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TRANSGENIC *LYCOPERSICUM ESCULENTUM MILL* (TOMATO) PLANT AS BIOREACTOR FOR THE PRODUCTION OF HUMAN NEUTROPHIL PEPTIDE-1 (HNP-1): A USEFUL PROTEIN BASED PHARMACEUTICAL

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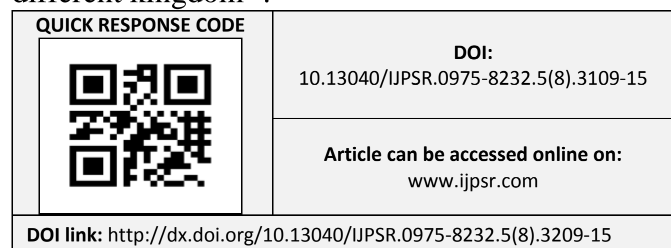
ABSTRACT: The term 'transgenic plant' is defined as the plants that are produced in the lab by artificial insertion of genes sometimes from the same species and sometimes entirely from the different kingdom. A major advantage of transgenic plant because of which it gains a lot of importance in today's world is developing it for producing pharmaceutically useful compounds. Hence, they are acting as a natural bioreactor for producing therapeutic proteins and peptides in large amount. The present work deals with the investigation of producing a Human Neutrophil Peptide-1 (HNP-1) which is an antimicrobial peptide and commonly known as Defensin in *Lycopersicum esculentum* Mill. plant by *Agrobacterium tumefaciens* mediated genetic transformation technique. The cloning of HNP-1 was successfully done in a plant expression vector 'pGreenI 0029 with 35S CaMV promoter' by authors and described earlier. In this work we would like to describe transformation of pGreenI 0029 with HNP-1 gene and 35S CaMV promoter, inside *Agrobacteium tumefaciens* cells and then genetic transformation of *Lycopersicum esculentum* Mill. plant via recombinant *Agrobacteium tumefaciens* by seed cotyledon method. The transgenic tomato plants were screened for the presence of HNP-1 gene. The isolated total soluble proteins from tomato leaves were subjected for *in vitro* antimicrobial activity and showed antimicrobial activity against various pathogenic microorganisms like *S. aureus, B. subtillis, E. coli* and *Candida albicans*.

INTRODUCTION: The term 'transgenic plant' is defined as the plants that are produced in the lab by artificial insertion of genes sometimes from the same species and sometimes entirely from the different kingdom [1].



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Some of the important advantage of producing transgenic species are they give biotic and abiotic stress resistance to the plant [2-3]. The biotic stress resistance includes resistance to various pathogenic organisms [4]. The abiotic stress includes genes which are inserted into the plant that gives resistance for extreme heat, cold or salinity because of which plant shelf life increases [5]. A major advantage of transgenic plant because of which it gains a lot of importance in today's world is developing it for producing pharmaceutically useful compounds [6].

Hence they are acting as a natural bioreactor for producing therapeutic proteins and peptides in large amount. The aim of the present work was producing a Human Neutrophil Peptide-1 (HNP-1) which is an antimicrobial peptide commonly known as Defensins in *Lycopersicum esculentum* Mill. plant by *Agrobacterium tumefaciens* mediated genetic transformation technique. The Defensin was selected as a model drug to be expressed inside the transgenic *Lycopersicum esculentum* Mill. (Tomato) plant since they are small 3-4 kDa antimicrobial peptides found in the neutrophil cells of human blood and have shown to exert broad range of pharmacological activities [7-9].

They are active against various gram positive and negative bacteria, fungi and *Mycobacterium tuberculosis* [10, 11]. They are also shown to be cancer biomarkers and in treatment of cancer since they initiate apotosis in cancerous cells [12, 13]. They also increase immunity to the patient against various infections and therefore useful for patient with HIV infection [14]. The plant *Lycopersicum esculentum* Mill. (Tomato) was selected on the basis of some of the characteristics like popular vegetable, fruit does not contains toxic substances, easily processed, easily propagated by means of seed or by tip or shoot cuttings [15]. There is high plant biomass and it can be easily grown under containment.

