LITERATURE REVIEW

Taxonomic position of tea

The generic name *Camellia* was derive from Kamel, George Joseph Kamel, a German Missionary stationed in the Philippines, also wrote about plants found in Asia during the latter half of the seventeenth century. In 1753 Linnaeus had given the name of tea plant *Thea sinensis* in Vol. I of his 'Species planterum' while describing the two ornamental species as *Camellia sasanqua* and *Camellia japonica* in Vol.II. Lastly, in its session of the Botanical Congress held in Amsterdom during 1935, decided to unite the two genera *Thea* and *Camellia* into a single genus, *Camellia.* The correct scientific name of the tea plant is *Camellia sinensis* (L.) O. Kuntze since it gives recognition to the authority responsible for the union of the old name *sinensis* with the new genus *Camellia.* The internationally accepted scientific nomenclature is *Camellia sinensis* (L.) O. Kuntze. The genus *Camellia* of which tea is a member belongs to the family Theacee, tribe Gordonieae (Barua, 1989). All tea plants were placed under one species, *Camellia sinensis.* Earlier taxonomists describe the tea plant as *Camellia sinensis* (L.) for the whole tea population ignoring its varietals status. Later taxonomists (Wight, 1962; Barua, 1963 and Bezbaruah, 1971) recognized two main varieties namely China type (all narrow leaved China types of plants) and Assam type (covering the broad leaved Assam type of plants) on the basis of morphological and anatomical description of the tea plants. Two main varieties of tea were also grouped as *C. sinensis* var. *senensis* (L). and *Camellia sinensis* var. *assamica* (Masters). The Assam variety was first described by Masters (1844) as *Thea assamica.* The Assam and China varieties have different morpohological atrributes, markedly differ in their growth rate and other features, leaf size, shape, texture *etc.* Then a third type of tea plant was found which was considered as a Cambod (Cambodiensis) or southern form as described by Planchon. This plant did not differ much from the Assam type plant. Later on it was described as *Camellia assamica* sub sp. *lasiocalyx* (Planchon MS.). This third variety has been approved by different scientists (Kingdon-Ward, 1950; Sharma and Venkataramani, 1974). The present status of the tea has been considered as China type, Assam type and Cambod type as distinct variants or varieties of *Camellia sinensis* (L.) O. Kuntze, based on their distinctive morphological and morphmetric features (Bezbaruah, 1976; Wight, 1962). The morphological traits of

the three main variants of tea are therefore of considerable practical importance, not only because of their agronomical importance in providing yield and quality, but also because they provide the genetic sources for selection of better plant types in which features of agronomical significance can be incorporated.

Tea growing regions of India

South-East Asia is the original birth place of tea and China is the first country to use tea as a beverage. From this region, tea spread to different countries of the world such as China, Japan, Taiwan, Bangladesh, Indonesia, Srilanka, U.S.S.R., Kenya, Turkey, Iran, Africa, India and others. In India tea plantation is mainly concentrated into two traditional regions, one in north-east and other in South-India. In addition, tea is grown in a very limited area in Kangra (Himachal Pradesg) and Dehra Dun (Uttar Pradesh) valleys of North-West India (Figure 1 and Table 1).

Table 1. Tea growing regions of India.

Regions	Tea areas
1. North-East India (N.E.India)	Assam valley, Barak valley, Cachar, Dooars, Terai, Darjeeling, Sikkim, Orissa, Bihar.
2.North-West India	Kangra Valley (H.P), Dehra Dun (UP)
3.South India (Kamataka,Kerala & Tamil Nadu)	Annamalai (Cinchona), Travancore (Vandiperiyar), High Ranges (Munnar), Nilgiris (Coonoor), Wyannad (Mepppadi).

Genetic resources and natural variability

Tea plant belongs to the genus *Camellia,* family theaceae. The other ten genera under this family are *Stuartia, Schima, Franklinia, Yunnanea, Gordonia, Tutcheria, Laplacea Pyrenaria, Eurya, Ternstroiemia* and *Adenanthera* (Chang, 1981). Chang (1981) reported over 200 species under the genus *Camillia,* which is the most important of all the genera.

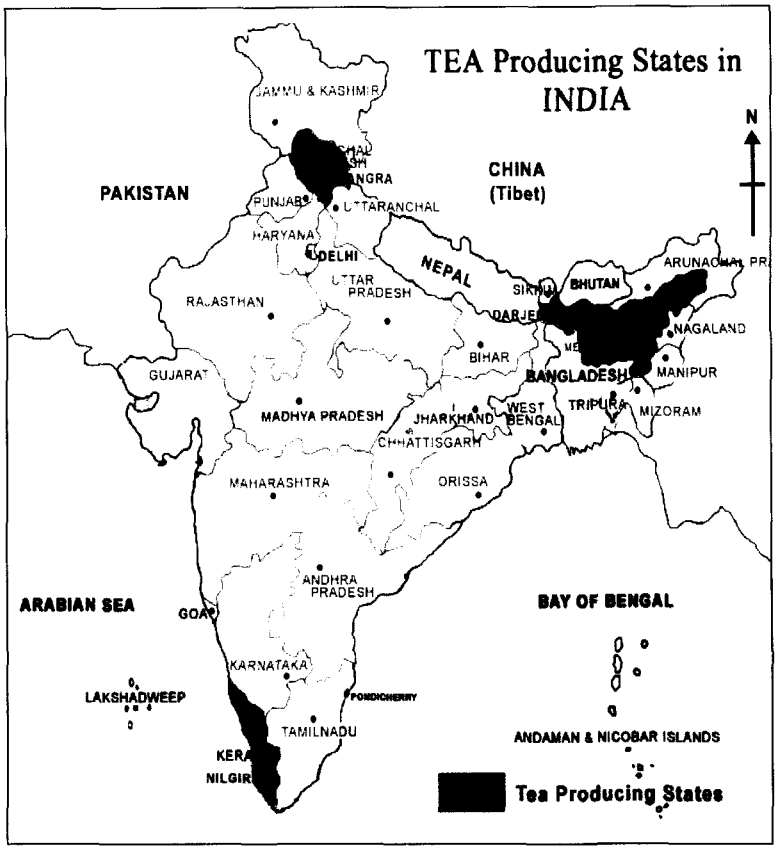

Figure 1. Map of India showing the three main tea producing regions.

Tea population of today are highly heterozygous because of free cross-pollination for many years among all the taxa owing to their cross compatible and inter- fertile nature. Species hybrids, at least involving a number of related species exist in cultivated tea population (Barua, 1965 and Bezbaruah, 1974). Quality trait of tea particularly Darjeeling tea might be due to incorporation of a number of genes from other species including non-tea or wild species growing in the same habitat. Barua (1965) reported that the complex hybrids of the three cultivated forms of tea one or more of the wild species of *Camellia* occur in the tea population particularly in the Darjeeling district of north-east India, which is considered as "Champenge of tea" for its distinctive quality and flavour. Tea was hybridized with non-tea species *Camellia irrawadiensis, C. taliensis, C. lutescens* to develop caffeine deficient tea but industrially was not viable. The above three non-tea species are devoid of caffeine.

The cultivated population can broadly be classified on the basis of morphological, anatomical and biochemical proximity to the main taxa into three types-Assam type (*Camellia assamica* Masters), China type (*Camellia sinensis* L.)

and Cambod type or Southern form (*Camellia assamica* ssp. *lasiocalyx* Planchon. MS). Since, China is centre of origin of *Camellia*, where maximum genetic diversity and most of the species of China type and others are found in the Yunnan province of South-west China. The north-east India mainly Assam region bordering Burma is the origin of Assam tea plant where maximum diversity will respect to genetically and morphological characters are found. Other *Camellia* species prevailed in this region (north-east) are *Camellia caudata, C. caduca, C. irrawadiensis, C. taliensis, C. kissi, C. drupifera* and related genera like *Eurya, Pyrenaria, Schima and Gordonia.*

So, genetic resources of tea composed of cultivated varieties, non-cultivated or wild species and genera, land races, improved clones, breeding hybrids and seed stocks. In the hybrids, considerable species introgression has also taken place. For commercial use, tea clones must carry the desirable characters for cultivation like cup quality, disease, pest and drought resistance *etc.* Quality differs from one kind of tea to another. The China kind of tea has a quality different from that of the Assam kind. Darjeeling tea is made from Chinery bushes. Different kinds of tea are made in different parts of the world from more or less morphologically distinct populations.

Tea Germplasm in India

Unfortunately no central pool of tea germplasm exists anywhere in the world. India can claim to be one of the pioneers in scientific approach to tea cultivation in the world. Collection of tea germplasm was started in India at Tocklai Experimental Station (TES), Jorhat, Assam in 1900, soon after the discovery of the indigenous tea plant in north-east India. It is the largest single collection of *Camellia* germplasm anywhere in the world (Singh and Bera, 1994). The collection is enriched by 14 species with 2532 accessions maintained at three main centres *viz.* Jorhat, Nagrakata and Darjeeling (table 2 and 3). Maximum diversity is found in *Camellia assamica* followed by *C. assamica* ssp. *lasiocalyx*, mainly collected from the North-East areas of India.

The collection of tea germplasm virtually started with the discovery of wild tea in Assam by Robert Bruce in 1823 and the collection was extended to Vietnam, Cambodia, Japan and China by various scientists. It is observed that over 60% of the world tea acreage received basic planting materials directly or indirectly from Indian tea germplasm. The geographical distribution of more important *Camellia* species (Table 2) suggests the possibilities of existence of wild varieties in the region

bordering Assam-Burma and China. There is a wide genetical base for wild tea as expressed in its phenotypic variation.

Indeed, most existing tea cultivars had their origins linked to one or the other of these genetic materials and they still offer opportunities for selecting a wide range of superior cultivars with greater genetic diversity. The tea germplasm is important not only because it supplies genes to modify and improve cultivars and hybrids but also because in it the mutant genes that originated naturally have accumulated over thousands of years. This natural resource must therefore be saved in order to delve deeper into the molecular level to develop molecular blueprints for tea cultivars endowed with potentialities for high yield and quality. The desirable genetic characters can be introduced by inter-specific hybridization only on the basis of knowledge of the evolutionary organization of the tea gene pool differentiation. So, the characterization of germplasm and its preservation can only ensure the availability of the wide range of gene pool in tea for future use in tea improvement.

Table 2: Collection of tea germplasm in North-East India. A total of 2532 accessions have been collected and preserved in the field Gene bank at the three main locations.

Sl. No	Location	No. of Accessions
1	Tocklai Experimental Station, Jorhat, Assam	**1724**
	i) Primitive seed sources, natural variants	1279
	ii) Improved seed/clonal cultivars	196
	iii) Polyploids	174
	iv) Wild and related *Camellia* species.	75
2	Nagrakata Sub-station, West Bengal	**555**
	i) Natural variants and breeding stocks	490
	ii) Improved seed/clonal cultivars	65
3	Clonal Proving Station, Ging Tea Estate, Darjeeling	**253**
	i) Natural variants and breeding stocks	208
	ii) Improved seed/clonal cultivars	45
		Total: 2532

Table: 3: *Camellia* germplasm available in the Tocklai Experimental Station, Jorhat, Assam, North-East India.

Species	Source of Collection	No. of Accessions
Camellia assamica	Assam, Manipur, Sri Lanka, South India.	2337
Camellia sinensis	China, Darjeeling Hills	35
C. assamica ssp *lasiocalyx*	Indo China, Mayanmar, Assam	60
C. kissi (drupifera)	Meghalaya	50
C. caudata	Assam	-
Eurya japonica	North-East India	7
E. acuminata	North-East India	2
Gordonia excelsa	North-East India	2
G. imbricata	Sri Lanka	2
C. japonica	USA, Japan	-
C. sasanqua	USA, Japan	-
C. irrawadiensis	Upper Mayanmar	2
C. japonica var. *kyoniski*	Japan	1
C. rosiflora	Sri Lanka	1
Total		2507

Source: Singh and Bera (1994).

Tea gene pool

It has been suggested that the tea might have arisen from the same basic genome because most of the species of the genus *Camellia* maintain a comparable chromosomal structure and numbers (table 4). The cultivated varieties contain diploid chromosome number 2n=30 but except a seed population in Vietnam showed triploid Chromosome constitution 2n=3x=45 (Bezbaruah, 1971). The wild species *Camellia*

caudata, *C. kissi* and *C. irrawadiensis* have diploid chromosome numbers as like the cultivated varieties, 2n=30. The *C. sasanqua* is a hexaploid having 2n=6x= 90.

