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RESEARCH ARTICLE

Genetic Transformation of Tomato with Enhanced Resistance against *Fusarium* Wilt Using Mulberry Chitinase Gene

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Abstract

Different strategies have been adopted to control diseases in tomato crops caused by various fungal pathogens. These are cultural, biological, chemical and biotechnological approach. Recently biotechnological approach is gaining attention as suitable control measures for disease resistance. Gene transformation in the plant to improve resistance is effective measures. Chitinase genes effectively increase resistance in plants against fungal pathogens and insects. In this study a chitinase gene *MnChi10* from mulberry (*Morus notabilis*) tree was isolated, characterized, amplified and transformed into tomato plant for effective control of *Fusarium* wilt disease. Chitinase *Mn-Chi10* gene showed homology with family 19 of glycosyl hydrolases. Furthermore, the *MnChi10* gene was transformed and expressed in tomato plants through a modified *Agrobacterium*-mediated transformation. Transformed tomato plants showed higher chitinase activity than control plants, as well as better growth and increased resistance against *Fusarium* wilt disease. It was observed that transformed plants suppressed fungal growth and continued to thrive even under heavy fungal infestation. It is concluded that chitinase gene *MnChi10* showed promising results in resistance against *Fusarium* wilts. These tomato plants, in future, may be used as resistant variety for commercial growing.

Keywords: Tomato, Transgenic resistance, Chitinase, *MnChi10*, pCAMBIA 1301, Fluorescent *In Situ* Hybridization

Introduction

Crop plants are susceptible to biotic and abiotic factors which are pathogen, diseases and stress. These factors are involved in

yield reduction of crops and affect quality and quantity of agricultural commodities (Mwangi, et al. 2023). Crop diseases are most economical and devastating pathogens (Doehlemann, et al. 2017). Fungi are notorious and most dominant casual agents of plant diseases. Among these notorious fungi, some can kill their host while others are not (Fauteux, et al. 2005). Crops mainly affected by fungi are tomato, potato, cucumber, pepper, cotton, wheat and other crops (Thambugala, et al. 2020, Panno, et al. 2021). Tomato (*Solanum lycopersicum* L.) is an important and extensively grown crop of Mediterranean region throughout the world (Brahimi, et al. 2017). Tomatoes are a good source of important nutrients and minerals. These are lycopene, potassium, iron, folate and vitamin C (Staniaszek, et al. 2014). Besides vitamins, tomatoes are also a source of antioxidants. β -carotene, phenolic compounds (flavonoids), hydroxycinnamic acid, homovanillic acid, ferulic acid and chlorogenic (Ullah, et al. 2017). Tomato crop is grown all over the world as winter and summer vegetable and is prone to various serious diseases i.e. attacked by a series of pathogens (Collins, et al. 2022). Tomato crop is highly susceptible to attack by fungal pathogens, *Fusarium* and *Alternaria* (Nazarov, et al. 2020). *Fusarium oxysporum* is an important pathogen, soil fungus and exists as saprophyte. It has the ability to degrade lignin and is an endophyte of plant roots (Chitwood-Brown, et al. 2021). More than 120 different forms of *Fusarium oxysporum* are commonly found causing various diseases (Arie, 2019). *Fusarium oxysporum* penetrates into plant roots and colonize the vascular tissues (Wang, et al. 2020). There are no effective chemical control measures for *Fusarium* wilts (Díaz and Jiménez-Gasco, 2011). In previous era, when the agriculture is started, the growers had to tackle the damaging organisms. These damaging organisms are controlled by physical and biological ways (Recep, et al. 2009). In developing countries, chemical control pollutes the water with poisons and carcinogenic materials. Multipurpose pesticides are available in the market that not only destroys target pathogen but also beneficial species (Chaerani and Voorrips, 2006).