For expression of antimicrobial Defensin protein inside *Lycopersicum esculentum* Mill. (Tomato), plant expression vector 'pGreenI 0029 with 35S CaMV promoter' was selected [16]. Since pGreenI 0029 vector as such does not contain plant promoter cassette, it was decided to clone the 35S CaMV promoter inside the pGreenI 0029 plasmid. The Defensin protein was cloned inside CaMV promoter using restriction enzyme site BamHI/EcoRI which is present inside the CaMV promoter cassette.

MATERIALS AND METHODS: pGreen vector with 35S CaMV promoter was used for expression study, *Lycopersicum esculentum* Mill. (Tomato) plants were used as a host for expression. All other reagents like Tomato regeneration medium, MSO medium, SOB medium and other tissue culture media and apparatus were purchased from Hi Media.

All the experiments were conducted after the Institutional Bio Safety Committee (IBSC) approval.

Genetic Transformation of *Lycopersicum esculentum* Mill. (Tomato) plants via *Agrobacterium tumefaciens EHA 105* [17-19]:

1. **Preparation of competent cells of Agrobacterium tumefaciens EHA 105 by $CaCl_2$ method:** A freshly grown colony of *Agrobacterium tumefaciens EHA 105* was picked up and inoculated into 50 ml of Luria Bertani (LB) medium with Rifampicin (5µg/ml). The flask was incubated at 28^0C with vigorous shaking for 48 hours. 2 ml of the grown culture was reinoculated in to 50 ml of the same medium with Rifampicin (5µg/ml). The incubation was continued will the O.D. reaches to 0.5 and chilled in ice. The cells were then transferred aseptically to 50 ml ice cold centrifuge tube and centrifuged at 4000 rpm for 10 minutes in refrigerated centrifuge. The supernatant was discarded and cells were suspended in 5 ml ice cold 20 mM $CaCl_2$ solution. The centrifugation was repeated and supernatant was discarded. Finally the cells were resuspended in 1 ml of ice cold 20mM $CaCl_2$ containing 10% glycerol. Aliquots of the cell were made in 100µl quantity in 1.5ml centrifuge tubes. The cells were stored at -70ºC for future use.

2. **Transformation of Agrobacterium tumefaciens EHA 105 with recombinant pGI0029 with 35 CaMV promoter and HNP-1 gene:** 10µl (~1µg) of pGI0029 recombinant plasmid was added into 100µl of competent cells. A control sample without plasmid was also kept along with this. Both the tubes were kept in liquid nitrogen for 5 minutes for freezing. The frozen tubes were taken out and thawed for 10 minutes at room temperature. The content of the tubes were then transferred to the culture tubes containing 2 ml of LB and incubated with shaking at 28^oC for 4 hours. The cells were pelleted by spinning for 2 minutes at high speed in refrigerated ultracentrifuge. The supernatant was discarded and the pellet was resuspended in 0.5 ml of SOB medium containing Kanamycin (50 µg/ml).

100µl of the above mixture was then plated on SOB Agar media containing Kanamycin (50µg/ml) for selection.

3. **Transformation of Tomato with *Agrobacterium tumefaciens EHA 105* containing recombinant pGI0029 [15, 20]:**

   a. **Sterilization and Preparation of seeds:** Dry seeds of tomato were sterilized in 20% household bleach and 0.1% Tween 20 for 15 minutes followed by three times rinsing with sterile distilled water. The sterilized seeds were germinated on ½ × MSO medium in sterile petridish under dark box. They were grown at 26°C for 10-14 days.

   b. **Transformation:** When seedling comes out, cotyledons were used for transformation. The top of the seedling were cut off and floated on the liquid ½ MSO medium. Cotyledons were cut near the proximal end and while submerged in ½ MSO medium. These cotyledons were placed upside down on petridish containing D1 medium. 5 ml of a 20 fold diluted 2 day old culture of *Agrobacterium tumefaciens EHA 105* containing pGreen plasmid with 35S CaMV promoter was poured over the cotyledons on the surface of the plate and incubated for 2 hours. After this, the excess culture was removed with the help of a pipette. The plates were also swirled to ensure the touching of the *Agrobacterium tumefaciens EHA 105* with the cut surface. The plates were then closed and incubated at 26^{0}C and light (16L/8D) condition in tissue culture station for two days.

   c. **Co-Cultivation and transfer of plants to selection medium:** After two days of co cultivation, the cotyledons were transferred to deep plates (Tissue Culture Jars) containing selection medium with 500 pg/ml Carbenicillin and 100 pcg/ml Kanamycin. The cotyledons were transferred carefully to the selection medium with upside down position.

