TRANSGENE INTEGRATION, EXPRESSION AND INHERITANCE IN TEA PLANTS

THESIS

By

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AUM NAMAH SHIVAY

Is there anything I can say,

anything I can give

or do for you.....

Because all that I'm

all that I have

I owe to you.....

Affectionately Dedicated

to my

Revered Parents

Who have always sacrificed

their present

to make my future better

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CERTIFICATE I

This is to certify that the thesis entitled **"Transgene integration, expression and inheritance in tea plants"** submitted in partial fulfilment of the requirements for the award of the degree of **Master of Science (Agriculture)** in the subject of **Agricultural Biotechnology** of CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur is a bonafide research work carried out by **Mr. Rajash Koul (A-2006-30-08)** son of **Shri Girdhari Lal Koul** under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

Amita Bhattacharya

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Chairman
Advisory Committee

Place: Palampur
Dated: 16 July, 2009

CERTIFICATE II

This is to certify that the thesis entitled **"Transgene integration, expression and inheritance in tea plants"** submitted by **Mr. Rajash Koul (A-2006-30-08)** son of **Shri Girdhari Lal Koul** to the CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur, in partial fulfilment of the requirements for the degree of **Master of Science (Agriculture)** in the subject of **Agricultural Biotechnology**, has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

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Needless to say, all omissions and errors are mine.

Place : Palampur
Dated: 16th July, 2009

(Rajash Koul)

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ABBREVIATIONS USED

%	Percent
µg	Microgram
µl	Microlitre
µm	Micrometer
°C	Degree centrigrade
bp	Base pair
cDNA	Complementary DNA
cm	centimeter
CTAB	Cetyl trimethyl ammonium bromide
DEPC	Diethyl pyrocarbonate
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxy ribonucleoside triphosphate
dsRNA	Double stranded ribonucleic acid
DTT	Di thio triol
E	East
e.g.	exempli gratia (for example)
EDTA	Ethylene diamine tetra-acetic acid
et.al.	idest (that is)
FA	Formaldehyde agarose
g	Gram
gus	β- glucuronidase gene
H_2O	Water
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
hr	Hour(s)
M	Molar
m	Meter(s)
min	Minute(s)
ml	Milliliter
mM	Millimolar
mRNA	Messenger RNA

MW	Molecular weight
N	North
ng	Nano gram
PCR	Polymerase chain reaction
pH	Power of hydrogen ions
PVP	Polyvinyl pyrrolidone
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RT	Reverse Transcriptase
RT-PCR	Reverse transcriptase PCR
SDS	Sodium dodecyl sulphate
sec	Second(s)
TAE	Tris-acetate EDTA
Taq	*Thermus aquaticus*
TE	Tris-EDTA
UV	Ultra-violet
V	Volt
W	West
w/v	Weight by volume

Introduction

Chapter I

INTRODUCTION

Tea (*Camellia sinensis* L.) is an evergreen shrub widely cultivated throughout the tropics and sub-tropics, especially in the hilly or mountainous regions. The tender leaves of tea or 'two and a bud' have rejuvenating properties (Hodgson *et al.*, 1999). It is one of the most widely used beverages in the world, second only to water. It is an important cash crop for all the tea growing areas of the world. The area under tea cultivation reached 2.461 million ha resulting in 3.196 million ton production world-wide during 2005 (Anonymous, 2006). These figures clearly demonstrate the importance of tea and also reflect the potential economic benefits that can be realized with increment in productivity. This plant has received considerable interest in recent years as a medicinal agent with its well-documented anti-bacterial (Vijaya *et al.,* 1995), anti-ulcer (Maity *et al.,* 1995), anti-cancer (Weisburger and Chung, 2002; Jousha *et al.*, 2003) and anti-inflammatory (Chattopadhyay *et al.*, 2004; Chaudhuri *et al.*, 2005) properties.

The primary center of origin for tea is South East Asia i.e. at the point of intersection between 29°N (latitude) and 98°E (longitude) near the source of the Irrawaddy river at the confluence of North-East India, North Burma, South West China and Tibet (Wight, 1959). However tea is now distributed in more than 50 countries of the world within the latitudinal ranges between 45°N to 34°S (Sana, 1989). There are three main types of cultivated tea namely, *Camellia sinensis* or China type, *C. assamica* or Assam type and *C. assamica* sub spp. *lasiocalyx* or

Cambod type. The 'China type' plants are normally 1 to 3 m in height with branches that arise from the base of the plant. The leaves of these plants are erect, relatively small, thick and leathery with sunken stomata. The 'Assam type' plants are 10-15 m high with ramifying branch systems and thin, glossy, relatively large (8-20 cm × 3.5-7.5 cm), broadly elliptic leaves with more or less acuminate apex and distinct marginal veins. The 'Cambod type' plants are 6-10 m high with more or less equally developed ascending main stems and glossy, intermediate sized leaves that change colour from light green to coppery-yellow or pinkish red during autumn (Mondal, 1999).

Tea is becoming increasingly important for its pharmacological properties and its biological constituents viz. polysaccharides, essential oils, vitamins, minerals, purines, flavonoids, alkaloids like caffeine and polyphenols like catechins. Importantly, the vitamin C content in green tea is comparable to that of lemon. Hong *et al.* (2002) and Hara (2003) listed several industrial and medicinal uses of tea products and their constituents. Tea leaves are processed in three different manners and each yields a distinct beverage that has antibacterial and free radical capturing (anti-oxidising) activities (Chu, 1997). While the flavonoids have antioxidant, anti-inflammatory, anti-allergic, antibacterial and antiviral effects with an ability to strengthen veins and decrease permeability, the tannins or catechins are basically detoxifying agents.

Despite the fact that tea is gaining immense popularity as a 'health drink' all throughout the world, yet the total produce is not sufficient enough to meet the demands of the world market (Bora and Deka, 1999). While the yield of tea is

further reduced by several biotic and abiotic stresses, the scope for extending the cultivable land at the expense of important food crops is also greatly limited even in the countries where tea is significantly important. Unlike other crops where higher yield per unit area is of primary importance, tea not only demands higher yield but also better adaptability and cup characters (Bhattacharya and Ahuja, 2003). In this regard, vertical crop improvement has gained importance as the conventional crop improvement programs cannot cater to these targets fully in the limited available land and because of some inherent problems of tea (Mondal *et al.,* 2004). Thus, it is not surprising that almost all biotechnological research in tea, today is focused towards transgenic tea production. However, proper commercial utilization of a transgenic plant requires stable transgene integration and expression over successive generations. Microprojectile bombardment of plant transformation is one of the most successfully employed methods after *Agrobacterium* and the transgenes delivered by this method are generally inherited as single Mendelian factors (Pawlowski *et al.*, 1996). Many a times, however, their expression over successive generations are impaired due to more than one copy of the transgene, their position, re-arrangement, methylation, sterility of T_0 plants or non-transmission of the intact transgenes to some or all the progenies (Vain *et al.,* 2002). Variable transgene expression in plants is also often observed within populations of transgenic plants transformed with the same construct and under identical transformation conditions (Meyer, 1998). This inter-transformant variation highly complicates phenotypic analysis and the production of transgenic commercial crops with stable and predictable performance.

Variation in gene expression and silencing are the other major problems experienced during the production of stable transgenic plants (Matzke and Matzke, 1998; Bregitzer and Tonks, 2003). Variation in transgene expression levels is undesirable for the application of transgenic plants in research, development of genetically modified products and biosafety for regulatory perspectives. It reduces the predictability and efficiency of genetic transformation and necessitates the establishment and analysis of many independently selected transformants to obtain the desired phenotype with the appropriate stability of transgene expression (Conner and Christey, 1994).

It is therefore, necessary to understand the structure, integration and expression of the transgene both at T_0 and T_1 levels. Studies on plant development characteristics like growth, fertility and yield are also important as the transgenic plants have to bear the burden of an additional gene which may have considerable effects on the growth and metabolism of the plants.

So keeping these in view, studies were undertaken with the following objectives:

i. Analysis of the transgene integration patterns in the T_0 and T_1 plants with respect to copy number, silencing due to methylation etc.
ii. Assessment of transgene expression in T_0 and T_1 plants and phenotypic analysis with respect to plant development characteristics like growth, fertility, morphology and yield in terms of catechins and caffeine.

Review of Literature

Chapter II

REVIEW OF LITERATURE

The production of transgenic crop plants is an expanding component of agricultural biotechnology. However, it is crucial that the introduced genes be transmitted faithfully through successive generations in a predictable manner for the commercial success of all sexually propagated plants. Unfortunately, this does not always hold true, as transgene inactivation is a frequently observed phenomenon in transgenic plants. Though not well understood, certain factors affecting transgene inactivation include multiple-copy integration (Assaad *et al.*, 1993), differential base composition between transgene and the integration site (Allen *et al.,* 1993), hyperexpression of transgenes (Elmayan and Vaucheret, 1996) and environmental factors (Meyer *et al.,* 1992). Although single-copy transgenes can be silenced, there is a much higher incidence of gene instability correlated with high transgene copy number (Finnegan and McElroy, 1994). Complex integration sites may undergo structural instability such as intra-chromosomal recombination between multiple copies, resulting in loss of the transgene (Srivastava *et al.,* 1996) or chemical modification such as DNA methylation (Matzke *et al.,* 1994).

In order to obtain more predictable transgene expression levels, single-copy transformants are generally preferred. However, they are not guaranteed to produce predictable transgene expression levels (Meza *et al.,* 2002; De Buck *et al.,* 2004). It is commonly generalized that transgenic plants obtained by direct

DNA-transfer methods (e.g. biolistic and electroporation) contain a large number of transgene copies (even up to 100 copies) whereas *Agrobacterium* mediated transformation leads to the insertion of fewer transgene copies (<10) and a higher occurrence of single-copy integrants (Reddy *et al.,* 2003). *Agrobacterium*-mediated transformation method is therefore, preferred over direct gene transfer as multiple transgene copies are associated with low transgene expression levels (Muskens *et al.,* 2000). However, this method has also been shown to consistently yield 1-3 copies of the transgene (Romano *et al.,* 2003). Hence, transgene copy numbers may be highly variable, irrespective of the transformation method and are probably dependent on numerous other factors that are yet to be identified.

To date, no sound correlations between copy number and transformation methods/parameters have been made. However, a transformation procedure that could be used to consistently generate transgenic plants with single copy integration would be of great value. Hence, rigorous testing is required to optimize methods for simple integration patterns. Moreover, it is also important to ensure the stability of a transgene by insulating it with matrix-attachment regions, avoiding repetition of promoter or transgene sequences, particularly the inverted repeats (Stam *et al.,* 1997), and using moderate promoters to avoid hyperexpression (Matzke and Matzke, 1995).

2.1 Biolistic mediated transformation

Biolistic mediated transformation is the method of choice for the production of transgenic plants where *Agrobacterium* mediated transformation is not effective. This technique was developed by Sanford and co-workers (Klein *et*

al., 1987) and involves the coating of microcarrier (tungsten or gold particles) with DNA. These particles are then accelerated by a variety of techniques viz. gun powder, compressed gas or other means to energies at which they can penetrate a plant cell. Once in the cell, the DNA released from the particles are either expressed transiently or stably incorporated into the plant genome. Such cells can then give rise to stable transgenic plants (Portrykus, 1991; Gray and Finer, 1993).

Microprojectile bombardment has several advantages over *Agrobacterium* mediated transformation. Since there is no host range limitation, microprojectile bombardment is an attractive technique for the transformation of recalcitrant species and DNA can be introduced directly into a range of organized morphogenic tissues such as embryos, meristems and pollens. This technique has led to the development of transgenic plants like maize (Gordon-Kamm *et al.,* 1990), rice (Christou *et al.,* 1991), soyabean (McCabe *et al.,* 1988), wheat (Vasil *et al.,* 1992) etc. Haploid transgenic plants of *Nicotiana rustica* have also been generated from bombarded pollen cells by this method (Morikawa *et al.,* 1994).

Biolistic mediated transformation of tea was attempted by many workers. Akula and Akula (1999) reported a high transient GUS expression after bombardment of tea somatic embryos with gold particles (1.5-3.0 µm) coated with plasmid p2K7DNA. However, it was Sandal *et al.* (2001) who succeeded in producing transgenic tea plants with stable integration of *gus* and *nptII* genes. Sandal (2003) emphasized the importance of the right combination of transgene DNA concentration, target distance, macro-carrier flight distance, gap distance and burst pressure.

Shan *et al.* (2005) also used particle delivery system for tea callus. They compared several pre-bombardment culture media and showed the highest transient gene expression in the media with PVP. They also observed that bombardment of tea callus at a target distance of 5 cm was suitable for both GUS activity and regeneration.

2.2 Transgene integration

Plant transformation by microprojectile bombardment results in transgene integration patterns that generally exhibit multiple transgene copies and extensive rearrangements of the introduced DNA (Pawlowski and Somers, 1996). The mechanisms involved in genomic integration of transgenic DNA delivered by microprojectile bombardment are not well understood but can probably be considered in two stages. The first stage includes preintegration rearrangements in the introduced DNA as it is delivered into the nucleus, whereas the second stage involves the process of integration into the host genome.

Plasmid DNA, generally used in microprojectile bombardment experiments in plants may either be subjected to mechanical shearing during particle preparation and bombardment processes, or may also be degraded by host-cell nuclease activity. In addition, nuclease activity may "nibble" the ends of linear DNA molecules (Riggs and Bates, 1986). Ligation of these broken DNA fragments into arrays of transgenic DNA is thought to precede genomic integration (Bates *et al.,* 1990). On the other hand, very little is known about the actual process of transgene integration via the microprojectile bombardment or

other direct DNA delivery methods. The process is difficult to monitor while integration is occurring because of the low frequency of stable transformation events. However, the study of transgene integration patterns in stable transformants was analysed to elucidate the integration process. This is because direct DNA delivery usually results in transgenes integrating at one genomic locus (Christou *et al.,* 1989; Spencer *et al.,* 1992) and consists of multiple, intact, and rearranged copies of transgenes (Fromm *et al.,* 1990; Wan and Lemaux, 1994). In many instances, head-to-head or head-to-tail concatenation of the introduced transgenes have been reported (Kartzke *et al.,* 1990) further supporting the concept that contiguous arrays of transgenic DNA are integrated into a single genomic site. It has also been reported that transgene loci in rice plants transformed by microprojectile bombardment may have host DNA sequences separating the closely linked transgene sequences (Kohli *et al.,* 1998). Transgene integration patterns may often affect the qualitative yield of crops. Thus, an evaluation of the transgene integration patterns in the transgenic plants is required.

2.3 Transgene expression

Although there has been great progress in transformation technologies in recent years, yet transgene expression in plants remains largely unpredictable. There is considerable variation in expression levels and stability between independently transformed plants (Walters *et al.,* 1992). Different integration sites, copy numbers, transgenic locus configurations as well as epigenetic silencing mechanisms can all contribute to this variability (Iyer *et al.,* 2000).

Experimental procedures such as transformation systems (*Agrobacterium* or direct methods), construct configuration (Breyne *et al.,* 1992), promoters (Mlynarova *et al.,* 1995), coding sequences, terminators, selection strategy (Bhattacharyya *et al.,* 1994), flanking Matrix Attachment Regions (MARs) (Mlynarova *et al.,* 1994) or the targeted plant tissue (Ulker *et al.,* 1999) have been reported to influence transgene structure or expression in plants. The multiplicity of these factors or their interactions contributes strongly to the unpredictability, variability and instability of transgene expression in plants. This problem is particularly acute in plants generated by direct transfer methods (electroporation, particle gun, silicon carbide fibers etc.) as complex transgenic loci are created in the plant genome. The numerous and uncontrolled transgene rearrangements, high gene copy number and systematic transgene linkage (Pawlowski and Somers, 1996) all favour variable and unstable transgene expression (Hansen and Chilton, 1996; Matzke and Matzke, 1998).

2.4 Variations in transgene expression

The prominent source of variation in transgene expression is the inconsistency of transgene copy numbers. Multiple copies of the transgene DNA tend to integrate in one or a few insertion sites as a result of extra-chromosomal ligation of the transgenic DNA fragments prior to integration (De Buck *et al.,* 1999). Theoretically, an increase in transgene copy number results in an increase of transgene expression level. However, multiple copy integration patterns are associated with low-level transgene expression, especially complex integration patterns such as tandem repeat (Wang and Waterhouse, 2000) and

inverted repeat (IR) structures (Muskens *et al.,* 2000). This reduced expression seems to result from the interactions between homologous sequences of multiple transgene copies and has been termed as homology-dependent gene silencing (HDGS) (Meyer and Saedler, 1996). HDGS may manifest when multiple copies of the transgene are inserted at one locus or at unlinked sites. This puzzling silencing phenomenon is believed to reduce expression of multiple transgene copies either at the transcriptional (transcriptional gene silencing; TGS) or post-transcriptional level (post-transcriptional gene silencing; PTGS). Over the years, PTGS and TGS have become the major determinants of transgene expression in plants.

