsal systems (Nawaz et al. 2014; Yuen & Christopher 2013). As a molecular switch, secondary messengers such as cyclic nucleotide monophosphates (cNMPs; 3',5'-cAMP and 3',5'-cGMP) and Ca^{2+}/calmodulin (CaM) can regulate CNGCs,; those messengers are activated by directing binding of cyclic nucleotides as well as arebeing inhibited by binding of CaM to the CaM binding domain (Borsics et al. 2007; Defalco et al. 2016; Kaplan et

al. 2007; Saand et al. 2015b).

As CNGCs are key components for plant development, many previous research~~es~~ studies have been ~~were~~ conducted (citations?). With the application of bioinformatics tools, the identification of *CNGC* gene family members in *Arabidopsis*, rice and other plants were carried out. So far, *CNGC* genes have been identified in many plants, with 120 for *Arabidopsis*, 16 for rice, 18 for tomato and 26 in *B. oleracea*. (Bridges et al. 2005; Chen et al. 2015; Guo et al. 2017; Kakar et al. 2017; Nawaz et al. 2014; Saand et al. 2015a; Ward et al. 2009; Zelman et al. 2013; Zelman et al. 2012). Most *CNGC*s have been characterized by genetic methods and found to be related to plant physiological and molecular functions, including playing vital roles in multiple physiological processes which are involved in signal pathways, plant development, and environmental stresses. For example, *AtCNGC18* is expressed primarily in *Arabidopsis* pollen (Frietsch et al. 2007); phot1 and phot2 increases cytosolic Ca^{2+} in *Arabidopsis* leaves (Harada et al. 2003); *AtCNGC2* play key roles in stress signaling pathways, including changes the cytosolic free Ca^{2+} in *Arabidopsis* and *CNGC4* is permeable to both K+ and Na+ and activated by both cGMP and cAMP (Balague 2003; Reddy et al. 2011; Tracy et al. 2008). Meanwhile, the structure of *Arabidopsis* CNGCs have six transmembrane domains with a pore domain, also have a cyclic nucleotide-binding domain and CaM-binding domains in the C-termin~~al~~us, and these domains have diverse functions (Chin et al. 2009; Hua et al. 2003; Köhler & Neuhaus 2000; Talke et al. 2003). For example, *Arabidopsis CNGC6*, *CNGC7*, *CNGC8*, *CNGC9*, *CNGC10* and *CNGC16* participate in the pollen development (Gao et al. 2012; KW et al. 2006; Tunc-Ozdemir et al. 2013; Wang et al. 2013); *CNGC2*, *CNGC4*, *CNGC11* and *CNGC12* are activated in response to pathogenic microorganisms (Dodd et al. 2010); and, *CNGC6*, *CNGC10*, *CNGC19* and *CNGC20* are involved in abiotic stress (Kugler et al. 2009; Mosher et al. 2010).

In recent years, efforts had been made in studying the CNGC family in plants, but as one of the most important food crops and source of industrial materials in the world, the maize CNGC gene family was rarely reported. In this study, with the benefit from genome-wide sequence information in maize and research information on *Arabidopsis* and rice CNGC families, we conducted genome-wide identification of CNGCs in maize through comprehensive bioinformatics analyses. Furthermore, comprehensive analyses were conducted including multiple alignments, gene structure, conserved motifs of ZmCNGCs, and prediction of cis-acting regulatory

elements, GO analysis, expression profiles of *ZmCNGC* genes and a co-expression network between ZmCNGC and other maize genes. ~~It~~This is the first systematic~~ally~~ study of CNGC genes in maize and will provide the basis for further research on the *ZmCNGC* gene family.

