Turkish Journal of Botany

Volume 48 | Number 5 Article 4

9-25-2024

Genome-wide identification, phylogeny, and pharmacological analysis of date palm cyclic nucleotide-gated ion channel (CNGC) gene family in response to fungi

MUMTAZ ALİ SAAND

MARINA MEMON

AMEER AHMED MİRBAHAR

QURBAN ALİ MAGSI

ATHAR ABBAS BABAR

See next page for additional authors

Recommended Citation

SAAND, MUMTAZ ALİ; MEMON, MARINA; MİRBAHAR, AMEER AHMED; MAGSI, QURBAN ALİ; BABAR, ATHAR ABBAS; and GHANGHRO, SAHIB ABBAS (2024) "Genome-wide identification, phylogeny, and pharmacological analysis of date palm cyclic nucleotide-gated ion channel (CNGC) gene family in response to fungi," *Turkish Journal of Botany*: Vol. 48: No. 5, Article 4. https://doi.org/10.55730/1300-008X.2813

Available at: https://journals.tubitak.gov.tr/botany/vol48/iss5/4

Genome-wide identification, phylogeny, and pharmacological analysis of date palm cyclic nucleotide-gated ion channel (CNGC) gene family in response to fungi

Authors
MUMTAZ ALİ SAAND, MARINA MEMON, AMEER AHMED MİRBAHAR, QURBAN ALİ MAGSI, ATHAR ABBAS BABAR, and SAHIB ABBAS GHANGHRO

TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2024) 48: 249-261

doi:10.55730/1300-008X.2813

Genome-wide identification, phylogeny, and pharmacological analysis of date palm cyclic nucleotide-gated ion channel (*CNGC*) gene family in response to fungi

Mumtaz Ali SAAND*, Marina MEMON, Ameer Ahmed MIRBAHAR, Qurban Ali MAGSI, Athar ABASS, Sahib GHANGHRO
Department of Botany, Shah Abdul Latif University, Khairpur, Sindh, Pakistan

Received: 11.01.2024 • **Accepted/Published Online:** 12.08.2024 • **Final Version:** 25.09.2024

Abstract: Cyclic nucleotide-gated ion channels (CNGCs) are suggested to be important calcium-conducting channels and are involved in various biological functions such as plant development, phytohormone responses, and plant–pathogen interactions. Nevertheless, *CNGC* gene family identification has not yet been conducted for date palm crops. In this study, genome-wide identification and pharmacological analysis of date CNGCs were conducted against fungal pathogens including *Aspergillus* and *Fusarium* spp. Twenty-six *PdCNGC* genes were identified using extensive bioinformatics tools. Gene Ontology analysis revealed the signal transduction, transmembrane transport, and ion-binding proteins to be the major regulators in the biological processes and molecular functions of PdCNGCs. Protein–protein interactions of PdCNGCs and their patterns confirmed an integrated role that may be related to nitric oxide and calcium ion-mediated signal transduction and plant–pathogen interactions. Extensive phylogenetic analysis provided clear evidence that group IV had ancient origins. This was supported by the fact that the entire sets of CNGCs from both nonvascular plants (moss) and vascular nonflowering plants (*Selaginella*) were clustered together with groups IVa and IVb, emphasizing the evolutionary antiquity of this group. Furthermore, a pharmacological assay suggested that the application of cGMP and cAMP as CNGC activators may enhance susceptibility against *Aspergillus* spp. in the leaves and stems of date palm, respectively. Treatment with $LaCl_3$ as a calcium channel blocker reduced the resistance of date fruit to *Fusarium* spp. These results suggest a valuable role for CNGC and calcium ions, which may be negatively regulated in date palm against fungal pathogens. This study provides a theoretical basis for the further functional analysis of date palm CNGCs and their molecular mechanisms in the context of pharmacological assessments of plant disease resistance.

Key words: Date palm, cyclic nucleotide-gated ion channel, phylogeny, Gene Ontology, pharmacological assay, fungi

1. Introduction

Calcium signaling transduction via Ca^{2+} channels is an important mechanism used by plants to respond to internal and external stimuli (Reddy et al., 2011) and it is involved in various biological processes, including plant development (Frietsch et al., 2007), hormone responses (Munemasa et al., 2007), plant–pathogen interactions (Qi et al., 2010), and salt stress (Tracy et al., 2008). The cyclic nucleotide-gated ion channel (CNGC) is presumed to be one of the essential calcium-conducting channels in signaling transduction (Talke et al., 2003). CNGCs are ligand-gated nonselective cation protein channels localized in the cell membrane of both plant and animal systems (Liu et al., 2021). A recent study identified three CNGC homologs (CNGC15a/b/c) in *Medicago truncatula* localized in the nuclear membrane of the cell (Charpentier et al., 2016). *Arabidopsis thaliana* CNGC 19 and 20 proteins were also identified in the vacuolar membrane (Yuen and Christopher, 2013). It is suggested that CNGCs are activated by cyclic nucleotides and hampered by calmodulin (CaM) protein (Baloch et al., 2021). Their biological roles have been thoroughly elucidated in animals, but fewer recent studies have revealed the roles of plant CNGCs and more such roles remain to be discovered (Talke et al., 2003). Plant CNGCs consist of six transmembrane domains having a pore region (P) between the S5 and S6 domains, and the C-terminus is composed of cyclic nucleotide-binding (CNB) and CaMB domains (Zelman et al., 2012). The cyclic nucleotide-binding domain (CNBD) in plant CNGCs is highly conserved with a phosphate binding cassette (PBC) and hinge region (Zelman et al., 2012; Saand et al., 2015a, 2015b).

Plant CNGCs were first identified at the individual and genome levels in barley and Arabidopsis, respectively (Schuurink et al., 1998; Mäser et al., 2001). Additionally, *CNGC* gene families have been identified at the genome

* Correspondence: mumtaz.saand@salu.edu.pk

level in *Oryza sativa* (Nawaz et al., 2014), *Pyrus bretschneideri* (Chen et al., 2015), *Solanum lycopersicum* (Saand et al., 2015a), *Selaginella moellendorffii* (Zelman et al., 2012), *Physcomitrella patens* (Zelman et al., 2012), *Brassica rapa* (Baloch et al., 2021), *Brassica napus* (Liu et al., 2021), *Gossypium* spp. (Zhao et al., 2022), *Citrus* spp. (Zia et al., 2022), *Saccharum* spp. (Zhang et al., 2023a), *Helianthus annuus* (Oranab et al., 2024), *Glycine max* (Cui et al., 2023), *Solanum melongena* (Jiang et al., 2023), and *Mangifera indica* (Zhang et al., 2023b).

The Arabidopsis gene family contains 20 *CNGC* genes and may be clustered into five groups (I, II, III, IVa, and IVb) according to phylogeny (Mäser et al., 2001). Studies have revealed that numerous members of the Arabidopsis *CNGC* gene family are involved in various biological functions including the development of plants under stress tolerance (Kaplan et al., 2007), plant growth and seed germination (Gobert et al., 2006), pollen growth and fertility under stress (Frietsch et al., 2007; Tunc-Ozdemir et al., 2013), Ca^{2+} signaling in plant immunity and disease resistance against various pathogens (Ma and Berkowitz, 2011), and other abiotic stress responses (Yuen and Christopher, 2010). The discovery of three isoforms of *Medicago truncatula* CNGC15 (MtCNGC15a/b/c) constituted a significant breakthrough in plant biology. These isoforms play essential roles in regulating nuclear calcium (Ca^{2+}) levels and orchestrating symbiotic responses, particularly with nitrogen-fixing bacteria. These findings shed new light on the molecular mechanisms governing plant–microbe interactions and highlighted the importance of calcium signaling in plant systems (Charpentier et al., 2016). Various *CNGC* gene family members in rice were expressed in response to multiple stimuli, including hormonal, biotic, and abiotic stress tolerance (Nawaz et al., 2014). Recently, two studies addressed the roles of CNGCs in eggplant and mango in response to cold stress (Jiang et al., 2023; Zhang et al., 2023b).

We previously tested pharmacological effectors such as cAMP and cGMP as channel activators and alloxan and $LaCl_3$ as channel blockers to identify the role of CNGCs in protection against fungal pathogens in tomato crops (Saand et al., 2015a). That study revealed that cGMP and cAMP could reduce the diameter of necrosis significantly in the treatment of fungal pathogens compared to control plants treated with water whereas $LaCl_3$ as a calcium channel inhibitor showed susceptible results for fungal pathogen inoculations (Saand et al., 2015a). Since then, no other reports have demonstrated the roles of such pharmacological effectors for plant CNGCs against biotic stress. There is a need for more scientific evidence regarding the role of calcium channel and CNGC blockers/activators and identification of the functions of CNGCs in response to fungal pathogens. Therefore, in this study, we aimed to determine whether those calcium channel and CNGC blockers and activators regulate CNGCs in date palm fruits and leaves in response to fungal pathogens.

Date palm (*Phoenix dactylifera* L.) is an economically important crop belonging to the family Arecaceae and it is cultivated worldwide (Abul-Soad et al., 2015; Al-Dashti et al., 2021). Egypt is the largest date fruit-producing country in the world while Pakistan ranks sixth among date fruit-producing countries (Al-Dashti et al., 2021). Date palm is generally understood to be a drought- and saline-tolerant crop; nevertheless, various abiotic and biotic stresses may damage the date fruit and reduce the economy of the country. For example, fungal pathogens damaged the date fruit crop grown in Khairpur in the Sindh province of Pakistan (Maitlo et al., 2014). Recently, the genome of the date palm cultivar 'Barhee' (BC4) (PRJNA322046) was sequenced and released via the National Center for Biotechnology Information (NCBI) (Hazzouri et al., 2019). In light of this information, the present study aimed to identify the *CNGC* gene family in the date palm genome. The in silico characterization of date *CNGC* genes and proteins was undertaken by applying various bioinformatics tools. Subsequently, protein motifs, phylogeny, Gene Ontology (GO) results, and protein–protein interactions were analyzed for the date palm *CNGC* gene family. This study further aimed to evaluate the actions of pharmacological effectors such as cAMP, cGMP, alloxan, and $LaCl_3$ against fungal pathogens to better understand the role of CNGCs in date palm crops in response to biotic stresses such as fungi.

2. Materials and methods

2.1. Identification of *CNGC* genes in the *Phoenix dactylifera genome*

For identification of candidate date palm CNGC sequences, we used the CNGC protein sequences of Arabidopsis, a model taxon whose genome has been sequenced and completely annotated (Cheng et al., 2017). A total of 20 Arabidopsis CNGC protein sequences were obtained from the Arabidopsis Information Resource (TAIR) database.[1] The 45 candidate *CNGC* genes were subsequently retrieved from the date palm (*Phoenix dactylifera*) genome using the Arabidopsis CNGC protein sequences as a method via the website of the NCBI.[2] The E-value score in the BLASTp results ranged from 0 to 6e-130 against all Arabidopsis protein query sequences. All retrieved sequences were collected to analyze the domain architecture by utilizing the SMART (simple molecular architecture research

[1]The Arabidopsis Information Resource (TAIR). TAIR database [online]. Website https://www.arabidopsis.org/ [accessed 19 October 2021].

[2]National Center for Biotechnology Information (NCBI) [online]. Website http://www.ncbi.nlm.nih.gov/ [accessed 25 October 2021].

tool), CDD (conserved domain database), and Pfam databases. Entire candidate CNGC sequences were found with either a transmembrane/ion transport protein domain (TMD/TPD; PF00520) or effector domain of the CAP family (CAP_ED; cd00038)/cNBD (PF00027) and cNMP (SM000100) domains. The ITD/TM domains were replaced by the PLN03192 superfamily (cl33658) in a majority of date palm protein sequences at the CDD. Finally, a total of 26 date palm CNGC proteins were identified based on CNGC-specific proteins (PBC and hinge regions) following the criteria proposed by Zelman et al. (2012) and Saand et al. (2015a). Sequences that were lacking CNGC-specific motifs were discarded.

2.2. Multiple alignments and phylogenetic analyses

Multiple sequence alignments for 15 plant species including *Phoenix dactylifera*, *Arabidopsis thaliana*, *Solanum lycopersicum*, *Oryza sativa*, *Brassica rapa*, *Brassica napus*, *Gossypium hirsutum*, *Helianthus annuus*, *Mangifera indica*, *Citrus sinensis*, *Nicotiana tabacum*, *Ziziphus jujuba*, *Zea mays*, *Physcomitrella patens*, and *Selaginella moellendorffii* were performed for full-length protein sequences using the ClustalW program (Larkin et al., 2007) and phylogenetic trees for individual and/or multiple species were generated using MEGA 5.0 with the maximum likelihood (ML) method and 1000 bootstraps (Tamura et al., 2011). To visualize the phylogenetic trees, the online iTOL program (version 5) was used.[3] The analysis of CNGC-specific motifs within the CNB domain was performed following the methods proposed by Nawaz et al. (2014).

