Expression of a chitinase family protein at4g01700 from *Arabidopsis thaliana*

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ABSTRACT

Fugal pathogens destroy most of the crop plants causing huge loss, fungus resist due to the chitin present in their cell wall; chitin is the second most abundant polysaccharide in nature. Chitin is also reported to be present as the exoskeleton element of some animals including worms and arthropods which also act as pathogen to the crop plants. Chitinase are the hydrolytic enzymes which acts on the chitin present on the cell wall of the fungal pathogen and act as an antifungal protein hence identification and characterization of the putative gene and the protein is very useful in understanding its role in plant defence. For this purpose Chitinase gene At4g01700 was isolated, characterized and expressed from *Arabidopsis thaliana* (ecotype *Arabidopsis Columbia*). We wanted to characterize a putative chitinase of *Arabidopsis thaliana*, At4g01700 which resembled most with the chitinase of *Nicotiana tobaccum*. The gene was amplified using the cDNA of the plant *Arabidopsis thaliana*, cloned and expressed using PET EK/LIC vector in different hosts like BL21 (DE3), BL21-pLysS, Rosetta and Rosetta DE3 into a protein up to the size of 31kd. Further we carried out the homology modeling of the chitinase family protein At4g01700 for the three-dimensional protein structure modelling to gain the structural insight. With the help of the predicted homology model we can further identify the position of the conserved regions of the chitinase protein which can further help us in the identification of the putative active sites, binding pockets and ligand. Further the homology model predicted can help us in formulating the hypothesis of structure-function relationship. Information obtained from further characterisation of this protein chitinase gene (At4g01700) which the group is continuing with, can be used to develop the transgenic plants resistant to the diseases caused by the fungal pathogens.

KEYWORDS: Chitinases, *Arabidopsis thaliana*, TAIR database, Pet46EK/LIC vector, Chitinase gene At4g01700.

1. INTRODUCTION

Various proteins like chitinase are reported to be produced by plants; instead of having their own immune system (John, 1991). Chitinases (EC 3.2.1.14) are the enzymes that catalyze the hydrolysis of the β-1, 4-N-acetyl-D-glucosaaminidic linkages of the polysaccharide chitin, (Zamir, 1993) found in the cell wall of most fungi, some algae and in the exoskeleton of arthropods and crustaceans. Chitinase plays different role in different organisms like for growth and molting by insects, for availing carbon and energy source by bacteria. Higher plants and animals produce chitinase to defend themselves from infection of pathogenic fungi (John, 1991; Zamir, 1993). "Plant chitinase have both scientific and economic interest". Mostly the plant chitinase are endo-type chitinase which randomly hydrolyze the internal β-1, 4-N-acetyl-D-glucosaminidic linkages of chitin producing chitooligosaccharides. Thus plant chitinase genes can be used to develop the disease resistant transgenic plants that show the increased resistance to the fungal pathogens due to the degradation of chitin present on fungal cell wall by chitinase genes (Broglie, 1991; Nishizawa, 1999). There are data showing the up-regulation of chitinase in response to fungal elicitors, ethylene and other stress (Boller, 1988; Watanabe, 1999). Apart from the antifungal activity, chitinase also play important role towards regulation of normal plant development and regulation of legume response to rhizobial nod factors (Zhong, 2002; De Jony, 1993). Chitinase are usually involved in active or passive defence against pathogens and they are also known to regulate growth and development by generating or degrading signal molecule and through programmed cell death (PCD). Generally plants synthesize a number of closely related chitinase which are encoded by a gene family. Chitinases have been divided into six classes on the basis of their amino acid sequences (Collinge, 1993; Beintema, 1999; Meins, 1994). Plant chitinases are generally the monomeric proteins between 22 and 40 kDa in size. Various *in vitro* studies have demonstrated a growth inhibitory effect of chitinase against fungi containing chitin in the cell wall (Brokert, 1988). Researchers have primarily focused on chitinases from crop plants and ornamental plants (Beintema, 1999; Meins, 1994; Shishi, 1990). Although chitinases have been purified and characterized from a variety of sources, very little is known about the enzyme from *Arabidopsis thaliana* (John, 1991). Simple genome organization and short life cycle have made it a model organism in plant research and offers important advantages for basic research in genetic and molecular biology, the small size of its genome makes *Arabidopsis thaliana* useful for genetic mapping and sequencing with about 157 mega base pairs and fine chromosomes, gene encoding chitinase have also been used to genetically engineered plants to enhance their protection against fungal pathogens (Meyerowitz, 1990). *A. thaliana* on the other hand produces a single basic chitinase, encoded by single copy gene (John, 1991, Samac, 1990). In spite of being known as model organism, a very less work has been reported from *A.thaliana* plant. Since 1991, till date there has been one report on purification and characterization of chitinase from *A.thaliana.* Chitinase gene At4g01700 (John, 1991). Chitinase gene At4g01700 expresses a chitinase family protein. It is involved in carbohydrate metabolic process, cell wall macromolecule catabolic process, it is mainly located in the cell wall and is expressed in 18 plant structure; this gene is expressed during 13 growth stages. Homology modeling is a computational approach for the three-dimensional protein structure modeling and prediction. Uncharacterized proteins structures can be modeled using the homology modeling. This model builds an atomic model based on experimentally determined known structures that have the sequence homology of more than 40% with the target molecule. Modeling structures with less than 40% template similarity would result in less reliable models and hence ignored. The basic advantage of the homology modeling is that it can find the

