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AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION OF GINGER (*Zingiber officinale* Rosc.)

By

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THESIS

Submitted in partial fulfilment of the requirement for the degree of

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2006

DECLARATION

I hereby declare that this thesis entitled "***Agrobacterium* mediated genetic transformation of ginger (*Zingiber officinale* Rosc.)**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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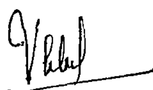

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Dedicated to
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ABBREVIATIONS

BA	- benzyl adenine
2,4-D	- 2,4-dichlorophenoxy acetic acid
NAA	- naphthalene acetic acid
BAP	- Benzylaminopurile
GA_3	- Gibberellic acid
min.	- minute
h	- hour
cm	- Centimetre
MS	- Murashige and Skoog's medium
Kana	- kanamycin
Cefo	- cefotaxime
Hygro	- hygromycin
Carb	- carbenicillin
O.D.	- optical density
μM	- micro molar
mM	- milli molar
mg l^{-1}	- milligram per litre
ppm	- parts per million
GUS	- Beta glucuronidase
npt II	- neomycin phospho transferase
PCR	- Polymerase chain reaction
Ti	- Tumour inducing

Introduction

INTRODUCTION

Ginger (*Zingiber officinale* Rosc.) belongs to the family Zingiberaceae and natural order Scitaminae. In earlier times, ginger was more valued for its medicinal properties and played an important role in primary health care in ancient India and China. In European medicine, ginger is one of the most highly valued mild carminatives and it is a component of many pharmaceutical preparations. It is a major spice crop of the Country. Though ginger is produced in almost all states, the major producers of ginger are Kerala, Orissa, Meghalaya and West Bengal. Kerala state has the maximum share in area (18.8%) and production (19%) in the Country. Wherever ginger is grown soft rot and bacterial wilt are the major field problems limiting the cultivation. Both the diseases are so devastating that their severe occurrence on conducive climate can completely wipe out the crop (Rajan and Agnihotri, 1989). Resistance breeding for the aforesaid maladies are hampered by non availability of natural resistance in cultivated types and absence of natural seed set. In this context, biotechnological approaches for inducing biotic stress tolerance/resistance become important.

The applicability of such biotechnology related techniques depends on the ability to regenerate plants effectively through *in vitro* cultures. The plant tissue culture technique combined with genetic engineering opened up new vistas in crop improvement through gene manipulation.

Plant transformation is performed using a wide range of tools such as *Agrobacterium* Ti plasmid vectors, microprojectile bombardment, microinjection, chemical (PEG) treatment and electroporation of protoplasts. All these methods have advantages unique to each of them. Transformation using *Agrobacterium* and microprojectile bombardment are currently the most extensively used methods.

Agrobacterium based DNA transfer system offers many unique advantages in plant transformation due to its simplicity, the precise transfer and integration of DNA sequences with defined ends, a linked transfer of genes of interest along with the transformation marker, high frequency of stable transformation with many single copy

insertions, reasonably low incidence of transgene silencing and the ability to transfer long stretches of T-DNA.

However for long, the inability of *Agrobacterium* to transfer DNA to monocotyledonous plants was considered its major limitation. With effective modifications in Ti plasmid vectors and finer modifications of transformation conditions, a number of monocotyledonous plants including rice, wheat, maize, sorghum and barley could be transformed. Now, *Agrobacterium T-DNA* transfer is viewed as universal based on successful transformation of yeast, *Aspergillus* and human cells.

In this context, an attempt for *Agrobacterium* mediated genetic transformation would be of great relevance. Achievements in this line would produce types with specific traits.

The present investigations were undertaken to study and optimize the process of gene transfer in ginger using *Agrobacterium tumefaciens* as a vector, with the following objectives:

1. Standardization of regeneration protocol and suitable explants for transformation.
2. Evaluation of ginger cultures for sensitivity to antibiotics.
3. Establishment of bacterial culture and production of recombinants.
4. Standardization of the optimum conditions required for facilitating transformation and the technique of transferring desirable genes into ginger using *Agrobacterium tumefaciens* as a vector.

Review of Literature

2. REVIEW OF LITERATURE

Edible ginger, a member of the Zingiberaceae family, has been an important tropical crop over the past several centuries and generally is propagated from mature rhizome seed pieces. The multiplication rate of rhizome is low compared to many other seasonal crops. Yield of ginger decreases extremely when infested by diseases. The plant is a perennial, commercially grown as an annual and produces no viable seed. Biotechnological means is the only way for introducing novel genes into the cultivated types. The literature on various aspects relevant to *Agrobacterium* mediated gene transfer and regeneration techniques of ginger are reviewed here under:

2.1 ORIGIN AND DISTRIBUTION

Zingiber officinale is not seen in a wild state, hence the Country of origin is not known with certainity. The Sanskrit name 'Singabera' gave rise to the Greek name Zingiberi and to the late Latin Zingiber. It might have originated in South East Asia, especially in India, or China and later introduced to countries like Japan, Sierra Leone, Nigeria, Australia and West Indies (Purseglove, 1978). It was the Spaniards who introduced ginger into Jamaica which is now famous for the quality of its produce (Purseglove, 1978).

2.2 SPECIES AND VARIETIES

Ginger (*Zingiber officinale* Rosc.) is a monocot belonging to the family Zingiberaceae of the order Zingiberales. The family Zingiberaceae consists of two subfamilies i.e. aromatic Zingiberoideae and non aromatic Costoideae. The sub family Zingiberoideae include important genera like *Aframomum, Amomum, Curcuma, Elettaria, Hedychium, Kaempferia* and *Zingiber*. The principal genus of Costoideae is *Costus* (Purseglove, 1978). Burkill (1966) mentioned general species that were used in native medicines in South East Asia, of which the most important seems to be *Zingiber cassumnae* Roxb. and *Z zerumbet* L. According to Purseglove *et al.* (1981), the genus, Zingiber consists of about 80 to 90 species of perennial rhizomatous herbs distributed throughout South East Asia and extending to Queensland and Japan.

Ginger is always propagated vegetatively and the number of clones available are limited. Several clones have been recognized in India which differ in the fibre content of the rhizome and in yield. Important ones are Maran, Nadia, Narasapattam, Kuruppampady and Himachal. (Paulose, 1973). The introduced Rio-de-Janeiro is a high yielding variety under Kerala conditions (Kannan and Nair, 1965; Thomas, 1966; Thomas and Kannan, 1969; Muraleedharan and Kamalam, 1973 and Nybe, 1978).

2.3 *IN VITRO* CULTURE TECHNIQUES IN GINGER

2.3.1 Surface sterilization of explants

A principal requirement for *in vitro* technique is the maintenance of reasonable sterility in cultures. Various surface sterilization techniques without affecting viability of explant were tried by many workers.

Rao *et al.* (2000) reported that fungicide treated (Dithane M-45 at 0.1%) rhizomes germinated in clean pre-sterilised sand and the emerging buds were excised and cleaned using a mild detergent for five minutes. Surface sterilization was carried out with a solution of 0.08 per cent mercuric chloride for 10 to 12 min. followed by rinsing in sterilized distilled water under aseptic conditions and by suspension of explants in 0.1 per cent solution of antibiotic, preferably streptopenicillin for 30 min. The explants were inoculated onto culture media with or without streptopenicillin (1000 ppm). The antibiotic medium reduced the contamination rate to less than 15 per cent.

The sprouted buds were subjected to pre-treatment (18-20 h) with a solution containing bavistin 0.25 per cent and chloramphenicol 0.5 per cent on a rotary shaker (100 rpm) followed by washing with distilled water (4-5 times) prior to surface sterilization with 0.1 per cent $HgCl_2$ (Jasrai *et al.*, 2000).

Bhagyalakshmi and Singh (1988) reported the use of 0.2 per cent $HgCl_2$ as the surface sterilizing agent was also reported by Babu *et al.* (1992).

2.3.2 The culture medium

The success of shoot development from young buds mostly depends on the formulation of an appropriate medium which will support the shoot multiplication and root development. MS medium was found the best medium for ginger according to (Babu *et al.*, 1992; Bhagyalakshmi and Singh, 1988; Rao *et al.*, 2000).

2.3.3 Micro propagation in ginger

Investigations on tissue culture in ginger are mostly confined to micro propagation from shoot-tip culture (Bhagyalakshmi and Singh, 1988; Noguchi and Yamakawa, 1988; Wang, 1989; Balachandran *et al.*, 1990). *In vitro* micro propagation methods for this spice crop have been reported by Hosaki and Sagawa (1977); Pillai and Kumar (1982); Ilahi and Jabeen (1987); Sato *et al.* (1987) and Babu *et al.* (1992). But most of them reported low multiplication ratio of less than 1:4.

Rao *et al.* (2000) reported that MS medium containing high level of benzylaminopurine (3 mg l^{-1} and above) promoted tiny multiple shoots but inhibited root initiation. A significant decrease in shoot number per explant was observed at lower levels (less than 1 mg l^{-1}). An average of 1:6 shoots could be obtained from an explant over a period of 28 days on MS medium containing BAP at 2 ppm concentration.

2.3.4 Effect of growth regulators and supplements

Addition of cytokinin (BA) to basal MS media not only enhanced shoot growth but also supported root induction, at a concentration of 2 mg l^{-1}. MS media supplemented with NAA were comparatively less effective for shoot elongation and root induction. Maximum bud elongation and regeneration of multiple shoots occurred on MS medium supplemented with BA 2 mg l^{-1} and as an average 20-25 plantlets of 7-8 cm height could be obtained from a single apical meristem segment within 6-8 weeks (Jasrai *et al.*, 2000). They also reported that the cytokinin BA had been reported to be the most generally effective cytokinin for meristem, shoot tips and bud cultures of various species followed by kinetin. Earlier studies recommended a combination of

BA and kinetin for bud cultures in ginger (Hosoki and Sagawa, 1977; Nadgauda *et al.*, 1980).

2.3.5 Callus mediated organogenesis

Ilahi and Jabeen (1987) successfully produced plantlets through callus mediated organogenesis. They reported that half strength of MS inorganic was more favourable for micro propagation of ginger than full strength. Choi and Kim (1991) also reported callus mediated organogenesis in ginger from explants of pseudostem containing one leaf blade. Callus was produced in MS medium containing 0.5 mg l^{-1} NAA. Shoot and root regeneration was obtained in a medium containing 0.1 to 1.0 ppm NAA and 1 ppm BA. Babu *et al.* (1992) also reported callus mediated regeneration in ginger. The presence of 2,4-D in the medium 9 to 22.6 µm resulted in callus growth. Organogenesis and plantlet formation occurred when the concentration of 2,4-D was reduced to 0.9 µm with the addition of 44.4 µm BA into the medium. Malamug *et al.* (1991) reported plant regeneration by organogenesis in ginger. The highest degree of callus formation was obtained on medium supplemented with 0.5 mg l^{-1} 2,4-D and 1 mg l^{-1} BA from shoot tips. Shoots were successfully regenerated on media with 3 mg l^{-1} BA.

2.3.6 Somatic embryogenesis

Kackar *et al.* (1993) reported somatic embryogenesis in ginger. Embryogenic cultures of ginger were induced from young leaf segments taken from *in vitro* shoot cultures. Dicamba at 2.7 µM was most effective in inducing and maintaining embryogenic cultures. Efficient plant regeneration was achieved when embryogenic cultures were transferred to MS medium containing 8.9 µM benzyladenine.

Somatic embryos of myrtle (*Myrtus communis L.*) were induced from mature zygotic embryos cultured in MS medium supplemented with 2,4-D. The best frequencies of induction were obtained in a medium containing 2.26 µM 2,4-D in which 97.3 per cent of the explants produced somatic embryos (Jorge *et al.*, 1999).

Ma Guohua (1998) reported that, MS medium containing cytokinin induced cassava shoot organogenesis while auxin (2,4-D, 18.1 μM) stimulated somatic embryogenesis.

Zygotic embryo and shoot tip explants of *Phoemix canariensis* were cultured on MS basal medium supplemented with various growth regulators. The explants after 12 weeks in darkness at 28°C produced embryogenic callus with very compact, pale yellow, nodular structures on MS basal with 2.26 μM 2,4-D, 0.83 μM kinetin and 2 μM abscisic acid (Le Thi Lan Huong *et al.*, 1999). Plant regeneration via.somatic embryogenesis was achieved from callus, derived from immature cotyledons of *Acacia catechu* on WPM supplemented with 13.9 μM kinetin and 2.7 μM 1-naphthalene acetic acid. The addition of 0.9 - 3.5 mM L-proline to the medium influenced development of somatic embryos and also promoted secondary somatic embryogenesis (Rout *et al.*, 1995).

The use of synthetic auxin 2,4-dichloro phenoxy acetic acid played an important role in the production and maintenance of totipotent cereal callus. Vigorously growing, friable and embryogenic callus formed at a concentration of 13.6 μM of 2,4-D (Bregitzer *et al.*, 1995).

Embryogenic suspensions of grapevine were initiated from somatic embryos from proembryonic masses that proliferated without differentiation in a medium containing 2,4-D. Somatic embryos mature and regenerated into plants in MS basal medium containing 3 per cent sucrose (Jayasankar *et al.*, 1999).

Eryngium foetidum was regenerated through somatic embryogenesis from mature leaf explants. Callus was produced on LS medium containing 1 mg l^{-1} 2,4-D and 1 mg l^{-1} BAP. Somatic embryos were induced from embryo-forming callus cultures on MS medium supplemented with 0.1 mg l^{-1} 2,4-D, 2 mg l^{-1} BAP and 1.0 mg l^{-1} GA_3. Subsequently, conversion of these somatic embryos into plantlets occurred on MS medium supplemented with 1.0 mg l^{-1} GA_3 or 0.1 mg l^{-1} BAP. The regenerated shoots were rooted and elongated on MS medium with 0.1 mg l^{-1} IAA and 1.0 mg l^{-1} GA_3 (Ignacimuthu *et al.*, 1999).

2.4 GENETIC TRANSFORMATION STUDIES

The progress in gene transfer technology paved way for manipulation of the hitherto stable nucleus of prokaryotic bacterium to eukaryotic higher plants. The progress in plant tissue and organ culture led to development of transgenic plants with desirable traits, altered metabolism and altered qualitative and quantitative traits.

Though there are several direct methods like electroporation, liposome mediated transformation, microinjection and particle gun technology, the *Agrobacterium* mediated transformation is the widely used system. Using this system, commercial transgenic products were developed and a few products are already available for the end users. *Agrobacterium* based transformation is the most widely used system for introducing genes into dicotyledons (such as tobacco, potato and Arabidopsis) whereas particle bombardment is the most frequently used method for monocotyledons (such as maize, wheat and rice). All the techniques have both advantages and disadvantages (Day and Lichtenstein, 1992).

The first transgenic plants were produced through *Agrobacterium* mediated transformation of *Nicotiana tabacum* leaf explants (Horsch *et al.*, 1985; De Block *et al.*, 1984). Since then, many plant species have been genetically engineered by various techniques.

2.4.1 Transformation vectors

Based on the knowledge about the mechanism underlying the T-DNA transfer, essentially two classes of vectors for gene cloning and subsequent transfer to plants were developed (White, 1993). Both the kind of vectors contain selectable or screenable marker or both. The two classes of vectors are

1. Co-integrate vectors

2. Binary vectors.

2.4.2 Co-integrate vectors

The standard co-integrate vector contains convenient sites for insertion of gene of interest, antibiotic selectable marker gene or genes active in both *E. coli* and *A. tumefaciens*, a plant functional selectable marker gene and an *E. coli* functional origin of replication that does not operate in *Agrobacterium*. Identification of *Agrobacterium* harboring the desired gene of interest-Ti plasmid hybrid is accomplished by selection for antibiotic resistance provided by the marker contained within the cointegrate vector. The colE1 origin of replication common to most cointegrate vectors does not function in *Agrobacterium* making stable maintenance of the GOI linked antibiotic resistance marker dependent upon cointegrate formation. During *Agrobacterium* mediated transfer of the chimeric T-DNA to the target plant genome, the plant functional selectable marker gene of the cointegrate vector is co-transferred, providing a mechanism for the direct selection of genetically transformed plants (An *et al.*, 1985).

2.4.3 Binary vectors

Binary vectors obviate the need for *in vivo* recombination. The binary vectors are designed to improve significantly the ease and efficiency of transforming *Agrobacterium* with plant-ready gene constructs (Hoekema *et al.*, 1983 and An *et al.*, 1988).

The standard components of a binary vector are: a multiple cloning site, a broad-host-range origin of replication functional in both *E. coli* and *Agrobacterium tumefaciens*, selectable markers for both bacteria and plants, transfer functions for conjugation based *Agrobacterium* transformation and T-DNA border sequences. Binary vectors have been constructed with multiple cloning site within a 2-galactosidase α-segment, allowing blue/white screening for fragment insertion (Becker *et al.*, 1992).

2.4.4 Improved vectors for monocot transformation

The development and use of super virulent binary vectors containing virulence genes (*vir* B, *vir* C and *vir* G) from pTibo543, Ti plasmid of the super virulent *Agrobacterium* strain A281, have been the keys to the success of monocot

transformation, including rice. Toki (1997) developed a new pSMABuba binary vector for rice transformation, which is a modified version of pSAMB70. Ku *et al.* (1997) used EHA101 strain containing the pSMABuba vector for high efficiency rice transformation of an intact maize *phosphoenolpyruvate catboxylase* gene (8 kb). A Bin 19 derived binary vector pKGH4, was used by Cheng *et al.* (1998) to transfer *CryIA* (b) and *CryIA* (c) genes into rice through *Agrobacterium* (LBA4404 and EHA105) transformation.

Chin *et al.* (1999) constructed *Ac* and gene trap *Ds* vectors and introduced them into rice through *Agrobacterium* transformation, the *Ac/Ds* mediated gene trap system can be used for analysis of gene function. Ye *et al.* (2000) developed pB19hpc, pZPsC and pZLeyH vectors carrying the genes of provitamin A (β-carotene) biosynthetic pathway enzymes, these vectors were electroporated separately into *Agrobacterium* strain LBA4404. The resultant strains were successfully used for rice transformation and to engineer the provitamin A biosynthetic pathway into rice endosperm.

In conjunction with an improved system for *Agrobacterium* mediated plant transformation, a new binary bacterial artificial chromosome (BIBAC) vector has been developed that is capable of transferring at least 150 kb of foreign DNA into the tobacco nuclear genome. The BIBAC vector has the minimal origin of replication of both *E. coli* and *A. rhizogenes* Ri plasmids, and it can replicate as a single-copy plasmid in both *E. coli* and *A. tumefaciens*. The large BIBAC T-DNAs, in conjunction with the helper that carries additional copies of virulence genes, *virG* and *virE*, have been used for high frequency transformation in dicots. A modified BIBAC vector (BIBAC4) containing 60 kb or 90 kb rice BAC clone has been similarly used for rice transformation. The ability to introduce high molecular weight DNA into plant chromosomes should accelerate gene identification and could lead to new approaches for studying genome organization (Hamilton, 1997).

Plant expression vectors (pPLEX), constructed from the gene regulation signals from subterranean clover stunt virus (SCSV) genome, has previously been used in dicot transformation. Insertion of an intron between the promoter and the transgene open reading frame increased transgene expression 50 fold in rice

(Schunmann *et al.*, 2003). The pPLEX series have the potential to be useful in monocot research the biotechnology.

2.5 *AGROBACTERIUM* - A PLANT PATHOGEN WITH A TALENT FOR TRANSFERRING GENES

Agrobacterium tumefaciens, a gram negative soil bacterium causes crown gall disease in a number of dicotyledonous plants (Smith and Townsend, 1907). Crown gall, a neoplastic disease is characterized by disorganized growth on the stem of plants near the surface of soil. White and Braun (1942) successfully cultivated gall tissue free of inducing bacterium in the laboratory on a completely defined medium lacking phytohormones. While normal plant cells or tissues grow axenically only when plant hormones are supplied, the gall tissue grew rapidly in the absence of any exogenous supplementation of phytohormones (Aerts *et al.*, 1979). Crown gall cells differ from normal plant cells in another aspect. Crown gall cells are capable of synthesis of a novel class of amino compounds called opines which are generally not found in normal plant cells or tissues (Menage and Morel, 1964). The opines are derivatives of common metabolic intermediates eg. octopine is a conjugate of arginine and pyruvic acid and nopaline is a conjugate of arginine and 2-ketoglutaric acid.

The opine synthesized in the tumors do not depend on the species of the host plant but on the strain of the bacterium which induces the tumor. Menage and Morel (1964) proposed that the bacterium must insert a gene governing the synthesis of an opine into the genome of plant cell and the tumor inducing principle must be DNA. It was postulated that the DNA must be on some mobile element.

2.5.1 Ti plasmid and T-DNA

Ti plasmid is a large, circular, double stranded DNA molecule of size 150-250 kb (Day and Lichtenstein, 1992). The plasmid will be lost if the bacteria are grown at high temperature (at or above 36°C), thus producing a virulent strains. The Ti plasmids have five regions of homology (Walden, 1988).

1. T-DNA region
2. Virulence (*vir* region)
3. Replication region (ori)
4. Opine catabolism region
5. Conjugal transfer region

2.5.2 T-DNA region

This is only 14-42 kb in size, which make up about 10 per cent of the plasmid. It is the only portion of the Ti plasmid which is transferred and integrated into the genomic DNA of the host plant and hence is named as transfer DNA (Chilton *et al.*, 1977; Zambryski, 1988; Kado, 1991; Fosket, 1994; Deblaere *et al.*, 1985). This T-DNA contains general genes responsible for tumour production ('onc' genes) opine synthesis ('OS' genes) and several other genes (whose function is not yet understood clearly), flanked on either side by two 25 bp, imperfect, direct repeats designated as right and left T-DNA border sequences.

2.5.3 Essential requirements for T-DNA transfer

Transposon mutagenesis and complementation studies helped a lot in identifying three important genetic components in T-DNA processing and transfer.

2.5.3.1 Chromosomal loci

Some bacterial chromosomal loci were involved/implicated in the establishment of successful *Agrobacterium* - plant interaction. Most of these loci are involved at the initial stages of *Agrobacterium* - plant cell interaction. Some of these were implicated in the synthesis of bacterial cell surface components. Chromosomal loci chv A and chv B govern the ability of *Agrobacterium* to bind to plant cell. Chv A mutants lose their ability to secrete polysaccharide into the medium while chv B mutants do not synthesize 1, 2, 2-D-glucan (Douglas *et al.*, 1982, 1985; Puvanesarajah *et al.*, 1985).

Other chromosomal loci *att* and *cel* are involved in attachment of bacterial and plant cell. Some proteins are absent from the outer membrane of *att* mutants (Robertson *et al.*, 1988). Mutations in *cel* locus result in altered attachment ability of the bacteria to the plants cells. The mutants lack the ability to synthesize cellulose fibrils (Matthyssee, 1983; Sykes and Matthysse, 1986). The mutants in the locus *exo* C (PSC A) fail to produce succinylglycan, a major exopolysaccharide and such mutants also fail to bind to plant cells (Marks *et al.*, 1987; Thomashow *et al.*, 1987).

2.5.3.2 Border sequences

Sequence analysis of different T-DNAs revealed the existence of imperfect 25 bp directly repeated border sequences (Barker *et al.*, 1983; Wang *et al.*, 1984; Yadav, *et al.*, 1982). These sequences delimit the border of T-DNA and are the only sequences required in cis for the mobilization of T-DNA to plant cells. Transfer of T-DNA is not affected by deletion of the internal portion of native T-DNA (Leemans *et al.*, 1982).

2.5.3.3 Vir region

Another part of the Ti plasmid (other than border sequences and T-DNA) involved in T-DNA processing and transfer is called the virulence region or *vir* region. This region approximately spans 35 kb. Mutation and complementation analysis showed that the *vir* region could be divided into six distinct complementation groups which were named as *vir* A, B, C, D, E and G. Information regarding the organization and functions of some of the *vir* genes are now available. The *vir* genes are expressed at very low levels in *Agrobacterium* under normal conditions. Where *Agrobacterium* was exposed to plant cells, *vir* genes are induced to varying degrees (Stachel *et al.*, 1985). The first inducer to be characterized was acetosyringone (Stachel *et al.*, 1985), a naturally occurring metabolite of the wound inducible phenyl-propanoid pathway (Kahl, 1982). Other phenolic compounds that can also serve as *vir* inducers include sinapinic acid, P-hydroxybenzoic acid and vanillin (Bolton *et al.*, 1986).

Efficient transformation can occur only upon induction of *vir* genes. If the particular explant used in co-cultivation does not happen to exude the appropriate signal compounds, it is unlikely that significant transformation will occur. The basic regulatory mechanism underlying *vir* gene expression is now established. Stachel and Zambryski (1986) in a series of experiments showed that *vir* induction by plant inducer is mediated through the action of the *vir* A and *vir* G protein. While both *vir* A and *vir* G genes are expressed constitutively, the level of expression of *vir* B, C, D and E are inducible and under optimal conditions can be induced more than several fold of

their normal expression level. It was proposed that *vir* A might serve to sense or transport the signal molecule thereby activating *vir* G which in turn is a positive transcriptional regulator of the *vir* loci (Stachel and Zambryski, 1986; Winans *et al.*, 1986; Rogowsky *et al.*, 1987).