MATERIALS AND METHODS

Identification of *CNGC* genes in maize genome

To identify the maize *CNGC* genes, 20 *Arabidopsis* and 16 rice *CNGC* protein sequences were obtained from the TAIR10 database (http://www.arabidopsis.org/index.jsp) and the RGAP database (http://rice.plantbiology.msu.edu/), respectively. Then, two methods were used to search against the maize protein sequences, one ~~is~~was build~~t~~ using a Hidden Markov Model (HMM) to search against maize protein sequences, and another~~one is~~ used the local BLASTP method with the e-value set to 1e-5. After that, the putative non-redundant protein sequence of maize *CNGC* genes were retrieved. To further confirm whether the ZmCNGC proteins ~~whether have~~contained the CNBD domain, those putative *ZmCNGC* protein sequences were summited to SMART (http://smart.embl-heidelberg.de/) (Letunic & Bork 2018) and NCBI-CDD (https://www.ncbi.nlm.nih.gov/cdd/) (Marchler-Bauer et al. 2017)~~,~~; genes without the CNBD domains or the amino acids of a size ~~of~~ below ~~<~~200 were removed and the *ZmCNGC* genes were confirmed. The information of chromosome distribution of ZmCNGCs and the sequences including DNA sequences, CDS, cDNA, up-stream 1500bps of *ZmCNGC* genes were obtained from results of the BLASTN search in the Ensembl Plant database (http://plants.ensembl.org/index.html) (Bolser et al. 2016).

Analyses of genes structure, conserved motifs, and cis-acting regulatory elements and GO annotation of ZmCNGCs

The gene structure of *ZmCNGC* genes were performed by the Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/) (Hu et al. 2015) using CDS and DNA sequences. The conserved motif domins were identified using the MEME software algorithn (http://meme-suite.org/index.html) (Bailey et al. 2015) with the maximum number of motifs obtained set at 9 and the optimum wid~~e~~th of motifs ranging from 6 to 200 amino acids. The up-stream 1500bps of *ZmCNGC* genes were used to find cis-acting regulatory elements by using 'Signal Scan Search' programs in the NEW

PLACE database (https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi?lang=en) (Higo et al. 1999). The PI (theoretical isoelectric point), MW (molecular weight), and GRAVY of ZmCNGCs were predicted by ExPASy (http://web.expasy.org/protparam/) (Artimo et al. 2012). ~~And t~~The prediction subcellular location of ZmCNGCs were identified using the CELLO v.2.5 (**http://cello.life.nctu.edu.tw/**) package. The gene ontology (GO) annotation of *ZmCNGC* genes were submitted to the Monocots PLAZA 4.0 (Van Bel et al. 2017) database, then were visualized and plotted by BGI WEGO (Ye et al. 2006).

Multiple alignments and phylogenetic analysis

Multiple sequences alignments were performed using T-COFFEE (http://tcoffee.crg.cat/apps/tcoffee/index.html) (Di Tommaso et al. 2011) and visualized by ESPript (Robert & Gouet 2014). An un-root phylogenetic tree was **c**onstructed with 1000 bootstrap replication using MEGA 7 (Kumar et al. 2016) based on the full-length protein sequences alignment.

Expression profiles of *ZmCNGC* genes by RNA-seq datasets and network interaction analysis

For understanding the expression of *ZmCNGC* genes in different tissues, two RNA-seq datasets of *Zea mays* obtained from the Expression Atlas ~~datasets~~database were obtained (https://www.ebi.ac.uk/gxa/home/) with the accession numbers E-MTAB-3826 and E-MTAB-4395. Those data were used to analyze the expression of *ZmCNGC* in 6 different tissues (including ear, embryo, endosperm, pollen, root and tassel) and different development stages in embryo, endosperm, and seed, respectively. The FPKM values were ~~used to~~ calculated for each of the ZmCNGC genes. The interaction network was constructed ~~on the~~ based on the orthologs between maize and *Arabidopsis* using the AraNet v2 (Lee & Lee 2017) annotations and visualized by the Cytoscape v3.4.0 (Shannon et al. 2003).