Table 4. Chromosome numbers in *Camellia* species.

Species	Chromosome number (2n)
Camellia sinensis	
var. *assamica*	30, 45
var. *sinensis*	30
C. kissi	30
C. caudata	30
C. irrawadiensis	30
C. japonica	30,45
C. rosaeflora	45
C. sasanqua	90
Camellia spp.	30, 45

Source: Bezbaruah, 1971.

Genetic variability utilized

Existing wide natural genetic variability present in the tea populations are due to free hybridization among the tea species and varieties (Singh and Bezbaruah, 1988; Singh and Bera, 1994; Barua, 1965). Elite tea clones are selected or created using the available existing genetic variability in tea germplasm. There is no scope for further genetic evolution to occur in the clones (asexual reproduction). Seeds can be used for the creation of evolution naturally through sexual reproduction. It is this feature of the seed which provides variability for the selection of clones. The inherited characters such as quality and cup strength are positively associated with some morphological features like pubescence (Venkataramani and Sharma, 1974) phloem index and vascular index. However, these factors are subjected to large phenotypic or environmental variations. The selection of right bushes and the development of improved tea clones is a laborious method and take 7-10 years to release a commercial planting clone (Bezbaruah, 1974, 1984; Singh, 1996; Barua, 1965). The clones are selected for good yield and cup quality in addition tolerance to pests, diseases, water stresses (drought, water logging) and rooting behaviour. Many clonal cultivars over 165 have been developed by Tocklai Experimental Station, Jorhat for commercial cultivation in North-East India (Singh, 1992, 1989) and 40 clones in

South India (Sharma and Satyanarayana, 1987, 1989) and two in Himachal Pradesh (table 5).

Table 5. Release of clonal cultivars in India.

Region	No. of Clones selected from		Total
	Natural variability	Created variability	
N.E India			
Plains	125	8	136
Darjeeling Hills	27	2	29
Total	155	10	165
South India	40	-	40
Himachal Pradesh	2	-	2
Grand Total	197	10	207

After Singh, 1994.

Natural as well as created genetic variability has been exploited for the development of these clones. Singh and Handique (1991) was selected a clone tolerant to drought and water logging from old seed grown regions. Usually it takes 10-15 years to select a clone, which is quite long; it needs to be reduced by developing more efficient selection criteria. The modern techniques of molecular biology and biotechnology may be used for early screening of selected clones. Genetic variability was created through recombination, mutation, polyploidy and tissue culture to some extend, and thus more efforts can be given to create genetic variability all over the world by tea breeders. Selection of seedlings for yield and other attributes holds a great potential and should be exploited for genetic improvement of tea. Genetic markers for identifying superior clones in the population may be introduced. The local Assam types crossed freely with the plants brought from China and Indo-China, giving India an edge in terms of great variability in germplasm collection, which has been a breeders' delight. Enterprising tea planters made initial selections of desirable seed material for raising plantations suited to their tea estates. Exploitation of a wide genetic variability in the early years by mass selection gave a number of distinct seed varieties or jats. Considering the present state

of tea plantation in the world and current tea improvements, total displacement of seed plants by vegetative clones can possibly be ruled out. It is more likely and desirable that development of seed varieties and selection of clones should proceed simultaneously. Indian tea plantations, the tea improvement scheme is commonly referred to as the "clone-seed cycle". Clonal plantings are gaining more popularity in major tea growing countries, where old seedling teas are being uprooted and replaced with a few popular clones, whose cultivation is a dead end to evolution. It is leading to narrowing of genetic base of tea plantations. It needs to be arrested by preserving interesting bushes in the old-seed grown sections besides planting diverse clones. Development of polyclonal (similar to composites in corn) or biclonal (double cross hybrids) have the advantage of genetic adaptability and uniformity within a narrow plant type. So, conservation of fast depleting, valuable germplasm present in the existing old plantations is strongly advocated. It assumes greater significance in view of a narrowing genetic base by depending upon a few clonal varieties in breeding programs, which is fraught with danger of genetic vulnerability.

Thus the first clone was selected and released as cultivar code No. 19/29/13- later named as TV1 (Tocklai vegetative 1) in the year 1949. Since then clones have been released with the last clone TV30 in 1993. The clone TV_1 is a hybrid of Assam and China type tea, with good rooting efficiency and drought resistant capability. The bio-chemical characteristics are summarized in table 6.

There are several major diseases of tea plant that threatens production such as red rot, blister blight, root rot, caused by several pathogens. The pathogen infects only the economically important, tender shoots which lead to enormous crop loss, estimated up to 50% depending on the severity of infection (Table 7). Blister blight incited by a fungal pathogen *Exobasidium vexans* Massee is the most important leaf disease in India affecting both the quality and productivity of tea (35-50%). The protections against pest and pathogens have been achieved (40-68%) by using some conventional fungicide and pesticides but it hampered the export quality. So we need high yielding, disease resistant, quality full cultivars for more production to promote export. There are some strategies have been developed by the various researchers to enhance the defense mechanism of the plant through the manipulation of genes that are related to stresses (abiotic and biotic) tolerance (Table 7).

Table 6. Bio-chemical characteristics of TV1.

Green Leaf		**Made Tea**	
Moisture %:	79.68	Theaflavins %:	1.77
PPO activity (QO_2 µl/mg):	15.82	Thearubigins %:	14.04
Total Oxygen uptake (µl/mg) :	18.03	Crude fibre % :	9.80
Total polyphenolics %:	26.10	Total polyphenolics %:	16.23
Tannin %:	20.20	Tannin %:	14.25
Water soluble solids %:	42.85	Caffeine %:	3.49
Caffeine %:	3.52	Chlorophyll a (mg/g):	0.051
Chlorophyll a (mg/g):	0.361	Chlorophyll b(mg/g):	0.004
Chlorophyll b(mg/g):	0.185	Ash %:	6.41
Total Carotenoids (mg/g):	0.89	Total colour%:	6.06

Source: Special Bulletin, Tocklai Vegetative Clones, 1994, TRA, Calcutta.

The transgenic tobacco plant was developed by inserting the glutathione-S-transferase gene of cotton and showed enhanced resistance to methyl viologen (Yu *et al.*, 2003). A novel superoxide dismutase gene was overexpressed in Alfalfa plant which showed increased level of winter survival (abiotic stress) without a detectable increase in photosynthetic oxidative stress (McKersie *et al.*, 2000). In order to optimize disease and pest management strategies it is important to understand the genetic capabilities of tea germplasm to defend itself against diseases.

Table 7. Crop loss due to diseases and pests in tea plantation and their genetic improvement.

Stresses	Causal organism	% yield loss
Weeds	*Mimosa pudica* *Amaranthus spinosus* *Solanum khasianum* *Ageratum conyzoides*	9
Insect pests	Lepidopterans and Hemipterans	6-14
Diseases Leaf diseases	*Exobasidium vexans* *Corticium theae* *Pestalotia theae* *Colletotrichum theae-sinensis*	35-50
Stem diseases Root disease	*Tunstallia aculeate* *Poria hypobrunnea* *Phomopsis theae* *Hypoxylon sepens* *Ustulina zonata* *Hypoxylon asarcodes* *Armillaria melloa* *Xylaria* species	35
Abiotic stresses	Oxidative stress, salinity, drought, cold.	10

DNA-based Molecular Markers: During past two decades, a series of DNA based markers techniques have been developed and used for germplasm characterization. Fundamental differences among the different categories of DNA-based markers are summarized below. These markers can be primarily classified in two major categories:

I) *Hybridization based*-especially primed markers from known and characterized regions of the genome.

RFLP (Restriction Fragment Length Polymorphism),

The development of this marker requires extensive cloning and sequencing of the target sequences to design a specific probe. RFLP is a co-dominant single locus marker and therefore highly expensive during application also. This marker could be developed from coding as well as from non-coding regions of the genome.

II) PCR-based-arbitrarily primed anonymous markers: Some are dominant markers others are co-dominant markers. The examples of dominant markers are-

RAPD (Randomly Amplified Polymorphic DNA),
ISSR (Inter Simple Sequence Repeats),
AFLP (Amplified Fragment Length Polymorphisms) markers,
The examples of co-dominant markers are-
CAPS (Cleaved Amplified Polymorphic Sites),
STMs (Sequenced Tagged Microsatellite Sites), and
SCAR (Sequence Characterized Amplified Regions) markers.

These markers are very commonly used because no prior sequence information is required prior to their use. In addition, these markers are multilocus markers and they provide information from several loci per assay, which reduces the cost per locus.

These markers may direct single locus, oligolocus, or multiple locus differences, and the markers detected may be inherited in a presence/absence, dominant, or co-dominant manner. Brief descriptions of each of the DNA markers are available from the information contained in the Plant Genome website (http//www.nal.usda.gov/pgdic/tutorial/lesson4.htm) and recent reviews of molecular markers useful in mapping plant genomes (Karp *et al.*, 1997).

Botstein *et al.* (1980) heralded the ear of DNA based molecular genetic markers by proposing the Restriction Fragment Length Polymorphisms (RFLP) technique which provides the basis of a new type of genetic diagnostic tool that permits direct identification of a genotype in any tissue and developmental stage, in an environment-independent manner. RFLP-based linkage maps have since been developed in a number of plant and animal species. This genetic diagnostic tool can provide great benefits to plant breeders (Tanksley *et al.*, 1989) through which one can linkup the trait of interest to the DNA markers. Quantitative traits, particular interest to breeders, could be resolved into Mendelian factors and diagnosed with linked RFLP markers in a breeding population (Paterson *et al.*, 1988).

DNA fingerprinting is a term that was first coined by Jeffreys *et al.*(1985) to describe a multilocus RFLP pattern for the identification of individual on the basis of

polymorphisms of minisatellite sequences which are called as variable number of tandem repeats (VNTRs, Nakamura *et al.*, 1987). Reviews of DNA fingerprinting with these methods have been provided by Nybom (1991) and by Weising *et al.* (1989). The report of RFLP loci in humans with as many as 80 different alleles suggested the possibility of greatly enhanced informativeness per locus. Such VNTR loci consist of sets of tandemly repeated oligonucleotide core sequences that were termed "minisatellites" by Jeffreys *et al.* (1985). The core sequences vary in length from 11 to 60 base pairs and the repetitive region is flanked by conserved endonuclease restriction sites. Thus, the length of the restriction fragment produced by this type of genetic locus is proportional to the number of oligonucleotide core units it contains. The DNA fingerprinting in plants was first referred by Ryskov *et al.* (1988) who reported the use of the M13 repeat phage probe. Human minisatellite probes and synthetic simple repeat oligonucleotide probes have also been utilized.