PTGS, generally referred to as RNA silencing, is a conserved eukaryotic surveillance mechanism thought to defend the plant against viruses, protect the genome from transposons and regulate gene expression (Baulcombe, 2004). The RNA-silencing pathways that exist in fungi (quelling), animals (RNA interference or RNAi) and plants (PTGS) have similar genetic requirements and biochemical features. However, there are also certain significant differences in the silencing pathways engaged by these various organisms (Susi *et al.,* 2004). Besides complex arrangements of multiple transgenic DNA insertions such as IRs, other triggers of PTGS in plants include dsRNA, concurrent expression of sense and antisense genes, homology between transgenes and endogenous genes, aberrant RNA production (premature transcripts, breakdown products or antisense RNA) and high levels of transgene expression exceeding a certain threshold level (Matzke *et al.,* 2002). As such, RNA silencing may play a dominant role in the establishment of variation in transgene expression.

TGS is like PTGS based on recognition of nucleic acid sequence homology. However, in this case no transcripts are consequently formed at the transcriptional level. TGS is frequently associated with methylation of promoter sequences of the transgene and is usually found to be meiotically irreversible (Park *et al.,* 1996). For a long time, TGS and PTGS were believed to involve two distinct mechanisms. In recent years however, the idea that small interfering RNAs, the key actors of RNA silencing, may also operate in the nucleus as regulators of gene expression at transcriptional level via chromatin remodeling or RNA-directed DNA methylation has gained tremendous momentum (Pickford and Cogoni, 2003; Matzke *et al.,* 2004). Hence, the neat distinction between TGS and PTGS has blurred and TGS is more and more viewed as the nuclear side of RNA silencing.

Another source of inter-individual variation of transgene expression is attributed to epigenetic position effects, i.e. the position within the genome into which the foreign DNA integrates. It is credited with the ability of transgenes to express (Matzke and Matzke, 1998). In contrast to prokaryotes and lower eukaryotes such as yeast, integration of transgenic DNA by homologous recombination is infrequent in higher eukaryotic organisms where DNA integrates mainly via non-homologous end joining or illegitimate recombination (Hohn and Puchta, 2003). Consequently, the integration of foreign DNA in the plant genome can take place at virtually any site i.e. regions with higher or lower transcriptional activity, those surrounding endogenous regulatory sequences, such as transcriptional enhancers and inhibitors (Francis and Spiker, 2005).

The transgene expression is also affected by factors other than copy numbers, RNA silencing and transgene integration site. A major cause of variation in transgene expression is somaclonal variation. This variation is generally defined as genetic or phenotypic variation in clonally propagated plants of a single donor clone (Kaeppler *et al.,* 2000). The specific regulatory sequences, like promoters and terminators may also affect transgene expression but this mechanism is poorly studied (De Bolle *et al.,* 2003).

In summary, different factors including copy numbers, RNA silencing, position effects, somaclonal variation and regulatory sequences represent potential hurdles in desirable and predictable transgene expression levels in plants. It would be naive to assume that transgene expression is influenced by only one of these factors. On the contrary, all these and possibly several yet unknown factors may interact in ways that have already been elucidated or ones that are yet to be discovered.

2.5 Transgene inheritance

The understanding of the stability and inheritance of the newly introduced transgenes is of great importance in determining the value and application of genetically engineered organisms in agriculture. Characterization of the transgene locus/ loci, transmission of the transgene and segregation analysis of the transgene-encoded phenotype in the subsequent progenies allow insight into the nature of 'transgene inheritance'. Integration of transgenes at a single Mendelian locus, regardless of copy number, is typically observed in transformants produced both by direct DNA delivery (Register *et al.,* 1994) and

by *Agrobacterium*-mediated transformation (Deroles and Gardner, 1988). Multiple complete and partial transgene copies inherited as digenic or multigenic Mendelian traits have also been studied (Cluster *et al.,* 1996). The non-Mendelian segregation occurs at a frequency between 10% and 50% of lines either due to unstable transmission of the transgene or poor expression (McCabe *et al.,* 1999; Limanton-Grevet and Jullien, 2001).

Transgene transmission as well as its expression is a main prerequisite for the production of new cultivars in sexually propagated plants. *Agrobacterium*-mediated transformation usually produces transgenic plants with a low copy number of transgenes that are transmitted to the progeny according to Mendelian (Budar *et al.,* 1986) and in some cases non-Mendelian inheritance (Deroles and Gardner, 1988). The characteristic features of the transgene integration pattern resulting from DNA delivery through particle bombardment often include integration of the full-length transgene as well as rearranged copies of the introduced DNA. Copy numbers of both the transgene and rearranged fragments are often highly variable. Multiple transgene copies most frequently are inherited as a single locus. A variable proportion of the transgenic events exhibit a Mendelian ratio *vs* events exhibiting segregation distortion (Pawlowski and Somers, 1996).

In some cases, the transgenic locus is not stably inherited and deletion of a transgene locus as well as rearrangement of inserted T-DNA with either retention or loss of expression have been reported (Srivastava *et al.,* 1996). Duplication or amplification of transgenes (Cannell *et al.,* 1999) as well as

epistatic interaction between different loci and/or allelic interaction within a single locus also exist (Matzke and Matzke, 1995; Nap *et al.,* 1997). Furthermore, mitotic/meiotic recombinations have also been observed at transgenic loci in various plant species (Tovar and Lichtenstein, 1992). Thus, analysis of transgene inheritance pattern is particularly important for complete utilization of a target crop. Transgene inheritance studies are also important for complementary biosafety measures.

Thus, it is not surprising that such studies have and will continue to constitute a major objective in all important food crops like rice, wheat, maize etc., cash crops like tea, coffee, rubber, bamboo and other industrially important crops.

2.6 Biochemical constituents of tea

The constituents of green tea leaf include carbohydrates, proteins, polyphenols, caffeine, theanine, vitamins and minerals. Polyphenols and caffeine are more important than the other constituents because of their role in the development of liquor characteristics and therapeutic attributes of tea.

2.6.1 Tea catechins

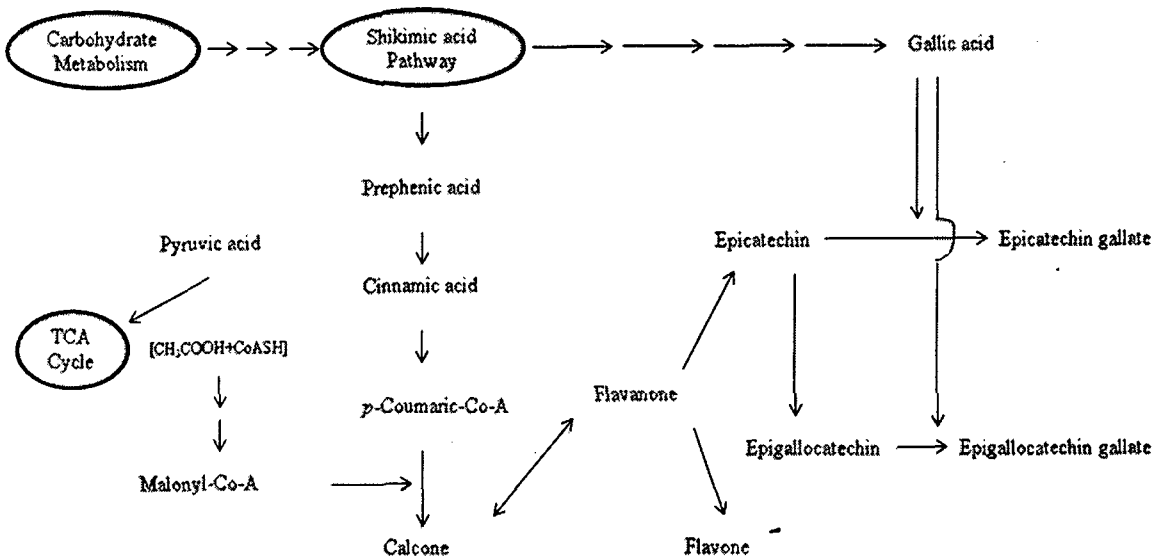
In case of tea, catechins – a group of primary phenols constitute an important class of secondary metabolites that help in the defense mechanism of tea plants against pathogens and diseases. These polyphenolic compounds in tea are different from other polyphenols of plant origin as most of them are found only in the leaves of tea plants (Chu and Juneja, 1997). The catechins have been

widely and intensively investigated for their bioactivity and utilization. Four major catechins namely epigallocatechin gallate, epigallocatechin, epicatechin gallate, and epicatechin constitute around 90% of the total catechin fraction while catechin and gallocatechin constitute about 6% of the fraction. There are also some minor catechins that constitute less than 2% of the total catechins.

The catechins are the substances responsible for the astringency and bitterness of green tea. These are also the precursors of theaflavins in black tea. During processing of fresh leaves and manufacture of orthodox or black tea, the catechins are transformed into dimeric theaflavins and polymeric thearubigins responsible for tea liquor characteristics. However, inactivation of polyphenol oxidase enzyme in green tea leaves prevents the catechins from undergoing the transformation. Catechins and their derivatives have been reported to have numerous therapeutic properties against cancer (Michels *et al.,* 2005), cholesterol (Maron *et al.,* 2003; Vinson *et al.,* 2004), diabetes (Vinson and Zhang, 2005), cerebral damage (Suzuki *et al.,* 2004) and pathogenic bacteria (Yoda *et al.,* 2004; Friedman *et al.,* 2005).

2.6.1.1 Catechin biosynthesis

Catechin is synthesized in tea leaves through malonic acid and shikimic acid metabolic pathways. Gallic acid is derived from an intermediary product produced in the shikimic acid metabolic pathway **(Fig. 2.1)**.



.Fig. 2.1 Biosynthetic pathway of catechins

2.6.2 Caffeine

Caffeine is a naturally occurring compound that belongs to a family of chemical substances called "xanthine alkaloids". It is a trimethyl derivative of purine 2, 6- diol and is synthesized mainly in leaves of the tea plant.

Tea contains 2-5% caffeine in fresh flush. The caffeine contained in tea flush is higher in spring and gradually decreases with the growth of leaves. The caffeine contents in 1st and 2nd leaf (3.4% in dry weight) are higher than in the mature leaf (around 1.5% in dry weight).

Caffeine is produced by a variety of plants, including coffee, tea, kola nuts, guarana berries, yerba mate and cacao beans. While caffeine is toxic for the majority of living organisms, mankind is relatively insensitive, perhaps because of high degrading ability in human systems. Rather, caffeine is used as a source of dietary stimulation. One of caffeine's pharmacological properties is to stimulate the central nervous system, often causing high respiration, high heart rate and

diuretic effects that are not normally serious. However, over-consumption can sometimes cause health problems, including insomnia, palpitation and increase in blood pressure.

Caffeine is also effective as a repellant and pesticide for slugs, snails and insects. For example, spraying of tomato leaves with over 1% caffeine solution drastically deters feeding by tobacco hornworms (Nathanson, 1984). Caffeine also reduces the reproductive potential of several species of moths (Mathavan *et al.,* 1985). While a recent study showed that slugs fed significantly less on 'Napa' cabbage leaves sprayed with only 0.01% caffeine solution, a topical treatment with over 0.1% caffeine solution was lethal to snails (Hollingsworth *et al.,* 2002). These results are consistent with the idea that the physiological function of caffeine is to constitute a chemical defense system against pathogen attack and herbivores (Ashihara and Crozier, 1999).

2.6.2.1 Caffeine biosynthesis

The major caffeine biosynthetic pathway is a four step process consisting of three methylation and one nucleosidase reactions **(Fig. 2.2)**. The xanthine skeleton of caffeine is derived from purine nucleotides.

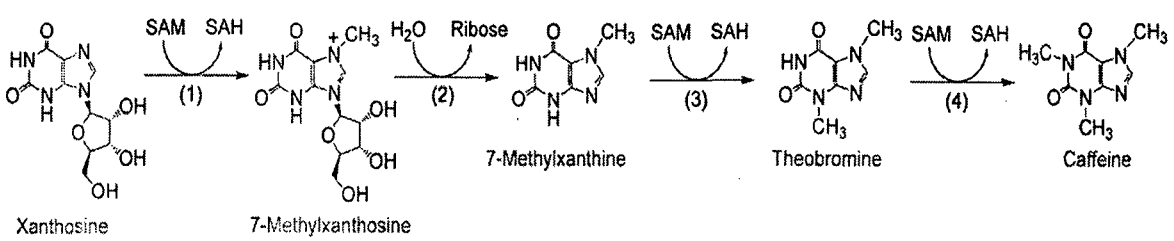


Fig. 2.2 Biosynthetic pathway of caffeine: (1) 7-methylxanthosine synthase (xanthosine N-methyltransferase); (2) N-methylnucleosidase; (3) theobromine synthase (monomethylxanthine N-methyltransferase); (4) caffeine synthase (dimethylxanthine N-methyltransferase); SAM: S-adenosyl-L-methionine; SAH: S-adenosyl-L-homocysteine.

The first step in the biosynthetic pathway of caffeine from xanthosine is the conversion of xanthosine to 7-methylxanthosine. This reaction is catalysed by 7-methylxanthosine synthase (xanthosine 7N-methyltransferase). The genes encoding 7-methylxanthosine synthase, CmXRS1 and CaXMT1 were isolated from *Coffea arabica* (Mizuno *et al.,* 2003; Uefuji *et al.*, 2003). The recombinant proteins obtained from these genes exhibit 7-methylxanthosine synthase activity *in vitro*.

The second step of caffeine biosynthesis involves a nucleosidase which catalyses the hydrolysis of 7-methylxanthosine. Recent detailed structural studies on coffee 7-methylxanthosine synthase suggest that the methyl transfer and nucleoside cleavage may be coupled and catalysed by a single enzyme (McCarthy and McCarthy, 2007).

The last two steps of caffeine synthesis are also catalysed by SAM-dependent N-methyltransferase(s), but this enzyme is different from the N-methyltransferase that catalyses the first step in the pathway. The enzyme, assigned the name caffeine synthase, catalyses the last two steps of caffeine biosynthesis, i.e. the conversion of 7-methylxanthine to caffeine via theobromine. The gene encoding caffeine synthase was cloned from young tea leaves by Kato *et al.* (2000).

Materials and Methods

Chapter III

MATERIALS AND METHODS

3.1 Plant material

The four years old transgenic tea plants developed earlier by the biolistic method and presently growing under contained polyhouse conditions at Institute of Himalayan Bioresource Technology, Palampur (1300 m elevation; 32° 06' N; 76° 33' E), Himachal Pradesh, India were studied in the present thesis. These were maintained with a distance of 2 ft. between row to row and 3 ft. between plant to plant. The environmental conditions in the contained polyhouse were un-controlled and were directly influenced by the seasonal variations in temperature.

3.1.1 Constructs

The transgenic plants harbouring osmotin and chitinase genes were raised by Saini (2007) from somatic embryos with the constructs depicted in **Fig. 3.1a** and **3.1b.** The construct depicted in **Fig. 3.1c** was used by Sandal *et al.* 2001) to bombard the first leaves of newly emerged shoots and produce transgenic plants with gus gene.

