MATERIAL AND METHODS

3.1. Plant material

Tea, *Camellia senensis* (L.), belongs to the family Theaceae. The cultivated taxa comprise of three main natural hybrids, which are: *Camellia sinensis* (L.) O. Kuntze (China type), *Camellia sinensis* var. *assamica* (Masters) (Assam type) and *Camellia sinensis* sub sp. *lasiocalyx* (Planchon MS.) (Cambod or Southern type). Tea plant is an evergreen, perennial tree (Plate 1, fig. A) but for commercial use it is pruned in regular intervals (Plate 1, fig. C). Leaf is the main criterion by which three types of tea are classified, briefly, Assam type has biggest leaves, China type has smallest leaves and Cambod type leaves size are in between of Assam and China type.

3.1.1. Collection

Twenty-one tea genotypes from three different varieties (*Camellia sinensis* var. *sinensis*; *Camellia sinensis* var. *assamica* and *Camellia sinensis* sub species *lasiocalyx*) of tea were used for the present study (Table 9). These genotypes were collected from the different tea germplasm centre of India (Darjeeling Tea Research Centre (DTRC), Kurseong, West Bengal; The United Planter's Association of Southern India (UPASI), Valparai, Tamil Nadu and Tocklai Experimental Station (TES), Jorhat, Assam and are presently being maintained under similar cultural practices at Tea Germplasm Bank, Department of Botany, North Bengal University, West Bengal, India (Plate 1, fig. B) by propagating through single stem node cuttings to maintain genetic identity according to Banerjee (1993).

3.1.2. Clonal propagation

Tea plants are mostly vegetatively propagated through single-node cutting. Fresh clonal cuttings of the tea varieties were propagated in sleeves containing sandy soil (3:1 of sand and soil) with a pH ranging from 4.5- 4.8 and were maintained in a green Agro-net house. In the early stage, limit of light tolerance was at 25% and after rooting progressed 50% light tolerance was maintained according to Banerjee (1993).

3.1.3. Maintenance in glasshouse

Clonal propagated tea plants (T383) of 6 months old were transferred to 6 cm and 12 cm diameter plastic pots as well as 24 cm earthen pots containing soil supplemented with 20% green manure and were maintained in the glasshouse for

experimental purposes in optimum day light (11 h), relative humidity (55-75%) and temperature (30-35°C), at which the photosynthetic rate was maximum, were provided to these plants according to Banerjee (1993).

Table 9. Tea genotypes from three different tea varieties.

Varieties	Tea genotypes
i)*Camellia sinensis* var. *sinensis*	**China type**
Sl-1	T383
Sl-2	T78
Sl-3	T135
Sl-4	HV39
Sl-5	TeenAli17
Sl-6	AV2
Sl-7	BS/7A/76
ii)*Camellia sinensis* var. *assamica*	**Assam type**
Sl-8	UPASI-3
Sl-9	UPASI-9
Sl-10	UPASI-26
Sl-11	TV20
Sl-12	TV21
Sl-13	TV27
Sl-14	TV28
iii)*Camellia sinensis* sub spp. *lasiocalyx*	**Cambod type**
Sl-15	TV18
Sl-16	TV19
Sl-17	TV22
Sl-18	TV23
Sl-19	TV25
Sl-20	TV26
Sl-21	TV30

Sl: Serial no.

3.2. Genomic fingerprinting by RAPD and ISSR markers

For characterization of genome (fingerprinting): A variety of molecular marker techniques based on hybridization or PCR are available for characterization of genome (fingerprinting). In the present investigation for the fingerprinting of twenty one tea clones PCR-based marker development method *i.e.* Randomly Amplified Polymorphic DNAs (RAPD) and Inter Simple Sequence Repeats (ISSR) were used.

Plate 1 [Fig. A-C]: (A) Tea plant grown without pruning; (B) Tea germplasm maintained by pruning and (C) Commercial tea plantation in plain lands of Northern Region of West Bengal, India .

3.2.1. Preparation of genomic DNA extraction buffer

The following buffers for DNA extraction were prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH using 1N HCl or 1N NaOH solution in all the cases.

CTAB buffer
2% CTAB (Cetyltrimethylammonium bromide)
1.5% PVP K-30 (polyvinylpyrrolidone)
1.4M NaCl
20mM EDTA
100mM Tris-HCl (pH 8.0)
0.1% β-mercaptoethanol (add before use)

10x Tris EDTA (TE buffer)
100mM Tris-HCl (pH 8.0)
10mM EDTA (pH 8.0)

10x TAE electrophoresis buffer

Tris base	48.5g
Glacial acetic acid	11.42ml
0.5M EDTA (pH 8.0)	20ml
H_2O to make fmal vol.	1000ml

6x Gel loading buffer (DNA)
0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF
40% (w/v) sucrose in H_2O

Sodium acetate (3M, pH 5.2)
40.83g Sodium acetate 100ml
Adjust pH with glacial acetic acid

Ethidium bromide (10mg/ml)
1g EtBr 100ml H_2O

1% Agarose gel solution
250 mg agarose dissolved in 25ml 1x TAE buffer

Proteinase K stock (20 mg/ml) 10ml

Proteinase K	200mg
50mM Tris (pH 8.0)	
1.5mM Calcium acetate	
Make final volume	10ml
Use 100µg/ml in reaction	

Proteinase K reaction buffer
0.01M Tris-Cl (pH 7.8)
0.005 M EDTA
0.5% SDS
50µg/ml Proteinase K

RNase A stock solution (10mg/ml)

RNase A	100mg
0.01 M Sodium acetate	
(pH 5.2) added to make	
Final volume	10ml
Heated to 100°C	15 min
Stored at	-20°C

RNase A treatment buffer
10mM Tris-Cl (pH 8.0)
0.1 M EDTA (pH 8.0)
0.5% (w/v) SDS
Added RNase A stock solution
20µg/ml

3.2.2. Genomic DNA isolation

Tender unfolded leaf samples (from twenty one tea genotypes) were collected from the clonally propagated plants and stored immediately at -80 °C for DNA extraction. The total genomic DNA was extracted from the stored leaf samples using

the modified CTAB method of Murray and Thompson (1980). Fresh unfolded tender leaf tissue (1g) was ground in a precooled mortar and pestle using liquid nitrogen. The pulverized material thus obtained, was transferred to 15ml polypropylene tube containing 4ml of preheated CTAB extraction buffer (65°C) with 20 µl Proteinase K stock solution. Then it was incubated at 65 °C in a water bath for 30 minutes with occasional gentle shaking after adding 1 ml of 10% w/v SDS. Equal volume of phenol: chloroform: isoamylalcohol (25:24:1) mixture was added, and the mixture was shaken vigorously. The mixture was centrifuged at 10,000 rpm for 10 minutes and supernatant was transferred to a new centrifuge tube. Again added equal volume of a mixture of chloroform: isoamylalcohol (24:1) and centrifuged at 10,000 rpm for 8 minutes and the supernatant was transferred to a new tube. DNA was precipitated by adding double volume of chilled (-20°C) absolute ethanol followed by addition of $1/10^{th}$ volume of 3M Sodium acetate (pH 5.5) and kept at -20°C for overnight for total DNA precipitation.

3.2.3. Genomic DNA purification

DNA purification was done according to the method of Sambrook and Russell (2001). The precipitated DNA was spooled out and washed 2-3 times in 70% ethanol and dissolved in 500µl RNase A treatment buffer containing 20µg/ml RNase A solution for purification in a 1.5 ml eppendorf tube. It was incubated at 37 °C for 1h to remove the RNA contamination. Then extracted with equal volume of chloroform: isoamylalcohol (24:1) and centrifuged at 10,000 rpm for 8 minutes and the supernatant was transferred to a new tube. DNA was precipitated by adding double volume of chilled (-20°C) absolute ethanol followed by addition of $1/10^{th}$ volume of 3M Sodium acetate (pH 5.5) and kept at -20°C for overnight for total DNA precipitation. The purified DNA pellet was collected by centrifuging at 5000 rpm for 5 minutes in a microfuge. The excess ethanol was air dried and purified DNA was finally dissolved in 250µl TE buffer (pH 8.0) for further use in PCR amplification.

3.2.4. Measure DNA concentration using Spectrophotometer

For quantitating the amount of DNA, readings were taken at wavelengths of 260 nm and 280 nm in a UV-*vis* Spectrophotometer (Shimadzu-160). The reading at 260 nm allows calculation of the concentration of DNA in the sample. An OD of 1 corresponds to ~ 50 µg/ml for double stranded DNA. The ratio between the readings

at 260 nm and 280 nm (OD_{260}: OD_{280}) provides an estimate of the purity of the DNA. A pure preparation of DNA has OD_{260}: OD_{280} value of 1.8. Concentration of the purified genomic DNA in each case was adjusted to 10 ng/µl in a different aliquots and stored at -20°C for further use.

