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CLONING AND SEQUENCING OF CHITINASE GENE SPECIFIC PCR AMPLIFIED DNA FRAGMENT FROM TEA PLANT *(CAMELLIA SINENSIS)* AND ANALYZED THE NUCLEOTIDE SEQUENCE USING BIOINFORMATICS ALGORITHMS

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ABSTRACT

Plant chitinases belong to a family of pathogenesis-related (PR) proteins, which are over-expressed by plants in response to a pathogen attack. Chitinases catalyze the hydrolysis of the β-1, 4 linked N-acetylglucosamine polymers that form chitin chains, a major component of fungal cell walls. Chitinase gene specific DNA fragment from tea plant (*Camellia sinensis*) has been amplified in PCR reaction using gene specific primers. The PCR product of 366bp is cloned into TA cloning vector (pGEMT Easy vector) and sequenced bi-directionally. The nucleotide sequence information of 366bp has been analyzed through the different BLAST programme of bioinformatics algorithm for its molecular analysis in order to obtain genetic information carried by the DNA fragment. The chitinase gene specific fragment of 366bp of tea is deposited into the GenBank of NCBI (Accession # EU373553).

Keywords: TA-cloning, tea, PR-proteins, Chitinase gene, pGEMT vector, bioinformatics.

INTRODUCTION

Globally, tea [*Camellia sinensis* (L.) O. Kuntze] is one of the oldest common non-alcoholic beverages used since ancient times. India is the largest producer and exporter of processed tea with over 30% share in global tea production. The processed tea is mainly coming from the different varieties of cultivated teas (from three species) namely, *Camellia sinensis* (China type), *Camellia assamica* (Assam type) and *Camellia assamica* spp. *Lasiocalyx* (Cambod type), which are taxonomically recognized in India based on leaf morphology and growth habit (Wight, 1962) under the family Theaceae. The ever-increasing demand for higher productivity and better quality of tea has resulted in the need for mass multiplication of elite clones. Tea is propagated largely through seeds or cuttings, but a major concern associated with seed raised populations has been the occurrence of high genetic variability. Although, vegetative propagation of elite clones has been used for large scale multiplication of plants with desirable attributes such as yield and quality. They are with some drawbacks that over the years in the field the yield parameters is changed under adverse/stress conditions. Moreover, these clonal plantations are relatively prone to pathogens in comparison to seed grown plantations (Barua, 1989). The major pathogenic diseases of tea plants are root diseases- such as brown root rot, black root rot and red root rot; stem diseases- such as wood rot, collar canker, and die back; leaf diseases- such as blister-blight, black rot, red dust and bird's eye spot etc. The synthetic chemicals (fungicides/pesticides) are generally used to control the diseases and pests in tea plantation. Among these bromopropylate, cartap, dicofol, endosulfan, ethion, parathion-methyl, permethrin, propargite, phosalone, tetradifon, hexaconazole are widely used chemicals in disease management in tea plantation. But main drawback of using these synthetic chemicals is its residual toxicity. Residue of pesticides/fungicides/chemicals applied to tea crops are causing concern the world over. The presence of residue beyond the 'Acceptable Daily Intake (ADI) level in the food items creates problems. As such, regulations and standards on the subject/chemicals are formulated for reasons of health and environmental safety. For tea, separate Minimum Residue level (MRL) are available for some chemicals. Tea producers are trying to restrict or minimize the use of synthetic chemicals by introducing Integrated Pest Management (IPM) systems. Pest resistant cultivars are the least expensive, safest, and most practical way to combat pest damage. Pest resistant cultivars that have the combined attributes of high yield and good quality help to curtail the build-up of noxious pests, at no additional cost. Resistant/tolerant cultivars can be screened from the available tea germplasm on the basis of some genetical parameters such as immunity of a plant against pathogen with respect to plant's own defense system. Plant has its own defense strategies they produce pathogenesis-related protein/enzyme (PR-proteins) to combat the pathogenic infection. So, elite tea clone can be identified on the basis of the chitinase gene structure- a PR-protein gene. Many attempts have already been made to analysis the crop plant germplasm on the basis of chitinase gene but not in tea plant. Plant chitinases belong to a family of pathogenesis-related (PR) proteins, which are over-expressed by plants in response to a pathogen attack (Giazinazzi, 1987; Boller, 1983; Collinge *et al.*,