RESULTS AND DISSCUSSIONS

Identification of CNGC genes in *Zea mays*

To identify a complete overview of CNGC genes in maize, we firstly used 20 *Arabidopsis* and 16 rice *CNGC* protein sequences to ~~blas~~ align ~~t~~BLAST ~~to~~ against maize protein sequences. After ~~blast~~BLAST alignment, a total of 18 putative

ZmCNGC genes were identified in the maize genome. Then, to confirm those 18 putative *ZmCNGC* genes, we used the SMART and NCBI CDD to ~~found~~find whether ~~have~~they contained the CNGC-specific domains (CNBD and transmembrane). After removing redundancy genes, a total of 12 *ZmCNGC* genes were detected. As shown in Table 1, five of them were located in chromosome 5, others were unevenly located in chromosomes 1, 2, 4, 6, 7, and 8. The physiological and biochemical properties of these 12 *ZmCNGC* genes ~~were~~are listed in Table 1. The protein lengths ranged from 326 to 745 aa with average of 612~~.42~~ aa. The molecular weight of these proteins ranged from 38.63 kDa (GRMZM2G129375) to 85.52 kDa (GRMZM2G135651) and the pI value ranged from 8.92 (GRMZM2G023037) to 9.75 (GRMZM2G090528). Subcellular localization analysis indicated that all of ZmCNGCs localized in the plasma membrane except for GRMZM2G066269 which was localized in the nuclear fraction, this result is consistent with *Arabidopsis*~~,~~; for example, previous studies have revealed that CNGCs are majorly localized in the plasma membrane~~, i~~. In addition, some are distributed in vacuole membrane and nuclear envelope (Borsics et al. 2007; Christopher et al. 2007; Yuen & Christopher 2013).

To further access the existence of ZmCNGCs we identified, all the expressed sequence tags (EST) ~~were blast to~~which aligned to ZmCNGC genes using the BLASTN program ~~in~~from NCBI~~,~~; results found only GRMZM2G066269 showed no EST hits, other ZmCNGCs had more than 13 representative matches to ESTs.

Phylogenetic analysis of CNGC genes in maize, *Arabidopsis* and rice

To further understanding the evolutionary relationship of *CNGCs*, an unrooted neighbor-joining phylogenetic tree was generated based on the full-length protein~~s~~ alignments of *ZmCNGCs*, *AtCNGCs* and *OsCNGCs* (Maser et al. 2001; Nawaz et al. 2014). Thus, 20 from *Arabidopsis*, 20 from rice, and 12 from maize were used for constructing un-rooted phylogenetic tree. As shown in Figure 1, the phylogenetic tree clustered the *CNGCs* into four major groups, including group I, II, III, and IV. Of those four groups, group I, II and III are monophyletic, group IV ~~were~~was divided into subgroups IVa and IVb. Among them, group I contains three maize *CNGC* genes (GRMZM2G066269, GRMZM2G148118, and GRMZM2G129375), six in *Arabidopsis* and two in rice. Group II contain~~s~~ed two maize CNGC genes (GRMZM2G077828, GRMZM2G023037), five in *Arabidopsis*, and three in rice. Similarly, group III contains three maize CNGC genes (GRMZM2G090528,

GRMZM2G074317, and GRMZM2G858887). ~~And gG~~Group IV embrace~~s~~d seven in rice and seven in *Arabidopsis*, form~~ing~~ the largest GNGC group with four members in maize ~~*CNGC* genes~~ , which includ~~inged~~ three in IVa (GRMZM2G068904, GRMZM2G005791 and GRMZM2G135651) and one in IVb (GRMZM2G141642). Based on the phylogenetic tree among *ZmCNGCs*, *AtCNGCs* and *OsCNGCs*, we also grouped maize *CNGC* genes into four sub-groups (Figure 3A).