To control devastating effects of wilts fungi, biotechnological approach is mandatory through genetic modifications (Anderson, et al. 2004). The second most abundant biopolymer in the earth is chitin, composed of β 1, 4-N-acetylglucosamine (GlcNAC) (Hamid, et al. 2013). Outer skeleton of insects, crabs, lobsters, shrimps and cell wall of fungi, yeast, algae while the internal structure of few invertebrates is made of chitin (Rathore and Gupta, 2015). Chitin occurs in three polymorphic forms i.e. Alpha chains are arranged in antiparallel fashion. Beta chains are arranged in parallel fashion while in the gamma form of chitin chains are arranged in both forms. Alpha chains are most abundant forms of chitin (Wubie, et al. 2014). Chitin is degraded by chitinases in two steps, first the chitin oligosaccharides are formed by cleavage of chitinase while in second step further cleavage cause formation of N-acetylglucosamine and monosaccharide (García, et al. 2001). About 16% of dry weight of filamentous fungi and basidiomycetes consists of chitin. The precursor for synthesis of chitin is uridine diphosphate N-acetyl-D-glucosamine that synthesizes chitin by the help of enzyme chitin synthase (Suginta, et al. 2000). As chitin is important part of the cell wall of fungi there must be a system by which it can be broken down and remodeled to give it some degree of plasticity that is important in budding and elongation of hyphae (Dana, et al. 2006). Chitinolytic enzymes are categorized in 3 families of glycosyl hydrolases i.e., family 18, 19 and 20. This grouping is done on sequence similarity of amino acids (Kumar, et al. 2018).

The plant chitinases are endo-chitinases and are present in stem, seed, tuber and flowers. Plant chitinases are categorized in 5-6 classes based on amino acid structure (Cletus, et al. 2013). Plant chitinases are produced in response to the pathogen or contacting with elicitor or growth regulators. There are reports that chitinases are expressed in response to abiotic stress such as high salt concentration, drought and cold (Sivaji, et al. 2014). There are different roles of cellular and secretory chitinases in plant defense pathway. Apo plastic chitinases block the growth of fungal hyphae by induced response and are considered as part of initial response (Cao J and Tan, 2019). It also stimulates the defense pathway by releasing of fungal elicitors. Chitinases are released when the fungal hyphae invade, penetrate and affect cell integrity (Collinge, et al. 2008). Class I chitinase are vacuolar and are induced by ethylene and jasmonate pathway while chitinase II are induced systematic response (Davis, et al. 2002). The role of chitinases in defense against fungi has been strongly established by genetic transformation experiments (Di, et al. 2016). The role of chitinase gene transformation in tomato plant is documented to control fungal pathogens. Resistance in tomato plant is built up by genetic engineering techniques against fungal pathogens but there is a lack of data related to chitinase genes confers resistance against fungal pathogens (Gai, et al. 2017). The main objectives of our study were to isolate and characterize the chitinase gene from mulberry tree (*Morus notabilis*), development of transgenic tomato plants and analyze the overall resistance of transgenic tomato plants against wilt fungi. In future, the project will help to understand the use of chitinase gene resistance against fungal pathogens.

Materials and Methods

Sample collection

Mulberry (*Morus notabilis*) tree leaves based on morphology are collected from University of the Punjab, Canal Road Lahore, Pakistan.

Extraction and purification of Mulberry DNA

Total DNA from mulberry leaves is extracted by CTAB (Cetyltrimethyl ammonium bromide) method and purified.

Amplification of chitinase gene

Chitinase gene extracted from mulberry tree leaves was amplified by using a specific set of primers listed below (Tab. 1).

Table 1. List of Primer for mulberry chitinase gene

Primers	Sequence	Product size
Pair 01	CGCCAACACCTTCTACACCT AGTAGTCGCTCTGTGGGCTA	982 BP

Cloning of chitinase gene in pET30a vector

For mulberry chitinase gene cloning in prokaryotic expression vector, the chitinase gene primers was indulged with NcoI and BgIII sites in both reverse and forward. pET30a vector cloning is used for protein isolation and characterization.

Cloning of chitinase gene in pCAMBIA 1301 vector

Amplification of chitinase primer for expression: The mulberry chitinase gene was optimized and synthesized for transformation in tomato variety Rio Grande. The codon optimized gene and promoter 35 s upstream and the GUS reporter downstream was synthesized from BioBasic Canada in multiple cloning sites of pCAMBIA 1301 with HindIII and EcoRI enzymes were used.

Transformation of chitinase gene construct in *Agrobacterium*: *Agrobacterium* strain LB4404 was used for chitinase gene transformation in tomato plant.

***Agrobacterium* competent cells preparation:** *Agrobacterium* strain LB4404 was used in competent cells making and transforming in pCAMBIA 1301 plasmid with chitinase gene in it. YEP media is prepared and single colony was inoculated on it, placed it for 48 hours. YEP broth was diluted and incubated for 600 nm OD at 30°C. Centrifuge the cells for 15 minutes, the pellet was washed with HEPES 1 mM. It was washed with chilled 10% glycerol for 10 minutes. After washing, the pellet was suspended in YEP broth containing glycerol with final concentration of 10%.