3.2 Analysis of the transgene integration patterns in T_0 plants

The transgene integration patterns of 24 plants of *osmotin,* 18 plants of *gus* and 18 plants of *chitinase* (chi11) along with control I (plants subjected to transformation process without the gene of interest) and negative or control II

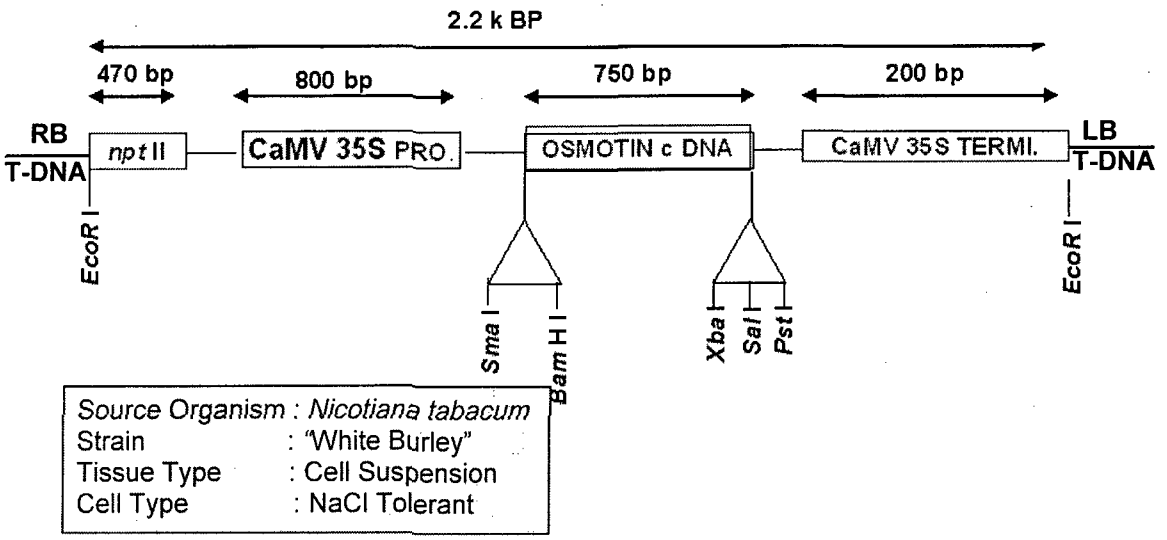


Figure 3.1a: Construct map of the plasmid pBin19: The *osmotin* gene from *Nicotiana tabacum* was inserted into the binary vector pBin19 under the direct control of 35SCaMV promoter and CaMV terminator. The vector also harboured a dominant selectable marker gene that confers high level of resistance to kanamycin.

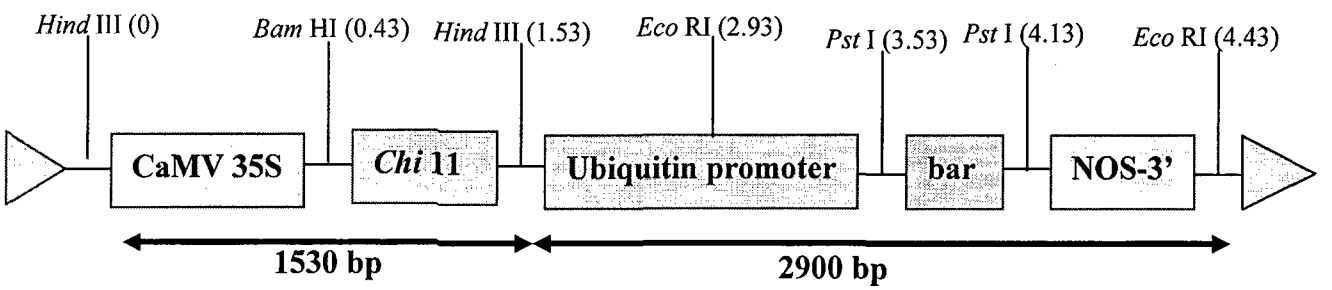


Fig. 3.1b: Construct map of pAHG11: The plasmid pAHG11 of 7.3 kbp contains a rice *chitinase* gene (*chi*11) and the *bar* gene under the control of the CaMV35S and maize ubiquitin promoters respectively. The selectable *bar* gene of *Streptomyces hygroscopicus* encoding phosphinothricin acetyltransferase (PAT) inactivates phosphinothricin (PPT), the active component of bialaphos. The rice *chitinase* gene *chi* II was obtained from a rice genomic clone (Huang *et al.*, 1991) and reconstructed by Lin *et al.* (1995) in the pGL2 vector. Gu (1996) introduced the 1.5 kb *Hind*III fragment containing the *chi*11 gene and the CaMV35S promoter from pGL2 into pAHG20 (Christensen and Quail, 1996) to obtain pAHG11.

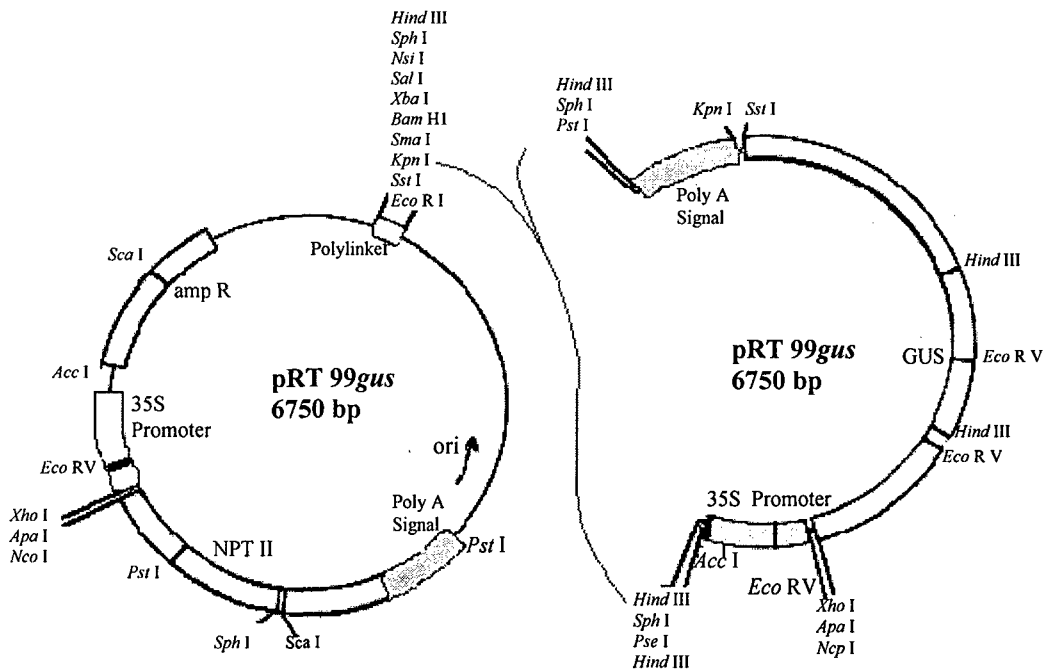


Fig. 3.1c: Construct map of pRT99gus: The plasmid pRT99gus (Topfer *et al.*, 1988) is a 6.71kb pUC18 derivative containing the *neo* gene coding for neomycin phosphotransferase (*npt*II) and *gus*A gene coding for β-glucuronidase (*gus*), both of which are driven by the CaMV35S promoter and flanked by the nos terminator

(un-transformed or wild type) plants were studied using PCR and RT-PCR. For this, genomic DNA and RNA were first isolated from tender fresh leaves randomly collected from 15 tea bushes. The protocols used were as follows:

3.2.1 Isolation of genomic DNA

The CTAB method (Doyle and Doyle, 1990) was used to isolate the genomic DNA from tender young leaves collected from tea bushes. Care was taken to wrap the leaves in an aluminum foil immediately after plucking and these were then immersed into liquid nitrogen.

1. About 0.1 g frozen leaf tissue was ground to a fine powder with the help of a pestle and mortar.
2. The powder was then transferred to an eppendorf tube containing 700µl of DNA extraction buffer **(Appendix 1)** that was pre-warmed at 65°C in a water bath.
3. The mix was vortexed gently and incubated again for 1 hr at 65°C with occasional mixing.
4. Equal volume of chloroform: iso-amyl alcohol (24:1) was added to the above, mixed gently for 2 min and centrifuged at 10,000 rpm for 10 min at 4°C.
5. The upper layer (aqueous phase) was transferred to a fresh eppendorf tube.
6. The step 4 was repeated 2-3 times until the upper aqueous phase became clear and transparent.

7. Pre-chilled 0.6 volume of isopropanol was added to the aqueous phase collected as above, mixed gently for 2 min and centrifuged at 10,000 rpm for 10 min.
8. While the supernatant was discarded, the pellet was washed with 500 µl chilled 70% ethanol for the precipitation of DNA.
9. The ethanol was drained off and the pellet was air dried at room temperature for 1 hr by keeping the eppendorf upturned on tissue paper.
10. Finally, DNA was dissolved in 80 µl TE buffer **(Appendix 2)**.
11. To this, 1 µl of RNase A was added, mixed well and incubated overnight at 37°C.
12. The DNA was checked on 0.8% agarose (GENE I) gel and stored at -20°C after quantification.

3.2.1.1 Agarose gel electorphoresis

Agarose gel (0.8%) was prepared **(Appendix 3)**, samples were loaded on the gel **(Appendix 5)** and subjected to electrophoresis using a gel elctrophoresis unit of BioRad, USA (Power Pac 300). The electrophoresed gel was stained with ethidium bromide (0.5 µg/ml) and visualized using UV trans-illuminator and photographed using AlphaDigidoc™ (Alpha Innotech Corporation, USA).

3.2.1.2 DNA quantification

Nano Drop spectrophotometer (ND 1000) was used for the quantification of the isolated DNA. From each sample of the isolated DNA, 2 µl was used and the readings were recorded.

3.2.2 Isolation of total RNA

Total RNA was isolated from 0.1 g of frozen leaf tissue using the *iRIS*™ method of Ghawana *et al.* (2004) as follows:

1. Firstly, contaminating RNases in all the glasswares, plasticwares and equipments to be used were inactivated **(Appendix 7)**.
2. Then the frozen leaf tissue (0.1 g) was ground to a fine powder with the help of a pestle and mortar.
3. A homogenous mixture of the frozen powder was made using 2 ml of *iRIS*™ solution I with intermittent grinding for complete thawing of the mixture.
4. After adding 800 µl of *iRIS*™ solution II, the mixture was further ground for a while and the homogenate was transferred to eppendorf tubes and incubated for 10 min at room temperature.
5. 200 µl of chloroform was added to each tube and vortexed briefly.
6. These were then incubated for 10 min at room temperature and centrifuged at 13,000 rpm for 10 min at 4°C.
7. The upper aqueous phase was transferred to fresh tubes and 0.6 volume of isopropanol was added, vortexed briefly and incubated again for 10 min at room temperature.
8. These were centrifuged again at 13,000 rpm for 10 min at 4°C and the supernatant was discarded.
9. The pellets that were obtained were washed with 70% ethanol (in DEPC water) and air dried at room temperature by overturning the tubes on tissue paper. Care was taken to avoid over drying.

10. RNA pellets thus, obtained were finally dissolved in 20-30 µl of DEPC autoclaved water and stored at -80°C.

3.2.2.1 Concentrating the RNA

RNA was precipitated as per requirements by adding 4 volumes of chilled ethanol in the presence of 0.1 volume of 3 M sodium acetate (pH 4.8) followed by incubation at -70°C for 3 hr. The RNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C, rinsed with 70% chilled ethanol and finally dissolved in RNases free water. The RNA was quantified by using Nano Drop spectrophotometer (ND 1000).

3.2.2.2 Denaturing agarose gel electrophoresis of RNA

The integrity of the RNA was checked on a 1% formaldehyde-agarose gel by monitoring distinct 28S and 18S rRNA bands **(Appendix 8)**.

3.2.3 Re-confirmation of transgene integration through PCR amplification

The protocols optimized by Sandal *et al.* (2001) and Saini (2007) were employed for the PCR amplification of the transgenes. Five DNA samples (40 ng) from each of the controls **(Section 3.2)** as well as transgenic lines i.e. *osmotin, gus* and *chitinase* were amplified with gene specific primers described below. These gene specific primers were obtained from Sigma-Genosys.

osmotin gene:

Forward: '5-CTGCCACTATCGAGGTCCGA-3'

Reverse: '5-CCACTTCATCACTTCCAGGC-3'

gus gene:

Forward: '5-GGTGGGAAAGCGCGTTACAAG-3'

Reverse: '5-TGGATCCCGGCATAGTTAAA-3'

chi11 gene:

- Forward: '5-TAAGGGCTTCTACACCTACGA-3'

  Reverse: '5-CGTCTGCTCGGATCAAATATCAAC-3'

25 µl reaction mixture(s) were prepared in 0.2 ml PCR tubes as described in **Appendix 9**, placed in a thermocycler (BioRad, USA) and then amplified using the different cycles given below:

Cycles	*osmotin*	*gus*	*chi*11
Initial cycle	94°C for 4 min	94°C for 4 min	94°C for 4 min
Denaturation	94°C for 30 sec	94°C for 30 sec	94°C for 30 sec
Annealing	57°C for 30 sec	55°C for 30 sec	54°C for 30 sec
Extension	72° C for 50 sec	72° C for 40 sec	72° C for 40 sec
Total Cycles	**35**	**35**	**35**
Additional, before its rapid cooling to room temperature	72°C for 7 min	72° C for 7 min	72° C for 7 min

The PCR products thus, obtained were separated on a 1% agarose gel, viewed under a UV trans-illuminator and captured on AlphaDigidoc™ (Alpha Innotech Corporation, USA).

3.2.4 Confirmation of transgene expression or silencing by RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) is a process by which RNA serves as the template for the synthesis of cDNA. This conversion of RNA into cDNA is catalyzed by the reverse transcriptase enzyme which is nothing but a RNA dependent DNA polymerase. The SuperScript III Reverse Transcriptase kit of Invitrogen was used for this work. 26S *rRNA* gene specific primers were used for RT-PCR as internal control for the transgene expression studies (Singh *et al.*, 2004).

3.2.4.1 First strand cDNA synthesis

The following components were taken in a nuclease free eppendorf tube of 0.2 ml volume.

Components	Concentration	Amount taken
oligo(dT)$_{12-18}$	250 ng	0.5 µl
Total RNA	1 µg	Varied as per concentration of RNA
dNTP mix (pH 7.0)	10 mM	1 µl

1. The volume of the above mixture was made up to 14 µl with autoclaved DEPC water.
2. The mixture was heated to 65°C for 5 min and then incubated on ice for 1 min.
3. The mixture was centrifuged briefly to settle the contents in the eppendorf tubes.
4. To this, 4 µl of 5X 'First strand buffer', 1 µl of 0.1 M DTT and 1 µl of SuperScript™ III RT (200 units/µl) were added and mixed thoroughly by pipetting.
5. This was then incubated at 25°C for 5 min followed by incubation at 50°C for 1 hr. The reaction was inactivated by heating at 70°C for 15 min.

3.2.4.2 PCR amplification of the cDNA

The cDNA was then used for the amplification of the respective transgenes i.e. *osmotin, gus* and *chi*11 with their respective gene specific primers. However, a total of 25 cycles were used instead of 35 cycles.

3.3 Assessment of transgene expression patterns in T_0 plants

3.3.1 Phenotypic analysis

Growth in the transgenic lines for *osmotin*, *gus* and *chi*11 growing under contained polyhouse conditions was compared with that of the controls as mentioned in **Section 3.2**. Observations were recorded on the parameters like shoot height, internode length, and size, shape, colour and texture of leaves.

3.3.2 Histology of shoots of transgenic lines

Stems from five different plants belonging to the different transgenic lines and controls were excised. Then, 2.5 cm pieces of the stem explants comprising of nodal region were fixed in FAA (Formaldehyde: acetic acid: 50% ethanol, 1:1:18) for five days, dehydrated in a tertiary-butyl alcohol series followed by infiltration in paraffin wax and finally embedded in paraffin wax block. Sections (15 µm thick) were cut with the help of microtome (Shandon Finesse ME). Gellatin jelly was used as an adhesive and the sections were stretched on the slides and placed on a hot plate at 50°C. The sections were subjected to de-waxing in xylene followed by staining with Safranine-Fast green. The slides were finally mounted in DPX (Distrene, 8-10 g, 5 ml dibutylpthalate and 35 ml xylene). The photographs were taken under a microscope (Nikon Biophot No. 78508, Japan) at 20X magnification using a digital camera (Nikon DXM 1200).

3.3.3 Analysis of parameters governing reproduction

The reproductive calendar of the different transgenic lines of *osmotin*, *gus* and *chi*11 was compared with that of the controls **(Section 3.2)**. Observations were recorded on initiation of flower buds, fully opened flowers,

fruit and seed set, and seed maturity at regular intervals of 20 days. Observations were also recorded on the rate of flower, fruit and seed abscission and also fruit dehiscence.

3.3.4 Assessment of quality in terms of catechins and caffeine

Fresh leaves i.e. 'two leaves and a bud' were collected from controls as well as transgenic lines of *osmotin, gus* and *chi*11**(Section 3.1)**. The collected leaves were dried immediately to a constant weight in a domestic oven (BPL-Sanyo-Micro-Convection model BMC 900 T) at power level 7 and kept in desiccators till further use. Total catechins and caffeine content was then estimated in the dried leaves as described below.