3.2.5. Agarose gel electrophoresis to check DNA quality

1% (w/v) agarose gel was cast by melting 250 mg agarose in the presence of 25 ml 1xTAE buffer in a microoven until a clear, transparent solution was achieved. The melted solution was then poured into a mold after adding ethidium bromide solution to a final concentration 0.5 µg/ml, set appropriate comb and allowed to harden. Upon hardening the gel, it was placed in the Mini-submarine gel electrophoresis tank (Bio-Rad, Sub-Cell GT) and added the 1x TAE buffer sufficient to cover the gel to a depth of ~1 mm (according to Sambrok and Russell, 2001).

3.2.6. Preparation of DNA samples for electrophoresis

Taken 20 µl purified DNA from each samples and mixed with 4 µl of 6x DNA gel loading buffer. The DNA sample mixed gently and slowly loaded 25 µl sample into the slots of the submerged gel using a micropipette. Low range DNA ruler (Bangalore Genei) also loaded into the slot on either of the sides as a DNA size marker.

3.2.7. Run gel electrophoresis for DNA fractionation

The electrical lead of the gel tank was attached firmly and applied electric supply at constant current 90 mA and voltage 75 Volt (Bio-Rad Power Pac-300) at least for 90 minutes. The DNA was migrated from cathode to anode (positive anode). Run was continued until the bromophenol blue and xylene cyanol FF have migrated an appropriate distance through the gel. Then turn off the electric current and removed the gel from the tank and examined the gel on UV-transilluminator and photographed for analysis.

3.2.8. Preparation of buffers for genomic fingerprinting

The following buffers for PCR amplification were prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH using 1N HCl or 1N NaOH solution in all the cases.

10x PCR Amplification buffer
500mM KCl
100mM Tris-HCl (pH 8.0)
15mM $MgCl_2$
0.1% Gelatin

Taq storage and dilution buffer
20mM Tris-HCl (pH 8.0)
0.1 mM EDTA
1mM dithiothreitol (DTT)
100mM KCl
0.5% Tween-20
0.5% Igepal
50% Glycerol (v/v)

PCR reaction mixture (Final volume 25µl)

10x Amplification buffer	2.5µl
10mM dNTPs (pH 8.0)	2.0µl
20µM Random primer	5.0µl
Taq polymerase 1U	1.0µl
Template DNA (30-35ng)	10.0µl
Sterile H_2O	4.5µl

3.2.9. PCR amplification for RAPD fingerprinting

Twelve RAPD primers (L5, OPA-1, OPA-4, OPA-9, OPA-11, OPA-12, OPA-13, OPA-16, OPA-18, OPA-19, OPB-10 and OPB-20) (Table 10) were used for PCR amplification of the genomic DNA of twenty one tea clones according to Williams *et al.*(1990). RAPD reactions were performed in a 25µl reaction mixture containing 10 µl template DNA, 2.5 µl PCR buffer, 2 µl dNTPs mixture, 5 µl of single 10-mer random primer, 1µl of 1 Unit Taq DNA polymerase (all chemicals and primers are procured from Bangalore Genei, Bangalore, India) and final volume was adjusted by adding sterile distilled water. A negative control with no DNA was included in each PCR run. The PCR amplification reactions were performed in a Gene Amp PCR System 2400 (Perkin-Elmer) programmed for an initial denaturation step of 94 °C for 4 minutes followed by 35 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C. After the last cycle, a final step of 7 min at 72 °C was added to allow complete extension of all amplified fragments and reaction was ended at 4 °C forever.

Table 10. RAPD primers used for the detection of polymorphism in *Camellia sinensis.*

Sl. Nos.	Primer code	Sequences (5' to 3')
1.	L5	acgcaggcac
2.	OPA-1	caggcccttc
3.	OPA-4	aatcgggctg
4.	OPA-9	gggtaacgcc
5.	OPA-11	caatcgccgt
6.	OPA-12	tcggcgatag
7.	OPA-13	cagcacccac
8.	OPA-16	agccagcgaa
9.	OPA-18	aggtgaccgt
10.	OPA-19	caaacgtcgg
11.	OPB-10	ctgctgggac
12.	OPB-20	ggacccttac

3.2.10. PCR amplification for ISSR fingerprinting

Seven ISSR primers (ISSR13, ISSR14, ISSR17, ISSR18, ISSR814.1, UBC830 and UBC825) were used in this profiling. The PCR amplification of the total genomic DNA from 21 tea genotypes (Table 9) was done according to the method of Tsumura *et al.*(1996). Amplification was carried out in 25 µl of reaction mixture same as RAPD amplification containing 10 µl template DNA, 2.5 µl PCR buffer, 2 µl of dNTPs mixture, 5 µl of single ISSR primer, 1µl of 1 Unit Taq DNA polymerase. PCR reactions were performed using an Perkin-Elmer's Gene Amplification Thermal Cycler programmed for an initial denaturation step at 94 °C for 4 minutes followed by 35 cycles of 1 min at 94 °C for denaturation, 1 min at 50 °C for primer annealing and 2 min at 72 °C for primer extension. A final step of 7 min at 72 °C was carried out for polishing the ends of PCR products and reaction was ended at 4 °C forever.

3.2.11. Fractionation of PCR products in 1% agarose gel

Both the PCR amplified products (RAPD and ISSR) were size fractionated in 1% agarose gel and electrophoresed in 1X TAE buffer (0.04 M Tris-acetate, 1 mM EDTA, pH=8.0) at 75 V, 90 mA current for 90 minutes (Power Pac-300, Bio-Rad) in a Sub-Cell-GT electrophoresis system (Bio-Rad). Ethidium bromide (0.5µg/ml final

concentration) was added into the agarose gel during its solidification to stain the DNA. The banding patterns were visualized with a UV-transilluminator (UV~312nm) and documented by a Canon digital camera. A low range DNA ruler was used as a molecular size marker. The amplification was repeated twice and only reproducible clear bands were scored for genetic analysis.

3.3. PCR amplification for chitinase gene

Chitinase gene specific DNA fragment of 201 bp was PCR amplified using chitinase gene specific primer pair. Genomic DNA sample was taken from tea clone T383.

3.3.1. Preparation of gene amplification buffer solution

Gene specific PCR amplification mixture buffer was prepared as given below and the desired pH was adjusted using 1N HCl or 1N NaOH solution in all the cases.

Gene specific PCR reaction mixture (Final volume 25µl)

10x Amplification buffer	2.5µl
10mM dNTPs (pH 8.0)	2.0µl
20µM forward primer	2.5µl
20µM reverse primer	2.5µl
Taq polymerase 1U	1.0µl
Template DNA (30-35ng)	10µl
Sterile H_2O	4.5µl

3.3.2. DNA isolation

The tender unfolded leaves of T383 cultivar was used to extract and isolate the genomic DNA according to the CTAB method of Murray and Thompson (1980) as already described in section 3.2.2.

3.3.3. DNA purification

The same methodology was used for DNA purification which has already been described in section 3.2.3.

3.3.4. Measure DNA concentration using Spectrophotometer

The protocol as stated in section 3.2.4. was followed to quantify the genomic DNA concentration.

3.3.5. Chitinase gene specific PCR amplification

The chitinase gene specific 20-mer primer pair was used to amplify the genomic DNA. Total volume of 25 µl PCR reaction mixture contains the following chemical ingredient: 30-35ng genomic DNA of tea clone T383, 2.5 µl of 10x PCR buffer, 20 µM of each primer pair (forward and reverse primer), 2 µl of dNTPs and 1U Taq DNA polymerase and final volume 25 µl was adjusted with sterile water. PCR conditions for gene specific amplification was as follows- 35 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C, at last a final extension at 72°C for 7 min was given for preparing same length of PCR products.

3.3.6. Fractionation of chitinase gene product

Chitinase gene specific PCR products were fractionated on 1% agarose gel electrophoresis and visualized on UV-transilluminator and photographed, the procedure was already described in section 3.2.11.