Electroporation of chitinase gene construct in *Agrobacterium*: Bio-Rad Electroporation device (#165-2105) was used for Electroporation of chitinase gene containing plasmid in *Agrobacterium*. Electroporation device was set to capacitance of 25 µF, 2.2 KV voltage and resistance of 200 ohms for a constant time. 80 µl of competent cells were mixed with 100 ng or 3 µl construct and place it on ice. 500 µl of YEP broth was immediately added to cuvette and placed on ice for 5 minutes. Thereafter the cuvette was shifted in a fresh vial and incubated for 2 hours at 30°C. YEP agar containing kanamycine is prepared and transformed culture was spread on it and kept for 48 hours at 30°C.

Transformations of tomato plant with chitinase construct: After confirmation of vector transformation, a single node of tomato cultivar Rio-Grande was used in transformation of construct.

Dissecting of intermodal part of tomato plant stem: A node of tomato plant was dissected with sterile surgical blade in a laminar flow chamber. About 1 cm of part was prepared as explants for transformation.

***Agrobacterium* mediated transformation:** The *Agrobacterium* strain LB4404 containing chitinase gene plasmid was streaked on solidified agar medium containing 50 µg/ml kanamycin and incubated at 30°C for 24 to 48 hours. A single colony was inoculated in 5 ml YEP broth containing kanamycin in 50 µg/ml concentration in 50 ml culture tube. The samples were incubated at 30°C for 24 hours with 200 rpm on rotary shaker. After incubation, the bacterial suspension was directly used in co-cultivation with internodes of the stem. The tubes were placed on the orbital shaker for 30 minutes. The internodes were dried on blotting paper, placed on MS plates and kept in dark for 48 hours. After dark 48 hours, the internodes were transferred to MS plates containing cefatoxime 250 µg/ml antibiotics and allowed to grow for 6-8 weeks.

Molecular analysis of transformed tomato cultivar: Regenerated tomato plantlets were multiplied by single node cuttings onto MS basal medium and subsequently analysed for sorting positive transgenic plants and PCR.

Antifungal analysis of transgenic plantlets

***In-vitro* fungal bioassay:** The experiments were further analyzed for antifungal activity against already grown *Fusarium oxysporum* on potato dextrose agar. A block of agar 5 mm with mycelium growth was placed on base of 4 week old transgenic plants and non-transgenic plants. The inoculated vessels were incubated at room temperature for 3 weeks. The experiment was repeated in a triplicate expression of chitinase gene.

Real time PCR: The best grown plants were used for expression at different intervals at 0 h, 12 h, 24 h, 36 h, 48 h and 72 hours when inoculated with *Fusarium oxysporum*. RNA was isolated and cDNA was synthesized. SYBR green dye (ROX, qPCR Master Mix 2X) was used. The threshold cycle (Cq) was normalized to the Cq of housekeeping gene, namely beta actin and was amplified with the samples.

Fluorescent *in situ* hybridization (FISH) analysis

Probe labeling and detection: Probe for fluorescence is labeled for chitinase gene detection.

Results

The study aims to develop transgenic tomato lines tolerant to wilt fungi (*Fusarium oxysporum*). Codon optimized chitinase gene was used in the study to evaluate gene expression.

Detection and identification of mulberry chitinase gene

Chitinase gene of 982 BP was isolated and characterized (Fig. 1).

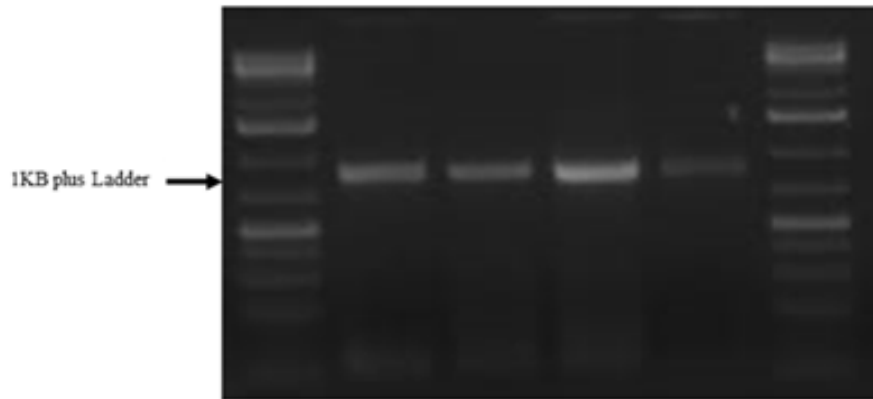


Figure 1. Amplified chitinase gene of mulberry RNA, Band size of 982 BP. Lane 1=1 kb plus ladder, Lane 2-4=samples.