   d. **Transfer to Tomato regeneration medium (Shoot and Root Organogenesis):** After 3 weeks, cotyledons were transferred to Tomato regeneration medium with 500µg/ml Carbenicillin and 100 pcg/ml Kanamycin for shoot organogenenis. Here care was taken to transfer entire explants. After three weeks, plants were excised away and transferred to the rooting medium (MSO + Carbenicillin + Kanamycin). Under this condition plant was rooted for 7 to 10 days.

   e. ***Transfer to Soil Pot:***The plants were then transferred to very wet soils. These pots were incubated under low light conditions for around 5 days. They were left here till they achieve reasonable size for future work.

Molecular Analysis of transgenic tomato plants [21, 22]:

1. **Isolation of genomic DNA of tomato plant:** Fresh tomato plant leaves and small portions of shoots were homogenized with 2% Sucrose (as cryo protectant) and lyophilized for 18 hours in Lyophilizer. 1 gram of dried powder was mixed with 25 ml EB (extraction buffer) in a 50 ml capped centrifuged tube. The tube was placed into 65^{0}C water bath and incubated for one hour. The contents of the tube were mixed by inversion several times during the hour. The tube was removed from water bath and allowed to cool for 10 minutes at room temperature. 20 ml of chloroform was added to the tube and then capped properly and mixed by inversion until the contents are thoroughly mixed. The tube was then centrifuged at 5000 rpm for 10 minutes to separate out the layers. Using micropipette, the top layer was removed carefully in fresh autoclaved capped centrifuged tube and 2/3rd volume of isopropyl alcohol was added to this. The contents were mixed gently by upside down when cottony mass of DNA was precipitated. The tube was centrifuged for 5 minutes at 3500 rpm to pellet down the DNA. The supernatant was removed and pellet was washed with Wash Buffer (80% ethanol, 15 mM ammonium acetate). The DNA was transferred to micro centrifuge tube and again washed with 70% ethanol. Finally the DNA was dissolved in 750µl of TE buffer.

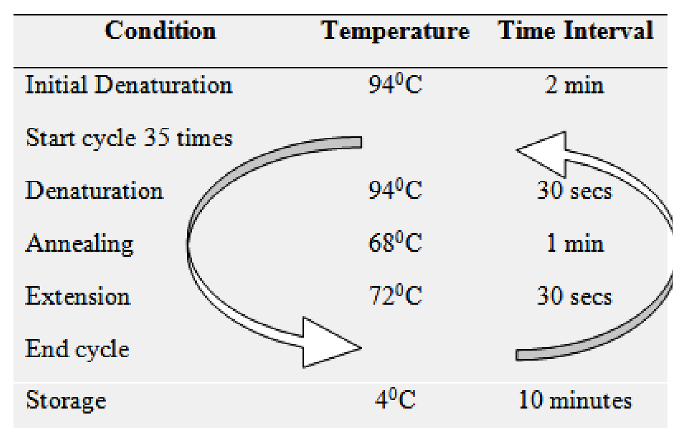
2. **Gel Electrophoresis of genomic DNA of tomato leaves:** A 0.8% Agarose gel was casted and isolated DNA was allowed to run in 0.5X Electrophoresis Buffer to check the quality of genomic DNA of tomato plant. The gel was stained with Ethidium Bromide (10 mg/ml) and visualized under UV transilluminator.

3. **PCR Analysis of genomic DNA:** To detect the presence of Defensin gene, a Polymerase Chain Reaction (PCR) was carried out. A 20µl reaction was set (Table 1). Different temperatures of PCR i.e. Denaturation, Annealing and Extension were also set (Table 2).