Such two operon activation systems of various processes regulated by environmental conditions in bacteria is very common (Miller *et al.*, 1989). For example, the envZ/ompR, ntrB/ntrC, phoR/phoB are some of the gene systems known in *E. coli* which respond to changes in osmolarity, nitrogen and phosphate concentration respectively. While products of envZ, ntrB and phoR all are membrane associated proteins and are likely to sense and detect environmental changes, ompR, ntrC and phoB gene products act as positive regulator of transcription. In the case of ntrC it was shown that ntrC gene product exists in two forms, nonphosphorylated and phosphorylated forms and ntrB converts ntrC into an active form by phosphorylation (Nixon *et al.*, 1986). *Vir* G shares strong homology with ompR, phoB and ntrC while *vir* A gene product is homologous to envZ, phoR and ntrB respectively. Part of the *vir* D operon encodes products which are specific endonucleases that recognize the T DNA border sequences and generate single stranded nicks at these points (Yanofsky *et al.*, 1986). *vir* D_2 protein attaches covalently to the T-strand and presumably targets it to the plant nucleus. One of the *vir* E gene products was a single stranded DNA binding protein and could be involved in protecting the single stranded T-DNA intermediate from degradation (Kado, 1991) during the transfer of T-DNA from bacteria to plant cells.

2.5.3.4 Effect of Acetosyringone on transformation

Wounded plants secrete a number of phenolic compounds including acetosyringone which elicit the activation of virulence genes of *Agrobacterium* (Show *et al.,* 1988). Acetosyringone does not exhibit a uniform effect on frequency of transformation in different plants (Stachel *et al.*, 1985). It had a varied effect on transformation depending upon the *Agrobacterium* strains/plant species combination

and the cocultivation conditions. Enhancement of transformation percentage in the presence of acetosyringone was reported for the first time by Sheikholeslam and Weeks (1987) in *Arabidopsis thaliana*. The frequency of transformation ranged from 55-63 per cent when acetosyringone was added to an *Agrobacterium tumefaciens* culture prior to cocultivation of explants as against the control without acetosyringone where the transformation frequency was only 2-3 per cent. De Kathen and Jacobsen (1990) reported that acetosyringone was found to decrease the rate of transformation in *Pisum sativum*. Godwin *et al.* (1991) reported that the effect of acetosyringone on *Agrobacterium* mediated transformation varied according to the plant species. Explants of five plant species (*Allium cepa*, *Glycine max, Brassica campestris, Antirhinum majus* and *Nicotiana tabacum*) were cocultivated with *Agrobacterium* strains to assess the effect of acetosyringone on its tumor inciting ability. Tumors were incited on all these species by strain N2/73 and A 281. The presence of acetosyringone during cocultivation generally enhanced the virulence of these strains most markedly N2/73 on Antirhinum majus and Glycine max, and A 281 on *Glycine max*. Strain Ach 5 was virulent on *Nicotiana tabaccum* only in the absence of acetosyringone. These workers concluded that acetosyringone may enhance virulence in some plant species/strain interaction and suppress the virulence in some others.

2.5.3.5 Genome of Agrobacterium tumefaciens

The microbe *Agrobacterium tumefaciens* is harmful to plants and useful to scientists for the same reason: It transfers DNA into plant genomes. Found in soil worldwide, *A. tumefaciens* causes disease in plants by transferring its own DNA into plant cells. But in the laboratory, the ability to move all sorts of genes into plants has made the microbe the standard tool for investigating plant genetics and modifying crops. Two teams of researchers (Wood *et al.*, 2001 and Goodner *et al.*, 2001) independently have sequenced the *A. tumefaciens* genome.

The *A. tumefaciens* genome has a very unusual structure. Some 5,400 genes reside on four DNA elements - a circular chromosome, a linear chromosome, and two smaller circular structures called plasmids. Many bacteria have circular

chromosomes and some have linear chromosomes, but *Agrobacterium* is the only bacteria known to have both structures together. The genome contains 5.67 million base pairs.

2.6 PRESENT STATUS OF *AGROBACTERIUM* MEDIATED MONOCOT TRANSFORMATION

Despite the availability of highly efficient gene transfer methods having practically no host range limitation, *Agrobacterium tumefaciens* mediated T-DNA transfer is widely sought after. Firstly because it is a poor man's vector (Veluthambi *et al.*, 2003) and requires no high-tech expertise and secondly due to the presence of 25 bp border sequences, it guarantees a clean introduction of the desired DNA package, thus making it feasible for novel genes to cross generic and inter kingdom barriers (Table 1).

Agrobacterium tumefaciens has been routinely utilized in gene transfer to dicotyledonous plants, but monocotyledonous plants including important cereals were thought to be recalcitrant to this technology as they were outside the host range of crown gall. Various challenges to infect monocotyledons including rice with *Agrobacterium* has been made in many laboratories, but the results were not conclusive till 1990's. Efficient transformation protocols mediated by *Agrobacterium* were reported for rice in 1994 and 1996. It is now clear that *Agrobacterium* is capable of transferring DNA to monocotyledons if tissues containing 'competent' cells are infected. The studies of transformation of rice suggested that numerous factors including genotype of plants, types and ages of tissues inoculated, kind of vectors, strains of *Agrobacterium,* selection marker genes and selective agents, and various conditions of tissue culture, are of critical importance (Hiei *et al*., 1997). Some recent works indicated that monocots susceptible to *Agrobacterium*-mediated transformation as indicated by various parameters like opine detection, expression of the introduced genes and T-DNA integration.

Table 1. *Agrobacterium* mediated monocot transformation

Sl. No.	Family/plant	Explant	Opines	Transient expression	T-DNA integration	Transient F_1	Reference
1	*Dioscorea bulbifera*	Bulbil	+	-	+	-	Hooykass *et al.* (1984)
2	*Hordeum vulgare*	Inflorescence spikes	+	-	-	-	Graves *et al.* (1988)
3	*Triticum aestivum*	Inflorescence spikes	+	-	-	-	Deng *et al.* (1988)
		Embryogenic callus	-	+	-	-	Bytebier *et al.* (1987)
4	*Oryza sativa*	Seedlings	+	-	+	-	Raineri *et al.* (1990)
		Seedlings segment	-	+	+	+	Li *et al.* (1992)
		Embryogenic callus	-	+	+	+	Hiei *et al.* (1997); Dong *et al.* (1996)
5	*Zea mays*	Immature embryos	-	+	+	+	Chan *et al.* (1993)
		Embryogenic callus	-	+	+	+	Hiei *et al.* (1994)
		Shoot	+	-	-	-	Graves *et al.* (1988)
6	*Cordyline terminalia*	Stem	+	-	-	-	Matzke and Matzke (1991)
7	*Narcissus*	Stem	+	-	-	-	Meyer *et al.* (1992)
8	*Gladiolus* sp.	Corm disks	+	-	-	-	Feng *et al.* (1988)
9	*Commelina communis*	Stem	+	-	-	-	Suseelan *et al.* (1987)
10	*Asparagus officinale*	Stem	+	+	+	+	Schafer *et al.* (1987)
11	Bermuda grass	Stolon nodes	+	+	+	+	Wang and Yaxin (2005)
12	*Elaeis guineensis*	Immature embryo	+	+	+	+	Abdullah *et al.* (2005)
13	*Oryza sativa*	Immature embryo	+	+	+	+	Bajaj and Mohanty (2005)

2.6.1 Opine detection

Opines are the chemical mediators of parasitism and their presence in cereal cells can be assumed to a certain extent to reveal T-DNA presence as their synthesis is under eukaryotic transcription. Opines have been detected in maize seedlings (Graves *et al.*, 1988) rice protoplasts, rice callus (Raineri *et al.*, 1990) tumors of barley and wheat (Deng *et al.*, 1988), and wheat immature embryo callus (Mooney *et al.*, 1991). It has however be seen that some non-transformed plants also show presence of opines and thus there is need for caution while interpreting such data.

2.6.2 Transient gene expression

Expression of the introduced gene can be a good indicator of DNA uptake and therefore many workers have checked the transient expression of the marker genes delivered by *Agrobacterium* to the monocot species. Expression of the *npt*II gene was shown in rice protoplasts (Baba *et al.*, 1986), kanamycin resistance in rice calli (Raineri *et al.*, 1990) and GUS activity monitored in the seedling explants with multiple copies of *vir* G (Li *et al.*, 1992). Similarly, expression of *gus* gene was observed in immature embryos of maize via shoot apex transformation in maize seedlings (Shen *et al.*, 1993; Gould *et al.*, 1991). Simultaneous expression of the *npt*II and gus was also observed in rice root calli and plants (Chan *et al.*, 1993) and in various tissues and genotypes of wheat (Mahalakshmi and Khurana, 1997; Hess *et al.*, 1990).

2.6.3 Factors influencing plant-microbe interaction

For a successful plant-microbe interaction to occur, various types of interactions between the two organisms need to take place i.e. chemotaxis, attachment, induction of *vir* loci, strain compatibility. There are reports emphasizing the importance of these varied interactions occurring at different stages of host-pathogen association with special reference to monocots.

2.6.4 Bacterial Attachment

Agrobacterium-mediated transformation of plant cells is believed to begin with bacterial attachment occurring at specific sites on the plant surface (Lippincott *et al.*, 1977). Efficient attachment of bacteria to plant cells requires the products of chromosomally encoded genes and not the type of Ti plasmid as shown by the attachment of *Agrobacterium* to wheat cells (Dale *et al.*, 1989). The infrequent incidence of monocot transformation was initially attributed to the lack of bacterial attachment. Later, bacterial binding on cut surfaces of maize and wheat seedlings with preferential binding occurring near vascular bundles in maize and wheat (Graves *et al.*, 1988) and on both wounded and unwounded surfaces in immature embryos of wheat, were shown to be plasmid independent. It thus appears that bacterial attachment on the plant cell is not a limiting factor.

2.6.5 *Agrobacterium* strains

Nopaline strains of *A. tumefaciens* were found superior in their DNA transferring ability as well as in agro infectivity in wheat, maize and rice (Marks *et al.*, 1987; Raineri *et al.*, 1993; Chan *et al.*, 1993). The difference in the efficiency of DNA transfer was neither due to the failure of octopine strains causing attachment nor to the *vir* inducing ability (Jarchow *et al.*, 1991). Nopalin strains agroinfection in maize was reported by (Raineri *et al.*, 1993). It was also shown that octopine and napaline type have *tzs* gene which is inducible by AS and octopine strains have *vir* F which forms part of the *vir* regular. Involvement of *vir* A gene products resulting in high levels of *vir* A proteins differ in their pH requirements for optimal *vir* induction.

Besides the type of the bacterial strain, it has also been observed that binary vectors are more efficient in T-DNA transfer than the co-integrate types as seen in maize (Shen *et al.*, 1993).

2.6.6 Age and the physiological status of the plant

It has been seen that meristamatic tissues, explants from young plants and cells undergoing differentiation are the choice material for *Agrobacterium*-mediated

transformation. The age and physiological status of the plant thus play an important role during plant-microbial interactions. Usually, 3-4 days old seedlings are used for agro infection and meristematic tissues at or close to the apex give higher frequencies of agro infection in maize, wheat and other cereals (Dale *et al.*, 1989) than dry dissected seeds with exposed apical meristems.

2.6.7 Selection of the genotype

Usually, genotypes which are highly embryogenic in culture and readily regenerable are selected. Agro infection of maize was found essentially genotype independent, but high frequencies of agro infection were obtained from the embryogenic line (Schlappi and Hohn, 1992), which emphasizes the difference in the genotype susceptibility to *Agrobacterium.*

2.6.8 Presence of *vir* inducers

Acetosyringone, the *vir* inducer is invariably used at levels ranging from 20-200 µM to induce *Agrobacterium* during co-cultivation to enhance the efficiency of T-DNA transfer. Its presence was necessary in maize, rice and wheat explants co-cultivated with *Agrobacterium*, although the T-DNA transfer occurred even in its absence (Raineri *et al.*, 1990; Lu *et al.*, 1992). Till date, *vir* inducers identified in cereals are ethyl ferulate from wheat suspension cultures which is far more potent than acetosyringone and organic molecules in rice which are equally effective as acetosyringone (Xu *et al.*, 1993). However, the efficiency of agro infection was independent of the presence of *vir* inducers and was comparable in both monocots and dicots (Ashby *et al.,*1988).

2.7 PERSPECTIVES AND PROSPECTS FOR COMPATABILITY BETWEEN MONOCOTS AND *AGROBACTERIUM*

2.7.1 Competence of plant cells

Plant tissues are composed of cells competent for different responses. Cells may exist with potential competence for regeneration and the most effective trigger for shifting the potentially competent cells to the competent state is wounding. Upon wounding, cells of the monocots differentiate rapidly and thus at any particular time

only a few cells at the wound site are in a competent stage to respond to *Agrobacterium* infection (Binns and Thomashow, 1988).

2.7.2 Wound response of *Agrobacterium* strains

It is well known that wounding is necessary for bacterial invasion and wounded cells are the ones susceptible to infection as these are the cells that produce the *vir* inducers (Messens *et al.*, 1990). In monocots, wounding is followed not by extensive cell divisions but the cells rather differentiate into lignified or sclerified cells at the wound, thus providing a ring of hardened cells at the wound site. Thus most of the so called *vir* induction process of the monocots is part of the lignification/ sclerification process and lack of/or reduced cell divisions, which is not very conducive for bacterial invasion. Rather, the wound site is quickly sealed thus providing the bacteria very little time to invade the plant. However, exceptions do exist and some members of the monocots like *Asparagus* and *Dioscorea,* respond to wounding by initiating active cell divisions and have proven more susceptible to *Agrobacterium*-mediated transformation.

2.7.3 Totipotency

The response to wounding is essentially the fundamental basis of totipotency/regeneration ability of somatic cells of plants. Since different plant species differ in their wound response, difference in their regenerative abilities is observed. There is a conscious preference for cocultivating explants with a higher regeneration ability. This poses serious limitations to the success with *Agrobacterium* co-cultivation experiments.

2.7.4 Endogenous inhibitors and cell wall composition

It affects the *Agrobacterium* infection. In maize homogenates, a heat-labile inhibitor was identified by Sahi *et al.* (1990), which had bacteriostatic action and inhibited induction of *vir* genes. There are considerable differences in the cell wall structures of the monocots, compared to dicots. During differentiation, the cell wall accumulate, a threonine-rich protein may leads to specific attachment sites as well as differences in the *vir* inducers identified in the monocots.

2.7.5 *Vir* inducers

Agrobacterium mediated plant transformation is wound mediated, and wounded cells are responsible for the leaching of diffusible substances capable of *vir* induction. These *vir* inducers are mostly phenolic compounds chiefly being acetosyringone and catechols. Some initial works to characterize the probable *vir* inducers from monocots by assaying for the T-DNA circularization event and reported that the monocots in all probability lack these *vir* inducers (Usami *et al.*, 1988). However, on subsequent investigation, it was found that the monocots do produce *vir* inducers (Messens *et al.*, 1990). The inducer molecule in wheat was reported to differ from the phenol inducer in dicots by being hydrophilic and having a high molecular weight. Later, Messens *et al.* (1990) characterized a small molecular *vir* inducer ethyl ferulate from *T. monococcum* suspension cells and found the same more active than acetosyringone in induction of *vir* gene expression. The importance of one of the *vir* genes *vir* A in maize was demonstrated by Raineri *et al.* (1993). The *vir* A protein is known to phosphorylate the *vir* G protein which in turn activates the transcription of the *vir* genes. It was seen that different *Agrobacterium* strains contains different allelic forms of the *vir* A gene, some of them coding for proteins insensitive to the *vir* inducers of monocots. These findings thus suggest that not only the *vir* inducers in the monocots may be different from the dicots but so may be the case with bacterial receptor proteins and thus resulting in lack of compatability between bacteria and the plants.

2.7.6 Key factors involved in *Agrobacterium tumefaciens* mediated transformation of monocotyledons

2.7.6.1 Use of meristematic tissue for co-cultivation

The success of transformation usinjg embryonic or meristematic tissues could be attributed to the following: (i) *vir*-inducing substances are produced by embryonic tissues, (ii) low production of bacteriotoxic substances, (iii) favourable endogenous hormone levels, (iv) availability of receptors for attachment of *Agrobacterium* (Chan *et al.*, 1993), and (v) these cells are actively dividing and host DNA synthesis is occurring. DNA synthesis may be required for T-DNA integration (Grimsley *et al.*, 1988). This may be significant because most differentiated monocotyledon cells fail to divide and thus cannot produce tumors. Many researchers

believe that meristematic target cells are essential (Gould *et al.*, 1991). These factors may all contribute to cellular competence to receive foreign DNA.

2.7.6.2 Vir gene action

Many monocotyledons may not produce the phenolic factors to activate the expression of the *vir* genes on the Ti plasmid (Usami *et al.*, 1988). Therefore, addition of these compounds to the co-cultivation mixture is probably beneficial. Monocotyledons may produce these compounds from meristematic, undifferentiated cells.

2.7.6.3 Monocotyledon gene promoters

Tumors may not form in monocotyledons because of the lack of transcription of the *onc* genes or because of a different endogenous hormone physiology as compared with dicotyledons. The use of monocotyledon promoters such as actin, ubiquinone, and α-amylase has been shown to enhance gene expression significantly than 35S promoter by monocotyledon cells.

2.7.6.4 Wide host range A. tumefaciens Strains

Success in monocotyledon transformation most certainly will depend on using the correct, compatible *A. tumefaciens* strains. In this regard, wide host range bacterial strains and new helper plasmids derived from them will be useful (Hood *et al.*, 1993) as well as C58 (Grimsley *et al.*, 1988).

2.8 SELECTABLE MARKERS

One of the most important advances in the development of plant transformation vectors was the construction of genetic markers applicable to plant tissue. Irrespective of the method employed to achieve transformation, a marker is a must to detect a transformation event and to select transforments. These markers in the transforments demonstrate not only entry, integration and expression of the foreign gene but are also useful in establishing the inheritance of the foreign DNA in the next generation. Moreover, the development of dominant selectable markers functional in plant tissues allow the direct selection of transgenic cells by their ability to grow under selection pressure. The marker gene can either be a dominant selectable marker, so as

to allow selection directly for the growth of transgenic tissue or it can be a screenable marker which is used to investigate levels of gene activity.

In majority of cases, the enzymes encoded by the marker gene are not present in the plant cells. The marker genes that have been developed are generally gene chimaeras where the coding sequence for the marker gene is flanked at the 5' end by DNA sequences which act as a promoter in plant cells and at the 3' end by a DNA sequence carrying the poly A signal. The promoters that have been routinely used are the CaMV35S promoter from cauliflower mosaic virus or the opine synthase promoters for octopine synthase or nopalin synthase gene from the T-DNA itself. These promoters are generally constitutive in their expression. It is necessary if the marker is to provide dominant selection because selection might be applied to cells at different levels of differentiation or development. The agents used for selection are generally antibiotics or anti metabolites which do not instantaneously kill the susceptible cells. Sudden death of plant cells releases chemicals which retard the growth of resistant cell also. However, choice and efficacy of selection agents differs between the various plant species. Some of the commonly employed dominant selectable markers are listed in Table 2.

Table 2. Selectable marker genes

Genes for	Agents used for selection	Reference
1. Neomycin phosphotransferase (*npt* II)	G 418, Kanamycin, Neomycin	Bevan *et al.* (1983) Herrera-Estrella *et al.* (1983)
2. Dihydrofolate reductase (Dhfr)	Methotrexate Trimethoprim	Herrera-Estrella *et al.* (1983)
3. Hygromycin phospho-transferase (hpt)	Hygromycin B	Vanden Elzen *et al.* (1985)
4. Aminiglycoside acetyl transferase (AAC 3)	Gentamycin	Hayford *et al.* (1988)
5. Gene for bleomycin resistance	Bleomycin	Hille *et al.* (1986)
6. Enolpyruvyl shikimato 3-phosphate synthase (aro A)	Glyphosate (Round up)	Shah *et al.* (1986)

Some of the commonly employed dominant selectable markers are neomycin-phosphotransferase (*npt* II) encoded by the bacterial transposon Tn 5

detoxifies aminoglycosides like G 418 and kanamycin by phosphorylation. It has become an important marker for use in transformation studies largely because selection for resistance to kanamycin can be applied to isolated cells, calli, tissue explants and whole explants (Herrera-Estrella *et al.*, 1983; DeBlock *et al.*, 1984).

Under ideal conditions, all plantlets that are regenerated under the selection pressure are transferred. But sometimes depending on the selection and the plant species there may be many escapes. This problem can be obviated if there is an independent means of readily identifying transformed plants. For this reason the presence of a screenable marker is desirable. β-glucuronidase (GUS) derived from *E. coli* is one of the most extensively used scorable markers. This enzyme is a hydrolase that cleaves a wide variety of β-glucuronidase. The enzyme is very stable, active in a variety of cellular millicea and there is a little or no β-glucuronidase activity in plants (Hu *et al.*, 1990). Moreover, the enzyme can tolerate large amino terminal additions. GUS is a useful marker largely because of its ability to cleave a variety of commercially available substrates for a variety of spectrophotometric, fluorometric and histochemical assays. These assays are particularly useful in investigating the tissue specific expression of a particular gene construct (Jefferson *et al.*, 1987). The ability to quantify gene expression through the routine use of enzyme assays greatly enhances precision and resolution of analyses of transgenotes. It allows accurate estimation of the quantity of chimaeric gene product even over an intrinsically fluorescent background.

2.9 ANTIBIOTIC SENSITIVITY OF CULTURED TISSUES

2.9.1 Kanamycin sensitivity

Kanamycin resistance is the most widely used selectable marker for plant transformation and the sensitivity of a particular species or explant to kanamycin is a key element in the development of any new transformation system in which a kanamycin resistance gene will be employed. Some monocotyledons have a high level of natural resistance to kanamycin concentrations. Greater than 500 mg l^{-1} are needed to completely inhibit the growth of rice callus (Dekeyser *et al.*, 1989) and more than

800 mg l^{-1} is required to inhibit the growth of cell suspension cultures of several species of Gramineae (Hauptmann *et al.*, 1988). In contrast, only 35 mg l^{-1} totally inhibits shoot differentiation from co-cultivated thin cell layer explants of *Brassica napus*. Sensitivity affects the recovery of transformed plants and varies widely among tissues and species. Kanamycin sensitivity should be determined in the initial stages of developing a plant transformation system. Baribault *et al.* (1989) were able to select transformed grape callus on only 10 mg l^{-1} kanamycin after co-cultivation with *A. tumefaciens* but it was completely inhibited by 100 mg l^{-1} a concentration used routinely to select transgenic tobacco the lowest concentration required to completely inhibit callus growth, adventitious shoot differentiation and root initiation. If growth in the presence of a normally inhibitory concentration is taken as an indicator of *npt* II enzyme activity, this would represent the lowest concentrations appropriate for the selection of kanamycin resistant tissues in *Agrobacterium* co-cultivation experiments.

Alternative selection schemes are being used in an increasing number of species where kanamycin selection is ineffective due to the resistance of plant species including *Arabidopsis thaliana* (Lloyd *et al.*, 1986), *Lolium multiflorum* (Potrykus *et al.*, 1985) and *Oryza sativa* (Shimamoto *et al.*, 1989). In such cases, other selection agents such as hygromycin and methotrexate are used as effective selection agents for many species but they inhibited the growth at extremely low levels. In cases where kanamycin resistance is an efficient selectable marker, selection concentrations typically range from 50 to 300 mg l^{-1}. Transgenic shoots are commonly selected from tobacco leaf discs with 300 mg l^{-1} (Horsch *et al.*, 1985) and in the case of transgenic tomato, shoots are selected on 100 mg l^{-1} (Fillatti *et al.*, 1987a). This range of concentrations has also proven successful for several woody species including apple (James *et al.*, 1993), poplar (Fillatti *et al.*, 1987) and walnut (Mc Granahan *et al.*, 1988).

Species and explant specific kanamycin sensitivities are important considerations in choosing an appropriate selectable marker for a new gene transfer system. While natural resistance to kanamycin has impeded the selection of transformants in some species, too much sensitivity has also hampered the recovery of

transformed tissues (Sheila and Carole, 1990). Dose response experiments with kanamycin and other selective agents should be an essential step in the development of new transformation system.