Recombinant chitinase gene expression

Full length 982 BP mulberry chitinase gene was successfully amplified. Sequence of chitinase gene from recombinant TA clone was analyzed and submitted in gene Bank NCBI with accession number PP951523. Fig. 2 indicates restriction digestion of mulberry chitinase gene in pET30a vector.

The amplified mulberry chitinase gene was cloned directionally in pET30a vector with T7 promoter and restriction enzymes BgIII and NcoI were used. Successful cloning was indicated by digestion of recombinant plasmid of pET30a.

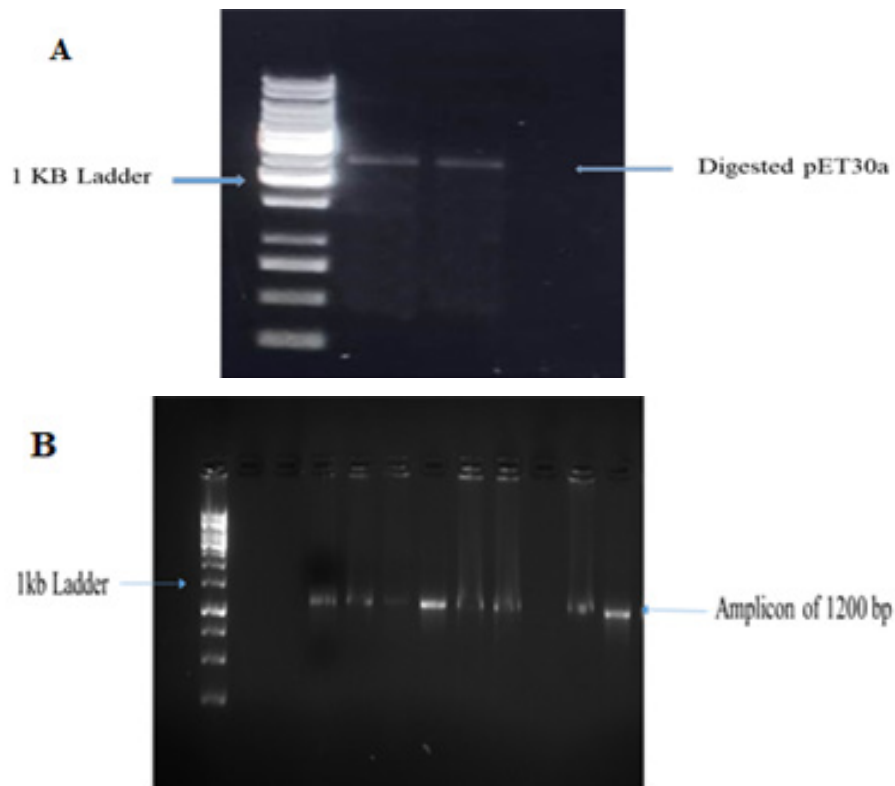


Figure 2. (A) Restriction digestion by EcoR1 and BamH1, Lane 1=1 kb ladder, Lane 2-3=samples of digested vector, (B) Confirmation of chitinase gene ligation in pET30a vector.

Mulberry chitinase gene transformation in tomato plant

Rio-grande tomato variety was transformed with mulberry chitinase gene, *MnChi10* via *Agrobacterium* mediated transformation. Fig. 3 illustrated regeneration, transformation and development of tomato plants. A total of 400 plant nodes were transformed by *Agrobacterium* mediated transformation. *Agrobacterium* strain used was LB4404. A total of 180 plantlets regenerated into complete plants. The transformed regenerated plants were confirmed by PCR amplification of chitinase gene in PCR reaction.