2.3. Localization, Gene Ontology (GO), and protein–protein interaction (PPI) analysis for date palm CNGCs

GO results and subcellular localizations for date palm CNGC proteins were observed using CELLO2GO (Yu et al., 2014). The functional PPIs for PdCNGCs were determined using STRING database (version 11.5)[4] to obtain interaction scores (medium confidence: 0.400) (Szklarczyk et al., 2023).

2.4. Plant material, sample preparations, and pathogen inoculation

Five-month-old date palm seedlings (cvs. Kurh and Gajjar) grown at the Date Palm Research Institute (DPRI) of Shah Abdul Latif University (SALU) in Khairpur, Pakistan, were used for the pharmacological assay. Leaf, stem, and root samples were obtained from healthy seedlings for pharmacological effectors and water was used for control treatments. The leaf, root, and stem samples of date palm were hard to puncture with a needless syringe (Saand et al., 2015a). Subsequently, the samples were spotted/dotted with a sterilized syringe as the chemicals and/or water could infiltrate into the samples and they were kept in petri dishes to which chemicals (pharmacological effectors) or water were added for 30 min. Samples were then removed from the petri dishes and immediately inoculated with fungal pathogens such as *Aspergillu*s and *Fusarium*, which were cultured on potato dextrose agar (PDA) media. The PDA media preparation contents included potato (infusion form) at 200 g, dextrose at 20 g, and agar-agar at 15 g, all dissolved in 1000 mL of water (H_2O) (Saand et al., 2015a). Cultures of *Aspergillus* 3 to 5 days old with black mycelia in the initial phase of the conidia with a slightly smooth-walled appearance of the colony were used for inoculation. Cultures of *Fusarium* spp. 3 to 4 days old (acquired from the DPRI of SALU, Khairpur) with white cottony texture, before the appearance of macroconidia, were used for the inoculation process. For inoculations, PDA plugs (2 mm in diameter) for both *Aspergillus* and *Fusarium* spp. were taken from the corners of plates (active and young mycelia) and put on samples (leaves, stems, and roots) of date palm with the mycelial side facing downwards (Saand et al., 2015a). The inoculated samples were kept in a box where humidity was maintained by covering them with a transparent thin polyethylene sheet and were incubated at 25 °C. The treated samples were observed and photographed, and disease symptoms were analyzed at each time point. Date fruits (khalal stage) of some early varieties (cvs. Gajjar and Kurh) were collected from trees grown in the orchard of the DPRI of SALU. A similar method was applied for date palm fruit treatments and inoculations.

2.5. Fungal isolate culturing and identification

Fungal isolates were taken from infected leaves and fruits of date palm cultivated in Khairpur, Sindh, Pakistan. The samples were surface-washed with sodium hypochlorite and cultured on PDA media until mycelium development. Samples were then incubated in an incubator at 27 °C until the visible growth of mycelium and the development of spores. The desirable mycelium growth and spore development for fungal isolates appeared within 1 week (Barker et al., 2022). Microscopy was performed using a confocal electron microscope at the Center for Biodiversity and Conservation, SALU, Khairpur. The fungal isolates were identified as *Aspergillus* species following the keys and taxonomic characteristics described by Samson et al. (2014).

2.6. Pharmacological assay

Pharmacological effectors and chemicals (SolarBio, Beijing, China) such as dibutyryl-cAMP (db-cAMP), dibromo-cGMP (db-cGMP), lanthanum(III) chloride hydrate ($LaCl_3$), and alloxan monohydrate were dissolved

[3]Interactive Tree Of Life (iTOL). iTOL program (version 5) [online]. Website https://itol.embl.de/ [accessed 25 January 2022].

[4]STRING database. STRING version 11.5 [online]. Website https://string-db.org/ [accessed 03 January 2022].

in sterilized distilled water (dH_2O) as stock solutions. cAMP, alloxan, and $LaCl_3$ were diluted with sterilized dH_2O to a final concentration of 1 mM, whereas cGMP was diluted with sterilized water to a final concentration of 100 μM (Qi et al., 2010; Saand et al., 2015a). For the control, sterilized dH_2O was used to treat the samples. The pharmacological/chemical treatments and fungal pathogen inoculations were performed as described in subsection 2.4.

3. Results and discussion

3.1. Identification of *CNGC* genes and CNGC-specific motif in date palm genome

Initially, genome-wide identification was carried out for the date palm genome via the NCBI database. To obtain the candidate CNGC protein sequences of *Phoenix dactylifera* L., a total of 20 AtCNGC protein sequences were used for BLASTp via the NCBI. A total of 45 protein sequences were obtained through the BLAST search method. Subsequently, domain analysis revealed that entire sequences were determined by important domains such as (CNBD)/cNMP/CAP_ED and/or ITPD/(TMD) across all three domain databases (Table S1). Of 45 full-length proteins, only 26 CNGC proteins were characterized by CNGC-specific motifs in date palm. Figure S1 depicts the PBCs and hinge region conservations, which were already identified for tomato and Arabidopsis CNGC proteins. The key shown at the top of Figure S1 was constructed following the criteria for Arabidopsis and tomato CNGCs (Mäser et al. 2001; Saand et al., 2015a).

The alignment of date palm CNGC-specific motifs was more conserved at 90% compared to Arabidopsis, tomato, and rice CNGCs (Zelman et al., 2012 Nawaz et al., 2014; Saand et al., 2015a). PdCNGC14/15/16/17/18 possessed 52 amino acids, whereas the rest of the PdCNGCs contained 42 amino acids (Figure S1). Importantly, the motifs for PdCNGC proteins revealed more conservation, being conserved at >90% with the 11 amino acids of GELWLPSTEFL (Figure S1) compared to the same motifs for tomato, in which only the 8 amino acids of GELWPSEF are conserved (Figure S2; Saand et al., 2015a). The results showed that two amino acids (LL) were conserved at the PBC and hinge region, respectively, in date palm CNGCs. One amino acid (T) positioned between the PBC and hinge region was also conserved in date palm compared to tomato CNGCs. To observe the conservation in comparison to Arabidopsis, we aligned the PdCNGCs with AtCNGC proteins and the results predicted even less conservation of amino acids compared to tomato CNGCs. The alignment for AtCNGCs and PdCNGCs entailed conservation of only 7 amino acids (GLPSEFL) at >90% alignment (Figure S3). The rice and date palm CNGC protein motifs were conserved with 7 amino acids at >90% alignment and those were GELW in the PBC and FL in the hinge region, whereas T was conserved between the PBC and hinge region (Figure S4). Interestingly, rice and date palm CNGC motifs were conserved at different positions compared to those in Arabidopsis. In the case of Arabidopsis and date palm, the SE amino acids were conserved, while the EWT amino acids were observed for rice and date palm CNGC motifs (Figures S3 and S4). This may be due to differences in monocot and eudicot clades with sequences conserved accordingly.

3.2. Phylogeny of the date palm *CNGC* gene family

To evaluate the phylogeny, a phylogenic tree was constructed in MEGA 5.0. The phylogenetic analysis revealed proteins in five major groups (I. II, III, IVa, and IVb) for date palm CNGCs (Figure S5). The phylogenetic trees together with Arabidopsis CNGCs were also clustered into five major groups (Figure 1). These results are consistent with those of Mäser et al. (2001) for AtCNGCs and Saand et al. (2015) for tomato CNGCs (Figure S6). The phylogenetic tree for date palm together with rice crops also revealed the logical clustering of all group members into five clades for both species (Figure S7).

In detail, PdCNGC groups I and II have three and two genes, respectively (Figure S5), whereas group III possesses eight genes. Moreover, groups IVa and IVb, respectively, contain five and eight *CNGC* gene members in date palm (Figure S5). Compared to the phylogeny of Arabidopsis, groups I and II possess six and five genes, respectively, while the same groups from the date palm phylogenetic tree contain three and two genes. Thus, there is a loss of three genes in both groups. There were eight and five genes in groups III and IVa of the date palm *CNGC* gene family, respectively. The same groups (III and IVa) in Arabidopsis possess five and two genes, respectively. These results revealed that groups III and IVa both gained three more *CNGC* genes during the course of evolution in the date palm genome. Finally, PdCNGC group IVb possesses eight genes while the same group for Arabidopsis contains two CNGCs. These results demonstrate that group IVb gained five more genes compared to Arabidopsis during evolution (Figures 1 and S5).

Regarding the rice CNGC tree, groups I, II, and IVb have three genes. In comparison, in the date palm CNGC phylogenetic tree, group I has three, II has two, and IVb has eight *CNGC* genes. Thus, group I has the same number of genes in both species. One gene has been lost in group II of the date palm gene family compared to rice *CNGC*s (Figure S7). The other groups of the date palm *CNGC* gene family have greater numbers of genes compared to rice *CNGC*s.

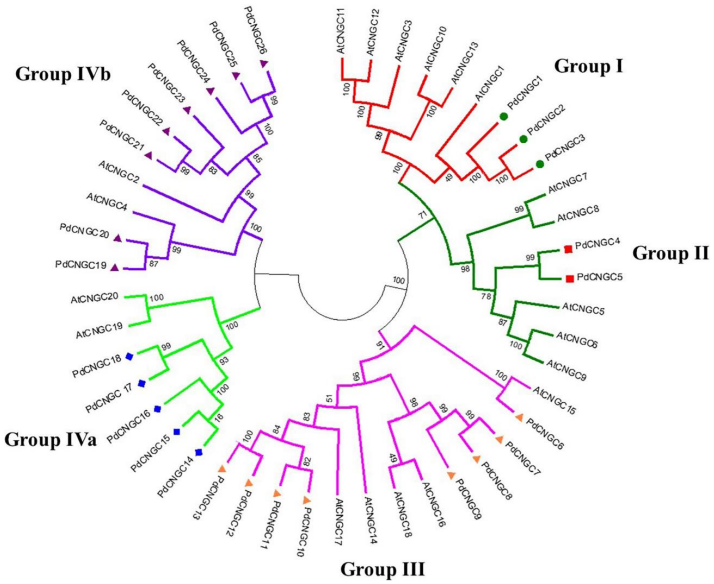

Figure 1. Phylogenetic tree of PdCNGC and AtCNGC gene families. The tree was generated using MEGA 5.0 software using criteria including the maximum likelihood method at 1000 bootstraps as suggested by Saand et al. (2015a). The five groups are represented in different colors i.e. red (having three PdCNGC labeled with green circles), dark green (having two PdCNGC with red squares), pink (having eight PdCNGC with orange triangles), light green (having five PdCNGC with blue rhombus) and blue (having eight PdCNGC with purple triangle) proteins with AtCNGCs in each group, respectively.

To predict the phylogenetic relationships of plant CNGCs with date palm CNGCs, we conducted a comprehensive phylogenetic analysis. In addition to dicots and monocots, two lower nonflowering species of pteridophyte and moss were also included in the phylogenetic analysis. Fifteen plant species including date palm (this study), Arabidopsis (Mäser et al., 2001), rice (Nawaz et al., 2014), tobacco (Nawaz et al., 2019), jujube (Wang et al., 2020), pear (Chen et al., 2015), *B. napus* (Baloch et al., 2021), cotton (Zhao et al., 2022), mango (Zhang et al., 2023b) tomato, maize, *B. rapa*, citrus (Saand et al., 2015b), and *Selaginella moellendorffii* and *Physcomitrella patens* (Zelman et al., 2012) were included in the phylogenetic analysis for their *CNGC* gene families (Figure 2). The phylogenetic tree for these 15 plant species possessing 361 CNGC proteins was divided into the five logical groups of I, II, III, IVa, and IVb. Groups I and III were found to be largest with 98 and 95 CNGCs, respectively (Figure 2), followed by groups IVb, II, and IVa.