location of alpha carbons of key residue inside the folded protein. It can help to guide the metagenesis experiments, or hypothesize the structure function relationships. The position of the conserved regions of the proteins surface can help to identify the putative active sites, binding pockets and ligands binding sites. In our experiment Arabidopsis thaliana plant was used for the expression of chitinase family protein At4g01700 which may play an important role in the protection of plants from the fungal and other pathogens which bears chitin in their exoskeleton thus can further serve in making the transgenic plants.

2. MATERIALS AND METHODS

2.1. c-DNA synthesis from *Arabidopsis thaliana:* c-DNA was prepared from the total RNA according to the manufacturer's instructions using the Omni script Reverse Transcription kit (QUIAGEN). In the reaction mixture we took 10X buffer RT 2µL, DNTP 2µl, Oligo dT primer 2µl, RTase 1µl, water 11µl, RNA 2µl, and finally the total reaction mixture was 20µl. The reaction mixture was mixed thoroughly and carefully by vortexing for not more than 5 sec and incubated the mixture at 37ºC for 60 min.

2.2. Identification of uncharacterized genes of chitinases and primer designing: As per the protocol published by Xu et al, 2007 (Chitinases in oryza sativa spp. japonica and *Arabidopsis spp.*) and Doxey et al, 2007, we separated out different chitinases on the basis of the information given in TAIR data base and using BLAST tool we have short listed At4g01700, At4g19820, At1g56680 as uncharacterized gene . Selected genes were analyzed in TAIR data base and finally primers of all these genes were designed. Putative Arabidopsis family 1 members were identified by searching public databases at the National Centre for Biotechnology information (http://www.ncbi.nlm.nih.gov/BLAST/) and The Arabidopsis Information Resource (http://www.Arabidopsis.org/Blast/). The genome sequence of selected β-1, 3 glucanase genes and the selected c-DNA region was taken and pasted the 24-28 base pair sequence from start codon in oligodt analyser and sigma primer calculator and checked their primer length, GC content, dimer formation and secondary structure.

2.3. Amplification of the desired gene, Gel extraction and purification: PCR was done using 2µl of c-DNA as template respectively, using the Hot star Hifidelity polymerase **(QUIAGEN)** and primers for different cycles with modification in annealing temperature and denaturation time using primer of AT4g01700 gene. For gel extraction 50µl of the amplified PCR product was loaded on 0.7% agarose gel and the part of gel containing the amplified PCR product was cut under the uv-transilluminator. Further the gel-purification was done following the instruction manual of QIAquick GEL Extraction kit **(QIAGEN).** The gel purified was then stored at -40ºC temperature.

2.4. Cloning of AT4G01700 gene and Plasmid Isolation: The ligation reaction was performed by using **NOVAGEN kit** which performed the directional cloning without restriction digestion; it contained **PET 46 EK/LIC** vector for ligation with desired gene, after performing the ligation and annealing of the desired gene with vector it was transformed in competent cell of DH5α and the colonies were observed on the next day. Similarly transformation was also done using BL21DE3, Rosetta, Rosetta DE3, BL21plysS competent cells. Plating was then done on plates containing antibiotics accordingly- BL21DE3 on LB amp plate, Rosetta DE3 on LB chlor+amp plate, BL21-plysS on LB amp plate and incubated overnight at 37ºC temperature. Plasmid from DH5α culture was isolated following the instructions of **MINI-PREP method** of plasmid isolation.