2.9.2 Plant hormone effect of antibiotics on the transformation efficiency of plant tissues by *Agrobacterium tumefaciens*

Agrobacterium mediated transformation procedure involves the inoculation of explants with *Agrobacterium* cells followed by the transfer of infected explants to a selective medium for regeneration of transgenic plants. The selective medium contains both antibiotics for eliminating *Agrobacterium* cells and chemicals for selecting plant tissues which express the foreign DNA in the plant cells. Although many antibiotics have been used for the effective elimination of *Agrobacterium* cells, carbenicillin and cefotaxime have minimal toxicity on most plant tissues and efficiently eliminate *Agrobacterium* cells (Okkels and Pedersen, 1988). Carbenicillin and cefotaxime have become the most widely accepted antibiotics for performing *Agrobacterium* mediated transformation. However, both antibiotics showed plant hormone like effects on plant tissue, especially when the plant tissues were grown in a medium containing low concentration of carbenicillin and cefotaxime (Mathias and Boyd, 1986). It is critical to have a defined ratio of auxin and cytokinin in order to establish a successful regeneration system in plant tissue culture (Akama *et al.*, 1992). The plant hormone like effects of antibiotics may complicate the hormone ratio in the culture media and influence the transformation efficiency by reducing the efficiency of regeneration.

2.9.3 Toxicity of carbenicillin on plant tissues

Cefotaxime is more effective in inhibiting *Agrobacterium* cell growth than carbenicillin. Carbenicillin is the antibiotic of choice in *Agrobacterium* mediated transformation because cefotaxime has shown high toxicity in many different plant tissues (Konez *et al.*, 1992). In the presence of 2,4-D, carbenicillin (250 $\mu g\ ml^{-1}$) shows tissue necrosis. The toxicity of combinations of carbenicillin and 2,4-D may be caused by the addition of an auxin like activity supplied by carbenicillin.

2.9.4 Plant hormone effect of carbenicillin

Plant hormone effects of carbenicillin were observed in carrot tissues at low concentration. The regeneration of shoots were decreased as the concentration of carbenicillin increased. However, the formation of callus increased as the concentration of carbenicillin increased. Similar results were obtained from the addition of different concentrations of cefotaxime, however callus formation was not as significant as on carbenicillin (Okkels and Pedersen, 1988).

The chemical structure of auxins such as 2,4-D and α-naphthalenacetic acid (NAA) contains either a phenolic or benzyl group connecting to a side chain of an acetic acid group. Carbenicillin also has an auxin related structure as well as lactam structure, which has a bactericidal effect. The presence of the auxin related structure of carbenicillin and hormonal activity in tissue culture strongly suggested that carbenicillin has plant auxin effects in addition to its well known bactericidal effects. The auxin effect of carbenicillin was described as breakdown products like phenylacetic acid by Holford and Newburg (1992).

2.9.5 Effect of antibiotics on elimination of *Agrobacterium* strains

An important prerequisite for *Agrobacterium* transformation is the determination of efficient antibiotics to eliminate the *Agrobacterium* as soon as its presence is no longer required. Because efficient regeneration is necessary for successful transformation and because some antibiotics have either a positive or a negative effect (Barret and Cassells, 1994; Mathias and Mukasa, 1987; Pollack *et al.*, 1983).

Generally, β-lactams are considered to be non-toxic to plant cells due to their specific action on bacterial cell walls (Pollack *et al.*, 1983; Mathias and Boyd, 1986) and in some cases their breakdown products in the culture medium stimulate plant cell growth (Holford and Newburg, 1992). However, the phytotoxicity of antibiotics can vary markedly between plant species and also depends on their concentrations used (Okkels and Pedersen, 1988; Yepes and Aldwinckle, 1994;

Hammerschlag *et al.*, 1997). As widely found, the β-lactams, such as carbenicillin and ticarcillin are not efficient in eliminating *Agrobacterium* since they are sensitive to β-lactamases produced by bacteria. Cefotaxime is highly resistant to β-lactamases, but it did not eliminate the super virulent strains, either alone or in combination with carbenicillin or cefotaxime (Hammerschlag *et al.*, 1997). Cooke *et al.* (1992) reported inhibition of *A. tumefaciens* and elimination of this bacterium from infected *Rosa* shoot cultures on media of pH 4.0 or below. (Hammerschlag *et al.* (1997) found that, by combining a short-term exposure to an acidic (pH 4.0 or below) medium with cefotaxime, *Agrobacterium* contamination could be reduced to an acceptable level. The addition of kanamycin further reduced the *Agrobacterium* contamination. An antibiotic effective in eliminating *A. tumefaciens* and also increasing transformation efficiency when combined with kanamycin is used for transformation experiments.

The elimination of *Agrobacterium* usually achieved by adding one or more antibiotics to the culture medium is quite important because the continued presence of *Agrobacterium* can present a problem for identifying transforments or interfere with the growth and development of the transformed plant cells or cause death of cultures (Matzk *et al.*, 1996; Cooke *et al.*, 1992; Martin *et al.*, 1990). The use of antibiotics for eliminating *Agrobacterium* is limited by the fact that some antibiotics such as carbenicillin and ticarcillin can be inactivated by β-lactamases produced by bacteria while cefotaxime is highly resistant to β-lactamases but inhibit plant regeneration (Yepes and Aldwinckle, 1994; Hammerschlag *et al.*, 1997; Shackelford and Chlan, 1996; Ling *et al.*, 1998). These are however several reports that another antibiotic, timentin (ticarcillin coupled with a specific β-lactamase inhibitor clavulanic acid) can efficiently eliminate *Agrobacterium* without inhibiting plant regeneration (Chevreau *et al.*, 1997; Cheng *et al.*, 1998; Ling *et al.*, 1998).

2.10 ANALYSIS OF TRANSFORMATION

2.10.1 β-glucuronidase - a sensitive and versatile gene fusion marker in higher plants

Control of gene activity can be manifested at many levels including the initiation of transcription or translation and the processing, transport or degradation of

mRNA or protein. The use of precise gene fusions can simplify analyse of these complex processes and delineate the contribution of transcriptional control by eliminating the specific signals for post-transcriptional controls and replacing them with sequences from a readily assayed reporter gene. The two useful reporter genes are the bacterial genes chloramphenicol acetyl transferase (CAT) and neomycin-phosphotransferase (*npt* II) which encode enzymes with specificities not normally found in plant tissues (Bevan *et al.*, 1983; Fraley *et al.*, 1983; Herrera-Estrella *et al.*, 1983). In addition, *npt* II can tolerate amino-terminal fusions and is enzymatically active, making it useful for studying organelle transport in plants (Vanden Broeck *et al.*, 1985). However, both CAT and *npt* II are relatively difficult, tedious and expensive to assay (Gorman *et al.*, 1982; Reiss *et al.*, 1984). Competing reactions catalized by endogenous esterases, phosphatases transferases and other enzymes also limit sensitivity and make quantification of CAT or *npt* II by enzyme kinetics very difficult.

E. coli β-glucuronidase gene is a reporter gene system for transformation of plants and GUS makes it a superior reporter gene system for plant studies. β-glucuronidase (GUS, EC 3.2.1.31), encoded by the *uid*A locus (Novel and Novel, 1973) is a hydrolase that catalyses the cleavage of a wide variety of β-glucuronides (Stoeber, 1961) many of which are available commercially as spectrophotometric, fluorometric and histochemical substrates. The β-glucuronidase gene was cloned and sequenced and encodes a stable enzyme that has desirable properties for the construction and analysis of gene fusions (Jefferson, 1987; Jefferson *et al.*, 1987). Glucuronidase is easily, sensitively and cheaply assayed *in vitro* and can also be assayed histochemically to localize GUS activity in cells and tissues.

Polymerase chain reaction

The polymerase chain reaction which is commonly known as PCR is a very powerful method used for *in vitro* cloning and also to amplify a single molecule of DNA to million folds in short time. This is achieved by an enzyme Taq DNA polymerase which is thermo stable and is isolated from a thermostable bacterium

Thermus aquaticus. The enzymatic reaction using this enzyme can synthesise microgram quantities of DNA of specified length and sequence from picogram quantities of complex template in a few hours time. This technique first described by Saiki *et al.* (1988) has a lot of application in various fields of molecular biology, especially in studies of genes which are expressed poorly and in low amounts and in RFLP mapping or study of DNA polymorphism.

PCR is specific and simple and can be used for analyzing gene expression in many different tissues and stages following transgenic plants and animals. PCR techniques have been effectively used for the analysis of putative transgenics using specific primers in a number of crops like coffee (Hatanaka *et al.*, 1999), rubber (Sobha *et al.*, 2003), almond (Miguel and Oliveira, 1999) and egg plant (Milkos *et al.*, 1995).

Materials and Methods

3. MATERIALS AND METHODS

The investigations on *Agrobacterium* mediated genetic transformation of ginger, *Zingiber officinale* Rosc. were carried out at the Department of Plantation Crops and Spices and Plant Tissue Culture Laboratory, CPBMB, College of Horticulture, Vellanikkara during 1999 to 2005. The study was aimed at developing a procedure for *Agrobacterium tumefaciens* mediated genetic transformation in ginger. The details regarding the experimental materials and methodology adopted for conducting various experiments of the study are presented in this chapter.

3.1 PLANT MATERIALS

The cultivar Rio-de-Janeiro from the Department of Plantation Crops and Spices was used for the study and rhizomes were collected for developing aseptic cultures.

3.1.1 Vectors and bacterial strains

Three *Agrobacterium* strains namely EHA 105, LBA 4404 and GV 2260 were collected, recombinants produced and used for the study.

3.2 CULTURE MEDIUM

3.2.1 Chemicals

The major and minor elements required for the preparation of media were of analytical grade and procured from M/s Sisco Research Laboratories (SRL) and M/s Merck India Ltd. The amino acids, vitamins and plant growth regulators were obtained from M/s Merck India Ltd. and Sigma Chemicals, USA. The antibiotics were from Himedia.

3.2.2 Glassware

Borosilicate glassware of Cornings/Borosil brand were used for the experiment.

3.2.3 Composition of media

Basal MS medium (Murashige and Skoog, 1962) with various plant growth regulators were used for plant tissue culture.

3.2.4 Preparation of the medium

Standard procedures (Gamborg and Shyluk, 1981) were followed for the preparation of the plant tissue culture media. After mixing appropriate quantities of the stock solutions and making up the volume to the required quantity by using distilled water, pH of the medium was adjusted between 5.6 and 5.8 using 0.1N NaOH/HCl. After adding agar at the rate of 7.5 g l^{-1}, the medium was heated to melt the agar. The medium was then dispensed to test tubes/conical flask and were plugged with non-absorbent cotton. Autoclaving was done at 121°C at 15 psi (10.5 kg/cm²) for 20 minutes (Dodds and Roberts, 1982) to sterilize the medium. The medium was allowed to cool to room temperature and stored in a culture room until used.

3.3 TRANSFER AREA AND ASEPTIC MANIPULATIONS

All the aseptic manipulations were carried out under the hood of a clean laminar airflow cabinet.

3.4 CULTURE CONDITIONS

The cultures were incubated at 25±2°C in an air conditioned culture room with 16 h photoperiod (1000 lux) supplied by fluorescent tubes unless otherwise mentioned. Humidity in the culture room varied between 60 and 80 per cent according to the climatic conditions prescribed.

3.5 STANDARDIZATION OF SURFACE STERILIZATION PROCEDURE

The shoot buds collected from ginger rhizomes carry a variety of microorganisms on them. Inorder to formulate an effective surface sterilization technique, three experiments were conducted as follows:

3.5.1 Effect of surface sterilants on culture establishment

The young shoot buds collected in sterile water were subjected to surface sterilization treatments as given in Table 3.

Table 3. Surface sterilization treatments for culture establishment

Sterilant	Concentration (%)	Duration
1. Mercuric chloride	0.1	5 min, 10 min
2. Ethanol + Mercuric chloride	50 + 0.1	Wipe (ethanol) + 10 min
3. Ethanol	95	10 sec

The sterilization treatments were carried out under aseptic conditions in a laminar flow cabinet and the sterilized shoot buds were inoculated in ½ MS medium supplemented with BA 2 mg l^{-1} and 3 mg l^{-1}, which had been reported by Rao *et al.*, (2000) as ideal for clonal propagation of ginger. The percentage survival was recorded 20 days after incubation.

3.5.2 *In vitro* evaluation of antibiotics against *Ralstonia solanacearum*

The bacterium from the contaminated cultures was isolated and identified as *Ralstonia solanacearum.* The *in vitro* efficacy of ampicillin, chloramphenicol, nalidixic acid, tetracyclin, streptomycin and cefotaxime in inhibiting the growth of the bacteria was tested by the standard filter paper disc method. The chemicals and their composition tried are given in Table 4.

Table 4. Details of antibiotics evaluated against *Ralstonia solanacearum*

Chemicals	Concentration (mg l^{-1})
1. Ampicillin	250, 500, 750, 1000
2. Chloramphenicol	250, 500, 750, 1000
3. Nalidixic acid	250, 500, 750, 1000
4. Tetracyclin	250, 500, 750, 1000
5. Streptomycin	250, 500, 750, 1000
6. Cefotaxime	250, 500, 750, 1000

Sterile filter paper discs of 10 mm diameter were dipped in the solutions and placed over Triphenyl Tetrazolium Chloride Agar medium (Kelman, 1954) seeded with 48 h old cultures of the bacterium. Filter paper discs dipped in sterile distilled water served as control. The test was replicated thrice and the discs were incubated at 30°C. The diameter of zone of inhibition was measured 24 h after inoculation and expressed in mm.

3.5.3 Management of bacterial interference in cultures

The antibiotic which was found most effective in the sensitivity test was used for treating the explants and their efficacy in controlling the bacterial interference in *in vitro* cultures were evaluated. The treatment details are given in Table 5.

Table 5. Treatment details of management of bacterial interference in culture

Sterilant	Concentration (%)	Duration
1. Mercuric chloride	0.1	10 min
2. Streptocyclin + $HgCl_2$	500 mg l^{-1} + 0.1	1 hr, 10 min
3. Streptocyclin + $HgCl_2$	1000 mg l^{-1} + 0.1	1 hr +10 min
4. Chloramphenicol + $HgCl_2$	500 mg l^{-1} + 0.1	1 hr + 10 min
5. Chloramphenicol + $HgCl_2$	1000 mg l^{-1} + 0.1	1 hr + 10 min
6. $HgCl_2$ + Streptocyclin (incorporated in media)	0.1 + 1000 mg l^{-1}	10 min
7. $HgCl_2$ + Chloramphenicol (incorporated in media)	0.1 + 1000 mg l^{-1}	10 min
8. $HgCl_2$ + Cefotaxime	0.1 + 250 mg l^{-1}	10 min + 1 hr
9. $HgCl_2$ + Cefotaxime (incorporated in media)	0.1 + 250 mg l^{-1}	10 min

3.6 *IN VITRO* ESTABLISHMENT OF GINGER CULTURE

The rhizomes of ginger cultivar Rio-de-Janeiro was thoroughly washed in water and treated with 0.1 per cent mercuric chloride for 15 min and again washed in water. Kept in Indofil M-45 0.25 per cent for one hour and dried under shade. The treated rhizomes were incubated in sterilized sand at room temperature to initiate sprouting. For surface sterilization, the pale yellow sprouts were treated with cefotaxime 250 mg l^{-1} for one hour followed by mercuric chloride 0.1 per cent for 5 min under aseptic condition. After this treatment, the sprouts were washed thoroughly.

in sterile distilled water. The buds were placed onto, MS medium containing cefotaxime 250 mg l^{-1} + BA 2 mg l^{-1} / 3 mg l^{-1} gelled with 0.8 per cent agar to induce multiple shoots. The multiple shoots were subcultured onto the same medium at 25 ± 1°C under 16/8 h photoperiod.

3.7 STANDARDISATION OF MEDIA FOR INDUCING CALLUSING

Young shoot buds, leaf and pesudostem explants isolated from four weeks old shoot cultures were cut into small segments and were inoculated into each culture tube with different growth regulator combinations. The following media compositions were tried (Table 6).

Table 6. Media combination for inducing callus

1. MS + 0.0 mg l^{-1} 2,4-D
2. MS + 0.5 mg l^{-1}2,4-D
3. MS + 1.0 mg l^{-1}2,4-D
4. MS + 1.5 mg l^{-1}2,4-D
5. MS + 2.0 mg l^{-1}2,4-D
6. MS + 3.0 mg l^{-1}2,4-D
7. MS + 6.0 mg l^{-1}2,4-D
8. MS + 8.0 mg l^{-1}2,4-D
9. MS + 0.5 mg l^{-1} NAA
10. MS + 1.0 mg l^{-1} NAA
11. MS + 2.0 mg l^{-1} NAA
12. MS + 3.0 mg l^{-1} NAA
13. MS + 6.0 mg l^{-1} NAA
14. MS + 8.0 mg l^{-1} NAA
15. MS + Dicamba 0.5 mg l^{-1}.
16. MS + Dicamba 1.0 mg l^{-1}
17. MS + Dicamba 1.5 mg l^{-1}
18. MS + Dicamba 2.0 mg l^{-1}
19. MS + Dicamba 2.5 mg l^{-1}
20. MS + Dicamba 3.0 mg l^{-1}
21. MS + 0.0 mg l^{-1} BA + 0.0 mg l^{-1} 2,4-D
22. MS + 0.5 mg l^{-1} BA + 0.5 mg l^{-1} 2,4-D
23. MS + 0.5 mg l^{-1} BA + 1.0 mg l^{-1} 2,4-D
24. MS + 0.5 mg l^{-1} BA + 1.5 mg l^{-1} 2,4-D
25. MS + 0.5 mg l^{-1} BA + 2.0 mg l^{-1} 2,4-D
26. MS + 0.5 mg l^{-1} BA + 3.0 mg l^{-1} 2,4-D
27. MS + 0.5 mg l^{-1} BA + 6.0 mg l^{-1} 2,4-D
28. MS + 0.5 mg l^{-1} BA + 8.0 mg l^{-1} 2,4-D

Four weeks after inoculation, observations were recorded at monthly intervals per explant in each treatment (Number of replication - 12; subculturing at 25 days interval).

3.8 ESTABLISHMENT OF CALLUS CULTURES

Media composition as well as the suitable explant were standardised and utilized for the establishment of callus cultures. Young shoot buds from the *in vitro* cultures were used as the explant. The young buds were cultured in test tubes in the medium consisting of full MS + cefotaxime 250 mg l^{-1} supplemented with 1.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA. Callus cultures were maintained by subculturing every 25th day on fresh medium containing the same growth regulator combination. The cultures were kept in dark at 25 ± 2°C for callus induction (four weeks) and then to light (16/8 h photoperiod).

The observations on time taken for callusing, number of explants callused and callus growth were recorded. Based on percentage of explant callused and callus growth score, callus index (CI) was calculated as given below:

$$CI = P \times G$$

where P is the percentage of explant callusing and G is the average growth score of the callusing explants. The maximum growth score of four was given when the surface of the media in the tube was fully covered by callus (Score one for $1/4^{th}$ coverage; score two for ½ coverage; score three for $3/4^{th}$ coverage).

3.9 PROLIFERATION OF CALLUS

Callus cultures were maintained in the medium consisting of full MS + cefotaxime 250 mg l^{-1} supplemented with 1.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA. Different periods of dark conditions (2weeks and 4 weeks) and a series of sub cultures were also tried to induce proliferation.

3.10 INDUCTION OF REGENERATION

Different combinations of BA and 2,4-D were tried for induction of regeneration from the callus induced (½ MS supplemented with BA 0, 1.0, 2.0, 3.0 mg l^{-1}) in combination with 2,4-D (0, 0.5 mg l^{-1}).

3.11 EVALUATION OF SENSITIVITY OF GINGER TISSUES TO VARIOUS ANTIBIOTICS

Leaf segments of size 1.0 cm² and young buds were tested for their sensitivity to various antibiotics. A preculturing of 3-4 days was given.

The callus induction medium viz. full MS supplemented with 1.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA was prepared in conical flasks of suitable size and stored in culture room until use. On the day of the experiment, the medium was melted and then cooled to a temperature of 40° to 50°C. It was then taken to the laminar flow cabinet where the required quantity of sterilized antibiotic was added and mixed thoroughly by vigorous shaking. The medium containing the antibiotic was dispensed to sterile disposible petriplates and allowed to cool and solidify. When the medium cooled down to room temperature, the explant was inoculated.

The experiments were repeated three times to evaluate the antibiotic sensitivity of ginger tissues. Details of the experiments are given in Table 7. Observation on induction of callus were recorded.

Table 7. Details of experiments conducted for evaluating antibiotic sensitivity of leaf/bud tissues of ginger

Antibiotics mg l^{-1}			
Kanamycin	Hygromycin	Cefotaxime	Carbenicillin
Control	Control	Control	Control
10	10	200	200
20	20	300	300
40	40	-	-
60	60	-	-
80	-	-	-
100	-	-	-
120	-	-	-
140	-	-	-
160	-	-	-
180	-	-	-
200	-	-	-
Control	-	-	-

Explants: Leaf, young buds
Subculturing interval: 21 days

3.12 BACTERICIDAL EFFECTS OF CARBENICILLIN AND CEFOTAXIME

Single colonies of *Agrobacterium tumefaciens* EHA 105, LBA 4404, GV 2260 were resuspended in 5 ml of YM broth and incubated at 30°C for 36-48 h. Then 20 µl of culture was transferred to 3 ml of YEM broth containing different concentrations of carbenicillin and cefotaxime and the cultures were incubated at 30°C on a shaker operating at 250 rev./min for 36-48 h. The concentration of cells was determined by measuring the optical density at 550nm$_{(OD\ 550)}$.

Table 8. Details of treatments tried for evaluating the bactericidal effect of carbenicillin and cefotaxime

Treatments	*Antibiotic concentration mg l^{-1}
1	Blank
2	Control
3	50
4	100
5	200
6	400
7	600
8	800
9	1000
10	1200
11	1400
12	1600
13	1800
14	2000
15	2200

* Same concentration for both antibiotics.

3.13 COLLECTION AND MAINTENANCE OF *AGROBACTERIUM* STRAINS

Agrobacterium tumefaciens EHA 105 (Hood *et al.*, 1993) containing the binary plasmid P35S GUS INT (Vancanneyt *et al.*, 1990) was used as one of the vector systems for transformation. *A. tumefaciens* EHA 105 is a disarmed derivative of *A. tumefaciens* A 281 (Hood *et al.*, 1993). The T-DNA contains the NOS pro-NPT II-NOS ter cassette as selectable marker and the 35 S pro-GUS INT-35 S ter cassette as reporter marker. The intron inserted in the protein coding region of the GUS INT gene prevents the translation of the corresponding mRNA in *A. tumefaciens* (Fig.1). Bacteria were cultured overnight on a shaker at 28°C and 200 rpm in YEM medium containing Rifampicin 20 mg l^{-1} and Kanamycin 50 mg l^{-1}.

Fig. 1. Schematic representation of *Agrobacterium tumefaciens* EHA 105 containing the binary plasmid p35SGUSINT

NPT II - Neomycin phosphotransferase
GUS - β glucuronidase
NOS - Nopaline synthase promoter

RB - Right border
LB - Left border
35S - 35S Promoter

3.13.1 Production of *Agrobacterium* transformants

3.13.1.1 Agrobacterium transformation by triparental mating

For triparental mating (Ditta *et al.*, 1980), the binary plasmid from *E. coli* was transferred into *Agrobacterium* by a conjugation process.

1. *Agrobacterium* strain LBA 4404: Harbours a derivative of Ti plasmid that lacks T-DNA region but contains the *vir*-region (*vir* helper strain), grows at 30°C on AB minimal medium + rifampicin 20 µg/ml.

2. Binary plasmid pBZ 100 in *E. coli* contains the TDNA borders and multiplies in *E. coli* and *Agrobacterium*. *E. coli* harbouring this plasmid grows on LB with 50 µg/ml kanamycin. pBZ 100 is not self transmissible from *E. coli* to *Agrobacterium* (Fig.2). It is possible with the help of pRK 2013 (helper plasmid).

3. pRK 2013 in *E. coli*: This plasmid when introduced into *E. coli* strain harbouring pBZ 100, mobilizes pBZ 100 into *Agrobacterium*. *E. coli* harbouring pRK 2013 grows on LB medium with 50 µg/ml kanamycin.

3.13.1.2 Triparental mating

Day 4 (-) LBA 4404 is streaked to get single colonies on AB minimal medium with 20 µg/ml rifampicin at 28-30°C.

Day 1 (-) The two *E. coli* strains were streaked on LB plates with 50 µg/ml kanamycin.

Day (+1) Took 100 µl of LB medium (liquid) in a sterile Eppendorf tube, dispensed one colony each from three of the above cultures and kept overnight.

Day (+2) Took an antibiotic free LB plate and plated the above suspension on 2/3 portion and the remaining 1/3 was streaked with *Agro*. LBA 4404, *E. coli* (1) and *E. coli* (2).