Variable Number of Tandem Repeats (VNTRs): All eukaryotic genomes contain some regions of highly repetitive DNA, some of them are tandemly repeated and varies in repeat number so, named as variable number tandem repeat (VNTR) which have higher mutation rates than that of the single copy DNA. These repetitive regions have been categorized into three classes-

i) Satellite: The first class of repeated DNA sequence elements was discovered by density gradient centrifugation of sheared total genomic DNA from various eukaryotic organisms. This revealed small 'satellite' bands above and/or below the main mass of DNA. This satellite DNA was found to be composed of long tandem repeats of 100 to 5000 bp core sequences clustered at chromosome ends (telomeres) and centromeres.

ii) Minisatellite: Jeffreys *et al.* (1985) described this class of repetitive DNA as 'minisatellites', with 11-60 bp core repeats occurring in tandem arrays of up to 1000 units, distributed throughout the genome. Variation in the number of tandem repeats of minisatellite DNA (VNTRs) have been used as molecular markers to detect high levels of polymorphism, even between closely related individuals and populations of a single species. VNTRs are generally assayed by a multilocus RFLP approach that involves digesting genomic DNA with a restriction endonuclease, blotting to a membrane, and hybridizing with a labeled probe. The probe can be a copy of any minisatellite repeat unit, but a set of

probes developed by Jeffreys *et al* gave good results across widely divergent groups of species. The multiple, 'anonymous' fragments generated with a VNTR probe are very useful for determining parentage or identity (genetic fingerprinting), but do not allow for the identification of alleles or the determination of genotypes. Thus these markers are difficult to use for measurements of relatedness among individuals or for phylogenetic analyses.

iii) Microsatellite: An even more ubiquitous class of repetitive DNA, which is composed of 1-6 bp core units that are tandemly repeated throughout the genome in arrays of few to many thousands of copies. These are less polymorphic than minisatellites, but occur more frequently in the genome. The abundance of this type of marker was suggested by the occurrence of a dinucleotide repeat such as $(CA)_{n>10}/(GT)_{n>10}$ as many 50,000 times in the human genome. This type of reiterated sequence has been termed as a Short Tandem Repeat (STR; Edwards *et al.*, 1991), a microsatellite (Litt and Luty, 1989) or a Simple Sequence Repeat (SSR; Weber and May, 1989). As is generally the case with VNTR loci, the DNA sequence flanking SSRs are conserved, allowing the selection of PCR primers that will amplify the intervening SSR in all genotypes of the target species. The major source of variation is replication slippage resulting in different array length. Microsatellites currently gain tremendous importance as markers for genetic mapping and fingerprinting. Microsatellite oligonucleotides are excellent DNA fingerprinting probes. Differences in length at an SSR locus are detected by DNA amplification *via* PCR, using a pair of oligonucleotide primers that complement unique sequences flanking the microsatellite (Weber and May, 1989). The sizes of the amplified products are precisely determined by acrylamide gel electrophoresis. This type of assay generates single-locus data that provide alleles and genotypes. Evolutionary distances can be calculated between the various alleles at a locus based on the assumption of a stepwise mutation process that results in the alleles closest in size being most closely related by descent from common progenitor. They showed Co-dominant Mendelian inheriantance. The primary drawback of SSR-based markers is the effort and cost required to develop specific primer pairs for each polymorphic locus. This involves the cloning and sequencing of large numbers of genomic DNA fragments containing SSRs. However, much progress is occurring in this area, so

costs associated with marker isolation and characterization is likely to diminish. Another problem is that microsatellites with four base-repeats might not be randomly distributed in a genome but clustered at the centromeres or telomeres. This limits the usefulness of such mutilocus probes in genetic linkage analysis and population studies.

Inter Simple Sequence Repeats (ISSR): Inter Simple Sequence Repeats (ISSR) marker was developed by Zietkiewicz *et al.*, (1994). Instead of SSR, the ISSR markers can be used to circumvent the problems associated with the SSR markers. ISSR is a technique in which primers that anneal to microsatellites during PCR lead to amplification of the piece of DNA between two primers. For this system two primers are constructed to anneal to microsatellites [(CA)n or (GT)n]with one extra nucleotide in the 3′-end. The extra nucleotide will permit amplification only if the primer binds to a 5′-end of a microsatellite with a suitable first nucleotide in the flanking sequence. Such extra nucleotides are termed anchors and they assure that amplification will always start from the 5′-end of the microsatellite. Amplification will only take place when two suitable microsatellites with suitable first flanking nucleotides are in a distance suitable for PCR amplification. Because of the many microsatellites in most genomes of higher plants, ISSR will often amplify 25 to 50 ISSR products in one reaction. Polymorphisms are mostly of the dominant type because of changes in the anchoring nucleotides. There is no need of cloning and sequencing of any DNA part of the genome under investigation.

RFLP is the most reliable polymorphism which can be used for accurate scoring of genotypes. RFLP are co-dominant and can identify unique locus. When cDNAs with known gene function are used as markers, the chromosomal position of the specific gene or genes can be identified on the chromosome. Using RFLP markers, genetic maps have been developed in many plant species. The polymorphism detected by RFLP markers is very reliable as it involves the recognition by specific restriction enzymes and hybridization with a specific probe (according to Southern, 1975). RFLP markers facilitate the selection of progenies with desirable genotypes in a shorter span of time. However, RFLP analysis is labour intensive and time consuming. One of the major problems in using RFLP marker technology in breeding programs is the expense involved. The expense includes not only the material and supplies but also less definable costs such as quality technical

support and radioisotope permits (Tanksley *et al.*, 1989) and it requires relatively large amount of DNA (5-10μg) and is refractory to automation. While variation in length or sequence of diagnostic DNA is used to measure degree of relatedness between individual organisms or inheritance in progeny populations, the identification of these molecular markers requires prior knowledge of DNA sequence, cloned and characterized probes, and considerable experimental manipulation.

To circumvent these limitations in the RFLP technique, a novel DNA amplification-based strategy was conceived. Single, arbitrary primer-based DNA amplification techniques (DAF, RAPD and AP-PCR) were developed (Williams *et al.*, 1990; Welsh and McClelland, 1990; Caetano-Anolles *et al.*, 1991a, 1991b, 1992a), extending the utility of Polymerase Chain Reaction (Mullis, 1986) to general genome analysis. Because of a plethora of terms, a general acronym was proposed as MAAP (Multiple Arbitrary Amplicon Profiling, Caetano-Anolles *et al.*, 1992b, 1993). The discrete amplifiable DNA regions, also termed amplicons in the PCR are determined by primer annealing to partially or perfectly complementary sites on each DNA strand. Strategies like random amplified polymorphic DNA (RAPD) analysis (Williams *et al.*, 1990), arbitrarily primed PCR (AP-PCR; Welsh and McClelland, 1990), and DNA amplification fingerprinting (DAF; Caetano-Anolles *et al.*, 1991a) target multiple annealing sites without the requirement of prior knowledge of template sequence were developed independently and apparently concurrently in the three laboratories. They have been widely used for the construction of genetic maps (Reiter *et al.*, 1991), for simplified identification of markers linked to traits of interest (Michelmore *et al.*, 1991), for genetic fingerprinting (Welsh *et al.*, 1991), for genetic diagnostics (Horn *et al.*, 1989; Horn and Rafalski, 1992), and in population genetics (Arnold *et al.*, 1991).

I. **AP-PCR** (Arbitrarily Primed-PCR) developed by Welsh and McClelland (1990), which uses PCR-primers of length 18 to 32 nt of arbitrary sequence to amplify target DNA under low stringency (*i.e.*, low annealing temperature) conditions for two amplification cycles followed by a series of cycles of high stringency amplification. Amplification product was resolved by polyacrylamide gel electrophoresis and was detected by autoradiography.

II. **RAPD** (random amplified polymorphic DNA) procedure was invented by Williams *et al.*, (1990), in which an arbitrary primer of either 9 or 10 nt in length was used to produce PCR products. RAPD products are routinely resolved on agarose gel electrophoresis and visualized by ethidium bromide staining on UV light. RAPD is widely used because of its simplicity and low-cost instrumentation.

III. **DAF** (DNA Amplification Fingerprinting) developed by Caetano-Anolles *et al.* (1991a, b). Of all MAAP procedures, DAF utilizes the shortest primer, down to 5 nt in length. The optimal length was found to be 8 nt a length which does not produce efficient amplification with RAPD. DAF products are routinely separated by polyacrylamide gel electrophoresis, and stained with an improved silver staining method for visualization of bands.

There are several examples of genetic diversity and relationship studies on different plant species by different workers (Joshi and Nguyen, 1992, 1993; Tanaka *et al.*, 1996; Tsumura *et al.*, 1996; Wachira *et al.*, 1995 and 1997; Paul *et al.*, 1997; Wolfe *et al* ., 1998; Lai *et al.*, 2001; Wendel and Liu, 2001; Vijayan and Chatterjee, 2003; Roy, 2004; Vijayan *et al.*, 2004; Nagaraju *et al.*, 2002, 2004; Matsumoto *et al.*, 2004; Eapen *et al.*, 2004; Roy and Chakraborty, 2007).

RFLPs were the first DNA based co-dominant markers used for genetic diversity studies. Matsumoto *et al.*, (1994) cloned the phenylalanine ammonia-lyase (PAL) cDNA from tea, and used it as RFLP probe. They were able to distinguish Assam hybrids and Japanese tea varieties with low and high levels of catechins. The PAL gene was shown to be single copy and multiallelic. The major disadvantage of RFLPs is that they are highly expensive and time consuming both during development and application. The advent of PCR (Polymerase Chain Reaction) led to development of another co-dominant marker, known as CAPS (Cleaved Amplified Polymorphic Sites) or PCR-RFLP. Kaundun and Matsumoto (2003) analysed the genetic diversity of *sinensis* and *assamica* cultivars of tea using CAPS markers for three genes, namely PAL, CHS_2 (Chalconesynthase) and DFR (Dihydroflavonol 4-reductase), which are involved in phenylpropanoid pathway leading to biosynthesis of catechin and tannin. The study showed that polymorphisms in PAL and DFR genes was due to indels in the introns of both genes as well as due to point mutations as revealed by restriction

digestion of amplicons. Although, they did not find any particular CAPS profile specific to *seninsis* or *assamica*, the combined data set from all the CAPS markers could group the cultivars according to their respective varietals status.

A greater level of variation was observed in CHS_2 gene, which is expected due to fact that CHS is a multigene family comprising of three non-allelic genes with high sequence similarity (Takeuchi *et al.*, 1994). As more and more genes from tea are being cloned and sequenced, there is great future potential for CAPS markers. These expressed sequence tags (EST) projects in tea have produced a large number of sequence data (Park *et al.*, 2004). Presently, there are over 2000 ESTs for tea available in NCBI data Bank. CAPS markers based on these ESTs can also be used for linkage analysis and marker-assisted selection in tea because these are from the expressed regions of the genome.

Although majority of these are uncharacterized sequences, these can still be used for development of CAPS markers in future. Several important genes controlling quality traits in tea such as beta-primeverosidase (Mizutani *et al.*, 2002), caffeine synthase (Kato *et al.*, 2000) and polyphenol oxidase (GenBank Accession no. AF 269192) have been already cloned and sequenced. Identification of polymorphisms in these genes can help in developing varieties with improved quality traits through marker-aided selection. STMs markers are another type of co-dominant markers, which are the most desirable markers for genetic studies due to inherently high levels of allele density (Tripathi and Negi, 2006). These markers are based on flanking sequences of microsatellites, which are tandem repeats of 2-5 base pairs. Development of STMs markers is highly expensive because it requires isolation, cloning and sequencing of portions of genome containing these repeats. Till date, only 15 microsatellite sequences from *C. sinensis* have been reported (Freeman *et al.*, 2004). Few microsatellites sequences from *C. japonica* have also been reported earlier (Ueno *et al.*, 1999). These markers are non-transferable between species except very closely related ones and therefore need to be developed for each species separately. Kaundun and Matsumoto (2002) attempted to transfer four nuclear microsatellites from *C. japonica* and seven chloroplast microsatellites from *Nicotiana tabacum* into *C. sinensis*. The CAPS markers have also been used in tea characterization (Kaundun and Matsumoto, 2003).

Molecular markers highlighted important differences in the partitioning of diversity within and among population. The potential use of different molecular markers such as RFLP (Matsumoto *et al.*, 1994), RAPD (Lee *et al.*, 1995; Wachira *et al.*, 1995; Kaundun *et al.*, 2000; Kaundun and Park, 2002) and AFLP (Paul *et al.*, 1997) have been reported in tea. These markers are useful for characterization, estimation of genetic relatedness and determination of genetic diversity among the tea germplasm. Comparison between RAPD, AFLP and SSR markers in different plants species has revealed that co-dominant SSRs detect the highest level of polymorphism per locus and it surveys the hyper-variable microsatellite regions of the genome, hence they have higher information content (Powell *et al.*, 1996; Maughan *et al.*, 1996). Sometimes, AFLP and RAPD despite their dominant nature can estimate relationship with a high resolution due to their multilocus approach (Powell *et al.*, 1996; Russell *et al.*, 1997; Teulat *et al.*, 2000).

Mishra and Sen-Mandi (2004) has developed a DNA marker, which is associated with drought tolerance in tea. The usefulness of molecular techniques, including RAPD, in the characterization and identification of plant germplasm has great advantage over the phenotypic characterization because of their independence from environmental variations (Varghese *et al.*, 1998). This RAPD marker has been utilized extensively for the characterization of tea genetic resources and estimation of genetic diversity were reported earlier by many workers (Bera and Saikia, 1999; Wachira *et al.*, 1995, 1997; Paul *et al.*, 1997; Raina and Marimuthu, 1999; Tanaka *et al.*, 1996). The RAPD methodology has provided informative data consistent with other markers, especially at the intraspecific level (Dos Santos *et al.*, 1994; Lerceteau *et al.*, 1997) and it is cost effective for large-scale population genetic analysis.