3.3.4.1 Estimation of total catechins and caffeine

Dried leaves from each of the control I and transgenic lines were ground to a fine powder with the help of a pestle and mortar. From each of these, 0.1 g of powdered sample was taken in an oakridge tube containing 2 ml of 70% methanol and vortexed for 1 min. The sample was then centrifuged at 4000 rpm for 10 min at 15°C. The supernatant was collected by filtering through a wad of cotton wool, whereas, 1.5 ml of 70% methanol was again added to the residue. The pellet with the methanol was vortexed for 1 min and centrifuged as above. This was repeated again. Every time, the supernatant was filtered using cotton wool and pooled together to form a total of 5 ml of supernatant. This was finally filtered through a filter of 0.45 µm. Different dilutions were made and total catechins and caffeine were estimated using HPLC (Merck-Hitachi Lichrom).

3.3.4.2 Expression of genes involved in caffeine and catechin biosynthetic pathways in T_0 plants

Total RNA was isolated and quantified as described in **Section 3.2.2** and **3.2.2.1** from three plants each of the controls and transgenic lines for *osmotin*, *gus* and *chitinase*. Using 1 µg of total RNA from each sample, cDNA was prepared as described in **Section 3.2.4.1**. Equal quantity of cDNA was used for RT-PCR analysis of caffeine biosynthetic pathway gene (caffeine synthase) (Mohanpuria *et al.*, 2007) and catechin biosynthetic pathway genes (lar and f3h). The sequences of the gene specific primers viz. lar and f3h have been submitted to NCBI (accession nos. *lar*-AY641729, *f3h*-AY641730) in a separate work of another group at IHBT. The amplified products of the transgenic lines were compared with that of controls. 26S *rRNA* gene specific primers were used for RT-PCR as internal control for the gene expression studies (Singh *et al.*, 2004).

3.3.5 Viability and germination of seeds collected from T_0 plants

In another study, mature seeds were collected both from plants as well as the ground below. The pericarps of the freshly collected fruits were removed carefully to extract the seeds. The seeds were then soaked in distilled water for 24 hr. The 'sinkers' or the seeds that sank to the bottom were considered viable and used for germination. The seeds were removed by carefully breaking the testa and the de-coated seeds were then washed in sterile water containing Tween-20 for 5-10 min. After rinsing with distilled water for 5-6 times, the seeds were also treated with a solution of antibiotics (0.02% of tetracycline and ampicillin along with 0.04% streptomycin and two drops of Tween 20). This was

shaken gently for 25 min, rinsed with distilled water and surface sterilized for 10 min using 4% (w/v) calcium hypochlorite and two drops of Tween 20 as sterilant. The seeds were finally washed free of the sterilant by 3-5 rinses in autoclaved distilled water and blotted dry on autoclaved filter paper for 5-10 min. The intact de-coated seeds were finally inoculated on agar (0.7%) solidified half strength basal MS (Murashige and Skoog, 1962) medium **(Appendix 10)** containing 3% sucrose. Observations were recorded at regular intervals of 7 days and all contaminated cultures (bacterial or fungal) were discarded.

3.4 Analysis of T1 plants

3.4.1 Generation of T_1 plants from T_0 seeds

After germination, the seedlings were allowed to grow until the leaves unfolded. The plants with fully expanded leaves and healthy roots were transferred to soil in 4 inch pots and hardened by covering them with magenta boxes. After one month, the plants were exposed to environmental conditions and were used for analysis of T_1 plants.

3.4.2 Assessment of transgene integration and expression in T_1 plants

Integration and expression of transgenes (osmotin, gus and chi11) were assessed as described in **Sections 3.2** and **3.3.**

3.4.3 Phenotypic analysis of T_1 plants

Observations were recorded on seedling development. The leaf morphology with respect to shape, size, colour and texture were also recorded.

Results

Chapter IV

RESULTS

4.1 PCR analysis of transgenic T_0 plants

Considerable variations were observed in the obtained amplified products when the different transgenic lines were subjected to PCR analysis **(Table 4.1 & Plate 4.1)**. No amplification was observed in the negative (untransformed) controls, the positive control i.e. plasmid DNA showed the expected bands of 650, 700 and 400 bp for gus, osmotin and chitinase genes, respectively (not shown).

The amplified product of *gus* transgenic lines corresponded exactly to the expected sharp band of 650 bp. However, of the total lines tested, only 17% showed a single sharp band of 650 bp. The remaining 83% lines showed extra two to four bands ranging between 400 to 1000 bp besides the desired sharp band of 650 bp **(Plate 4.1a)**.

In the osmotin transgenic lines, a 700 bp fragment was obtained as expected in 83% of the total lines. In addition to this, two bands of 150 and 300 bp were also obtained in 50% of the tested lines. Besides 150, 300, 700 bp bands, a very faint band of 900 bp was also seen in 33% of the tested lines. Out of the total lines, 17% failed to show any amplification **(Plate 4.1b)**.

In case of chitinase, only 17% lines yielded a single expected band of 400 bp. While 33% of the lines showed an additional 650 bp fragment, all the remaining lines failed to show any amplification **(Plate 4.1c)**.

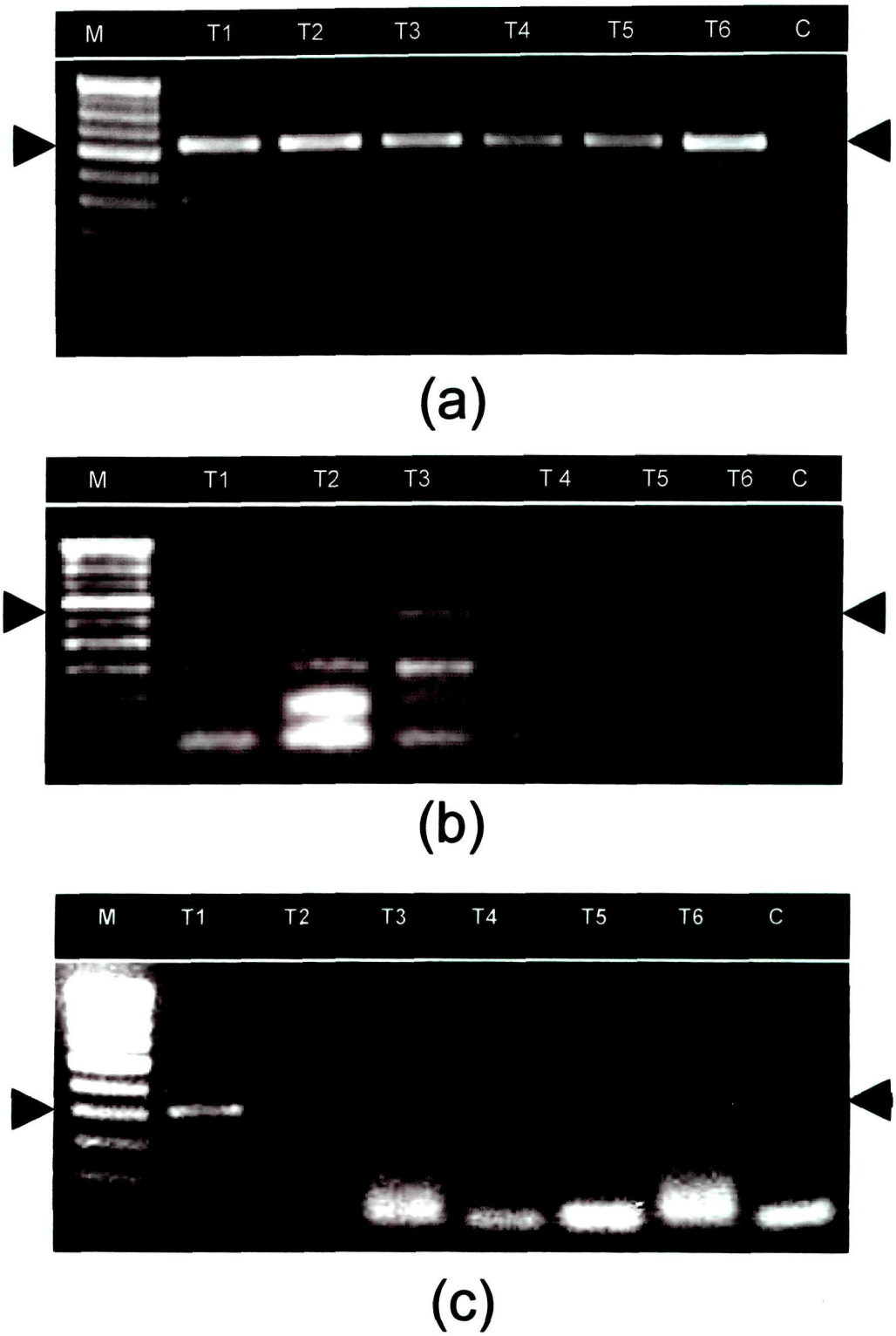


Plate 4.1: PCR of T_0 transgenic lines. M – 100 bp marker; C – negative control; T1, T2, T3, T4, T5 & T6 – transgenic lines of (a) *gus* (b) *osmotin* (c) *chi*11. Arrow shows the desired size of fragments

Table 4.1: PCR analysis of transgenic lines growing under contained polyhouse conditions

Transgenic lines for	Expected size (bp)	% Transgenic lines		
		With only the expected amplification product	With additional bands besides the expected amplification product	No amplification product
gus	650	17 (650 bp)	83 (400,500,800 & 1000 bp)	-
osmotin	700	83* (700 bp)	50(150 & 300 bp) + 33 (150, 300 & 900 bp)	17
chitinase	400	17 (400 bp)	33 (650 bp)	50

* Both expected and additional bands

4.2 Confirmation of transgene expression or silencing by RT-PCR

When the transgenic lines for *gus*, *osmotin* and *chi*11 were analyzed through RT-PCR for the confirmation of transgene expression, the 26S *rRNA* was expressed as desired. However, different results were obtained in the transgenic lines **(Plate 4.2)**. While most of the *gus* and *chi*11 lines failed to show any expression, only one line of each (gus and chi11) showed the desired expression **(Plate 4.2a & c)**. On the other hand, expression of variable intensities was observed in the *osmotin* lines **(Plate 4.2b)**.

4.3 Assessment of transgene expression patterns in T_0 plants

4.3.1 Phenotypic analysis

Distinct phenotypic differences were observed between the transgenic (osmotin, gus, chi11) and control plants **(Table 4.2)**. Among the different lines, the height of the control plants was maximum followed by that of *chi*11 transgenic lines. On the other hand, the height of the *osmotin* lines was the lowest.

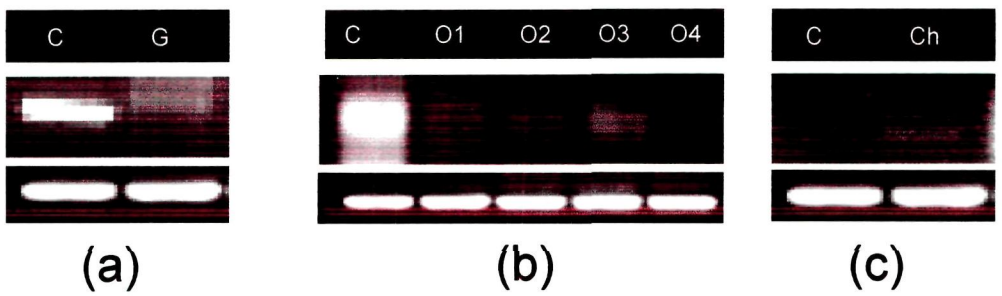


Plate 4.2 : RT-PCR of T_0 transgenic lines (a) *gus* (b) *osmotin* and (c) *chi*11, with 26S *rRNA* as internal control in the lower panel. C – control; G – *gus* line; O1, O2, O3 & O4 – *osmotin* lines; Ch – *chi*11 line

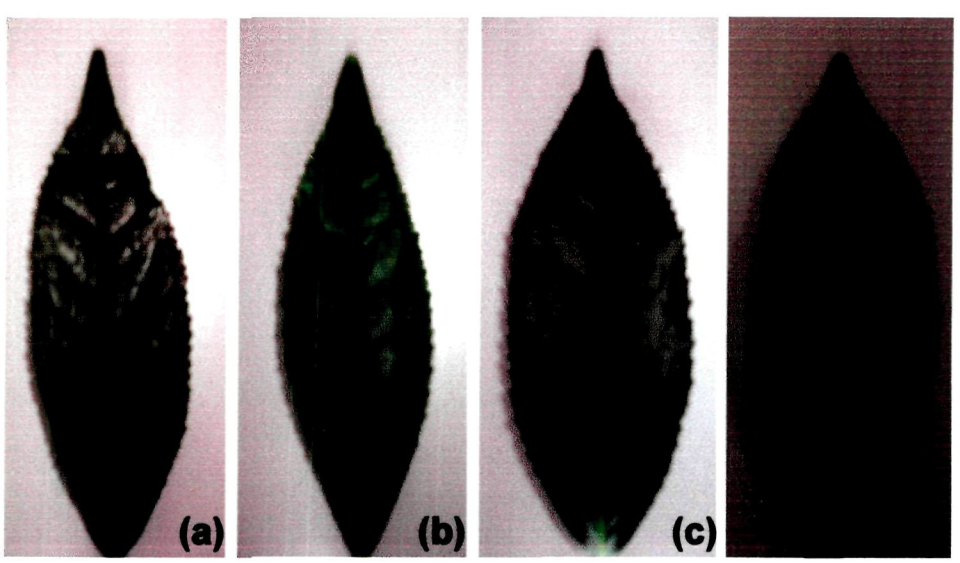


Plate 4.3: Leaves of T_0 plants (a) control (b) *osmotin* (c) *gus* (d) *chi*11

While the inter-node length of the *osmotin* and *chi*11 lines was almost at par (3.7 cm), that of the control and *gus* transgenics was 4.3 and 4.9 cm, respectively.

Although the shape and margin of the leaves remained unchanged in the transgenic lines and controls, other characteristics like leaf size, colour and texture were found to differ between the transgenic lines **(Plate 4.3)**. While the leaves of the *osmotin* lines were the longest followed by that of *chi*11, the *gus* leaves were the smallest. The *osmotin* leaves were tender and lush green in colour **(Plate 4.3b)**, whereas those of *chitinase* were blackish green with papery, thick texture **(Plate 4.3d)**.