2.5. Restriction digestion: Restriction digestion was done using the Hind III enzyme **(NEB).** We used HINDIII restriction enzyme because Hind III is the only single cutter enzyme present in gene AT4G01700 and it has no other cutting site within the PET EK/LIC vector so it became linear when visualized on the 1% agarose gel. For the preparation of the reaction mixture we took 10X buffer II 8.0µl, Hind-III 2.0µl, nuclease free water 58.0µl and the total mixture was 68.0µl. Added 17µl of the master mix to each 0.5ml 4 eppendrof tubes and 3µl plasmid DNA to each eppendorf tube. And in one 1.5ml eppendorf added 58.0µl of the nuclease free water, 2.0µl of the Hind-III, and 10 X buffer 9NEB) 8µl. Divided them equally i.e. 17µl to the 4 separate autoclaved eppendrof tubes (0.5ml) each and added 3µl of the plasmid DNA to each tube. Two fresh 0.5mleppendrof tubes were taken for uncut mixture, Added 17µl of nuclease free water in each tubs and add 3 µl of plasmid DNA and incubated the tubes in incubator for 5-6hrs at 37ºC and visualized the digested sample on agarose gel.

2.6. Optimisation for efficient transformation: The plasmid having the insert gene was used to transform BL21 (DE3) competent cells **(Novagen),** Rosetta competent cells DE3, BL-21 pLysS competent cells. In competent cells (100µl) 2 µl plasmid was added with gene of interest. Incubation on ice was given for 5 min. Heat shock was given at 42ºC for 45 sec. Again incubated on ice for 2 min. Added 250µl SOC media in each eppendrof and incubated at 37ºC for 1 hour and plating was done on the selection media plates having ampicilline and incubated at 37ºC overnight. The transformed colonies were observed the next day.

2.7. Induction of target protein at different conditions:

2.7.1.Using IPTG, BL21, DE3 as expression vector: Initially we took 5ml of the LB broth with 2µl of amp in it, and inoculated one colony each from the transformed plate in the LB media and incubated it overnight at 37ºC in incubator shaker. Next day 250µl primary culture was inoculated in 10ml LB broth as the OD reaches to 0.4, 1ml culture was taken in a separate eppendorf as uninduced sample (control) and in remaining media added 1mM IPTG to the final concentration of 0.2mM and 1mM and incubated in incubator shaker at 37ºC temperature 250 rpm. After one hour of IPTG induction 1ml of the culture was taken and centrifuged at 15000 rpm for 1 min, discarded the supernatant and stored the pellet at -40 º C temperatures. Similarly pelleted down 1ml IPTG for every 2, 3, 4 and 6 hour and freeze all the samples at -40ºC.Visualized on the (12%) SDS gel.

2.7.2: Using IPTG, BL21 (pLysS) and Rosetta as a expression vector: The plasmid containing targeted protein was transformed into expression vector BL21 and Rosetta and visualized on 12% SDS gel.

2.7.3. Using IPTG, BL21DE3 as expression host with at different temperature, IPTG concentration, time.

The media was inoculated with transformed BL21 DE3 STRAIN and incubated it overnight at 37ºC, 200 rpm. For secondary culture 50ml LB media was taken with 1%glucose (40% stock) and added 1 ml primary culture, incubated it in the incubator shaker at 200rpm, 37°C temperatures. After the OD reaches to 0.6, 1ml of the culture was taken in an autoclaved eppendorf for in induced sample and to the rest culture induced with the two different concentration of IPTG a) 0.2 mM IPTG b) 1mM IPTG.

And induced the culture at two different temperature i.e. 17ºC and 37ºC for both genes, for 2, 4, 6, 8 and 24 hour. 1ml culture was taken after the given interval of time, pelleted down at full rpm for 1 min and stored at -40ºC temperature and visualised on 12% SDS gel.

2.8. Analysis of proteins on SDS PAGE gel (12%): The samples were taken out from -40ºC and added 45µl of the sample buffer already kept at -40ºC and mixed the pellet well to the sample. The buffer was added and boiled at 100ºC for 10min. 10µl of the boiled samples was loaded in the gel (12% PAGE) along with 5µl of the protein marker and run. After 2-3 hours when the samples are completely run on gel, the gel was took out and was put on the staining solution (as mentioned above) for 30 min in the rocker, after 30 min decant the staining solution and put the gel in the distaining solution for 5-10 min in the rocker. After 5-10 min decant the distaining solution and added the autoclaved distilled water to the gel and visualized.

