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Identification and characterization of at CNGC19 for its role in salt stress regulation in *Arabidopsis thaliana*

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Abstract

Cyclic nucleotide gated ion channels (CNGCs) in plants, animals and prokaryotes have very important role in signaling and development. Structural analysis of cyclic nucleotide gated ion channels showed that CNGC 4, 5, 6 and 9 had long untranslated introns, while CNGC 7, 8, 13 and 16 had fully translated exons and introns. The insertion was confirmed in STC13 on chromosome 3 through TAIL PCR and the expression of AtCNGC19 was determined by RT PCR, which was activated twice in control and about 5 times under 150mM NaCl. The expression of gene was significantly reduced in knock out lines. The over expression lines were generated for expressing AtCNGC19 under 35: S constitutive promoter. The calli showed salt tolerance at 150mM NaCl compared with control. AtCNGC19 showed significant enhancement under salt stress in microarray. CNGCs expression under abiotic stresses showed that CNGC 19 of group IVa was highly expressed in roots under salt stress after 6h. These results provide new evidences for role of AtCNGC19 undersalt stress regulation in *Arabidopsis thaliana*. The study for them explores new insights of AtCNGCs helpful for functional genomics of CNGCs in plants and for their roles during biotic and abiotic stress.

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Introduction

The production of crop plants with increased resistance to abiotic and biotic stresses is necessary for sustained and equitable global food security (Munns and Tester, 2008). Salinity is a global problem and a multidisciplinary approach is required to solve this. There are different ways for remediation and appropriate utilization of saline soils including agronomic practices, use of salt tolerant crop varieties and phytoremediation (Hasanuzzaman *et al*., 2014).

As salinity became a major issue for agricultural productivity, that's why some mechanisms became knowledgeable for agricultural researchers to address this in saline conditions (Munns and Tester, 2008). High level of sodium chloride in plants render two different stress components, an osmotic and an ionic (Munns and Tester, 2008). The productivity of plants and their distribution is determined by the two environmental abiotic factors salinity and drought. Cellular redox homeostasis can be disturbed by the enhanced production of ROS by increasing oxidative processes like membrane lipid peroxidation, protein oxidation, enzyme inhibition and DNA and RNA damages (Bartels and Sunkar, 2005).

The CNGCs in plants are involved in salinity tolerance and can control Na^+ uptake (Bridges *et al*., 2005). CNGCs have signaling pathways leading to HR (hypersensitivity response) resistance. These are considered as significant calcium transporters in plants (Talke *et al*., 2003). CNGCs are very well conserved in plant species like *Arabidopsis* containing 20 members of CNGCs and tomato containing 18 members of CNGCs (Saand *et al*., 2015).

Cyclic nucleotides linked with calcium signals through cyclic nucleotide gated ion channels. The calcium dependent kinases (CDPKs) are coded by large gene family having important functions in environmental stress tolerances like salinity, drought and cold (Talke *et al*., 2003). AtCNGC19 and AtCNGC20 are located on chromosome 3 and placed in subgroup IVA of AtCNGCs. Both of these genes are 73% identical to each other and responded to signal of higher expression under salinity.

Different cells within shoots comprised different concentrations of ions for example barley and tomato having different concentration of K^+ and Cl^- (James *et al*., 2006). In *Medicago citrine* and *Ricinus communis* Na^+ was preserved in older leaves but prevented transport to newly developed mature organs. It was also relocated from there to petioles and leaf margins to provide barrier against excessive supply of Na^+. CNGCs provide linkage between cyclic nucleotide and calcium signals.

The calcium dependent kinases (CDPKs) are coded by large gene family having function in tolerance to the environmental stress such as cold, salinity and drought (Talke *et al.,* 2003). The direct and reversible binding of cAMP and cGMP to cyclic nucleotide binding domain (CNBD) is responsible for the activation of plant CNGCs.

