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Research Article

CLONING OF ENDOGLUCONASE GENE OF *RALSTONIA SOLANACEARUM INTO* E COLI.: BL21DE3PLYS E CELLS

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A B S T R A C T

Ralstonia solanacearum a proteobacterium, is responsible for causing wilting in a wide host range of plants specially to solanaceae family including potato, tomato, etc. *R. solanacearum* has many proteins that act as virulence factors and cause wilting by colonizing the xylem vessels functioning as cellulase enzyme thereby blocking the water transport and finally facilitating wilting. To control the crop loss caused by the organism, an identification kit for its early detection is being manufactured. The kit will work on the principle of antigen-antibody interaction where the causing agent i.e. the Egl protein will be taken as antigen and monoclonal antibodies will be produced against it. Therefore, the Egl gene is cloned here which would be further expressed in order to produce a bulk amount of protein for antibody preparation.

INTRODUCTION

Ralstonia solanacearum, a β-proteobacterium, is a soil-borne pathogen infecting over 200 plant species belonging to over 50 different botanical families. The pathogen affects not only Solanaceous plants, like tomato and potato, but many weeds, crops, shrubs, and trees belonging to([1]) both monocots and dicots, extending from annual plants to trees and shrubs **([2])** Bacterial wilt is taken into account among the foremost destructive bacterial plant diseases due to its extreme aggressiveness, world-wide geographic distribution, and broad host range. The common name for the disease varies with the host that's attacked. In tobacco, it's called bacterial wilt or wilt and Moko disease in banana. it's sometimes called southern wilt or southern bacterial wilt within the hemisphere ([3])

The bacterium enters plant roots, invades the xylem vessels, and spreads rapidly to aerial parts of the plant through the system. The vascular dysfunction induced by this extensive colonization causes wilting symptoms and eventual plant death. Signs of the pathogen infection include slimy, sticky ooze which forms a tan-white to brownish beads where the plant tissue is cut **([4])** If one amongll|one amongst|one in every of the cut ends is suspended in a clear container of unpolluted water, bacterial ooze will form a thread within the water. Typical disease symptoms include browning of the xylem, chlorosis, stunting, wilting and also the infected plants usually die rapidly **([5]).**

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R. solanacearum species complex (RSSC). A species complex is defined as a cluster of closely related isolates whose individual members may represent over one species. The term "species complex" was first applied to R. solanacerum by Gillings and Fahy to reflect the phenotypic and genotypic variations within the species. Later the R. solanacearum species complex concept was expanded to incorporate two closely related organisms, the blood disorder bacterium (BDB) and Ralstonia syzygies as both of these organisms were found to fall within the range of R. solanacearum as defined by 16s rDNA sequence analysis. Studies of DNA-DNA homology of R. solanacearum strains have revealed that the relatedness between isolates of this species is commonly below the 70% strength commonly expected within a species. R. solanacearum has been widely accepted as a model organism for the study of bacterial virulence and pathogenicity in plants. To date, many factors are found to contribute to the virulence of R. solanacearum. Some major virulence factors during this pathogen are:-Extracellular Polysaccharide I (EPS I) - A heterogeneous polymer of N-acetylated extracellular polysaccharide I (EPS I); with a trimeric repeat unit of N-acetyl galactosamine, 2-N-acetyl-2-deoxy-L-galacturonic acid, and 2-N-acetyl-4-N-(3-hydroxybutanoyl)-2-4-6-trideoxy-D-glucose **([6]).** hrp genes - The hrp genes are clustered on the megaplasmid **([7]),** and encode components of a T3SS and effector proteins. Type III secretion system (T3SS), a syringe-like membrane appendix that injects the so-called 'effector proteins' (type III effector proteins, or T3E into the plant cell cytosol to favor infection ([8]). Cell-Wall-Degrading Enzymes (CWDEs) - Phytopathogenic bacteria have often developed

enzymes to hydrolyze plant cell membrane components to get nutrients and energy, which are involved within the early stages of the infective process, favoring the entry and advance of the pathogenic agent in host tissues. R. solanacearum secretes several CWDEs, including three polygalacturonases (PehA, PehB, and PehC) ([9]). an endoglucanase (Egl) ([10]), a pectin methylesterase (Pme) ([11]), and a cellobiohydrolase (CbhA) ([12]). Other pathogenicity determinants - lipopolysaccharide (LPS) and lectins ([13]). Two genes acrA and dinF encoding multidrug efflux pump also contribute to its virulence ([14]).