Table 4.2: Phenotypic characteristics of the transgenic lines growing under contained polyhouse conditions

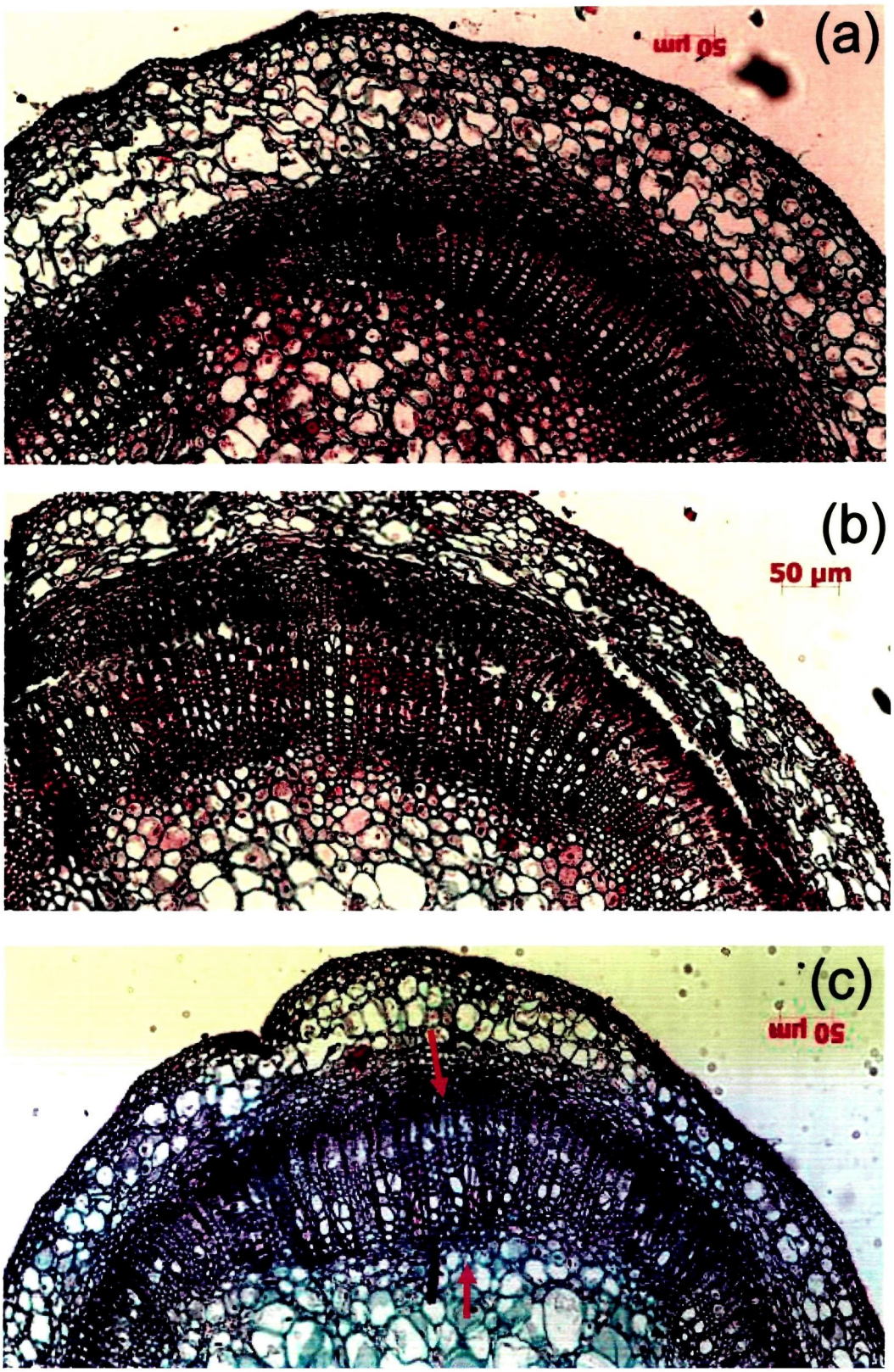
Phenotypic traits	Transgene			
	Control	***osmotin***	***gus***	***chi* 11**
Shoot height (cm)	160	104	116	121
Number of branches	7	8	9	10
Inter-node length (cm)	4.3	3.7	4.9	3.7
Leaf shape	Ovate	Ovate	Ovate	Ovate
Leaf colour	Green	Lush green	Dark green	Blackish green
Leaf size (cm)	8.3 x 3.5	8.8 x 3.2	8.1 x 3.3	8.5 x 2.7
Leaf margin	Serrate	Serrate	Serrate	Serrate
Leaf texture	Tender	Tender	Tender	Papery

4.3.2 Histology of shoots of transgenic lines

The histological studies of the controls and transgenic lines for *osmotin, gus* and *chi*11 revealed distinct differences in the development of vascular elements **(Plate 4.4)**. Incompletely formed xylem and phloem elements were observed in the control I plants subjected to the transformation process without the gene of interest **(Plate 4.4a)**. However, in the wild type or control II plants, normal vascular development, complete with small protoxylem, large metaxylem and phloem cells were observed **(Plate 4.4b)**. Interestingly, the *gus* transgenic lines were completely differentiated with well formed phloem, protoxylem and metaxylem cells **(Plate 4.4e)**. In the *osmotin* lines, however, only partial differentiation of vascular elements was observed. While the protoxylem and metaxylem elements were formed, the phloem cells were not **(Plate 4.4c)**. The *chi*11 lines were totally undifferentiated with two layers of only tiny phloem cells **(Plate 4.4d)**.

4.3.3 Analysis of parameters governing reproduction

The reproductive calendar of the transgenic lines as well as controls growing under contained polyhouse conditions was documented for three years and the overall the reproductive behaviour is depicted in **Plate 4.5**. The reproduction of both the controls and transgenic lines was similarly affected by season and flowering was found to occur throughout the year. Irrespective of the transgenic lines or controls, flower buds were initiated during the first quarter of the year (January-March). However, maximum number of flower buds was formed by the second quarter of the year (April-June). The flowers opened fully


(a)
50 µm
(b)
50 µm
(c)
50 µm

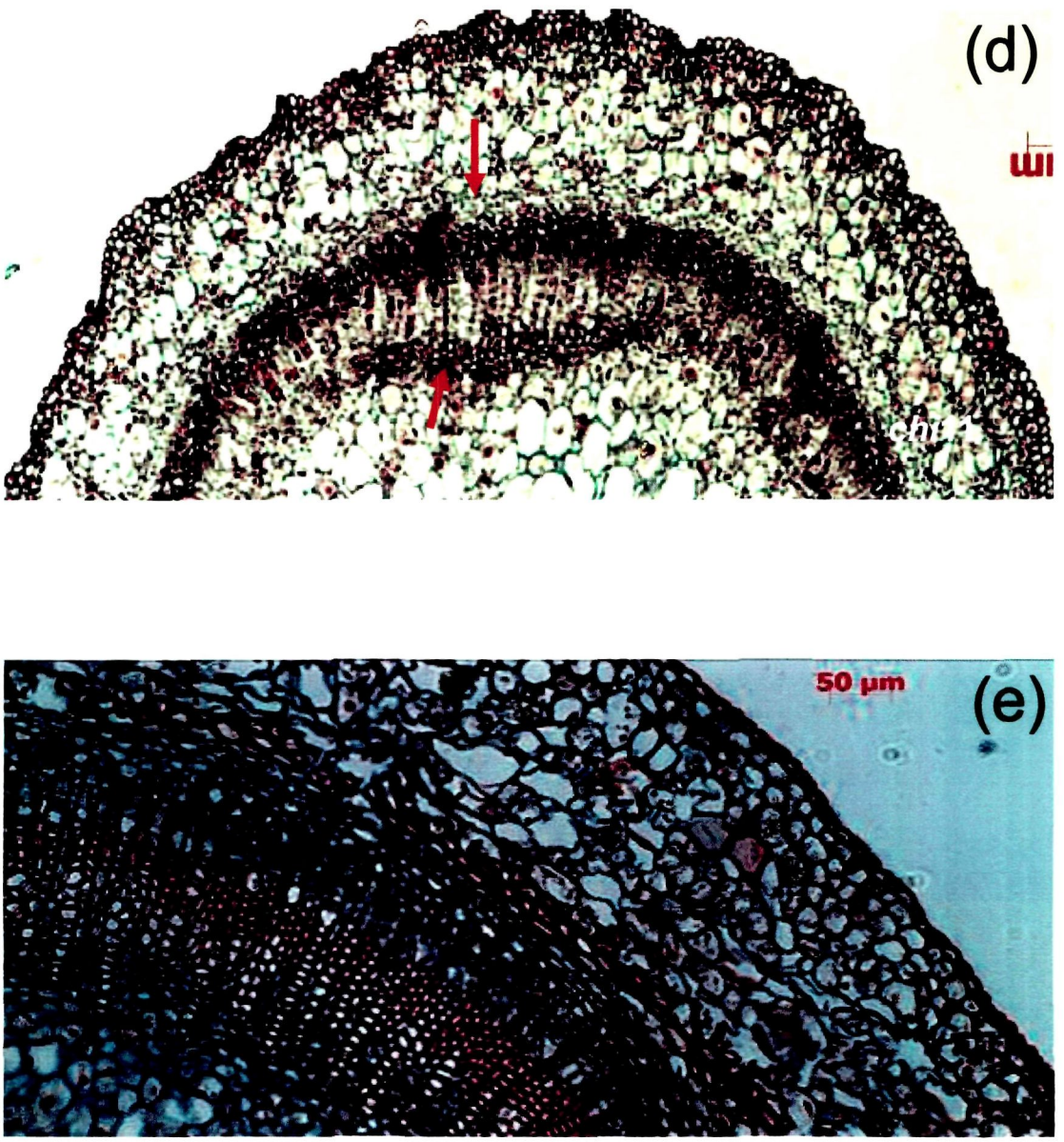


Plate 4.4: Histological studies showing differentiation of vascular elements (a) control I (b) control II (c) *osmotin* (d) *chi*11 (e) *gus*

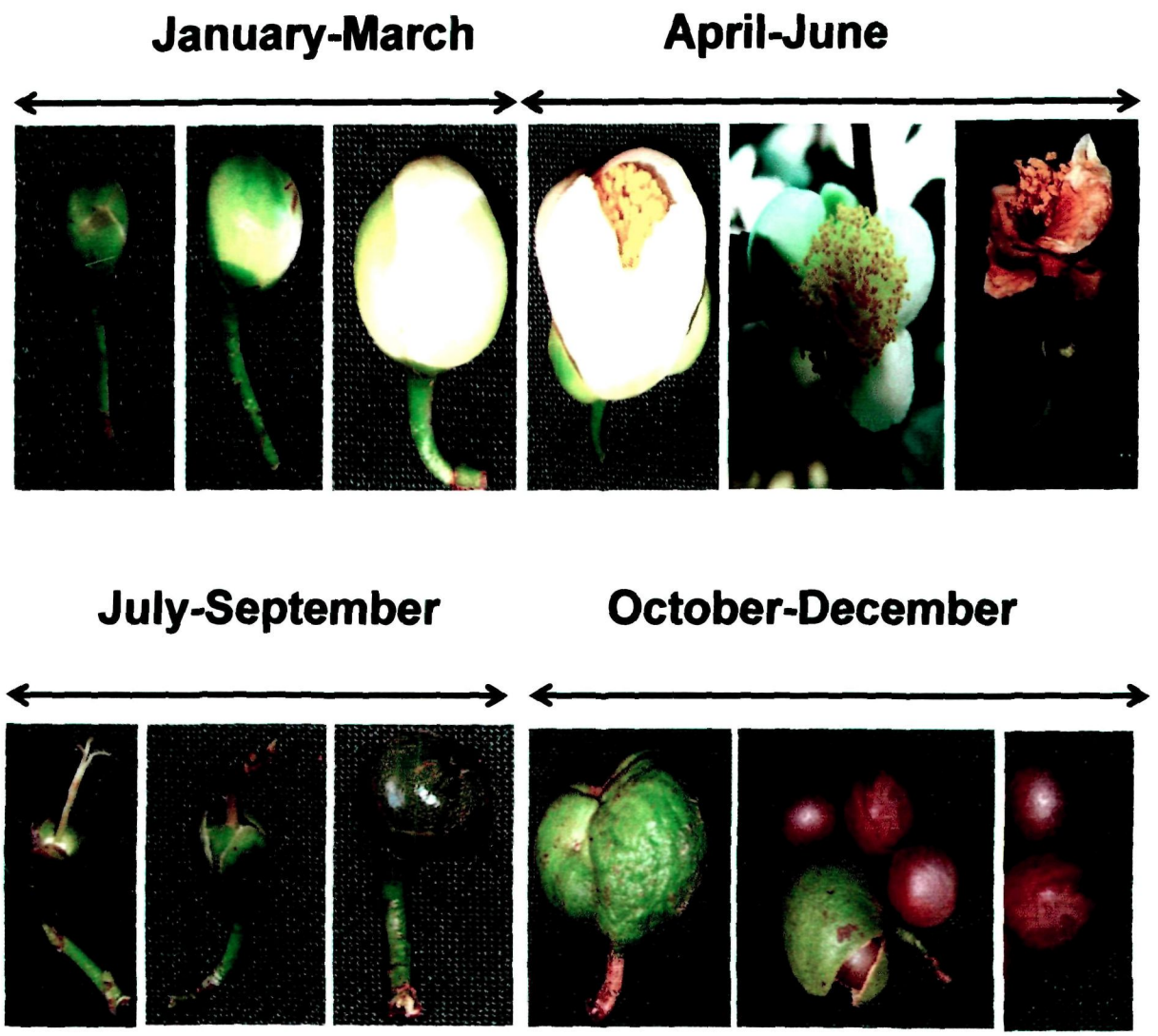


Plate 4.5: Reproductive calendar of transgenic and control plants under contained polyhouse conditions

during the third quarter (July-September) and continued till the last quarter (October-December). Very high rates of flower abscission were observed during the third quarter and it continued up to the last quarter. Thus the flowers that opened during the last quarter were not high enough to compensate for the large proportion of bud abscission that occurred by the third quarter. Despite the high rates of abscission, seed set ranged between 54.36 to 76.49% during the first quarter of the year. By the second quarter however, seed drop was observed and this continued further till the third quarter **(Table 4.3)**.

When the reproductive behaviour of the transgenic lines was compared, maximum flowering followed by highest abscission was recorded in the *osmotin* lines. Abscission of flower buds in the *gus* lines was 48.68%. Although the flowering in *chi*11 lines was high (next only to *osmotin*), the abscission was even lower (36.65%) than control (52.6%).

Percent seed drop also varied between the transgenic lines and control plants **(Fig. 4.1)**. Seed drop during the second quarter (April-June) was highest in *osmotin* lines (93.43%) followed by *chi*11 (87.48%) and *gus* (60.24%). The lowest seed drop was observed in case of control plants (36.31%). However, in the third quarter, further drop was observed in the seeds remaining from the second quarter. In the third quarter (July-September), maximum seed drop was observed in the *gus* lines (47.56%) followed by *chi*11 lines (21.73%). The seed drop in *osmotin* and control plants was 12.80 and 10.96% respectively. Consequently, maximum seeds were available only in the control lines by the last or fourth quarter.

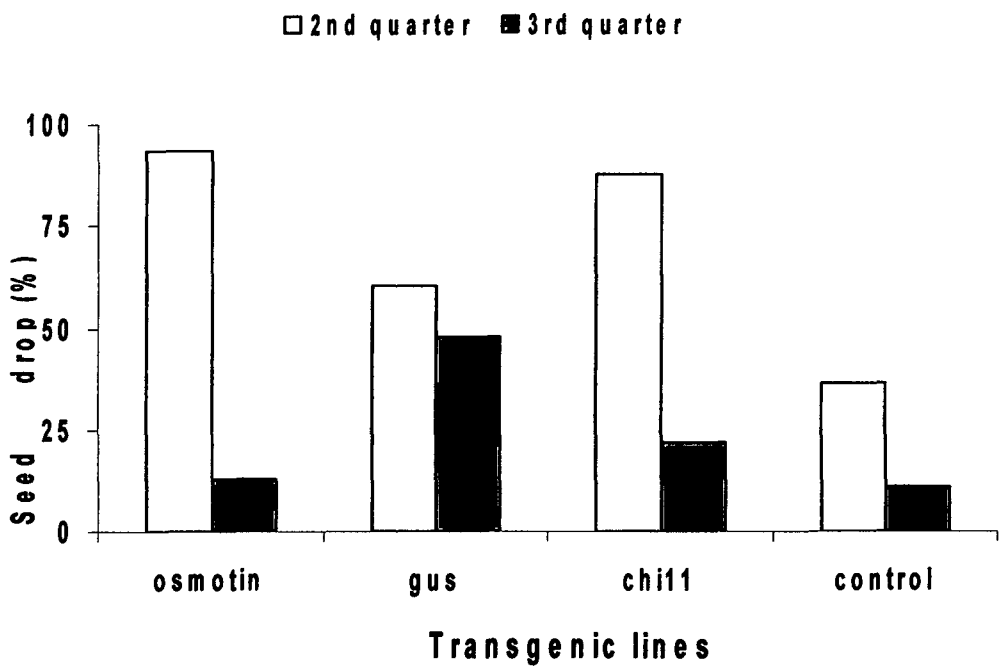


Fig. 4.1: Seed drop (%) in the transgenic and control lines during the 2nd quarter and further drop of the remaining seeds during 3rd quarter

Table 4.3: Reproductive calendar of transgenic and control plants growing under contained polyhouse conditions

Transgenic line	Flowering buds (no.)	Abscission (%)	Seed set (%)	Seed drop (2nd quarter)	Seed drop (3rd quarter)
gus					
Jan-Mar	652	48.68	54.36	60.24	47.56
Apr-June	4040				
July-Sept	1362				
Oct-Dec	2073				
osmotin					
Jan-Mar	766	75.0	63.9	93.43	12.80
Apr-Jun	6397				
Jul-Sep	1095				
Oct-Dec	1596				
chitinase					
Jan-Mar	1306	36.65	76.49	87.48	21.73
Apr-June	5006				
July-Sept	2241				
Oct-Dec	3171				
Control					
Jan-Mar	674	52.6	71.11	36.31	10.96
Apr-Jun	3658				
Jul-Sep	1172				
Oct-Dec	1731				

4.3.4 Assessment of yield and quality in terms of catechins and caffeine

Considerable variations in the percentage of catechins and caffeine were observed between the transgenic lines (i.e. with different transgenes as well as between the transgenes).