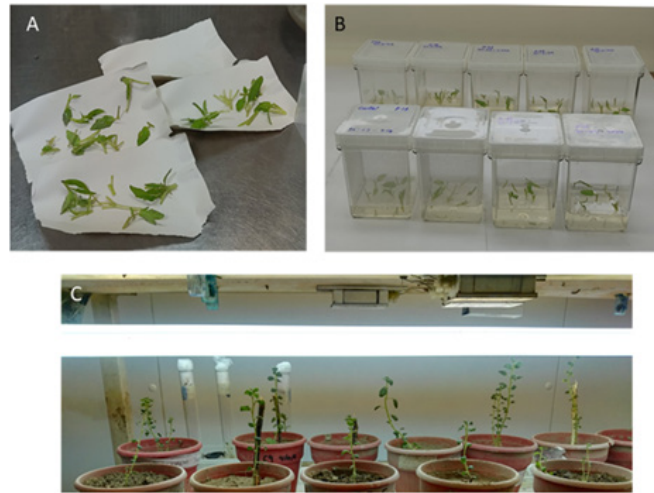


Figure 3. Steps in tomato transformation and regeneration. (A) Tomato internodes (B) Regeneration of tomato internodes after transformation in MS medium (C) Acclimatized tomato plants in pots.

Fish and molecular analysis of transformed tomato plants

In Fluorescence *In Situ* Hybridization (FISH) assay where only one fluorescent signal was detected, it indicates the successful genetic integration of the *MnChi10* gene into the genome of tomato. This result is pivotal for confirming the presence of *MnChi10* gene in tomato chromosome, which can have significant implications for enhancing wilt resistance in tomato crops (Fig. 4).

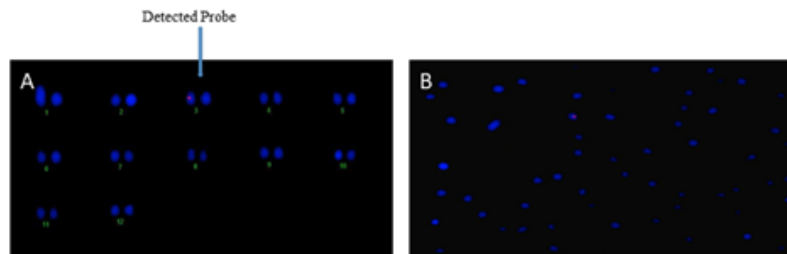


Figure 4. (A) Chitinase gene integration with tomato chromosome, (B) Hybridization of probe with tomato chromosome.

Among 180 transformed regenerated plants, only 50 plants were PCR positive. Among 50 plants 5 plants were showed stable integration of chitinase gene (Fig. 5).

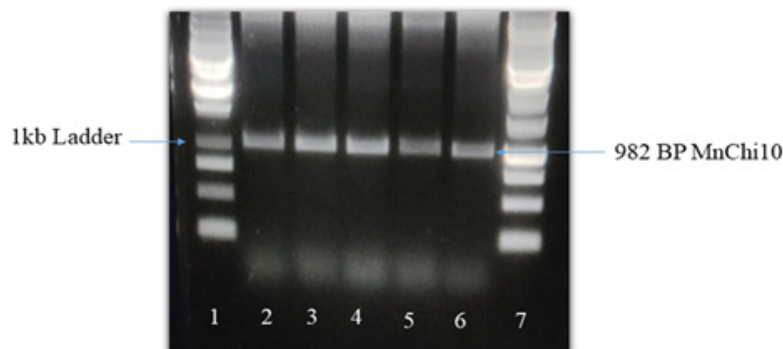


Figure 5. Molecular analysis of transformed tomato plant. PCR of positive tomato plants showed chitinase gene integrated in tomato genome.

Fungal Bioassay

Purified and recombinant chitinase protein was used in this experiment. Fungal inhibition experiment showed that the recombinant chitinase protein of mulberry was quite effective in controlling the said fungi. Fig. 7 showed the significant retardation of growth of inoculated fungi, *Fusarium oxysporum* at 40 µg protein concentration and it was evident from the experiment that fungal inhibition was effective as compared to 10 µg protein concentration. In the *in-vitro* antifungal assay, %age reduction of hyphal growth of *Fusarium oxysporum* was 51.8% at protein concentration of 30 µg, 60% at 50 µg, 95% at 70 µg, 97.5% at 100 µg protein concentration. 70 µg and above protein concentrations did not allow the fungal growth across the well (Fig. 6).