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1993; Legrand *et al.*, 1987). Chitinases catalyze the hydrolysis of the beta-1,4 linked N-acetylglucosamine polymers that form chitin chains, a major component of fungal cell walls and is involved in the inducible defenses of plants. Those with lysozyme activity also cleave ß-1, 4 linkages between GlcNAc and *N*-acetylmuramic acid. They are present in a broad range of organisms including bacteria, fungi, plants, and animals. Recently eleven families of PR proteins (PR-1 to PR-11) were recognized and classified in different plant species (tobacco and tomato, cucumber and parsley (Van Loon *et al.,* 1985, 1994, 1997, 1999). Plant chitinases and lysozymes are likely to have arisen from one coancestor by divergent evolution (Monzingo *et al*., 1996). The consequences of plant disease caused by fungal pathogens can be significant losses in crop quality and yields. Some researchers are trying to address the question whether manipulation of defense signaling pathways, either through genetic engineering or through application of defense signal-mimicking plant protectants, will boost the plant's immunity to potential invaders or will be a burden in crop protection strategies. Researchers have tried to isolate different chitinase gene from different plant species to use it in crop improvement program in order to increase the plant's own immune system (Sekeli *et al*., 2003; Eilenberg *et al.,* 2006; Metraux *et al*., 1989; Dominique Roby *et al*., 1991; Samac *et al.,* 1990; Datta, *et al.,*1999; Broglie *et al*., 1991; Sharma and Kumar, 2005; Schlumbaum *et al.,* 1986; Huynh *et al.*, 1992; Wu *et al.*, 1994; Passrinho and De Vries, 2002).

There is no report on chitinase gene isolation and cloning from tea plants and no initiative has been taken for the improvement of Indian tea crop by manipulating the defense protein like chitinase. The present study is aimed to partially clone the chitinase gene of tea (*Camellia sinensis*) for its characterization at the molecular genetic level using bioinformatics algorithm.

MATERIALS AND METHODS

Tea leaf samples were used for the extraction of genomic DNA for the present investigation was collected from the Tea Germplasm Bank of the Department of Botany, NBU, India.

DNA extraction and PCR amplification

The tender unfolded leaves of TV30 cultivar were used to extract and isolate the genomic DNA according to the CTAB method of Murray and Thompson (1980). The chitinase gene specific 34-mer primer pair was used to amplify the genomic DNA. Total volume of 25µl PCR reaction mixture contains the following chemical ingredient: 10-50ng genomic DNA of tea clone TV30, PCR buffer (10mM Tris-HCl, pH9, 50mM KCl, 0.1% TritonX-100 and 2mM $MgCl_2$), 100nM of each primer, 200mM of each dNTP and 1U Taq DNA polymerase. The gene specific primer amplifies a fragment of 201bp in PCR reaction using genomic DNA as template. PCR conditions for gene specific amplification was 35 cycles of 1min at 94°C, 1min at 60°C and 2min at 72°C, at last a final extension at 72°C for 7min was given for preparing same length of PCR products. The PCR products were fractionated on 1.5% agarose gel electrophoresis and visualized on UV-transilluminator after staining with 0.01% ethidium bromide and photographed (Fig. 1). The chitinase gene specific primer pair (forward and reverse) was designed from the available gene sequences of different plant species from the Gene Bank. The forward primer sequence was as such 5′-CAAATGGCCACTGGGTGTAGCAGTCCAAGTTATC TC-3′ and sequences of reverse primer were like as 5′ GATAACTTGGACTGCTACACCCAGTGGCCATTTG 3′. The PCR amplified DNA fragment was purified and cloned into TA cloning vector (Promega, Madison, WI) before sequencing. PCR amplified products were purified using phenol extraction procedure. Pool the PCR products from the replicates about 240µl and added equal volume of Tris-HCl saturated phenol and mixed gently. Centrifuge the mixture at 8000rpm for 5min and collected the upper aqueous phase containing DNA in a fresh eppendorf tube (1.5ml). The equal volume of 24:1 (v/v) chloroform:isoamylalcohol was added and mixed thoroughly. After centrifugation at 10,000rpm for 5min, the supernatant was taken in a fresh tube and 0.1volume of 3M sodium acetate (pH5.5) and precipitated DNA by adding double volume of chilled absolute ethanol. Incubate for 1-2 hours at -20°C for total precipitation of the DNA fragments. Centrifuge samples at 10,000rpm for 7min to remove supernatant and wash the DNA pellet with 70% ethanol twice. Carefully remove ethanol and allow the DNA pellet to air dry in the hood to remove remaining ethanol. Resuspended DNA pellet in 30µl TE buffer, pH8.0 (10mM Tris-HCl, pH8.0, 1mMEDTA, pH8.0) and stored at 4°C for further manipulation.