3.3.7. Purification of Chitinase gene fragment

The PCR amplified DNA fragment of 201bp was purified and cloned into TA cloning (pGEM-T *Easy*) vector (Promega, Madison, WI) before sequencing. PCR amplified product was purified using phenol extraction procedure. Pool the PCR products from the replicates about 150 µl and added equal volume of Tris-HCl saturated phenol and mixed gently. Centrifuge the mixture at 8000 rpm for 5 min and collected the upper aqueous phase containing DNA in a fresh eppendorf tube (1.5ml). The equal volume of chloroform: isoamylalcohol (24:1, v/v) was added and mixed thoroughly. After centrifugation at 10,000 rpm for 5min, the supernatant was taken in a fresh tube and added $1/10^{th}$ volume of 3M sodium acetate (pH 5.2). DNA was precipitated by adding double volume of chilled (-20° C) absolute ethanol and incubated for 2 h at -20°C for total precipitation of the DNA fragments. DNA samples were centrifuged at 10,000 rpm for 7 min to remove supernatant and washed the DNA pellet with 70% ethanol twice. Carefully removed ethanol and allowed the DNA pellet to air dry in the hood to remove remaining ethanol. DNA pellet was resuspended in 30µl TE buffer, pH 8.0 (10 mM Tris-HCl, pH8.0, 1 mM EDTA, pH 8.0) and measured the concentration by Spectrophotometry. Made different aliquots in 0.2 ml tube at a concentration of 10 ng/µl and stored at 4°C for cloning in pGEM-T *Easy* vector.

3.4. T/A Cloning of chitinase gene fragment

T/A cloning vector pGEM-T *Easy* of Promega was utilized in the present PCR cloning system because it is most suitable for PCR product cloning due to presence of a single overhanging 3′ deoxythymidine residue (T) (Plate 7, fig. A & C). This 3′-T overhanging easily makes complementary base pair with the 3′-A overhang of the PCR product. Since *Taq* DNA polymerase has a non-template dependent terminal transferase activity, which adds a single 3′ deoxyadenosine (A-residue) to the ends of PCR products, 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 3-4 days old PCR product). The cloning efficiency ranging between 60-80 clones per plate. The optimal ratio of PCR/vector is 3:1. The pGEM-T *Easy* vector is 3015bp in size.

3.4.1. Preparation of buffer for T/A cloning

The following buffers for T/A cloning and transformation were prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH using 1N HCl or 1N NaOH solution in all the cases.

10x Vector ligation buffer

0.66M Tris-HCL (pH 7.6)
50mM $MgCl_2$
50mM DTT
10mM ATP

Vector ligation mixture (Total 12µl)

10x ligation buffer	1.2µl
pGEM-T Easy vector	1.0µl
T4 DNA ligase	1.0µl
PCR product (50ng DNA)	8.0µl
Sterile H_2O	0.8µl

LB medium (Luria-Bertani medium)

Tryptone	10g
Yeast ectract	5.0g
NaCl	10g
Make final volume with H_2O	1000ml

Sterilized by autoclaving at 15 psi 20 min
For agar plate add 15g/l Bacto agar

SOC medium composition

Tryptone	10g
Yeast ectract	5.0g
NaCl	10g
KCl (250mM)	10ml
$MgCl_2$ (2M)	5.0ml
Glucose (1M)	20ml

H_2O to make final vol. 1000ml

IPTG (20% w/v, 0.8M) solution

IPTG	2g

H_2O to make final volume 10 ml
Filter through 0.22µm disposable filter

X-gal solution (2% w/v)

X-gal	2g
H_2O	final volume 100 ml

3.4.2. Ligation into pGEM-T *Easy* vector

Chitinase gene fragment of 201 bp was ligated into the linear pGEM-T *Easy* vector before transformation into the *E. coli* host cell strain JM109 for cloning the recombinant vector carrying the foreign gene fragment, according to the manufacturer's protocol (Promega). For the recombinant vector preparation, two tube of 0.2 ml was prepared, first tube containing 8 µl purified chitinase gene specific PCR product (50-80 ng DNA of 201bp) and second tube was prepared without PCR product but water 8µl (a control that will indicate the background of vector self-ligation). Added to each tube 1 µl pGEM-T *Easy* vector (50 ng), 1.2 µl 10X ligation buffer, 1 µl T4 DNA ligase (0.25unit/µg of DNA for cohesive end ligation), and 0.8 µl H_2O to make total volume 12 µl. The ligation reaction mixture was incubated overnight at 16°C to ligate the chitinase gene fragment with the linear pGEM-T *Easy* vector to make them circularized. Then the ligated products were used for standard transformation assay. Transformation of chemically competent *E. coli* host cell strain JM109 was done with the PCR fragment ligated pGEM-T *Easy* vector.

3.4.3. Transformation of *E. coli* host JM109

The following media were prepared for the transformation purposes- LB medium, LB agar medium and LB agar plus 50 µg/ml ampicillin plates. The day of transformation, puts LB (Luria-Bertani) agar (plus 50 µg/ml ampicillin) plates in 37°C to dry for an hour. Then added 40 µl of 20 mg/ml chromogenic substrate, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) + 8 µl of 200 mg/ml IPTG (isopropyl β-D-thiogalactoside) with a sterile spreader under the laminar hood to absorb the reagent into medium for blue-white colour screening. Put back at 37°C till needed. Thaw 50 µl host cells and added 2 µl of ligation mixture on ice while cells are thawing, pipette very gently to mix, and incubated on ice 30 minutes then proceed to one shot chemical transformation. Host cells were given heat shock in 42°C water bath for 1 minute. Immediately transferred the tube to ice for 2 minutes. Added 250 µl SOC medium, capped the tube tightly and shaked horizontally (200 rpm) at 37°C for 1 h prior to plating. Spreaded 50-100 µl of the transformed cells (SOC medium) on prewarmed selective LB agar plate (LB agar containing ampicillin + Xgal + IPTG) and incubated the plate at 37°C overnight (12-19 h) in inverted position. Removed the plates from the incubator and stored it at 4°C for 6 h to develop blue colour to its full

extent. Then the colonies carrying recombinant plasmids were identified through blue-white colour screening.

3.4.4. Screening of recombinant positive colonies

Positive colonies carrying recombinant plasmid were identified by α-complementation test (Sambrook and Russell, 2001). The *E. coli* host strain JM109 synthesizes ω-fragment of β-galactosidase and complement with the α-fragment of the β-galactosidase, which encoded by the empty (non-recombinant vector) plasmid vector. Colonies that carry intact plasmid (non-recombinant) produced active β-galactosidase and were blue in colour in the IPTG/X-gal selective medium. Colonies that carry recombinant pGEM-T *Easy* vector (with 201 bp chitinase gene fragment) could not produce active β-galactosidase and were white in colour in the selective medium. White recombinant colonies were picked with autoclaved toothpicks and inoculated into 1.5 ml of LB/ampicillin medium in an eppendorf tube for multiplication by keeping it for overnight at 37°C with 200 rpm agitation.

3.4.5. Isolation of plasmid DNA for sequencing

The plasmid vectors (recombinant pGEM-T *Easy* vector) containing PCR insert of 201 bp was isolated from the transformed cells according to Sambrook and Russell (2001). The bacterial culture in eppendorf tube was centrifuged at 5000 rpm for 5 min to collect the bacterial cells. Resuspend the bacterial pellet in 100 µl of ice-cold alkaline lysis solution I (50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA) and shaked vigorously. Added 200 µl alkali lysis solution II (0.2 N NaOH and 1% (w/v) SDS), then added 50 µl alkali lysis solution III (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml H_2O to made final volume 100 ml). Closed the tube tightly and disperse the lysis solution through inverting the tube several times then kept on ice for 5 minutes. Bacterial lysate was centrifuged at 10,000 rpm for 5 minutes at 4° C in a microfuge. Transferred the supernatant in a fresh tube and added equal volume of phenol: chloroform (1:1, v/v). Centrifuged at 10,000 rpm for 5 minutes and taken the aqueous upper layer to a fresh tube. Precipitated the plasmid DNA by adding 2 volumes of chilled (-20° C) ethanol and kept at 4° C for 1 h. Then the precipitated plasmid DNA was collected by centrifugation at 5000 rpm at 4° C in a microfuge. DNA pellet was washed twice with 70% ethanol then recovered the DNA by centrifugation at 5000 rpm for 5 minutes and

air dried the plasmid DNA pellet by evaporating the ethanol (10-15 minutes) at room temperature. Dissolved the DNA pellet in 50 µl TE (pH 8.0) containing 20 µg/ml RNase A (DNase-free) and kept at 37° C for 1 h to made it RNA contamination free. The DNA solution was vortex gently for a few seconds and stores the DNA solution at -20° C for future use. The amplified product was analyzed on 1% agarose gel to check the quality according to the protocol already described in section 3.2.11.