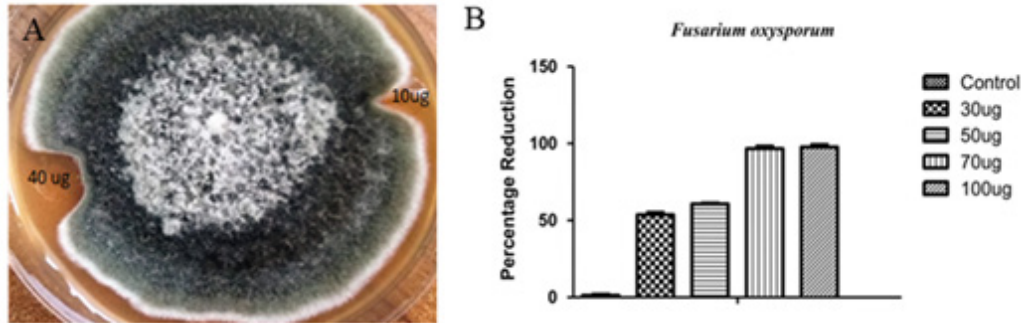


Figure 6. (A) Effect of recombinant chitinase protein on growth of *Fusarium oxysporum*. Zone of inhibition produced by purified recombinant chitinase protein applied along the growing border of *Fusarium oxysporum*. (B) Percentage reduction in the hyphal growth of *Fusarium oxysporum* exhibited by the purified recombinant chitinase protein. The data was obtained in *in-vitro* quantitative antifungal assays. Control sample didn't exhibit any reduction. The results showed that there was significant difference among control and different concentrations in standard value. ($P < 0.01$; $n = 3$).

Chitinase gene expression studies (RT PCR)

Real-time PCR is performed and results showed that the expression of chitinase gene is detected at first after infection by the pathogen than it increases within the passage of time. Increase in %KD value will decrease the gene expression, as %KD value is inversely proportional to gene expression. Initially at time interval 0 h, the *Fusarium oxysporum* fungi attacked tomato plant for disease infection and chitinase gene exhibits its activity but with the passage of time, in time interval 3 h, 6 h, 9 h, 12 h, 18 h, 24 h, 36 h and 72 h chitinase gene expression increases accordingly (Fig. 7).

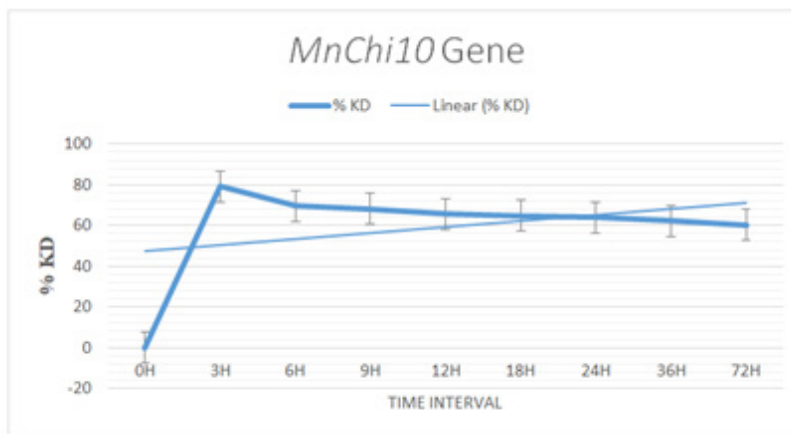


Figure 7. Relative quantification of mRNA knockdown expression of chitinase gene in transformed tomato plants.

Purified chitinase gene structure

Primary structure:

MNMIMIHKMASSKRSCVRVLCILTLTILSCLAWPGMGTRSSSSPTARLISEQLFNTFFLHKDDACAPANTFYTYDAFITATKCF-
PRFASTGSLSTRKREIAAFLAQISHETTGGWPTAPDGPYSWGLCFKEEISPQSDYCDSSNTQWPCSPGKSYKGRGPIQLSWNYNYGPA-
GEALHFDGLGSPEVVANDSVVAFKTAIWFWMTPRRPKPSCHQVMGVEYVPTRDDVAANRTAGYGLVTNIINGGLECGIPNDARVNDRI-
GYFRRYAGLLNVDTPNLDENQKPF

Secondary structure: PSIPRED (<http://bioinf.cs.ucl.ac.uk/psipred/>&uuid=e55400e2-5afb-11ef-b81e-00163e100466) was used for protein secondary structure prediction. Following are its results (Fig. 8 and Fig. 9):



Figure 8. Chitinase gene secondary structure.