The cyclic nucleotides cannot bind to CNBD in the presence of calcium and CNGCs remain inactive (Kohler and Neuhaus, 2000). These ligand gated calcium permeable channels are often localized in plasma membrane and effectively activated by direct binding of cyclic nucleotides and are complexly regulated by binding of calmodulin (CaM) to the CaM binding domain (Ma *et al*., 2009; Wang *et al.,* 2013; Gao *et al*., 2014; Zhang *et al*., 2018).

Physiological processes in which these signaling molecules are involved comprise various developmental processes, photomorphogenesis and tolerance to salt stress, gibberellic acid-induced signaling in barley and phytochrome signaling (Nawaz *et al*., 2014). The objectives of this study is to explore the potential role of CNGCs under different abiotic stresses with focus on AtCNGC19 under salinity and drought stress, phylogenetic relationship of AtCNGC19 with other members of their family showed close relationship of AtCNGC19 with AtCNGC20. In the mutant, a gene named salt tolerant callus 13 (stc 13; AtCNGC19) was activated with salt stress. Here we show that AtCNGC19 is involved in cold, salt and drought stress. The knocks out plants were sensitive to different level of NaCl, and over expression lines were tolerant to NaCl.

Materials and methods

Evolutionary Relationship of Arabidopsis CNGCs

The amino acid sequences of 20 CNGCs were extracted from (The *Arabidopsis* Information Resource) TAIR (www.arabidopsis.org) and aligned by Clustal W (Thompson *et al.*, 1994) program of Molecular Genetic Analysis (MEGA version 6.0) software suite. Phylogenetic tree based on the protein sequences of the CNGCs was constructed using the MEGA 6 software (www.megasoftware.net) with Neighbour- joining criteria (Saitou and Nei, 1987) 1000 bootstrap replicates. The structure of introns and exons of CNGCs were analyzed by using database Dicots plaza 3.0 (https://bioinformatics. psb.ugent.be/plaza/versions/plaza_v3_dicots/gene).

Genome wide expression of CNGCs under abiotic stresses

According to the different public data all CNGCs expressed differentially under different stresses. The expression of five groups of CNGCs were examined under nine abiotic stresses including cold, osmotic, salt, drought, genotoxic, oxidative, UV-B, wounding, and heat stress. The expression data of group IVA CNGCs (CNGC19 and CNGC20) under these stresses in shoots and roots of *Arabidopsis thaliana* for 6 and 12 h were extracted from database *Arabidopsis* eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb. cgi/primaryGene), and then displayed it in the form of graphs.

Expression Analysis of AtCNGC19 under Salt Stress

Transformation and Selection of Mutants

Activation tagged mutant lines were generated from wild type *Arabidopsis thaliana* (ecotype Col-0) plants. Surface sterilized seeds were kept in dark at 4°C for three days and sown on solidified Murashige Skoog (MS) medium containing 2.2% Gelzan, and incubated in growth chamber (20°C with fluorescent light). Fifteen to twenty seedlings were transferred into flask containing liquid MS medium and subsequently grown with shaking at 80 rpm for two weeks. Green tissues (stem and leaves) from cultured roots, were cut into small pieces and transferred into callus induction medium (CIM) for incubation in growth chamber up to 5 days.

The roots of these plants were infected with *Agrobacterium tumefaciens* GV3101 harboring binary vector pRi35ADEn4 for activation tagging. The roots were washed with liquid CIM (supplemented with 0.1mg/mL of cefotaxime) and incubated for three weeks on CIM (with 0.2mg/mL vancomycin and 0.1mg/mL cefotaxime) for inhibition of *A. tumefaciens*. Then transformants were selected on MS medium supplemented with 0.1µg/mL chlorosulfuron. Mutants were selected on MS media containing 150mm NaCl.