2.9.Building a 3D model of *Arabidopsis Thaliana* AT4GO1700 protein and its refinement: *Arabidopsis thaliana* AT4G01700 protein sequence with the length of about 280 amino acids was obtained using the UNIPROT database in the FASTA format. Further for the selection of the templates based on the sequence identity and similarity the protein sequence was BLAST against the PDB database. Six out of the most similar sequence from the BLAST hits were selected. The table below shows the maximum identity which was above 40% of the selected sequence from BLAST. Multiple sequence alignment (MSA) of the target sequence along with the selected template sequence was performed using CLUSTAL O and saved the alignment file from MSA result. The 3D structure of the target sequence was predicted using the best template having high similarity with the target sequence using the SWISS-MODEL (alignment mode).

Table 1: Showing the maximum identity of the query sequence and the template sequence

Template	PDB Id	Max. Score or Total Score	Query Coverage	E-value	Max.Ident
01	3CQL_A	317	86%	9e-108	60%
02	2DKV_A	299	86%	5e-100	59%
03	4DWX_A	308	86%	2e-104	58%
04	3W3E_A	304	87%	5e-103	58%
05	2BAA_A	294	86%	8e-99	58%
06	1CNS_A	293	86%	1e-98	58%

By using the SWISS- MODEL (alignment mode) with template (2DKV_A) we got three models.To ensure the model evaluation and refinement, predicted structures are further checked for loop modelling, side chain refinement using the PRIME tool of the Schrodinger software. Further energy minimization was done by Optimized Potential for Liquid Simulation 2005(OPLS 2005) force field parameters using the SCHRODINGER software. Further to evaluate the quality of refined predicted structure RMSD values were calculated by superimposing the predicted model with most similar template with the help of PyMol tool and QMEAN Z Score were calculated by submitting predicted structure to the QMEAN web server (swissmodel.expasy.org/qmean).

3. RESULTS

3.1. Amplification of c DNA and genomic DNA using primer of AT4G01700 Gene: For amplification of c DNA and genomic DNA, in the PCR reaction the annealing temperature (55˚C) was changed and genomic DNA was taken as template, normal taq polymerase was taken instead of the hot start and visualized on 1% agarose gel. We got band of the amplified gene. After getting success with PCR on genomic DNA we modified conditions for c- DNA for this we used Hi Fidelity Hot start DNA Polymerase for increasing specificity with modification like addition of Q solution and $MgSO_4$ in reaction mixture. We got an amplified product of about 843 bp (Figure 1).

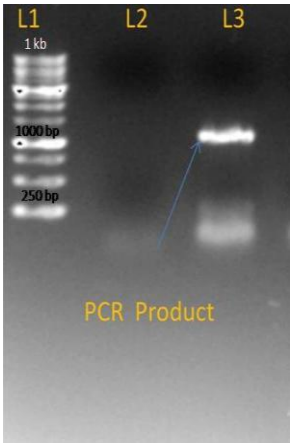


Figure.1.L1-I kb ladder, L2-Negative control, L3-PCR-product.

3.2. Gel elution and purification of PCR product of AT4G01700: After conformation of product size the product was eluted with the help of sharp blade under UV transilluminator, from 0.7% agarose gel and finally purified the product from gel by gel elution kit (QIAGEN). After elution the PCR product was purified and was finally visualized on agarose gel (1%). Purified PCR product of the gene AT4G01700 is shown in lane L1 **(Figure 2).**