The comprehensive phylogenetic analysis suggested that plant CNGCs belonging to group IV (both a and b) were most ancient as all nonvascular moss data (*Physcomitrella patens*-PpCNGCs) and nonflowering vascular data (*Selaginella moellendorffii*-SmCNGCs) clustered within those subgroups (Figure 2). The five moss PpCNGCa/c/e/g/h proteins and four SmCNGCs (D8SCK7_SELML, D8SGT07_SELML, D8QYM9_SELML, and D8RB40_SELML) were clustered in group IVa, while the remaining three, namely PpCNGCb/d/f (moss), and one D8T0W5_SELML (nonflowering vascular plant) protein clustered with group IVb. These results showed that group IV of plant CNGCs is the most ancient among all considered CNGC groups. These results contradict the plant CNGC phylogeny proposed by Saand et al. (2015b) and Nawaz et al. (2019). Evaluation of the phylogenetic relationship of tobacco CNGCs with 20 plant species revealed that four nonvascular CNGCs (PpCNGCa/c/e/h) clustered with group III. Previously, all nonflowering vascular proteins (SmCNGCs) were clustered with both VIa and VIb (Nawaz et al., 2019). Intriguingly, our results are consistent with those of Nawaz et al. (2019) for nonflowering vascular proteins (SmCNGCs) that clustered with group IV plant CNGCs (Figure 2). Our previous analysis of 20 plant species' CNGCs revealed that two SmCNGCs were clustered in a group II but the rest of the lower nonvascular and nonflowering vascular CNGCs were assembled in group IV (Saand et al., 2015b). Thus, we conclude that the CNGCs belonging to group IV are the most ancient and all other groups evolved thereafter during the course of

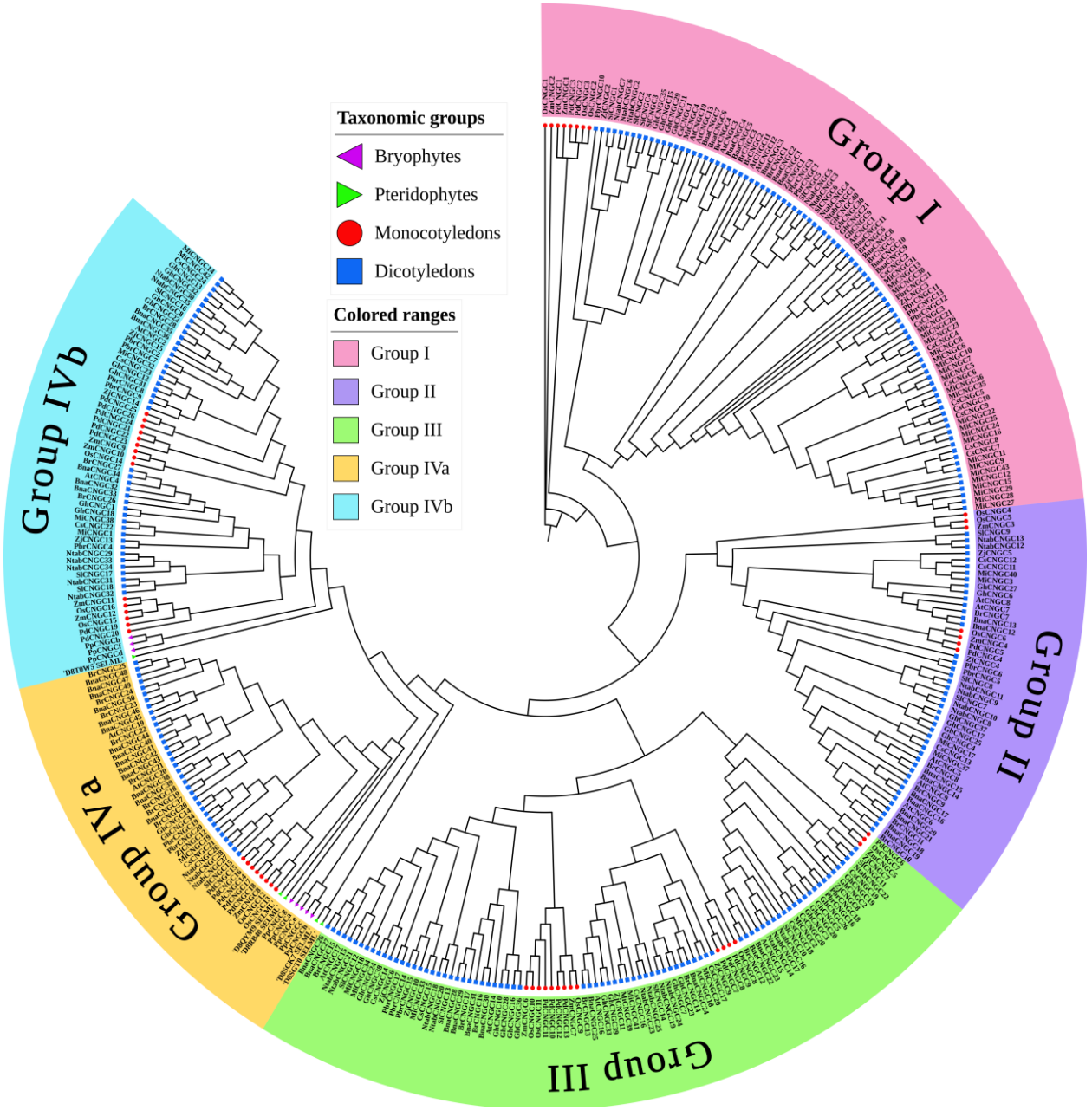

Figure 2. Phylogenetic relationships of date palm CNGCs with other plant CNGC families. The tree was generated for 361 CNGC proteins form 15 plant species by using MEGA 5.0 software following criteria maximum likelihood method at 1000 bootstraps as suggested by Saand et al. (2015a). In this tree, the taxonomic groups are presented in different colors and shapes including nonvascular plants (moss) in a pink triangle, vascular nonflowering (lycophyte) in a green triangle, monocots in the red circle, and eudicots in a blue square. Each group is also shown in different colors along with names.

the evolution of the CNGCs of land plants (Figure 2). This contradiction can be explained by the recent identification of *CNGC* genes in the genomes of several additional plant species such as *B. rapa*, *Mangifera indica*, and *Citrus* spp. (Baloch et al., 2021; Zia et al., 2022; Zhang et al., 2023b). Further studies may further elucidate the evolutionary history and perspectives of plant CNGCs.

3.3. Predicted protein–protein interactions of data palm CNGCs

PPIs were predicted among the date palm CNGCs using the STRING program with a medium confidence score (0.4). Only 15 CNGC proteins were found to show a connection in the network with each other and their partner interactors (Figure 3). PdCNGC1 and three members of group I interacted

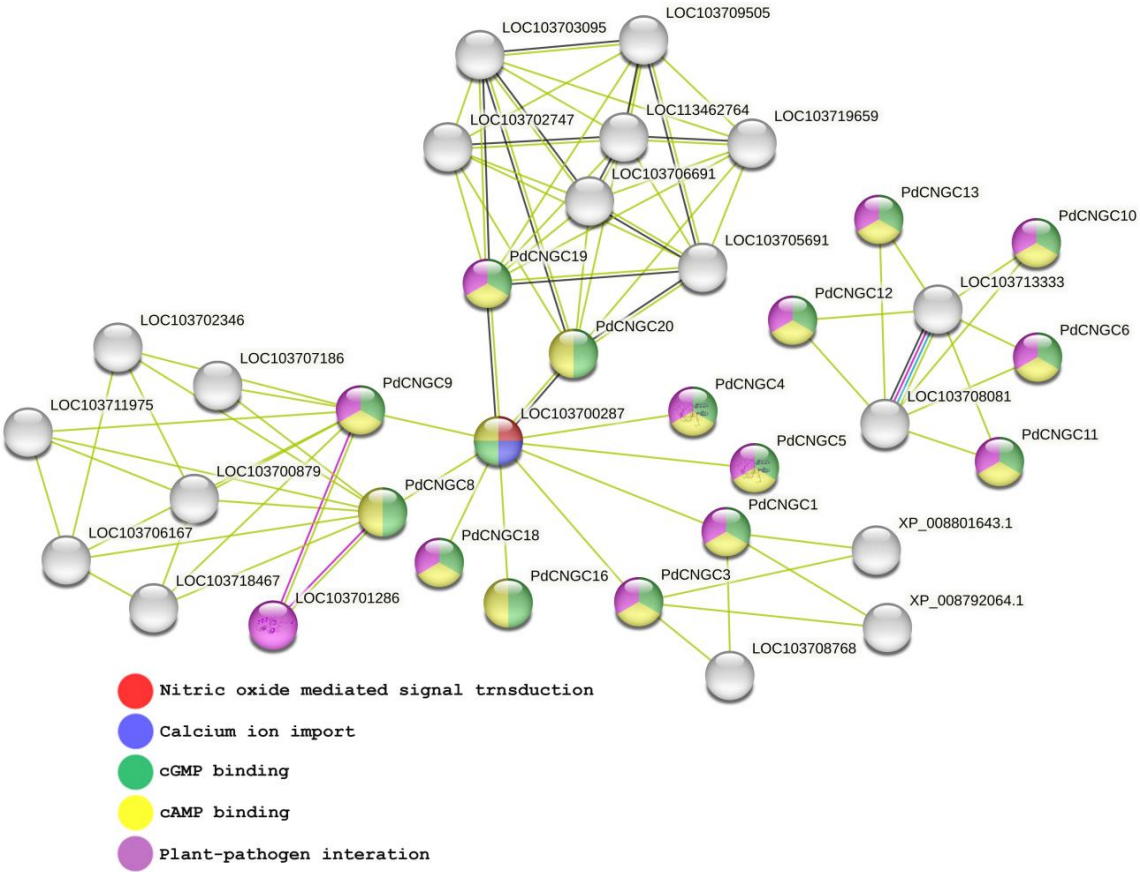

Figure 3. Predicted protein-protein interaction network in date palm CNGCs. The different colors demarcate the predicted biological process, molecular function, and KEGG pathway analysis for PdCNGCs and their partner protein interactors. The protein interaction analysis was observed at the String database.

with three partner proteins including XP_008801643.1, XP_008792064.1, and LOC103708768, with these three proteins belonging to ABC transporter C family member 3. The ABC transporter C family members were previously reported as key players in the abiotic stress response of oil palm crops (Saand et al., 2022). Members of group III such as PdCNGC6, 10, 11, 12, and 13 interact with each other and two partner proteins (LOC103713333, plastid-lipid associated protein; LOC103708081, DNA repair protein). PdCNGC19 and 20 (members of group IVa) were predicted to interact with seven proteins, including the omega-3 fatty acid desaturase and alpha carbonic anhydrase proteins (Table S2). LOC103700287 (cyclic nucleotide-gated ion channel 2-like, partial) predicted a major partner interaction with ten PdCNGCs, namely PdCNC1/3/4/5/8/9/16/18/19/20. Additionally, PdCNGC8/9 interacted with seven proteins. Among them, one protein, LOC103701286 (calcium-dependent protein kinase 28-like; CDPK28), was experimentally determined and it interacted directly with PdCNGC8/9. CDPK28 has been reported to be involved in plant disease resistance and immunity in Arabidopsis and cotton (Monaghan et al., 2014; Wu et al., 2021). Previous PPI network analysis demonstrated that several BoCNGC members of *B. oleracea* were associated with CaM proteins including CaM4, CaM6, and CaM7 (Kakar et al., 2017). The involvement of date palm CNGC8/9 with the CDPK protein suggests that the CaM and CDPK proteins may generally be involved in fine-tuning the CNGC-mediating Ca^{2+} signaling pathway in plant immune responses (Ma and Berkowitz, 2011). Moreover, previous protein–protein network analysis showed strong connectivity and interactions among CNGC proteins as well as with their homologous proteins in citrus, maize, and cotton (Hao and Qiao, 2018; Zhao et al., 2022; Zia et al., 2022). A strong connection was found between citrus CNGCs with syntaxin-121 and vesicle-associated membrane-related proteins (Zia et al., 2022). These results are inconsistent with the results for the PPI network analysis of PdCNGC proteins. Several PdCNGCs and their partners were involved in nitric oxide-mediated signal transduction and calcium ion import (biological process) and cGMP and

cAMP binding (molecular function), as observed in GO analysis. Moreover, all PdCNGCs (except PdCNGC8/16 and 20) and one partner, calcium-dependent protein kinase 28 (LOC103701286), were predicted to be involved in plant–pathogen interactions in the KEGG pathway (Table S2; Figure 3). A recent study revealed that CNGC1 was involved in anion channel activity and interacted with the cGMP-binding defense response in eggplant during protein–protein network analysis (Jiang et al., 2023). These results suggest that PdCNGCs may regulate a wide range of functions including plant disease resistance to biotic and abiotic stress responses.