Confirmation of T-DNA Inserts by PCR

Genomic DNA was isolated from transformed calli for PCR analysis through specific primer for AtCNGC19 to amplify a fragment of 200 bp for confirmation of transformants. The PCR product was subjected to gel electrophoresis using 3% (w/v) agarose and DNA bands were analysed through UV analysis.

Determination of Insertion Location on Chromosomes by TAIL-PCR

The genomic DNA was isolated from mutants and subjected to TAIL-PCR using AD and T-DNA end primers. Purified fragments following tertiary PCR were sequenced directly and the flanking sequences were subjected to BLAST search using.

The *Arabidopsis* Information Resource (TAIR, http//:www.arabidopsis.org). Specific primers were designed and used in combination with T-DNA-specific primers to amplify specific fragments which were sequenced to confirm the insertion sites.

Real-time PCR Analysis

Total cellular RNA was extracted treated with RNase-free DNase. RNA was then subjected to cDNA synthesis using the first strand cDNA synthesis kit. Real-time PCR was conducted in a reaction mixture containing 2µL of diluted cDNA, 10µL of SYBR green PCR master mix and 10 pmol each of the forward and reverse primers in a final volume of 20µL. The PCR conditions comprised 45 cycles at 95°C for 5 s and 60°C for 20s. The amplification was followed by a thermal denaturation step to generate dissociation curves which verified the amplification specificity.

Results and discussion

Evolutionary Relationship of Arabidopsis thaliana CNGCs

The identified 20 *Arabidopsis* CNGC paralogues were classified into five groups, I, II, III, IVa and IVb (Fig. 1). All 20 CNGCs were divided into two main clads A and B. The clad A was divided into A1 and A2, the clad A1 was larger as compared to A2 and further divided into A1a and A1b. The clad A1a comprised CNGCs of group 1, CNGC (1, 3, 10, 11, 12, 13) and was divided into a sub clad A1aI and a separate branch carried only CNGC1. This showed that CNGC1 was dissimilar than the other members of group I, The sub clad A1aI was divided into two sub-sub clads A1aIi and A1aIii. CNGC11 and CNGC12 were located on the same clad while CNGC3 was positioned on a separate branch of the clad A1aIi, while CNGC10 and CNGC13 were located on the same clad A1aIii. A1b carried all CNGCs of group II (5, 6, 7, 8 and 9), and was divided into A1bI and A1bII. CNGC7 and CNGC8 were located on the same clad of A1bI, while A1bII was divided into a clad carried CNGC6 and CNGC9, and a separate branch carrying CNGC5. The sub clad A2 carried all CNGCs of group III, and it was divided into a sub clad A2a and a separate branch carried CNGC15. The sub clad A2a was further divided into two sub sub clads A2aI and A2aII. CNGC14 and CNGC17 were located on the clad A2aI while CNGC16 and CNGC18 were located on clad A2aII. The clad B carried all CNGCs of group IV, The clad B divided into two sub clads B1 and B2, CNGC2 and CNGC4 were located on clad B1, while CNGC19 and CNGC20 were located on clad B2 (Fig.1), showed that group IV is quite distinct from rest of the CNGCs groups. All 20 CNGCs of *A. thaliana* are unevenly distributed on 5 chromosomes, according to which maximum number of CNGCs i.e., 6 CNGCs were located on chromosome number 2, while chromosome number 5 contained 5 CNGCs and chromosome number 1, 3 and 4 contained 3 CNGCs on each (Fig. 1).