4.3.4.1 Catechins

Of the five catechins studied, epigallocatechin gallate (EGCG) was found to be the highest followed by epigallocatechin (EGC), irrespective of transgenic lines (with different transgenes or between the transgenes). The catechin (C) on the other hand, was found to be the lowest **(Fig. 4.2)**.

When the mean percent catechins were compared between the transgenes, highest percent of catechin (C) was found in the *chi*11 and *gus* lines as compared to control, whereas, that of *osmotin* lines was slightly lower than control. The percent epicatechin (EC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG) and epigallocatechin (EGC) were highest in the *gus* lines followed by *chi*11 as compared to control. However, these constituents were lower than control in the *osmotin* lines. Irrespective of the transgenic or control lines, all components of catechins studied were lowest in the *osmotin* lines **(Table 4.4)**.

Table 4.4: Mean catechins (%) in the control and different transgenic lines

Transgenics	Catechin (C)	EC	EGCG	ECG	EGC
Control	0.27	0.52	3.20	0.66	1.73
gus	0.35	0.86	5.06	0.92	3.02
osmotin	0.25	0.23	2.27	0.33	1.09
*chi*11	0.36	0.60	4.44	0.73	2.56

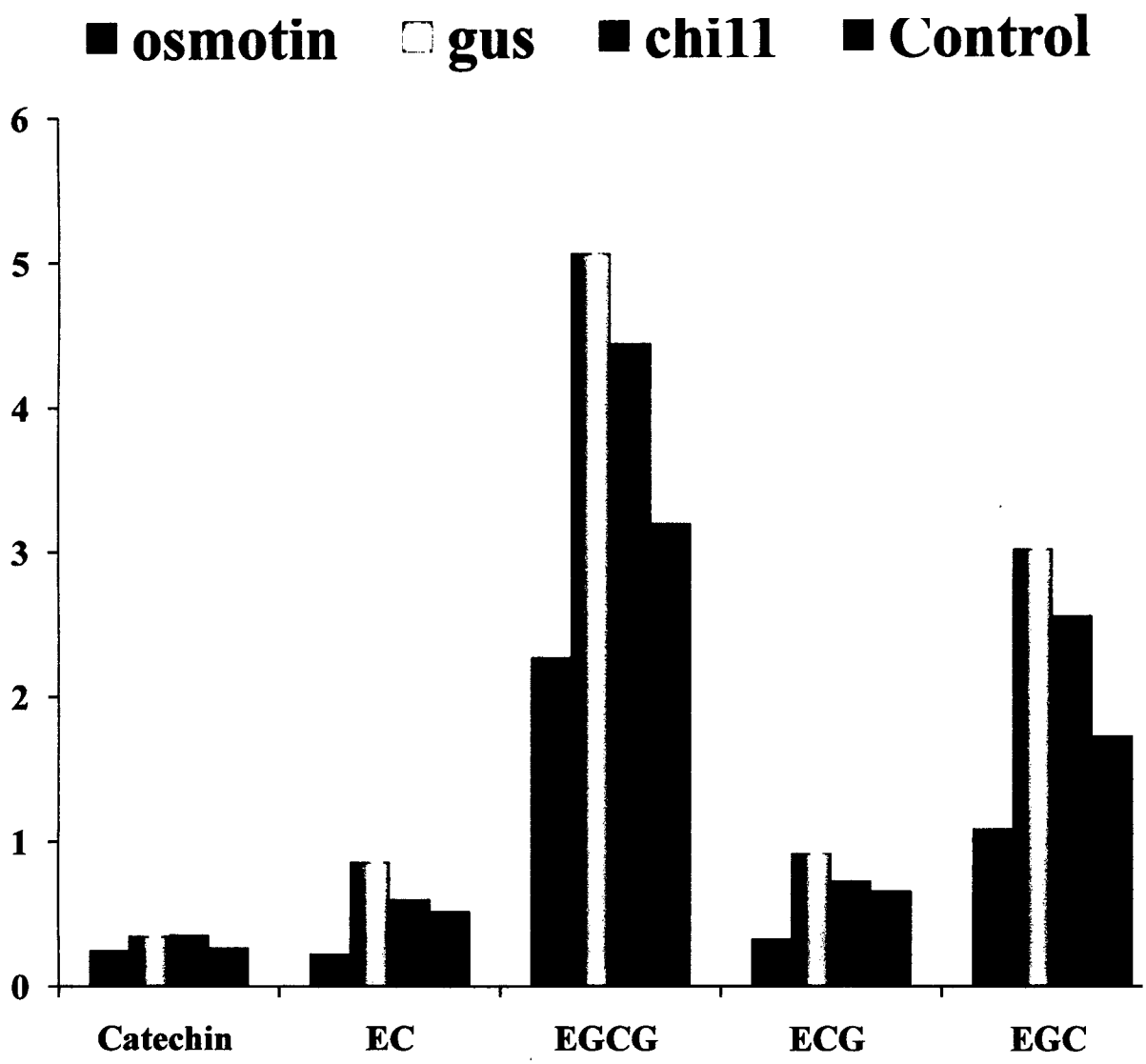


Fig. 4.2 Mean catechins (%) in the control and different transgenic lines

4.3.4.2 Caffeine

The mean percent caffeine was highest in the *gus* lines followed by control, whereas, that of the *osmotin* lines was lowest **(Fig. 4.3; Table 4.5)**.

Table 4.5: Mean caffeine (%) in the control and transgenic lines

Transgenic	Caffeine (%)
Control	2.17
gus	2.77
osmtoin	1.26
*chi*11	1.96

4.3.4.3 Caffeine and catechin biosynthetic pathway gene expression

4.3.4.3.1 Caffeine synthase gene expression in RT-PCR

A 376 bp band was observed after RT-PCR of the control I as well as transgenic T_0 lines with 'caffeine synthase gene' specific primers. However, the intensity of the expression of the endogenous 'caffeine synthase gene' in both the controls as well as transgenic lines varied among the plants of each transgenic line and also between the transgenes **(Plate 4.6)**. Of the transgenic lines with different genes, two out of three tested *chi*11 lines showed strong to very strong signals **(Table 4.6)**. In the *gus* and *osmotin* lines, two out of three tested lines showed faint signal. In control I plants, only one out of three tested lines showed strong signal.

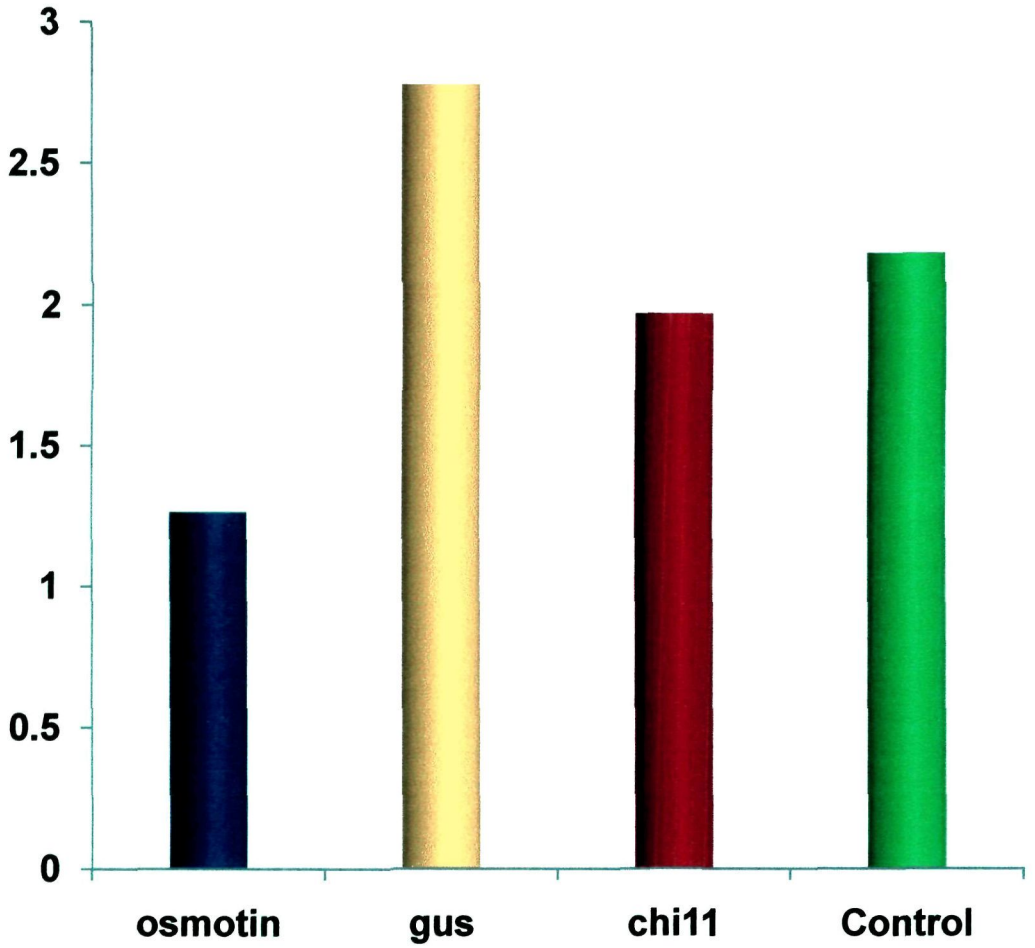


Fig. 4.3 Mean caffeine (%) in the control and transgenic lines

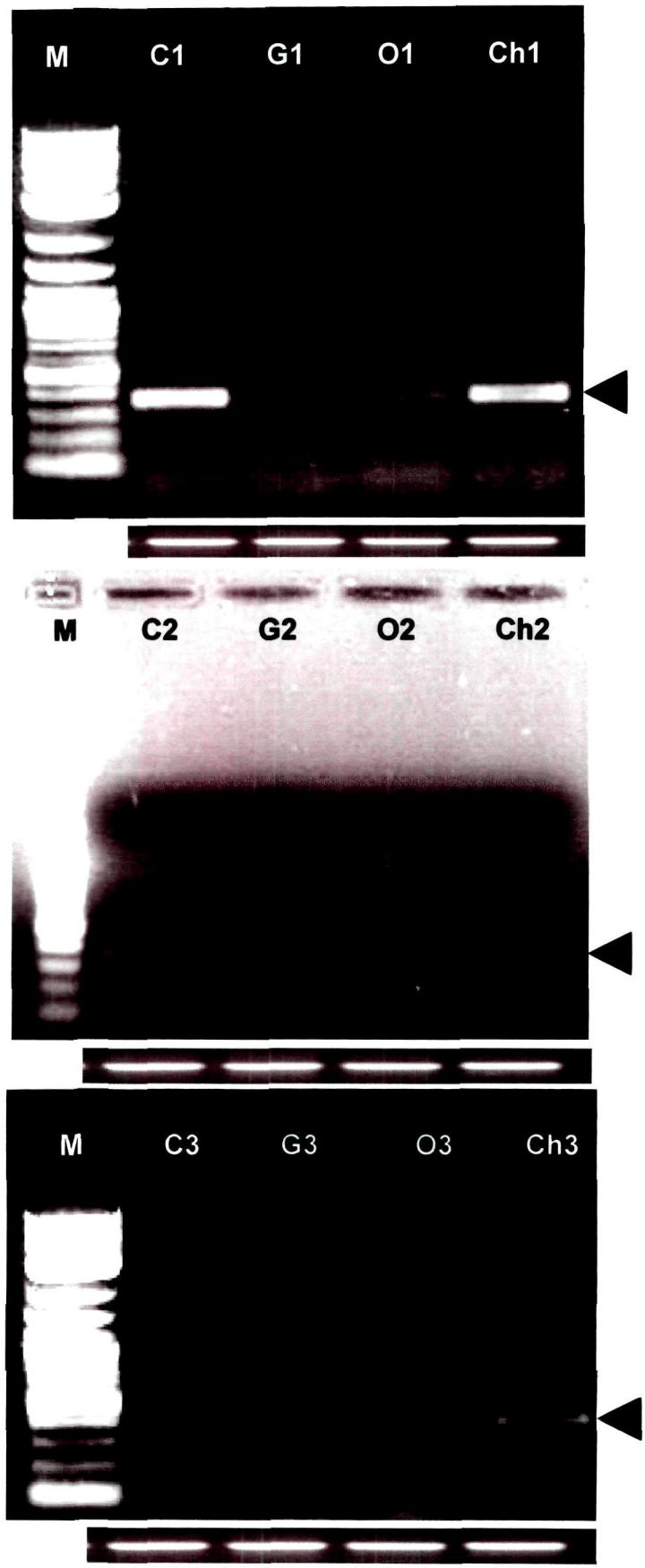


Plate 4.6: RT-PCR expression of endogenous caffeine synthase gene with 26S *rRNA* as internal control in the lower panel. M – 0.1-10 kb ladder; C1, C2 & C3 – Control I lines; O1, O2 & O3 *osmotin* lines; Ch1, Ch2 & Ch3 – *chi*11 lines; G1, G2 & G3 – *gus* lines

Table 4.6: RT-PCR expression of caffeine synthase gene in the control I and transgenic lines

Lines	Signal intensity		
	1	2	3
Control I	++++	++	++
gus	++	+	++
osmotin	++	+	++
*chi*11	++++	++	+++

++++ = very strong; +++ = strong; ++ = faint signal; + = very faint signal

4.3.4.3.2 Leuco-anthocyanidine reductase (lar) and Flavanone 3-hydroxylase (f3h) gene expression in RT-PCR

The intensity of expression of the endogenous lar gene (involved in the catechin biosynthetic pathway) varied within as well as between the transgenic lines **(Plate 4.7)**. No expression was observed in two out of the three *gus* lines and one out of three *chi*11 lines. Even in one of the three control I lines bombarded without the transgene, no expression was observed **(Table 4.7)**. However, the expression was down-regulated in all the three *osmotin* lines as evident from the faint bands observed in **Plate 4.7**. While a very strong band indicated an over-expression in one of the control I lines, a strong band in one of the *chi*11 line indicated appreciably good expression **(Table 4.7)**.

The endogenous f3h gene failed to express in all the three *gus* lines as evident form RT-PCR expression **(Plate 4.8)**. While no expression was observed in one of the three lines of *chi*11 and control I, all the three osmotin lines showed only faint expression in RT-PCR **(Plate 4.8; Table 4.7)**. None of the bands was as strong as one of the controls which showed a band of strong intensity, thereby indicating up-regulation of f3h gene expression.