Multiple alignments, gene structure and conserved motif of ZmCNGCs

~~Due to t~~The CNBD domain ~~is the gene~~is a structur~~e~~eural feature element in plant CNGCs which contain the PBC and the hinge region (Diller et al. 2001). As shown in Figure 2, after aligning the CNBD region of maize CNGCs, the putative PBC and hinge domain were also identified, which were consistent with rice CNGCs (Nawaz et al. 2014). Results showed that glycine (G), acidic residue glutamate (E) ~~and,~~ leucine~~s~~ (L) and aromatic tryptophan (W) inside the PBCs were 100% conserved. As well ~~as,~~ the aliphatic alanine (A), aromatic phenylalanine (F) and leucine~~s~~ (L) were the most conserved within the~~in~~ hinge region. Compared to rice and Arabidopsis (Supplemental File 2), we found that glycine (G) and leucine~~s~~ (L) were found to be conserved ~~at~~ 100% in CNGCs PBC domains, while aromatic phenylalanine (F) and leucine~~s~~ (L) were 100% conserved in the hinge domain. Furthermore, gene structure analysis could add better understanding to the gene functions and evolution. As a whole, the number of exons ranged from 1 to 8 while GRMZM2G077828 was intronless (Figure. 3B). In addition, eight putative motifs were characterized and named as motif 1 to motif 8 in ZmCNGCs. The relative positions of motif in the four groups were found to have various patterns (Figure. 3C). The conserved domain of ~~the~~ most ZmCNGCs harbor motif 1, representing it is the typical ZmCNGC domain.

Prediction of cis-acting regulatory elements and GO analysis of ZmCNGC proteins

~~For~~To better understand the possible biological processes of these ZmCNGCs involved, 1.5 kb upstream of *ZmCNGC* genes genomic sequences were used to identify cis-regulatory elements and these were submitted to the NEW PLACE web tool. ~~There are~~ 137 different putative cis-elements were found to be ~~presented in at least one~~associated with indentified *ZmCNGC* gene~~s~~ and only 12, including CACTFTPPCA1, EBOXBNNAPA, DOFCOREZM, MYCCONSENSUSAT,

CAATBOX1, GTGANTG10, WRKY71OS, GT1CONSENSUS, ROOTMOTIFTAPOX1, POLLEN1LELAT52, MYBCORE, and OSE2ROOTNODULE, ~~out of them~~ were apparently~~appeared~~ in the promoter region of all *ZmCNGC* genes (Supplemental File 1: Table S1). Additionally, five cis-elements were gene-specific, such as ACGTCBOX, TATABOX3, HDZIP2ATATHB2, ABREMOTIFAOSOSEM and MRNA3ENDTAH3 and were unique to GRMZM2G005791, GRMZM2G068904, GRMZM2G074317 and GRMZM2G135651, respectively [5 compared to 4? one missing].

Also, some cis-elements were involved in different abiotic/biotic stimuli, including those such as hormone~~s~~-response (abscisic acid, auxin, ethylene, etc.), stress-related (drought, temperatures, disease, etc.) and development-related (mesophyll specific, tissue specific, etc.), indicat~~ed~~ing that these *ZmCNGC* genes ~~might~~may be involve~~d~~ in regulating divers~~ity~~e stress~~es~~ response~~s~~. Different cis-elements presenting in *ZmCNGC* genes indicated that they may relate to different regulatory networks.

Furthermore, gene ontology (GO) terms were used to predict the functions of ZmCNGCs by classifying them into categories with three independent ontologies including those for biological process (BP), molecular function (MF), and cellular components (CC) (Consortium 2017). As shown in Figure 4, the biological process of ZmCNGCs were involved in cellular process~~es,~~ for establishment of localization and transmembrane transport. The molecular function ZmCNGCs participated in substrate-specific and transmembrane transport~~er~~. Further, cellular component analysis revealed the localization of ZmCNGCs in the cell and membrane.