3.4. Localization of date palm CNGCs

The subcellular localization of date palm CNGC protein sequences was analyzed using CELLO2GO, an online database (Yu et al., 2014). Subcellular localization results revealed that 90% of all PdCNGCs were localized in the plasma membrane (PM) (Figure 4A). PdCNGC16 was predicted to be present in the extracellular and/or nuclear membrane. All PdCNGCs were shown to be localized in the mitochondrial and nuclear membranes except PdCNGC20 and PdCNGC23. PdCNGC10 was present in the vacuolar membrane. However, few PdCNGCs had predicted localizations in the ER, peroxisomes, chloroplasts, or Golgi bodies (Figure 4A). Previous reports predicted the localization of plant CNGCs to the PM (Duszyn et al., 2019). All citrus CsCNGC proteins were found to be localized in the PM (Zia et al., 2022). Entire BnaCNGCs were found in the PM, except for 11 *B. napus* BnaCNGC proteins that were localized in the chloroplasts (Liu et al., 2021). One study found that the three CNGC15a/b/c homologs in *Medicago truncatula*

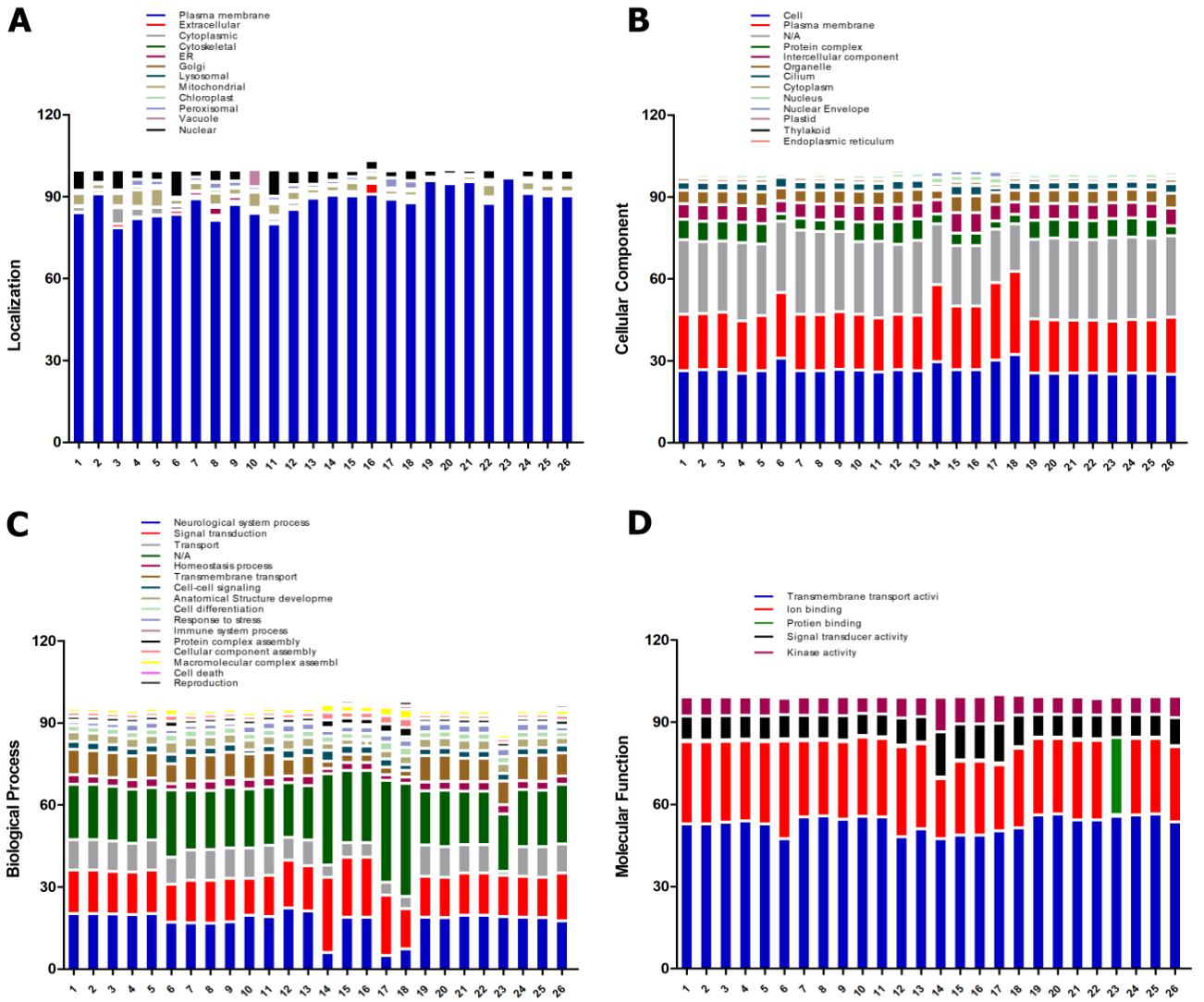

Figure 4. GO and localization of PdCNGCs. A shows localization. B, C, and D represent the cellular component, biological process, and molecular function under gene ontology (GO) analysis for date palm CNGCs.

were localized in the nuclear membrane (Charpentier et al., 2016). Another study reported that AtCNGC19 and AtCNGC20 were localized in a vacuolar membrane (Yuen and Christopher, 2013). Our previous study based on the Protein Subcellular Localization Prediction Tool (PSORT) showed that OsCNGC3, OsCNGC7, and OsCNGC11 were localized in the cytoplasm, chloroplast thylakoid membrane, and mitochondrial inner membrane, respectively (Nawaz et al., 2014), while all other OsCNGCs were found in the PM (Nawaz et al., 2014). Further studies may confirm the localization of PdCNGCs in subcellular components such as the ER, peroxisomes, chloroplasts, mitochondria, and Golgi bodies.

3.5. Gene Ontology analysis for date palm CNGC proteins

GO categories such as cellular components, biological processes, and molecular functions were also analyzed for PdCNGCs. The cellular component results for PdCNGCs revealed a rate of more than 45% for the cell and plasma membranes. All PdCNGCs had very low rates for protein complexes, intercellular components, organelles, cilia, cytoplasm, and nuclei (Figure 4B). Previous studies have shown that cells, membranes, and membrane parts were significantly enriched among the CC GO terms for *Citrus sinensis*, *Brassica oleracea*, and *Gossypium hirsutum* (Kakar et al., 2017; Zia et al., 2022; Kirungu et al., 2023). Additionally, more than 25% remained unknown, marked as N/A (not applicable), in the course of cellular component detection. The neurological system process, signal transduction, transport, and transmembrane transport were the top-ranking results for biological processes in PdCNGCs. The homeostasis process was also revealed as a biological process for PdCNGCs (Figure 4C). More than 20% of the CNGC proteins were undetected and marked as N/A. Cell death, response to stress, and the immune system process were revealed as biological processes for PdCNGCs. The cotton CNGCs were enriched in terms of localization, biological regulation, response to stimuli, and several processes such as metabolic, developmental, and multicellular organism processes among the BP GO terms (Kirungu et al., 2023). Previous research demonstrated that the majority of *CNGC* genes were enriched in terms of the cellular process and transport in *Brassica oleracea* (Kakar et al., 2017), whereas a few *CNGC* genes were assigned to cell death and response to stimuli for the BP GO terms of *Brassica oleracea*. These results indicate that the BP data for date palm CNGCs are partially consistent with *Brassica oleracea* and partially consistent with cotton CNGC BP GO enrichments (Kakar et al., 2017; Kirungu et al., 2023). On the contrary, we found that the neurological system process and signal transduction were the two major components in BP GO enrichment. The allocation of neurological system process enrichment in biological processes for date palm CNGCs is unclear but it is possible that CNGCs may mediate Ca^{2+} signal transduction in response to stimuli (Ma and Berkowitz, 2011). Generally, these results suggest that PdCNGCs could play important roles in plant immunity and may respond to various stresses.

Additionally, binding and activity functions such as transmembrane transport activity, ion binding, signal transducer activity, and kinase activity were dominant in the GO category of molecular function for most PdCNGC proteins. Protein binding, structural and molecular activity, and nucleic acid binding transcription factor activity were also observed as PdCNGC protein GO molecular functions (Figure 4D). The data suggested that PdCNGCs may regulate various molecular functions via protein ion binding, signaling, and transport activities. These results are supported by previous findings; for example, one study identified that binding, transport, and catalytic activity were associated with MF GO enrichments in cotton CNGCs (Kirungu et al., 2023). Notably, transporter activity was significantly enriched among MF GO terms, followed by binding and molecular transducer activity in *Brassica oleracea* (Kakar et al., 2017). Most genes were involved in ion channel activity and voltage-gated potassium channel activity for *Citrus sinensis* CNGCs (Zia et al., 2022). These results are also partially consistent with the MF GO enrichments for PdCNGCs. Functional analysis may reveal the molecular functions of PdCNGCs in future studies.

3.6. Pharmacological analysis of date palm CNGCs in response to *Aspergillus and Fusarium* spp.

To analyze the roles of pharmacological effectors in date palm, a set of CNGC blockers and activators were used in response to fungal pathogens. For this purpose, CNGC activators (cAMP and cGMP), an adenylyl cyclase blocker (alloxan), and a calcium channel blocker ($LaCl_3$) were added to date palm fruits, leaves, stems, and roots in response to fungi (Qi et al., 2010; Saand et al., 2015a). The date palm fruits and leaves were prone to damage from fungal infections during the monsoon rain in Khairpur District, Sindh, Pakistan (Figure S8). Subsequently, the fungal isolates were identified as *Aspergillus niger* from date palm fruits and leaves in the Department of Botany of SALU, Khairpur (Figure S9). *Aspergillus* spp. are reported as devastating fungal pathogens of date palm crops, causing black mold disease in date fruits and flowers (Cohen et al., 2021).

The effect of these fungal isolates was determined in date palm leaves against pharmacological effectors. The results revealed that necrosis symptoms were more prominent with cGMP, alloxan, and $LaCl_3$ compared to the control against *Aspergillus* inoculations in leaves at 5 days after the infiltration of pharmacological effectors (5

dpi) (Figure 5A). cAMP showed fewer symptoms while cGMP had more leaf necrosis symptoms in response to the *Aspergillus* fungal pathogen (Figure 5A). Previously, we found that both cAMP and cGMP had smaller necrosis diameters in response to the fungus *Sclerotinia sclerotiorum* in tomato leaves (Saand et al., 2015a). Treatment with $LaCl_3$ led to susceptibility to *S. sclerotiorum* in tomato leaves at 44 h after inoculation (Saand et al., 2015a). Thus, the present results contradict our previous results as it was found that treatment with cGMP led to susceptibility to the *Aspergillus* fungal pathogen while cAMP showed less necrosis compared to the control in a tomato crop (Saand et al., 2015a). These inconsistent results could be attributed to the effects of CNGC activators (cAMP and cGMP) against different fungal pathogens such as *S. sclerotiorum* and/or *Aspergillus* varying in different plant species. These results suggest that cAMPs may have a role in regulating CNGCs against *Aspergillus* in date palm leaves.

Furthermore, the effect of the same fungal pathogen was investigated with the administration of the same pharmacological effectors in date palm fruits, stems, and roots. In the case of date palm fruits and roots, there was no significant result in any of the treatments, including the control, regarding the development of spores and/or necrosis at 2 and 5 dpi (Figure 5B). Spore development and disease symptom findings were the same in stems for all treatments except cAMP including the control at 5 dpi. On the other hand, the stem was severely damaged with

