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INSILICO ANALYSIS OF CorA SUPERFAMILY MAGNESIUM TRANSPORTERS OF FUNGI AND IDENTIFICATION OF MAGNESIUM TRANSPORTER STMg WITH GQN MOTIF FROM *NEUROSPORA CRASSA*

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ABSTRACT The Neurospora crassa genome encodes four putative CorA transporters Tmg-1, Tmg-2, Tmg-3, and Tmg-4. The GQN motif is found on the Tmg-4 transporter encoded by the NCU07816.5 (tmg-4) gene in contrast to the conserved GMN motif which is essential for the structure and function of CorA magnesium transporters. We were intrigued to see the wide distribution of variant motif GQN in place of GMN across the fungi. Using various bioinformatics tools, we catalogued around 300 protein sequences with tentative CorA superfamily features from 100 fungal species belonging to all phyla. Surprisingly, the proteins with the GQN motif and CorA domain are mostly present in the Sordariomycetes class of fungi and are named STMg. In STMg proteins the GQN motif is flanked by phenylalanine residues, and the glycine of the motif is present in the first transmembrane helices, and the remaining two residues glutamine and aspargine are present in the loop. Phylogenetic analysis of STMg proteins and other CorA proteins resulted in four clades namely, ALR, MNR2, MRS2, and STMg. The predicted model Tmg-4 of N.crassa was subjected to structural comparison with its closest homolog, TmCorA of Thermotoga maritime. The alignment of Tmg-4 and TmCorA structures resulted in good superimposition which is indicated by a RMSD value of 0.96Å. Insilico analysis of four clades and structural superposition of the crystal structure of CorA (GMN motif) with a homology model of Tmg-4 (GQN motif) confirms GQN as a novel motif and suggests that it may be involved in magnesium transport in *Neurospora crassa*.

KEYWORDS : Tmg-4, GQN, Variant, STMg, GMN, CorA Superfamily magnesium transport Proteins, Neurospora crassa

INTRODUCTION

Magnesium (Mg^{2+}) bioavailability, its abundance in the earth crust and physicochemical properties cumulatively might have enforced the nature to use it handsomely. It plays a pivotal role in both prokaryotic and eukaryotic cells as it is involved in the organization of various cellular organelles such as chlorophyll, ribosome, nucleus, mitochondria, plasma membrane and also stabilizes the structure of macromolecules which includes nucleic acids, proteins and lipids (Anastassopoulou and Theophanides 2002). As a cofactor, it binds with high energy molecules (ATP, GTP), participates in cellular processes such as replication, transcription, translation, cell cycle, cell division, apoptosis, and also in enzymatic reactions like glycolysis, krebs cycle, -oxidation of lipids (Cowan 1998; Elin 1994; Guo et al 2016; Shaul 2002; Williams and Salt, 2009).

The transport of Mg^{2+} is unique when compared to other divalent cations because of its different hydration chemistry (Moomaw and Maguire, 2008). Many Mg^{2+} transporters have been identified in both prokaryotes (CorA, MgtE and MgtA/B) and eukaryotes (Mrs2, ALR1&2, MNR2, LPE10, SLC41 and MGT family) (Gregan et al 2001; Kehres et al 1998; Knoop et al 2005; Kolisek et al 2008; Li et al 2001; Pisat et al 2009; Snavely et al 1989). In fungi, especially intracellular needs of magnesium are quenched by a group of dissimilar transporter proteins that belong to CorA superfamily and are decorated in the plasma membrane. The best-characterized eukaryotic homologues ALR1 and ALR2 are found in the plasma membrane of yeast *Saccharomyces cerevisiae* and their overexpression confers resistance to aluminium ion (MacDiarmid and Gardner, 1998). On the other hand MRS2 and LPE10 proteins are functional CorA homologues located in the inner mitochondrial membrane (Gregan et al 2001). The crystal structure of CorA has been resolved from *Thermotoga* maritima (TmCorA) characterized by pentameric cone with two transmembrane helices and universally conserved GMN motif in the first transmembrane domain (Eshaghi et al 2006; Lunin et al 2006). Each and every amino acid residue in the GMN motif is very crucial for magnesium transport and integrity of the pentamer (Szegedy and Maguire 1999; Palombo et al 2013).