Figure 9. Extracellular or periplasmic regions, signal peptide in targeting the protein and membrane interaction in protein.

The amino acids highlighted in yellow indicate extracellular or periplasmic regions. The ones in pink denote the involvement of signal peptide in targeting the protein. Moreover, amino acids spanning from positions 234 to 249 are involved in membrane interaction (Fig. 10 and Fig. 11).



Figure 10. (A) Blue blocks represent the confidence score for each residue in the protein's secondary structure. The higher the peaks, the higher the confidence score is, indicating a greater likelihood of the aforementioned residues being present at the desired positions. (B) Pink colored blocks represent the alpha helices. (C) It represents the fate of each residue, whether it will form alpha helix, beta strand or coil. (D) This row represents the amino acid sequence of protein. (E) Yellow colored blocks represent the beta strands.

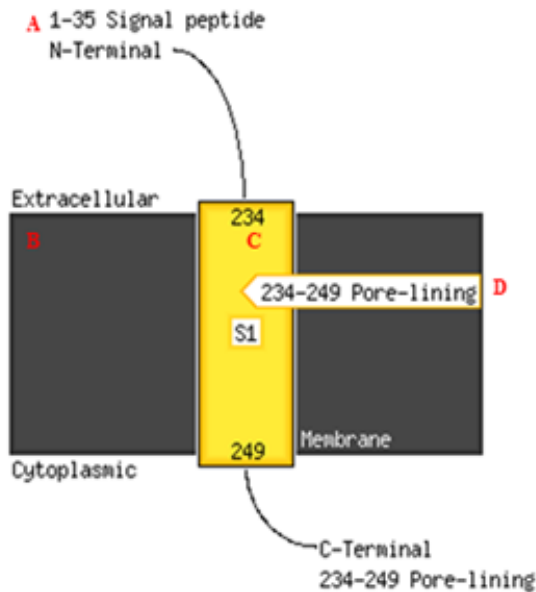


Figure 11. (A) Signal peptide is present at N-terminal or beginning of the protein sequence. (B) Lipid bilayer forming cell membrane. (C) Transmembrane helix. (D) Amino acids residing this region form the lining of the channel, which is present in the membrane.

Tertiary structure: SWISS-MODEL (<https://swissmodel.expasy.org/interactive/sFB2dy/models>) was used for protein tertiary structure prediction. Following are its results (Fig. 12 and Fig. 13):

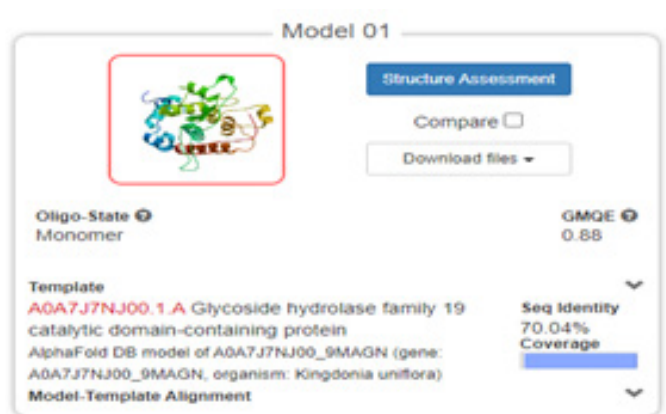


Figure 12. As the GMQE value is 0.88 i.e., close to 1, it represents that the predicted structure is accurate and it is a reliable model.

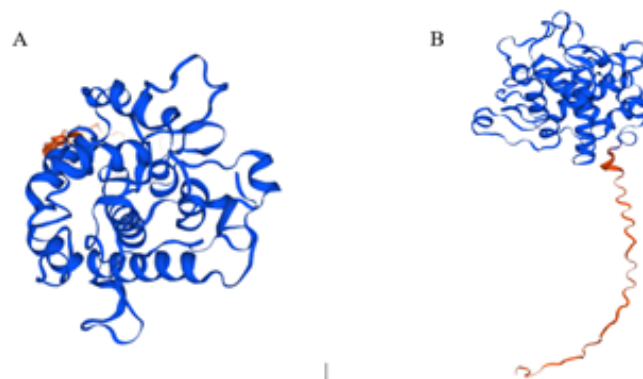


Figure 13. (A and B) Tertiary structures of protein.