R. solanacearum β-1,4-endoglucanase is supposed to be a 43-kDa protein that has proved to be involved in pathogenicity. The protein will be used as an antigenic marker for Ralstonia solanacearum infection in plants. The presence of this marker protein is detected by specific antibodies raised against the recombinant variety of β-1, 4-endoglucanase through hybridoma technology which might be wont to develop immune-based detection systems.

MATERIALS AND METHODS

Primer designing for PCR based cloning & standardization of annealing temperature

The gene sequences of Endogluconase gene from 6 different strains (PSI07, GMI1000, RS 10 244, RS 09 161, CMR15, CFBP2957) of *Ralstonia solanacearum* were retrieved from NCBI- Gen Bank. The retrieved gene sequences were aligned using the offline tool CLC-Sequence Viewer. Primers (Forward and Reverse) were designed such that they bind specifically to the Endogluconase gene. The target specificity of the designed primer sequences against *Ralstonia solanacearum* was determined by performing NCBI-BLAST i.e. PRIMER-BLAST analysis. The presence of secondary structures within and between the two primers was analyzed using bioinformatics software Oligo analyzer. The designed oligonucleotides EglpET22bF (forward primer) and EglpET22bR (Reverse Primer) as given in Table 1 results in a PCR product of 1263bp. Gradient PCR master mix was prepared using *R. solanacearum* (G1C1b) DNA at temperature range (50-65)^{0}C to determine the annealing temperature. Appropriate Restriction Digestion sequences were added at the 3' and 5' end of the primers after performing Insilco digestion of the EGL sequence with restriction enzymes in multiple cloning sites in pET 22b vector.

Table 1 Constructed primers

Primer	Primer sequence	Length
EglpET22bF	5'-AAGGAATTCGCTGGTTGCCGCTTC -3'	24
EglpET22bR	5'-CAGAAGCTTCTTCAGGTACGGCGC-3'	24

Bacterial cultures used, Enzymes, Chemicals, Genomic DNA extraction

The previously characterized bacterial *Ralstonia solanacearum* (G1c1b) strain used was obtained from the culture collection at DIBER, halwani and *Escherichia coli* BL21DE3plysE strain was purchased from Qiagen (Germany). Taq DNA polymerase, dNTPs, $MgCl_2$, Betaine, Ampicillin, Chloramphenicol, LB Agar, SMSA media, pET-22b cloning vector and other chemicals (Sigma, Himedia) were procured. The culture of *R. solanacearum* standard strain G1c1b was maintained on SMSA plates from which a single colony was picked and inoculated in 5ml LB broth and grown overnight at 37^{0}c. Well grown culture was harvested and DNA was extracted using HiPurA Bacterial Genomic DNA Purification Kit as per the manufacturer's instructions. The concentration and purity of DNA was checked using absorbance at 260 and 280 nm's. After performing the PCR, the products were electrophoresed in 1.2% agarose gel and the bands were visualized to obtain the annealing temperature.