In *N.crassa* magnesium starvation reported decrease in nucleic acid synthesis (Viotti et al 1971), whereas high concentration of magnesium resulted in reversal of metal toxicity (Cobalt, Nickel and Zinc) attributing to suppression of the uptake of toxic metal ions (Sastry et al 1962). The lacunae in the knowledge of magnesium transport, distribution, trafficking is hampering the physiological and developmental relevance of the magnesium, Hence we undertook the insilico study to unveil the genes and their proteins responsible for magnesium homeostasis in fungi and *N. crassa*. In the present study apart from confirming four gene models, a novel motif called GQN motif was discovered in one of the models instead of GMN, a hallmark of motif of CorA superfamily. Furthermore we identified that the homologues of transporter protein with GQN motif are present majorly in Sordariomycetes class of fungi. Tmg-4 bearing GQN motif is a novel variant of CorA superfamily magnesium transporter could be involved magnesium accumulation in *N. crassa*.

MATERIALS AND METHODS

Sequence Retrieval, Generation Of Dataset Of Magnesium Transporter Proteins

Protein sequences coding for magnesium transporters in

N.crassa (Kiranmayi and Mohan, 2006) were retrieved from GenPept database and subjected to sensitive BLAST homology searches (Altschul 1990) at the NCBI (http://www.ncbi.nlm.nih.gov/) to first identify sequence homologues for each of the functionally characterized proteins: ALR, MNR2 and MRS2. Apart from these three clusters, a sequence variant was observed and BLAST homologs were identified for this variant originating a new cluster named as STMg (Sordariomycetes Transporters of Magnesium). Distantly related proteins were then used in reiterated searches to identify additional homologues. From these results a dataset was created with four categories of proteins namely ALR, MNR2, MRS2 and STMg. All the proteins in our dataset were checked for the CorA domain using SMART tool http://smart.embl-heidelberg.de/ and for 2 or 3 C-terminal TM stretches with the PRED-TMR server (Letunic I and Bork, 2018; Pasquier et al 1999). Furthermore MEME http://meme-suite.org/ and GLAM2 Scan http://bioinformatics.org.au/tools/glam2/ were utilized to scan the motifs for the presence of conserved G[x]N that is likely to be involved in the function of protein (Bailey et al 2009). The proteins that showed overall sequence similarity and fulfilled our criteria defined above were selected for our analysis.

Multiple Sequence Alignment And Phylogenetic Analysis

Multiple sequence alignment was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) for CorA superfamily protein sequences across different genus including yeast and several filamentous pathogenic and non-pathogenic fungi. In several cases, redundant identical or nearly identical protein sequences from different species of the same genus were not included in our data collection. All alignments were checked for the correct alignment of the G[x]N motif. The PHYML web server was used for construction of phylogenetic trees based on Maximum Likelihood method considering alignment of full length sequences of CorA proteins (Guindon et al 2003). The tree was constructed with the default parameters and 100 bootstrapped data sets (Dereeper et al 2006). Trees were visualized with the iTOL server https://itol.embl.de/ (Letunic and Bork, 2007)

Structure Prediction And Superimposition

Using I-TASSER (Iterative Threading ASSEmbly Refinement) (server https://zhanglab.ccmb.med.umich.edu/I-TASSER/ three dimensional structures of Tmg-3 and Tmg-4 protein of *N. crassa* were predicted by submitting their aminoacid sequences online (Roy et al 2008). I-TASSER modeling starts from the identification of structure templates from the PDB by LOMETS threading approach and uses only the best templates of the highest significance from the threading alignments. I-TASSER simulations generate a large group of structural conformations, called decoys. which are clustered by SPICKER program to select final models. The confidence of each model is quantitatively measured by C-score and were recorded. The models with higher C-score were selected and high resolution structures were made using PyMOL Molecular Graphics System. Structural alignment was carried out using PyMOL Plugin ALIGN tool (Cohen 1997). Predicted Tmg4 model was first superposed with the TmCorA crystal structure (PDBID:2BBJ chain A) and then with modeled Tmg3 structure. In addition to this the effect of substitution on protein stability in Tmg-4 structure was studied by DUET server http://biosig.unimelb.edu.au/duet/ (Pires et al 2014). To run prediction on this server, we submitted Tmg-4 structure in PDB format, mutation information which included residue position of 586, wild type and mutant residue codes as glutamine and methionine respectively.