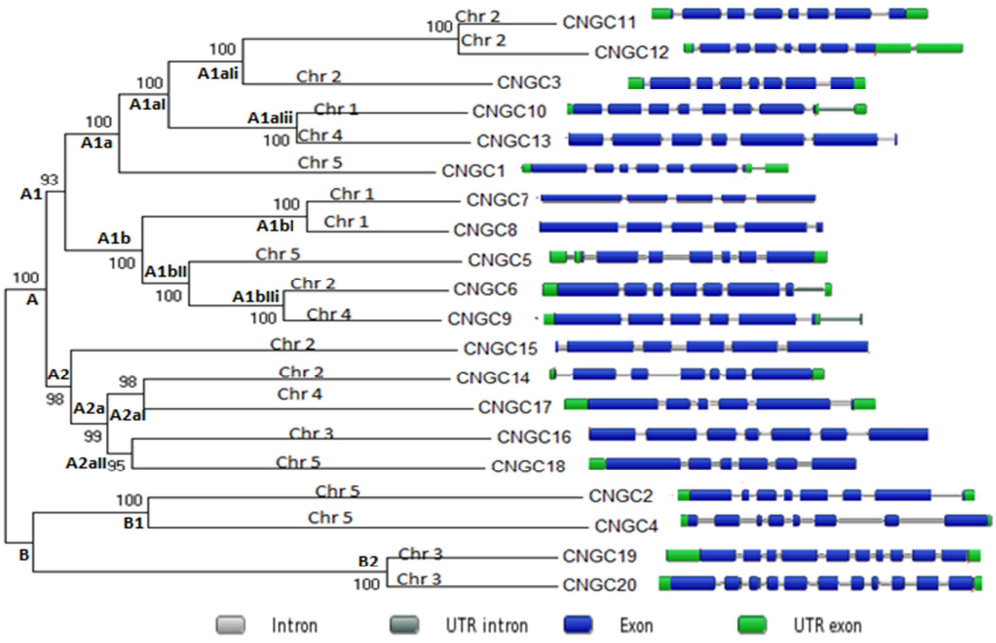

Fig. 1. Phylogenetic tree of 20 CNGCs of *Arabidopsis*.

CNGCs have variable number of exons, introns, UTR introns (untranslated region) and UTR exons (Fig. 1). It was shown that chromosome number 1 carries three CNGCs including (7, 8 and 10), CNGC 7 and 8 contain fully translated exons and introns while CNGC10 has two small untranslated exons and one large untranslated intron. Chromosome number 2 carries CNGCs (3, 6, 11, 12, 14 and 15), CNGC 15 contains all translated exons and introns, while CNGC 3, 11 and 14 have untranslated exons on the edges, CNGC6 has two untranslated exons and one untranslated intron, CNGC12 has two untranslated

exons on one side and one on other side. CNGC 17, 19 and 20 are present on chromosome number 3 containing untranslated exons on both edges. CNGC9, 13 and 17 are present on chromosome number 4. CNGC13 has not any UTR exon and intron, CNGC 9 has two UTR exons and one long UTR intron, while CNGC17 has UTR exons on both sides. CNGC2, 4, 5 and 18 are present on chromosome number 5. CNGC4 has two small UTR exons on both edges and two large UTR introns and 5 short UTR introns. CNGC 5 has three UTR exons and seven UTR introns, and CNGC 18 has one UTR exon and five UTR introns, while CNGC2 has two UTR exons on both sides and has no UTR intron.

The neighbor joining method was used to deduce the evolutionary history of all 20 CNGCs in *Arabidopsis* (Saitou and Nei 1987). The optimal tree with the sum of branch length is 3.35170352.

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein 1985).

Whole Genome Expression of CNGCs under Abiotic Stresses

The expression of group IVA of CNGCs of *A. thaliana* under nine abiotic stresses including cold, osmotic, salt, drought, genotoxic, oxidative, UV-B, wounding, and heat stress in root and shoot of *A. thaliana* was examined after 6 and 12h time intervals. The expression of CNGC19 was very low in shoots under all stresses after 6h. The expression of CNGC20 was slightly higher under osmotic and UV-B stress while it was lower under rest of stresses in shoots after 6h. The expression of CNGC19 and 20 was very higher under salt stress in roots after 6h. Indeed, the expression of CNGC19 was maximum under salt stress as compared to other stresses. CNGC 19 again showed slightly higher expression under salt stress in roots after 12 h, while it showed very inconsiderable expression under all the rest of stresses in shoots and roots after 12 h. The expression of CNGC 20 was slightly higher under osmotic stress in shoots and under salt stress in roots after 12h, and showed very little expression under the rest of stresses in shoots as well as roots after 12h (Fig. 2).