PCR amplification and cloning of Endogluconase gene

Primers for Endogluconase gene of *R. solanacearum* were *in silico* designed using CLC Sequence viewer and Oligo analyzer, on the basis of sequences retrieved from NCBI-GenBank. These primers were chemically synthesized from Integrated DNA Technology (IDT, USA). The PCR reaction was performed by 50ul reaction mixture containing 1X PCR buffer,1.5mM $MgCl_2$, 200uM Nucleotide mix, 3unit/100ul Taq DNA polymerase, 20pmol/25ul of EglpET22bF and EglpET22bR each , 10ng/ul of DNA template. Total 35 cycles were performed with following conditions : preheating to 95^{0}C for 3 mins, 1 min denaturation at 95^{0}C, followed by 20-sec annealing at 57°C and 1 min and 15-sec's extension at 72^{0}C with final extension of 3 min at 72^{0}C. The PCR amplicon (Amplified EGL gene) was visualized on 1.2% agarose gel electrophoresis using 5 ul of the PCR product in reference to a 1-kb ladder. The PCR product was purified using QIAquick PCR Purification Kit as per the noted instructions. Competent cells were prepared using the procedure described by Cohen in (1972) i.e. by Calcium chloride method. Restriction digestion of the pET22b vector and the EGL gene for insert was done by specific restriction endonucleases **[EcoR1, Hind III]** specific for their restriction sites **[GAATTC, AAGCTT]** to create sticky ends for further ligation process. After digestion both the vector and the insert were run on Low Melting 0.6% Agarose Gel ,the separated bands obtained were cut from the gel and were extracted from within the gel for ligation process. The purified DNA was ligated with the pET-22b cloning vector at 4^{0}C in a 3 day incubation procedure using T4 DNA Ligase enzyme. The constructed recombinant cells after ligation were transformed in plysE cells through the CaCl2 method. Screening of transformants was done in two steps. Primarily a Colony PCR was performed by picking colonies from the master plate with (ampicillin, chloramphenicol) and dispersing in 500µl water each and finally using it as template for PCR (1ul) each reaction. Secondly the plasmid DNA was isolated from the primary confirmed colonies using the Plasmid DNA extraction kit and PCR was done taking those as templates (1ul) each. The PCR product obtained was visualized on 1.2% agarose gel to confirm the presence and size of the cloned insert in the plasmid.

RESULTS

Primer Designing for PCR based Cloning & Standardization of PCR Parameters

Cloning primers were designed using sequences retrieved from NCBI-GenBank of 6 different strains of *Ralstonia solanacearum* coding for EGL gene, these sequences were aligned in CLC Sequence Viewer. From the consensus sequence obtained after multiple sequence alignment a length of 15 bp nucleotide sequence which had the maximum

similarity in the alignment with least no. of mismatches, was selected as the primer sequence.

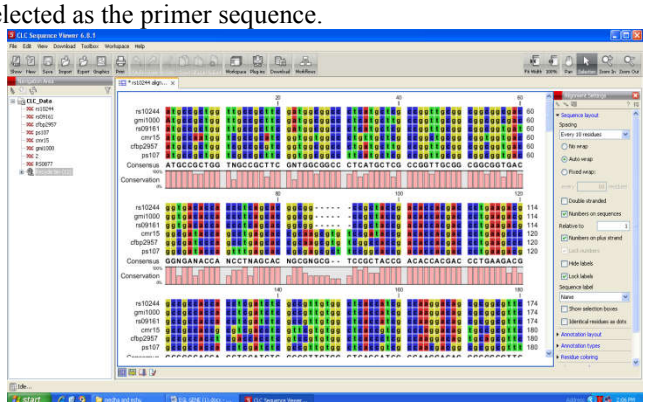

Figure 1. This image exhibits a screenshot of CLC Sequence Viewer software showing alignment in Endogluconase gene sequences from 6 different strains of *Ralstonia solanacearum* (PSI07, GMI1000, RS 10 244, RS 09 161, CMR15, CFBP2957) indicating consensus sequence in them.

The probability of secondary structure formation in the primer itself and also with the sequence to be amplified (Endogluconase gene) was checked using the tool Oligo Analyser.