PCR cloning and transformation

PCR fragments ligation into pGEMT Easy vector and transformation into *E. coli* host strain JM109 has been carried out according to the manufacturer's protocol (Promega).

Ligation into pGEMT Easy vector

For the ligation reaction sets the three 200µl tubes containing 8µl PCR product (50ng DNA) in the first tube, a 1:10 dilution of the PCR product of 8µl into the second tube and third tube without PCR product but water 8µl (a control that will indicate the background of vector self-ligation). Add to each tube 1µl pGEMT Easy vector (50ng), 1.2µl 10X ligation buffer (0.66M Tris-HCl, pH7.6, 50mM $MgCl_2$, 50mM DTT, 10mM ATP), 1µl T4 DNA ligase (0.25unit/µg of DNA for cohesive end ligation), and 0.8µl H_2O to make total volume 12µl. Incubate the reaction mixture overnight at 16°C. Then

used this ligated product for standard transformation assay. Transformation of chemically competent *E. coli* host cell strain JM109 was done with the PCR fragment ligated pGEMT Easy vector. The pGEM-T vector is compatible with JM109 chemically competent cells of Promega.

Preparations for cloning

The following media were prepared for the transformation purposes- LB medium, LB agar medium and LB agar plus 50µg/ml ampicillin plates. The day of transformation, puts LB (Luria-Bertini) agar (plus 50µg/ml ampicillin) plates in 37°C to dry for an hour. Then add 40µl of 20mg/ml Xgal + 8µl of 100mg/ml IPTG (isopropyl β-D-thiogalactoside) with a sterile spreader under the laminar hood. Put back at 37°C till needed. Thaw 50µl host cells and add 1µl of ligation mixture on ice while cells are thawing, pipette very gently to mix, and incubate on ice 30-45minutes then proceed to one shot chemical transformation. Heat shock cells in 42°C water bath for 1minute. Immediately transfer the tube to ice for 2minutes. Add 250µl SOC medium (1ml SOC: 18µl 20% dextrose, 10µl 1M $MgCl_2$, 10µl 1M $MgSO_4$ and 962µl LB medium), cap the tube tightly and shake horizontally (200rpm) at 37°C for 1h prior to plating. Plate 50-100µl SOC medium (transformed cells) on prewarmed selective plates (LB agar ampicillin + Xgal + IPTG) and leave at 37°C overnight or longer. Selection and screening of positive clones: Colonies containing insert will be white and without insert is blue in this selective medium. White recombinant colonies are picked with autoclaved toothpicks and inoculate into 50-80µl of LB/amp medium for multiplication by keeping it for overnight at 37°C with 200rpm agitation. The transformed cells containing PCR insert of 366bp was sequenced bi-directionally (Bangalore Genei, Bangalore, India).