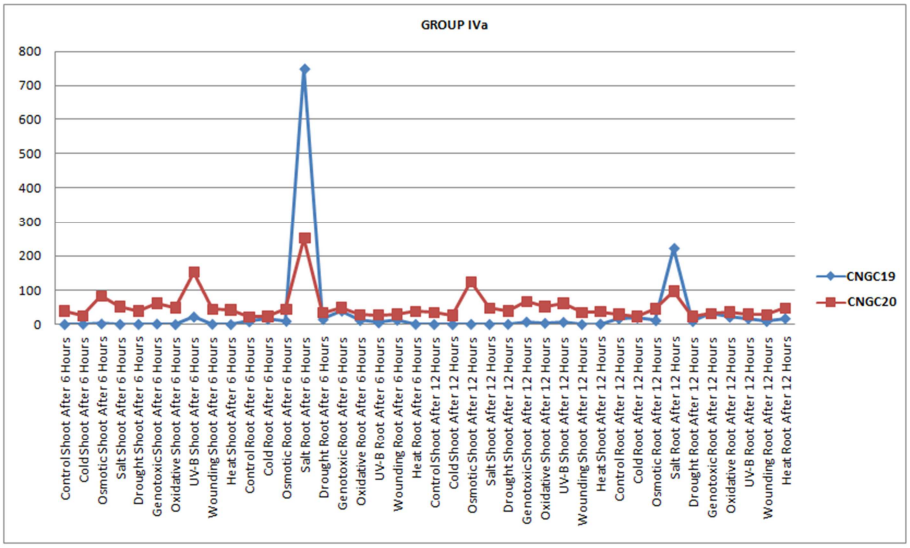

Fig. 2. Group IVa expression under abiotic stresses.

Selection of Mutants

The key gene involved in salt tolerance was found out at cellular levels of *Arabidopsis thaliana* by generating mutant calli on 150 mM NaCl stress level. Activation-tagged calli were screened and 40 mutants were selected from 62,000 transformants. These lines showed different levels of salt tolerance. Following a second screening at 150mm NaCl, 18 mutants were selected for analysis. It was observed that the survival and proliferation rates of mutant calli were significantly high at different NaCl stress levels compared with untransformed calli.

T-DNA Insertion in stc13 and Expression analysis

The increasing expression of gene, T-DNA was incorporated in genome of *Arabidopsis thaliana*. TAIL PCR was performed to determine location of T-DNA (Genome wide screening). As mutation was located on chromosome 3 CNGC19 was found 20 bp away from T-DNA. Real time PCR was done to identify activated genes.

The (Fig. 3) showed comparison of mutant lines with its wild type under normal condition and in 150mm NaCl, and difference was about twice but under salinity difference was increased about 3-4 fold. This concluded that CNGC19 was activated under salinity. Screening of overexpression of callus was shown in (Fig. 4). The vector was transformed into Callus and grown on CIM with 150 Mm NaCl. A and C part were showing strong evidence for vector transformation because callus was grown in them while, B and D were indication of dead and weak callus because vector was not transformed.

The knockout lines of CNGC19 showed no expression under salt stress of 150 mM NaCl (Fig. 5)

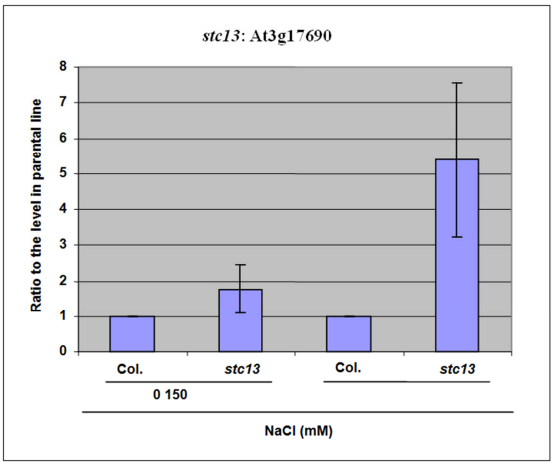

Fig. 3. In Fig. 3, 0 and 150 should be distant apart. 150 should be under the next line carrying Col. and stc13.