3.4.6. DNA sequencing

The above purified recombinant plasmid DNA was used for sequencing purposes. DNA sequencing was done bi-directionally using universal primer by ABI-PRISMS Big-Dye terminator cycle sequencing method and separated on Applied-BioSystem (ABI-3700-96) DNA sequencer to get sequence information of the 201 bp chitinase gene fragment (By Bangalore Genei, Bangalore, India).

3.4.7. DNA sequence analysis

DNA sequence information of the above 201 bp was analyzed using the alignment software of BLASTS algorithm (http://ingene2.upm.edu.my/Blast, Altschul *et al.*, 1997) for the different characteristics of DNA sequences. The other software program such as GENSCAN was also run to analyze the DNA sequences for the identification of the putative chitinase gene sequence with respect to peptide information.

3.4.8. Submission to GenBank (Accession no. EF673751)

Chitinase gene specific sequence information of 201 base pair was deposited to NCBI GenBank through BankIt procedure and approved as a sequence of chitinase gene after complete annotation and given the accession no. EF673751.

3.5. Caffeine synthase gene amplification by PCR reaction

Caffeine synthase gene specific genomic DNA sequence was amplified using gene specific primer in polymerase chain reaction. DNA samples from tea clones HV39 and T383 were used in this gene specific PCR amplification.

3.5.1. Preparation of DNA isolation buffer

As described in section 3.2.1.

3.5.2. Genomic DNA isolation

As described in section 3.2.2.

3.5.3. Purification of genomic DNA

As described in section 3.2.3.

3.5.4. Preparation of PCR amplification buffer

As described in section 3.3.1.

3.5.5. Caffeine synthase gene specific PCR amplification

The forward (TCS1, Tea caffeine synthase 1) and reverse (TCS2) primer (oligonucleotide) pair of caffeine synthase gene was used for partial amplification of the caffeine synthase gene from genomic DNA of two tea clones (T383 and HV39). The sequence of the caffeine synthase gene specific primer was 5′ - tcttcaaaggcctgtcgtct-3′ for forward and 5′- tccccttgtttaatgccaag-3′, for reverse primer. The primer pair was so designed (from GenBank Accession no-AB031280 of caffeine synthase gene of 1438bp cDNA) as to give amplification product of the internal sequences of the caffeine synthase gene of approximately 200-202 bp. PCR reaction mixture was set in 0.2 ml PCR tube containing 2 U Taq DNA polymerase, 10 mM Tris-HCl (pH= 8.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 5μl each of forward (TCS1) and reverse (TCS2) primer at a final concentration of 30 pmol and 50 ng template genomic DNA in a final volume of 25μl. Each reaction mixture was overlaid with 50 μl mineral oil to prevent evaporation. Samples for enzymatic amplification were subjected to an initial denaturation programme of one cycle at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min. Finally the reaction mixture was allowed to complete an additional extension of 7 min at 72°C.

3.5.6. Fractionation of PCR product in 1% agarose gel

As described in section 3.2.11.

3.5.7. PCR product purification

As described in section 3.3.7.

3.5.8. DNA sequencing of caffeine synthase gene fragment

As described in section 3.4.7.

3.5.9. Analysis of caffeine synthase gene sequence

As described in section 3.4.8.

3.5.10. Submission to GenBank (Accession no. AY599069 and AY601112)

Caffeine synthase gene sequences of 202 bp (from clone HV39) and 200 bp (from clone T383) were submitted to the GenBank of NCBI. Two accession numbers were approved by the GenBank after the verification and annotation of the submitted DNA sequences. Accession no. AY599069 was given for 202 bp of tea clone HV39 and AY601112 for 200 bp of tea clone T383.

3.6. *In vitro* tissue culture

Plant tissues and organs are grown *in vitro* on artificial media, which supply the nutrients necessary for growth and development. The success of plant tissue culture as a means of plant propagation is greatly influenced by the nature of the culture medium and plant sources used.

The callus tissues from the stem explants and shoots regenerated from the somatic embryos were used in the differential gene expression in induced systemic resistance (ISR) following induction with an inducer, methyl jasmonate.

3.6.1. Plant material for *in vitro* tissue culture

Four tea clones, TV30, UPASI-26, T383 and HV39 were used for the present *in vitro* tissue culture for somatic embryogenesis and callusing. All the tea clones are being maintained in the Tea Germplasm Bank, Department of Botany, University of North Bengal. Somatic embryogenesis was initiated from the immature and mature cotyledons from tea seeds. Callus culture was initiated from the stem segment as explants. Green capsules were collected in September for immature cotyledon from immature seeds (9-11 mm diameter) and mature seeds from open mature capsules were collected in November for mature cotyledon. Mature seeds (13-18 mm diameter, Plate 8, fig. G & H) were extracted from the mature capsules and floated overnight in water to discern viable from nonviable seed. Seeds that floated were discarded whilst those that sank were used in the present study. Immature cotyledon was collected from the fruits of September. Mature and immature cotyledon was used for somatic embryogenesis and regeneration of plantlets. Plantlets were also regenerated from *in vitro* grown stem callus through morphogenesis.

3.6.2. Composition of tissue culture media

The principal components of most plant tissue culture media are inorganic nutrients (macronutrients and micronutrients), carbon source (s), organic supplements,

growth regulators and a gelling agent. For the induction of organogenesis and regeneration of plantlets from the cultured tissues, MS (Murashige and Skoog, 1962) and B5 medium (Gamborg *et al.*, 1968) was used in the present study. The B5 medium differs from MS medium in having much lower amounts of nitrate in the form of ammonium. The compositions of MS and B5 media are given in table 11 and 12 which are used in the present study.

Table 11. Composition of MS and B5 plant tissue culture media (mg/l).

Constituent	MS medium Murashige and Skoog, (1962)	B5 Gamborg *et al.* (1968)
Macronutrients (>0.5 mM /l)		
$MgSO_4\ 7H_2O$	370	250
$NaH_2PO_4.H_2O$	-	150
$CaCl_2.2H_2O$	440	150
KNO_3	1900	2500
NH_4NO_3	1650	-
KH_2PO_4	170	-
$Ca\ (NO_3)_2.\ 4H_2O$	-	-
$(NH_4)_2SO_4$	-	134
Micronutrients(< 0.5 mM/l)		
$FeSO_4.\ 7H_2O$	27.8	-
$MnSO_4.4\ H_2O$	22.3	-
$MnSO_4.H_2O$	-	10
KI	0.83	0.75
$CoCl_2.6H_2O$	0.025	0.025
$ZnSO_4.\ 7H_2O$	8.6	2
$CuSO_4.5H_2O$	0.025	0.025
H_3BO_3	6.2	3
$Na_2MoO_4.2H_2O$	0.25	0.25
EDTA disodium salt	37.3	-
EDTA-Na ferric salt	-	43
Organic Supplements		
Vitamins		
m-ionositol	100	100
Thiamine HCl	0.5	10
Pyridoxine HCl	0.5	1.0
Nicotinic acid	0.5	1.0
Others		
glycine	2	-
Cysteine	-	10
Sucrose	30,000	20,000
pH	5.8	5.5

Table 12. Concentration of different inorganic salts (mM) in MS and B5 media.

Salt	MS	B_5
Macronutrients (mM)		
Ca	3	1.0
Cl	6	2.2
K	20.1	25
NH_4	20.6	2
NO_3	39.4	25
PO_4	1.25	1.1
SO_4	1.8	-
Mg	1.5	1.0
Micronutrients (mM)		
B	100	50
C	0.11	1.0
Cu	0.1	0.1
Fe	1.0	1.0
I	5.0	4.5
Mn	92.5	60
Mo	1.0	1.0
Zn	30	7.0

3.6.3. Preparation of stock solution for MS medium (macro and microsalts)

Preparation of macro and micronutrient stock solutions: Four different stock solutions were prepared using double distilled water to prepare MS basal medium as shown in table 13.

a) Major salts stock solution I (20 X concentrated), b) Minor salts, stock solution II (200 X concentrated), c) Iron stock solution III (200 X concentrated) and d) Stock solution IV, organic nutrients except sucrose (200 X concentrated).

While dissolving the nutrients in double distilled water, one compound was added at a time to avoid precipitation. For macronutrient stock solution, calcium chloride was dissolved separately in water and then added to the rest of the solution to avoid precipitation. To prepare 1 l of medium, the following amount of stock solutions were taken, 50 ml of stock solution I, 5 ml of stock solution II, 5 ml of stock solution III and 5 ml of stock solution IV. The stock solution III was prepared freshly by dissolving $FeSO_4$ and $Na_2EDTA.\ 2H_2O$ separately in 450 ml distilled water by heating and constant stirring. Then mixed the two solutions, adjusted pH to 5.8 and added distilled water to make up the final volume to 1 l. The pH of the medium was adjusted to the required value (pH 5.8 for MS) by dropwise addition of 1N NaOH or 1N HCl with constant stirring.