The PCR based anonymous markers have also been used in genetic diversity studies on tea. The markers are as such: RAPD, ISSR and AFLP (Wachira *et al.*, 1995; Paul *et al.*, 1997; Lai *et al.*, 2001; Balasaravanan *et al.*, 2004). The PCR based markers has been used to create a genetic linkage map in tea by Hackett *et al.* (2000). Using RAPD, Kaundun *et al.* (2000) observed higher level of genetic diversity in Korean cultivated accessions of tea as compared to accessions from Japan and Taiwan. However, the intra-population genetic diversity of six Korean tea populations was found to be relatively lower than the expected diversity of natural populations (Kaundun and Park, 2002). This was attributed to narrow genetic base of the tea

samples introduced from China and considerable reduction in population size following mass destruction of tea populations during fourteenth century.

Wachira *et al.* (1995) used RAPD to estimate genetic diversity and taxonomic relationships in 38 clones belonging to the three tea varieties, China, Assam and Cambod type. Extensive genetic variability was detected in these clones and showed that 30% of the total diversity resided among population while 70% diversity resided within populations based on Shannon's diversity index. Cluster analysis using RAPD data could separate the three populations in a manner consistent with both the present taxonomy of tea and with the known pedigree of some clones. The study also showed the usefulness of RAPD in discriminating the commercial clones, even those that cannot be distinguished on the basis of morphological and phenotypic traits. Wachira *et al.*, (1997) studied the genetic relationship of 20 species of the genus *Camellia* using RAPD and organelle-specific PCR. The result was generally consistent with the classification based on morphology and the relationship revealed from comparative photochemical data.

This implied that the results of RAPD and ISSR studies were highly congruent. However, ISSRs seemed to be more discriminative in cultivar identification then RAPDs because cultivars that could not be distinguished by RAPDs markers were separable based on ISSR markers. This finding suggests that the evolution rate of ISSRs might be faster than that of RAPDs in the tea samples studied. ISSR markers are, therefore, suitable for use in the study of genetic diversity and determination of genetic relationships of closely related tea cultivars. Other studies also indicated that ISSR markers exhibit higher levels of polymorphism than RAPD markers (Yang Quiros, 1993; Nagaoka and Ogihara, 1997; Parsons *et al.*, 1997; Esselman *et al.*, 1999). The observed difference between ISSR and RAPD might be explained in terms of functional constraints since some RAPD bands may be associated with functionally important loci (Penner, 1996).

The fast evolutionary rate and the hyper variability of ISSR may suggest that ISSR bands represent neutral markers (Esselman *et al.*, 1999). Results showed that Assam tea had the smallest within group diversity while native wild tea had the largest diversity for both RAPD and ISSR markers. Three clones of Assam tea were included in the present study. These clones represent only a small portion of the genetic variation of Assam tea. This might account for the small within group

diversity of Assam tea observed. Six samples of native wild teas were studied, and the diversity within native wild tea was larger than China tea, which consisted of 21 cultivars. The result indicated that the native wild tea is highly variable and the germplasms of native wild tea might be useful in breeding programs.

Linkage maps of canola have been constructed using isozymes, RAPDs, RFLPs, (Thormann *et al.*, 1994) and to a limited extent using microsatellites (Kresovich *et al.*, 1995). The RFLP markers generally represent single and low copy sequences. SSRs are widespread in the genome and the average heterozygosity index of these markers has been reported to be higher than any other single locus approach (Roder *et al.*, 1995; Powell *et al.*, 1996). AFLP approach has the ability to expedite the construction of a saturated linkage map as it detects a high level of polymorphisms in DNA in a single lane. The segregating loci mostly map on the independent locations. AFLP maps are currently being made in a number of crops.

The AFLP technology developed by Vos *et al.* (1995) is a more reliable and robust technique. This marker technology has received great attention in genetic diversity studies during recent years. The first paper on genetic diversity of tea using AFLP was by Paul *et al.* (1997), who studied diversity of 32 tea clones comprising of Indian and Kenyan origin. A total of 73 bands generated with 5 AFLP primer combinations revealed that the intramorphotype genetic diversity was higher in Chinary types than in Assam or Cambod types.

Shannon's index of diversity was used to partition the total variation, and they found that most of the diversity was detected within populations, with 79% of the variation being within and 21% being between populations of India and Kenyan tea. A dendrogram constructed on the basis of band sharing separated the tea samples into China, Assam and Cambod types. The Pearson's correlation coefficient between similarity matrices based on RAPD and ISSR was 0.811. The Mantel test revealed that the correlation was highly significant ($P<0.001$). The clustering obtained in this study was consistent with the morph-types of the accessions used in this study. The principal component analysis revealed that the Assam clones from India and Kenya were highly related which supports the history of migration of tea from India to Kenya (Singh and Bera, 1994).

Balasaravanan *et al.* (2003) studied the genetic diversity of 49 tea accessions from UPASI comprising of Assam, China and Cambod types. A total of 1555 bands obtained with 3 primers combinations supported the findings of Paul *et al.* (1997) revealing an overall higher genetic diversity with China type accessions than Assam types. The study also revealed a very low diversity within South Indian cultivars, which may be due to selection from a narrow gene pool.

Towards the ultimate goal of genetic improvement of tea, efforts are required to construct a high-density linkage map using molecular markers and integration of economically important traits on this linkage map. A beginning has been made by Hackett *et al.* (2000), who used RAPD and AFLP markers for construction of a linkage map of *C. sinensis*. As tea is highly cross-pollinated, conventional mapping populations such as F_2 selfed or BC_1 cannot be developed. Alternative approaches such as pseudo test cross that have been used in other tree species can also be used in tea (Grattapaglia *et al.*, 1996; Hackett *et al.*, 2000). The biclonal seed stock populations, which are readily available in various tea germplasm institutes, can be used for this purpose. Clonal seed orchards or seed baris can be established using genetically diverse accessions identified based on genetic diversity and morphological data. This will generate excellent raw material for future selection of clones with desirable combination of traits and for further use in research such as QTL (Quantitative Trait Loci) mapping and marker-assisted breeding.

It is relatively easy to turn the RAPD assay into a secondary PCR assay through DNA sequencing of the RAPD band of interest and conversion to allele-specific PCR, allele-specific ligation (Landergren *et al.*, 1988) or a sequence characterized amplified region (SCAR) assay (Paran and Michelmore, 1993). Additional sequence polymorphisms may be detected in RAPD bands using restriction enzyme divergence, single strand conformational polymorphism assay (SSCP; Orita *et al.*, 1989), or denaturing gradient gel electrophoresis (DGGE; Myers *et al.*, 1986) may be applied to individual RAPD bands.

The advent of polymerase chain reaction (PCR) favored the development of different molecular techniques such as random amplified of polymorphic DNA (RAPD), simple sequence repeats (SSR or microsatellite), sequence tagged sites (STS), random amplified microsatellite polymorphism (RAMP) and inter simple sequence repeat polymorphism (ISSR) and so on (Saiki *et al.*, 1988; Welsh and

McCleland 1990; Williams *et al.*, 1990; Akkaya *et al.*, 1992; Tragoonrung *et al.*, 1992; Zietkiewicz *et al.*, 1994; Wu *et al.*, 1994a; Nagaoka and ogihara, 1997).

Molecular markers successfully developed during the last two decades have largely overcome the problems that are associated with phenotype-based classification. Initially, isozymes (Chengyin *et al.*, 1992) and Restriction Fragment Length Polymorphisms (RFLPs) (Botstein *et al.*, 1980; Beckmann and Soller 1986; Tanksley *et al.*, 1989; Miller and Tanksley, 1990; Wang *et al.*, 1992) served as reliable markers for genetic analyses in plants. But PCR based techniques developed in recent years such as Random Amplified Polymorphic DNA (RAPDs) Williams *et al.*, 1990; Welsh and McClelland 1990), Inter Simple Sequence Repeats (ISSR) (Zietkiewicz *et al.*, 1994), Amplified Fragment Length Polymorphisms (AFLPs) (Vos *et al.*, 1995), and Simple Sequence Repeats (SSRs) (Weber and May, 1989), also called microsatellites, sequence tagged sites (STS), random amplified microsatellite polymorphism (RAMP) provide DNA markers that are dispersed throughout plant genomes (Saiki *et al.*, 1988; Tragoonrung *et al.*, 1992; Wu and Tanksley, 1993; Wu *et al.*, 1994) and are easier to reproduce and analyze.

High levels of polymorphism and their co-dominant nature have made SSRs ideal markers for studying genetic diversity in plants (Akkaya *et al.*, 1992; Morgante and Olivieri, 1993; Plaschke *et al.*, 1995). However, the time and cost of identifying SSR motifs and designing primers for regions flanking SSRs have restricted the widespread use of microsatellites in plants (Beckmann and Soller, 1990; Roder *et al.*, 1995). ISSR markers, which show dominant inheritance, use SSR repeat-anchored primers and are being used as an alternate tool in diversity studies. ISSR markers are useful in detecting genetic polymorphisms among accessions by generating a large number of markers that target multiple microsatellite loci distributed across the genome.

Further, they are simpler to use than the SSR technique as prior knowledge of the target sequences flanking the repeat regions is not required (Zietkiewicz *et al.*, 1994; Tsumura *et al.*, 1996; Nagaoka and Ogihara, 1997; Nagaraju *et al.*, 2002; Bornet *et al.*, 2002). It has been shown that different markers might reveal different classes of variation (Powell *et al.*, 1996; Russell *et al.*, 1997). It is correlated with the genome fraction surveyed by each kind of marker, their distribution throughout the genome and the extent of the DNA target which is analyzed by each specific assay

(Davila *et al.*, 1999). Of these techniques, RAPD has several advantages, such as simplicity of use, low cost, and the use of small amount of plant material. ISSR markers, which involve PCR amplifications of DNA using a primer, composed of a microsatellite sequence anchored at 3' or 5' end by 2-4 arbitrary, could be used to assess genetic diversity (Qian *et al.*, 2001). ISSRs have been used for cultivar identification in maize (Kantety *et al.*, 1995; Pejic *et al.*, 1998), potatoes (Prevost and Wilkinson, 1999), wheat (Nagaoka and Ogihara, 1997), cotton (Dongre and Parkhi, 2005) and barley (Fernandez *et al.*, 2002; Tanyolac, 2003).

Restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) have been applied successfully and have provided considerable genetic information in a number of plant species (Zhang *et al.*, 1992; Vos *et al.*, 1995; Xu *et al.*, 2000). The above two techniques are slow and expensive and are not amenable for assessment of genetic variation in large number of genetic analysis. More recently, PCR-based, random amplified polymorphic DNA (RAPD) marker, developed by Williams *et al.* (1990) have been employed widely in cultivars identification and characterization (Halward *et al.*, 1992; Sharma *et al.*, 1995; Lai and Hsiao, 1997; De Bustos *et al.*, 1998; Mandolino *et al.*, 1999). Inter-simple sequence repeat polymorphism (ISSR) markers developed by Zietkiewicz *et al.*, (1994) can also be employed for the study of genetic variation among the cultivated tea species (Wolff *et al.*, 1995, Tsumura *et al.*, 1996; Fang and Roose, 1997; Chen *et al.*, 1998).