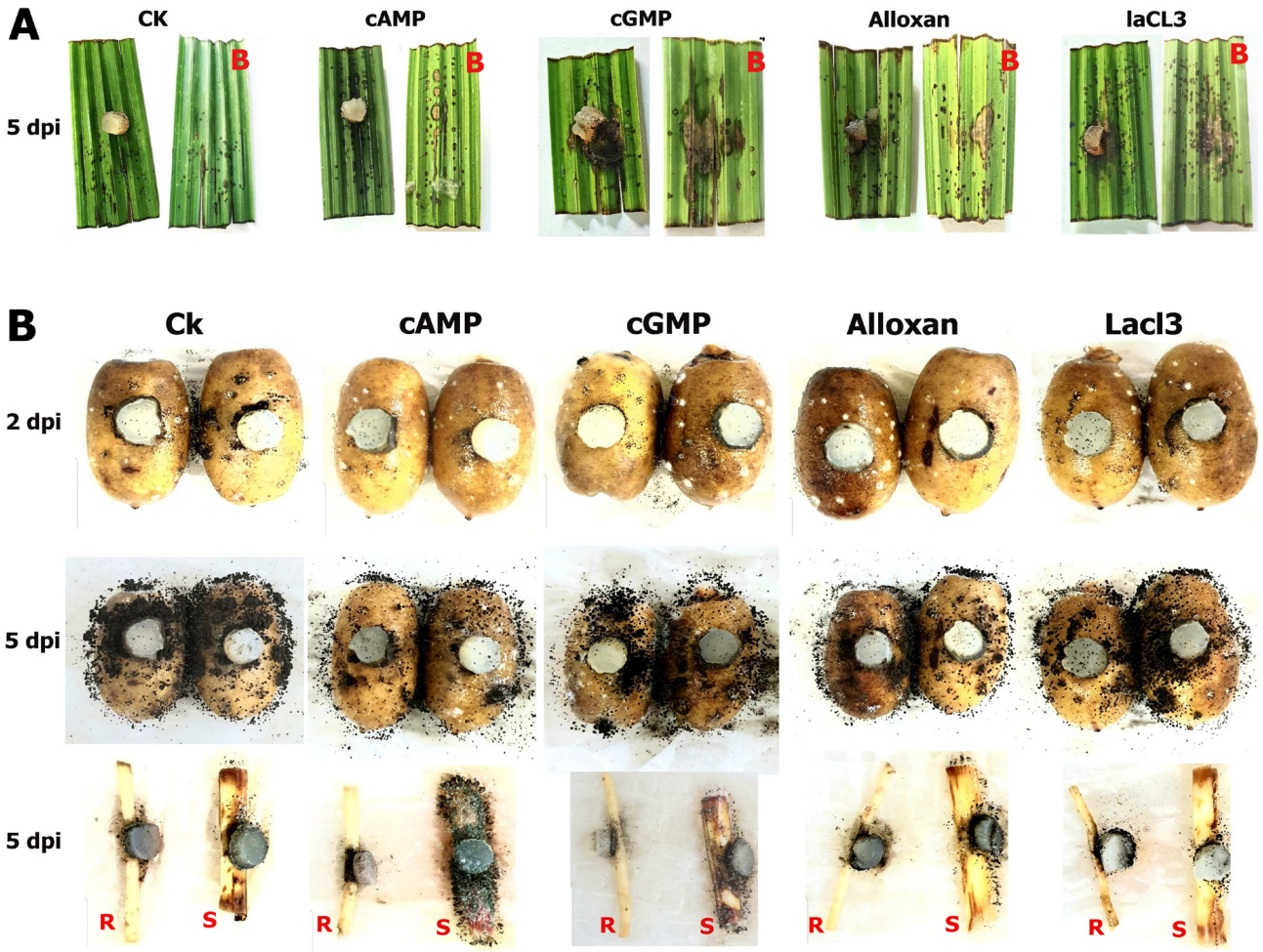

Figure 5. Pharmacological assay in date palm (cv. Kurh) **crop in response to *Aspergillus* spp.** A Shows the pharmacological effectors' analysis in leaves of date palm. B refers to pharmacological effectors' infiltrations in fruit, roots, and stems of date palm. The names of the control and each treatment are shown above the leaf sample. The capital letter "B" (in red color) on leaves refers to the backside of the leaves that were treated and inoculated with pharmacological effectors and fungal pathogens. Capital letters R and S in red colors demarcate the roots and stems of date palm seedlings. The time points are indicated on the left side with 2 dpi and 5 dpi (days postinfiltration/inoculations) of treatments and pathogens. Samples after each treatment i.e. CK (water), cAMP (1 mM), cGMP (100 µM), alloxan (1 mM), and LaCl3 (1 mM) were inoculated with the fungus.

the development of *Aspergillus* spores following cAMP treatment compared to the other treatments (cGMP, alloxan, and $LaCl_3$) and the control at 5 dpi (Figure 5B). These results indicate that cAMP may play a role in susceptibility to infection by *Aspergillus* species in the stems but not the roots while regulating CNGCs in date palm crops.

Fusarium wilt has been reported as a syndrome of sudden decline, also known as Bayoud disease and caused by *Fusarium* spp. in date palm crops worldwide (Alwahshi et al., 2019). Therefore, *Fusarium* was tested against the administration of these chemicals to date fruit (local cv. Gajjar). The results showed that treatment with $LaCl_3$ augmented fungal mycelium development and symptoms while there was no significant difference among the other treatments (cAMP, cGMP, and alloxan) and the control for the *Fusarium* inoculation at 5 dpi (Figure S10). These results demonstrated that $LaCl_3$ as a calcium channel inhibitor may impart disease susceptibility in date fruit. Overall, these results suggest that calcium and CNGC activators and/or inhibitors may play vital roles against disease resistance or susceptibility to fungal pathogens while regulating CNGCs in date palm crops. Further studies are required to better understand the role of pharmacological effectors in regulating calcium channels and CNGCs through the silencing of *CNGC* genes in date palm crops.

4. Conclusion

A total of 26 CNGC genes were identified in the date palm genome in the present study. Extensive bioinformatics analysis of the PdCNGCs revealed the conservation of the CNGC-specific motif compared to other plant CNGCs. GO analysis suggested that PdCNGCs may regulate a wide range of functions including protein binding, signaling transduction, and involvement in stress responses. The comprehensive phylogenetic analysis of 15 plant species showed that group IV was the most ancient among all groups in the course of the evolution for the CNGCs of land plants. The PdCNGCs interacting with various protein partners may be involved in cAMP/cGMP-binding and plant–pathogen interactions and may regulate abiotic and biotic stress responses. Subsequently, a pharmacological assay revealed that calcium and CNGC activators (cAMP and cGMP) may play roles in susceptibility against *Aspergillus* via CNGCs in date palm leaves and stems. Further studies are required to understand the mechanisms and regulation of CNGC activators in response to fungal pathogens in date palm crops. Functional analysis through gene silencing may also confirm the role of pharmacological effectors for calcium-mediating CNGC activation in date palm in response to biotic stresses in the future.

Funding

This project was funded by the Sindh Higher Education Commission (SHEC) under Research Support Program-Thematic Area Agriculture FY-2020-21 to principal investigator Dr. M.A. Saand (Project Number/ Code: SHEC/SRSP/Agr-2/10/2020-21). The authors are very thankful and acknowledge the SHEC of Pakistan for financial and technical support.

References

Abul-Soad AA, Mahdi SM, Markhand GS (2015). Date palm status and perspective in Pakistan. In: Al-Khayri JM, Jain SM, Johnson DV (editors). Date Palm Genetic Resources and Utilization. Dordrecht, the Netherlands: Springer, pp. 153-205. https://doi.org/10.1007/978-94-017-9707-8_5

Al-Dashti YA, Holt RR, Keen CL, Hackman RM (2021). Date palm fruit (*Phoenix dactylifera*): effects on vascular health and future research directions. International Journal of Molecular Sciences 22 (9): 4665. https://doi.org/10.3390/ijms22094665

Alwahshi KJ, Saeed EE, Sham A, Alblooshi AA, Alblooshi MM et al. (2019). Molecular identification and disease management of date palm sudden decline syndrome in the United Arab Emirates. International Journal of Molecular Sciences 20 (4): 923. https://doi.org/10.3390/ijms20040923

Baloch AA, Raza AM, Rana SSA, Ullah S, Khan S et al. (2021). *BrCNGC* gene family in field mustard: genome-wide identification, characterization, comparative synteny, evolution and expression profiling. Scientific Report 11: 24203. https://doi.org/10.1038/s41598-021-03712-y

Barker KR, Kus JV, Normand A-C, Gharabaghi F, McTaggart L et al. (2022). A practical workflow for the identification of *Aspergillus*, *Fusarium*, *Mucorales* by MALDI-TOF MS: database, medium, and incubation optimization. Journal of Clinical Microbiology 60 (12): e01032-22. https://doi.org/10.1128/jcm.01032-22

Charpentier M, Sun J, Martins TV, Radhakrishnan GV, Findlay K et al. (2016). Nuclear-localized cyclic nucleotide-gated channels mediate symbiotic calcium oscillations. Science 352 (6289): 1102-1105. https://doi.org/10.1126/science.aae0109

Chen JQ, Yin H, Gu J, Li L, Liu Z et al. (2015). Genomic characterization, phylogenetic comparison and differential expression of the cyclic nucleotide-gated channels gene family in pear (*Pyrus bretchneideri* Rehd.). Genomics 105 (1): 39-52. https://doi.org/10.1016/j.ygeno.2014.11.006

Cheng C-Y, Krishnakumar V, Chan AP, Thibaud-Nissen F, Schobel S et al. (2017). Araport11: a complete reannotation of the *Arabidopsis thaliana* reference genome. The Plant Journal 89 (4): 789-804. https://doi.org/10.1111/tpj.13415

Cohen Y, Shulhani R, Rot Y, Zemach H, Belausov E et al. (2021). *Aspergillus niger*, the causal agent of black mould disease in date fruits, infects and colonizes flowers and young fruitlets. Plant Pathology 70 (5): 1195-1208. https://doi.org/10.1111/ppa.13358

Cui Y, Wang J, Bai Y, Ban L, Ren J et al. (2023). Identification of CNGCs in *Glycine max* and screening of related resistance genes after *Fusarium solani* infection. Biology 12 (3): 439. https://doi.org/10.3390/biology12030439

Duszyn M, Świeżawska B, Szmidt-Jaworska A, Jaworski K (2019). Cyclic nucleotide gated channels (CNGCs) in plant signalling—current knowledge and perspectives. Journal of Plant Physiology 241:153035. https://doi.org/10.1016/j.jplph.2019.153035

Frietsch S, Wang Y-F, Sladek C, Poulsen LR, Romanowsky SM et al. (2007). A cyclic nucleotide-gated channel is essential for polarized tip growth of pollen. Proceedings of the National Academy of Sciences of the United States of America 104 (36): 14531-14536. https://doi.org/10.1073/pnas.0701781104

Gobert A, Park G, Amtmann A, Sanders D, Maathuis FJM (2006). *Arabidopsis thaliana* cyclic nucleotide gated channel 3 forms a non-selective ion transporter involved in germination and cation transport. Journal of Experimental Botany 57 (4): 791-800. https://doi.org/10.1093/jxb/erj064

Hao L, Qiao X (2018). Genome-wide identification and analysis of the *CNGC* gene family in maize. PeerJ 6: e5816. https://doi.org/10.7717/peerj.5816

Hazzouri KM, Gros-Balthazard M, Flowers JM, Copetti D, Lemansour A et al. (2019). Genome-wide association mapping of date palm fruit traits. Nature Communications 10: 4680. https://doi.org/10.1038/s41467-019-12604-9

Jiang Z, Du L, Shen L, He J, Xia X et al. (2023). Genome-wide exploration and expression analysis of the *CNGC* gene family in eggplant (*Solanum melongena* L.) under cold stress, with functional characterization of SmCNGC1a. International Journal of Molecular Sciences 24: 13049. https://doi.org/10.3390/ijms241713049

Kakar KU, Nawaz Z, Kakar K, Ali E, Almomeafy AA et al. (2017). Comprehensive genomic analysis of the *CNGC* gene family in *Brassica oleracea*: novel insights into synteny, structures, and transcript profiles. BMC Genomics 18: 869. https://doi.org/10.1186/s12864-017-4244-y

Kaplan B, Sherman T, Fromm H (2007). Cyclic nucleotide-gated channels in plants. FEBS Letters 581 (12): 2237-2246. https://doi.org/10.1016/j.febslet.2007.02.017

Kirungu JN, Magwanga RO, Shiraku ML, Okuto E, Cai X et al. (2023). Genome-wide identification and analysis of the CNGC gene family in upland cotton under multiple stress conditions. Journal of Cotton Research 6: 17. https://doi.org/10.1186/s42397-023-00152-z

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA et al. (2007). Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947-2948. https://doi.org/10.1093/bioinformatics/btm404

Liu W, Kang Y, Ren R, Liu Y, Li W et al. (2021). Identification and expression analysis of *BnaCNGC* family gene in the response to phytohormones, abiotic and biotic stresses in *Brassica napus*. Journal of Plant Interactions 16: 575-586. https://doi.org/10.1080/17429145.2021.2007303

Ma W, Berkowitz GA (2011). Ca^{2+} conduction by plant cyclic nucleotide gated channels and associated signaling components in pathogen defense signal transduction cascades. New Phytologist 190 (3): 566-572. https://doi.org/10.1111/j.1469-8137.2010.03577.x

Maitlo WA, Markhand GS, Abul-Soad AA, Lodhi AM, Jatoi MA (2014). Fungi associated with sudden decline disease of date palm (*Phoenix dactylifera* L.) and its incidence at Khairpur, Pakistan. Pakistan Journal of Phytopathology 26 (1): 67-73.