3.6.4. Preparation of B5 medium

The B5 medium was prepared by making three different solution mixtures. Desired amounts of the B5 components were added into each of the three mixtures in a 500 ml flask containing 250 ml double distilled water. Mixture I, containing all the macronutrients, mixture II containing all the micronutrients and mixture III containing organic supplements including vitamins. Added all the three mixtures slowly in a 1l flask and then added 20g sucrose and 8.0 g Difco agar. The medium components were dissolved by heating them in a water bath. The medium was supplemented with the different plant growth regulators such as BAP, IAA, PBOA, NAA, 2,4-D, TDZ and Kin (1-5 mg/ml), or 10-15% CM either singly or in combinations, for induction of somatic embryos from mature and immature cotyledons and callusing from stem explants. After mixing well, the pH of the medium was adjusted to 5.5 using 1N NaOH and 1 N HCl. The final volume of the medium was made up to 1000 ml with double distilled water.

Table 13: Stock solutions for Murashige and Skoog's (1962) medium (MS).

Constituents	Amount (mg/l)
Stock solution I (20X)	
NH_4NO_3	33000
KNO_3	38000
$CaCl_2.2H_2O$	8800
$MgSO_4.7H_2O$	7400
KH_2PO_4	3400
Stock solution II (200X)	
KI	166
H_3BO_3	1240
$MnSO_4.4H_2O$	4460
$ZnSO_4.2H_2O$	1720
$Na_2MoO_4.7H_2O$	50
$CuSO_4.5H_2O$	5
$CoCl_2.6H_2O$	5
Stock solution III (200X)	
$FeSo_4.\ 7H_2O$	5560
$Na_2EDTA.\ 2H_2O$	7460
Stock solution IV (200X)	
myo-Inositol	20000
Nicotinic acid	100
Pyridoxine. HCl	100
Thiamine. HCl	20
Glycine	400

3.6.5. Preparation of stock solution for plant growth regulators

All the growth regulators are not soluble in water. Solubility of different growth regulators is given in Table 14. The compound was dissolved in few ml of solvent and then slowly added water to make up to the requisite volume.

Table 14. Preparation of stock solutions for plant growth regulators.

Compound	Abbreviation	Molecular weight(MW)	Solubility	mg/100ml (1 mM)
Auxins Indol-3 acetic acid	IAA	175.18	1N NaOH	17.51
Indole-3 butyric acid	IBA	203.23	1N NaOH	20.32
2,4-Dichlorophenoxy acetic acid	2,4-D	221.00	Alcohol	22.10
Naphthalene acetic acid	NAA	186.20	1N NaOH	18.62
Phenylboronic acid (Novel auxin)	PBOA	121.93	1N NaOH	12.19
Others: Gibberellic acid	GA_3	346.37	Alcohol	34.63
Brassin	Brassin	480.68	Alcohol	48.06
Cytokinins				
Benzyl adenine or 6-benzyl amino purine	BA or BAP	225.2	1N NaOH	22.52
Kinetin	KIN	215.2	1N NaOH	21.52
n-phenyl-N-1,2,3-thiadiazol-5-Urea (thidiazuron)	TDZ	220.2	1N NaOH	22.02

Table 15. Time duration for media sterilization at 15 psi

Volume (ml)	Sterilization time (min) at 121°C and 15 psi (pressure per square inch)
1 - 200	15
200 - 1000	30
1000 - 2000	40

3.6.6. MS medium preparation and sterilization

The sequences of steps involved in preparing MS medium were as follows: 30g sucrose and 8g agar powder were weighed and dissolved in 750 ml double distilled water in a 1 l flask, by heating them in a water bath. Appropriate quantities of the stock solutions were added (I, II, III and IV) sequentially. The medium was supplemented with the different plant growth regulators such as BAP, IAA, PBOA, NAA, 2,4-D and Kin (1-5 mg/ml), or 10-15% CM either singly or in combinations, for the induction of somatic embryos and embryo conversion to plantlets or effect on morphogenesis from callus. After mixing well, the pH of the medium was adjusted to 5.8 using 1N NaOH and 1 N HCl. The final volume of the medium was made up to 1000 ml with double distilled water. About 50 ml medium was dispensed into 250 ml Erlenmeyer flask and 15 ml in a 25 x 150 mm culture tube. Thermolabile plant growth regulators were filter sterilized through a 0.22 μm-0.45 μm disposable filter. Filter sterilized solutions were then combined with other nutrient substances sterilized in the autoclave to give a complete medium. Mouth of the culture vessels (flask) were closed with non-absorbent cotton wrapped in cheese-cloth (such closures exclude microbial contaminants but allow free gas exchange) and culture tube with plastic caps. The culture vessels containing medium were transferred to appropriate baskets, covered with aluminum foil to check wetting of plugs during autoclaving and sterilized by autoclaving at 121°C (15 psi) for 18 minutes (Table 15). The medium was allowed to cool at room temperature in 250 ml flasks but for slant preparation by keeping the tubes tilted during cooling in a slanted plastic rack. Such slants provide a larger surface area for tissue growth. The prepared medium was used 3-4 days after preparation, so that if it is not properly sterilized, contamination will start appearing and this medium can be discarded. Media were stored at 4°-10°C.

3.6.7. Preparation of undefined supplement (coconut milk)

The coconut milk (CM) was used to promote the growth of certain calli and organs. The liquid endosperm of *Cocos mucifera* fruits was used in the present investigation as an undefined organic supplement. Coconut milk from the fresh fruits was usually strained through cloth and deproteinized by being heated to 80-100°C for about 10 minutes while being stirred. It was then allowed to settle and the supernatant was separated from the coagulated proteins by filtration through paper. The liquid was stored frozen at-20°C and used as and when required.

3.6.8. Sterilization of explants

Mature and immature cotyledon of tea seeds from tea clone T383 was used for somatic embryogenesis and 5-7 mm long stem segment for callusing were used as explants in the present investigation from four tea clones, T383, TV30, HV39 and UPASI-26. Surfaces of the plant explants carry a wide range of microbial contaminants. To avoid this source of infection the plant explants were thoroughly surface sterilized before inoculation into the medium by different disinfectant (Table 16).

The following sequences of steps had been followed in the initiation of aseptic tissue culture from four tea clones. Mature and immature fruits were collected in a beaker and washed them in tap water with few drops of liquid detergent (Teepol) and then thoroughly washed in running tap water. After washing, fruits were treated with 3% hydrogen peroxide for 2 min and then with 70% alcohol for 2 min in a beaker, and then decanted the alcohol. Fruits were transferred to a screw cap bottle containing 20% solution of sodium hypochloride (1% active chlorine content) with a small amount of a suitable surfactant (2-3 drops of Triton-X100) and after capping the screw bottle was shaken gently for 5 minutes under Laminar Air flow. Then the cap of the bottle was removed and the liquid poured out and an adequate quantity of sterilized, distilled water was poured onto the seeds and the cap replaced. After shaking a few times, the water was discarded. Such washings with sterile distilled water were repeated 5 times. The seeds were removed from the fruits using sterile scalpel and forceps.

The seeds were then transferred to a pre-sterilized Petri-dish to remove the seed coat from the seeds and collected the cotyledon by removing the embryo and used as initiation explants. The intact mature de-embryonated cotyledonary endosperm cut into pieces (0.5-1.0 cm long) before placed into the somatic embryo induction medium. Green capsule collected in September were sterilized by the same procedure before the immature seeds (9-11 mm in diameter) were isolated and then each of immature de-embryonated cotyledon placed into the same medium for somatic embryogenesis. The cotyledon explants of various size and maturity are transferred onto the different medium in a culture tube of 25 x 150 mm in size and in 250 ml flask containing 50 ml medium, the neck of the culture vial flamed and the closure replaced in quick succession. For flame sterilization of the small instruments (Scalpels, forceps, razor) these were soaked in 95% ethyl alcohol followed by flaming

on a spirit lamp in the laminar airflow hood, which was repeatedly done while aseptic manipulation work was in progress. Young shoots of 10 cm long were also collected from the germplasm field of four tea clones for callus initiation and morphogenesis. Young shoots were washed in tap water with few drops of liquid detergent (Teepol). Young shoots were treated with 10% calcium hypochloride for 5 minutes. Then young shoot was cut into 5-7 mm long stem segment to be used as explants and treated with 70% ethanol in a screw cap bottle for 2 minutes. Stem explants was then transferred to sterile tissue paper in a petri plate and then inoculated into the MS and B5 medium with different concentration of plant growth hormones.