RESULTS AND DISCUSSION

The figure 1 shows the photograph of agarose gel containing the DNA bands of gene specific PCR product of about 366pb in lane 1, lane 2- DNA ladder as marker, and lane 3 genomic DNA of tea clone TV30. The PCR product was purified according to the Sambrook *et al.* (2001) and used in TA cloning procedure. The TA cloning vector pGEMT Easy of Promega was utilized in the present PCR cloning system because it is most suitable for PCR product cloning. Since Taq DNA polymerase has a non-template dependent terminal transferase activity, which adds a single 3′ deoxyadenosine (A) to the ends of PCR products, this linearized pGEMT Easy vector has a single overhanging 3′ deoxythymidine residue (T), which allows for increased ligation efficiency with the vector. It is always prefer to use new PCR product during cloning otherwise the terminal 3′ deoxyadenosine residue has a tendency to fall off over time (from 3-4days old PCR product). The cloning efficiency ranging between 60-80 clones per plate (Fig. 2). The optimal ratio of PCR/vector is 3:1. The pGEMT Easy vector is 3015bp. PCR amplified chitinase gene specific DNA fragment of about 366bp is cloned into pGEMT-Easy vector in *E. Coli* host cell strain JM109. Transformed cells are selected on LB-ampicillin medium with chromogenic substrate Xgal and IPTG (isopropyl-β-D-thiogalactoside, inducer of LacZ gene). Blue colonies are with non-recombinant vector without PCR insert but white colonies contain recombinant vector with PCR insert (Fig. 2). Blue colonies are produced because the intact LacZ gene on the plasmid synthesizes active β-galactosidase enzyme which converts the colourless Xgal (5-bromo-4chloro-3-indolyl-β-d-galactoside) to blue indolyl compound and helps to distinguish the recombinant from non-recombinant vector containing clones. The cloned DNA was sequenced bi-directionally using the T7 and SP6 vector specific primer using Big Dye Terminators (Applied Biosystems by Bangalore Genei, India). The 366bp DNA sequence information of the chitinase gene has been deposited in the GenBank of NCBI. Members of the chitinase gene family are found in all plants, which express them inducibly as PR proteins and constitutively in tissues vulnerable to pathogen attack (Samac *et al.,* 1990; Collinge *et al.*, 1993). Several lines of evidence indicate that chitinases play a direct role in plant defense by attacking chitin, a β-1, 4-linked polymer of N-acetyl-D-glucosamine and a major component of fungal cell walls. Purified chitinases can inhibit hyphal growth *in vitro* (Broglie *et al.*, 1991) and chitinolytic breakdown products induce the production of defense compounds (phytoalexins) and systemic acquired resistance. These antifungal properties are greatly enhanced in the presence of β-1,3-endoglucanase, another PR-protein that attacks

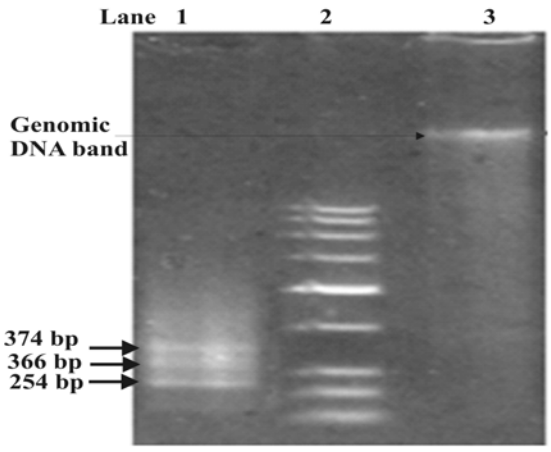

Fig. 1. PCR amplified chitinase gene specific products are fractionated on 1.5% agarose gel electrophoresis and visualized on UV-Transilluminator after staining with ethidium bromide. Lane 1, three PCR bands of 374bp, 366bp, and 254bp; lane 2, low range DNA ruler marker and lane 3, genomic DNA of tea clone TV30.

Fig. 2. Cloning of PCR product in TA cloning vector. PCR amplified chitinase gene specific DNA fragment of 366 bp is cloned into pGEMT Easy vector using *E. coli* host cell strain JM109. Transformed cells are selected on LB ampicillin medium with X-gal and IPTG. Blue colonies are with non-recombinant vector without insert but white colonies contain recombinant vector with 366 bp PCR insert.

the glucan matrix in which chitin is embedded (Collinge *et al.*, 1993). The acidic and basic chitinase genes have isolated and characterized fully in *Arabidopsis thaliana* (Samac *et al.*, 1990). Genes for chitinases have been analyzed at the molecular evolutionary level in maize and family poaceae (Peter, 2004). Plant-pathogen co-evolution is analyzed in *Arabis* sp, in relation to class-I chitinases (Bishop *et al.*, 2000). Sekeli *et al*. (2003) have isolated and cloned chitinase-I gene from winged bean seed and characterized its structure in relation to defense system. Chitinase gene fragments were isolated from a winged bean seed cDNA library using two sets of degenerate primers corresponding to the conserved regions of chitinase class I and IV proteins. Isolation and characterization of chitinase genes from pitchers of the carnivorous plant *Nepenthes khasiana* has been conducted by Eilenberg *et al*. (2006). Metraux *et al*. (1989) have studied in details the chitinase gene isolation and cloning. Dominique Roby *et al*. (1991) studied the regulation of chitinase *in vitro* in protoplast culture inducing the defense system with ethylene and other elicitors. Samac *et al*. (1990) have critically demonstrated the isolation and cloning of chitinase gene in model plant *Arabidopsis thaliana*. Datta *et al*. (1999) has shown that transgenic rice plant over expressing PR-5 gene can be more resistance against sheath blight disease pathogen *Rhizoctonia solani.* Plants over-expressing chitinases under the control of a strong constitutive promoter have been engineered and have shown improved resistance against fungal pathogens under laboratory conditions (Broglie *et al*., 1991). Sharma and Kumar (2005) have studied the PR-5 (Pathogenesis related protein) induction in the drought conditions in tea plant. These enzymes can inhibit the growth of fungal hyphae *in vitro* (Schlumbaum *et al.,* 1986; Huynh *et al.*, 1992). Some chitinases are induced following pathogen infection (Wu *et al.*, 1994) and the overexpression of at least some chitinases in transgenic plants causes significant reductions in pathogen damage (Broglie *et al.*, 1991). Taken together, these observations support the notion that a primary function of plant chitinases is in defending plants against attack by fungal pathogens, although there is also evidence that chitinases may function as lysozymes degrading bacterial cell walls and may play a role in developmental processes (Passrinho and De Vries, 2002). The production of transgenic plants with enhanced resistance to attack by the fungal pathogen *Rhizoctonia solani* has been developed (Broglie *et al.*, 1991).