Several types of molecular markers have been employed previously to quantify the genetic diversity within tea germplasm collections in different countries (Tanaka *et al.*, 1996). Wachira *et al.* (1995, 1997) have used this molecular marker system to determine genetic diversity and differentiation within and between cultivated tea and related *Camellia* species. Paul *et al.* (1997) studied the diversity and genetic differentiation of Indian and Kenyan tea using AFLP markers. Lai *et al.* (2001) studied the genetic relationships in cultivated tea clones and native wild tea in Taiwan using RAPD and ISSR markers. A few Tocklai variety (TV clones) has also been characterized on the basis of RAPD analyses by Bera and Saikia (1999). Matsumoto *et al.* (1994) characterized the genetic diversity in tea cultivars on the basis of PAL (phenylalanine ammonia lyase) cDNA probe analysis. Devarumath *et al.* (2002) evaluated the genetic integrity of micropropagated diploid and triploid elite tea clones on the bases of RAPD, ISSR and RFLP fingerprinting. Kaundun *et al.* (2000)

also studied the genetic diversity among elite tea accessions using RAPD markers. Six Korean tea populations were analysed on the basis of RAPD-PCR to reveal their genetic diversity among the tea populations by Kaundun and Park (2002). The difference in the generation of bands per primer was probably due to the differences in the primer sequence and primer-template DNA interaction (Weir *et al.*, 1997). According to Rafalski *et al.* (1991) variation in the number of bands in RAPD profiles is independent of the complexity of the genome. ISSR markers, which involve PCR amplifications of DNA using a primer composed of a microsatellite sequence anchored at 3′ or 5′ end by 2-4 arbitrary, could be used to assess genetic diversity (Qian *et al.*, 2001). ISSRs have been used for cultivar identification in maize (Kantety *et al.*, 1995; Pejic *et al.*, 1998), in potatoes (Prevost and wilkinsom, 1999), in barley (Fernandez *et al.*, 2002, Tanyolac, 2003).

So far only a few attempts have been made to characterize the genetic diversity in tea by using molecular markers. These include AFLP based marker analysis (Paul *et al.*, 1997), RFLP (Wachira *et al.*, 1995; 1997; Devarumath *et al.*, 2002; Matsumoto *et al.*, 2004), RAPD profiles (Kaundun *et al.*, 2000; Lai *et al.*, 2001; Park *et al.*, 2002; Luo *et al.*, 2004; Roy, 2004; Li *et al.*, 2005; Chen *et al.*, 2005; Roy and Chakraborty, 2007; Cheng-Wen *et al.*, 2008), ISSR based analysis (Lai *et al.*, 2001; Mondal and Chand, 2002 and Devarumath *et al.*, 2002; Liang *et al.*, 2007). In the current study we report the use of RAPD and ISSR markers for assessing the genetic diversity and relationships among 21 cultivated clonal genotypes from three tea varieties.

Tissue Culture for Genetic Improvement

The tea plant is generally propagated vegetatively by rooting of the single stem node cutting. Although, vegetative propagation of elite clones has been used for large scale multiplication of plants with desirable attributes in respect to yield and quality. But it has some drawbacks of such clonal plantations, noted over the years in field, is yield variability under adverse/stress conditions. Vegetative clonal plantations are also relatively prone to pathogens in comparison to seed grown plantations (Barua, 1989). Quite often, the regenerated cuttings gradually lose their vigour in the long run and if the source plant itself is infected, the cuttings also carry the disease.

Like may other woody species, it has traditionally been propagated vegetatively because it is highly heterozygous and seedlings are too variable for commercial use (Banerjee *et al.*, 1992). 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. Tissue culture technique can be employed to eliminate the problem. Tissue culture technology and mass clonal propagation with advancements in the field of genetic engineering are likely to make a major impact on the tea industry. *In vitro* tissue culture techniques include rapid mass clonal propagation of selected clones, production of pure breeding lines, germplasm storage and exchange, interspecific and intergeneric hybridization, development of haploids, polyploids and somaclonal variants, somatic embryogenesis leading to the overall improvement in yield and quality of tea.

Plantlets were regenerated *in vitro* from callus by Wu and Li (1976). A number of reports appeared on *in vitro* culture of tea using almost all the plant parts *viz.* leaf, node, internode, cotyledon, embryo, root, anther, and pollen. Tissue culture provides a method of rapid multiplication of selected genotypes (Nakamura, 1989; Kato, 1986, 1996; Arulpragasam and Latiff, 1990; Rajkumar and Ayyappan , 1992; Vieitez and Borciela, 1992; Sood *et al.*, 1993; Akula and Dodd, 1998; Mondal *et al.*, 2004). The application of tissue and organ culture for multiplication of elite tea clones has been advocated. Tissue and cell culture technology along with advancements in the field of genetic engineering are likely to make a major impact on the industry in the areas of rapid and mass clonal propagation of selected clones, in disease elimination and development of stress (biotic and abiotic) tolerant clones, production of pure breeding lines through haploid culture technology, germplasm storage and exchange, mutant selection and development and for the overall improvement in quality and yield of tea (Jain and Newton, 1990).

The plantlets were transferred to rooting medium for root initiation, after which they were hardened or acclimatized. However, only a few hardened tissue culture derived plants were transferred to field. The use of modern technology for mass multiplication of tea for commercial plantings is limited largely due to problems of rooting, hardening and survival after field transfer of tissue culture raised plants.

Phukan and Mitra (1984) obtained shoot buds from the nodal segments without an intervening callus phase. Banerjee and Agarwal (1990) studied rooting in vitro in *Camellia sinensis*. Micropropagation of tea was first reported by Kato (1985) followed by several others (Arulpragasam and Latiff, 1986; Akula and Dodd, 1998; Saratchandra *et al.*, 1988; Jha *et al.*, 1992). Micropropagation has not yet been commercially adopted as a means of propagating tea due to the fact that tea is easily propagated vegetatively by single leaf cuttings. The tissue culture method is not season bound, and is suitable for production of large number of plantlets and can be profitably used in the development of gene banks and for multiplication of novel elite plants obtained through other biotechnological approaches. Moreover, meristem cultures can be used for regeneration of disease free explants which can be transported from one place to another without quarantine problems. While plant tissue culture is essentially used as a method of clonal (true-to-type) propagation, tissue culture induced variation also provides an additional tool for enhancing genetic variability, including the incorporation of desirable traits like high yield. Variation in cultured cells and plants regenerated from these is referred as somaclonal variation (Larkin and Scowcroft, 1981; Evans *et al.*, 1981).

There have been several studies aimed at the production of haploid tea plants using anther culture (Raina and Iyer, 1983). Haploid plants thus obtained can be diplodized to obtain homozygous diploids for use in breeding programmes and for developing inbred lines. If homozygosity achieved at all loci, which would greatly improve the process of selection and helps in screening mutants. Hence, haploid technology has great potential for initiating investigations on genetic and genetic engineering for the improvement of tea plant. Haploids obtained by anther and pollen culture can be depolarized to get homozygous diploids for use in the breeding program to get inbreeds lines.

The first observation of *in vitro* somatic embryogenesis was made in *Daucus carota* (Reinert, 1958, 1959; Steward *et al.*, 1958). Somatic embryo formations in *Camellia* sp. were investigated by many workers (Vieitez and Borciela, 1990; Jha *et al.*, 1992; Kato, 1996; Wachira and Ogunda, 1995; Ponsamuel *et al.*, 1996).

Tea (*Camellia sinensis*) plants are perennial, and highly heterozygous, requiring many years of selection to incorporate favourable traits. Nakamura (1988) has induced adventitious embryo formation on the tea cotyledon of *Camellia sinensis*

and other *Camellia* sp. The adventitious bud formation was investigated in tea stem culture by Nakamura (1988). The somatic embryogenesis in tea (*Camellia sinensis*) has been investigated by Akula and Akula (1999). A novel plant growth regulator, betaine was used in tea tissue culture for rapid induction of somatic embryogenesis (Akula *et al.*, 2000). Somatic embryogenesis from immature leaves has been investigated by Kato (1996). Mondal *et al.* (2001b) have shown the *in vivo* somatic embryogenesis in tea (*Camellia sinensis*) from cotyledons. Transgenic tea plant has been developed by *Agrobacterium*-mediated transformation of somatic embryos in tea (*C. sinensis*) (Mondal *et al.*, 2001). The somatic embryo has been obtained from embryonic tissues of *Camellia japonica* L. (Vieitez and Barciela, 1990).

The RAPD marker has been used appropriately for a number of crop species to detect genetic integrity among micropropagated plants (Isabel *et al.*, 1993; Rani *et al.*, 1995; Rani and Raina, 2000; Mondal and Chand, 2002). Genetic variation may occur in the tissue culture raised tea plant as a result of mutation during micropropagation (Mondal and Chand, 2002). The other markers RAPD, ISSR and RFLP were used to examine the genetic integrity of micropropagated tea plants, UPASI-26, UPASI-3 and UPASI-27 (Devarumath *et al.*, 2002).

The micropropagated plants of both the UPASI-3 and UPASI-27 (Assam type) revealed complete stability but the regenerated clone UPASI-26 (China type) showing lack of complete genetic integrity. That means China type clone UPASI-26 is prone to genetic changes during propagation. Two Assam type clones UPASI-3 and UPASI-27 exhibited complete stability during *in vitro* propagation. It is indicating that the genetic changes in tea clones are genotype dependent rather than culture condition dependent (Devarumath *et al.*, 2002). Somaclonal variation has been detected in micropropagated plants of a number of species for commercial importance as well as genetic integrity was maintained in the tissue culture derived plants (Isabel *et al.*, 1993; Rani and Raina, 2000; Derarumath *et al.*, 2002). Hashmi *et al.* (1997) has detected somaclonal variants in each regenerated plants from tissue culture using RAPD markers, that reveals that genetic changes occur during *in vitro* culture in peach.

Genomic Constitution as Revealed by Chromosome Karyotype

The taxonomic status of the present day cultivated tea is a subject of much debate and controversy. Cultivated tea has been classified into three types i) China type, ii) Assam type and iii) Cambod type on the basis of morphology *i.e. C. sinensis* var. *sinensis*, *C. sinensis* var. *assamica* and *C. assamica* sub sp. *lasiocalyx* (Cambod type). All the three types of tea are almost self-sterile and outbreeding in nature coupled with frequent spontaneous hybridizations that take place between different types of tea, resulting in highly heterozygous plants ranging from China type to Assam type, has further complicated the taxonomic status of tea. The cultivated tea is cytogenetically very poorly understood. The investigations are largely restricted to mere determination of zygotic numbers. Although preliminary meiotic analysis have been conducted in few cases but the available information is not sufficient to draw any conclusion or sometimes conflicting (Bezbaruah, 1971).

Except for the few natural triploids and polyploids reported by Bezbaburah (1971), the cultivated tea plant is a diploid with a chromosome number of 2n = 30. The chromosome number in tea was determined by many investigators (Bezbaruah, 1971). Root-tip method was generally used for mitotic studies which necessitated digging up of roots. To avoid this, Bezbaruah adopted the shoot-tip method for examination of mitotic chromosomes in tea and other related species after necessary modification and standardization. Bezbaruah (1971) made detailed karyotype analysis of 30 tea clones belonging to the three races of tea, ten from each race, as well as of plants of a few allied species. The somatic chromosome number of 30 was common to all the tea clones and plants of the allied species. The chromosome of tea, as of other *Camellias*, is short but there is a gradation of size from the longest to the shortest. The chromosomes have median to sub-median primary constrictions. In a few clones, one or two pairs of chromosomes may have secondary constrictions. Clones belonging to the three races of tea do not reveal any major differences in their karyotype, although some minor differences exist. However, none of the minor differences could be correlated with any morphological feature of the plants. No irregularities are observed in the meiosis of any of the clones. In many genera and species, the process of evolution is accompanied by different types of karyotypic changes whereas in others morphological difference and divergence occur without any visible change in chromosome morphology.

This phenomenon is attributed to cryptic gene mutation. Close morphological similarity of the tea chromosomes suggests that the observed differences in growth and form of plants of the different races of tea are the results of mutative changes of the genes as found in *Pinus* and *Quercus*, where the karyotype is closely similar although they are different species. Karyotype analyses of different cultivars of tea showed a smooth decrease in size of chromosome from the longest to the shortest. Naturally evolved polyploids in tea are very rare. Only a few natural triploids (2n=45) have so far been reported from Japan, South India (Venkataramani and Sharma, 1974). Ackerman (1977), and Bezbaruah (1971) mentioned that the ploidy level in the genus *Camellia* ranged up to hexaploid with 2n=90 chromosomes. Ackerman (1977) proposed that segmental allopolyploid might have originated during the course of evolution in diploid level and continued in the polyploids through inter-crossing among them. Bezbaruah (1971) reported that the chromosomes of tea plant were primitive but other classified them as of advanced status depending on the symmetry and asymmetry of chromosomes respectively. The length of tea chromosomes ranged from 1.24μm to 4.20μm. The 'r' value (ratio of long arm to short arm) for all the 15 pairs of chromosomes ranged from 1.00 to 1.91. The chromosomes were classified morphologically on the basis of relative lengths, position of the centromere and presence or absence of secondary constriction. The meiotic division in Assam and hybrid tea were regular with 15 bivalents at diakinesis and telophase I and the segregation was regular (Bezbaruah 1971). Bezbaburah (1971, 1976) observed two satellite pairs of chromosomes in the complement of *C. assamica*. Lack of predominant satellites indicated tea as being inherent in low activity of the nucleolus organizer, which restricts the chromosomes to produce secondary constrictions.