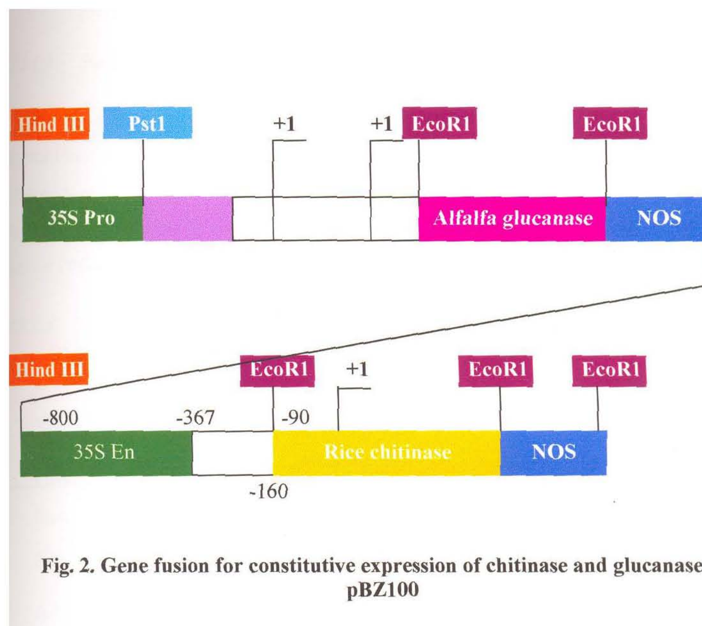

Fig. 2. Gene fusion for constitutive expression of chitinase and glucanase in pBZ100

Day (+3) The bacterial growth was scraped out and streaked on LB plate with kanamycin 50 + rifampicin 20 on 2/3 portion. 1/3 portion were split into three and streaked with LBA 4404, pBZ 100 and pRK 2013.

Day (+4) The recombinants alone will grow and single loop streaked on YEP/AB medium with kanamycin 50 + refampicin 20 and kept at 30°C under dark.

3.13.1.3 Verification of plasmid presence and structure

Isolation of plasmid from *Agrobacterium* by Alkaline lysis mini preparation method (Sambrook *et al.,* 1989) was used to confirm both the presence and structure of vector sequences within transformed *Agrobacterium* colonies. With binary vectors, alkaline lysis mini preps from *Agrobacterium* will produce sufficient plasmid DNA to enable diagnostic restriction digestion and gel electrophoresis.

3.13.1.4 Agrobacterium **mini plasmid preparation**

1. Inoculated 5 ml culture (YEP medium supplemented with antibiotics) and shaked at 28°C to early log phase (approx. 36 h).

2. Centrifuged 1.5 ml of the culture at 12000 x g in a microfuge for 10 min. Resuspended the bacterial pellet in 100 µl cell suspension solution (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA (pH 8.0).

3. Added 20 µl of 20 mg/ml lysozyme solution, mix well and incubate at 37°C for 15 min.

4. Added 200 µl of cell lysis solution (0.2 M NaOH, 1% SDS) mix completely by repeated gentle inversion of the tube.

5. Added 50 µl phenol equilibrated with 2 vol. of cell lysis solution and vortex to mix.

6. Added 200 µl of neutralization solution (3 M sodium Acetate) pH 5.2 mix completely by repeated inversion of the tube.

7. Centrifuged at 12000 x g for 5 min, transfer the upper aqueous phase to a second microfuge tube, add 2.5 volumes 95% ethanol and place on ice for 10 min.

8. Centrifuged at 12000 x g for 5 min to spindown the DNA/RNA pellet and re-ethanol precipitate from 400 µl TE (20 mM Tris/HCl, 0.1 mM EDTA (pH 7.8).

9. Resuspended the pellet in 50 µl TE.

10. Extracted the resuspended pellet once with phenol: chloroform.

11. Removed aqueous phase and pellet DNA by addition of 0.5 vol. of 7.5 M CH_3COONH_4 and 2.5 vol. of 100 per cent ethanol for 1 h at -20°C.

12. Washed with 70 per cent alcohol, dissolve in TE (pH 8.0) check on 0.6 per cent gel.

3.13.2 *Agrobacterium* transformation by freeze thaw method (An *et al.*, 1988)

Bin AR is a Bin 19 derivative (binary vector: M Bevan (84) NAR 12, 8711) containing expression cassette for constitutive expression of chimaeric genes in plants. Expression cassette cloned into EcoRI/Hind III sites of Bin 19(Fig.3).

Cassette: 770 bp EcoRI/Hind III fragment containing the CaMV 35 S promoter, a partial pUC 18 polylinker and the OCS terminator.

Unique polylinker sites: Kpn I/Sma I/Bam HI/Xba I/Sal I

The recombinant plasmid named as pBin AR osm (13.25 kb) contains osmotin cDNA between Bam HI and Xba I sites. Xba I site is lost during ligation and therefore cDNA (0.75 kb) can be taken out by restriction with Bam HI and Xba I. The recombinant plasmid Bin AR which was available in *E. coli* DH 5α cells. The *Agrobacterium* strain GV 2260 (Deblaere *et al.*, 1985) is an octopine type virulent strain carrying pGV 2260.

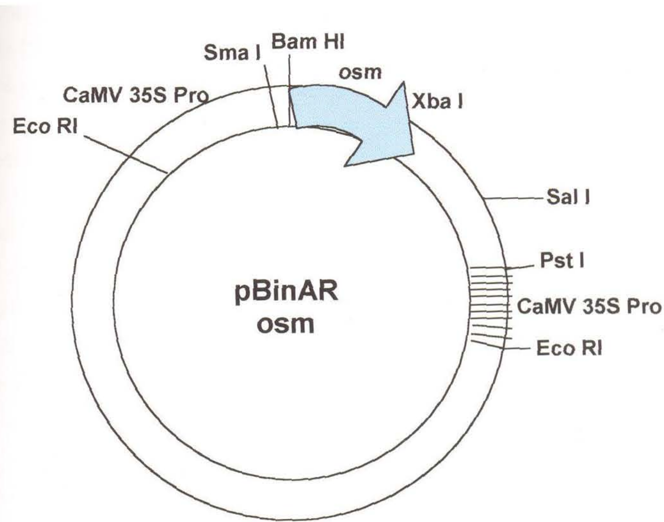

Fig. 3. Osmotin construct with 35 S promoter in pBin-AR

3.13.2.1 Confirmation of the presence of recombinant plasmid in E.coli DH5∝ cells

3.13.2.1.1 Isolation of plasmid from E.coli DH5∝ cells

The plasmid was isolated from *E. coli* cells harbouring the plasmid BinAR by alkaline lysis method (Birnboim and Dolly, 1979). Single colonies were picked up and inoculated in 25ml Luria broth (see media and solutions) with the antibiotic kanamycin (50 mg l^{-1}) contained in a 100ml autoclaved conical flask. It was incubated in the shaker at 37°C at 200 rpm overnight. 1.5 ml of overnight grown culture was taken in a 2 ml Eppendorf tube and centrifuged at 12,000 rpm for 30 seconds at 4°C. The supernatant was discarded and the pellet was suspended in 200μl of ice-cold solution-I (see media and solutions). Lysozyme (2mgl^{-1}) was added immediately before use. The cells were mixed thoroughly by vortexing immediately after adding solution-I and kept on ice for 5 min. Then freshly prepared solution-II was added and gently mixed by inverting the tubes. It was kept on ice for 5 min. 300 μl of ice-cold solution-III was added and mixed by inverting the tubes several times. It was kept on ice for 5 min. and then spin at 12000 rpm for 5 min at 4°C. The pellet was discarded and the supernatant was transferred to a fresh eppendorf tube. To the supernatant, twice the volume of isopropanol was added and kept on ice for 20 min. It was centrifuged at 12000 rpm for 15 min at 4°C. The supernatant was removed by gentle aspiration and the pellet was rinsed with 70 per cent ethyl-alcohol. The pellet was dried for 20 min in air, till the last drop of liquid was removed. Subsequently, the pellet was suspended in 30 μl of TE (pH 8.0). Quantification of the plasmid DNA was done by spectrophotometry.

3.13.2.1.2 Restriction digestion and checking of the insert

The plasmid DNA was restricted with BamHI and XbaI enzymes. The reaction mixture containing 15 μl of plasmid DNA, 2.0 μl of BamHI enzymes, 2.0 μl of XbaI enzyme 2.0 μl of multicore buffer and 4.0 μl of double distilled water, was incubated in water bath at 37°C for 4 h. The digest was loaded onto 0.8 per cent agarose gel. The electrophoresis was carried out at 55V for 1 h. The presence of two

bands, one corresponding to the vector (12.5 kb) and the other corresponding to the insert (0.75 kb) was checked.

3.13.2.1.3 Transformation of Agrobacterium tumefaciens by Freeze thaw method (An et al., 1988)

The *Agrobacterium* strain (GV 2260) was grown in 50ml yeast manitol broth containing rifampicin (50 mgl^{-1}) and carbenicillin (100 mg l^{-1}) antibiotics and incubated at 28°C under shaking at 200 rpm for 16-18 h to reach ($O.D_{600nm}$ 0.5-0.6). The cells were pelleted by centrifugation at 10000 rpm for 5 min at 4°C and suspended in 0.5 ml of 85% (v/v) 100 mM $CaCl_2$ and kept on ice for 30 min and centrifuged. 100 µl of aliquot was taken and about 2 µl of plasmid DNA was added and cells were quickly frozen in liquid nitrogen for 2 min followed by thawing at 37°C for 5 min and kept on ice for 10 min. About 1 ml of YEM was added and incubated at 28°C for 16 h with shaking. After 16-18 h of incubation, 100µl of the cells was spread on plates containing YEM agar, kanamycin (50 mg l^{-1}) rifampicin (50 mg l^{-1}) and carbenicillin (100 mg l^{-1}). Colonies were observed after two days.

3.13.2.1.4 Isolation, restriction digestion and checking of the plasmid from Agrobacterium tumefaciens

To confirm the transfer of the binary plasmid from *E. coli* to *Agrobacterium*, the plasmid was first isolated by adopting the alkaline lysis procedure (Birnboin and Dolly, 1979) as outlined earlier. After isolation, the plasmid was digested with both BamHI and Xba-I enzymes. The reaction mixture in an eppendorf tube containing 15 µl of plasmid DNA, 1.5 µl of BamHI enzymes, 1.5 µl of Xba-I enzyme, 2 µl of multicore buffer and 2 µl of double distilled water was incubated at 37°C for 4 h. The digest was then loaded onto 0.8 per cent agarose gel. The gel electrophoresis was carried out at 50V for 2 h. The presence of 2 bands, one corresponding to the vector (12.5 kb) and the other corresponding to the insert (0.75 kb) were checked on the gel as earlier done with the plasmid DNA from *E. coli* cells.

3.14 CULTURE AND MAINTENANCE OF *AGROBACTERIUM* STRAINS

The YEP/YEM medium was prepared in required volume in conical flask and kept in culture room until use. On the day of use, the medium was melted and then cooled to 40-50°C. Inside a laminar flow cabinet, the corresponding sterilized antibiotics were added to the medium and mixed thoroughly by vigorous shaking. The medium was then distributed on to sterile petriplates and allowed to solidify and attain room temperature.

EHA 105 → Kanamycin 50 + Rifampicin 20

LBA 4404 (R.C) → Kanamycin 50 + Rifampicin 20

GV 2260 (R.C) → Kanamycin 50 + Carbenicillin 100 + Rifampicin20

Using a sterile bacterial loop, a single colony was scooped from the previous bacterial culture plate and streaked onto freshly prepared solidified medium. The newly streaked bacterial plate was sealed with parafilm and incubated in inverted position at room temperature (28°C). The bacterial culture developed in two days. The bacterium was subcultured once in 21 days.

3.15 *AGROBACTERIUM* INOCULATION TECHNIQUE FOR GINGER PLANT TISSUES

3.15.1 Explant source material

The ginger transformation protocol utilized young buds and embryogenic calli as explant for transformation.

3.15.1.1 Procedure

1. Shoot bud cultures were raised 4-6 weeks prior to transformation in ½ MS medium containing BA 2 mg l^{-1}. The multiple shoots were subcultured onto the same medium at 25±1°C under 16/8 h photoperiod.

2. Young buds produced aseptically on 4 weeks old cultures were used for transformation.

3.15.2 Explanting and preculture

3.15.2.1 Procedure

1. Preculture plates were prepared by plating callus induction medium in a 90 mm petriplate.

2. The aseptically produced shoot buds were cut using scalped blade and explants were placed on each preculture plates.

3. The preculture plates containing the explants were sealed using parafilm and kept in dark in a culture room for different preculture periods.

3.15.3 *Agrobacterium* inoculation and co-culture

3.15.3.1 Procedure

1. *Agrobacterium* liquid cultures were prepared by inoculating 2 ml of liquid YEM with 2-3 bacterial colonies obtained from an *Agrobacterium* culture grown on solid YEM. Inoculated the liquid culture from a plate of *Agrobacterium* grown at 28°C for 2-3 days. The streaked plate could be reused for approximately four weeks if kept refrigerated.

2. The liquid *Agrobacterium* cultures were grown overnight at 26-28°C on a shaker at 200 rpm then subcultured by adding 200 µl of the overnight culture to 25 ml of liquid YEM and grown upto 16 h in order to ensure that the culture was actively growing. In some species, log phase *Agrobacterium* cultures yield a higher transformation response.

3. The bacterial suspension was taken in a sterile centrifuge tube and centrifuged at 10,000 rpm for 15 min, pelleted, washed and resuspended in liquid MS and dilutions were made.

4. Prior to preparing the final explant inoculum the concentration of the bacteria in the final subculture was determined. A portion of the final subculture was diluted 1:10 in MS liquid medium. The optical density (O.D) at 660 nm of the dilution

was obtained, MS medium without bacteria was used as the blank. To determine the bacterial cell number of the final subculture, the obtained O.D of the 1:10 dilution was multiplied by a standard conversion number (1.0 $O.D_{550\ nm} = 3 \times 10^9$ cells) and then multiplied by 10 to compensate for the dilution factor.

5. The final bacterial inoculum was prepared by diluting the final subculture in MS liquid so that the bacterial concentration could be adjusted to 1×10^9 cells/ml.

6. The explants were then removed from the pre-culture plate and placed in a sterile petriplate. The *Agrobacterium* inoculum was added so that it covered the explant. After the incubation of explants and *Agrobacterium* for 5 min and 10 min, the liquid was pipetted off. The explants were then sandwiched between 2 layers of dry, sterile filter paper to remove excess inoculum and then returned down to the original preculture plates.

7. The explants were allowed to co-culture with the *Agrobacterium* for different periods. Co-culture occurred on the same medium and environmental conditions used for preculture.

3.16 SELECTION AND PLANT REGENERATION

After co-culture, *Agrobacterium* growth was inhibited and explant regeneration was induced. *Agrobacterium* growth was inhibited by the addition of bacteriostatic antibiotics such as carbenicillin/ cefotaxime to the regeneration medium. The regeneration medium also contain a selective agent to ensure the preferential growth of transgenic cells.

3.16.1 Procedure

1. Immediately after co-culture, explants were placed on callus induction medium containing kanamycin for selection and cefotaxime to eliminate continued *Agrobacterium* growth.

2. The explants were kept at dark for 2-4 weeks and then in 16/8 h photoperiod.

3. Regular subculturing of the explants were done at 21 days interval.

4. The embryogenic callus produced on the selection medium were transferred to shoot induction medium.

3.17 GENETIC TRANSFORMATION OF GINGER

Genetic transformation experiments with were carried out using *Agrobacterium tumefaciens* strains EHA 105 p35SGUSINT for standardizing optimum conditions using young buds as explants. Each experiment consisted of different treatment combinations along with two controls, which were common for the experiment. In one of the controls, the explants without bacterial infection were placed in callus induction medium containing antibiotics for selection and the other control consisted of explants without bacterial treatment placed in the callus induction medium without antibiotics. Sub culturing was carried out at 21 days interval.

3.17.1 Assessment of factors affecting transformation

3.17.1.1 Influence of preculturing and density of the initial bacterial inoculum

It consisted of 9 treatment combinations and two controls. The nine treatment combinations varied among themselves with respect to preculture of explants (1, 2, 3, 4, 5 days) and bacterial dilution (1;0, 1:5, 1:10, 1:20 v/v). The precultured explants were treated with their respective induction media having a bacterial density of $O.D._{550\ nm} = 0.8$. After co-cultivation of two days, the explants were washed, dried and transferred to callus induction medium containing 100 mg l^{-1} kanamycin and 300 mg l^{-1} cefotaxime sub culturing was done at 21 days interval. The explants at random were checked for the transient gus expression and the treatments compared.

3.17.1.2 Effect of infection time on growth of Agrobacterium

This consisted of five treatment combinations and two controls, with varied infection time (5, 10, 15, 20, 30 minutes). After the preculture of three days, the explants were treated with a bacterial dilution of 1:20 (v/v). The explants were then

blot dried and cocultivated with bacteria for two days. The explants were subcultured after three weeks.

3.17.1.3 Influence of co-cultivation period on transformation of ginger

In this experiment, five treatment combinations viz. different co-cultivation periods (15 min, 1, 2, 3, 4 days) were tried. The explants were precultured for three days and treated for 5 min in their induction media having a bacterial dilution of 1:20 (v/v). The cocultivated explants were transferred to selection media and subculturing were done at 21 days interval.

3.17.1.4 Effect of post cultivation in darkness

This experiment included four treatments and two controls. Treatment consisted of different periods of exposure of explants to dark condition viz. 0, 2, 4, 8 weeks.

The explants were precultured for three days in callus induction medium and treated for 5 min with induction media having a bacterial dilution of 1:20 (v/v) and co-cultivated for two days. The explants were subcultured at an interval of 21 days.

3.17.1.5 Standardisation of acetosyringone concentration for increasing the efficiency of transformation

The effect of acetosyringone in increasing the transformation efficiency of the optimized general protocol for transformation was studied.

Incubation periods tried in liquid medium were 0, 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 h. Acetosyringone concentrations were 0, 100, 200, 300, 400, 500, 1000 μM.

Agrobacterium tumefaciens strains EHA 105 were grown overnight at 28°C in AB minimal medium at pH 7.0 (Chilton *et al.*, 1977) containing 5 g l^{-1} sucrose, kanamycin 50 mg l^{-1} and 200 μm acetosyringone to activate *vir* genes. Co-cultivation of suspension cells with 200 μm acetosyringone treated *A. tumefaciens* was carried out using a two step co-cultivation method.

The first step involved, the co-cultivation of young buds of ginger with 200 μm acetosyringone treated *A. tumefaciens* strain EHA 105. The bacteria were pelleted and resuspended in YEM liquid medium 1:20 v/v dilutions were made and incubated in a shaker at 28°C. The explants were co-cultivated after 0 h, 0.5 h, 1.0 h, 2.0 h, 4.0 h, 6.0 h and 8.0 h. After co-cultivation the explants were air dried and placed on to callus induction medium and kept under dark condition for two days.

In the 2nd step, the explants were co-cultivated with 200 μm acetosyringone treated *A. tumefaciens* strain EHA 105. Before co-cultivation, the bacterium cells were co-cultivated for 8 h in 1:20 diluted bacterial suspension with YEM liquid medium. Callus induction medium containing acetosyringone (0, 100, 200, 300, 400, 500 and 1000 μM) were prepared and the air dried explants after infection were inoculated and kept in the dark for two days. Number of GUS spots per 100 mg cell clumps were calculated and compared with the treatment.

3.17.2 Genetic transformation of embryogenic callus and buds

After optimizing the condition for transformation, experiments were conducted using *Agrobacterium tumefaciens* strains with useful genes in embryogenic calli and young bud as explants.

Embryogenic calli produced from young buds were selected, cut into small pieces of size 1.0 cm and used as explant for transformation. Two *Agrobacterium tumefaciens* strains EHA 105 and GV 2260 (R.C) were used. The calli were immersed for 5 min in the bacterial culture.

The calli were air dried over a sterile filter paper and cocultivated for two days and transferred to selection medium containing cefotaxime 300 mg l^{-1} and kanamycin 100 mg l^{-1}. The explants were subcultured into fresh screening medium at an interval of two weeks. After two subculturing, transferred to regeneration medium (1/2 MS+ 3 mgl^{-1} BA) containing antibiotics.

Transformation of young buds were also carried out using the optimum conditions.

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3.18 GUS ASSAY

Explants were assayed for expression of gus A INT gene following the histochemical procedure described by Jefferson *et al.* (1987).

3.18.1 Staining leaves for GUS activity

1. Prepared X-Gluc solution (Appendix - VI)
2. Incubated explant in the above solution for 24-48 h at 37°C in dark.
3. Following overnight incubation, explants were cleaned and fixed in 95 per cent (v/v) ethanol: 1 per cent (v/v) acetic acid.
4. Transient gus A INT expression was measured immediately after co-cultivation by counting the number of GUS expressing zones appearing as blue spots on a white background after the staining procedure.
5. The number of GUS expressing calli that developed on kanamycin selection medium after 4-5 weeks was determined by counting the number of calli with blue zones after X-Gluc incubation.
6. GUS assays were replicated 3 times with a minimum of 5-10 explants per treatment per replication.
7. The test was repeated on leaves of regenerated plants.

3.19 MOLECULAR CHARACTERIZATION OF TRANSGENIC PLANTS

The presence of the transgene in the putative transgenic plantlets was analysed by PCR.

3.19.1 Isolation of plant DNA

For subsequent molecular analysis, DNA was isolated from leaf tissues of the putative transgenic plants, following the method by Doyle and Doyle (1987).

Procedure

1. Pre warmed the lysis buffer in 30 ml centrifuge tube.
2. Ground 0.5 g plant tissue with liquid nitrogen, 3 ml extraction buffer using a mortar and pistle.
3. Poured the homogenate to a fresh 30 ml tube containing 3 ml lysis buffer, pre warmed and maintained at 65°C and add 1 ml saxosine.
4. Mixed the content by slight inversion.
5. Kept the centrifuge tube at 50-65°C for 15 min.
6. Added equal volume (7 ml) of chloroform : Isoamyl alcohol mixture (24:1).
7. Mixed gently by slight inversion (5 times)
8. Centrifuged at 10,000 rpm for 15 min at 4°C
9. Transferred the upper aquous phase to new centrifuge tube
10. Added 0.6 volume chilled isopropanol
11. Mixed gently and keep at -20°C for half an hour
12. Collected DNA pellet by centrifugation at 10,000 rpm for 15 min at 4°C
13. Discarded isopropanol, wash the pellet with 70 per cent absolute ethanol centrifuge, collect pellet after each wash.
14. Finally air dried pellet and dissolved in very minimum quantity TE buffer.
15. Did RNAse treatment
16. Electrophoresed on 1 per cent agarose.

3.19.2 Polymerase chain reaction (PCR)

PCR was performed to identify the presence of the marker gene (*npt*-II) in the transgenic plants. DNA isolated from the plant tissue was used as template. Primers of *npt*-II gene were used to amplify the fragment of interest with the reaction conditions as indicated below:

Primer *npt*-II (Bangalore Genei)

Forward primer 5' CAA TCG GCT GCT CTG ATG CCG 3'

Reverse primer 5' AGG CGA TAG AAG GCA ATG CGC 3'

Reaction mixture for PCR

Components	Actual volume used
1. Template DNA	1 µl
2. Forward primer	1 µl
3. Reverse primer	1 µl
4. d NTP mix	1 µl (10 mM)
5. Taq buffer	2.5 µl
6. Taq DNA polymerase	2.0 µl (0.6 units)
7. Sterile double distilled water	16.5 µl
Total volume	25 µl

PCR programme (For 30 cycles)

DNA amplification was carried out on PCR machine (MJ Research) with the following cycles.

Condition	Temperature in °C	Time (in minutes)
1. Initial denaturation	94	2
2. Denaturation	94	1
3. Primer annealing	54	1
4. Primer extension	72	2
5. Hold go to '2' (repeat 29 cycles)		

Final extension 72°C for 10 min

A control was also maintained with non-transformed plantlet.

The PCR products were visualised in 0.8 per cent ethidium bromide stained agarose gel along with 1 kb DNA ladder mix as marker.

Results

4. RESULTS

The results of the experiments on *Agrobacterium* mediated genetic transformation of ginger (*Zingiber officinale* Rosc.) conducted at the Department of Plantation Crops and Spices and CPBMP, College of Horticulture, Vellanikkara during the period from 1999 to 2005 are presented in this chapter.

4.1 STANDARDISATION OF *IN VITRO* REGENERATION PROTOCOL IN GINGER

4.1.1 Standardisation of surface sterilization procedure

Three experiments were done for this purpose. The results of these are described as follows:

Table 9. Effect of various surface sterilants on culture establishment

Sl. No.	Sterilant	Concentration (%)	Duration	Contamination due to bacteria (%)	Survival (%)
1	Mercuric chloride	0.1	5 min	100	00
2	Mercuric chloride	0.1	10 min	90	10
3	Ethanol	95	10 sec	100	00
4	Ethanol + Mercuric chloride	95 + 0.1	10 sec + 5 min	100	00

Medium - ½ MS + sucrose 3% + BA 2 mg l^{-1}
Explant - rhizome buds
(Average of 12 observations, 20 days after inoculation)

The ginger buds collected were subjected to various surface sterilization treatments and the results obtained are presented in Table 9. Treating the ginger buds either in ethanol 95 per cent for 10 seconds or mercuric chloride 0.1 per cent for 5 min or in combination were not effective in controlling the bacterial contamination as no survival was registered in both the treatments. Mercuric chloride 0.1 per cent for 10 min registered 10 per cent survival of the cultures. The bacterium present in the culture was isolated and identified as *Ralstonia solanacearum* [Yabuuchi (Smith)] It was gram negative, rod shaped and motile with one or more polar flagella or non motile without flagella.