Table16: Disinfectants used for sterilizing explants material

Sterilizing agents	Concentration used (%)	Duration (minutes)
Calcium hypochloride	10	5
Sodium hypochloride	3% (of 20% v/v commercial bleach)	5
Hydrogen peroxide	3	3

3.6.9. Transfer of the explants

All kinds of transfer operations were carried out under strictly aseptic conditions in the laminar airflow hood, to prevent the entry of any contaminant into the culture vial when its months was opened either for sub-culture or for planting fresh tissues (inoculation). Laminar airflow hoods were sterilized by switching the UV light to expose the working area for 20 minutes and then wiping the working surface with 95% ethyl alcohol 15 minutes before initiating inoculation operation under the hood. The velocity of the air coming out of the fine HEPA filter was 27± 3 m per min (removes particles larger than 0.3 μm) which was adequate for preventing the contamination of the working area. Hands were washed with 95% ethyl-alcohol before starting the inoculation experiment as a measure of precaution.

3.6.10. Initiation of callus culture

Callus culture was initiated in both the basal media of MS and B5 taking the stem segment from four tea clones as explants, TV30, HV39, T383 and UPASI-26. After four weeks of culture the callus was transferred to the MS medium only because growth was optimum in MS medium. The MS medium was supplemented with

different concentration of plant growth hormones and coconut milk (CM) for the study of effect of various growth factors in callus growth, development and regeneration of plantlets. The cultures were incubated at 25± 2°C under 16 h photoperiod of 2000 lux light intensity (from Phillip's fluorescent lamps), at the level of the cultures.

3.6.11. Initiation of somatic embryogenesis

Both the cotyledonary tissues of immature and mature seeds (of tea clone T383) were placed onto the surface of semisolid, full strength Murashige and Skoog (1962) medium. The intact mature de-embryonated cotyledonary endosperm was cut into pieces (0.5-1.0 cm long) before placed into the somatic embryogenesis medium. Immature cotyledons from the immature seeds (9-11 mm in diameter) were isolated and then each of immature de-embryonated cotyledons placed into the medium. The medium was supplemented with the different plant growth regulators such as BAP, IAA, PBOA, NAA, 2,4-D and Kin (1 –5 mg/l), or 10% CM either singly or in combinations, for induction of somatic embryos. The medium was solidified with 0.8% agar after adjusting to pH 5.8 and sterilized at 121°C for 15-18 min. All the MS media contained Fe-EDTA and micronutrient formula described by Murashige and Skoog plus 0.5 mg/l thiamine-HCl, 0.5 mg/l pyridoxine-HCl, 0.05mg/l, nicotinic acid, 100 mg/l, m-inositol 30 g/l, sucrose 30g/l and 8g/l Difco-Bacto-Agar, The cultures were maintained for 14 weeks at 25± 2°C under a 12/12 (day/night) photoperiod with light provided by cool-fluorescent tubes at a photon flux density of 52-μmol $m^{-2}s^{-1}$ and 60-65% relative humidity.

3.6.12. Regeneration of plantlets from somatic embryos and calluses

For tea plant regeneration, somatic embryos (of tea clone T383) were removed from cotyledons and cultured in MS and Gamborg's B_5 (1968) basal medium impregnated with various growth regulators like BAP (5 mg/l), IAA (2 mg/l), Brassin (2 mg/l) and GA_3 (1.5 mg/l), either singly or in different combinations, or without growth regulators. Plantlets were regenerated from callus of tea clone T383 in MS medium containing different levels of cytokinin (TDZ, BAP) and auxin (IBA and NAA). Shoot formation was initiated in MS medium containing 1 mg/l IBA and 4 mg/l BAP. The shoot formation was also initiated from callus while cultured in MS medium with TDZ (cytokinin). MS medium with 1 mg/l NAA and 3 mg/l BAP was

shown shoot formation. The shoots were then transferred to ½ MS medium with 1 mg/l IBA in filter paper bridge in liquid medium for rooting. Kept as such for 4-6 weeks and then transferred to ½ MS medium for normal growth and development. All the experiments were repeated three times. Regenerated plants after 15-16 weeks old with well-developed roots and leaves were transferred into the hardening chamber for hardening the plantlets.

3.6.13. Hardening of *in vitro* regenerated plantlets

Selection and preparation of plantlets prior to their transfer to pots. 15-16 weeks old plantlets of *Camellia sinensis* were obtained through *in vitro* somatic embryo germination and morphogenesis from callus, were used as ready source of material for present study. Healthy plantlets having a height of 4-6 cm with 5-6 well developed leaves and 3-4 roots were deflasked and washed very carefully with double distilled water. Utmost care was taken during washing so that no agar or media should remain attached with the plantlets. After that they were treated with fungicide captan (4 mg/l) and kept on a sterile Petri dish.

3.6.14. Transfer of plantlets to potting mixture

Plastic bags measuring 2□ diameter X 2.5□ height were brought from the market. The bags were filled with a mixture of peat and soil at a ratio of 1:1. After filling up the plastic bags (pots) with potting material the plantlets were very carefully transferred and planted in the pots. The pots were then covered with inverted beakers. Distilled water was regularly sprayed in the beakers to keep high humidity around the plantlets. The pots were kept in the hardening chamber at culture room conditions *i.e.* at 25± 2°C under 16 h photoperiod for 3 weeks. After that the pots were transferred to greenhouse and the beakers were removed. In the greenhouse the pots with plants were kept for another 2 weeks under natural photoperiod and humidity. Finally after 2 weeks the plantlets were transferred to large earthen pots filled with garden soil. Watering was done at regular intervals. Results showed that the humidity played important role in the hardening of the regenerated plantlets. The highest survival rate 61% was found in clone T383 at the relative humidity of 95%.

3.7. Genomic constitution as revealed by chromosome

Cultivated tea (*Camellia sinensis*) has been maintained for centuries, by vegetative propagation. An immense heterogeneity is existed in the commercial tea

populations, because of the polymorphic origin of the latter. Cultivated tea generally is a mixture of species of tea *Camellia sinensis* (L) O. Kuntze (China type); *Camellia assamica* (Masters) (Assam type) and *Camellia assamica* sub-species *lasiocalyx* Planchon MS. (Cambod type), and other species of *Camellia*, including those fall outside the purview of *Thea* section. Most of the cultivated tea of the world are diploid 2n=30 and highly heterogeneous as a result of free natural hybridization between geographical races during cultivation. Moreover, continued development and release of cultivated varieties have added to the genomic diversity of commercial tea. There are no serious and systematic attempts have been made for gaining detailed information about the genomic constitution of cultivated tea.

3.7.1. Plant material

Karyotype analyses in ten cultivated tea clones [(*Camellia sinensis* (L.) O. Kuntze] were investigated for their characterization. Ten tea cultivars, T78, T383, TV30, HV39, TeenAli17/1/54, TV23, TV25, TV29, TV26, and UPASI-26, were used in the present chromosome study. Six months old tea cuttings were maintained in the earthen pot for the collection and availability of root tips. Root tips were collected by inverting the whole pot and removing the pot from soil clumps. And then fresh root tips of 5 mm size were collected in distilled water with a scissor from the surface of the soil clumps. Root tips were collected in a day light between 10.00 am to 12 noon.

3.7.2. Preparation of solution and cytological stain

2% (w/v) Aceto-Orcein stain

2 g Orcein powder was dissolved in 90 ml of 45% acetic acid in a 250 ml flask and heated the solution for 10 minutes at a simmering point. Then allowed to cool at room temperature and filtered through Whatman filter paper. Final volume 100 ml was made up by adding required amount of 45% acetic acid and stored at room temperature for cytological preparation.

Saturated solution of ρ-dichlorobenzene

The saturated solution of ρ-dichlorobenzene was prepared by adding at least 300g ρ-dichlorobenzene in 500 ml distilled water and allowed overnight for saturation and then stored at 4°C.

Fixative chemicals

Aceto: ethanol (1:3) mixture was prepared by adding 25 ml glacial acetic acid and 75 ml absolute ethanol and stored at 4°C for further use.