In tea, preliminary studies on the detailed karyotypic analysis and nucleolar organizer carried out at Tea Research Foundation, Kenya, revealed that the nucleolar numbers correspond to multiples of the somatic chromosome number (2n=30). Nucleolar number per cell was shown to reflect ploidy. Some NORs and therefore, ribosomal gene clusters were observed to be located in telomeric regions of some chromosomes. Banding technique and differential staining of chromosomes to study the karyotypic differences between the species are of immense importance in the future strategy of cytological studies in tea. The predominant occurrence of trivalent in the triploid UPASI-3 indicates either autotriploid or segmental allotriploid nature of

polyploid. Based on the meiotic analysis of diploid tea clones, it might be considered to be segmental allotriploid in origin. More studies are, however, needed to establish some conclusion regarding the origin of three different types of tea varieties.

The cytology of cultivated tea is very poorly understood. The clones manifested a chromosomal homology in chromosomal pairing during meiosis, between the genomes of tea clones, which are known to be "highly heterozygous". It appears that the genomes of the involved taxa are not sufficiently differentiated but exhibit cryptic hybridity, which is sufficient to differentiate clones in their morphology as well as their agronomic traits. The occurrence of trivalent in the only triploid cultivated clone UPASI-3 (2n =3x =45) indicates that it might have originated as segmental allo-triploid.

Most of the teas under cultivation are diploid with two sets of basic chromosomes, though natural triploids and pentaploids have also been reported (Bezbaruah, 1971, 1976; Sharma and Venkataramani, 1971). Bezbaruah (1971) observed that the quality of the natural triploid and tetraploids are inferior to the diploids, because of the hardiness of the triploids and tetraploids. Natural polyploids have more diverse genetic variation in terms of desirable traits which may be incorporated by hybridizing them with better quality diploids (Bezbabaruah, 1976). Tea polyploids (triploids and tetraploids) have clearly demonstrated the superiority of over domesticated diploids in morphological and agronomical characteristics.

They showed increased plant vigour and winter hardiness compare to diploids. The triploids generally do not set fruits though they bear flowers, so the energy required for the development of fruit, is diverted towards the development of vegetative parts. Chromosome number is the basic criterion for differentiations of a species. Most of the chromosomal investigation carried out on tea (Sharma and Raina, 2006) till now of different species and varieties (Somatic and meiotic) which showed a remarkably constant number 2n = 30 in all cases except for a few natural triploids (Bezbaruah, 1971). Karyotype study revealed that the clones belonging to the three main races of tea (diploid) did not show any major differences in their gross morphology and karyotype, although some minor differences existed (Bezbaruah, 1971). The knowledge of the genomic constitution and genetic markers are pre-requisites for development of the elite improved tea varieties. Almost self-sterile and out-breeding nature of the tea plant, coupled with frequent and spontaneous

hybridization of different species and genera, resulted in highly heterozygous plants ranging from very small leaf China type to very large leaf Assam type. The hybridization is so extreme that sometimes molecular markers could not resolve correct identification of cultivated clones.

The tea varieties are essentially out breeders and a free exchange of gene pools accounts for an endless series of continuous morphological variability in many taxa. In spite of what little data accumulated on cytology of different members of tea, certain aspects of the cytology of some of the important cultivated varieties are yet to be investigated. In recent years it has been repeatedly discussed that the role of gene mutation in the evolution of species was over emphasized in the past. Consequently, due emphasis was not laid on structural alternation of chromosomes in the origin of species. Refined techniques, worked out lately by several workers have been adapted to trace karyotype differences between species. The importance of karyotype study in establishing phyletic relationship and evolutionary trends is well recognized. With the aid of improved chromosome techniques it has been possible to work out the chromosomal basis of intervarietal and even inter-strain differences (Datta, 1974). The chromosomes derived their prominence as a tool in taxonomy from their direct relation to the genetic system of which they are an integral part. The comparative organization of chromosomes may indicate the extent of relationship and a variety of ways in which the related individuals have evolved, besides commenting upon the adequacy of correct taxonomic placements.

In view of the scanty work on tea species and the importance of karyotype analysis in relation to phylogenetic relatedness, the present investigation was undertaken which includes detailed chromosome analysis in commercially important ten tea clonal varieties. Further, for a proper understanding of cytological situation in any plant material under *in vitro* conditions, it is imperative to consider the *in vivo* cytological constitution of the taxon used for induction and growth of the callus. It is also expected that the cytological situation in an explants tissue would be reflected in that of callus derived from it. For a proper assessment of *in vitro* cytological observations it seems to be justified to undertake a detailed cytological investigation on the experimental materials under *in vivo* conditions. The significance of *in vitro* cytology of explants source for a meaningful interpretation of its corresponding *in vivo* cytology. The varieties included in the present scheme of work are TV23, TV25,

T78, T383, TV30, HV39, TeenAli17, TV29, TV26, and UPASI-26 all of which are used to produce commercial tea in the tea industries.

Transgenic Tea through Genetic Engineering

Plant protection is a major challenge to agriculture world wild. One of the effective strategies for disease resistance in plants has been the incorporation of disease resistant genes into commercially acceptable cultivars. The most attractive initial candidates for manipulation of the single gene defense mechanism approach. The genes encoding for chitinase and β1,3- glucanase can be used because these two enzymes hydrolyze chitin and β-1,3-glucans, which are structural components of the cell walls of several fungi. Major emphasis has been given to the plant hydrolases such as β1,3-glucanase (PR-2) and chitinase (PR-3), as they are capable of clearing fungal cell walls resulting in pathogen growth inhibition (Neuhaus,1999) and moreover, the products of the hydrolysis can act as elicitors of further defense responses (Boller *et al.*, 1998). Both the β 1,3-glucanase and chitinase are highly abundant proteins in plant involved in diverse physiological and developmental processes .They can act either alone or in combination strengthening their antifungal activity. Their accumulation is not restricted only to resistant plants but is often observed in compatible plant-pathogen interactions or even non-pathogenic combination. Constitutive expression of individual PRs in transgenic plants can lead to reduced pathogen growth and symptom expression, consistent with a role of PRs in the expression of acquired resistance (Ryals *et al.*, 1994).

There is strong evidence that experience of β-1,3 glucanase transgene alone or in combination with chitinase transgene regulated by the strong CaMV 35S RNA promoter can reduce the susceptibility of plants to infection by certain fungi. Transgenic tobacco plants expressing a soybean β-1,3-glucanse can elicitor releasing β-1,3-glucanase or the tobacco class II β-1,3 glucanse PR -2b, that showed reduced symptoms when injected with *Alternaria alternata* or the oomycetes *Phytophthora parasitica* var. *nicotianae* and *Perenospora tabacina*. Wubben *et al.* (1996) reported on the tissue-specific expression of genes encoding the hydrolytic enzymes, β-1,3-glucanse and chitinase, as studied by means of *in situ* hybridization. No preferential accumulation of gene transcripts in tissue near penetrating hyphae was observed in compatible or incompatible interaction. Activation of natural weapons before

infection, called systemic acquired resistance (SAR) is initiated by pathogens, or pathogen-derived elicitors, as well as a number of chemical compounds. Among the main defense genes, which are switched on in response to pathogen infection, belong to those encoding PRs.

The association of PRs with SAR, but of with ISR, has led to the hypothesis that accumulation of PRs is not a pre-requisite for the induction of resistance, but that PRs contribute to the protective state (Van Loon and Van Strien ,1999). SAR is dependent on the accumulation of SA, but not JA or ethylene. It appears that only when increases in the levels of any of these signals occur, PRs become detectable in the infected plants. Spraying plants with solution of salicylic acid (SA) mainly induces the acidic PR-1 and acidic β-1,3-glucanase genes (Bol *et al.*, 1990). Salicylic acid is a naturally occurring compound in tobacco plants and the levels of endogenous salicylic acid rise after TMV infection, not only in the infected leaves but also in the uninfected parts of the plant, which show inducted resistance. Resistance can be local and restricted to the treated tissue or be induced systemically. The induction of resistance usually coincides with the accumulation of pathogenesis related proteins and their transcripts in resistant tissue (Kuc, 1982).

Despite major advances in the field of biotechnological approaches in case of tea improvement, the molecular markers, *in vitro* tissue culture as well as chromosomal studies and chitinase gene accumulation in tea remain elusive. In the years ahead, new genomic and proteomic technologies will assist in the identification and characterization of tea germplasm and transgenic gene expression for the creation of disease resistance improved tea. In view of the many limitations to horizontal improvement, transgenic technology is the most popular method for vertical crop improvement (Galun and Breiman, 1997). An ever moving goal, that the scientists of each of the tea regions try to achieve these goal through conventional/non-conventional breeding of tea plants that are not only high yielding with good cup characters but are also more tolerant to biological and abiological stresses (Bhattacharya *et al.*, 2004).

The conventional breeding programme can not fully achieve the target goal due to some inherent problems in tea (Mondal *et al.*, 2004). The tea characteristics like tolerance to stresses, yield and quality are multigenic characters and can not be dealt in a time effective manner with the conventional breeding technique. With the

consideration above all, transgenic tea development can be a more effective technology for pyramiding desirable genes into tea for better adaptation, quality and yield (Bhattacharya and Ahuja, 2001; Bhattacharya *et al.*, 2006). Production of transgenic plants requires cells or tissues that are not only competent for gene transfer and integration but also ones that would regenerate successfully into *in vitro* plants (Cahteau *et al.*, 2001; Gelvin, 2000).

Generally, preference has been given for the development of somatic embryogenesis or a proliferating callus from which adventitious shoot buds or somatic embryos can be regenerated in recurrent cycles. Preterminal meristemic tissues like the apical or the axillary buds are generally not preferred especially in the *Agrobacterium*-mediated gene transfer system in order to avoid the formation of chimeras or mosaic plants (Riva *et al.*, 1998). Since somatic embryogenesis is one of the most worked out regeneration system in tea (Jain and Newton, 1990) was employed in the gene transfer system through *Agrobacterium* harboring a binary vector pBIN19, containing the plasmid p35SGUSINT and developed first transgenic tea (Mondal *et al.*, 1999). Later Mondal *et al.* (2001a) optimized certain parameters to express the GUS gene (β-glucoronidase) in transformed somatic embryo and regeneration of plantlets by somatic germination. Micro-shoots of the transgenic tea plants was excised and further multiplied *in vitro* and later micro-grafted on the seedling derived root stocks of the same cultivar. Some other parameters for efficient transformation of tea explants have been investigated by many workers (Mondal *et al.*, 2004).

Agrobacterium tumefaciens mediated gene transfer technique has been used by several workers using different explants for genetic transformation in tea (Matsumoto and Fukui, 1998, 1999; Biao *et al.*, 1998; Luo and Liang, 2000). The putative transformed leaf callus with stable gene integration had been confirmed after molecular characterization through PCR and southern hybridization by these workers (Matsumoto and Fukui, 1989, 1999; Biao *et al.*, 1998). It is common to occur multiple insertion of transgene into the genome, rearrangement of the genes and/or deletions of the integrated transgene in the regenerated plants (Svitashev *et al.*, 2000). Biolistic mediated genetic transformation had also been initiated to transform tea callus by many workers (Akula and Akula, 1999; Prakash *et al.*, 1999).

The natural infection of tea plants with *Agrobacterium* has been reported *in* woody plants and attempted has taken in tea by several workers (Biao *et al.*, 1998; Matsumoto and Fukui, 1998, 1999; Luo and Liang 2000; Mondal *et al.*, 1999, 2001a, b) using different explants systems such as *in vitro* grown leaf, and somatic embryos. In view of all this, transgenic appear to be the most time effective technology for stacking desirable genes into tea for better adaptation, quality and yield (Bhattacharya and Ahuja, 2001) irrespective of the limitations of a specific region of a particular product quality.