4.1.1.1 *In vitro sensitivity of Ralstonia solanacearum to antibiotics*

The diameter of the inhibition zone made by each chemical was measured after 24 h of incubation and presented in Table 10. The study revealed that streptomycin, chloramphenicol and cefotaxime could inhibit the growth of bacteria.

Table 10. Screening of antibiotics towards sensitivity of *Ralstonia solanacearum*

Sl. No.	Chemicals	Inhibition zone (mm)				
		250 mg l^{-1}	500 mg l^{-1}	750 mg l^{-1}	1000 mg l^{-1}	Mean
1	Ampicillin	1.5	1.8	1.9	1.9	1.8
2	Streptomycin	2.7	2.8	2.9	2.9	2.8
3	Chloramphenicol	2.8	2.9	3.1	3.0	2.9
4	Nalidixic acid	1.1	1.3	1.2	1.4	1.3
5	Tetracyclin	1.6	1.6	1.7	1.8	1.7
6	Cefotaxime	3.0	3.1	3.1	3.2	3.1
7	Control	0	0	0	0	0

4.1.1.2 *Management of bacterial interference in cultures of rhizome bud*

The antibiotics streptocyclin, chloramphenicol and cefotaxime were selected from those tested against *Ralstonia solanacearum* under *in vitro* condition for management of bacterial interference in cultures and the results are presented in Table 11. Here no fungal contamination was noticed and cultures were destroyed by bacteria alone. Rinsing the ginger buds with 0.1 per cent mercuric chloride for 10 min followed by washing with sterile water three times did not give any control against bacterial infection as no survival of cultures was observed. The survival percentage was more in treatments containing chloramphenicol, higher concentration of 1000 mg l^{-1} being resulted in better survival percentage (30%) compared to lower concentrations. The treatment involving dipping the bud in Teepol containing water for 5 min followed by treatment with Indofil M-45 0.25 per cent for 30 min followed by washing in water and treatment with mercuric chloride 0.1 per cent for 10 min under aseptic condition and washing thoroughly in sterile water five times and placing on to medium containing chloramphenicol 1000 mg l^{-1} resulted in a survival of 70 per

cent compared to other antibiotics tested. Cefotaxime at 250 mg l^{-1} being the lowest concentration gave better control than other treatments. Cefotaxime 250 mg l^{-1} incorporated in the media resulted in the survival of 90 per cent of the cultures.

Table 11. Management of bacterial interference in cultures of ginger buds

Sl. No.	Treatments	Duration	Contamination due to bacteria (%)	Survival (%)
1	Mercuric chloride 0.1%	10 min	100	00
2	Streptocyclin 500 mg l^{-1} + $HgCl_2$ 0.1%	1 hr + 10 min	100	00
3	Streptocyclin 1000 mg l^{-1} + $HgCl_2$ 0.1%	1 hr + 10 min	95	5
4	Chloramphenicol 500 mg l^{-1} + $HgCl_2$ 0.1%	1 hr + 10 min	95	5
5	Chloramphenicol 1000 mg l^{-1} + $HgCl_2$ 0.1%	1 hr + 10 min	90	10
6	Streptocyclin 1000 mg l^{-1} (incorporated in media) + $HgCl_2$ 0.1%	10 min	95	5
7	Chloramphenicol 1000 mg l^{-1} (incorporated in media) + $HgCl_2$ 0.1%	10 min	30	70
8	Cefotaxime 250 mg l^{-1} + $HgCl_2$ 0.1%	10 min	90	10
9	Cefotaxime 250 mg l^{-1} (incorporated in media) + $HgCl_2$ 0.1%	10 min	10	90

Average of 12 observations, 20 days after inoculation
Medium - ½ MS + sucrose 3% + BA 2 mg l^{-1}
Explant - rhizome bud

4.2 ESTABLISHMENT OF GINGER CULTURES

4.2.1 Production of shoot bud cultures of ginger from young sprouts / buds

Following the surface sterilization procedure, young buds from rhizomes of ginger cultivar Rio-de-Janeiro was used for establishment of cultures for transformation studies. 100 per cent of the cultures produced shoots and roots on MS media containing BA (2 mg l^{-1} and 3 mg l^{-1}). The number of shoot buds produced was more (10-12 per tube) in media containing BA 3 mg l^{-1} (Table 12). The decapitated crown sections cultured in the same medium produced more number of shoots (15-20) within 14 days (Plates 1 and 2).

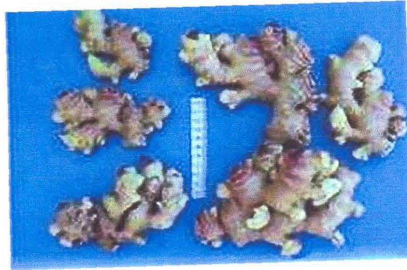


Plate 1. Ginger rhizomes showing buds

Plate 2. Shoot bud culture of ginger

Table 12. Effect of BA on shoot and root production by decapitated crown sections of ginger one month after initiation of culture in solid medium

Growth regulator concentration (mg l^{-1}) BA	Morphogenesis (%)		Production of shoots per pseudostem (No.)
	Shoot	Root	
0.0	10	10	1-2
2.0	100	100	8-10
3.0	100	100	10-12

4.3 STANDARDISATION OF CALLUS MEDIATED REGENERATION PROTOCOL

4.3.1 Effect of 2,4-D and NAA on leaf and pseudostem explants of ginger

Leaf and pseudostem explants taken from one month old aseptic cultures were cultured in full MS medium with two different growth regulator combinations of 2,4-D and NAA. The data on percentage of callusing are given in Table 13.

The results clearly show that, 2,4-D concentration ranging from 0.5 to 8.0 mg l^{-1} and NAA concentration ranging from 0.5 to 8.0 mg l^{-1} were not effective in inducing callusing for both explants. It was observed that at lower concentrations the leaf explants showed green colour and slight swelling but at higher concentrations, the explants became bleached after two subculturing.

Table 13. Effect of 2,4-D and NAA on leaf and pseudostem explants of ginger on callus induction

Auxin		No. of cultures callusing (%)		Callus index	
2,4-D (mg l^{-1})	NAA (mg l^{-1})	2,4-D	NAA	2,4-D	NAA
0.5	0.5	0	0	0	0
1.0	1.0	0	0	0	0
2.0	2.0	0	0	0	0
3.0	3.0	0	0	0	0
6.0	6.0	0	0	0	0
8.0	8.0	0	0	0	0

0 - No response; Number of replications - 12; Subculturing - 25 days interval

4.3.2 Effect of dicamba on callus induction of leaf and pseudostem explants of ginger

Leaf and pseudostem explants taken from one month old *in vitro* grown cultures were cultured in full MS medium with dicamba concentrations ranging from (0.5-3.0 mg l^{-1}). The data on percentage of callusing and callus index were given in Table 14.

It was observed that dicamba, concentration ranging from (0.5 to 3 mg l^{-1}), was not effective in inducing callusing in leaves whereas callus was produced from pseudostem at lower concentrations. At 0.5 mg l^{-1} and 1 mg l^{-1}, the callus produced was negligible. However, at a concentration of 1.5 mg l^{-1}, callus produced was more and 30 per cent of the cultures showed callusing.

Table 14. Effect of dicamba on leaf and pseudostem explants of ginger

Diacamba (mg l^{-1})	Callusing (%)		Callus index	
	Leaf	Pseudostem	Leaf	Pseudostem
0.5	0	35	0	35
1.0	0	25	0	25
1.5	0	30	0	60
2.0	0	0	0	0
2.5	0	0	0	0
3.0	0	0	0	0

0 = No response
Number of replications - 12

4.3.3 Effect of auxins on callus induction in bud explants of ginger

Bud explants from *in vitro* grown cultures were cultured in full MS medium under two different growth regulator combinations of 2,4-D and NAA. The data on percentage of callusing and amount of callus produced are given in Table 15.

Table 15. Effect of auxins on callus induction in bud explants of ginger

Auxin		Callusing (%)		Callus Index	
2,4-D (mg l^{-1})	NAA (mg l^{-1})	2,4-D	NAA	2,4-D	NAA
0.5	0.5	0	0	0	0
1.0	1.0	0	0	0	0
2.0	2.0	20	0	20	0
3.0	3.0	25	20	50	20
6.0	6.0	0	20	0	20
8.0	8.0	0	0	0	0

0 = No response
Number of replications - 12

2,4-D at concentrations ranging from 2.0 mg l^{-1} and 3.0 mg l^{-1} was effective in inducing callusing and the best concentration being 3 mg l^{-1} with 25 per cent callusing. NAA induced a slight amount of callusing at higher concentrations of 3.0 mg l^{-1} and 6.0 mg l^{-1}. The amount of callus produced was less compared to 2,4-D (callus index 20-50) (Plate 3).

4.3.4 Effect of 2,4-D and BA on leaf and pseudostem explants of ginger

Explants (pseudostem and leaf) taken from one month old axenic cultures were cultured under two different growth regulator combinations of BA and 2,4-D. The data on percentage of callusing and callus index are presented in Table 16. 2,4-D and BA had no effect on callus induction on leaves whereas callus was produced from pseudostem in the growth regulator combination of 2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA (70%) and 3 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA (60%). However, the amount of callus produced was negligible.

NAA 6.0 mg l^{-1}

2,4-D 3.0 mg l^{-1}

Plate 3. Effect of auxins on callus induction (bud explant)

Plate 4. Effect of Dicamba on callus induction (0.5 mg l^{-1})

Table 16. Effect of 2,4-D and BA on leaf tissue and pseudostem explants of ginger

Growth regulator concentration (mg l^{-1})		Callusing (%)		Callus index	
2,4-D	BA	Leaf	Pseudostem	Leaf	Pseudostem
0.5	0.5	0	0	0	0
1.0	0.5	0	0	0	0
2.0	0.5	0	70	0	140
3.0	0.5	0	60	0	120
6.0	0.5	0	0	0	0
8.0	0.5	0	0	0	0

0 - No response
Number of replications - 12

4.3.5 Embryogenic callus induction in bud explants of ginger

The data on callus induction in full MS in six combinations involving 2,4-D and BA, and dicamba are presented in Table 17. Among the combination tried, with 1.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA, 90 per cent of the explants produced embryogenic cultures. Callus induction was also noticed when dicamba was used at very low concentration of 0.5 mg l^{-1} (55%). All other concentrations showed hairy root growth (Plate 4).

Table 17. Effect of auxin and cytokinin on callus induction in bud explant of ginger

Auxin 2,4-D	Cytokinin BA	Explants producing callus (%)	Type of callus (%)		
			Embryogenic	Non-embryogenic	Mean
0.5	0.5	10	-	100	50
1.0	0.5	90	35	40	38
2.0	0.5	30	R	20	10
3.0	0.5	0	R	-	-
6.0	0.5	0	R	-	-
8.0	0.5	0	-	-	-
Diacamba					
0.5	-	55	25	30	27.5
1.0	-	10	R	-	-
1.5	-	0	R	-	-
2.0	-	0	R	-	-
2.5	-	0	R	-	-
3.0	-	0	R	-	-

R - roots (hairy roots)
0 - No response
Number of replications - 12

Table 18. Somatic embryogenesis from embryogenic callus of ginger

Sl. No.	Cultivar	Induction media (mg l^{-1})	Regeneration media (mg l^{-1})	Time taken for callusing	Time taken for germination of SE	Time taken for shoot initiation	Time taken for shoot differentiation	Time taken for root differentiation	Time taken for plantlets formation	No. of embryos developed / calli	Viability of the embryo (%)	Survival rate of embryo derived plant
1	Rio-de-Janeiro	1.0 2,4-D + 0.5 BA	3 BA	30 days	25 days	30 days	35 days	30	45 days	2-9	25-45	3-6

Media - MS + 1.0 mg l^{-1} 2,4-D + BA 0.5 mg l^{-1}

4.3.6 Somatic embryogenesis and plant regeneration

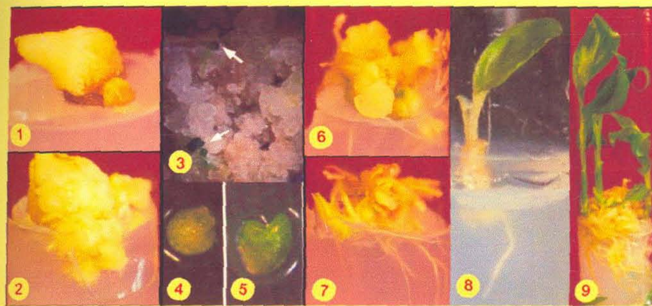
The data on somatic embryogenesis are shown in Table 18. After 14-18 days of culture, the bud became enlarged and showed callus initiation. Callus proliferation was noticed after 3-4 weeks. Callus induction and proliferation needed dark condition. The first sign of embryogenesis was marked by the appearance of white globular structures that were attached to the surface of nodular callus by a distinct stalk after 3-4 weeks. Further differentiation led to the formation of lateral notch which was the first stage of individualization of the embryogenic axis from the scutellum. Simultaneous development of shoot and root was observed in full MS medium containing 3.0 mg l^{-1} BA + 0.5 mg l^{-1} 2,4-D. It took four weeks for the germination of somatic embryo, two weeks for shoot and root differentiation and 40-45 days for the complete development of plantlets. At optimum conditions, 2-8 somatic embryos developed per culture tube and the survival rate of the embryo ranged between 25-75 per cent. It took 4-5 months from callus induction to plantlet formation (Plates 5, 6 and 7).

4.3.7 Proliferation of callus and plant regeneration

Among the different combinations tried for inducing callusing, MS medium supplemented with 1.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA was effective in inducing callusing. Two to three subculturing at an interval of 25 days in the same medium resulted in the proliferation of callus. At this concentration 90 per cent of the explants produced embryogenic callus.

The embryogenic calli were cultured on MS basal medium supplemented with varying levels of cytokinin (BA 0, 1, 2, 3 mg l^{-1}) and auxin (2,4-D 0, 0.5 mg l^{-1}) (Table 19).

Culturing of fresh calli on MS medium without growth regulators resulted in rhizogenesis. Half MS medium supplemented with cytokinin alone did not result in organogenesis even after four cycles of subculture. Addition of BA 3.0 mg l^{-1} in the presence of 2,4-D 0.5 mg l^{-1} resulted in embryogenesis and subsequent development of plantlets.



1 - 2 : Induction of friable embryogenic callus (FEC) on youngbuds after 4 weeks of culture on MS medium with 1 mg 1^{-1} 2, 4 - D + 0.5 mg 1^{-1} BA.

3 : Developing somatic embryos (SE s arrow) on FEC after 4 weeks of subculture bar 2.5 mm.

4 -5: Isolated SEs at different stages of development. 4. globular stage (bar 1 m.m.). 5. torpedo stage (bar 1 m.m)

6-7: Germinating SE with root and shoot development after 4 weeks.

8 - 9: SE derived plants.

Plate 5. Somatic embryogenesis and plant regeneration in ginger

Plate 6. Callus showing regeneration

Plate 7. Germinating somatic embryos

Table 19. Effect of auxin and cytokinin on callus proliferation and plant regeneration

Growth regulator concentration (mg l^{-1})		Morphogenic response	Cultures showing response (%)
BA	2,4-D		
0	0	Only root developed	100
1	0	Callus growth	100
2	0	Callus growth	100
3	0	Good callus growth	100
1	0.5	Callus growth	100
2	0.5	Shoot bud primordia developed	80
3	0.5	Well developed shoot formation	90

Explant - callus
Number of replications - 12

4.3.8 Effect of explants on plant regeneration through callus phase on MS basal medium

Table 20 shows the morphogenic response of the explants. The explants differed in their ability to form callus. The highest amount of callus was obtained from vegetative buds followed by pseudostem. The amount of callus produced was 3.1 g per tube from vegetative bud and 2.1 g per tube from pseudostem. The leaves did not show any response under various growth regulator combinations.

Table 20. Effect of explants on plant regeneration through callus phase on MS basal medium

Sl. No.	Explant	Callus production (g)	Morphogenic pathway	Days taken for morphogenesis	No. of plantlets /culture
1	Vegetative bud	3.1	Somatic embryogenesis	165	3-6
2	Leaf	0	0	-	-
3	Pseudostem	2.1	-	-	-

Number of replications - 12
Media - MS + 1.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA

4.4 EVALUATION OF GINGER CULTURES FOR SENSITIVITY TO ANTIBIOTICS

Successful transformation using *Agrobacterium* utilizes various antibiotics for the elimination of *Agrobacterium* from the culture. The optimum concentration of antibiotics are to be standardised and the toxicity of explants towards the lethal dose have to be evaluated.

Various experiments were carried out to find out the optimum level of antibiotics and results are presented hereunder.

4.4.1 Sensitivity of plant tissues to antibiotics

Carbenicillin and cefotaxime were found effective in inhibiting *Agrobacterium* growth. Cefotaxime is the antibiotic of choice in *Agrobacterium* mediated transformation because carbenicillin shows high toxicity. The toxic effects of both the antibiotics on ginger bud explants were examined by incubating the bud explant on MS medium (callus induction medium) with cefotaxime 200, 300 mg l^{-1} and carbenicillin 200 and 300 mg l^{-1}. No toxic effects were observed by incubating bud explants on MS plates containing cefotaxime 200 and 300 mg l^{-1}. At 200 mg l^{-1} concentration, 90 per cent of the explants showed callusing and 80 per cent showed shoot and root differentiation in the shoot induction medium. However, toxic effects on ginger bud explants were clearly seen when these explants were grown in MS medium containing carbenicillin. No callus initiation was noticed and the colour of the tissue turned into brown (Table 21). From these results, it could be concluded that cefotaxime at 300 mg l^{-1} could be effectively used for the elimination of bacteria during *Agrobacterium* mediated transformation and after two cycles of subculture, the concentration could be reduced to 200 mg l^{-1} (Plates 8 and 9).

Table 21. Sensitivity of buds to varying concentrations of carbenicillin and cefotaxime

Antibiotics	Dosage (mg l^{-1})	Explants showing calli (%)	Calli showing shoot and root differentiation (%)
Cefotaxime	200	90	80
	300	70	60
Carbenicillin	200	0	0 (bleached)
	300	0	0 (bleached)
Control	-	90	80

Culture duration - 8 weeks
Subculturing - 21 days interval
Media - MS + 1.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA

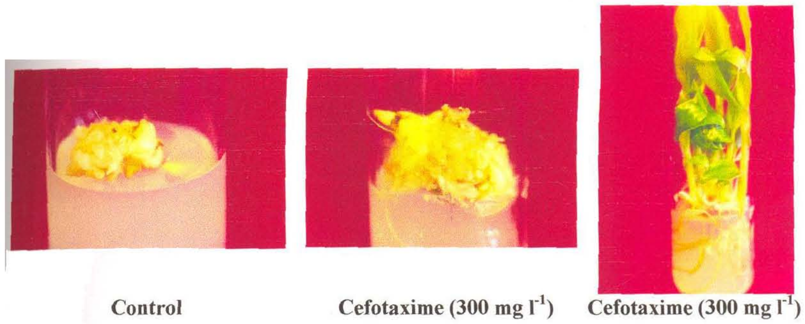
4.4.2 Sensitivity of cultured tissues of *Zingiber officinale* to kanamycin

Sensitivity of the leaf tissues to kanamycin at concentrations ranging from 10 to 200 mg l^{-1}, subcultured at an interval of 21 days was tested. The data after 8 weeks are given in Table 22.

Table 22. Sensitivity of leaves to kanamycin

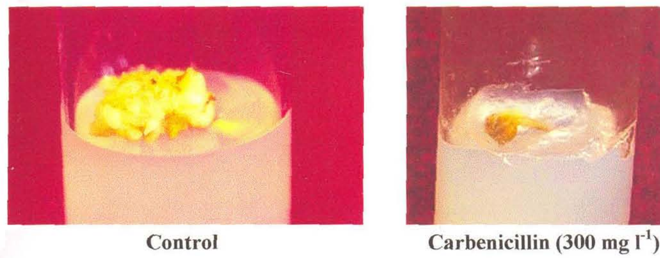
Dosage (mg l^{-1})	Percent of explants showing callus	Appearance
10	0	Green colour
20	0	"
40	0	"
60	0	"
80	0	"
100	0	Bleached
120	0	"
140	0	"
160	0	"
180	0	"
200	0	"
Control	70	Green colour

Culture duration - 8 weeks
Subculturing - 21 days interval
Media - MS + 1.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA



Control Cefotaxime (300 mg l^{-1}) Cefotaxime (300 mg l^{-1})

Plate 8. Sensitivity of buds to cefotaxime



Control Carbenicillin (300 mg l^{-1})

Plate 9. Sensitivity of buds to carbenicillin

When young leaf discs were used as explant, kanamycin treated leaf discs became white, the degree of bleaching increasing with kanamycin concentration. Control leaf discs gradually browned throughout the experiment (Plate 10).

Table 23. Sensitivity of buds to different concentrations of kanamycin

Dosage (mg l^{-1})	Percentage of explant showing callusing	Appearance
10	100	good callus
20	100	good callus
40	100	good callus
60	60	slow growing callus
80	0	bleached
100	0	bleached
120	0	bleached
140	0	bleached
160	0	bleached
180	0	bleached
200	0	bleached
Control	100	good callus

Culture duration - 8 weeks
Subculturing - 21 days interval
Media - MS + 1.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA

The kanamycin sensitivity of callus growth, shoot and root formation were studied in ginger to investigate the suitability of kanamycin resistance as a selectable marker for ginger. Kanamycin concentrations ranged from 0 to 140 mg l^{-1}. Cefotaxime was added to the medium in all experiments at a concentration of 300 mg l^{-1}.

From the results it could be observed that kanamycin decreased callus growth compared to control (Table 23). Callus formation was observed at 0, 10, 20, 40, 60 mg l^{-1}. The small increase in fresh weight observed at higher kanamycin concentration was presumably due to cell expansion. Kanamycin 100 mg l^{-1} completely inhibited callus initiation. Subsequently, shoot and roots developed from control (young buds grown without kanamycin and cefotaxime) (Plate 11).

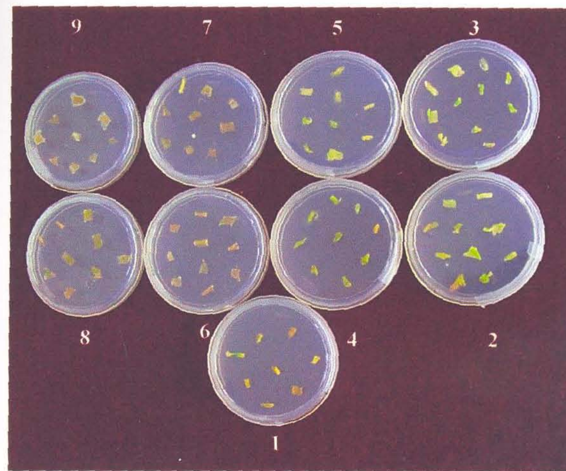


Plate 10. Sensitivity of leaves to kanamycin

1 - Control; 2 - Kanamycin 10 mg l^{-1}; 3 - Kanamycin 20 mg l^{-1}; 4 - Kanamycin 40 mg l^{-1}; 5 - Kanamycin 60 mg l^{-1}; 6 - Kanamycin 80 mg l^{-1}; 7 - Kanamycin 100 mg l^{-1}; 8 - Kanamycin 120 mg l^{-1}; 9 - Kanamycin 140 mg l^{-1};

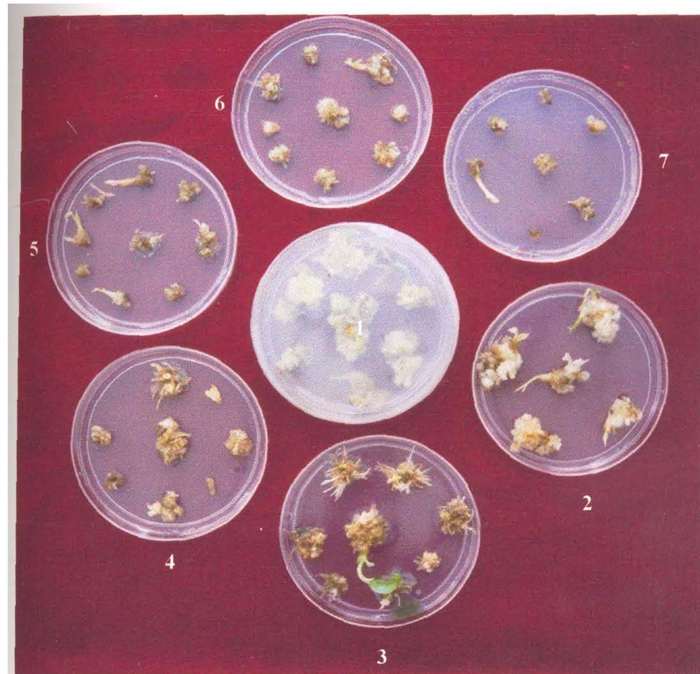


Plate 11. Sensitivity of buds to kanamycin

1 - Control; 2 - Kanamycin 10 mg l^{-1}; 3 - Kanamycin 20 mg l^{-1}; 4 - Kanamycin 40 mg l^{-1}; 5 - Kanamycin 60 mg l^{-1}; 6 - Kanamycin 80 mg l^{-1}; 7 - Kanamycin 100 mg l^{-1}

4.4.3 Sensitivity of different explants towards hygromycin

In this experiment, sensitivity of leaf and young buds to four different concentrations of hygromycin were tested. The data are given in Table 24. In control, all the explants seemed to be fresh and the buds showed callusing (90%), while hygromycin treated explants, irrespective of the type of explants used, showed bleaching after one week. The plant tissue is highly sensitive to hygromycin (Plates 12 and 13).