RESULTS

Insilico classification of CorA homologues and identification of novel variant with GQN motif

Bioinformatics analysis was performed to know the genes involved in the magnesium transport in *N.crassa*. Its genome encodes four putative magnesium transporters, whose gene loci are, NCU11312.5, NCU03312.5, NCU09091.5, and, NCU07816.5 named as *tmg-1, tmg-2, tmg-3* and *tmg-4* respectively. Among these Tmg-1, Tmg-2, Tmg-3 proteins have shown 72%, 64%, 69%,sequence identity with ALR ,MNR2, MRS2 magnesium transporters of *Podospora anserina*. In contrast, Tmg-4 possesses low sequence identity with characterized CorA magnesium transporters of fungi. This observation instigated us to catalogue and generates a dataset of fungal magnesium transporters and this dataset is employed to carry out *insilico* analysis. BLAST homology searches were first used to identify sequence homologues of *N.crassa* for each of the functionally characterized proteins: ALR, MNR2, MRS2 which altogether resulted in 299 proteins (Supplementary material; S1). All these proteins were examined for the conserved domains, TM stretches and the presence of signature motifs. These studies showed that 264 proteins belong to CorA superfamily with two transmembrane Segments (TMS) and GMN motif. The remaining proteins did not have conserved GMN motif inspite of possessing CorA domain. Through SMART analysis Tmg-4 of *N.crassa* and its homologous proteins from filamentous fungi were found to consist of CorA family domain starting at around 250th residue from N terminal and ending near the C-terminus of the proteins. The length of CorA domain is 250 aminoacids in Tmg-4 protein and its homologues (Fig. 1).

The motif organization in STMg suggests the presence of two motifs by MEME suite (Fig.2). The positions of the 1st and 2nd motifs in Tmg-4 homologous proteins are ~145-244 amino acids and ~390-585 amino acids respectively. From our observation we report that 2nd conserved segment could be a probable functional motif as it is a part of CorA domain showing lesser E-value than the first motif (Fig. 2). Furthermore GLAM2 analysis also predicted similar motifs in STMg proteins indicating that the 2nd motif of protein could be playing a role in magnesium transport. The multiple sequence alignment with ALR,MNR2 MRS2 and Tmg-4 homologous proteins reveal that the only region conserved among the proteins is G-M/Q-N (Fig. 3).In conjunction with domain organization, motif analysis and multiple sequence alignment we could identify a tentative novel motif (GQN) flanked by aromatic amino acids in Tmg-4. The homologues of Tmg-4 with GQN motif were majorly present in Sordariomycetes class of fungi and hence they are named as STMg whose sub cellular localization was predicted to be a plasmamembrane. The details of STMg proteins such as reference numbers, species, Transmembrane segments (TMS), GQN presence and subcellular localization were catalogued (S1). For a deeper understanding of similarities and dissimilarities of magnesium transporters in fungi, conserved residues around the GMN and GQN were searched in ALR, MNR2, MRS2 and STMg families using multiple sequence alignment strategy.

In fungi, despite the very low overall sequence similarity, automatic alignment was possible in which the GxN motif was consistently aligned over the sequences revealing that ALR, MNR2 ,MRS2, has FGMNV, WGMNV, majorly YGMNL sequences respectively whereas STMg possess FGQNF sequence (Fig. 3). From the analysis of PRED-TMR and Clustal omega it has been noticed that GM residues are positioned at the end of first transmembrane segment (TMS1) and N residue in loop of ALR, MNR2 and MRS2. While in STMg proteins only G residue is located at the end of TMS1 and QN residues lie in loop. Based on the localization of G[x]N residues reference numbers in the catalogue were represented in different colours (S1). Phylogenetic analysis of STMg proteins and other CorA proteins resulted in four clades namely, ALR, MNR2, MRS2 and STMg (Fig. 4) whose categorization was based on sequence identity, signature sequences and functional motifs. All these observations encouraged us to

functionally characterize the *tmg-4* gene of *N.crassa*.

Structure prediction of Tmg-3 and Tmg-4 proteins of *N.crassa* unveils significant structure similarity with CorA transporter proteins.