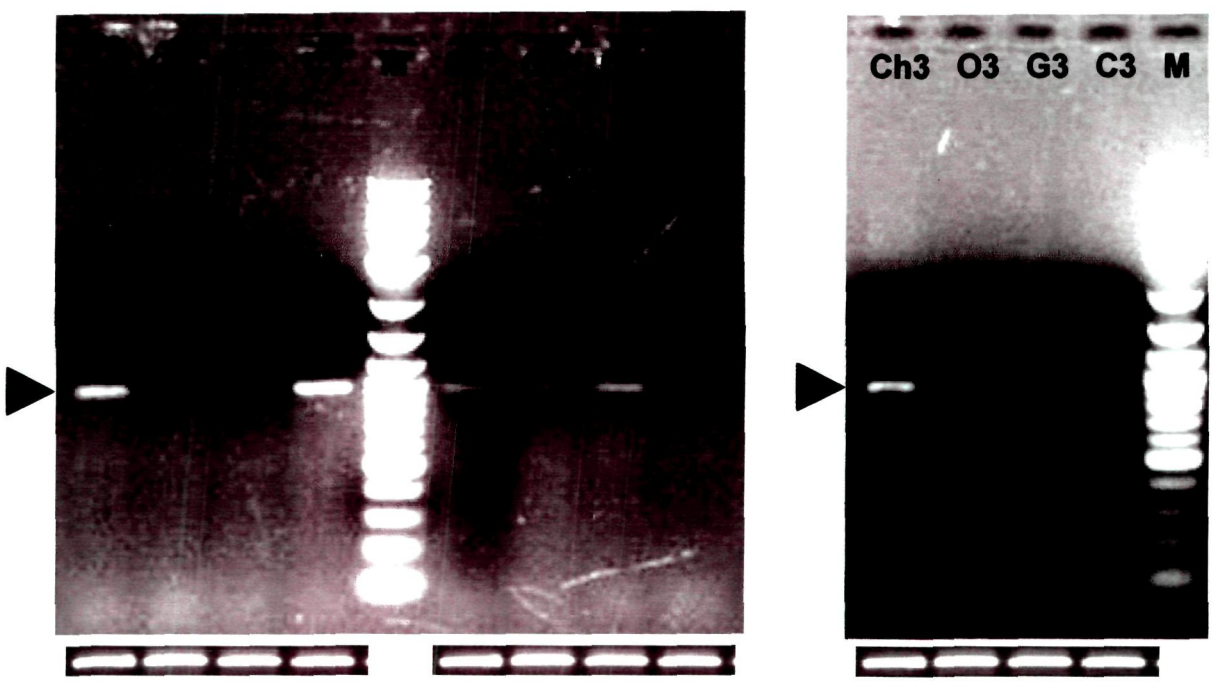


Plate 4.7: RT-PCR expression of endogenous lar gene with 26S *rRNA* as internal control in the lower panel. M – 0.1-10 kb ladder, C1, C2 & C3 – Control I lines; O1, O2 & O3 – *osmotin* lines; G1, G2 & G3 – *gus* lines; Ch1, Ch2 & Ch3 – *chi*11 lines

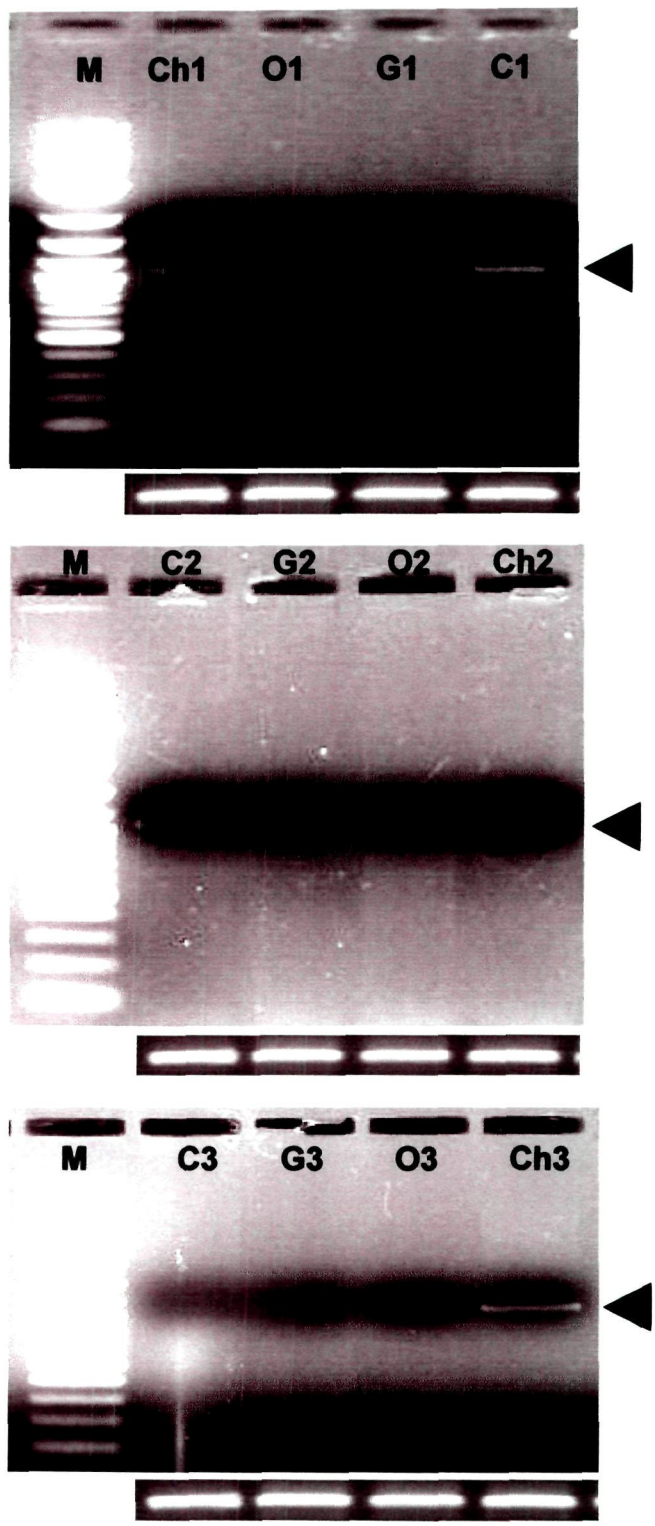


Plate 4.8: RT-PCR expression of endogenous *f3h* gene with 26S *rRNA* as internal control in the lower panel. M – 0.1-10 kb ladder; C1, C2 & C3 – Control I lines; O1, O2 & O3 – *osmotin* lines; G1, G2 & G3 – *gus* lines; Ch1, Ch2 & Ch3 – *chi*11 lines

Table 4.7: RT-PCR expression of endogenous lar and f3h genes in the control I and transgenic lines

Endogenous genes of catechin biosynthetic pathway	Lines	Signal intensity		
		1	2	3
lar	Control I	++++	++	-
	gus	-	+	-
	osmotin	+	++	++
	*chi*11	+++	-	++++
f3h	Control I	+++	++	-
	gus	-	-	-
	osmotin	++	++	+
	*chi*11	++	-	++

++++ = very strong; +++ = strong; ++ = faint signal; + = very faint signal

4.4 Analysis of T_1 plants

4.4.1 Viability and germination of seeds collected from T_0 plants

Irrespective of controls or transgenic, more than 75% of the T_0 seeds were floaters and were thus, non-viable. Only the sinkers were viable and a total of ten *chi*11 seeds germinated on half strength MS medium. However, only four seeds each of *gus* and *osmotin* germinated into T_1 seedlings under *in vitro* conditions **(Plate 4.9)**.

Plate 4.9: *In vitro* germination of (a) control (b) *chi*11 (c) *osmotin* (d) *gus* seeds

4.4.2 Generation of T_1 plants from T_0 seeds

Of the total ten *chi*11 seedlings, one died prior to transfer to soil, one died during hardening and one was infected by fungal infection. Of the control plants, only 50% of the seedlings survived. Three seedlings each of *osmotin* and *gus* survived to grow into healthy plants with three and four leaves, respectively **(Plate 4.10)**.

4.4.3 Assessment of transgene expression in T_1 plants

In RT-PCR, very low accumulation of *osmotin* transcript was observed in two T_1 plants. However, high transcript accumulation was observed in the *chi*11 plants of T_1 generation **(Plate 4.11)**. No expression was observed in case of *gus* and control I (bombarded without the gene of interest).

4.4.4 Phenotypic analysis of T_1 plants

Distinct phenotypic differences were observed in the seedlings from different transgenic lines **(Plate 4.12)**. The seven surviving *chi*11 seedlings were stout with blackish green, extremely thick, large rounded leaves (4.5 × 2.5 cm) with a yellow coloured prominently serrated margin. The root system of the seedlings was also robust and seedling growth was faster than the *osmotin*, *gus* and control seedlings. The leaves of *osmotin* seedlings was 2.5 × 1.2 cm in size, that of *gus* was 1.5 × 0.5 cm and control was 3.0 × 1.5 cm. Except for the leaves of *chi*11 seedlings, all the lines had yellowish green leaves.

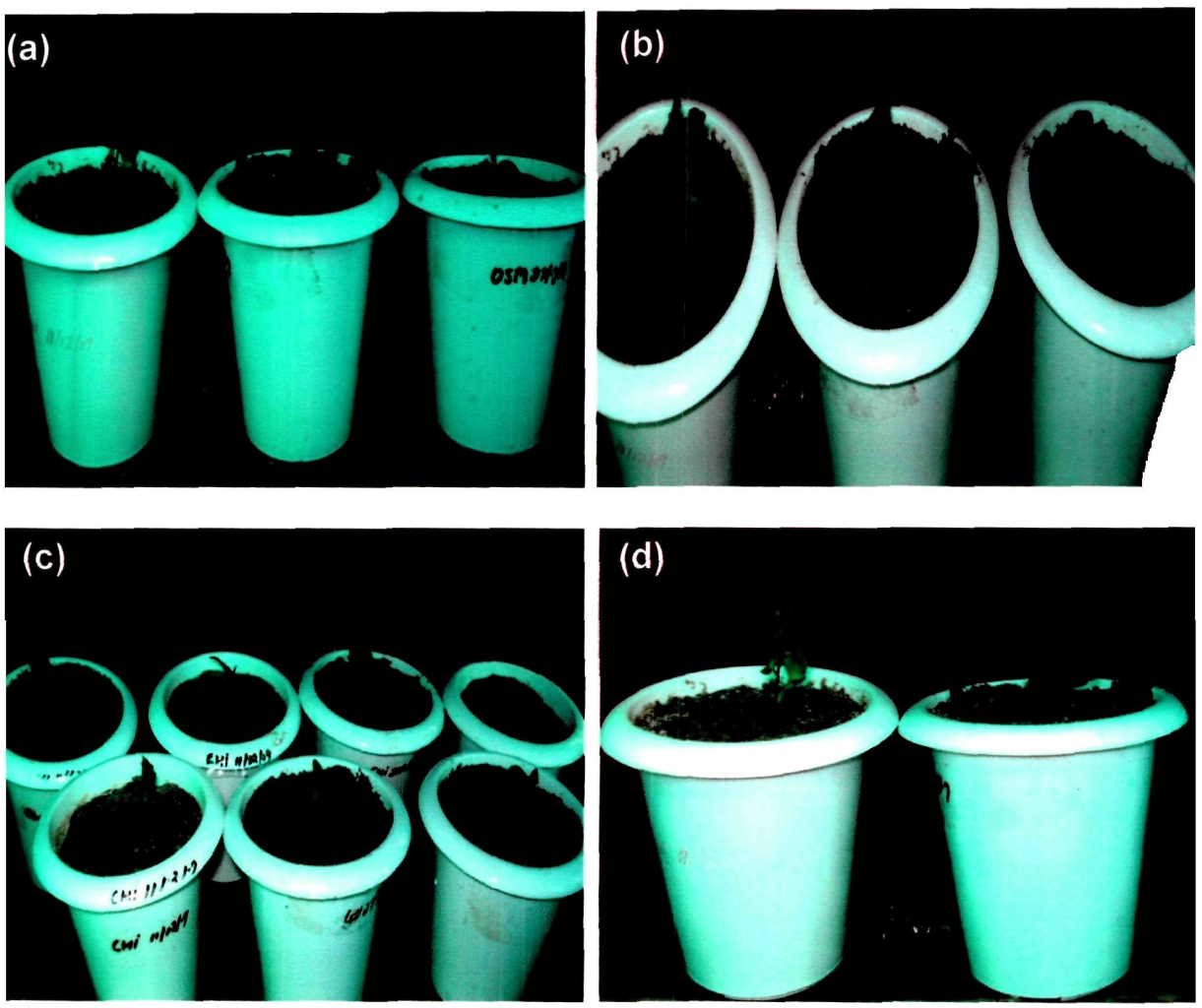


Plate 4.10 Generation of T_1 plants from T_0 seeds (a) *osmotin* (b) *gus* (c) *chi11* (d) control

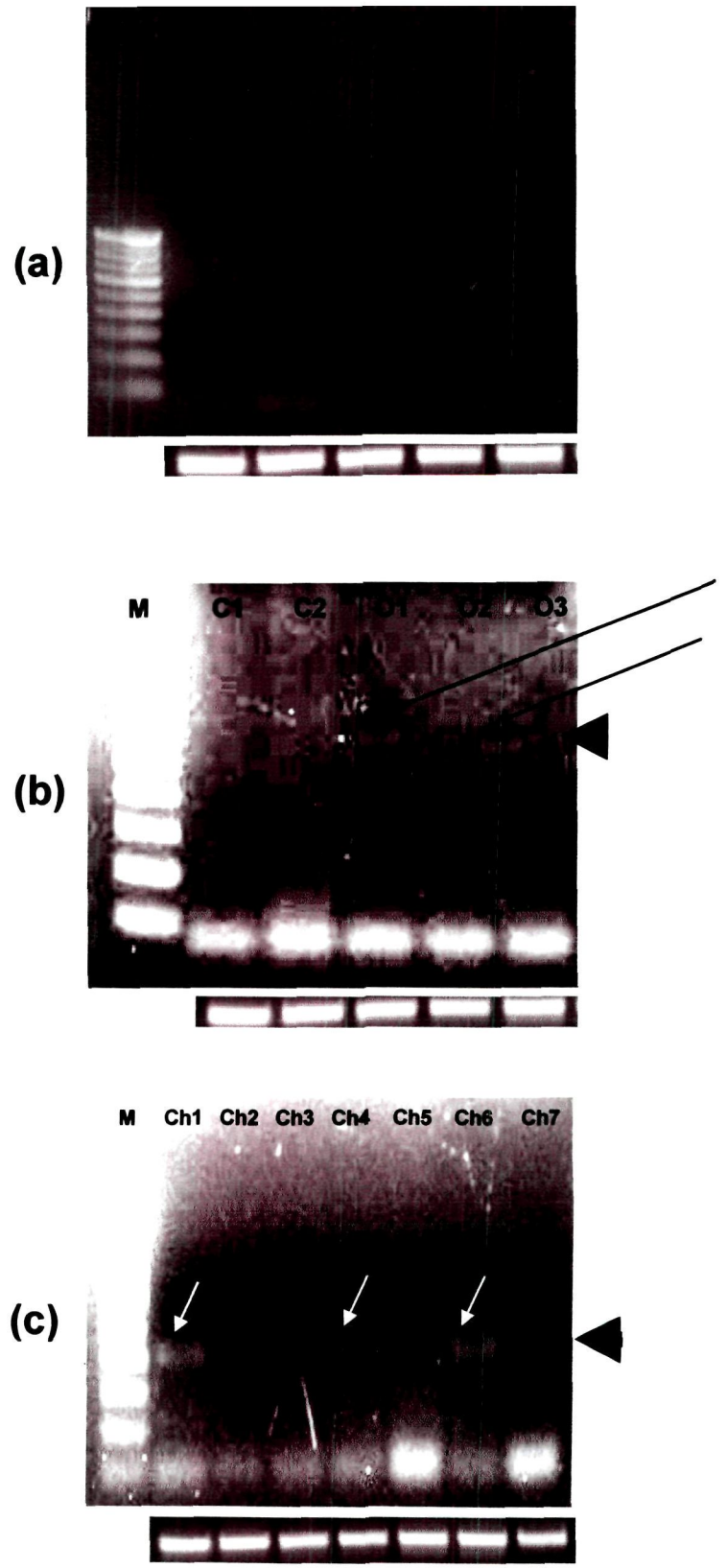


Plate 4.11: RT-PCR of T_1 transgenic lines (a) *gus* (b) *osmotin* and (c) *chi*11, with 26S *rRNA* as internal control in the lower panel. M – 100 bp marker; C1 & C2 – Control; G1, G2 & G3 – *gus* lines; O1, O2 & O3 – *osmotin* lines; Ch1, Ch2, Ch3, Ch4, Ch5, Ch6 & Ch7 – *chi*11 lines

Plate 4.12: Phenotypic variations of T_1 plants: (a) *osmotin* (b) *gus* (c) *chi11* (d) control

Discussion

Chapter V

DISCUSSION

In the past few decades, plant transformation has developed into a robust technology for producing transgenic plants expressing new and useful traits, and also for studying the expression of gene(s) endogenous to plants (Baszczynski *et al.*, 2003). However, this vast majority of the transformation work has continued to rely on random integration of transgene(s). This is because the production of almost all successful transgenic crops has continued to rely either on the *Agrobacterium* or the biolistic method of transformation. Both these methods bring about random integration, albeit at varying degrees (Jackson *et al.*, 2001). Random integration is a process implicated in numerous problems associated with stability of transgene expression, variable expression between independent transgenic events and other position related effects (Kooter *et al.*, 1999; Vanleeuwen *et al.*, 2001; Meng *et al.*, 2006).

Recently 'site specific recombination systems' have gained popularity for better control of integration, expression and manipulation of transgenes (Lyznik *et al.*, 2003; Butaye *et al.*, 2005). Yet, the optimization of standard procedures with respect to *Agrobacterium* or biolistic method has continued to play a major role in a vast majority of transgenic crops. In these procedures, precise selection of the right transgenic line(s) is most crucial because transgene expression may vary even between plants independently transformed with the same procedure and/or construct (Lessard *et al.*, 2002). There is also no

guarantee that the primary transformants showing transgene expression will ultimately give rise to progenies with the same characteristics (Yin *et al.*, 2004).