Table 24. Sensitivity of leaf and buds to varying concentrations of hygromycin

Antibiotic and their concentrations	Explant used	Percentage of explants showing growth
Hygromycin 10 mg l^{-1}	Leaf	0
	Buds	0
20 mg l^{-1}	Leaf	0
	Buds	0
40 mg l^{-1}	Leaf	0
	Buds	0
60 mg l^{-1}	Leaf	0
	Buds	0
Control	Leaf	0
	Buds	90

Subculturing at 21 days interval
Culture duration - 8 weeks
Media - MS + 1.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA

4.5 BACTERICIDAL EFFECT OF CARBENICILLIN AND CEFOTAXIME

In order to examine the antibacterial effects of carbenicillin and cefotaxime, three different strains of *A. tumefaciens* EHA 105, LBA 4404 and GV 2260 were incubated in YEM broth with different concentrations of the antibiotics. For carbenicillin, the inhibition of cell growth started at a concentration between 200 mg l^{-1} and 400 mg l^{-1} for all the three strains studied. For cefotaxime, the inhibition of cell growth started at a concentration of 200 mg l^{-1} for all the strains and the complete inhibition of cells growth occurred with 300 mg l^{-1} (Tables 25 and 26). These results demonstrated that *A. tumefaciens* cells were more sensitive to cefotaxime and carbenicillin (Figs. 5 and 6).

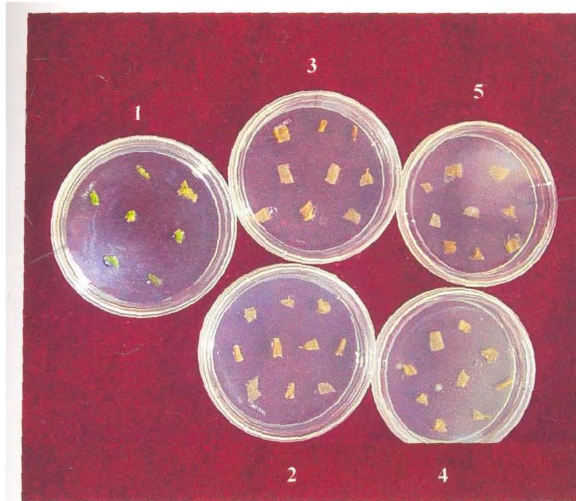


Plate 12. Sensitivity of leaves to hygromycin

1 - Control; 2 - Hygromycin 10 mg l^{-1}; 3 - Hygromycin 20 mg l^{-1}; 4- Hygromycin 40 mg l^{-1}; 5 - Hygromycin 60 mg l^{-1}

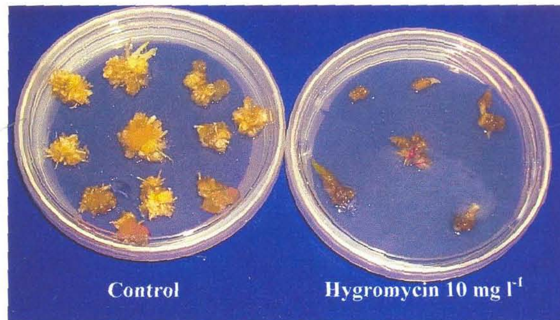


Plate 13. Sensitivity of buds to hygromycin

Table 25. Bactericidal effect of carbenicillin on *Agrobacterium* strains

Antibiotics (conc.mgl[1])	$OD_{(550nm)}$		
	EHA-105	GV 2260	LBA 4404
Blank	0	0	0
Control	1.380	1.050	0.503
50	0.502	0.498	0.501
100	0.402	0.489	0.500
200	0.400	0.480	0.510
400	0.030	0.040	0.030
600	0.040	0.030	0.020
800	0.030	0.021	0.030
1000	0.020	0.020	0.040
1200	0.020	0.028	0.039
1400	0.020	0.026	0.028
1600	0.020	0.020	0.026
1800	0.020	0.020	0.021
2000	0.030	0.022	0.023
2200	0.020	0.020	0.020

Table 26. Bactericidal effect of cefotaxime on *Agrobacterium* strains

Antibiotic concentration (mgl[1])	$OD_{(550nm)}$		
	EHA-105	GV 2260	LBA 4404
Blank	0	0	0
Control	1.380	1.050	0.503
50	1.080	1.020	0.501
100	0.050	1.010	0.498
200	0.020	0.020	0.020
400	0.022	0.020	0.020
600	0.014	0.013	0.020
800	0.027	0.012	0.050
1000	0.020	0.020	0.010
1200	0.040	0.040	0.028
1400	0.029	0.030	0.021
1600	0.031	0.021	0.022
1800	0.028	0.018	0.031
2000	0.021	0.019	0.031
2200	0.020	0.020	0.020

4.6 ESTABLISHMENT OF BACTERIAL CULTURE

4.6.1 Collection and maintenance of *Agrobacterium* strains

Agrobacterium strains were collected and maintained in the medium for further transformation studies.

Table 27. Description of *Agrobacterium* strains

Sl. No.	Name of the construct	Source	Plasmid	Selection marker
1	EHA-105	NRCPB, New Delhi	p35SGUS-INT	*npt*-II
2	GV2260	NRCPB, New Delhi	pBinARosm	*npt*-II
3	LBA4404	RGCB, TVM	pBZ100 (glucanase + chitinase)	*npt*-II

npt-II Neomycin phosphotransferace

Agrobacterium tumefaciens EHA105 containing plasmid p35SGUSINT capable of expressing GUS gene was used as the vector system for standardizing optimum condition for effecting genetic transformation in ginger. It contains genes for the scorable marker, β-glucuronidase (GUS) and the selectable marker *npt*-II. GUS is very stable and tissue extracts continued to show high levels of GUS activity after prolonged storage. Histochemical analysis was carried out to demonstrate the localization of gene activity in cells and tissues of transformed plants.

4.6.1.1 Production of Agrobacterium transforments

4.6.1.1.1 Agrobacterium transformation by triparental mating

Recombinants of *Agrobacterium tumefaciens* LBA4404 containing the binary plasmid pBZ100 were produced using the method triparental mating. The binary plasmid pBZ100 containing the gene construct (chitinase + glucanase) was mobilized into the native *Agrobacterium* strain LBA4404 using the helper plasmid pRK2013.

Presence of the binary plasmid in the recombinants were verified using *Agrobacterium* mini plasmid preparation method (Plate 14).

4.6.1.1.2 Agrobacterium transformation by freeze thaw method

Recombinants of *Agrobacterium tumefaciens* GV2260 containing the binary plasmid pBinAR were produced by freeze thaw method. The binary plasmid was isolated from *E. coli* DH5α cells and quantified (Plate 15). The plasmid DNA was restricted with BamH1 and xbaI enzymes. The presence of two bands one corresponding to the vector (12.5 kb) and the other corresponding to the insert (0.75 kb) was checked (Plate 16).

The plasmid containing the insert was mobilized to *Agrobacterium* strain GV2260 and the presence of the plasmid in the *Agrobacterium* was confirmed by restriction digestion and gel electrophoresis (Plate 17).

4.7 GENETIC TRANSFORMATION OF GINGER

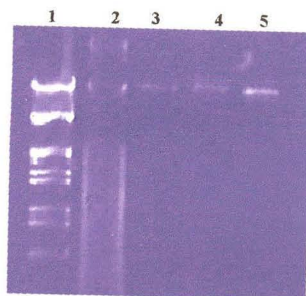
After standardization of the regeneration protocol and determination of the antibiotic sensitivity to plant tissues, genetic transformation experiments were carried out to optimise the conditions for effecting transformation. The effects of factors such as co-cultivation period, preculture of explants, bacterial density, infection time, post cultivation in darkness, use of inducers such as acetosyringone were assessed. The results of various experiments are as given under:

4.7.1 Assessment of factors affecting transformation

To evaluate the influence of diverse factors on the efficiency of T-DNA transfer, experiments were conducted to observe the levels of transient GUS expression in inoculated buds.

4.7.1.1 Influence of preculturing and density of the initial bacterial inoculum on transient GUS expression of ginger (bud explant)

In this experiment, different preculture period and different bacterial dilutions were tested and GUS activity scored as number of GUS spots/100 mg of cell



Lane 1 : Marker DNA
Lane 2,3,4,5: plasmid DNA of pBZ100

Plate 14. Isolation of plasmid DNA (pBZ100) from LBA 4404 (Recombinant) using Alkali mini prep method

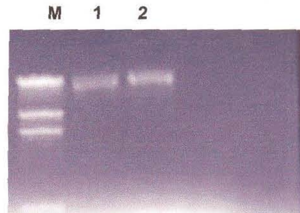
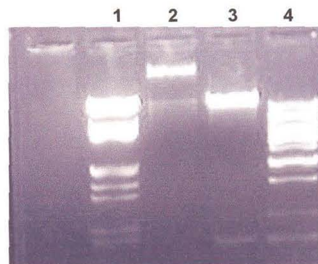


Plate 15. Isolation of plasmid from *E.coli* DH5∝ using Alkalin lysis method (Birnboin and Dolly, 1979)

M : Marker DNA, Lane 1 : plasmid DNA of pBinAR and Lane 2 : plasmid DNA of pBinAR



Upper band (12.5 kb) shows the vector pBinAR and lower band (0.75 kb) represents osmotin insert

Plate 16. Restriction digestion of pBinAR from *E.coli* cells with BamHI and Xba-I enzymes.

Lane 1 : Eco RI + Hind III marker, Lane 2 : Uncut plasmid DNA,
Lane 3 : Restricted plasmid DNA, and Lane 4 : 1 kb ladder

Upper band (12.5 kb) shows the vector pBinAR and lower band (0.75 kb) represents osmotin insert

Plate 17. Restriction digestion of pBinAR from *Agrobacterium* with BamHI and XbaI enzymes

Lane 1 : Eco RI + Hind III marker, Lane 2 : Uncut plasmid DNA,
Lane 3-4 : Restricted plasmid DNA, Lane 5 : 1 kb ladder

clumps. Among the different bacterial dilutions tested, 1:20 (V/V) was found optimum, since it gave optimum growth of bacteria after co-cultivation. GUS activity was highest in cells precultured for three days and with a bacterial dilution of 1:20 (6.5 GUS + spots) (Table 28).

Table 28. Influence of preculturing and density of the initial bacterial inoculum on transient GUS expression of ginger (bud explant)

Treat-ment	Pre-culture	Induction medium			Concentration of antibiotics in the selection medium		No. of GUS spots / 100 mg cell clumps
		Bacterial dilution v/v	Induct-ion time (min.)	Co-cultivat-ion (days)	Cefotaxime mg l^{-1}	Kanamycin mg l^{-1}	
1	1	1:0	5	2	300	100	0
2	1	1:5	5	2	300	100	1.0
3	1	1:10	5	2	300	100	2.0
4	1	1:20	5	2	300	100	2.0
5	2	1:0	5	2	300	100	2.0
6	2	1:5	5	2	300	100	2.0
7	2	1:10	5	2	300	100	2.0
8	2	1:20	5	2	300	100	2.0
9	3	1:0	5	2	300	100	2.5
10	3	1:5	5	2	300	100	3.0
11	3	1:10	5	2	300	100	3.5
12	3	1:20	5	2	300	100	6.5
13	4	1:0	5	2	300	100	2.0
14	4	1:5	5	2	300	100	2.0
15	4	1:10	5	2	300	100	2.0
16	4	1:20	5	2	300	100	2.5
17	5	1:0	5	2	300	100	2.5
18	5	1:5	5	2	300	100	3.0
19	5	1:10	5	2	300	100	3.0
20	5	1:20	5	2	300	100	2.5
21	Control W.A.				300	100	
22	Control W.O.A				300	100	

WA-with antibiotics, WOA-without antibiotics

Media - MS + 1.0 mg l^{-1} 2,4-D + BA 0.5 mg l^{-1}

Influence of preculturing on genetic transformation was studied and is presented in Table 29. Preculturing of explants prior to inoculation and co-cultivation with *Agrobacterium* was found to improve genetic transformation frequencies. Preculturing of ginger bud explants for three days on callus induction medium

increased the transformation frequency almost eight times compared to without preculturing.

Table 29. Influence of preculture of explants on transient GUS expression

Days to preculture	GUS + buds (%)	Mean number of blue spots / 50 explants
0	20	10
1	20	10
2	20	40
3	88	80
4	40	30
5	40	30

(50 buds were tested for each assay)
Media - MS + 1.0 mg l^{-1} 2,4-D + BA 0.5 mg l^{-1}

Callus formation also increased in precultured explants when co-cultivated with *Agrobacterium* after cutting. The preculture had a positive effect on the induction of competent cells for transformation (Fig. 7).

The influence of a wide range of initial bacterial inoculum on genetic transformation was studied and presented in Table 30. The frequency of genetic transformation was assessed by histochemical staining for GUS activity at two days after co-cultivation. An inoculam level of 1.0 x 10^9 cfu ml^{-1} was found optimum.

Table 30. The effect of density of inoculating *Agrobacterium* suspension on the transformation on ginger bud culture

Sl. No.	Inoculum level (cfu ml^{-1})	Transient GUS activity (GUS spots / explant)
1	12 x 10^9	2.0
2	3 x 10^9	2.5
3	1.3 x 10^9	3.5
4	1.0 x 10^9	6.5

Media - MS + 1.0 mg l^{-1} 2,4-D + BA 0.5 mg l^{-1}

4.7.1.2 *Influence of co-cultivation period on transformation of ginger*

Table 31 shows the influence of the co-cultivation period on transformation of ginger bud explants. Histochemical GUS assays indicated that transformation had occurred at specific zones of the explants, and each spot represented an independent transformation event. When the explants were transferred to selection medium immediately after infection with *Agrobacterium* (no co-cultivation), no transformation was observed. The transformation frequency was very low after one day of co-cultivation but increased rapidly when the co-culture was prolonged to two days, reaching a maximum at three days. Coculture duration had no apparent effect on the area of transformed tissue per transformation event. In ginger, beyond two days of co-cultivation resulted in abundant proliferation of the bacteria and it was found very difficult to eliminate the bacteria during selection. Therefore a two day co-cultivation was routinely used (Fig. 8).

Table 31. Influence of co-cultivation period on transformation of ginger

Treat-ment	Pre-culture	Induction medium			Concentration of antibiotics in the selection medium		No. of GUS spots*
		Bacterial density ($O.D_{550}$ nm)	Induct-ion time (min.)	Co-cultivat-ion (days)	Cefotaxime mg l^{-1}	Kanamycin 100 mg l^{-1}	
1	3	0.8	5	15 min	300	100	0
2	3	0.8	5	1	300	100	<5
3	3	0.8	5	2	300	100	50
4	3	0.8	5	3	300	100	60
5	3	0.8	5	4	300	100	60

* Average of 50 explants
Media - MS + 1.0 mg l^{-1} 2,4-D + BA 0.5 mg l^{-1}

4.7.1.3 *Effect of infection time and growth of Agrobacterium on bud explant*

Table 32 shows the effect of different infection time and growth of *Agrobacterium* on explants after co-cultivation. Varied timings from 5 min to 30 min were tried. Except treatment 1 and 2, all others showed over growth of bacteria in the selection medium. With an induction time of 10 min, 80 per cent of the explants

showed bacterial overgrowth and a time of 5 min was found optimum since it showed no overgrowth in the selection media.

Table 32. Effect of infection time on growth of *Agrobacteirum* (bud explant)

Treat-ment	Pre-culture (days)	Bacterial density ($O.D_{550}$ nm)	Bacterial dilution v/v	Infect-ion time (min.)	Concentration of antibiotics (mg l^{-1})		Percentage of explants with bacterial overgrowth
					Cefotaxime	Kanamycin	
1	3	0.8	1:20	5	300	100	Nil
2	3	0.8	1:20	10	300	100	80
3	3	0.8	1:20	15	300	100	100
4	3	0.8	1:20	20	300	100	100
5	3	0.8	1:20	30	300	100	100

Media - MS + 1.0 mg l^{-1} 2,4-D + BA 0.5 mg l^{-1}

4.7.1.4 Effect of post cultivation in darkness

As shown in Fig. 9, culture in darkness clearly improved callus induction. Callus formation and subsequent regeneration were progressively increased by maintaining the explants for two and four weeks in darkness and then transferring them to light. However, when the explants were kept in the dark for four weeks and eight weeks, the callus became white and failed to regain green colour under light conditions (Fig. 10).

From the result it could be observed that exposure of explants to darkness was useful in stimulating callus induction and two weeks was found optimum.

4.7.1.5 Influence of acetosyringone as transformation enhancer

Two preliminary experiments were conducted to clarify the effects of three factors namely (1) co-cultivation of suspension cells in 200 µM acetosyringone activated *A. tumefaciens* strain EHA 105. (2) inclusion of acetosyringone in the

co-cultivation medium and (3) duration of the co-cultivation period on transient GUS expression (Table 33).

Table 33. Effect of acetosyringone on co-cultivation period for *vir* induction

Co-cultivation period in liquid medium (h)	Number of GUS spots per 100 mg cell clumps		Average
	Experiment I	Experiment II	
0.0	2.0	2.0	2.0
0.5	2.0	2.0	2.0
1.0	3.0	2.0	2.5
2.0	3.0	3.0	3.0
4.0	3.0	3.5	3.3
6.0	3.0	4.0	3.5
8.0	5.0	5.5	5.3

(*A. tumefacieus* strain EHA 105 were pelleted and resuspended in YEM liquid medium (1:20 v/v) containing 200 μM acetosyringone, incubated for different period and 2 days of further co-cultivation on (0.8%) agar solidified callus induction medium)

The results showed that 200 μM acetosyringone treated *Agrobacterium* and a two step co-cultivation method i.e. 8 h co-cultivation of suspension cells in a 1:20 (V/V) suspension of *A. tumefaciens* (first step) immediately followed by two days co-cultivation on 0.8 per cent and agar solidified co-cultivation medium containing 200 μM acetosyringone (2nd step) were effective for increasing transient GUS expression of cells.

All the varieties were equally response to *Agrobacterium* infection, no varietal variation was noticed.

GUS activity scored as number of GUS spots per 100 mg of cells was the highest in cells co-cultivated with 200 μM acetosyringone treated *A. tumefaciens* using the two step co-cultivation method.

Inclusion of acetosyringone in the co-cultivation medium also increased the number of GUS spots with the highest number 11.5, obtained with 200 μM (Table 34). However, further increase in concentration reduced the GUS activity (Plates 18 and 19).

Table 34. Effect of acetosyringone on transient GUS expression in ginger

Acetosyringone (μM)	Number of GUS spots per 100 mg cell clumps		Average
	Experiment I	Experiment II	
0	-	-	-
100	4	3	3.5
200	12	11	11.5
300	5	6	5.5
400	5	4	4.5
500	3	4	3.5
1000	2	4	3.0
Without acetosyringone treated strain	3	4	3.5

(200 μM acetosyringone treated *Agrobacterium tumefaciens* strain EHA 105 was co-cultivated for 8h in 1:20 (v/v) diluted bacterial suspension with YEM, 2days of further co-cultivation on agar solidified medium (0.8%) with different concentrations of acetosyringone).

The co-cultivation period also affected transient GUS expression of ginger cells. Transient GUS expression was undetectable in cells co-cultivated for one day but could be observed in cells co-cultivated with two days in 0.8 per cent of agar solidified concentration medium. Extending the co-cultivation period to 3-4 days did not increase GUS activity but caused browning and death of cells on the selective medium.

Thus the above experiments indicated that a suitable transformation protocol for ginger included three days preculture of explants on callus induction medium, a bacterial dilution of 1:20 (v/v) infection time 5 min, co-cultivation of two days and post cultivation on callus induction medium with 100 mg l^{-1} kanamycin and 300 mg l^{-1} cefotaxime under darkness for two weeks, then in 16/8 h photoperiod.

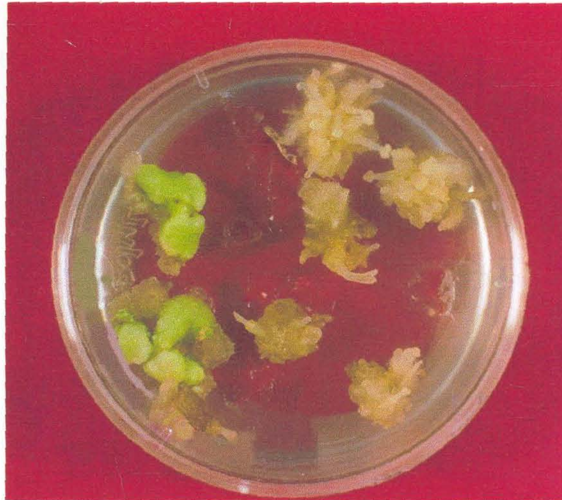


Plate 18. Acetosyringone treated buds showing callusing

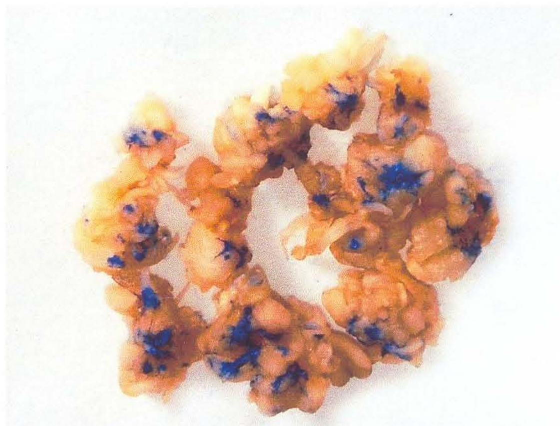


Plate 19. Acetosyringone treated buds showing GUS expression

4.7.1.6 *Transformation experiments using embryogenic calli*

Transformation experiments were conducted with standardised transformation protocol using embryogenic calli which were producing somatic embryos upon sub culture on to fresh MS (callus induction medium) and the results are presented in Table 35. Embryogenic calli were co-cultivated with two *Agrobacterium* strains EHA 105 and GV 2260 (R.C.). Each clump was considered as one independent event. In the selection medium, shoot induction was noticed after 4-5 weeks. The per cent response was between 10-20 per cent. The regenerated shoots showed bacterial growth in the selection medium after three subculturing and the shoot turned yellow and dead (Plates 20 and 21).

Table 35. Transformation of embryogenic callus of ginger with GV 2260 and EHA 105

Strain	Plasmid	No. of ECC co-cultivated	No. of ECC selected in the selection medium	No. of ECC regenerated in regeneration medium	Percentage response
Control		10	0	0	0
EHA 105	p35SGUS-INT	30	27	3	10
GV 2260 (R.C)	pBinAR (Osmotin)	30	28	6	20

ECC-embryogenic callus clumps
Media - MS + 1.0 mg l^{-1} 2,4-D + BA 0.5 mg l^{-1}

Using the optimum conditions, transformation of young buds were carried out and the data obtained are presented in Table 36. The transformation efficiency was very low, ranging from 1.1 to 2.2 per cent. The callus growth was very slow in the presence of antibiotics (Plates 22, 23 and 24).