Mäser P, Thomine S, Schroeder JI, Ward JM, Hirschi K et al. (2001). Phylogenetic relationships within cation transporter families of *Arabidopsis*. Plant Physiology 126 (4): 1646-1667. https://doi.org/10.1104/pp.126.4.1646

Monaghan J, Matschi S, Shorinola O, Rovenich H, Matei A et al. (2014). The calcium-dependent protein kinase CPK28 buffers plant immunity and regulates BIK1 turnover. Cell Host and Microbe 16 (5): 605-615. https://doi.org/10.1016/j.chom.2014.10.007

Munemasa S, Oda K, Watanabe-Sugimoto M, Nakamura Y, Shimoishi Y et al. (2007). The *coronatine-insensitive 1* mutation reveals the hormonal signaling interaction between abscisic acid and methyl jasmonate in *Arabidopsis* guard cells. Specific impairment of ion channel activation and second messenger production. Plant Physiology 143 (3): 1398-1407. https://doi.org/10.1104/pp.106.091298

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. https://doi.org/10.1186/1471-2164-15-853

Nawaz Z, Kakar KU, Ullah R, Yu S, Zhang J et al. (2019). Genome-wide identification, evolution and expression analysis of cyclic nucleotide-gated channels in tobacco (*Nicotiana tabacum* L.). Genomics 111 (2): 142-158. https://doi.org/10.1016/j.ygeno.2018.01.010

Oranab S, Ahmad HM, Fiaz S, Hussain A, Rizwan M et al. (2024). Genome-wide characterization and expression analysis of cyclic nucleotide-gated ion channels (CNGCs) gene family in *Arabidopsis thaliana* L. and *Helianthus annuus* L. for drought stress. Brazilian Journal of Botany 47: 885-900. https://doi.org/10.1007/s40415-023-00957-x

Qi Z, Verma R, Gehring C, Yamaguchi Y, Zhao Y et al. (2010). Ca^{2+} signaling by plant *Arabidopsis thaliana* Pep peptides depends on *AtPepR1*, a receptor with guanylyl cyclase activity, and cGMP-activated Ca^{2+} channels. Proceedings of the National Academy of Sciences of the United States of America 107 (49): 21193-21198. https://doi.org/10.1073/pnas.1000191107

Reddy ASN, Ali GS, Celesnik H, Day IS (2011). Coping with stresses: roles of calcium– and calcium/calmodulin-regulated gene expression. The Plant Cell 23 (6): 2010-2032. https://doi.org/10.1105/tpc.111.084988

Saand MA, Li J, Wu Y, Zhou L, Cao H et al. (2022). Integrative omics analysis of three oil palm varieties reveals (Tanzania × Ekona) TE as a cold-resistant variety in response to low-temperature stress. International Journal of Molecular Sciences 23 (23): 14926. https://doi.org/10.3390/ijms232314926

Saand MA, Xu Y-P, Li W, Wang J-P, Cai X-Z (2015a). Cyclic nucleotide gated channel gene family in tomato: genome-wide identification and functional analyses in disease resistance. Frontiers in Plant Science 6: 303. https://doi.org/10.3389/fpls.2015.00303

Saand MA, Xu Y-P, Munyampundu J-P, Li W, Zhang X-R et al. (2015b). Phylogeny and evolution of plant cyclic nucleotide-gated ion channel (CNGC) gene family and functional analyses of tomato *CNGCs*. DNA Research 22 (6): 471-483. https://doi.org/10.1093/dnares/dsv029

Samson RA, Visagie CM, Houbraken J, Hong SB, Hubka V et al. (2014). Phylogeny, identification and nomenclature of the genus *Aspergillus*. Studies in Mycology 78 (1): 141-173. https://doi.org/10.1016/j.simyco.2014.07.004

Schuurink RC, Shartzer SF, Fath A, Jones RL (1998). Characterization of a calmodulin-binding transporter from the plasma membrane of barley aleurone. Proceedings of the National Academy of Sciences of the United States of America 95 (4): 1944-1949. https://doi.org/10.1073/pnas.95.4.1944

Szklarczyk D, Kirsch R, Koutrouli M, Nastou K, Mehryary F et al. (2023). The STRING database in 2023: protein–protein association networks and functional enrichment analyses for any sequenced genome of interest. Nucleic Acids Research 51 (D1): D638-D646. https://doi.org/10.1093/nar/gkac1000

Talke IN, Blaudez D, Maathuis FJM, Sanders D (2003). CNGCs: prime targets of plant cyclic nucleotide signalling? Trends in Plant Science 8 (6): 286-293. https://doi.org/10.1016/S1360-1385(03)00099-2

Tamura K, Peterson D, Peterson N, Stecher G, Ne M et al. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28 (10): 2731-2739. https://doi.org/10.1093/molbev/msr121

Tracy FE, Gilliham M, Dodd AN, Webb AAR, Tester M (2008). NaCl-induced changes in cytosolic free Ca^{2+} in *Arabidopsis thaliana* are heterogeneous and modified by external ionic composition. Plant Cell Environment 31 (8): 1063-1073. https://doi.org/10.1111/j.1365-3040.2008.01817.x

Tunc-Ozdemir M, Tang C, Ishka MR, Brown E, Groves NR et al. (2013). A cyclic nucleotide-gated channel (CNGC16) in pollen is critical for stress tolerance in pollen reproductive development. Plant Physiology 161 (2): 1010-1020. https://doi.org/10.1104/pp.112.206888

Wang L, Li M, Liu Z, Dai L, Zhang M et al. (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 (191): 1-16. https://doi.org/10.1186/s12864-020-6601-5

Wu Y, Zhang L, Zhou J, Zhang X, Feng Z et al. (2021). Calcium-dependent protein kinase GhCDPK28 was dentified and involved in verticillium wilt resistance in cotton. Frontiers in Plant Science 12: 772649. https://doi.org/10.3389/fpls.2021.772649

Yu C-S, Cheng C-W, Su W-C, Chang K-C, Huang S-W et al. (2014). CELLO2GO: a web server for protein subCELlular LOcalization prediction with functional Gene Ontology annotation. PLoS ONE 9: e99368. https://doi.org/10.1371/journal.pone.0099368

Yuen CYL, Christopher DA (2010). The role of cyclic nucleotide-gated channels in cation nutrition and abiotic stress. In: Demidchik V, Maathuis F (editors). Ions Channels and Plant Stress Responses. Heidelberg, Germany: Springer, pp. 137-157. https://doi.org/10.1007/978-3-642-10494-7_7

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: 1-14. https://doi.org/10.1093/aobpla/plt012

Zelman AK, Dawe A, Gehring C, Berkowitz GA (2012). Evolutionary and structural perspectives of plant cyclic nucleotide-gated cation channels. Frontiers in Plant Science 3: 95. https://doi.org/10.3389/fpls.2012.00095

Zhang N, Lin H, Zeng Q, Fu D, Gao X et al. (2023a). Genome-wide identification and expression analysis of the cyclic nucleotide-gated ion channel (CNGC) gene family in *Saccharum spontaneum*. BMC Genomics 24: 281. https://doi.org/10.1186/s12864-023-09307-3

Zhang Y, Li Y, Yang J, Yang X, Chen S et al. (2023b). Genome-wide analysis and expression of *cyclic nucleotide–gated ion channel (CNGC)* family genes under cold stress in mango (*Mangifera indica*). Plants 12 (3): 592. https://doi.org/10.3390/plants12030592

Zhao J, Peng S, Cui H, Li P, Li T et al. (2022). Dynamic expression, differential regulation and functional diversity of the CNGC family genes in cotton. International Journal of Molecular Sciences 23 (4): 2041. https://doi.org/10.3390/ijms23042041

Zia K, Rao MJ, Sadaqat M, Azeem F, Fatima K et al. (2022). Pangenome-wide analysis of cyclic nucleotide-gated channel (CNGC) gene family in citrus *Spp*. Revealed their intraspecies diversity and potential roles in abiotic stress tolerance. Frontiers in Genetics 13: 1034921. https://doi.org/10.3389/fgene.2022.1034921

Supplementary data

Table S1. Summary of date palm CNGC gene family.

Group	Gene name	Gene locus	Protein size (aa)	Introns	Domain analysis		
					CDD	SMART	Pfam
I	PdCNGC1	XP_008783842.2	699	6	CAP_ED PLN03192	TM 5 cNMP	ITP cNBD
	PdCNGC2	XP_008788856.1	699	6	CAP_ED PLN03192	TM 7 cNMP	ITP
	PdCNGC3	XP_026661114.1	628	5	CAP_ED PLN03192	TM 4 cNMP	ITP cNBD
II	PdCNGC4	XP_008787797.1	727	6	CAP_ED PLN03192	TM 3 cNMP	ITP cNBD
	PdCNGC5	XP_026665885.1	727	6	CAP_ED PLN03192	TM 5 cNMP	ITP cNBD
III	PdCNGC6	XP_008791605.2	711	6	CAP_ED PLN03192	TM 3 cNMP	ITP cNBD
	PdCNGC7	XP_026660584.2	715	5	CAP_ED PLN03192	TM 5 cNMP	ITP cNBD
	PdCNGC8	XP_017698138.3	678	6	CAP_ED PLN03192	TM 5 cNMP	ITP cNBD
	PdCNGC9	XP_008781660.2	712	5	CAP_ED PLN03192	TM 5 cNMP	ITP cNBD
	PdCNGC10	XP_038981463.1	721	6	CAP_ED Ion_trans	TM 6 cNMP	ITP cNBD
	PdCNGC11	XP_008794413.1	722	6	CAP_ED PLN03192	TM 6 cNMP	ITP cNBD
	PdCNGC12	XP_008782078.1	718	6	CAP_ED PLN03192	TM 6 cNMP	ITP cNBD
	PdCNGC13	XP_008782071.2	718	6	CAP_ED PLN03192	TM 6 cNMP	ITP cNBD
IVa	PdCNGC14	XP_038983045.1	757	11	CAP_ED PLN03192	TM 6 cNMP	ITP
	PdCNGC15	XP_038983046.1	735	11	CAP_ED PLN03192	TM 6 cNMP	ITP
	PdCNGC16	XP_038983042.1	759	11	CAP_ED PLN03192	TM 6 cNMP	ITP
	PdCNGC17	XP_038987401.1	772	11	CAP_ED PLN03192	TM 5 cNMP	ITP
	PdCNGC18	XP_038987403.1	772	11	CAP_ED Ion_trans	TM 5 cNMP	ITP
IVb	PdCNGC19	XP_008812641.2	654	6	CAP_ED PLN03192	TM 7 cNMP	ITP
	PdCNGC20	XP_038982156.1	498	5	CAP_ED PLN03192	TM 4 cNMP	ITP
	PdCNGC21	XP_008785947.2	716	7	CAP_ED PLN03192	TM 5 cNMP	ITP cNBD
	PdCNGC22	XP_026659376.2	644	6	CAP_ED PLN03192	TM 3 cNMP	cNBD
	PdCNGC23	XP_008777824.2	716	7	CAP_ED PLN03192	Tm7 cNMP	ITP cNBD

Table S1. (Continued.)

	PdCNGC24	XP_038983452.1	740	7	CAP_ED PLN03192	TM 5 cNMP	ITP
	PdCNGC25	XP_038989982.1	754	7	CAP_EDPLN03192	TM 5 cNMP	ITP cNBD
	PdCNGC26	XP_038989983.1	749	7	CAP_ED PLN03192	TM5 cNMP	ITP cNBD

PdCNGC: phoenix dactylifera cyclic nucleotide-gated ion channels; CDD: conserved domain database; CAP_ED: calcium phosphate/effector domain of CAP family; SMART: simple modular architecture research tool; TM: transmembrane domain; cNMP: cyclic nucleotide-monophosphate binding domain; Pfam: protein families database; ITP: ion transport; CNBD: cyclic nucleotide-binding domain.