The study of transgene integration, expression and inheritance in tea was therefore, considered extremely important. Such study is required for the proper utilization of the earlier developed transgenic tea plants both in agriculture and industry. Correct integration and expression of transgene(s) is the final and the most important aspect of transgenic production in an agriculturally important crop like tea. This is particularly, so because the transgenic tea plants were developed by biolistic method of transformation. Although the recent improvements in the biolistic method have now enabled more precise control, multiple copies of transgene in the transgenic plants developed by the biolistic method continues to be a major problem (Romano *et al.*, 2003).

Therefore, a major objective of the present study was re-confirmation of the integration and expression of the 'biotic and abiotic stress tolerant' osmotin and chitinase genes in the T_0 generation. The present study also aimed at comparing the *osmotin* and *chitinase* lines with that of the lines harbouring the gus reporter gene. Thus, when the different T_0 transgenic lines for *osmotin, chitinase* and *gus* were subjected to PCR and RT-PCR analysis, variable results were obtained **(Table 4.1; Plate 4.1 & 4.2)**. While developing these transgenic lines, Saini (2007) had reported strong amplification products of expected size in the transformed somatic embryos on using the gene specific primers. Although the desired/expected fragments were amplified in the *osmotin, chi*tinase and *gus*

T_0 transgenic lines confirming the presence of the particular transgene, a number of differently sized PCR products were also observed in some of the lines. That, the transgenes (osmotin, chitinase and gus) were being differentially expressed was also evident from the differences in the intensity and the number of RT-PCR amplification products. The presence of variable sized fragments of the amplification products indicated the occurrence of multiple insertions, deletions and/or rearrangements even within the transgenic lines for a particular transgene (Kohli *et al.*, 1999).

Variations in plant height and leaf texture were also observed in the different transgenic lines as compared to control **(Plate 4.3; Table 4.2)**. This was probably due to the 'position effect' of the studied transgene(s). The site at which the transgene integrates has a profound influence not only on its expression in the transgenic plants (Matzke *et al.*, 2000) but also on the endogenous plant genes which comes under the influence of the transgene (Salvo-Garrido *et al.*, 2004). The phenotypic differences that were observed between the transgenic lines were probably due to both 'position effect' as well as the 'size of the transgene'. Generally, the size of the transgene governs the site at which the transgene will integrate in the plant genome. This in turn may either silence the 'endogenous gene' or 'transgene' completely or may influence the transgene expression to various extents. Proximal regulatory sequences of the plant genome are known to influence the expression of transgenes that integrate particularly, in the transcriptionally active regions (Lindsey *et al.*, 1993). This also

explains the overall suppression (>50%) as well as the differences in the reproductive capacity of the *osmotin, chi*11 and *gus* transgenic lines **(Table 4.3)**. Generally, it is desired that only a single copy of the transgene be targeted into a highly transcribed region of the plant genome such that the existing endogenous plant genes are not disrupted or silenced (Filipecki and Malepszy, 2006). However, in reality, this may not actually happen and often variations in both transgene and endogenous gene expression may be observed.

The effect of 'transgene size' and 'integration sites' was also evident from the differences in the development of vascular elements in the transgenic lines for *osmotin, chi*11 and *gus* **(Plate 4.4)**. While incomplete development was observed in case of *osmotin* and *chi*11 lines, that of *gus* was complete and comparable to the wild type controls. The size of the coding region along with the regulatory sequences in each of these cases was 2.2, 1.5 and 2.5 kb, respectively. Interestingly, the control I lines obtained from somatic embryos bombarded without the gene of interest also showed incomplete development of vascular elements. Probably, an integration of a large sized blank vector back-bone without the gene of interest was responsible for this phenomenon. Chance integration of vector backbone is often known to affect transgene expression in plants produced by the biolistic method.

Distinct differences in catechins and caffeine were also observed between and within the transgenic lines **(Table 4.4 & 4.5; Fig. 4.2 & 4.3)**. The transgenes (osmotin, chi11 and gus) and the site at which they had integrated

through bombardment had probably influenced the endogenous plant genes of the catechin and caffeine biosynthetic pathways. This was indeed proved true in the RT-PCR analysis of caffeine and catechin biosynthetic pathway gene(s) using 'tea plant gene specific primers'. Thus, even within a particular transgenic line, these genes were either up or down regulated or even silenced **(Table 4.6 & 4.7; Plate 4.6, 4.7 & 4.8)**. Such observations were also recorded between the transgenic lines (*osmotin, chi*11 and *gus*) and also in the bombarded control I lines (without the transgene). It is well known that trans or foreign gene integrates almost exclusively at random, non-homologous sites during transformation, particularly, during bombardment. While some integration may occur in condensed transcriptionally inert chromatin regions, others may occur at transcriptionally active chromatin environments (Mengiste and Paszkowski, 1999). Consequently, the function of a wild type or endogenous gene product may be changed either by over or under production of a variant gene product. This concept of functional inactivation of a gene has been proposed by Herskowitz (1987) and can be termed as 'dominant negative mutation'. Integration of transgene(s) in certain regions of the plant genome also does not result in the expression of the final product (Proles and Meyer, 1992). Transcriptionally active sequences contain isochore sequences (*cis* acting flanking regions) that facilitate DNA bending or elicit different *trans* acting factors necessary for adequate functioning of the genetic machinery. Any deviation from this isochore structure is recognized as a foreign element and may get eliminated or silenced (Meyer, 1995).

The transgenic lines that were analyzed in the present study had been earlier raised through biolistic mediated transformation (Sandal *et al.,* 2001; Saini, 2007). Therefore, the possibility of abnormal, modified or no transgene expression due to integration of more than one copies and/or high degree of rearrangement was expected (Svitashev et al., 2000). Consequently, T_0 lines with variable number of amplification products and also expression were observed in each of the three lines with *osmotin, chi*11 and *gus* **(Plate 4.1 & 4.2)**. While the *osmotin* and *gus* lines with more than one inserts showed strong expression in RT-PCR, no expression was observed in the *chi*11 lines. This can be explained by the fact that even a number of undetected minor re-arrangements can be responsible for many loss of transgene expression due to seemingly intact transgenes (Jackson *et al.*, 2001). Probable integration of the transgene in a transcriptionally inactive region could be another reason for the lack of expression in the T_0 *chi*11 line. However, such unpredictable patterns may be also be inherited by subsequent progenies (Kohli *et al.*, 1999) and may often pose difficulties in the containment of the transgenic plants. This in turn may lead to serious biosafety concerns particularly, if the transgenic plants are to be cultivated in a large scale in the open in 'seed baris'. Therefore, selection of appropriate transgenic lines through studies on inheritance is highly important for any future use.

Tea is cultivated only through vegetative propagation. Its seeds are used only in 'seed baris' for raising progenies in crop improvement programmes (Barua, 1989). Thus, the results of this study on T_1 generation are important for

its utilization in breeding programmes. It was found that despite being suppressed, reproduction of the T_0 lines of *osmotin*, *chi*11 and *gus* were capable of yielding T_1 lines with distinct phenotypic characteristics. While the *chi*11 seedlings were stout with blackish green, extremely thick, large rounded leaves and yellow coloured prominently serrated margin, the leaves of *osmotin* and *gus* were small, yellowish green with not so prominent margins **(Plate 4.12)**. Even the RT-PCR analysis of the T_1 transgenic lines showed high and low accumulation of *chi*11 and *osmotin* transcripts, respectively. No transcript accumulation in the T_1 *gus* lines despite appreciable expression in the T_0 lines is probably due to segregation of the transgene in the T_1 progeny. A probable silencing of the gus gene due to transcriptional gene silencing could be another reason for the absence of transcript accumulation in RT-PCR studies in some of the T_1 plants. Gene silencing generally occurs when a particular mRNA species is not synthesized. In such cases, the expression is blocked at the transcription level (Park et al., 1996; Ye and Singer, 1996).

In conclusion, it can be summarized that the findings of the present study are important as it provides valuable insight into the transgene integration, expression and inheritance patterns of the different transgenic lines for *osmotin*, *chi*11 and *gus*. The empirical assessments of inheritance and phenotypic expression of transgenics are important for the development of efficient breeding strategies for transgenic germ lines (Bregitzer and Tonks, 2003). The study also provides a basis for further studies required for biosafety regulations.

Summary

Chapter VI

SUMMARY

Tea is a non-alcoholic beverage with numerous nutritive and medicinal properties. While the caffeine in tea makes it one of the most refreshing drink, the catechins account for its antibacterial and antioxidant properties. Tea is also rich in vitamin C, amino acids and important flavonoids that make it an all purpose health drink that is fast gaining global acceptance. Thus, it is not surprising that tea is an important commercial crop and a major employment generator in several countries of the world. Despite an ever increasing demand for high quality tea, scarcity of land and labour has greatly limited its horizontal improvement. Thus, vertical crop improvement through the production of transgenic tea plants appears to be an attractive alternative. Consequently, almost all biotechnological research on tea improvement is currently focused on the production of transgenic plants with higher yield and better cup characteristics. It is thus, a small wonder that transgenic tea plants harbouring gus, osmotin and chitinase genes were earlier produced by the biolistic method of transformation. The biolistic method of transformation is popular for most crops that are resistant to *Agrobacterium* mediated transformation or where *in vitro* regeneration protocols are absent. However, this method is generally associated with high degree of transgene rearrangement and consequent variations in transgene expression. This makes screening and re-screening a number of transgenic lines produced by the biolistic method extremely important. This is

required for the selection of a line that will stably express the desired characteristics of the transgene over successive generations. It is also important to have line(s) where the expression of important house-keeping and desirable genes is not disrupted. This is imperative for proper commercial utilization of such transgenic plants. Therefore, in the present study, integration, expression and inheritance patterns of the gus, osmotin and chitinase genes were studied in the transgenic lines that are presently growing in IHBT's contained facility.

PCR is one of the simplest and time effective methods for screening transgenic lines. Therefore, the different T_0 and T_1 transgenic lines for *osmotin*, *chitinase* and *gus* were subjected to PCR using primers specific to these genes. Although the desired/expected fragments were amplified in the *gus, osmotin* and *chi*11 T_0 transgenic lines confirming the presence of the particular transgene, a number of differently sized PCR products were also observed in some of the lines. That, the transgenes (gus, osmotin and chi11) were being differentially expressed was also evident from the differences in the intensity and the number of RT-PCR amplification products. The occurrence of multiple insertions, deletions, and/or rearrangements associated with biolistic method of transformation probably accounted for the presence of variable sized PCR products within the transgenic lines for a particular transgene.

Generally, the biolistic mediated integration of transgene(s) occur randomly and often the position of the transgene may affect the function of one or more endogenous house-keeping or other important functional gene(s). Thus,

it is important to assess changes if any, in the phenotype and growth characteristics of the transgenic plants. True to this knowledge, distinct differences in the phenotypic and growth characteristics were observed between the *gus*, *osmotin* and *chi*11 transgenic plants and controls. This was further confirmed by the histological studies of the shoots of the transgenic and control lines. The *gus, osmotin* and *chi*11 transgenic plants and also the controls revealed distinct differences in the development of vascular elements. While normal development was observed in the wild type control and *gus* lines, incomplete development of either phloem or xylem elements was observed in the *osmotin, chi*11 and control I lines. That, the 'site of transgene integration' and also the 'size of the transgene' may have affected some functional genes was hypothesized based on the observation that the reproductive behaviour of the transgenic lines was considerably suppressed as compared to wild type control plants. Although a large number of flowers were produced in the different transgenic lines, high rates of flower and bud abscission in these lines resulted in very poor seed set. In contrast, appreciable numbers of seeds were produced in the wild type control plants. Of the few seeds that were finally harvested from each of the *gus*, *osmotin* and *chi*11 transgenic lines, a majority were floaters or non-viable. Consequently, only ten *chi*11 and four each of *gus* and *osmotin* lines finally germinated into T_1 lines with distinct phenotypic characteristics. The burden of harbouring the *gus*, *osmotin* and *chi*11 transgene(s) in the T_1 progenies were also probably responsible for the poor seedling growth. As a

result, only seven *chi*11 and three each of *gus* and *osmotin* lines survived as compared to the control plants, where about 50% survival was observed. That the expressible form of transgene(s) was transferred from the T_0 to the *chi*11 and *osmotin* T_1 progenies was also evident from the amplification products in RT-PCR analyses with gene specific primers.

The catechins and caffeine are the two main constituents that affect the quality and yield of tea. Any changes in these constituents bring about remarkable changes in tea yield and quality. However, distinct differences were observed between and within the transgenic lines when the amounts of these constituents were measured in the *gus*, *osmotin* and *chi*11 transgenic lines. This indicated that either the position or the size of the integrated transgene and their expression were probably affecting the endogenous plant genes that governed the synthesis of these products in the catechin and caffeine biosynthetic pathways. This was indeed true as differential expression was observed both within and between the *gus*, *osmotin* and *chi*11 transgenic lines when RT-PCR was performed with the caffeine synthase, *lar* and *f3h* gene specific primers.

In conclusion, the present thesis was successful in screening the different T_0 and T_1 transgenic lines. The different analyses that were conducted were actually useful in identifying the right transgenic plant for each of the gus, osmotin and chi11 genes. As a result, the way was paved for future analyses of subsequent generations of these lines.

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Appendices

Appendix 1. DNA extraction buffer

CTAB	2% (w/v)
Tris-HCl, (pH 8.0)	100 mM
EDTA, (pH 8.0)	20 mM
NaCl	1.4 M
PVP	1% (w/v) (MW 40,000)

Appendix 2. TE buffer (10:1)

10 mM Tris (pH 8.0)
1 mM EDTA (pH 8.0)

Appendix 3. Preparation of Agarose gel

First, the gel assembly was cleaned, dried and a comb with requisite number of wells was placed in the gel tray. Then a slurry was made by mixing 1% agarose with 50 ml of 1X (from 50X) TAE buffer **(Appendix 4)**. This was heated in the microwave and allowed to cool at room temperature. When the solution was luke warm, it was poured into the casting tray and allowed to set. Once set, the comb was removed, the gel along with casting tray was placed in the electrophoresis tank and filled with 1X TAE buffer.

Appendix 4. 50X TAE buffer

Tris base	242.1 gm
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

The volume was adjusted to 1 litre with dH_2O.

Appendix 5. Loading of samples on the gel

DNA or RNA was taken in PCR tubes (0.2 ml) and 1X (of 6X) gel loading dye **(Appendix 6)** was added to it. Each DNA sample and gel loading dye was mixed

well with a pipette and loaded into separate wells of the gel. The gel was finally run at 60 V (DNA migrates from cathode to anode). After considerable distance is covered by the visible dye, the gel was stained with ethidium bromide (0.5 µg/ml) and observed under UV transilluminator.

Appendix 6. 6X Gel loading dye

Ficoll 400	15%
Bromophenol blue	0.25%
Xylene cyanol	0.25%

Appendix 7. Inactivation of contaminating RNases

Each and every glass and plasticware was soaked overnight in DEPC-treated water at 37°C followed by autoclaving (1.05 kg/cm^2). Even the gel running apparatus was cleaned with 0.5% SDS solution, washed with DEPC-treated autoclaved water, treated with 3% H_2O_2 for 1 hr and finally rinsed thoroughly with DEPC-treated autoclaved water. All solutions were prepared in DEPC-treated water and then autoclaved or prepared in autoclaved DEPC-treated water as per the autoclaving requirements. Hand gloves were worn for all experiments in order to avoid contamination of RNases.

Appendix 8. Denaturing agarose gel electrophoresis of RNA

RNA (2 µg) was mixed with RNA loading dye and denatured by incubating the samples at 65°C for 10 min followed by chilling on ice for 5 min. Denatured samples were loaded onto 1% formaldehyde-agarose gel and electrophoresed at 70 V in 1X FA gel running buffer. The gel was viewed using a UV-transilluminator and captured on AlphaDigidoc™ (Alpha Innotech Corporation, USA).

Appendix 9. Composition of PCR reaction

Contents	Amount taken
Autoclaved dH_2O	19.7 µl
10X PCR buffer	2.5 µl
dNTP mix (10mM)	0.5 µl
Primer forward (10 µM)*	0.5 µl
Primer reverse (10 µM)*	0.5 µl
Taq DNA polymerase enzyme	0.3 µl
DNA template	1.0 µl
Total	**25.0 µl**

*these primers are gene specific.

Appendix 10. MS medium (Murashige and Skoog, 1962)

Components	Concentration (mg/l)
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.4H_2O$	8.6
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
Myo-inositol	100
Pyridoxine-HCl	0.5
Thiamine-HCl	0.1
Nicotinic acid	0.5
Glycine	2.0
Sucrose	30 g/l
Agar	8 g/l