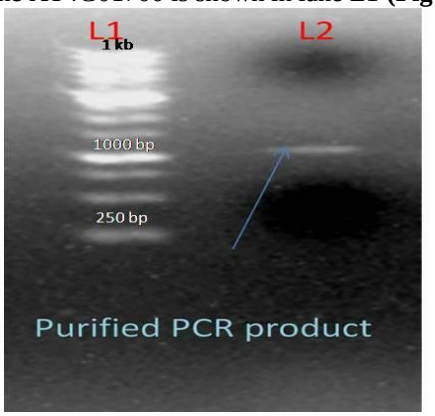


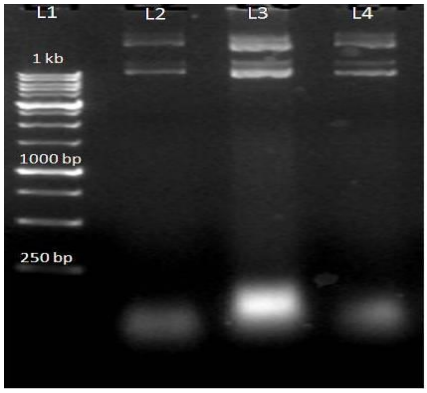
Figure 2 **Figure 3**

Figure.2.L1-1kb DNA Ladder L2-purify PCR product of AT4G01700 Gene

Figure.3.Transformed colony of DH5α

3.3. Ligation and Transformation: After the gel elution and purification of the PCR product was done thereafter the ligation reaction was performed by using NOVAGEN kit which performed directional cloning without restriction digestion, it contained PET46 EK/LIC vector for ligation with desired gene, after performing the ligation and annealing of the desired gene with vector it was transformed in competent cell of DH5α and colonies were observed. We got the number of transformed colonies of strain DH5α (Figure 3).

3.4. Plasmid isolation and restriction digestion: In the next step in order to confirm the positive colony, we isolated the plasmid from each transformed colony of gene AT4G01700 using Mini-Prep method. Further on running in gel we visualized the bands showing the plasmid isolated (Figure 4). After the plasmid isolation, restriction digestion was performed to confirm the ligation of the insert with the vector. In this we used HindIII restriction enzyme because Hind III is the only single cutter enzyme present in AT4G01700 and it has no other cutting site within the pET EK/LIC vector and so it become linear when visualized on the 1% agarose gel (Figure 5). Next after the confirmation of the ligation of insert with vector, we transformed the plasmid containing insert in to competent cells of BL21 (DE) and many other expression hosts like Rosetta, Rosetta (DE3),BL21(plysS). Further for the confirmation of the transformation we again isolated the plasmid following the Mini-Prep method and performed the restriction digestion by using HindIII enzyme. (Figure 6); showing restriction digestion with Hind III enzyme in which At4g01700 gene insert have single cutting site for HindIII enzyme, which becomes linear after digestion.



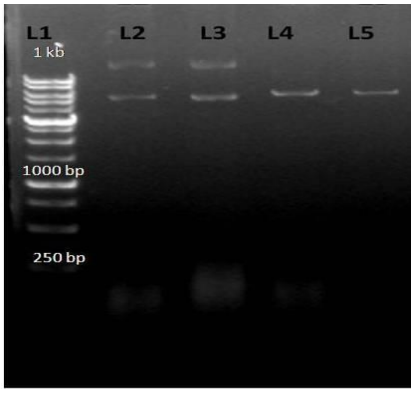
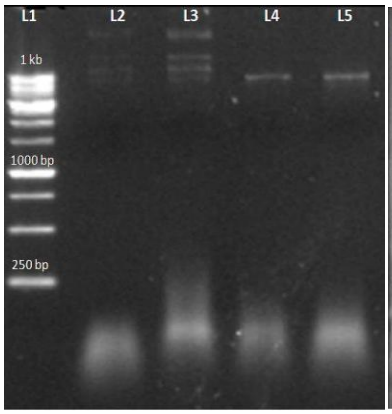


Figure 4 **Figure 5**

Figure 4-L1-1Kb ladder, L2-AT4G01700 (a) plasmid, L3-AT4G01700 (b) plasmid L4-AT4G01700(c) plasmid

Figure 5- L1-1 kb DNA ladder; L2-AT4G01700 (a) insert having no cutting site for Hind III (uncut); L3- AT4G01700(b) insert having no cutting site for Hind III(uncut) L4-AT4G01700(a) insert have single cutting site ; L5- AT4G01700(b) insert have single cutting site.



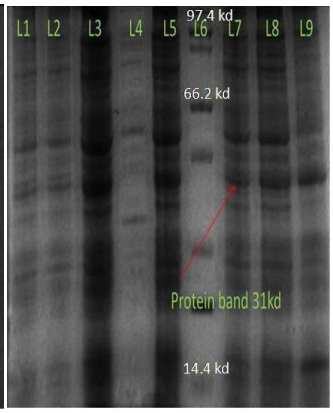


Figure 6 **Figure 7**

Figure.6. L1-1 kb DNA ladder L2-uncut (a)no cutting site for HindIII L3- uncut(b) no cutting site for HindIII L4-AT4G01700 (a)gene insert having single cutting site L5- AT4G01700(b) insert having single cutting site.