TABLE 1: REACTION COMPONENTS FOR PCR ANALYSIS OF HNP-1 GENE

Sr. No.	Name of Component	Conc^n Given	Conc^n Taken	Qty. taken for 20µl
1	10X Buffer + $MgCl_2$	10	1X	2µl
2	dNTPs	2.5 mM	0.25mM	2µl
3	Template	50ng/ml	100ng	2µl
4	FP	10 pMol/µl	30 pMol/µl	3µl
5	RP	10 pMol/µl	30 pMol/µl	3µl
6	Taq DNA polymerase	5 U/µl	2.5 U/µl	0.5µl
7	RO water	-	-	7.5µl

TABLE 2: REACTION CONDITIONS FOR PCR ANALYSIS OF HNP-1 GENE



Condition	Temperature	Time Interval
Initial Denaturation	94^0C	2 min
Start cycle 35 times		
Denaturation	94^0C	30 secs
Annealing	68^0C	1 min
Extension	72^0C	30 secs
End cycle		
Storage	4^0C	10 minutes

Extraction of soluble proteins from transgenic tomato leaves [23]: 5 grams of leaves were homogenized using 2% sucrose as cryoprotectant and lyophilized for overnight in lyophilizer. The dried powder was homogenized in extraction buffer (10mM KCl, 9.6mM, $NaH_2PO_4.2H_2O$, 15.2mM Na_2HPO_4, 150mM NaCl, pH 7, 10 ml) with a boiling for 10 minutes at $100^{\circ}C$. The mixture was centrifuged at 13,000 rpm for 30 minutes and supernatant was precipitated with Ammonium Sulfate at 70% relative saturation. The precipitated protein fraction was collected by centrifugation at $4^{\circ}C$ at 13,000 rpm for 30 minutes. The pellet was dissolved in 2 ml of ddH_2O and filter sterilized using 0.22 µm membrane filters.

Protein concentration was quantified using Bradford Assay with BSA as standard.

***In vitro* antimicrobial activity of isolated protein from transgenic Tomato plants [24]:** The crude soluble protein, isolated from Tomato plants were tested against different microorganisms like *E-coli*, *S. aureus & B. subtilis* by cup plate method to check its biological activity. The cultures of different microorganisms were inoculated one day before the testing of antimicrobial activity. The nutrient agar media was poured into sterile petridish aseptically under Laminar Air Flow (LAF). When the media gets solidified, cultures were inoculated by Swab method and four cups were made with the help of sterilized Borer and Lifter. Different dilutions of isolated protein solution (20, 40, 80 and 100 mgL^{-1}) was filled in four cups and kept in refrigerator for an hour for proper diffusion. The plates were then kept for incubation at 37^0C for 18 hours. Next day zone of inhibition was measured.

RESULTS AND DISCUSSIONS:

Genetic transformation of *Lycopersicum esculentum* Mill. (Tomato) plants via *Agrobacterium tumefaciens* EHA 105: The recombinant pGreenI 0029 plasmid with Defensin protein and 35S CaMV promoter was successfully transformed inside *Agrobacterium tumefaciens EHA 105* using 'Freeze Thaw' method (**Figure 1**). The recombinant *Agrobacterium tumefaciens EHA 105* was used to transform *Lycopersicum esculentum* Mill. (Tomato) by seed cotyledon method (**Figure 2 & 3**).

FIGURE 1: TRANSFORMATION OF COMPETENT *AGROBACTERIUM TUMEFACIENS EHA 105* WITH pGREENI 0029 CONTAINING DEFENSIN WITH 35S CAMV PROMOTER

FIGURE 2: GERMINATION OF STERILIZED TOMATO SEEDS IN ½ MSO MEDIUM

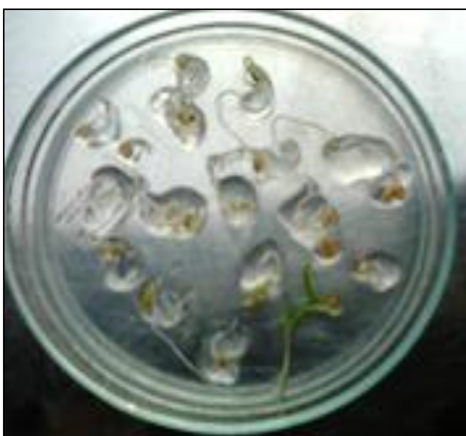


FIGURE 3: AGROBACTERIUM TUMEFACIENS EHA 105 INFECTION TO COTYLEDONS OF TOMATO PLANTS

The grown plants under tissue culture station was periodically transferred for root organogenesis and shoot organogenesis and allowed to grow for future analysis work (**Figure 4, 5 & 6**). For selection of the transformants, Kanamycin Antibiotic was used.