Although the first successful report on the regeneration of tea plantlets from the cotyledon derived calli was published by Wu and his team (Wu *et al.*, 1981). Kato (1986) and Yan and Ping (1983) have reported the induction of somatic embryos when mature cotyledon sections were cultured on MS medium supplemented with BAP and IBA. Then the higher rates of somatic embryogenesis and secondary somatic embryogenesis have been reported by several workers using immature cotyledons (Nakamura 1988; Akula and Dodd, 1998; Mondal *et al.*, 1999; Palni *et al.*, 2006). The artificial seeds have been produced successfully from the somatic embryos in cultivated tea by Mondal *et al.* (2000) and Akula *et al.* (2000). The inter-specific hybrid embryos were rescued from immature somatic embryos before abortion (Nadamitsu *et al.*, 1986). The plantlets are obtained either through the somatic embryogenesis route or by induction of adventitious shoots followed by rooting. Akula and Dodd (1998) used mixture of sand, peat and vermiculite (1:2:1) in nursery pots that were kept under moist conditions in a green house for successful acclimatization.

Caffeine is synthesized from the nucleoside xanthosine in four sequential reactions. The first step in the pathway is the methylation of the nucleoside xanthosine by S-adenosylmethionine, which is catalyzed by the enzyme xanthosine-7-methyltransferase (XMT). The product, 7-methlyxanthosine is hydrolyzed to 7-methylxanthine, and undergoes further methylations to theobromine and caffeine. Although there are some *Camellia* species (Tea) that produce low level of caffeine, but these are not readily available for commercial use. To achieve this some researchers have utilized the antisense RNA technology for the production of decaffeinated tea (Kato *et al.*, 2000; Stiles *et al., 2000*), for the tea drinkers those who prefer caffeine less tea. Because high caffeine consumption has some negative health

impacts, which include palpitation, gastrointestinal disturbances, increased blood pressure, insomnia, anxiety and tremor.

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.*, 1993; Legrand *et al.*, 1987). 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 and John,1989; 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).

Pathogenesis related Proteins and respective genes for Plant's Own Defense system

Since their discovery in tobacco leaves hypersensitively reacting to TMV by two independently working groups (Van Loon and Van Kammen, 1970; Gianinazzi, 1982; Van Loon, 1985, 1997), pathogenesis-related proteins (initially named "b" proteins) have focused an increasing research interest in view of their possible involvement in plant resistance to pathogens. This assumption flowed from initial findings that these proteins are commonly induced in resistant plants, expressing a hypersensitive necrotic response (HR) to pathogens of viral, fungal and bacterial origin. Later, however, it turned out that b-proteins are induced not only in resistant, but also in susceptible plant-pathogen interactions, as well as in plants, subjected to abiotic stress factors (Van Loon *et al.*, 1994).

The term "pathogenesis-related proteins" (PRs) was coined by Antoniw *et al.*, (1980), which have been defined as "proteins encoded by the host plant but induced only in pathological or related situations", the latter implying situations of non-pathogenic origin. To be included among the PRs, a protein has to be newly expressed

upon infection but not necessarily in all pathological conditions. Pathological situations refer to all types of infected states, not just to resistant, hypersensitive responses in which PRs are most common; they also include parasitic attack by nematodes, insects and herbivores. Induction only by abiotic stress conditions is not a sufficient criterion for inclusion as PRs. These considerations imply that the characteristics of the induction of PRs take priority over other identifying features, such as chemical properties or cellular localization (Van Loon *et al.*, 1994; Van Loon, 1999). Originally, five main groups of PRs (PR-1 to PR-5) were characterized by both molecular and molecular-genetic techniques in tobacco, numbered in order of decreasing electrophoretic mobility.

Each group consists of several members with similar properties (Bol *et al.*, 1990). Odjakova and Hadjiivanova (2001) have reviewed in details the defense system in plants. The present status of the Pr-protein and its role in plant defense system has been reviewed by Edreva (2005). PR-1 is the most abundant, reaching up to 1-2 % of total leaf proteins. PRs of group 5 share significant amino acid sequence homology with the sweet tasting protein in the fruits of the tropical plant *Thaumatococcus daniellii*, and have been named thaumatin-like (TL) proteins (Cornelissen *et al.*, 1986). By then seventeen families (PR-1 to PR-17, table 8) were recognized and classified for tobacco and tomato, with the families PR-8 and PR-10 being also present in cucumber and parsley, respectively (Christensen *et al.*, 2002).

PRs have dual cellular localization-vacuolar and apoplastic, the apoplast being the main site of their accumulation (Van Loon, 1999). Apart from being present in the primary and secondary cell walls of infected plants, PRs are also found in cell wall appositions (papillae) deposited at the inner side of cell wall in response to fungal attack (Jeun, 2000). Interestingly, they are detected in the cell walls of invading fungal pathogens and in the space formed between cell walls and invaginated plasma membrane of fungi (Jeun, 2000; Jeun and Buchenauer, 2001). Acidic and basic PRs are identified, each of these counterparts having both apoplastic and vacuolar localisation (Buchel and Linthorst, 1999). Earlier data show that acidic tobacco PR-1 is localized in the apoplast, whereas basic tobacco PR-1 accumulates in the vacuole (Bol *et al.*, 1990). This may be valid for one PR family (PR-1) in a host plant, such as tobacco, but cannot be generalized as a differential localization feature of acidic and basic proteins in plants.

Table 8. Recognized families of pathogenesis-related proteins

Families	Type member	Properties
PR-1	Tobacco PR-1a	antifungal
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P, Q	chitinase type I,II, IV,V,VI,VII
PR-4	Tobacco 'R'	chitinase type I,II
PR-5	Tobacco S	thaumatin-like
PR-6	Tomato Inhibitor I	proteinase-inhibitor
PR-7	Tomato P_{69}	endoproteinase
PR-8	Cucumber chitinase	chitinase type III
PR-9	Tobacco 'lignin-forming peroxidase'	peroxidase
PR-10	Parsley 'PR1'	'ribonuclease-like'
PR-11	Tobacco 'class V' chitinase	chitinase, type I
PR-12	Radish Rs-AFP3	defensin
PR-13	Arabidopsis THI2.1	thionin
PR-14	Barley LTP4	lipid-transfer protein
PR-15	Barley OxOa (germin)	oxalate oxidase
PR-16	Barley OxOLP	'oxalate oxidase-like'
PR-17	Tobacco PRp27	unknown

In the leaves PRs are present in mesophyll and epidermal tissues. They are also localized in the abscission zone of leaves and inflorescence, abscission zone at the stem-petiole junction, and vascular tissue of stems and petioles. PRs in plants are coded by a small multigene family. Since their discovery, regulation of PRs has been a highly active research area. Putative plasma membrane localized receptors of PRs inducers are suggested, and secondary signals of PRs induction, such as salicylic acid (SA), jasmonic acid and ethylene, are established. Many of these secondary signals are well-known inducers of PRs expression (Durner *et al.*, 1997; Poupard *et al.*, 2003).

Cross-talks are common between signaling pathways mediated by these secondary messengers. Thus, SA-independent/jasmonate dependent, and *vice-versa* pathways of PRs induction have been demonstrated. It has been proven that PRs synthesis is regulated at transcriptional level; the exact mechanisms of transcriptional regulation have been ones of the most active fields of PR gene studies. Several *cis-*

regulatory elements in PR-promoters mediating PR gene expression have been identified. These include Wbox, GCC box, G box, MRE-like sequence, SA-responsive element (SARE) (Zhou, 1999). New mutants are developed providing clues into the better understanding of the regulation of PRs (Delaney, 2000). PRs are synthesized following a long lag period (no less than 8 h) the synthesis proceeds *in situ*, *i.e.* PRs are not translocated from the site of their induction to other plant parts, as proven by elegant grafting experiments (Gianinazzi *et al.*, 1982). By using PRs patterns the origin of *N. tabacum* from the wild progenitors *N. sylvestris* and *N. tomentosiformis* was confirmed (Ahl *et al.*, 1982).

The amino acid sequence GHYTQVVW is a particularly well-conserved region in the two groups of proteins, suggestive of the functional role of this domain (Van Loon and Van Strien, 1999). Peter Tiffin (2004) has studied the comparative evolutionary histories of chitinase genes in the genus *Zea* and family Poaceae. Plants are exposed to very different attackers, including microbial pathogens and herbivorous insects by Van Loon (2001). To protect themselves, plants have evolved defensive strategies to counteract potential invaders. Recent advances in plant defense signaling research have revealed that plants are capable of differentially activating inducible, broad-spectrum defense mechanisms, depending on the type of invader encountered. The plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are major players in the network of defense signaling pathways. Cross-talk among SA, JA and ET dependent signaling pathways is thought to be involved in fine-tuning the defense reaction, eventually leading to the activation of an optimal mix of defense responses to resist the intruder. Genetic engineering of the biosynthetic pathways of these signaling compounds and the development of protective chemicals mimicking their mode of action provide useful tools for the development of new strategies for crop protection. However, there is evidence for antagonism between SA-dependent resistance to microbial pathogens and JA-dependent resistance to herbivorous insects: once a plant is conditioned to express resistance against microbial pathogens it may become more susceptible to attack by herbivores, and *vice versa*. Yet, the evidence for tradeoffs between pathogen and insect resistance is contradictory.

It has been focused on recent experimental evidence on the relationship between SA, JA and ET dependent induced resistance to microbial pathogens and

herbivorous insects. In addition, 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. 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 catalyses the hydrolysis of β-1,4-N-acetyl-D-glucosamine linkages of the fungal cell wall polymer chitin and is involved in the inducible defenses of plants. 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.

The poly-A mRNA was reversed transcribed and further amplified using RT-PCR to measure its expression. They isolated and cloned 1.1 Kb fragment of the chitinase gene. A nucleotide sequence comparison identified the fragment as a Class I basic chitinase cDNA; this fragment was subsequently used as a probe to screen for a full-length transcript from the cDNA library. Library screening resulted in the isolation of a 1324 bp clone, which is designated as CHRZP; encoding a polypeptide of 289 amino acids. Isolation and characterization of chitinase genes from pitchers of the carnivorous plant *Nepenthes khasiana* has been conducted by Eilenberg *et al.* (2006). Metraux and John (1989) have studied in details the chitinase gene isolation and cloning. 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) have shown that transgenic rice plant over expressing PR-5 gene can be more resistance against sheath blight disease pathogen *Rhizoctonia solani*.

Chitinases are enzymes that hydrolyse ß-1,4-*N*-acetyl-D-glucosamine (GlcNAc) linkages. 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. According to their primary structure, chitinases are divided into seven classes (classes I–VII). Plant chitinases and lysozymes are likely to have arisen from one coancestor by divergent evolution (Monzingo *et al.*, 1996). The protein genealogy of chitinases shows that class I and

class II chitinase genes evolved from the same ancestral gene (Shinshi *et al.*, 1990; Araki and Torikata, 1995). Moreover, a basic class II chitinase is a putative ancestor of basic class I and acidic class II chitinase genes (Ohme-Takagi *et al.*, 1998). It has also been proposed that chitinases in class IV, which are phylogenetically related to class I and II chitinases (Araki and Torikata, 1995; Hamel *et al.*, 1997; Gomez *et al.*, 2002), evolved from a class I chitinase gene by four deletions in the coding sequence (Araki and Torikata, 1995).

Among many other functions, chitinases can stimulate embryo development (van Hengel *et al.*, 1998) and seed development (Van Damme *et al.*, 1999). Besides chitin, the primary substrate for chitinase, which is not present in plants, chitinases can also hydrolyse arabinogalactan proteins (AGPs) (van Hengel *et al.*, 2001), rhizobial Nod factors (Staehelin *et al.*, 1994) and other lipo-chito-oligosaccharides (LCOs) (Dyachok *et al.*, 2002). Since the enriched pattern of proteins found in cell suspensions includes seed-specific proteins, it has been assumed that some somatic cells show endosperm properties (Kragh *et al.*, 1993). It has been hypothesized that endosperm and embryo interact during their development (Berger, 1999). The evidence supporting endosperm–embryo interaction is derived from the carrot system. The EP3 chitinase that is expressed in the endosperm of carrot, rescue somatic embryos of carrot *ts11* variant (van Hengel *et al.*, 1998).