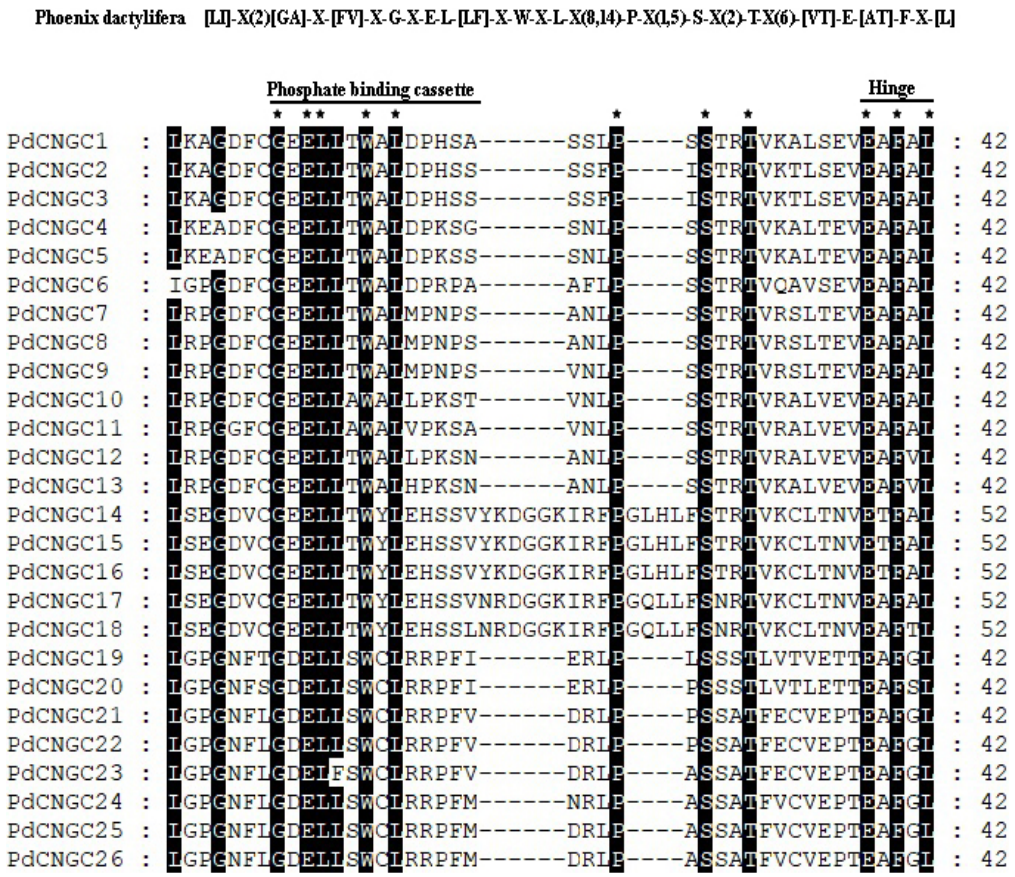

Figure S1. The CNGC-specific motif covers the phosphate binding cassette (PBC) and hinge region within the CNB domain of 26 PdCNGCs. The motifs were generated following the criteria for tomato CNGCs (Saand et al., 2015a). Square brackets "[]" denotes conserved amino acids in motif position, round brackets "()" indicate the number of amino acids, and "X" refers to any amino acid. To the left of the motif, the names are indicated for PdCNGCs and the total numbers of amino acids are mentioned on the right side of alignment. PdCNGCs were aligned at >90% and asterisks indicated 100% conservation developed by the ClustalW in the MEGA 5.0 program.

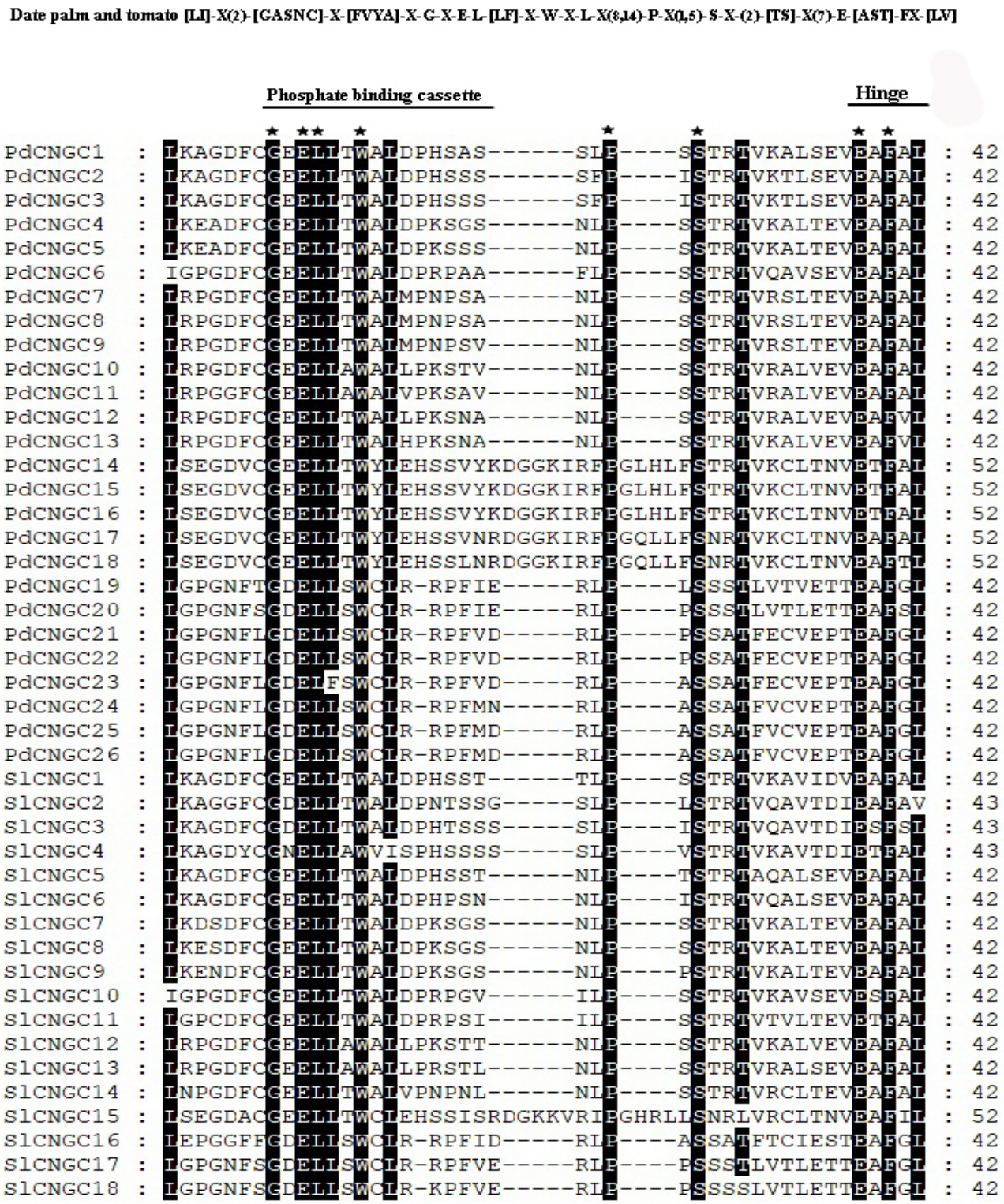

Figure S2. The CNGC-specific motif covers the phosphate binding cassette (PBC) and hinge region within the CNB domain of 26 PdCNGCs and 18 SlCNGCs. The motifs were generated following the criteria for tomato CNGCs (Saand et al., 2015a). Square brackets "[]" denotes conserved amino acids in motif position, round brackets "()" indicate the number of amino acids, and "X" refers to any amino acid. To the left of the motif, the names are indicated for PdCNGCs and the total numbers of amino acids are mentioned on the right side of alignment. PdCNGCs were aligned at >90% and asterisks indicated 100% conservation developed by the ClustalW in the MEGA 5.0 program.

Figure S3. The CNGC-specific motif that covers the phosphate binding cassette (PBC) and hinge region within the CNB domain of 26 PdCNGCs and 20 AtCNGCs. The motifs were generated following the criteria for Arabidopsis CNGCs (Zelman et al., 2012). Square brackets "[]" denotes conserved amino acids in motif position, round brackets "()" indicate the number of amino acids, and "X" refers to any amino acid. To the left of the motif, the names are indicated for PdCNGCs and the total numbers of amino acids are mentioned at the right side of alignment. PdCNGCs were aligned at >90% and asterisks indicated 100% conservation developed by the ClustalW in the MEGA 5.0 program.

Date palm and rice [LI]-X-(2)-[GA]-X-[FV]-X-G-X-EL-[LF]-X-W-X-[LM]-X-(8,14)-[PH]-X(1,5)-[SA]-X(2)-T-X(7)-[EQ]-X-F-X-L

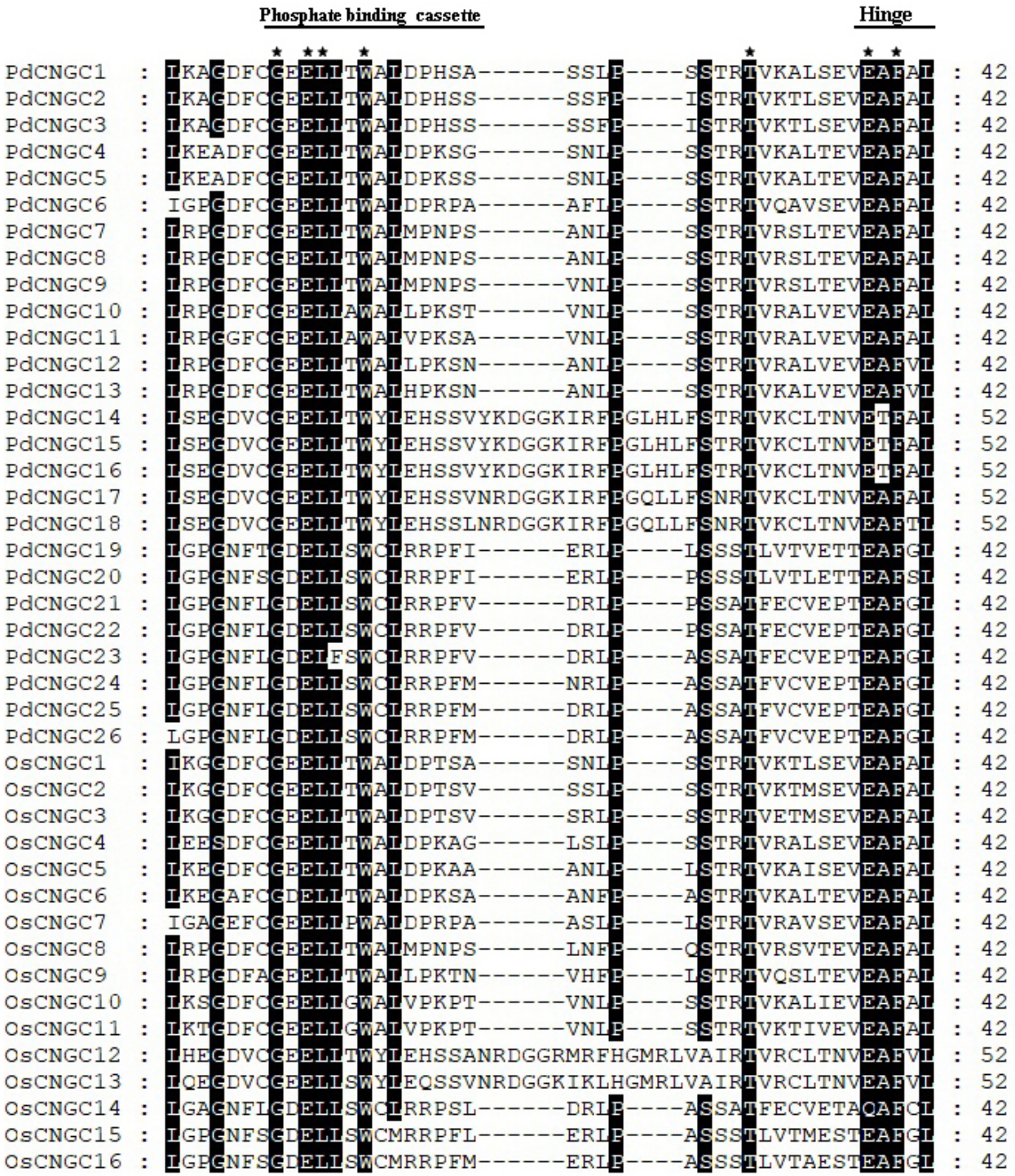

Figure S4. The CNGC-specific motif covers the phosphate binding cassette (PBC) and hinge region within the CNB domain of 26 PdCNGCs and 16 OsCNGCs. The motifs were generated following the criteria for rice CNGCs (Nawaz et al., 2014). Square brackets "[]" denotes conserved amino acids in motif position, round brackets "()" indicate the number of amino acids, and "X" refers to any amino acid. To the left of the motif, the names are indicated for PdCNGCs and the total numbers of amino acids are mentioned at the right side of the alignment. PdCNGCs were aligned at >90% and asterisks are indicated 100% conservation developed by the ClustalW in MEGA 5.0 program.

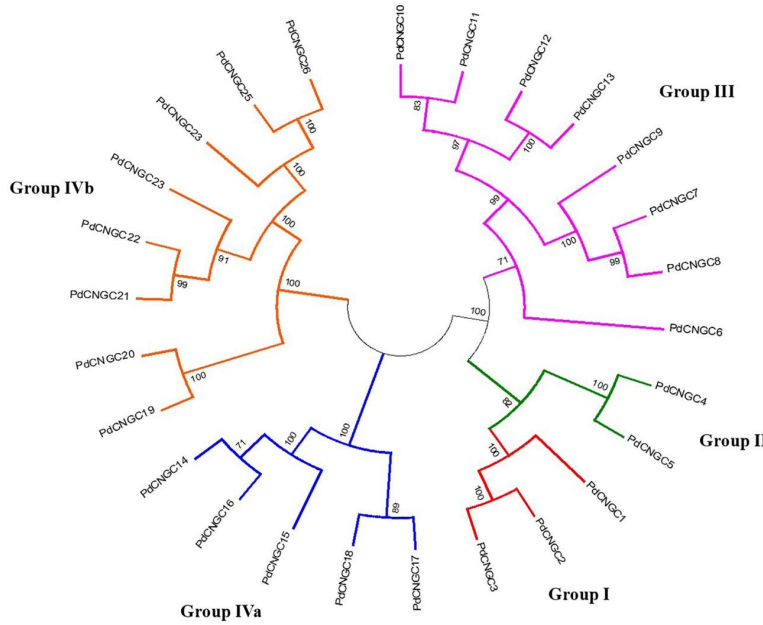

Figure S5. Phylogenetic tree of date palm CNGC gene family. The tree was generated using MEGA 5.0 software using criteria including the maximum likelihood method at 1000 bootstraps as suggested by Saand et al. (2015a). The five groups are represented in different colors i.e. red, green, pink, blue, and orange for group I (having three proteins), II (having two proteins), III (having eight proteins), IVa (having five proteins) and IVb (having eight proteins), respectively.