Expression of At3g17700 Cyclic nucleotide gated ion channel (CNGC) in parental line and mutant line salt tolerant calli 13 (stc13). Total RNA was extracted from the calli grown on normal CIM and CIM supplemented with 150 mM NaCl and expression was determined through real-time PCR.

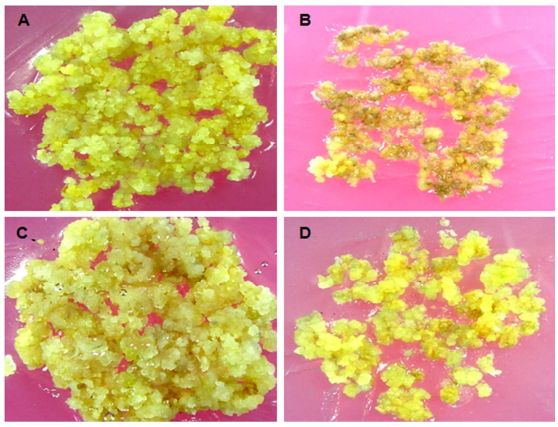

Fig. 4. (B and D) empty vector transformed calli, (A and C) 35S: CNGC transformed calli on CIM with 150 mM NaCl. The pictures were taken after three weeks of stress.

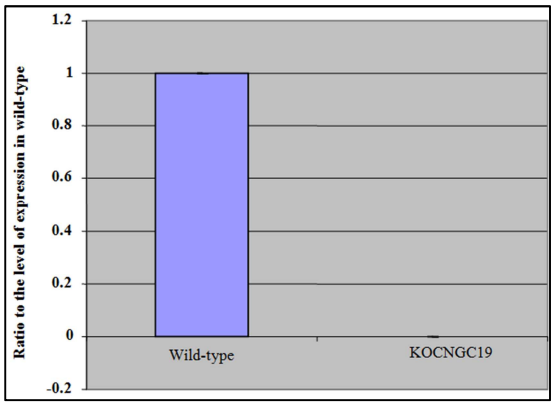

Fig. 5. At CNGC19 expression in wild type and knockout line under 150 mM NaCl stress

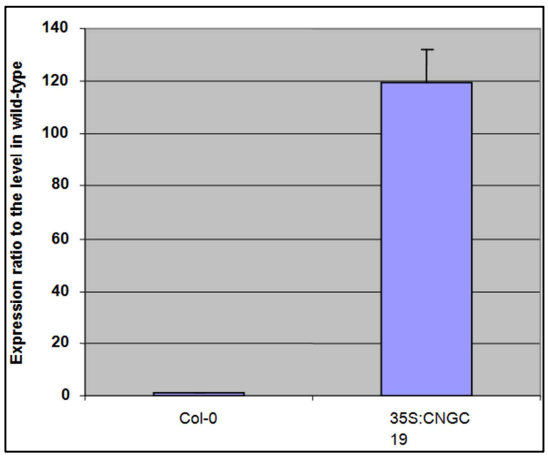

Fig. 6. AtCNGC19 expression in wild-type and over expressed line under 150 mM NaCl stress.

Total RNA was extracted from the calli grown on normal CIM for 3 weeks and expression was determined through real-time PCR using light cycler.