Wounding of plant tissues has been shown to induce complex molecular responses many of which are considered plant defense response (Aneja and Gianfagna, 2001; Leon *et at.*, 2001; Cheong *et al.*, 2002). Wounding can induce formation of jasmonates and ethylene, each of which are capable of independently inducing signal transduction pathways leading to resistance against plant pests. Jasmonic acid and related compound methyl jasmonate are part of the octadecanoid pathway in plants (Strassner *et al.*, 2002; Wasternack and Hause, 2002) and in some incidences contribute to resistance to insects (McConn *et al.*, 1997; Baldwin, 1998) and pathogen (Park *et al.*, 2001). Jasmonates have been shown to function as regulators of many different plant defense genes (McConn *et al.*, 1997; Park *et al.*, 2001). Ethylene is known to induce specific subsets of pathogenesis-related proteins in many plant species (Kitajima and Sato, 1999; Thomma *et al.*, 1999). The effects of ethylene have been associated with resistance to many different plant pathogens

(Abeles *et al.*, 1992; Thomma *et al.*, 1999; Park *et al.*, 2001) and insects (Abeles *et al.*, 1992; O'Donnell *et al.*, 1996; Argandona *et al.*, 2001; Bailey *et al.*, 2005).

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; Legrand *et al*, 1987; Boller, 1989; Collinge *et al.*, 1993). Chitinases catalyze the hydrolysis of the β-1,4 linked N-acetylglucosamine polymers that form chitin chains, a major component of fungal cell walls. Chitinases have been divided based on their structure into at least four classes (classes i to iv; Hamel, *et al.*, 1997). Even though chitinases have been shown to inhibit the hyphal tip growth of many fungi *in vitro.* A plant's natural defense mechanisms are often insufficient to prevent an invasion by the pathogen (Neuhaus *et al.*, 1999). The consequences of plant disease caused by fungal pathogens can be significant losses in crop quality and yields. 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).

Further, plants constitutively over-expressing a hybrid endochitinase exhibited improved tolerance to fungal diseases in field tests (Grison *et al.*, 1996). The expression of chitinases in plants is therefore useful to enhance resistance in plants against fungal pathogens. Chitinases expressed in plants are also reported to have anti-insect activity. Additionally, chitinases are useful in industrial processes aimed at the bioconversion of shellfish chitin waste. Although expression of chitinases can be useful to reduce infection by fungal pathogens and other pests, constitutive overexpression of foreign proteins in crop plants has a potentially yield-reducing metabolic cost. Moreover, it is commonly found that particular chitinases only have antifungal activity against a narrow range of fungal pathogens. Roby *et al.* (1991) have been studied the regulation of a chitinase gene promoter by ethylene and elicitors in bean protoplasts to improve the crop protection mechanism. Recent studies suggest that the production of enzymes capable of degrading the cell walls of invading phytopathogenic fungi may be an important component of the defense response of plants. The production of transgenic plants with enhanced resistance to attack by the fungal pathogen *Rhizoctonia solani* has been developed (Broglie *et al.*, 1993). Payne *et al.*, (1990) isolated the cDNA of chitinase gene from tobacco.

Jeyaramraja *et al.* (2005) have studied the role played by certain factors, physical barriers and chitinase enzyme, associated with blister blight resistance in tea. Sharma and Kumar (2005) studied the differential display-mediated identification of three drought-responsive expressed sequence tags in tea [*Camellia sinensis* (L.) O. Kuntze] and have shown the PR-5 like protein (pathogenesis related protein) induction in drought conditions. Kirubakaran and Sakthivel, (2007) had cloned and over expressed the barley chitinase gene in *E. coli*, which was believed to be involved in plant defense responses to pathogen infection. The molecular weight of chitinase is 35 kDa that was isolated and purified. Purified chitinase exerted broad-spectrum antifungal activity against *Botrytis cinerea* (blight of tobacco), *Pestalotia theae* (leaf spot of tea), *Bipolaris oryzae* (brown spot of rice), *Alternaria sp.* (grain discoloration of rice), *Curvularia lunata* (leaf spot of clover) and *Rhizoctonia solani* (sheath blight of rice). Due to the potential of broad-spectrum antifungal activity barley chitinase gene can be used to enhance fungal-resistance in crop plants such as rice, tobacco, tea and clover. Genetic transformation of pigeon pea with rice chitinase gene had been investigated by Kumar *et al.* (2004) for enhanced resistance activity against pathogenic attack.

Plants are endowed with various defense related genes and they express a variety of defense products in response to pathogen attack as a consequence of transcriptional activation. Accumulation of defense gene transcripts generally commences within minutes to hours around the infection sites, and several hours or days later at distant sites over the whole plant. During the local and systemic responses of plants, a large group of defense enzymes, PR-proteins and signal molecules are synthesized in high amounts to display a broad spectrum of antimicrobial activity. Though intensive studies, numerous genes involved in these processes have been identified (Hammnd-Kosack and Jones, 1996). Defense response genes are induced in both incompatible and compatible plant-pathogen interactions. However, mRNA accumulation for many plant defense genes is more rapid during interactions involving a plant expressing resistance to a particular pathogen (Davis *et al.*, 1993; Christensen *et al.*, 2002; Davis *et al.*, 2002). Polyphenol oxidases (PPO) are nuclear coded enzymes of almost ubiquitous distribution in plants (Mayer and Harel, 1979; Mayer, 1987).

However, there are very few reports of transcripts accumulation studies with regard to the role of defense enzyme like glucanase, chitinase, phenylalanine

ammonia lyase (PAL), peroxidase, chalcone synthase, PPO (Demeke and Morris, 2002) and LOX involved in blister blight infection. Therefore other examples of defense enzymes and PR proteins transcripts accumulation during induction of resistance with various other inducers and also during host-pathogen interactions is briefly presented here. The research progress on the molecular characterization of the defense enzyme PPO is also briefly outlined.

Pritsh *et al.* (2000) studied the pattern of transcripts accumulation of six-typical defense response genes, POX (peroxidase), PR-1, PR-2 (β-1, 3-glucanase), PR-3 (Chitinase), PR-4 & PR-5 (thaumatin like protein) in spray-inoculated panicles of both the susceptible cv. Wheatson and the resistant cv. Sumai3 of wheat. POX transcripts accumulation has been studied by Collinge and Boller (2001) in potato upon infection with *Phytophthora infestans*. They isolated two genes, a putative peroxidase. Fossdal *et al.* (2001) isolated cDNA encoding the putative defense related and basic plant peroxidase SPI2 (Spruce pathogen induced-2) with an estimated molecular mass of 34 kDa from roots of Norway spruce (*Picea abies*) seedlings. McFadden *et al.*, (2001) reported the expression of PRs- cadinene synthase, acidic chitinase, basic chitinase, β-1, 3-glucanase, phenylalanine ammonia lyase (PAL) and PR10 transcripts in cotton infected by *Verticillum dahlia*. Transcripts of defense response genes PR-1, PR-2 (β-glucanase), PR-3 (Chitinase), PR-4 & 5 (thaumatin like) and PR-9 (Peroxidase) transcripts accumulated in wheat spikes upon infection with *Fusarium graminareum* and treated with BTH (Yu and Muehlbauer, 2001). Christensen *et al.* (2002) reported the isolation of two cDNA clones from barley after inoculation with *Blumeria graminis f.sp.hordei*. The encoded protein of these cDNA clones belong to a new family of plant pathogenesis proteins designated as PR-17. Mohammadi and Kazemi (2002) showed the involvement of PPO in resistance against *Fusarium graminareum* during induced resistance. PPO catalyzes the oxygen dependent oxidation of phenols to quinines. Because of their conspicuous reaction products and their wound and pathogen inducibilities PPOs have frequently been suggested to participate in plant defense against pests and pathogens (Constabel *et al.*, 1995; Thipyapong *et al.*, 1995; Thipyapong and Steffins, 1997). However, the role of the enzymes in plant defense against pathogens has not been studied much.

There are only a few reports of showing the involvement of PPO during defense against plant pathogens. Overexpression of polyphenol oxidase in transgenic

tomato plants resulted in enhanced resistance to *Pseudomonas syringae* (Li and Steffens, 2002). PPOs appear to be nearly ubiquitous among plants (Sherman *et al.*, 1995), but the many possible roles they play have not been totally resolved. PPO activity is often thought of as a defense mechanism based on the appearance of PPO reaction products upon wounding or pathogenesis and the inducible nature of PPO upon wounding (Mayer and Harel, 1979; Constable *et al.*, 1995) showed that antisense down-regulation of constitutive and inducible PPO activity results in a hyper susceptibility to pathogens in tomato (*Lycopersicon esculentum*). Overexpression of PPO in tomato results in enhanced resistance to bacterial disease (Li and Steffens, 2002). These results strengthen the assertion of a defense role for the enzyme. Although there is speculation as to the mode of action involved in such defensive roles, the actual mechanisms have not been elucidated.

Various studies have reported the cloning and characterization of the multigene families encoding PPOs from different plant species, including faba bean (Cary *et al.*, 1992), potato (Hunt *et al.*, 1993), tomato (Newmann *et al.*, 1993) and Virginian pokewood (Joy *et al.*, 1995). In 1994, Dry and Robinson described the molecular cloning and characterization of grape berry PPO. Southern analysis suggested the presence of only one gene in the grapevines. Moreover, high level of gene expression was found in young, developing berries, whereas expression in mature tissues was low. More recently Boss *et al.* (1995) isolated a full length cDNA clone encoding apple PPO and described it as a multigene family. These studies have revealed a high degree of sequence conservation among the investigated PPOs. Leaf PPO sequences can be roughly divided into three domains, with a central domain containing the copper-binding sites (Van Gelder *et al.*, 1997).

Reverse-transcriptase-polymerase chain reaction (RT-PCR) has become an important tool to examine levels of mRNA transcripts because of its high sensitivity. The powerful amplification potential of PCR has assured its use in the detection of low-abundance mRNA in cells and tissues. This technique is particularly valuable in cases where the amount of an RNA species present is so minute that traditional RNA analysis methodologies such as Northern blot or RNase protection assay may not be adequate. It has been estimated that RT-PCR is thousands of times more sensitive than Northern blot analysis. The technique consists of two parts: synthesis of cDNA from RNA by reverse transcription, and amplification of a specific cDNA by

polymerase chain reaction (PCR). The method requires very little RNA and differs from Northern blotting because it is somewhat tolerant of degraded RNA, as long as the RNA is intact within the region of interest.

Because PCR targets only DNA sequences, mRNA is first converted by reverse transcriptase (RT) to single-stranded complementary DNA (cDNA), which can be amplified by PCR reaction. Avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (MMLV) reverse transcriptases are commonly used for cDNA synthesis. For reverse transcription to occur, mRNA must be primed at the 3' end. This can be easily achieved by using primers of random sequences that will hybridize to their respective complementary RNA sequences along the RNA molecule. Thus, this random-primed method can be used for any RNA species, including ribosomal and messenger RNA. For mRNA, most of which ends in 3' polyadenlyation, specific 3' priming can be effected by annealing with oligo(dT). If the 3' sequence of a target RNA is known, a 3' primer can be used to specifically prime the RNA, resulting in a cleaner PCR product profile. Priming with any of these three methods, followed by extension with reverse transcription will result in a cDNA template for the 5' sense primer in the PCR reaction, allowing the PCR to proceed.

As long as an RNA molecule can be reverse-transcribed to render a target sequence for an ensuing PCR reaction, even partially degraded RNA can be amplified and be detected. The quantitative measurement of specific mRNA species is of major importance for studies on gene expression. Northern blotting is a relatively insensitive method requiring microgram amounts of RNA. It is time consuming and semi-quantitative at best. Because of the limitations of Northern blotting, various strategies have been developed for quantitation of cDNA by polymerase chain reaction (PCR)-based methods (Schweitzer and Horikoshi, 1992), *i.e.* RT-PCR technique.

So, 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 investigation is aimed to partially clone the chitinase gene of tea [*Camellia sinensis*] for its characterization at the molecular genetic level using bioinformatics algorithm.