Figure.7. SDS gel image (12%) *AT4G01700* gene protein expression of (BL21DE3) L1- 1 hour induced L2- uninduced sample of 1 hour L3- induced sample of 2 hour L4- uninduced sample of 4 hour, L5- induced sample of 4 hour, L6- protein marker, L7- induced sample of 6 hour L8- induced sample of 8 hour L9- induced sample of 10 hour.

3.5. Expression of Targeted protein using IPTG: For the expression of the targeted protein we used the different expression host like BL21 (DE3), BL21pLysS, Rosetta, and Rosetta DE3. We also changed the different IPTG concentration at different OD and also gave temperature variation like 18ºC and 37ºC with IPTG concentration 0.2 to 1. The cells were also induced at lower temperature as has been for expressing *Arabidopsis* chitinase V (Ohnuma et al; 2011). To prevent the leaky expression we used 0.5% glucose in primary culture and 1% glucose in induced culture. In first experiment we used BL21 (DE3) as expression host. In this we induced with IPTG, when O.D. of the secondary culture reached 0.6. Started the sample collection after each hour until 8 hour, immediately centrifuged the sample and freezed the pellet at -40ºC. Resuspended the sample with sample buffer and boiled for 10 min and loaded on 12% SDS gel in SDS gel unit (Bio-Rad) (Figure 7).

Similarly on the expression of the AT4G01700 protein in Rosetta expression host with 0.2 mM IPTG concentration at temperature 18ºC temperature with 1% glucose in induced culture we obtained 31 kd of the protein. And on expressing the AT4G01700 gene protein in plysS expression host and Rosetta (DE3) with 1mM IPTG and 0.2mM concentration respectively at temperature 18ºC temperature we again obtained the protein of 31kd. Similarly for the expression of the AT4G01700 gene protein in Rosetta (DE3) expression host with 0.2mM and 1mM IPTG concentration at temperature 37ºC and 18ºC temperature we again obtained the protein of size 31 kd.

3.6. Homology modelling of chitinase family protein At4g01700: BLAST of chitinase family protein At4g01700 against the PDB database revealed that the query sequence have three conserved domains i.e. lysozyme like domain, glycoside hydrolase family 19 catalytic domain and predicted chitinase and all belongs to the Glycoside Hydrolase family 19(GH-19) (Figure 8). Out of the obtained significant hits, 3CQL-A, 2DKV-A, 4DWX-A, 3W3E-A, 2BAA-A, 1CNS-A were selected as template for the homology modelling. The multiple sequence alignment (MSA) of our query sequence with the selected significant hits from the blast result is shown in figure 9. After the model refinement and with the help of RMSD value and QMEAN Z score (table 2), we found that model-2(figure 10) is the best fitted 3D-model for At4g01700 chitinase protein. Further ProFunc

(http://www.ebi.ac.uk/thornton-srv/databases/profunc/) analysis of the best fitted model showed that it has 10 clefts (binding sites) for the ligand interaction and has 13-α helices and 21 beta turns.

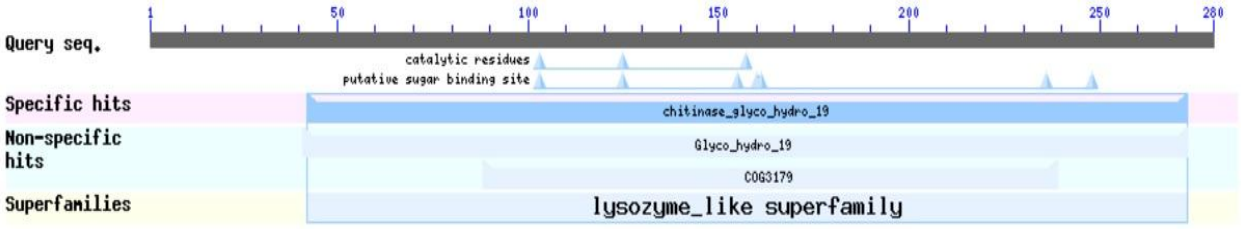


Figure.8.BLAST image showing different functional domains

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CLUSTAL O(1.2.1) multiple sequence alignment


tr|Q9ZSI6|Q9ZSI6_ARATH       ME--------KQISLLLCLLLFIFSI-SSSLH-----------ETEARKHNKYKPAPIMS
gi|442570458|pdb|3W3E|A      ------------------------------------------------------AEVGS
gi|195927481|pdb|3CQL|A      ------------------------------------------------------GIEK
gi|146386456|pdb|2DKV|A      MEQCGAQAGGARCPNCLCCSRWGWCGTTSDFCGDGCQSQCSGCGPTPTPTPPSPSDGVGS
gi|400977413|pdb|4DWX|A      ------------------------------------------------------MSVSS
gi|157834680|pdb|2BAA|A      ------------------------------------------------------SVSS
gi|1310888|pdb|1CNS|A        ------------------------------------------------------SVSS
                                                                                     : .