Figure 2 This image exhibits a screenshot of Oligo Analyser software indicating no chance of secondary structure formation in the constructed Forward Primer

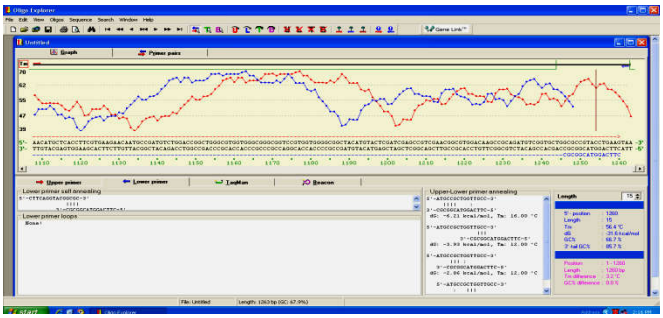

Figure 3 This image exhibits a screenshot of Oligo Analyser software indicating very fewer chances of self complementarity in the constructed Reverse Primer

The efficiency and target specificity of the constructed primers was checked using a BLAST tool specifically PRIMER BLAST. It checks for the target gene sequence to primer specificity.

On insilico digestion of the EGL gene it was found that the enzymes EcoR1 and Hind III were absent in the gene. Hence the sites GAATTC (Eco R1) and AAGCTT (Hind III) were added to the forward and reverse primers respectively.

The sequence of the constructed primers:

Primer	Sequence	Tm	Molecular Weight	GC Content	Length
Forward	5'-AAG**GAATTC**GCTGGTTGCCG CTTC 3'	60.8^{0}C	7353.9	58.3%	24
Reverse	5'-CAG**AAGCTT**CTTCAGGTACG GCGC-3'	59.1^{0}C	7359.9	54.2	24

It was found that no binding sites were available for the primers in *R. solanacearum* genome except the sites in EGL gene and the primer produces a theoretical amplicon of 1263bp as analyzed by NCBI PRIMER BLAST.

Cloning of Endogluconase gene into BL21DE3PlysE E.coli cells

For PCR amplification of EGL gene the genomic DNA from the G1C1b strain of *R.solanacearum* was extracted, and its concentration was determined using Multimode Plate Reader, at A_{260} which was found to be 55ng /µl. This isolated DNA was used for the determination of the annealing temperature of the constructed primers using it as template in PCR, employing thermostable *Taq* DNA polymerase, forward and reverse primers targeting the gene encoding the β-1,4-endoglucanase protein of *R.solanacearum* on variant temperature range i.e. [50^{o}c-65^{o}c]. The maximum amplification was found to be at 58.3^0 as seen in the 9th lane of figure 4. The PCR amplification resulted in 1263bp amplicon, which was visualized on 1.2% agarose gel.

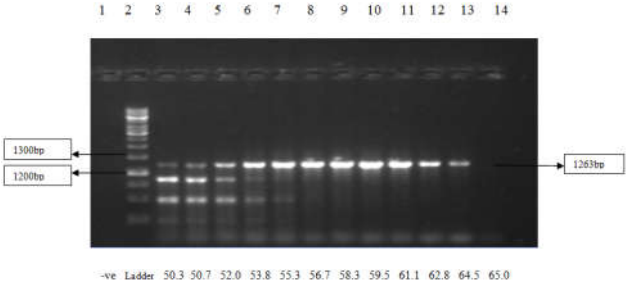

Figure 4 This image exhibits gradient PCR product of 1263bp showing Endogluconase gene amplification from *R.solanacearum* G1C1b strain.

Lane1- Negative control ,Lane2- 100bp ladder ,Lane3-50.3□C,Lane4-50.7□C ,Lane5-53.8□C ,Lane6-55.3□C,Lane7-55.3□C ,Lane8-56.7□C ,Lane9-58.3□C,Lane10-59.5□C,Lane11-61.1□C ,Lane12-62.8□C,Lane13-64.5□C,Lane14-65□C

The purified PCR product and vector pET22b were digested then ligated and finally transformed into *E. coli* BL21DE3PlysE strain by CaCl2 method. The transformed cells were then screened and selected on LB plates with ampicillin (50ng/µl) and chloramphenicol (50ng/µl) antibiotics. A total of 36 clones were seen on the plate with the spreading of 200µl of the transformed mix. Colony PCR was done of the five selected colonies from the above plate. The presence of the insert was confirmed by Colony PCR using the same primers used earlier. The PCR result revealed the presence of insert in all the 5 clones.