3.7.3. Somatic chromosome technique

The actively growing apical meristem part of the roots were collected between 10 am to 12 noon and properly washed in distilled water immediately after collection. About 5 mm long cut apical root meristems were pretreated in saturated solution of ρ-dichlorobenzene with a little amount of Aesculine for 3.3 h at room temperature and fixed in a fixative chemical, acetic: ethanol (1:3) for at least 24 h at room temperature. Following hydrolysis in 5N HCl for 1 h at room temperature, the root tips were transferred to 45% acetic acid for 10 min. The root tips were then stained in 2% aceto-orcein by heating the sample over spirit lamp for few seconds and allowed for 40 minutes to take the proper stain by the chromosomes. The temporary squash preparations were made in 45% acetic acid on grease free slide covered by square cover slips. The slides were sealed with wax to prevent air penetration into the chromosome preparation. The chromosome karyotypes from each of the clone were drawn using Camera-Lucida at the table magnification X 1500 and photomicrographs were taken in Lieze microscope at different magnifications and suitably enlarged and analyzed.

Each of the Karyotype was analyzed by the following index-

Centromeric index (F %), Total centromeric index (TF %), Disparity index (DI) and Total Haploid Chromosome Length (TCL) were calculated according to Huziwara (1962).

$$F\% = \frac{\text{Short arm length of the chromosome}}{\text{Total length of the chromosome}} \times 100$$

$$TF\% = \frac{\text{Total sum of short arm length}}{\text{Total sum of the chromosome length}} \times 100$$

$$D.I. = \frac{LCL - SCL}{LCL + SCL} \times 100$$

LCL = Longest chromosome length
SCL = Shortest chromosome length

3.7.4. *In vitro* cytology

Somatic chromosome constitution from the root tips of the regenerated plantlets (T383) was also studied to investigate the somaclonal variation at the chromosomal level. Same methods were applied as was *in vivo* cytological method. The apical meristems of root tips collected from one year old plantlets (T383) growing *in vitro* was used for the present cytological studies.

3.8. Abiotic induction of defense genes expression by methyl jasmonate (MeJa)

3.8.1. Preparation of test solution

A known plant defense inducer such as methyl jasmonate (0.2 mM) was prepared by dissolving 44.8 mg in 1000 ml distilled water. The solution was supplemented with a few drops of Tween-20 to ensure adhering to the plant leaves.

3.8.2. Foliar application

The 12 months old tea plants (clone T383) were grown in earthen pots and maintained in greenhouse in ambient light and temperature. Leaf development was separated into two stages- Stage I-young leaves, 2-4cm long (YL) and Stage II-mature leaves, 5-9cm long (ML). The above test solution, methyl jasmonate (MeJa) was sprayed as foliar application at a rate 40 ml/m^2 as to wet the leaves completely on both the ventral and dorsal surface using a hand sprayer.

Similarly tissues (tea clone T383) from different growth and developmental stages of *in vitro* grown calluses (2 months) and *in vitro* regenerated shoots of somatic embryos (6 months) were treated with test solution after filter sterilized through a 0.22 µm disposable filter. Controls were mock sprayed with distilled water plus Tween-20. Sprays were applied between 10.00 am to 11.00 am while stomata were remaining consistently open. The leaves, callus and shoots were harvested from different time courses (0, 0.3, 2, 6, 12, 16, 24, 36, 48, 72 h) and frozen in liquid nitrogen and stored at -80°C before RNA isolation. Experiments were repeated twice with 3 replicates.

3.9. Chitinase gene specific transcript accumulation

Differential gene expression (specifically chitinase gene, PR-3 protein gene) was studied during the induced systemic resistance (ISR) induced by methyl jasmonate. The above abiotically induced tea plants (T383) in different

developmental stages were used for mRNA transcript accumulation in different time course to observe the defense related gene expression levels.

3.9.1. Preparation of buffer for RNA isolation and gel electrophoresis

The following buffers for RNA isolation reactions were prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH using 1N HCl or 1N NaOH solution in all the cases.

10x MOPS electrophoresis buffer

0.2 M MOPS (pH 8.0)
20mM sodium acetate
10mM EDTA (pH 8.0)

RNA denaturing solution

10x MOPS electrophoresis buffer	2.0µl
Formaldehyde	4.0µl
Formamide	10.0µl
Ethidium bromide (200µg/ml)	1.0µl
RNA (up to 20µg)	2.0µl

10x Formaldehyde gel-loading buffer

50% glycerol (diluted in DEPC- water)
10mM EDTA (pH 8.0)
0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF

RNA extraction buffer (200ml)

2% CTAB(w/v)	4g
2% PVP K-30 (w/v)	4g
100mM Tris-HCl (pH 8.0)	2.42g
25mM EDTA	1.16g
2M NaCl	16.38g
Spermidine	0.1g
2% (v/v) β-mercaptoethanol	4ml

Lithium Chloride (LiCl, 7.5M)

LiCl 31.8g
Make final volume 100ml with H_2O

DNase I dilution buffer

10mM Tris-Cl (pH 7.5)
150mM NaCl
1mM $MgCl_2$

10x DNase I treatment buffer

100mM Tris-Cl (pH 8.4)
500mM KCl
15mM $MgCl_2$
0.01% gelatin

3.9.2. Total RNA isolation (transcripts)

Total RNA isolation was done according to Korban *et al.* (2004) from the above abiotically induced tea plant tissues. Briefly 1g leaf tissue was taken from each of the different tea samples (four treated and four controls). Samples were frozen and pulverized in liquid nitrogen, and added prewarmed (60°C) 5 ml RNA extraction buffer to make slurry using mortar and pestle. Vortex briefly and kept at 60°C for 15 minutes. Added equal volume of chloroform: isoamylalcohol (24:1) and immediately vortex for 2 minutes. Centrifuged at 10,000g for 10 minutes at 4°C and taken the upper aqueous supernatant into a fresh tube and repeated the extraction with an equal volume of chloroform: isoamylalcohol mixture. Again collected the upper supernatant carefully and transferred into a new tube. RNA precipitation was done by adding 1/3 volume of 7.5 M LiCl to each tube, mixed by inversion and kept at 4°C overnight for complete precipitation. Each of the RNA pellet was recovered by centrifugation at 12,000g at 4°C for 30 minutes and washed RNA pellet twice with 70% ethanol and air-dried at room temperature for 10 minutes. Each of the RNA samples was treated with DNase I, by the addition of 100µl DEPC water, 20µl 10x DNase I buffer and 4 µl DNase I to each tube. After a 30 minutes incubation at 37°C, the samples were extracted with an equal volume of 1:1 phenol:chloroform and centrifuged at 12,000g for 10 minutes at 4°C. Transferred the upper aqueous supernatant into a fresh tube and precipitated the RNA with 20µl 3M sodium acetate and 600µl absolute ethanol keeping at -80°C overnight. The samples were centrifuged 10 minutes at 12000g at 4°C. The RNA pellets were washed in 70% ethanol twice and air-dried at room temperature for 10 minutes. Dissolved the RNA in 80µl DEPC-treated distilled water and measured the quantity by UV-*vis* Spectrophotometer (Shimadzu, 160) at 260nm. Again RNA was precipitated by adding 2.5 volumes of 70% ethanol followed by addition of $1/10^{th}$ volume of 3M sodium acetate for 3 h at -80°C. RNA pellet was recovered by centrifugation at 12,000g for 30 minutes at 4°C and finally dissolved in 200µl DEPC-treated (diethylpyrocarbonate) distilled water and stored at -80°C.

3.9.3. Quantification of RNA by Spectrophotometry

The purified total RNAs were quantified with a UV-*vis* Spectrophotometer (Shimadzu, 160) at wavelength of 260nm and 280nm. The A_{260} value 1 corresponds to ~ 38µg/ml RNA. A pure preparation of RNA has $A_{260/280}$ value 2.0, which

indicates that the RNA is pure without any protein contamination. RNA concentration was adjusted at 20μg/μl with DEPC treated distilled H_2O. The integrity of total RNA was verified by running samples on 1.2% denaturing agarose gel.

3.10. Denaturing agarose gel electrophoresis for RNA

RAN was fractionated on 1.2% agarose gel containing 2.2 M formaldehyde (commercial formaldehyde is 12.3M) to check the quality and integrity.

3.10.1. Preparation of 1.2% denaturing agarose gels

To prepare 30 ml of a 1.2% agarose gel containing 2.2 M formaldehyde, added 360mg agarose to 21.7 ml of sterile distilled water. Dissolve the agarose by boiling in a microwave oven. Cool the solution to 55°C and added 3.0 ml 10x MOPS electrophoresis buffer and 5.3 ml of formaldehyde. The gel was set at room temperature for 1 h, and then transferred to the submarine gel electrophoresis tank for fractionation (according to Sambrook and Russell, 2001).