FIGURE 4: TRANSFER OF INFECTED COTYLEDONS TO TOMATO PLANT REGENERATION MEDIUM AFTER TWO DAYS (SHOOT ORGANOGENESIS)

FIGURE 5: TRANSFER OF PLANTS TO ROOTING MEDIUN CONTAINING KANAMYCIN (50MG/ML) AND CEFOTAXIM (100MG/ML)

FIGURE 6: GROWTH OF TRANSGENIC TOMATO PLANTS

Molecular Analysis of transgenic *Lycopersicum esculentum* Mill. (Tomato) plants: The molecular analysis of genetically transformed *Lycopersicum esculentum* Mill. (Tomato) plants detect the presence of Defensin gene by using Polymerase Chain Reaction. The isolated genomic DNA (**Figure 7**) of tomato plant leaves was used as template and gene specific primers were used to amplify the HNP-1 gene (**Figure 8**).

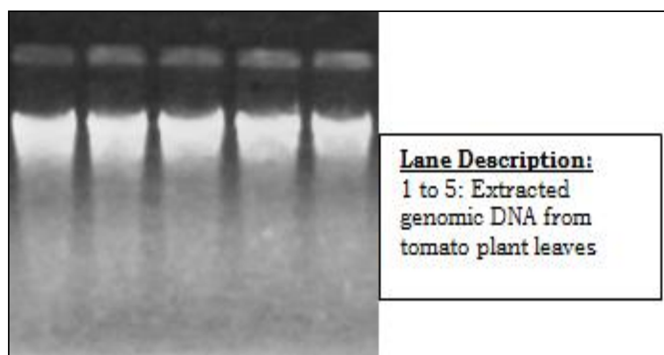


FIGURE 7: GEL ELECTROPHORESIS PICTURE OF EXTRACTED GENOMIC DNA OF TOMATO PLANTS

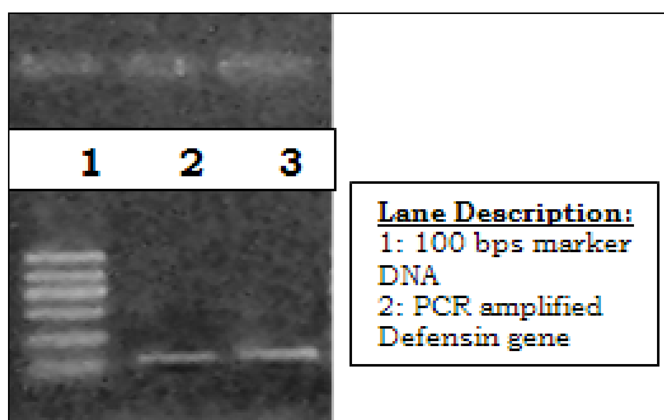


FIGURE 8: PCR TO DETECT THE PRESENCE OF DEFENSIN IN TRANSGENIC TOMATO PLANTS

***In-Vitro* antimicrobial activity of isolated proteins from transgenic *Lycopersicum esculentum* Mill. (Tomato) plants:** The total soluble proteins were isolated from the tomato plant and subjected to *in vitro* antimicrobial activity by 'Cup plate method'. The isolated protein showed antimicrobial effect against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* **(Figure 9)**.

FIGURE 9: *IN VITRO* ANTIMICROBIAL ACTIVITY OF TOTAL SOLUBLE PROTEIN FROM TOMATO LEAVES AGAINST *S. AUREUS*

FIGURE 10: *IN VITRO* ANTIMICROBIAL ACTIVITY OF TOTAL SOLUBLE PROTEIN FROM TOMATO LEAVES AGAINST *E. COLI.*

FIGURE 11: *IN VITRO* ANTIMICROBIAL ACTIVITY OF TOTAL SOLUBLE PROTEIN FROM TOMATO LEAVES AGAINST *B. SUBTILIS.*

DISCUSSION AND CONCLUSION: Transgenic plants for production of therapeutic peptides are promising tool for large scale production of Human Neutrophil Peptide-1 (HNP-1). The given peptide can be produced in a transgenic Tomato plant and which in turn lead to resistance of Tomato plants against plant pathogens. The Tomato plants will become more resistible for microbial infections. Since HNP-1 (Defensin-1) has role in increasing the immunity of the humans therefore plants with HNP-1 (Defensin-1) expression will surely increase the immunity of the individuals who will take it. The purified Defensins from transgenic tomato plants can also be used as a potential target to be developed as a drug for antimicrobial therapy against various infections.

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