tr|Q9ZSI6|Q9ZSI6_ARATH       LVPRTLYDQIFIHKDNNACPAKGFYPYEAFVEATRSFPKFGSVGNFWTRRREVAAFLAQI
gi|442570458|pdb|3W3E|A      VIGASLFDQLLKHRNDQACEGKGFYSYNAFITAARSFAAFGTTGDSNTRKREVAAFLAQT
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gi|157834680|pdb|2BAA|A      IVSRAQFDRMLLHRNDGACQAKGFYTYDAFVAAAAAFPGFGTTGSADAQKREVAAFLAQT
gi|1310888|pdb|1CNS|A        IVSRAQFDRMLLHRNDGACQAKGFYTYDAFVAAAAAFSGFGTTGSADVQKREVAAFLAQT
                             ::    ::::: *::: ** .:*** *:**: *: :*  ** .*.  .::*::****.*

tr|Q9ZSI6|Q9ZSI6_ARATH       SHETTGGWATAPDGPYAWGLCFKEEVSPQSNYCDASNKDWPCVSGKSYKGRGPIQLSWNY
gi|442570458|pdb|3W3E|A      SHETTGGAATSPDGPYAWGYCFVTERDKSNRYCDGS---GPCSAGKSYYGRGPIQLTHNY
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gi|1310888|pdb|1CNS|A        SHETTGGWATAPDGAFAWGYCFKQERGASSDYCTPS-AQWPCAPGKRYYGRGPIQLSHNY
                             ******* ::*** ::** **  *      **  *    **  *: * *******: *:

tr|Q9ZSI6|Q9ZSI6_ARATH       NYGQAGRALGFDGLQNPELVANNSVLAFKTALWFWMTEQTPKPSCHNVMVNRYRPTKADR
gi|442570458|pdb|3W3E|A      NYNAAGRALGVDLINNPDLVARDAVVSFKTALWFWMTPQGNKPSCHDVITNRWTPSAADK
gi|195927481|pdb|3CQL|A      NYGPCGEALRVNLLGNPDLVATDRVISFKTALWFWMTPQAPKPSCHDVITGRWQPSAADT
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gi|400977413|pdb|4DWX|A      NYGPAGRAIGVDLLRNPDLVATDPTVSFKTALWFWMTAQAPKPSSHAVITGKWSPSGADR
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gi|1310888|pdb|1CNS|A        NYGPAGRAIGVDLLANPDLVATDATVSFKTAMWFWMTAQPPKPSSHAVIVGQWSPSGADR
                             **  .*.*: .: : **:*** : .:.****:***** *  ***.* *:. :: *: **

tr|Q9ZSI6|Q9ZSI6_ARATH       AANRTVGYGLVTNIINGGLECGIPGDGRVTDRVGYFQRYAQLFKVTTGPNLDCENQRPFS
gi|442570458|pdb|3W3E|A      AANRVPGFGVITNIINGGLECGKGPTPASGDRIGFYKRYCDVFGVSYGPNLNCRDQRPFG
gi|195927481|pdb|3CQL|A      AAGRLPGYGVITNIINGGLECGKGPNPQVADRIGFFRRYCGILGVGTGNNLDCYNQRPFG
gi|146386456|pdb|2DKV|A      AAGRAPGYGVITNIVNGGLECGHGPDDRVANRIGFYQRYCGAFGIGTGGNLDCYNQRPFN
gi|400977413|pdb|4DWX|A      AAGRAPGFGVITNIINGGLECGHGQDSRVADRIGFYKRYCDILGVGYGDNLDCYNQRPFA
gi|157834680|pdb|2BAA|A      AAGRVPGFGVITNIINGGIECGHGQDSRVADRIGFYKRYCDILGVGYGNNLDCYSQRPFA
gi|1310888|pdb|1CNS|A        AAGRVPGFGVITNIINGGIECGHGQDSRVADRIGFYKRYCDILGVGYGNNLDCYSQRPFA
                             ** *  *:*::***:***:***        :*:*:::**.  : :  * **:* .****