Tertiary structure of Tmg-3 and Tmg-4 of *N.crassa* predicted by I-TASSER server reported five models for each protein and the confidence of each model was quantitatively measured by C-score. Best-fit model for Tmg-3 and Tmg-4 proteins were found to have C-score values of -3.40 and -3.51 respectively that are typically in the acceptable range of [–5, 2].In both the cases the most reliable models were considered for further structural analysis. I-TASSER used the TM-align structural alignment program to identify best templates in the PDB library. This section reported top 10 proteins from the PDB that have the closest structural similarity to the predicted I-TASSER model (TM score). TM score for Tmg-3 was found to be 0.46 and its structural analog in PDB was identified as 3RKG which is yeast Mg^{2+} channel MRS2. TM score for Tmg-4 protein was found to be 0.45 with its structural analog in PDB as 2IUB which is TmCorA. TM score >0.5 suggests the correct topology and can be used for determining the structure class or protein family of the predicted query protein structure. From these results it was confirmed that models were predicted accurately as they were belonging to CorA metal ion transporter family. Our results demonstrate that in Tmg-3 as well as in Tmg-4 protein most of the residues form alpha helix followed by random coils and least form beta strands (Fig. 5). In mutation analysis carried out by DUET server, predicted results are expressed as variation in Gibbs free energy (ΔΔG) and found to be 0.495 kcal/mol denoting stabilizing mutation. Thus Tmg-4 retained protein stability inspite of substitution of glutamine by methionine in the conserved GQN motif.

Structural alignment reveals large regions of global similarity in TmCorA and Tmg-4 inspite of GMN substitution by GQN motif

Comparitive studies play a pivotal role in bioinformatics. It has been demonstrated that structural resemblance of protein implies their functional similarity. To examine if the variation in motif lead to variation in structure, the predicted models Tmg-3 and Tmg4 were subjected to structural comparison with its closest homolog TmCorA. The alignment of Tmg-4 and TmCorA structures resulted in good superimposition which is indicated by RMSD value of 0.96 Å. However the conserved GMN/GQN motifs did not overlap eliciting different orientation of connecting loops. We also superposed Tmg-3 over the previous alignment which showed good overlap of all the structures over large regions but the GMN fold of Tmg-3 and TmCorA bears notable differences although both of them are in the vicinity of each other as shown (Fig. 6). These results concluded the conservation of CorA domain structures along with sequence similarity across the members of STMg family. Thus the superimposition of the functional CorA domains from Tmg-3 and Tmg-4 revealed remarkable structural similarity.

DISCUSSION

Preliminary studies noticed that the four gene loci belonging to the CorA superfamily are responsible for magnesium uptake (Maguire 2006). Three of four (*tmg-1, tmg-2 and tmg-3*) genes encoded proteins consist of 2TMS and GMN motif which are universally conserved and hallmark of CorA superfamily (Lunin et al 2006; Chen et al 2009). Whereas, *tmg-4* encoded protein has 2 TMS and no GMN motif. When Tmg-4 is subjected to sensitive blastp it aligns with prokaryotic, eukaryotic CorA and ZntB homologs resulting in twilight identity. The ZntB with GVN motif is a distant relative of CorA transporter and is involved in the efflux or influx of the zinc and cadmium (Worlock and Smith, 2002; Knoop et al 2005; Gati et al 2017). Thus we generated a catalogue of characterized and hypothetical magnesium transporter proteins and analyzed the data set using bioinformatic tools for the presence of mandatory domains and signature motifs to assign them as CorA superfamily members. From our dataset analysis it was found that surprisingly homologues of Tmg-4 protein has GQN instead of GMN motif and are mainly present in Sordariomycetes class of fungi and are so named as STMg. The multiple sequence alignment from CorA superfamily protein sequences showed the maximum conservation of amino acid residues around the CorA domain across the species. The characterized fungal CorA magnesium transporters such as ALR, MNR2, MRS2 and it homologues do have hydrophobic aminoacids (V/L) just after GMN motif where as in STMg proteins GQN is flanked by aromatic amino acid phenylalanine which similar to bacterial CorA counterparts. Phylogenetic analysis of representatives of catalogued fungal CorA transporter proteins reflects four clades such as ALR, MNR2, MRS2, and STMg. The assignment of these clades seems to correlate with alterations in the highly conserved sequence around G[x]N motif. PRED-TMR predicted the physical location of GMN in ALR, MNR2, MRS2 and GQN in STMg to be in first transmemebrane segment and in loop connecting second transmembrane segment of fungal CorA proteins. The strong conservation of core residues around the CorA domain region explains that least disturbances might have occurred during their evolution revealing their crucial functional role.