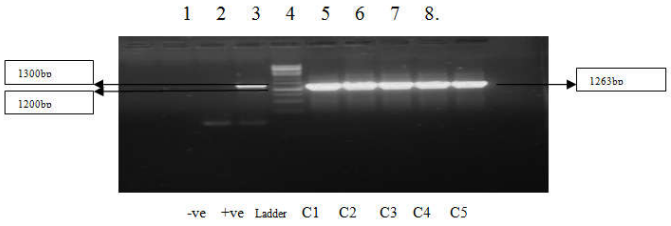

Figure 6 This image exhibits a 1.2% agarose gel with Colony PCR products of 1263bp obtained by applying EGL gene primers to the five screened and selected colonies picked from the (antibiotic+LB) master plate spread with transformants.

Lane1-Negative control, Lane2-Positive control, Lane3-100bp ladder, Lane4-C1, Lane5-C2, Lane6-C3, Lane5-C4, Lane8-C5.

Further plasmid DNA was isolated from the above 5 colonies and PCR was done using it as template ,which showed amplification in 1.2% agarose gel. Among the total 5 colonies giving the primary confirmation, only two colonies gave the optimum amplification after PCR confirming two pure clones only.

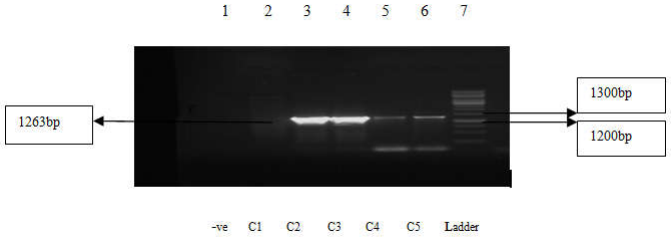

Figure 7 This image exhibits 1.2% agarose gel containing 1263bp PCR products obtained by using isolated plasmid DNA as a template from the five primarily confirmed colonies with EGL gene primers.

Lane1-Negative control, Lane2-C1, Lane3-C2, Lane4-C3, Lane5-C4, Lane6-C5, Lane6-C6

DISCUSSION

A 1.2 kb DNA fragment from R. solanacearum encoding cellulase activity is cloned in E. coli cells. PCR experiments demonstrated that the cloned DNA fragment contains the EGL gene that encodes a 43-kDa polypeptide which is the major endoglucanase excreted by many R. solanacearum strains. A possible role for the 43-kDa EGL in phytopathogenicity is implied by its potential to degrade plant cell wall components (i.e., glucans) thereby hindering water transport. However, the ability to produce enzymes that can destroy plant cell components is insufficient evidence to explain that such enzymes are involved in or are necessary for pathogenesis therefore detailed study of the endogluconase gene expression and understanding the exact mechanism and pathway involved is important .However in our study we have cloned the Endogluconase gene facilitating towards the construction of the aimed detection kit for early diagnosis of the wilting disease.

CONCLUSION

R. solanacearum,s β-1,4-endoglucanase is a 43-kDa protein that has proved to be involved in plant pathogenicity. The protein can be used as an antigenic marker for *Ralstonia solanacearum* infection in plants that lead to wilting disease. The presence of this marker protein can be detected by specific antibodies raised against the recombinant form of β-1,4-endoglucanase which can be used to develop immune-based detection systems.

In this work the gene encoding the β-1,4-endoglucanase is cloned in pET 22b vector and transformed into *Escherichia coli* BL21DE3 plys E cells**,** giving future targets of its expression and monoclonal antibody production against it.

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