tr|Q9ZSI6|Q9ZSI6_ARATH       ----------
gi|442570458|pdb|3W3E|A      ----------
gi|195927481|pdb|3CQL|A      ----------
gi|146386456|pdb|2DKV|A      SGSSVGLAEQ
gi|400977413|pdb|4DWX|A      ----------
gi|157834680|pdb|2BAA|A      ----------
gi|1310888|pdb|1CNS|A        ----------
```

Figure.9.Clustal O Result showing conserved Residues with colour

Table.2.The QMEAN Z-score and RMSD value

Model	QMEAN Z-Score	RMSD	Minimum Energy
Model_01	-0.14	1.237	-49715.8438 kJ/mol
Model_02	-0.07	1.072	-49431.9336 kJ/mol
Model_03	-0.84	1.804	-55259.6914 kJ/mol

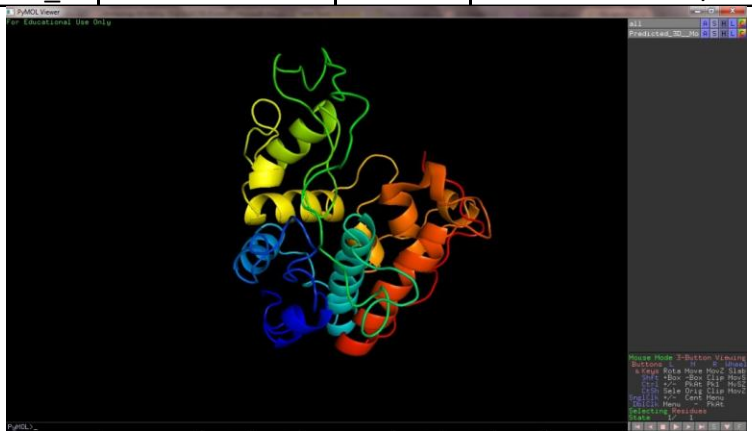

Figure.10.Showing the 3D- structure of protein

4. DISCUSSION

Since the chitinase is a hydrolytic enzyme and antifungal in nature, it acts on the chitin present on the exoskeleton of the invertebrates and chitin present on the cell wall of the fungal pathogens. Since fungal pathogens cause huge loss to the crops, hence chitin metabolisms have been considered as the suitable target to manage pests and pathogens. In the above experiment the uncharacterized gene AT4G01700 of *Arabidopsis thaliana* was isolated, characterized and cloned and the protein up to 31kd was expressed. This gene of chitinase i.e. AT4G01700 along with other genes can be characterized and if useful these genes can be used for the development of the transgenic plants to make them resistant against the fungal pathogen. Chitinase is often produced in higher plants as a general defence response after wounding or pathogenic attack. A putative role for chitinase in muskmelon seeds is defence against fungal pathogen (Witmer, 2007). So on the basis of research paper published by Xu etal and Doxey et al, 2007, we separated out different chitinases and on the basis of the information given in TAIR database and using BLAST tool we found the uncharacterized gene AT4G01700, AT1G56680, AT4G19820 and analyzed in TAIR database. Putative Arabidopsis family 1 members were identified by searching public databases at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) and The Arabidopsis Information Resource (http://www.Arabidopsis.org/Blast/). In the paper by Verburg et.al, 1991, purification and characterization of basic chitinase from *Arabidopsis Thaliana* was reported and the enzyme of 32 kilodalton was determined by SDS PAGE. We further tried to build the homology model of the protein which can help in the determination of the various ligand binding sites, catalytic domains etc. which can be further be useful in the protein expression as an antifungal protein. The chitinase gene (AT4G01700) characterized can be used to develop the transgenic plants resistant to the diseases caused by the fungal pathogens.

CONCLUSION

In the above experiment the uncharacterized gene At4g01700 of *Arabidopsis thaliana* was isolated, characterized and cloned and the protein up to 31kd was expressed and further we tried to develop a homology model of the chitinase family protein At4g01700, which can help in the determination of the various ligand binding sites, catalytic domains etc. The gene At4g01700 can be further purified and can be used as an antifungal protein to check the pathogen attack on plants, and depending upon the expression level in different parts of the plant we can lead further towards the development of transgenic plants through introducing such genes into different plant species susceptible to fungal pathogens.

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