Protein functionality is extremely structure specific recommending that protein function is determined more accurately by its structure rather than sequence of residue identities. Therefore we next predicted Tmg-3 and Tmg-4 structures in order to identify structural homologs. ITASSER server facilitated the generation of models for Tmg-3 and Tmg-4 protein of *N.crassa* precisely which is evident from C-Score and TM Score values. Additionally, TM-align results aid in classifying both the models to CorA superfamily, confirming the perfect prediction of models. We also predicted that Tmg-4 protein indeed exists as a pentamer whose oligomeric structure was generated by SWISSMODEL server (Waterhouse et al 2018). Proteins exhibit structural resemblance despite of possessing low sequence similarity elucidating that structures are 3-10 times more conserved than sequences (Illergård et al 2009). On the other hand the extremely small differences within protein folds are being recognized as determinants of functional specificity (Dessailly et al 2009). It has been illustrated that functional similarity can be better inferred from motif similarity when compared to sequence similarity. In order to achieve this we proceeded for structural alignment studies. From the superposed structures it is apparent that Tmg-3 and Tmg-4 of *N.crassa* retained global structural similarity with TmCorA.

Earlier studies reported that in GMN, Glycine and Aspargine side chains guard cation selectivity at the entrance while methionine governs the function and stability of TmCorA. Further results showed that conserved substitution at GMN reinforce that G is necessary for function, M is required for pentamer integrity in the putative open conformation, and N for both functions (Payandeh et al 2008; Palombo et al 2013). We tried to understand why nature allowed substitution of M by Q in STMg class of CorA superfamily. The reason might be M could be replaced with Q because when Q is substituted by M in Tmg-4 and checked for the stability by substitution mutational server (duet server) it revealed protein stability (ΔΔG value of 0.495 kcal/mol). This suggested that Tmg-4 protein structure is tolerant to substitution which in turn retains function too. CorA family magnesium transporters with GMN motif are widely distributed in fungi and plants including the pathogens. So here our studies congruently confirms and validates that *tmg-4* gene encoded protein is a novel variant of CorA superfamily magnesium transporters and reporting for the first time in fungi. These results help us to reorganize the fungal magnesium transporter of CorA superfamily into four

clades ALR, MNR2, MRS2 with GMN motif and STMg with a novel GQN motif. Further research is needed to address why existence of STMg in only one class of fungi and their physiological role.

CONCLUSION

In summary we conclude that the G-M-N motif, despite its high degree of conservation, can functionally replace methionine by glutamine. Through bioinformatic predictions we propose that the overall CorA structure appears to be retained in bacterial and eukaryotic homologs. In the present work, the characterization of structural and functional attributes through computational approaches suggests that Tmg-4 with a novel motif (GQN) may serve as magnesium transporter in *N.crassa*. We also discovered a new family called STMg and this study identifies CorA proteins with GQN motif constitute an attractive target for developing inhibitors that fight against fungal pathogens of this family.

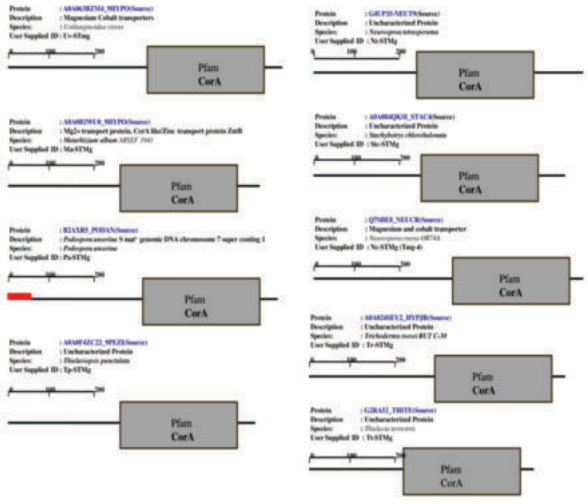

Fig 1. Prediction Of Domains In STMg Proteins By SMART Tool.