Discussion

The small genome of *A. thaliana* makes it ideal organisms for research in genetics and molecular biology in plants. It has of total 114.5 Mb/125Mb with 20 CNGC (Maser *et al.*, 2001), further divided into four groups (I-IV). The group II and IV are further distributed into two subgroups (IIA and IIB, IVA and IVB). Group I contains CNGCs from monocotyledonous and dicotyledonous angiosperm plants. Group II contains CNGCs from land plants and ancestors are same after separation from aquatic plants. Group IIB contains CNGCs from lower land plants such as mosses (bryophytes) and lycophytes. Group IIA and III contain CNGCs from embryophytes (Angiosperm and gymnosperm). Group IV contain CNGCs from vascular plants such as embryophytes (Li *et al.,* 2009).

In defense signaling, fluxes of Ca^{2+} and K^{+} are among the earliest detectable events, however, the mechanisms of defense responses and by which the ion channel mediates HR (hypersensitive response) is not well understood. The activation of many defense responses and the onset of HR have been correlated with Ca^{2+} influx and subsequent rise in cytoplasmic Ca^{2+} (Blume *et al.,* 2000). The secondary messengers such as Ca^{2+} is generated after the identification of stress stimulus lead to the onset of signal transduction pathways (De Silva *et al.,* 2011). AtCNGC2 can form an ion channel that mediates Ca^{2+} and K^{+} influxes but it does not allow substantial Na^{+} influx (Leng *et al.,* 2002). Many stresses result in accumulation of Ca^{2+} in cytosol, that plays an important role as a secondary messenger in accelerating of physiological processes and gene expression that finally lead to stress adaptation (Luan, 2008). Plants are more susceptible to damage by salinity at low level of Ca^{2+} (Hong-Bo *et al.,* 2005). High salinity conditions raised Ca^{2+} dependent signaling pathway, showed that Ca^{2+} conducting proteins are closely related to plant salt tolerance. CaM mediated to play their role in co-ordination with Ca^{2+} signals decoding elements in plant responses to salt stress (Shao *et al.,* 2008). Ca^{2+} is specific for this defect of growth because wild type and mutants are indistinguishable in their response to various changes of other ions. It is suggested that the defects in CNGC2 were exaggerated in elevated amount of external Ca^{2+} affect both vegetative and reproductive development (Chan *et al.,* 2003). Drought is also an important stress factor that restricts productivity, growth and overall development of plant. It was shown by different studies that Ca^{2+} has potential for drought tolerance, protection of plasma membrane, optimal photosynthesis and metabolism modulation of phytohormone and other important chemicals (Hasanuzzaman *et al.*, 2018), The over expression and knockout lines of this gene were studied under salt stress. The plants showed different expression pattern under different concentration of salts. This gene was also compared with its wild type and showed different expression pattern.

TAIL PCR was performed to determine location of T-DNA (Genome wide screening). As mutation was located on chromosome 3 CNGC19 was found 20 bp away from T-DNA. Real time PCR was done to identify activated genes. This comparison of mutant lines with its wild type under normal condition and difference was about twice but under salinity difference was increased about 3-4 fold This Concluded that CNGC19 was activated under salinity. The expression of knockout lines was compared with its wild type under salinity.

Conclusion

It is concluded from the above results that the Group IVA CNGCs of *A. thaliana* expressed differentially under different abiotic stresses, the expression of CNGC19 was higher under salt stress than CNGC20, while both of CNGCs are located on chromosome 3 with close vicinity. They might be complementing the effect of each other. The lab experiments also showed that CNGC19 has great influence in salt tolerance as compared to CNGC20. It was observed in the expression of real time PCR analysis, that the over expressed CNGC19 showed greater expression as compared to the wild type plants. CRISPR /Cas9 can be further used for the study of knock out CNGCs in plants. It could be proved very helpful for the efficiencies of CNGCs under different stress conditions. The affect of these interactions on the functionality of these channels can contribute to the

plant CNGC research significantly. The outcomes of these studies could further be used for the generation stress resistant crops important for food conservation and security.

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