Co-expression network between ZmCNGC and other maize genes

To get the detail~~ed~~ information about the interaction relationship between *ZmCNGC* genes and other maize genes, the co-expression network based on the orthology-based prediction~~s~~ follow~~ed~~ing the network in *Arabidopsis* were constructed. As shown in Figure 5, a total of 5 *ZmCNGC* genes including GRMZM2G068904, GRMZM2G077828, GRMZM2G005791, GRMZM2G141642 and GRMZM2G858887 with 76 gene pairs of network interactions were identified. GO annotations of interact~~ed~~ing genes were also performed (Supplemental File 1: Table S2). Some symbols such as SOS1, BPM2, SKOR and BPM4, which play an essential role in regulation of organ development and osmotic stress respon~~d~~se were shown. The co-expression network analysis of *ZmCNGC* genes may provide comprehensive

information for understanding ZmCNGC genes transduction pathways in maize.

Expression profiles of *ZmCNGC* genes in different tissues

We performed transcriptome sequencing to evaluate tissue-specific expression levels of *ZmCNGC* genes ~~at~~in different tissues based on previous stud~~y~~ies. As shown in Figure 6, the expression levels among *ZmCNGC* genes were tissue-specific in their express~~ed~~ion. For example, *GRMZM2G077828* and *GRMZM2G148118* were highly expressed in pollen and all IVb sub-group genes (*GRMZM5G858887*, *GRMZM2G074317* and *GRMZM2G090528*) were mainly expressed in embryo, while *GRMZM2G129375* and *GRMZM2G005791* were ~~lowly~~ expressed at low levels in pollen and embryo, indicted that IVb sub-group genes contribute to maize embryo development. We also evaluated some *ZmCNGC* genes in embryo, endosperm and seed expression in some days after pollination. As showed in Figure 6 B, C and D, the embryo specific-expression gene *GRMZM5G858887* is gradually went up with time, and *GRMZM2G090528* is highly expression in embryo, endosperm and seed.

Most researches showed that cyclic nucleotide-gated channels (CNGCs) have been related to pollen development and in response to environment stimulus. For example, *Arabidopsis CNGC16* is critical for pollen fertility under conditions of heat stress and drought stress (Tunc-Ozdemir et al. 2013), and *CNGC18* has been shown to function in pollen tube tip growth . In rice, OsCNGC13 promotes seed-setting rate by facilitating pollen tube growth in stylar tissues (Xu et al. 2017). *GRMZM2G148118* and *GRMZM2G077828*, two homologous gene of *CNGC16* and *CNGC18* in our study are mainly expressed in pollen, indicated they mainly involved in pollen development. Previous study showed that the AtCNGC3 promoter::GUS construct in transgenic plants revealed expression throughout plant development mainly in the embryo, leaves and roots, the expression level of *GRMZM2G023037* is consistent with *AtCNGC3* which highly expressed in plant development except pollen (Kaplan et al. 2007).

CONCLUSION

To study the CNGC gene family in the maize, we identified 12 *CNGC* genes distributed in 7 chromosomes which were classified into four major groups. Aligning the maize CNGCs and other plants showed that PBC and hinge domain is the most conserved in CNBD domain. Also, a total of 137 putative cis-elements were found

and related to hormones response, abiotic stress and organ development. GO analysis indicated that most of them are involved in various biological processes, including cellular process, establishment of localization and transmembrane transport. Furthermore, the co-expression network analysis of *ZmCNGC* genes may provide important information for the better understanding ZmCNGC transduction pathways. Expression profiles of *ZmCNGC* genes were tissue-specific expressed and related to pollen development. Taken together, our results provide a solid foundation for further evolutionary and functional investigations on ZmCNGCs.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

Lidong Hao performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, and wrote the paper, prepared figures and/or tables.

Xiuli Qiao conceived and designed the experiments, reviewed drafts of the paper.

Supplemental Information

Supplemental File 1

Table S1 Numbers of known stress-related elements in the promoter regions of ZmCNGCs

Table S2 Detail information of Network of ZmCNGC genes with other maize genes

Supplemental File 2

Multiple sequences alignments among maize, *Arabidopsis* and rice.

Supplemental File 3

The gene sequences used in this research.

Data Availability

The following information was supplied regarding data availability:

The raw data has been supplied as a Supplemental File 3.

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