STMg protein sequences of *Ustilaginoidea virens*, |*Metarhizium album*, *Podospora anserina*, *Thielaviopsis punctulata*, *Neurospora tetrasperma*, *Stachybotrys chlorohalonata*, *Neurospora crassa*, *Trichoderma reesei*, *Thielavia terrestris* were subjected to SMART analysis and all of them were predicted to possess conserved CorA domain

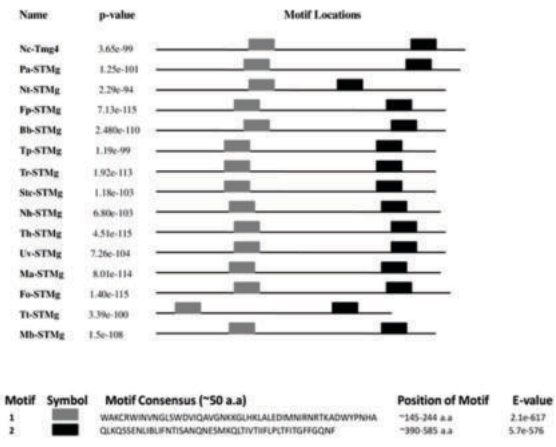

Fig 2. Motif identification in STMg proteins by MEME Suite.

STMg protein sequences of various fungal organisms such as *Nc-Neurospora crassa* (gi|758991088), *Pa- Podospora anserina* (gi|171687150), *Nt-Neurospora tetrasperma* (gi|698987050), *Fp-Fusarium pseudograminearum* (gi|685849049), *Bb-Beauveria bassiana* (gi|701769163), *Tp-Thielaviopsis punctulata* (gi|802100311) *Tr-Trichoderma reesei* (gi|589103375), *Stc-Stachybotrys chlorohalonata* (gi|667722362),*Nh-Nectria haematococca* (gi|302891893) ,*Th-Torrubiella hemipterigena* (gi|729181633), *Uv-Ustilaginoidea virens* (gi|632914022), *Ma-Metarhizium anisopliae* (gi|770393357), *Fo-Fusarium oxysporum* (gi|591474481) ,*Tt-Thielavia terrestris* (gi|367051194), *Mb-Metarhizium brunneum* (gi|743643740) were analysed by MEME suite. The output displayed sequences of two motifs, their position and E-value

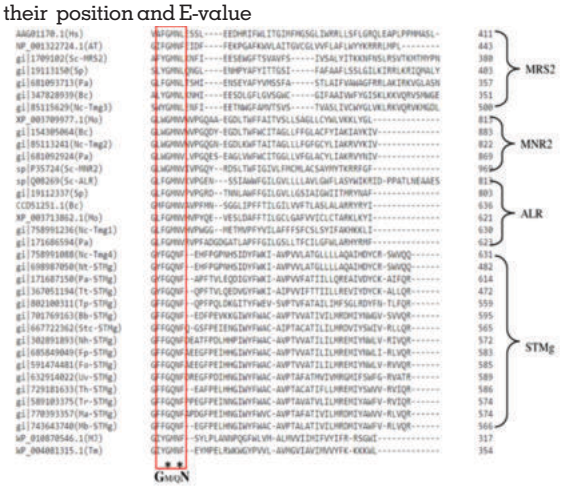

Fig 3. Identification of conserved residues in STMg and CorA proteins from different organisms using Clustal Omega.

Hs-Homo sapiens, *At- Arabidopsis thaliana*, *Sc-Saccharomyces cerevisiae*, *Pa- Podospora anserina* , *Sp-Schizosaccharomyces pombe* , *Nc-Neurospora crassa* , *Mo-Magnaporthe oryzae* , *Nt-Neurospora tetrasperma* , *Tt-Thielavia terrestris* , *Tp-Thielaviopsis punctulata* , *Bb-Beauveria bassiana*, *Stc-Stachybotrys chlorohalonata* , *Nh-Nectria haematococca*, *Fp-Fusarium pseudograminearum* , *Fo-Fusarium oxysporum* , *Uv-Ustilaginoidea virens* , *Th-Torrubiella hemipterigena* , *Tr-Trichoderma reesei* , *Ma-Metarhizium anisopliae* , *Mb-Metarhizium brunneum* , Bc-Botrytis cinerea *Tm-Thermotoga maritima*Mj-*Methanocaldococcus jannaschii*. '*' indicates G and N residues are fully conserved. On the contrary no symbol at M position in GMN indicates substitution of 'M' residue by an aminoacid 'Q' in STMg proteins