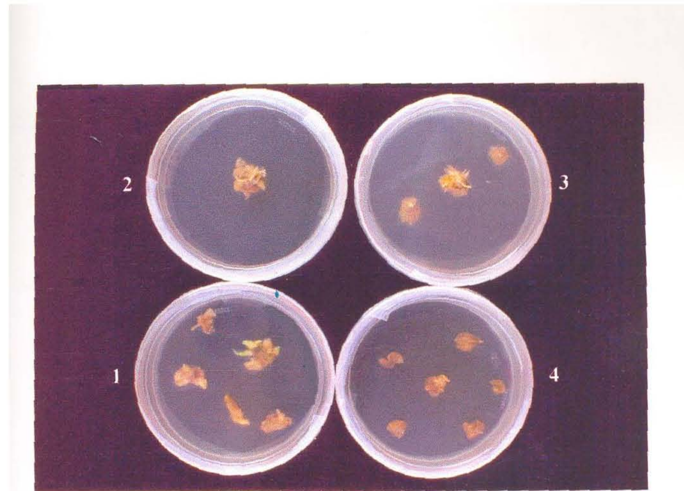


Plate 20. Transformed embryogenic calli with GV 2260

1 to 3 - Transformed calli; 4 - Control

Plate 21. Embryogenic calli showing regeneration

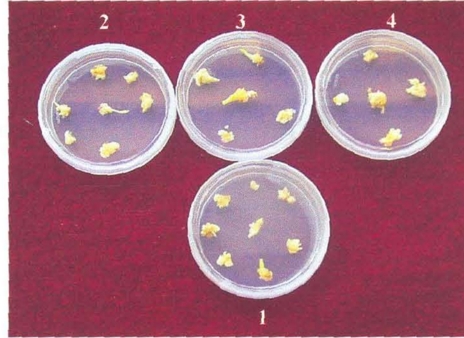


Plate 22. Transformed buds 2 days after co-cultivation

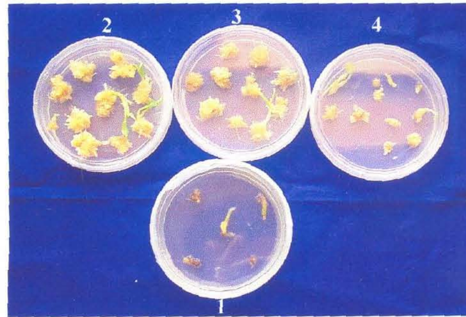


Plate 23. Transformed buds 40 days after co-cultivation

1 - Control; 2 - Transformed buds with GV 2260
3 - Transformed buds with LBA 4404
4 - Negative control (without antibiotics)

Plate 24. Transgenic shoots in regeneration media

Table 36. Transformation of young buds with EHA 105, GV 2260 and LBA 4404

Bacterial strains	Plasmid	Variety	No. of explants co-cultivated	No. of explants regenerated	No. of explants producing shoots	Per-centage
1. EHA 105	p35SGUS-INT	Rio-de-Janeiro	90	10	1	1.1
2. GV 2260	pBinAR (Osmotin)	Rio-de-Janeiro	90	8	2	2.2
3. LBA 4404	pBZ100 (glucanase + chitinase)	Rio-de-Janeiro	90	7	1	1.1
4. Control (non-inoculated)		Rio-de-Janeiro	30	-	-	-

4.8 ANALYSIS OF TRANSFORMED TISSUE

GUS histochemical assays were carried out according to standard protocol (Jefferson *et al.*, 1987) for the regenerated callus using EHA-105 (Plates 25 and 26). Stable expression was detected 40 days after co-cultivation in the calli developed on selective media (Plate 27). The test was repeated on leaves of regenerated plants. Three GUS positive plant clones derived from independent transformation events were regenerated. These plants did not show any morphological alteration under *in vitro* conditions.

Histochemical GUS assays on small leaf portions to detect the expression of GUS A in these shoots were also performed. One of the shoots always gave consistent GUS expression while others did not consistently show the characteristic blue staining in repeated assays (Plates 28 and 29).

4.9 CONFIRMATION OF TRANSGENIC GINGER SHOOTS

Molecular confirmation of the presence of *npt* II gene in the transformed plant was gained by PCR with specific primers. DNA isolated from the transgenic plant along with non transgenic plant as negative control were used in PCR analysis

with primers designed for the amplification of *npt* II gene. The plasmid vector was used as the positive control. The presence of *npt* II gene was confirmed by the amplification of 600 bp fragment amplicon in transgenic plant and in positive control. No amplification was detected in the untransformed plant (Plates 30, 31 and 32).

Plate 25. Transformed buds 40 days after co-cultivation

Plate 26. Transformed buds showing transient GUS expression

Plate 27. Transformed buds showing stable GUS expression

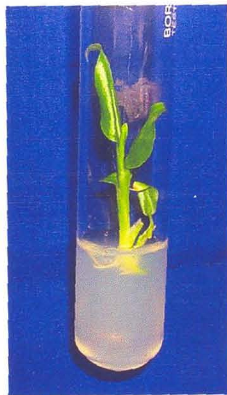
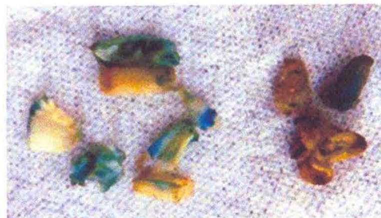


Plate 28. Transgenic shoots in regeneration media



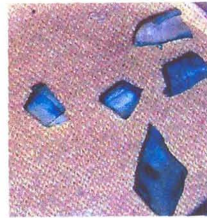
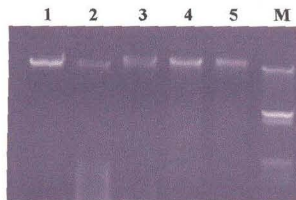
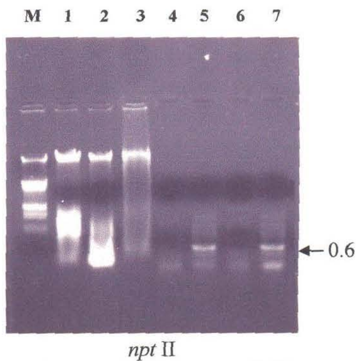


Plate 29. Leaves of the regenerated shoots showing GUS expression



M - Marker DNA;
Lane 1 to 4 - genomic DNA from Putative transgenic ginger shoots
Lane 5 - genomic DNA from untransformed ginger shoots

Plate 30. Electrophoretic analysis of the genomic DNA of ginger shoots



M - Marker DNA (1 kb DNA ladder)
Lane 1 & 2 - genomic DNA from Putative transgenic ginger shoots
Lane 3 - genomic DNA from untransformed ginger shoots
Lane 4 - untransformed control
Lane 5 - transformed
Lane 6 - Negative control
Lane 7 - Positive control

Arrow heads point to amplification of the 600 base pair fragment of the *npt* II gene

Plate 31. Detection of genes in transgenic ginger plants by PCR

Discussion

5. DISCUSSION

Ginger is one of the most important and most widely used spice world wide. Recent advances in plant tissue, cell and protoplast culture combined with genetic engineering have opened up new and exciting possibilities in propagation, gene manipulation, crop improvement and germplasm conservation in many plant species including spice crops.

Crop improvement through biotechnological methods has tremendous advantages in horticultural crops, especially those which are propagated vegetatively. This is especially relevant to ginger because the conventional breeding programmes are hampered due to lack of fertility and natural seed set. The applicability of biotechnology related techniques depends upon the ability to regenerate plants effectively through *in vitro* culture.

The progress made during the last decade has demonstrated that refinement of routine *in vitro* techniques coupled with recombinant DNA technology and genetic engineering have paved way for crop improvement (Potrykus *et al.*, 1985; Potrykus and Spangenberg, 1995). The *Agrobacterium* mediated gene transfer is successfully used in plants, but has limited applications in monocots due to host range limitations. Development of a reliable regeneration system would provide an opportunity to improve *Zingiber officinale* by genetic engineering. High frequency of transformation, broad host range and high rate of expression of inserted genes have made *Agrobacterium* based gene transfer system the most popular one.

Rio-de-Janeiro is a popular high yielding variety of ginger cultivated in Kerala. In the present study, an attempt was made to standardise the optimum conditions of *Agrobacterium tumefaciens* mediated transformation of this variety with the super virulent *Agrobacterium tumefaciens* strain EHA105 having selectable marker genes (*npt* II) and reporter gene (GUS). The results of the studies conducted are discussed in this chapter.

5.1 ESTABLISHMENT OF SHOOT BUD CULTURES OF GINGER

Half MS medium containing BA 3 mg l^{-1} promoted tiny multiple shoots. An average of 1:10 shoots was obtained from an explant over a period of 28 days on half MS medium containing BA 3 mg l^{-1}. When the culture period was prolonged to 40-50 days in the same medium, plantlets attained a height of 6-8 cm and prolific rooting was also observed (Plates 1 and 2).

Leafy aerial and decapitated crown sections cultured on solid MS medium supplemented with BA 3 mg l^{-1} produced an average of 5-10 shoot buds. This subculture technique could significantly contribute explants for transformation studies. Cytokinin and BA had been reported to be the most generally effective cytokinins for meristem, shoot tip and bud cultures of various species, followed by kinetin. Earlier studies recommended a combination of BA and kinetin for bud cultures of ginger (Hosoki and Sagawa, 1977; Nadgauda *et al.*, 1980). However, in the present study, BA alone in the medium supported multiple shoot production and elongation of the *in vitro* shoots. The cytokinin requirement for shoot multiplication reported in earlier studies was much higher (5 mg l^{-1}) for Wynad Local ginger (Hosoki and Sagawa, 1977; Nadgauda *et al.*, 1980). On the other hand, in the present study only 3 mg l^{-1} was found necessary for multiple shoot production.

5.2 STANDARDISATION OF EXPLANTS FOR TRANSFORMATION

Diverse plant tissues have been used for *Agrobacterium* mediated genetic transformation. In fact, one of the distinct advantages of *Agrobacterium tumefaciens* system is the wide spectrum of explants that can be used for transformation such as leaf, internodes, shoot tips, cotyledon, embryogenic callus etc.

Tissue culture investigations in ginger are mostly confined to micropropagation from shoot tip culture (Bhagyalakshmi and Singh, 1988; Noguchi and Yamakawa, 1988; Wang, 1989; Balachandran *et al.*, 1990). Malamug *et al.* (1991) and Babu *et al.* (1992) reported plant regeneration by organogenesis in ginger. In the present study leaves, pseudostem and young buds from one month old *in vitro* grown cultures were used for inducing callus and regeneration.

5.2.1 Callus induction

Many parts of the plant may have an ultimate potential to produce callus *in vitro*, but it is frequently found that callus cultures are more easily established from some specific organs. Young meristamatic tissues are most suitable and meristamatic areas in older parts of a plant can also give rise to callus (Bhaskaran and Smith, 1990). Monocotyledons react differently and are less likely to form callus than dicotyledons (Pierik, 1987). In most cereals, callus growth can be obtained only from organs such as zygotic embryos, germinating seeds, seed endosperm, seedling mesocotyl and very young leaves (Green and Philips, 1975; Dunstan *et al*., 1978).

Babu *et al.* (1992) reported that in ginger, callus was successfully induced from vegetative buds, young leaf bits, ovary and anther tissues on MS medium supplemented with various levels of NAA and 2,4-D.

For the production of callus during the present study, leaf, pseudostem and young buds from one month old axenic cultures were inoculated in full MS medium supplemented with 2,4-D, NAA, dicamba and combinations of 2,4-D and BA. Auxins in general induce callus formation, 2,4-D at a concentration of 3 mg l^{-1} was effective in inducing callus but produced only less amount of callus. But these cultures did not show further differentiation. NAA induced a slight amount of callus at higher concentrations of 3 mg l^{-1} to 6 mg l^{-1}. The amount of callus produced was negligible based on callus index and growth rate (Plate 3).

Callus induction was not noticed from leaves whereas pseudostem produced callus under the growth regulator combination of 2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA (upto 70%) and 3 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA (60%).

MS medium with a combination of 2,4-D 1 mg l^{-1} + BA 0.5 mg l^{-1} resulted in callus induction and embryogenic callus production in 90 per cent of the cultures. The explant differed in their ability to form callus. The maximum callus was obtained from the vegetative bud. The pseudostem explant gave the least amount of callus. In ginger, the callus was loose, friable and pale yellow in colour. In the subsequent

cultures, the callus formed some hard organised embryonic clumps within the mass of loose cells.

5.2.2 Somatic embryogenesis and regeneration

Somatic embryogenesis is the process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without fusion of gametes. This process occurs naturally in a wide variety of species from both reproductive tissues such as the nucellus and synergid cells and somatic tissues such as leaf margins (Karpoff, 1982).

Attempts were made to induce callus from leaf pseudostem and young bud as explants. Among the hormonal combinations tried, 2,4-D 1 mg l^{-1} + BA 0.5 mg l^{-1} induced embryogenic callus from young buds. Dark condition upto four weeks favoured callus induction and from callus initiation to plantlet formation it took 4-5 months time (Fig. 4). Leaves did not show any response in the presence of dicamba. Dicamba at 0.5 mg l^{-1} induced callus from young buds (Plate 4). Embryogenic callus cultures of ginger (var. Erattupetta) were induced from young leaf segments taken from *in vitro* shoot cultures in MS medium containing dicamba at 2.7 µM and efficient plant regeneration was achieved when embryogenic cultures were transferred to MS medium containing 8-9 µM BA (Kackar *et al.*, 1993).

Babu *et al.* (1992) also have reported that explants differed in their morphogenic response with regard to the morphogenic pathway as well as the plantlets regenerated. The plant regeneration was by organogenesis in leaf and anther derived callus, whereas it was by both organogenesis and embryogenesis in vegetative bud derived and ovary derived callus. In the present study also, similar results were noticed from vegetative buds. The embryo development followed typical monocotyledonous stages of development from globular, heart shaped to torpedo shaped embryos.

Somatic embryogenesis has the potential for rapid and efficient clonal propagation of plants. The yield and quality of somatic embryos produced in cell culture depends on the media constituents. The role of auxin for induction of somatic

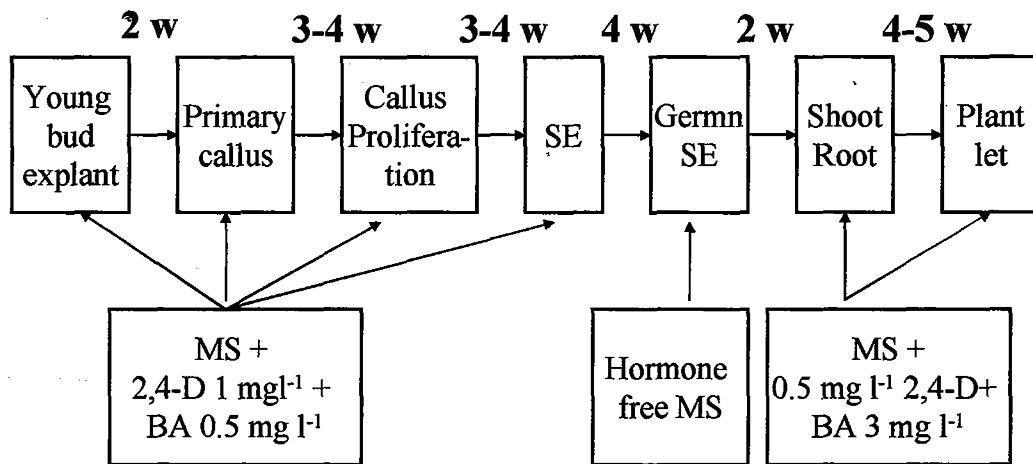


Fig. 4. Schematic representation of the successive stages of somatic embryogenesis of *Zingiber officinale* Rosc.

embryogenesis has been emphasized (Sharp *et al.*, 1980; Maheshwaran and Williams, 1986) and cytokinins have also been reported to improve somatic embryo yield in some systems, where endogenous auxin may inhibit embryo development (Stuart *et al.*, 1985).

Plant regeneration via. *in vitro* tissue is a basic system for achieving genetic transformation and there have been many reports involving genetic transformation via. somatic embryos in coffee (Hatanaka *et al.*, 1999); rubber (Sobha *et al.*, 2003), egg plant (Miklos *et al.*, 1995), cotton (Leelavathi *et al.*, 2004) and vigna (Karthikeyan *et al.*, 1996). Somatic embryogenesis protocol, if available in a crop has two distinct advantages in transformation work: Firstly because of its single cell origin, the development of chimaeric plants is avoided. Secondly, transformed embryogenic calli, if obtained can be multiplied rapidly and thousands of transformed plants can be raised within a short period of time.

5.2.3 Plant regeneration

The ratio of auxin to cytokinin concentration influences the balance between root and shoot organogenesis from cultured tissues. Generally high auxin concentration relative to that of cytokinin favours root formation and the converse situation favours shoot formation (Wanen, 1991). MS medium supplemented with 1 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA produced callus. 2,4-D at higher concentration resulted in rhizogenesis in combination with BA 0.5 mg l^{-1} (Table 17). Addition of BA (3 mg l^{-1}) in the presence of 0.5 mg l^{-1} 2,4-D resulted in embryogenesis and subsequent development of plantlets (Table 19). Once the morphogenic pathway of the cultures was determined, they continued to show their morphogenic potential in subsequent cultures even after two years.

5.3 *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION

5.3.1 Sensitivity of plant tissues to antibiotics

Agrobacterium mediated transformation is one of the well established procedures for introducing a foreign DNA into plant tissues (Horsch *et al.*, 1985, 1987

and Miki *et al.*, 1993). The procedure involves the inoculation of explants with *Agrobacterium* cells followed by the transfer of *Agrobacterium* infected explants to a selective medium for regeneration of transgenic plants. The selective medium contains both antibiotics for eliminating *Agrobacterium* cells and for selecting plant tissues which express the foreign DNA in the plant cells. Although many antibiotics have been described for the effective elimination of *Agrobacterium* cells, carbenicillin and cefotaxime have minimal toxicity on most plant tissues and efficiently eliminate *Agrobacterium* cells (Pollock *et al.*, 1983; Okkels and Pedersen, 1988). These antibiotics are the most widely accepted antibiotics for performing *Agrobacterium* mediated transformation.

In the present study, the sensitivity of leaf explants and young buds of ginger (*Zingiber officinale* Rosc.) to different antibiotics were evaluated. The evaluation was carried out with different doses of the antibiotics.

The toxic effect of cefotaxime and carbenicillin were examined and no toxic effects were observed by incubating bud explants on MS plates with callus induction medium containing cefotaxime 200 and 300 mg l^{-1}. The explants showed callusing, shoot and root differentiation compared to carbenicillin treated explants (Plates 8 and 9).

5.3.1.1 Kanamycin sensitivity of cultured tissues of ginger

Kanamycin resistance is the most widely used selectable marker for plant transformation and the sensitivity of a particular species or explant is a key element in the development of any new transformation systems in which a kanamycin resistant gene will be employed. Some monocotyledons have a high level of natural resistance to kanamycin, concentrations greater than 500 mg l^{-1} is needed to completely inhibit the growth of rice callus (Dekeyser *et al.*, 1989) and more than 800 mg l^{-1} is required to inhibit the growth of cell suspension cultures of several species of Gramineae (Hauptmann *et al.*, 1988). On the other hand, only 35 mg l^{-1} totally inhibits the callus growth in *Brassica napus* (Charest *et al.*, 1988). The sensitivity affects the recovery of transformed plants and varies widely among tissues and species. Kanamycin

sensitivity should be determined in the initial stages of developing a plant transformation system.

Ginger tissues are sensitive to kanamycin. Kanamycin decreased callus growth compared to the control. Kanamycin 100 mg l^{-1} completely inhibited callus initiation in buds in all the three varieties (Plate 13). When leaf discs were used as explants, with kanamycin concentration above 100 mg l^{-1}, the leaves became bleached while in the control, green leaf discs gradually turned brown during the course of the experiment. The degree of bleaching of kanamycin treated leaf discs was found to increase with increasing concentration (Plates 10 and 11).

5.3.1.2 Hygromycin sensitivity of cultured tissues of ginger

Leaf, pseudostem and young buds were found to show total inhibition of growth at different concentrations of hygromycin. All the explants showed complete arrest of callus induction at a minimum dose of 10 mg l^{-1} hygromycin (Plates 12 and 13).

Thus, the series of antibiotic sensitivity tests conducted under this study showed that ginger tissues are sensitive to kanamycin and hygromycin and these two antibiotics can be used as selection agents in transformation experiments.

Different strengths of antibiotics were used for selection in different crops. Karthikeyan *et al.* (1996) reported that a concentration of 50 mg l^{-1} kanamycin was effective for selection of transforments in *V. mungo*. During successful transformation of coffee, using embryonic callus from leaf, Hatanaka *et al.* (1999) reported to have used 100 mg l^{-1} kanamycin. In the case of *Brassica* species very low concentration of hygromycin or kanamycin (10-15 mg l^{-1}) could prevent morphogenesis of the explants (Kuvshinov *et al.*, 1999). This indicates that there is variation in the sensitivity/resistance to antibiotics depending on the genotype of the plants.

5.3.2 Bactericidal effect of carbenicillin and cefotaxime

Successful transformation using *Agrobacterium* depends not only on the efficiency of the plant regeneration system but also on the subsequent elimination of

Agrobacterium, usually achieved by adding one or more antibiotics to the culture medium, which is important because the continued presence of *Agrobacterium* can present a problem for identifying transformants or interfere with the growth and development of the transformed plant cells or cause death of the cultures (Martin *et al.*, 1990; Cooke *et al.*, 1992; Matzke *et al.*, 1996).

During the study, *A. tumefaciens* strains EHA105, LBA4404 (RC) and GV2260 (RC) were found effectively killed by carbenicillin at the strength of 200 to 400 mg l^{-1} when the antibiotic was added to pure bacterial cultures (Fig. 5 & 6). Hence a strength of 300 mg l^{-1} was found suitable for the elimination of the bacteria during the transformation work.

Cefotaxime at a concentration of 300 mg l^{-1} could be used for eliminating *Agrobacterium* (Table 26). It had been used successfully for elimination of *Agrobacterium* in transformation work in a number of crops. It was successfully used at a strength of 200 mg l^{-1} in eliminating *Agrobacterium* from the inoculated explant during transformation in white clover (Voisey *et al.*, 1994). In *Casuarina glauca* it was used at a strength of 250 mg l^{-1} for eliminating *Agrobacterium* (Le *et al.*, 1996). Cefotaxime was used at a concentration of 300 and 500 mg l^{-1} for eliminating *Agrobacterium* during transformation work in commercial melon (Valles and Lasa, 1994) and Phalaenopsis orchid (Belarmino and Mii, 2000) respectively.

The use of antibiotics for eliminating *Agrobacterium* is limited by the fact that some antibiotics such as carbenicillin can be inactivated by β-lactamases produced by bacteria, while cefotaxime was highly resistant to β-lactamases but its use inhibits plant regeneration (Yepes and Aldwinckle, 1994; Hammerschlag *et al.*, 1997; Ling *et al.*, 1998). There are many reports concerning the toxicity of antibiotics to callus growth and shoot regeneration, but only a few on their effect on somatic embryogenesis (Mathias and Boyd, 1986; Eapen and George, 1990; Sarma *et al.*, 1995).

Hence a better understanding of the toxicity of antibiotics to both *Agrobacterium* and callus/plant tissue growth is necessary. The toxicity of antibiotics

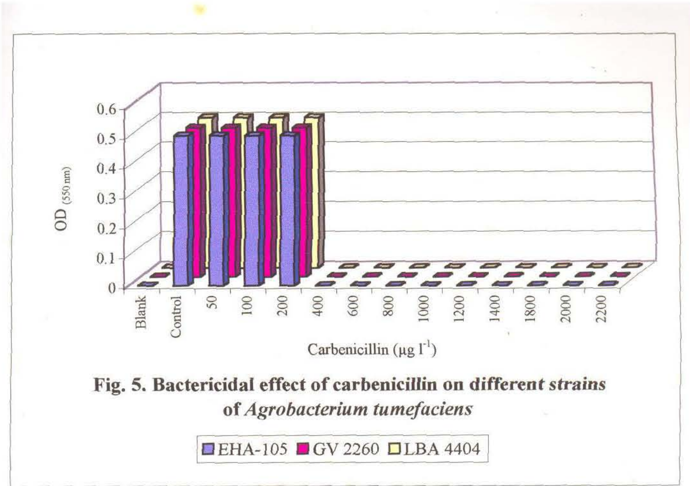


Fig. 5. Bactericidal effect of carbenicillin on different *strains* of *Agrobacterium tumefaciens*

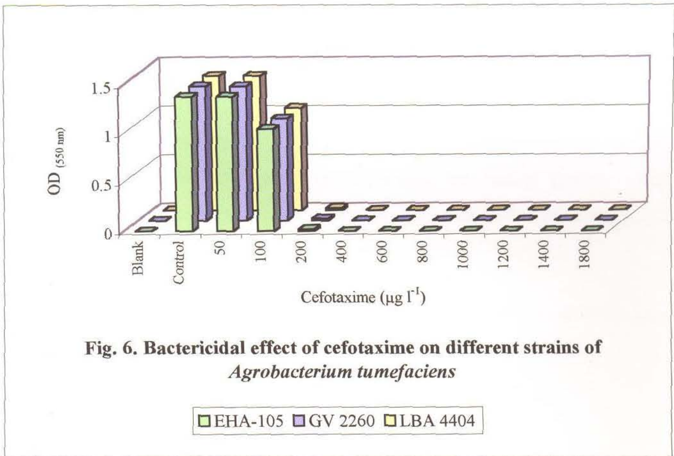


Fig. 6. Bactericidal effect of cefotaxime on different strains of *Agrobacterium tumefaciens*

towards plant tissue could also be studied before starting the transformation experiment.