trRosetta

To validate the prediction of protein's tertiary structure, trRosetta was also used (<https://yanglab.qd.sdu.edu.cn/trRosetta/output/TR153286/>). Following are its results (Fig. 14):

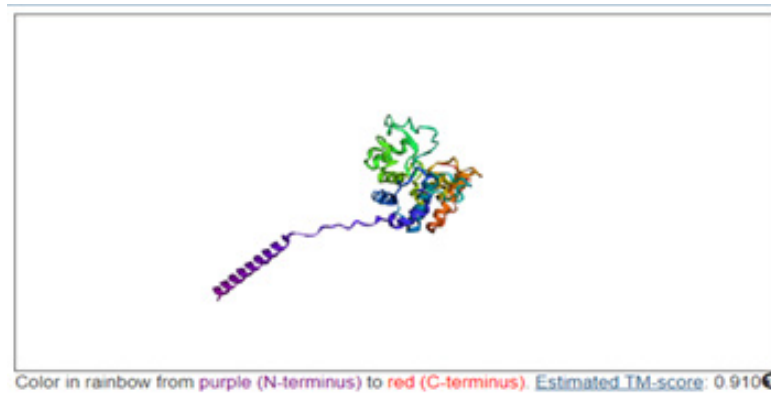


Figure 14. Estimated TM score of this model 0.910. As it is greater than 0.5, it indicates a model with correctly predicted topology.

TM score greater than 0.5 shows that the overall folding of protein is close to its native structure and has a correct topology.

Discussion

Plant chitinases are safe and biodegradable biocontrol agent that can be used in place of common fungicides (Kumar and Vivekanand, et al. 2018). Chitinases cleaves β -1,4-glucosidic bonds of chitin, which is main component in cell wall of fungi (Kumar, et al. 2018). Plants do not have the ability to produce Chitin by natural process that's why plant chitinases has been identified to discharge elicitors for plant defense by hydrolyzing the pathogen cell wall (Hibbett, et al. 2011). Chitinase gene is effectively transformed into other plants to enhance resistance against various fungal pathogens and insects (Fisher, et al. 2012). It has been reported that various chitinases from many plants and fungi were stable to different ranges of pH and temperature (Bussink, et al. 2007). Our study aimed to evaluate the expression of chitinase gene in tomato plants and overall efficiency of *MnChi10* gene to control *Fusarium* wilt disease. Overall functionality of the chitinase gene in transgenic product was analyzed through transfer of desired chitinase gene in tomato plant. Real time PCR was performed with transgenic plants to check chitinase gene level at various intervals of time. It showed that at time interval 0 h the chitinase gene expression in transgenic plants is detected after infection, from time interval 3 h to 72 h, the expression of chitinase gene is increasing continuously.

It depicted that as chitinase gene level is increasing, it helps the plant to cope the diseased fungi. Chromosomal studies by Fluorescent *In Situ* Hybridization (FISH) showed that the transformation of chitinase gene in tomato plant. Chitinase gene is successfully incorporated in the genome of tomato plant. The fluorescent probe is shown on chromosome pair 3 of the total chromosomes. In Fluorescence *In Situ* Hybridization (FISH) assay where only one fluorescent signal was detected, it indicates the successful genetic integration of the *MnChi10* gene into the genome of tomato. This result is pivotal for confirming the presence of *MnChi10* gene in tomato chromosomes, which can have significant implications for enhancing disease resistance in tomato crops. Chitinases genes are involved in plant safety against damaging organisms/plant pathogens (Downing and Thomson, 2000). Most of the transformed plants showed mild symptoms upon infection or didn't showed symptoms which showed successful integration of chitinase gene in tomato genome (Kabir, et al. 2016). Chitinases are revolutionary in agriculture sector because they alter the use of chemicals on the plants (Kumar and Vivekanand, et al. 2018, Dean, et al. 2012). Our results documented the transgenic plants with chitinase gene *MnChi10* recorded enhanced resistance against *Fusarium* wilts.

Conclusion

In conclusion, plant chitinases can be widely used to control fungal diseases and increase the resistance of plants. The tomato plant showed greater flexibility for incorporation the *MnChi10* gene from the. *MnChi10* gene showed promising results to build resistance in tomato against wilt fungi. There is a strong need to commercialize the transgenic tomato to support farmers' livelihoods and increase national income to boost the economy.

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Consent to Publish

All the authors declared they have no objection to publish manuscript.

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