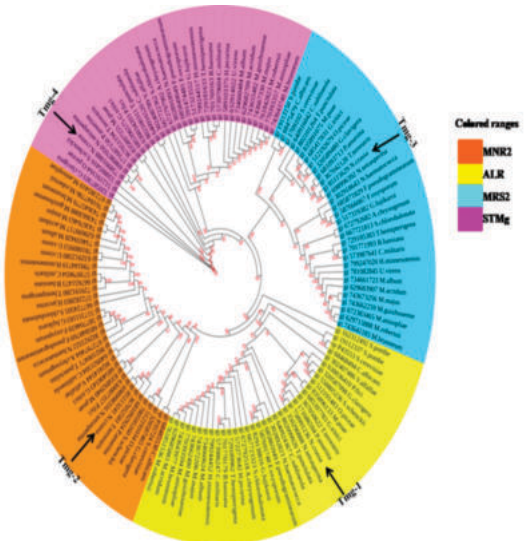

Fig 4. Phylogenetic Tree Of Fungal CorA Superfamily Magnesium Transporters: The evolutionary tree was constructed using PhyML based method and cladogram was depicted using iTOL.

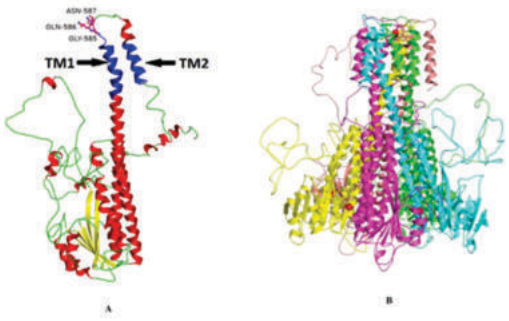

Fig 5. Structure Prediction:

A. Monomeric form of the predicted Tmg-4 structure; The secondary structural element alpha helices, -strands and loops are represented in red, yellow and green, respectively. Transmembrane helices (TM1 and TM2) are represented in blue B. Generated pentamer of Tmg-4 with the membrane domain at the top. Each monomer is colored independently with GQN motif represented as ball and stick and 5 Mg^{+2} as red spheres using PyMol.

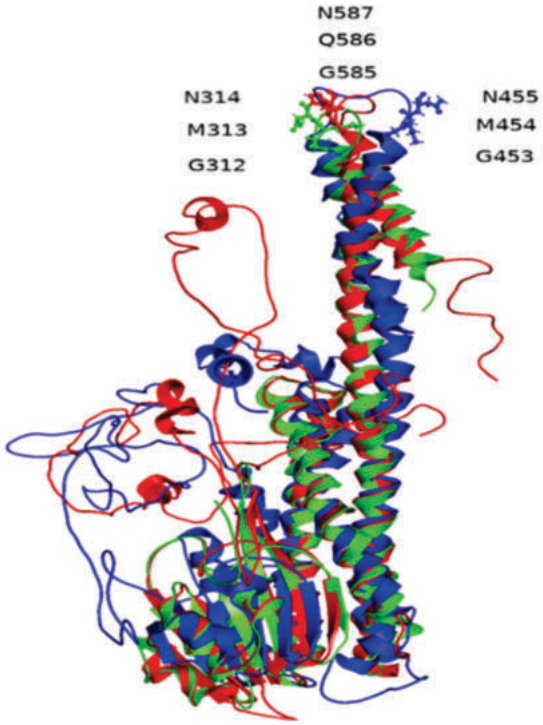

Fig. 6. Structural comparison of Tmg-3 and Tmg-4 models with TmCorA (PDBID-2BBJ).

A.Superimposition of Tmg-3 (blue) and Tmg-4 (red) of *N.crassa* on Tm-CorA (green) with an RMSD of 4.89Å and 0.87Å respectively. GMN/GQN motif is represented as ball and stick using PyMol.

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