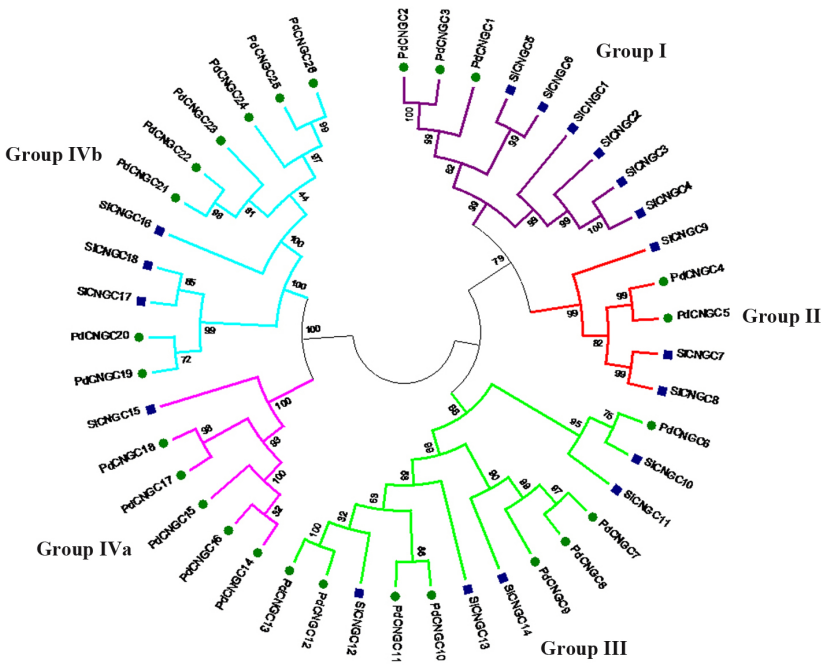

Figure S6. Phylogenetic tree of date palm and tomato CNGC gene family. The tree was generated using MEGA 5.0 software using criteria including the maximum likelihood method at 1000 bootstraps as suggested by Saand et al. (2015a). The five groups are represented in different colors i.e. red, green, pink, blue, and orange for groups I (having three proteins), II (having two proteins), III (having eight proteins), IVa (having five proteins) and IVb (having eight proteins), respectively.

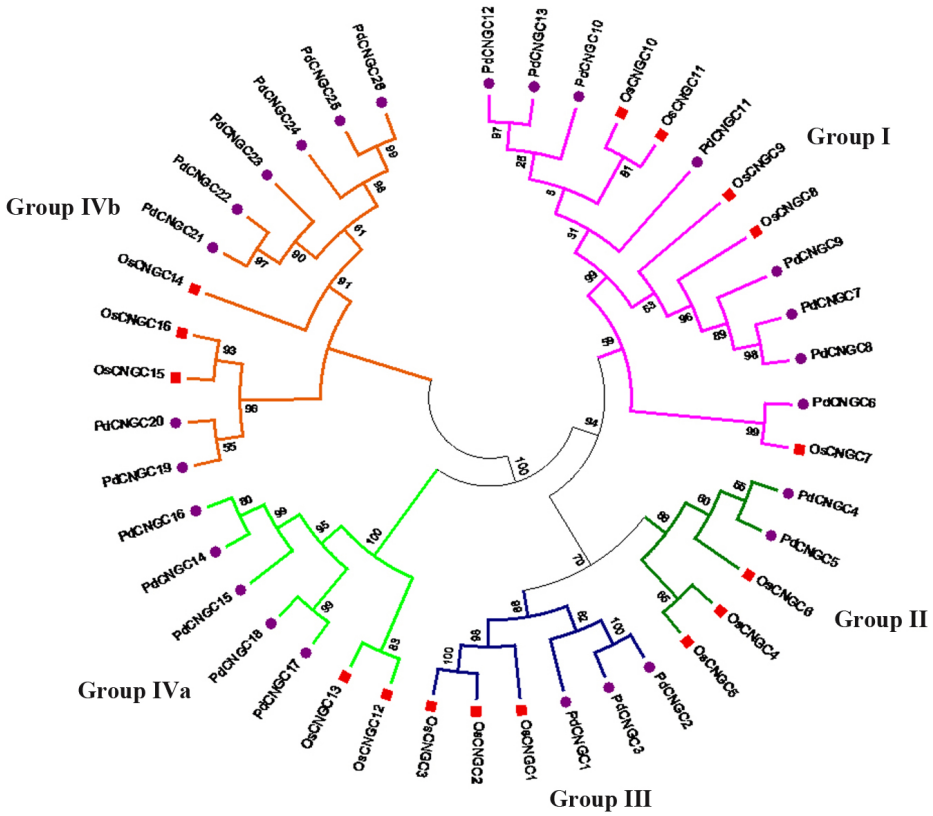

Figure S7. Phylogenetic tree of date palm and rice CNGC gene family. The tree was generated using MEGA 5.0 software using criteria including the maximum likelihood method at 1000 bootstraps as suggested by Nawaz et al. (2014). The five groups are represented in different colors i.e. red, green, pink, blue, and orange for groups I (having three proteins), II (having two proteins), III (having eight proteins), IVa (having five proteins) and IVb (having eight proteins), respectively.

Table S2. Information of protein interaction network among date palm CNGCs.

S. No	Accession number	Protein description	Interaction network
1	LOC103705691	Alpha carbonic anhydrase 1	PdCNGC19/20
2	LOC103706691	LOW QUALITY PROTEIN: omega-3 fatty acid desaturase	PdCNGC19/20
3	LOC103702747	Homeobox protein knotted-1-like 3	PdCNGC19/20
4	LOC113462764	Uncharacterized protein LOC103708257	PdCNGC19/20
5	LOC103719659	Homeobox protein knotted-1-like 3	PdCNGC19/20
6	LOC103703095	Alpha carbonic anhydrase 1	PdCNGC19/20
7	LOC103709505	Omega-3 fatty acid desaturase	PdCNGC19/20
8	LOC103708081	DNA repair protein rhp26 isoform x1 DNA excision repair protein CSB	PdCNGC6/10/11/12/13
9	LOC103713333	Probable plastid-lipid-associated protein 14	PdCNGC6/10/11/12/13
10	XP_008801643.1	ABC transporter c family member 3	PdCNGC1 and 3
11	XP_008792064.1	ABC transporter c family member 3-like	PdCNGC1 and 3
12	LOC103708768	ABC transporter C family member 3-like isoform X1	PdCNGC1 and 3
13	LOC103700287	Cyclic nucleotide-gated ion channel 2-like, partial	PdCNC1/3/4/5/8/9/16/18/19/20
14	LOC103701286	Calcium-dependent protein kinase 28-like	PdCNGC8/9

Table S2. (Continued.)

15	LOC103718467	GPI-anchored protein LLG1-like	PdCNGC8/9
16	LOC103700879	Actin-depolymerizing factor 5	PdCNGC8/9
17	LOC103706167	Actin-depolymerizing factor 5-like	PdCNGC8/9
18	LOC103707186	Low quality protein: phytosulfokine receptor 2-like	PdCNGC8/9
19	LOC103702346	GPI-anchored protein LLG1	PdCNGC8/9
20	LOC103711975	GPI-anchored protein LLG1-like	PdCNGC8/9

Figure S8. Date palm tree infected with fungal pathogens. The samples were collected from Shah Abdul Latif University (SALU), Khairpur Sindh, Pakistan. The samples and photos were taken during the postmonsoon rainy season in the area.

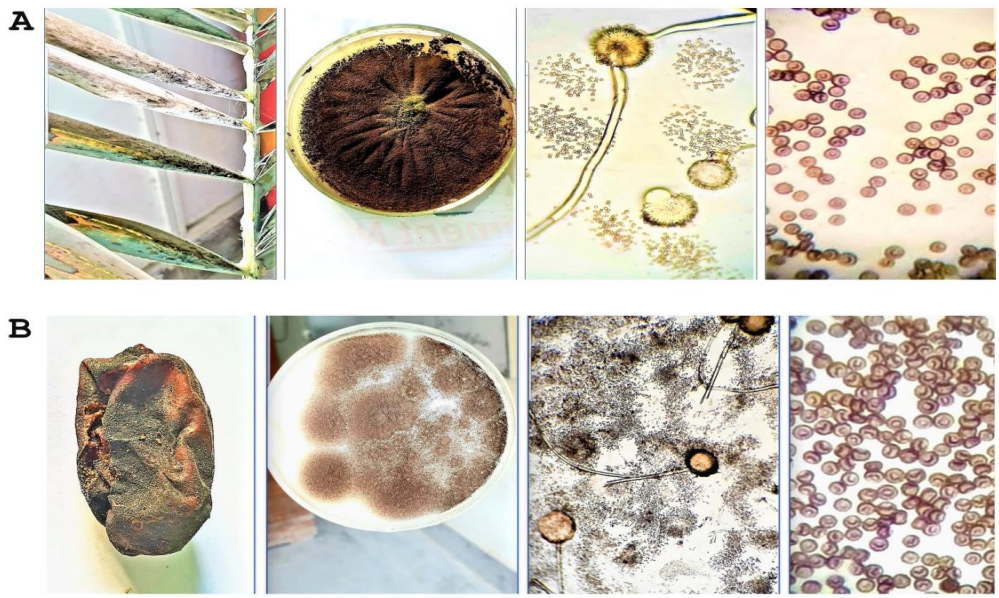

Figure S9. Identification of *Aspergillus* spp. from leaf and fruit of date palm. The samples were collected from trees grown at SALU, Khairpur, Sindh, Pakistan. A shows the samples with fungal disease symptoms on a leaf of date palm. B depicts the samples with fungal disease symptoms on the fruit of date palm. The samples were collected during the postmonsoon rainy season. The identification of fungal pathogens was carried out using methods described by Samson et al. (2014).

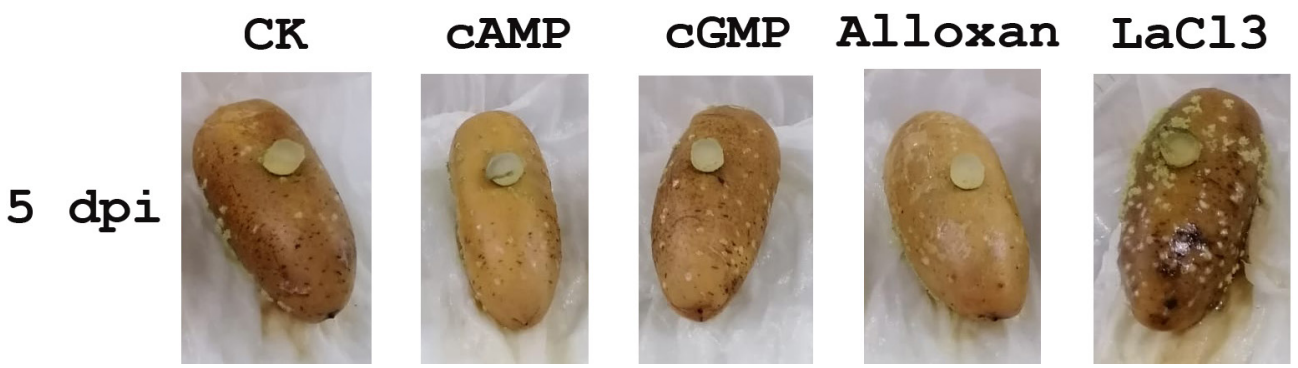

Figure S10. Pharmacological assay in date palm (cv. Gajjar) **fruit in response to *Fusarium* spp.** The time point is indicated on the left side with 5 dpi (days postinfiltration/inoculations) of treatments and pathogen. Samples after each treatment i.e. CK (water), cAMP (1 mM), cGMP (100 µM), alloxan (1 mM) and LaCl3 (1 mM) were inoculated with fungus.