The BLAST (Basic Local Alignment Search Tool) algorithm BLASTN gives the following significant alignment information about the 366bp DNA sequence of tea plant. The DNA sequence matches with basic chitinase gene of *Nepenthes khasiana* (1717bp, Accession no. gb|AY61883.1|) complete cds of 1717bp, Score = 65.8 bits (35), Expect = 1e-07, Identities = 35/35 (100%), Gaps = 0/35 (0%), Strand=Plus/Minus, as follows:

```
Query  2    CAAATGGCCACTGGGTGTAGCAGTCCAAGTTATCT 36
            ||||||||||||||||||||||||||||||||||
Sbjct 1709 CAAATGGCCACTGGGTGTAGCAGTCCAAGTTATCT 1675
```

The GenScan algorithm has predicted (81 amino acid coding sequences) the following peptide sequence(s) from

366bp DNA sequence of tea plant: Predicted coding sequence(s):
04:15:39|GENSCAN_predicted_peptide_1|81_aa,
MGAVEGYRIAGGPLGEVTDPLYPELKVKEIKNGRLAMFSMFGF
FVQAIVTGKGPLENLADHLADPVNNNAWAYATNFVPGK.
>04:15:39|GENSCAN_predicted_CDS_1|246_bp.

The TBlastX has shown the significant alignments with chitinase protein, Accession number AY618881.1 *Nepenthes khasiana* basic chitinase 1-1 gene, complete cds, Length=1572
Score = 37.7 bits (76), Expect = 4.1, Identities = 14/15 (93%), Positives = 14/15 (93%), Gaps = 0/15 (0%),
Frame = -2/+2
Query 46 STREITWTATPSGHL 2
ST EITWTATPSGHL
Sbjct 1520 STTEITWTATPSGHL 1564

tcaaatggccactgggtgtagcagtccaagttatcttcactttcctgggacaaagtt
tgtagcataagcccatgcattgttgttaacagggtcagcaaggtggtcagccagg
ttctccaatggtccctttcctgtcacaattgcctgaacaaagaacccaaacattgag
aacatggccagtcttccattcttgatctccttcaccttcagctctgcaaaagcctctg
gtcatcagctaggcccaatgggtcgaagcttcccaccggggtagagcgggtcg
gtcacctccccgagtggtccaccagcaatgcggtagccctcaacagcacccatc
aagataattggactacacccagtggccatttg

That means the isolated 366bp genomic DNA sequence (given above) of tea plant carries some functional genetic information. It can encode protein of 81 amino acids with the characteristics of plant Chitinase protein/enzyme. The present study has been conducted to look inside into the chitinase gene of tea plant. The deduced partial nucleotide sequence of chitinase gene will be used as a probe to clone the full length chitinase gene from the cDNA as well as genomic DNA library to be constructed from the tea plant. That will help the researcher to study the chitinase gene of tea plant in more details with respect to *exon-intron* boundary, promoter sequence and others. This will utilized in the improvement of tea production and quality by manipulating the chitinase gene in future in order to improve the plant immunity against the fungal pathogens to manage the disease manifestation with out applying any health hazardous chemical such as pesticides/fungicides.

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