3.10.2. RNA sample preparation and electrophoresis

Before loading into the gel, RNA was denatured in a denaturing solution at 55°C for 60 minutes. Samples were chilled for 10 minutes in ice water then added 2μl of 10x formaldehyde gel-loading buffer to each sample. RNA samples (20 μg RNA) were loaded into the slots of the gel and turn on the power supply at 90 mA constant current and 75 Volt for electrophoresis in 1x MOPS buffer for 2 h. The RNA bands were visualized by placing the gel on a UV-transilluminator and photographed.

3.11. Reverse transcriptase- Polymerase Chain Reaction (RT-PCR)

The above RNA was taken from each of the treated and untreated (control) samples after induction with methyl jasmonate for the quantization of transcription accumulation (mRNA transcription levels). Defense system related gene expression was induced in the tea plant (clone T383) by the inducer, methyl jasmonate. In the present study, chitinase gene specific expression level (mRNA transcripts) was measured by RT-PCR (Innins *et al.*, 1990).

3.11.1 Preparation of RT-PCR buffers

The following buffers for RT-PCR reactions were prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH using 1N HCl or 1N NaOH solution in all the cases.

10x PCR Amplification buffer
500mM KCl
100mM Tris-HCl (pH 8.0)
15mM $MgCl_2$
0.1% Gelatin

Taq storage and dilution buffer
20mM Tris-HCl (pH 8.0)
0.1 mM EDTA
1mM dithiothreitol (DTT)
100mM KCl
0.5% Tween-20
0.5% Igepal
50% Glycerol (v/v)

One-step RT-PCR reaction mixture (25µl)

2x RT-PCR reaction mix.	12.5µl
10mM dNTPs (pH 8.0)	2.0µl
20µM Forward primer	2.5µl
20µM Reverse primer	2.5µl
Taq polymerase 2U	1.0µl
Denatured RNA (15µg)	2.0µl
Reverse transcriptase (100U)	1.0µl
RNasin	1.0µl
Sterile H_2O	0.5µl

10x Reverse transcriptase buffer
500 mM Tris-Cl (pH 8.0)
750 mM KCl
30 mM $MgCl_2$

Proteinase K (20 mg/ml) 10ml

Proteinase K 200mg
50mM Tris (pH 8.0)
1.5mM Calcium acetate
Make final volume 10ml
Use 50µg/ml in reaction

Proteinase K reaction buffer
0.01M Tris-Cl (pH 7.8)
0.005 M EDTA
0.5% SDS
50µg/ml Proteinase K

3.11.2. One-step RT-PCR for chitinase gene specific transcript accumulation

Chitinase gene specific transcript accumulation (mRNA transcription level) was obtained through RT-PCR method. The one step M-MuLV RT-PCR kit of Bangalore Genei was used in the present study and reaction was carried out according to the manufacturer's instruction manual. This one-tube method is designed for the

reverse transcription (RT) and polymerase chain reaction (PCR) amplification of a specific target RNA from either total RNA or mRNA. In the present investigation total RNA was added in the reaction mixture. Two-enzyme system was added at a time for sensitive, quick and reproducible analysis of even rare RNAs. The M-MuLV reverse transcriptase (M-MuLV-RT) from moloney murine leukemia virus was used for first strand cDNA synthesis and the *Taq* DNA polymerase from *Thermus aquaticus* was used for second-strand cDNA synthesis and DNA amplification with a fidelity enzyme. The kit consists of two major components: RT-PCR enzyme mixture and 2x RT-PCR reaction mixture. The M-MuLV RT enzyme in the mixture can synthesize cDNA at a temperature range of 40°C to 50°C and detect product upto 3 kb with varied amount of starting material (10 ng to 2 µg) of total RNA.

During reverse transcription, the HotStart *Taq* DNA polymerase is completely inactive and does not interfere with the reverse transcription reaction. For RT-PCR reaction, prepared the master mixture1, by adding RNasin, 2x RT-PCR reaction mixture, RT-PCR enzyme mixture including a fidelity enzyme, dNTPs, primer pair (chitinase specific) according to RT-PCR kit, and kept on ice. Master mixture 2 was prepared separately by adding denatured RNA at a final concentration 15µg/25µl RT-PCR reaction mixture. Heated master mixture 2 at 65°C for 5 minutes to denature the template RNA and kept on ice. The required volume of the master mixtures 1 and 2 were dispensed into each 0.2ml PCR tube on ice according to the manufacturer instruction manual for reverse transcription reaction. Placed the PCR tubes in the thermal cycler and started the One-step M-MuLV RT-PCR reaction.

The first strand cDNA synthesis was performed using the above One-step RT-PCR reaction mixture by incubating the reaction for 30 minutes at 50°C. After reverse transcription by M-MuLV reverse transcriptase, reaction was heated to 95°C for 10 minutes to activate HotStart *Taq* DNA polymerase and to simultaneously inactivate the reverse transcritase. The cDNA obtained was PCR amplified in 35 cycles consisting of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min and 72°C for 7 min for final extension in a thermocycler using the primers 5'-TTTTCGTCGGAAAATGGAAG-3' (forward primer) 5'-ACCAGCTTCTTCTCGTCCAA-3' (reverse primer). The primer sequence was the same as that used for obtaining the 201bp chitinase gene specific DNA fragment from genomic DNA (T383). The second DOP-primer pair was constructed from the

chitinase protein sequences was also used in the RT-PCR to quantify the chitinase gene specific transcript accumulation in treated (induced) and untreated samples. Forward DOP-primer was 5'-CTGCTGCGGCAAGTGGkgytggwrytg-3' and reverse primer was 5'-CAGTAGCAGCAGCAGTTGCArywccarcmsma-3'. In case of DOP-primer, the cycling temperature for 35 cycles was as follows- 94°C for 1 min, 60°C for 1 min and 72°C for 2 min and 72°C for 7 min for final extension.

3.11.3. RT-PCR product quantification on gel

The amplified cDNA product obtained from the RT-PCR was analyzed by electrophoresis on 1% agarose gel (described in section **3.2.11**) for visual quantification of the chitinase gene specific transcript accumulation in different samples (treated and control). 15µl aliquot of each of the RT-PCR product was taken and mixed with 3µl of 6x DNA loading buffer and then loaded into the slots of agarose gel. The gel was then run at voltage 75 and current 90 mA for 1.3 h until the bromophenol blue and xylene cyanol FF migrated at the end of the gel. The gel was taken out from the buffer tank and examined the gel by UV light on UV-Transilluminator and photographed.

3.11.4. RT-PCR product purification for transcript accumulation

The cDNA product of RT-PCR was purified according to Sambrook and Russell (2001). 150µl RT-PCR product was pooled up from the reaction tubes and added 0.2 volume of 5x proteinase K buffer to a final concentration of 50µg/ml. Incubated the mixture for 60 minutes at 37°C. Enzyme activity of the proteinase K was inactivated by heating the reaction mixture to 75°C for 30 minutes. Then extracted the solution once with equal volume of phenol: chloroform (1:1) and once with chloroform only. The upper supernatant was transferred to a fresh tube after centrifugation at 10,000g for 5 minutes. Added 0.2 volume of 10 M ammonium acetate and 2.5 volume of chilled (-20°C) ethanol for DNA precipitation. Mixed the solution by gentle shaking and kept at 4°C for 30 minutes for complete precipitation of RT-PCR product (cDNA fragments). The precipitated DNA was recovered by centrifugation at 5000 rpm for 5 minutes at 4°C in a microfuge. Discarded the supernatant and washed the DNA pellet twice with 70% ethanol. The DNA pellet was collected by centrifugation the solution at 5000 rpm in a microfuge at 4°C for 5 minutes. The DNA pellet allowed drying in the air in room temperature. Finally

purified RT-PCR product (DNAs) dissolved in 50µl TE buffer (pH 8.0). It was assumed that approximately 80% of the RT-PCR product was recovered by this method.

3.11.5. Quantification of purified RT-PCR product (cDNAs) to measure transcript accumulation

The purified RT-PCR products (cDNAs) from each of the treated and untreated samples (six samples) during induced systemic resistance by methyl jasmonate were measured by spectrophotometry. In RT-PCR, cDNA was synthesized by converting the mRNA molecules present in the starting RNA samples by reverse transcriptase and then the cDNA was amplified by normal PCR method. Quantification of RT-PCR product simultaneously indicates the quantity of transcription accumulation (mRNA transcription) in a given time. That means, the chitinase gene specific transcript accumulation has also been measured. The amount of the DNA concentration estimated in each sample was compared with the band thickness on the agarose gel. Bands were proportionately thick on the gel slots wherever the mRNA concentration was more within the starting samples of 15µg total RNA.