5.4 EXPERIMENTS ON GENETIC TRANSFORMATION

In the present study, *A. tumefaciens* strain EHA105 containing the binary plasmid p35SGUS INT was used as the vector system for standardizing optimum conditions for transformation. It is a super virulent strain containing *npt* II (neomycin phosphotransferase II) gene as selectable marker which is present in the T-DNA, which confers resistance to kanamycin. It also contains GUS (beta-glucuronidase) as the reporter marker. The intron inserted in the protein coding region of the GUS INT gene prevents the translation of the corresponding mRNA in *A. tumefaciens*. Since the vector p35SGUS INT contains a chimeric gus A gene with a plant intron, the gus A INT reporter gene cannot be expressed in *A. tumefaciens* thus making it a useful tool for studying early events in transformation. Once the T-DNA of the bacteria is transferred to the plant genome, the transformed plant cells are expected to develop resistance to kanamycin and will be capable of normal growth in the medium containing these antibiotics in contrast to the untransformed cells which are susceptible to these antibiotics.

In order to develop an efficient and reliable transformation system for ginger that could be used for genetic improvement, the factors directly affecting *Agrobacterium tumefaciens* virulence and explant cell competence for transformation as well as factors affecting regeneration phase were studied.

The successful implementation of a transformation system requires optimization of the conditions.

5.4.1 Assessment of factors affecting transformation

In species where low transformation efficiencies are expected, the study of the effect of several factors by comparing the percentage of recovered transformed plants may prove unsuccessful because limited numbers of transformants are produced. Therefore, as a first approximation to evaluate the influence of diverse

factors on the efficiency of T-DNA transfer, experiments were conducted to observe the levels of transient GUS expression in inoculated buds. It is known that results of transient GUS expression, originating mainly from non-integrated T-DNA copies, may not necessarily correlate with stable transformation events. However, these studies can be used as a guide, and only major differences between tested parameters were taken into consideration for the establishment of transformation protocol in ginger.

5.4.1.1 Effect of bacterial strain, preculture period and bacterial concentration

Preculture of young buds of ginger on callus induction medium increased the transformation frequency to almost eight times compared to explants without preculturing (Fig. 7). It had a positive effect on the induction of competent cells for transformation. In the case of citrange, the preculture had a negative effect on the induction of competent cells for transformation. Preculturing explants prior to inoculation and co-cultivation with *Agrobacterium* has been shown to improve genetic transformation frequencies in some woody fruit plants such as plum (Mante *et al.*, 1991) and apricot (Laimer *et al.*, 1992). Sangwan *et al.* (1992) have also reported that in *Arabidopsis thaliana* explants, the number of putatively competent cells for transformation was greatly increased by a preculture treatment on a medium rich in auxins. Extended preculture may be deleterious for transformation in some species (Janssen and Gardner, 1993; De Bondt *et al.*, 1994).

β-glucuronidase activity was used to monitor transformation efficiency as affected by preculturing and bacterial dilution. Among the bacterial dilution tested, 1:20 (v/v) was found optimum in the present study. Blue staining was evident as soon as two days after co-cultivation of explants with EHA 105. Number of blue sectors/100 mg cell clumps ranged from 1 to 6.5. GUS activity was highest in cells precultured for three days and with a bacterial dilution of 1:20 (Table 28).

5.4.1.2 Influence of co-cultivation period on transformation

The explant response to *Agrobacterium* is affected by the coculture conditions. The coculture conditions that have the greatest influence on transformation

efficiency are medium salt composition and concentration, growth regulator composition, presence or absence of a cell suspension nurse culture and light intensity (Ritchie and Hodeges, 1993).

Figure 8 shows the influence of co-cultivation period on transformation of ginger bud explants. Histochemical GUS assays indicated that transformation had occurred at specific zones, and each spot represented an independent transformation event. When the explants were transferred to selection medium immediately after inoculation with *Agrobacterium*, no transformation was observed. GUS activity was observed from the explants co-cultivated for 2-3 days. Although explants which had undergone co-cultivation for 4-5 days showed GUS activity, the tissues were adversely affected due to the overgrowth of the bacteria. It is clear from the result that the co-cultivation time with *Agrobacterium* needed was 2-3 days to obtain efficient expression of GUS in ginger.

Although prolonged co-cultivation periods more than 3 days have been successfully used for certain plants (Jia *et al.*, 1989; Dong *et al.* 1991, Morgues *et al.*, 1996), 2-3 days co-cultivation has been routinely used in most reported transformation protocols, since longer co-cultivation periods frequently result in *Agrobacterium* overgrowth. In citrange, 5 days co-cultivation also resulted in abundant proliferation of the bacteria and a subsequent decrease in the regeneration frequency of transformed shoots (Cervera *et al.*, 1998). In rice also it was reported that co-cultivation for 2-3 days with *Agrobacterium* was required to obtain efficient expression of GUS (Rashid *et al.*, 1996).

In the case of blueberry, 3 days of co-cultivation with strain EHA 105 was required for efficient transfer of the gus A int gene (Cao *et al.*, 1998). Studies with apple have reported similar results with length of co-cultivation (De Bondt *et al.*, 1994), although greater than 4 days of co-cultivation is not recommended because of the difficulty in eliminating *A. tumefaciens* from the leaf tissue (De Bondt *et al.*, 1994). Hammerschlag *et al.* (1997) suggested that vacuum infiltration of explants with a low pH medium followed by short exposure to high levels of cefotaxime could be effective in eliminating *A. tumefaciens* from explants following prolonged co-cultivation.

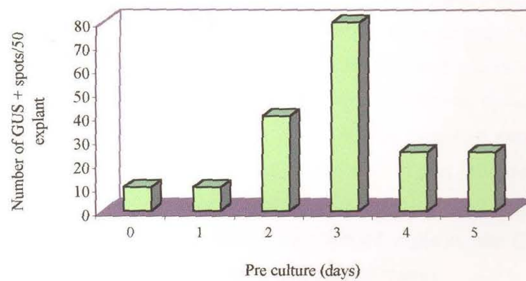


Fig. 7. Influence of pre culturing of explants on transformation

□ Mean number of blue spots / 50 explants

Bacterial dilution - 1:20 (v/v)
Co-cultivation time - 2 days
Infection time - 5 minutes

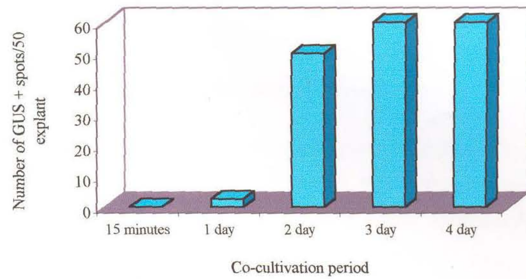


Fig. 8. Influence of co-cultivation period on transformation of ginger

□ Mean number of blue spots / 50 explants

5.4.1.3 *Post cultivation in darkness*

As shown in Figs. 9 and 10, culture in darkness clearly improved callus induction. Callus formation and consequent regeneration were progressively increased by maintaining the explant for two weeks in darkness and then transferring them to light. When the explants were kept in dark for 4 weeks and 8 weeks, the callus became white and failed to regain green colour. This is similar (negative control) to non inoculated explant and transformed explants. In the case of non inoculated explants, 70 per cent of the explants showed callus initiation, when it was kept under dark for 2 weeks and then under light. In the case of transformed explants, the GUS positive explants as well as callus formation increased from 2 to 4 weeks.

Exposure of explants to darkness has also been used to stimulate regeneration of transgenic shoots in some citrus species such as *Carrizo citrange* (Pena *et al.*, 1995) and lime (Pena *et al.*, 1997) and other woody fruit species such as apple (Maheswaran *et al.*, 1992; James *et al.*, 1993) and pear (Morgues *et al.*, 1996). Furthermore, the result from the present study suggest that the occurrence of etiolated shoots in the transformation procedure is probably due to excessive exposure of the explants to darkness (4-8 weeks). Moreover, it seemed that culture in the dark favoured callus formation.

5.4.1.4 *Activation of vir genes by phenolic compounds*

The bacterium is attracted to wounded plants presumably by following signal molecules released by the plant cell to which it attaches (Zambryski, 1992). Wounded tobacco (*Nicotiana* sp.) cells exude phenolic compounds such as acetosyringone and α-hydroxy acetosyringone that activate *vir* genes which are responsible for the transfer of T-DNA from *A. tumefaciens* to the wounded host cell (Stachel *et al.*, 1985). Monocotyledons, particularly the grasses, may not produce these compounds, or if they do, then not at sufficient levels to serve as signal molecules. Later on, it was found that monocots do produce *vir* inducers, but differ from the inducers in dicots (Messens *et al.*, 1990). Transformation of monocots is different not probably because they contain fewer competent cells, but in having a different wounding response, resulting in cellular necrosis as compared to cell divisions in the dicots and in having a different cell wall composition and may thus

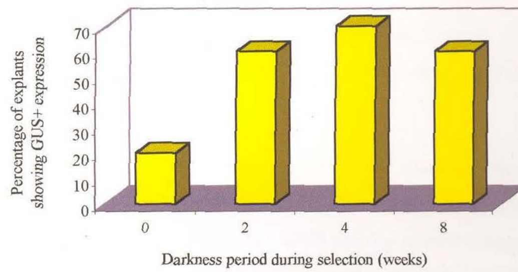


Fig. 9. Influence of exposure to darkness on the regeneration potential of *Agrobacterium* inoculated ginger bud explants

Pre culturing - 3 days
Bacterial dilution - 1:20 (v/v)
Infection time - 5 minutes
Co-cultivation - 2 days

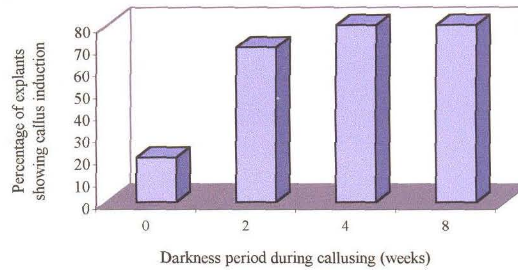


Fig. 10. Influence of exposure to darkness on the callus induction potential of non inoculated explant (Negative control without antibiotic)

have evolved a different mechanism to respond to *Agrobacterium* positively or negatively.

The use of acetosyringone during co-cultivation increase *Agrobacterium* mediated transformation frequencies (Sheikholeslam and Weeks, 1987) as was seen in the present study also.

In the present investigations, 200µM acetosyringone treated *Agrobacterium* and a two-step co-cultivation method were effective for obtaining transient GUS expression of cells (Table 31). GUS activity scored as number of GUS spots per 100 mg of cells was the highest in cells cocultivated with 200 µm acetosyringone treated *A. tumefaciens* using the two step co-cultivation method (Table 32). The *vir* gene activity must be sustained at a high level during the 8 h inoculation and two day co-cultivation period (Plates 18 and 19).

Acetosyringone is a phenolic compound produced during wounding of plant cells that induces the transcription of the virulent genes of *A. tumefaciens*. Its beneficial role has been demonstrated in the genetic transformation of some woody fruit species such as apple (James *et al.*, 1993), kiwifruit (Janssen and Gardner, 1993), citrange (Cervera *et al.*, 1998) and phalaenopsis (Belarmino and Mii, 2000).

5.5 PRODUCTION OF TRANSGENIC PLANTS

The different experiments conducted indicated that a suitable transformation protocol for ginger would include 3 day preculture of explants on callus induction medium, bacterial dilution of 1:20 (v/v) as the initial inoculum, an infection time of 5 min, 2 day co-cultivation with *Agrobacterium* and post cultivation on callus induction medium with 100 mg l^{-1} kanamycin and 300 mg l^{-1} cefotaxime under darkness for 2 weeks and then under 16/8 hour photoperiod.

Acetosyringone was effective at a concentration of 200 µm for *vir* induction incubated for 8 h and co-cultivation on callus induction medium containing 200 µm acetosyringone. Inclusion of acetosyringone increased the transient GUS expression in ginger. These results suggest that *vir* gene activity must be sustained at a high level during the 8 h inoculation and 2 day co-cultivation period.

These conditions were used in different experiments. In one of these experiments embryogenic calli were used as explants and 10-20 per cent of the explants showed regeneration. Due to the bacterial overgrowth in the culture medium, none of the shoots however survived.

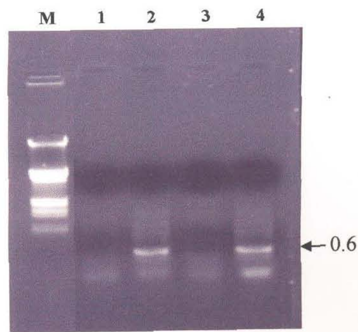
In another experiment conducted with young bud as the explant, a transformation frequency of 1.1 to 2.2 per cent was noticed. The callus growth was very slow in the presence of antibiotics.

5.6 ANALYSIS OF TRANSFORMATION

Histochemical GUS assay was performed on transformed callus and leaf tissue of the regenerated shoot. For plant transformation, the UidA (β-glucuronidase, GUS) intron containing gene was used to prevent bacterial GUS expression. It enabled testing of GUS activity at early stages of transformation. In the optimization experiments, GUS assays were conducted two days after co-cultivation with *A. tumefaciens*. Stable expression was detected in transformed calli 40 days after co-cultivation (Plates 26 and 27). All of the green regenerated shoots were checked for GUS activity, one of the shoot always gave consistent GUS expression while others did not consistently show the characteristic blue staining in repeated assays. All shoots from one clone showed strong GUS expression, while shoots from the other clones and control did not show any blue staining (Plate 29). GUS expression in GUS positive shoots was clearly visible in leaves, due to easier penetration of substrate into the tissue.

PCR assays were used to amplify *npt* II coding sequences in DNA extracted from kanamycin resistant shoots expressing GUS activity. A ~600 bp fragment amplicon was obtained in the case of putative transformants, confirming transformation. No amplification was obtained in non-transformed plantlets (Control) (Plates 31 and 32).

The future work in transformation of ginger with *Agrobacterium tumefaciens* may include using virulent strains reported to be effective in infecting monocots.



M	- Marker DNA (1 kb DNA ladder)
Lane 1	- untransformed control
Lane 2	- transformed
Lane 3	- Negative control
Lane 4	- Positive control (p35S GUS INTRON)

Arrow heads point to amplification of the 600 base pair fragment of the *npt* II gene

Plate 32. Electrophoretic analysis of the PCR products of transgenic ginger shoots with primers for *npt* II

Summary

6. SUMMARY

Investigations on *Agrobacterium* mediated genetic transformation of ginger (*Zingiber officinale* Rosc.) were carried out at the Department of Plantation Crops and Spices and Plant Tissue Culture Laboratory, CPBMB, College of Horticulture, Vellanikkara during 1999-2005. The objective of the study was to standardise the optimum conditions required for facilitating transformation and the technique of transferring desirable genes into ginger cultivar Rio-de-Janeiro using *Agrobacterium tumefaciens* as the vector.

Plant materials were collected, sufficient cultures were raised *in vitro* and explants were collected from axenic cultures for standardization of regeneration protocol and suitable explants for transformation work.

- Young buds from rhizomes of ginger were used for establishment of cultures.

- Surface sterilization procedure were standardised, the treatment involving dipping the bud in Teepol containing water for 5 min, followed by treatment with Indofil M-45 0.25 per cent for 30 min followed by washing in sterile water five times and placing them on medium containing cefotaxime at 250 mg l^{-1} could effectively control bacterial and fungal interference in cultures.

- Shoot bud cultures of ginger were raised using young sprouts collected from rhizomes in half MS medium containing BA 3 mg l^{-1}. The cultures were maintained by subculturing the decapitated crown section into the same medium.

- MS medium supplemented with different auxins and cytokinin such as 2,4-D, NAA, dicamba and combinations of 2,4-D and BA were tried on various explants such as leaf, pseudostem and young buds.

- Among different explants tried, young buds were found to be the best material for inducing embryogenic calli and plant regeneration.

- MS medium + 2,4-D 1 mg l^{-1} + BA 0.5 mg l^{-1} resulted in callus initiation (14-18 days) followed by callus proliferation and then by formation of green embryogenic structures. It took 4-5 months from callus induction to plantlet formation. At optimum conditions, 2-8 somatic embryos developed per culture tube and the survival rate of the embryo ranged from 25-75 per cent.

- Embryogenic callus regenerated in MS medium with BA 3 mg l^{-1} + 0.5 mgl^{-1} 2,4-D.

- Explant sensitivity to different antibiotics at varying concentrations were tested. When antibiotics were used along with subculturing at 3 week interval, ginger tissues were found to be sensitive to both kanamycin and hygromycin. Callus induction was completely suppressed with kanamycin and hygromycin.

- Kanamycin 100 mg l^{-1} was used to discriminate between transformed and non-transformed cells.

- Hygromycin at 10 mg l^{-1} concentration completely suppressed callus induction, inducing complete bleaching after one week.

- The antibactericidal activity of cefotaxime and carbenicillin on *Agrobacterium tumefaciens* strains were studied. Both the antibiotics were found to be effective for eliminating the bacteria from the cultures but carbenicillin was toxic to ginger tissue in the presence of 2,4-D in callus induction medium.

- Cefotaxime 300 mg l^{-1} added to the selection medium to eliminate the bacteria was found effective.

- *Agrobacterium tumefaciens* strain EHA 105 containing plasmid p35SGUSINT was used as the vector system for standardizing optimum conditions for effecting genetic transformation in ginger.

- A suitable transformation protocol would include three days preculture of explants, a bacterial dilution of 1:20 (v/v), infection time of 5 min co-cultivation of 48 h and post cultivation on callus induction medium with

100 mg l^{-1} kanamycin + 300 mg l^{-1} cefotaxime in darkness for 2 weeks and then under 16/8 h protoperiod.

- Use of acetosyringone in the co-cultivation medium increased the efficiency of transformation.
- 200 µm acetosyringone treated *Agrobacterium* and two step co-cultivation method were effective for obtaining GUS expression of cells.
- Embryogenic calli used as explants showed regeneration 4-5 weeks after co-cultivation.
- Bacterial over growth affects the survival of regenerated shoots.
- Young bud used as explants gives a transformation frequency of 1.1 to 2.2 per cent.
- Stable expression was detected in transformed calli 40 days after co-cultivation.
- One of the 3 clones developed consistent GUS expression compared to the others.
- PCR assays confirmed the presence of *npt* II coding sequences in DNA extracted from kanamycin resistant shoots expressing GUS activity.

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* Originals not seen

Appendices

A. Composition of media used in the study

APPENDIX - I

Composition of MS basal medium (Murashige and Skoog, 1962)

Components	Quantity ($mg\ l^{-1}$)
a) Major nutrients (Stock solution I)	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSo_4.7H_2O$	370
KH_2PO_4	170
b) Minor nutrients (Stock solution II)	
H_3BO_3	6.20
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
c) Stock solution III	
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
d) Organic constituents (Stock solution IV)	
Myoinositol	100
Pyridoxine HCl	0.5
Glycine	2.0
Thiamine	0.1
Nicotinic acid	0.5
e) Sucrose	30 g/L
f) Agar	8 g/l

APPENDIX - II

Media composition (YEP)

Peptone	- 10.0 g.
Yeast	- 10.0 g
NaCl	- 5.0 g
$MgCl_2$	- 1.0 g
Distilled water	- 1 litre
pH at 25°C	- 7.

APPENDIX - III

Luria Bertani (LB) medium

Tryptone	- 10.0 g
Yeast extract	- 5.0 g
NaCl	- 5 .0 g
Glucose	- 1.0 g
Distilled water	- 1 litre
pH	- 7.

APPENDIX - IV

Media composition (YEM)

K_2HPO_4	- 5.0 g
$MgSO_4.7H_20$	- 2.0 g
NaCl	- 1.0 g
Manitol	- 10.0 g
Yeast	- 1.0 g
Distrilled water	- 1 litre
pH	- 7.

APPENDIX - V

Media composition (AB)

AB medium (20x) Stock 1 litre

1. AB buffer (Solution I)

KH_2PO_4	- 60 g
NaH_2PO_4	- 20 g
pH	- 7

AB salts (Solution II)

NH_4Cl	- 20 g
$MgSO_4.7H_20$	- 6 g
KCl	- 3 g
$CaCl_2$	- 3 g
$FeSO_4.7H_20$	- 50 mg

Autoclave the solution separately and store

For 500 ml agar medium

Add 2.5 g glucose to 450 ml of water. Add 7.5 g Agar and Autoclave. After the medium cools to 50 to 60°C, add 25 ml of 20 x AB salts and 25 ml of 20 x AB buffer.
For liquid AB medium
For liquid medium for 100 ml90 ml 0.5 % glucose
5 ml AB salt
5 ml AB buffer

APPENDIX VI

Triphenyl tetrazolium chloride (TZC) medium

Peptone	- 10.0 g
Casein hydrolysate	- 1.0 g
Glucose	- 5.0 g
Agar	- 20.0 g
Distilled water	- 1 litre
pH	- 7.

1 per cent TZC was added to a final concentration of 5 ml l^{-1} after autoclaving.

APPENDIX - VII

Staining leaves for GUS activity

Prepare X-GLUC solution (20 µl) by mixing with

- 10 mg X-gluc in 100 µl dimethyl formamide
- 1 ml 1N Sodium phosphate pH 7.0
- 1 ml Triton (from a 20% stock solution)
- 18 ml H_2O

B. Buffers and solutions

APPENDIX VIII

Resuspension buffer (for plasmid isolation)

50 mM Tris Cl pH 8.0
10 mM EDTA
0.2 mg ml^{-1} R Nase A

Lysis buffer (for plasmid DNA isolation)

200 mM NaOH
1 per cent SDS

Neutralization buffer (for plasmid DNA isolation)

3 M potassium acetate pH 4.8

TES buffer

1 M Tris pH 8	- 5 ml
0.5 ml EDTA	- 10 ml
Sucrose	- 20.0 g
Water	- 200 ml

TE buffer

Tris	- 1.211 g
EDTA	- 0.372 g
Water	- 1 litre
pH	- 7.5 (with acetic acid)

Gel loading dye

Glycerol	- 60%
TAE buffer	- 30%
1% Bromophenol blue	- 10%

TAE buffer 50 x

Tris base	- 242 g
Glacial acetic acid	- 57.1 ml
0.5 M EDTA	- 100 ml
pH	- 8.0

Make up with distilled water to 1 litre.

AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION OF GINGER (*Zingiber officinale* Rosc.)

By

SUMA, B.

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

Doctor of Philosophy in Horticulture

Faculty of Agriculture
Kerala Agricultural University

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ABSTRACT

Investigations on genetic transformation in ginger (*Zingiber officinale* Rosc.) varieties Maran, Rio-de-Janeiro and Himachal using *Agrobacterium tumefaciens* strain EHA 105 harbouring antibiotic resistant selectable marker genes (*npt* II) and GUS reporter genes were carried out at the Department of Plantation Crops and Spices and Plant Tissue Culture Laboratory, CPBMB, College of Horticulture, Vellanikkara, Thrissur during the period from 1999 to 2005.

Axenic shoot bud cultures of ginger variety Rio-de-Janeiro was raised under *in vitro* condition to generate explants with reduced contamination for transformation. Half strength MS medium with BA 3 mg l^{-1} was found to be the best for establishing shoot bud cultures.

In order to standardise a regeneration protocol, MS medium supplemented with varying concentration of auxin and cytokinin were tried on different explants. Embryogenic calli were induced from bud explants of ginger supplemented with MS + 1.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA, followed by plant regeneration on MS medium + BA 3 mg l^{-1} + 2,4-D 0.5 mg l^{-1}.

Bactericidal effect of antibiotics towards different strains of *Agrobacterium* and sensitivity of ginger tissues to different antibiotics were also studied to standardise the optimum level of antibiotics. Cefotaxime at a concentration of 300 mg l^{-1} was selected for eliminating the bacteria after co-cultivation. Kanamycin 100 mg l^{-1} was used to discriminate between transformed and untransformed cells.

Agrobacterium strains were collected, recombinants were made and the presence of the construct confirmed in the native strains of *Agrobacterium* before starting transformation experiments. *Agrobacterium* strain EHA 105 p35SGUSINT was used for standardising the optimum conditions by comparing the levels of transient GUS expression in inoculated buds.

A suitable transformation protocol would include 3 days preculture of explants, bacterial dilution of 1:20 (v/v), infection time of 5 min, co-cultivation of 48 h and post cultivation on callus induction medium with 100 mg l^{-1} kanamycin + 300 mg l^{-1} cefotaxime in darkness for 2 weeks and then under 16/8 h photoperiod. Use of acetosyringone in the co-cultivation medium (200 μm) and *vir* induced *Agrobacterium* strain (200 μm), increased the efficiency of transformation.

Histochemical GUS assays were employed to study and compare the transient GUS expression, stable expression from putative transgenics. Further confirmation was made by PCR assays.

The regeneration protocol as well as transformation protocol could be effectively used for further transformation.