CONFIRMATION OF TRANSGENES IN PUTATIVE TRANSGENIC TISSUE OF WILD POMEGRANATE (*PUNICA GRANATUM* L.)

Thesis

by

RITIKA CHAUHAN

Submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

BIOTECHNOLOGY

COLLEGE OF HORTICULTURE
DR YASHWANT SINGH PARMAR UNIVERSITY OF HORTICULTURE AND FORESTRY, NAUNI, SOLAN-173 230 (H.P.) INDIA

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2005

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CERTIFICATE-I

This is to certify that the thesis entitled **"Confirmation of transgenes in putative transgenic tissue of Wild pomegranate (*Punica granatum* L.)"** submitted in partial fulfillment of the requirements for the award of degree of **MASTER OF SCIENCE** in **BIOTECHNOLOGY** to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Solan (H.P.) is a bonafide research work carried out by **Ms. Ritika Chauhan (H-2003-13-M)** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

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

Place: Nauni, Solan
Dated: 19th December, 2005

(Dr(Mrs) Kamlesh Kanwar)
Chairperson
Advisory Committee

CERTIFICATE – II

This is to certify that the thesis entitled **"Confirmation of transgenes in putative transgenic tissue of Wild pomegranate (*Punica granatum* L.)"** submitted by **Ms. Ritika Chauhan (H-2003-13-M)** to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Solan (H.P.), in partial fulfillment of the requirements for the award of degree of **MASTER OF SCIENCE** in **BIOTECHNOLOGY** has been approved by the Student's Advisory Committee after an oral examination of the same in collaboration with the external examiner.

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CERTIFICATE – III

This is to certify that all the mistakes and errors pointed out by the external examiner have been incorporated in the thesis entitled, **"Confirmation of transgenes in putative transgenic tissue of Wild pomegranate (*Punica granatum* L.)"** submitted by **Ms. Ritika Chauhan (H-2003-13-M)** to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Solan (H.P.), in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE** in Department of Biotechnology.

21/01/06

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I solely claim all responsibilities for the shortcomings and limitations in this work.

Ritika

(Ritika Chauhan)

Place: Nauni
Date: 19th December, 2005

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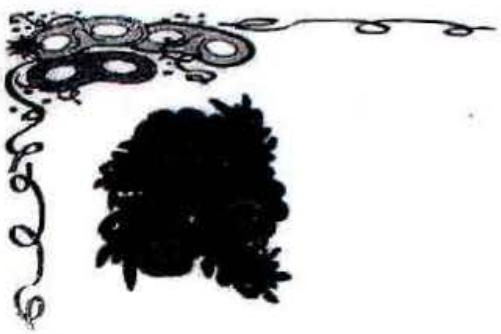
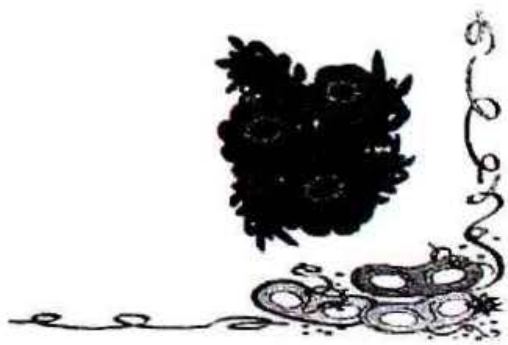
LIST OF ABBREVIATIONS

°C	degree Celsius
%	percent
IBA	3-indole butyric acid
IAA	indole acetic acid
M	molar
BAP	benzyl amino purine
cm	centimetre
EDTA	ethylene diamine tetra acetate
et al.	and others
g	gram
µl	microlitre
ml	milli litre
GA_3	gibberllic acid
hrs	hours
mg	milligram
MS	Murashige and Skoog
NAA	naphthalene acetic acid
Bt	Bacillus thuringiensis
C. D.	critical difference
DNA	deoxyribose nucleic acid
GUS	β-glucoronidase
PCR	Polymerase chain reaction
rpm	rotations per minute
UV	ultra violet
SE	standard error
ADW	autoclave distilled water
dNTPs	deoxy nucleotide triphosphate
F-primer	forward primer
R-primer	reverse primer
CTAB	cetyl tri methyl ammonium bromide



INTRODUCTION



Chapter-1

INTRODUCTION

Ecologically and economically, wild pomegranate (*Punica granatum* L.) commonly known as Daru or Darim is one of the most important agroforestry species that belongs to family Punicaceae. This species is considered to be native of Iran, Afghanistan and Baluchistan. In India, it can be seen growing wild in the hills especially between 900 – 1800 m above mean sea level (Anonymous, 1982). It is very common in the mid hill region of Western Himalayas.

On the basis of ecological requirements for a particular species, wild pomegranate grown well on slightly hot climate characterized by dry summer and fairly pronounced winters. This species provides a good income to the farmers and villagers particularly in the state of Himachal Pradesh, where it is found growing wild in Mandi, Kullu, Sirmour, Solan, Shimla, Bilaspur and parts of Chamba district.

Wild pomegranate is multi-purpose fast growing and of economic and ecological significance. It is an important social forestry cash crop tree of mid hill zone, being the rich source of vitamin-C and its fruit value for Anardana, thus, pushing up the economy of the farmers of areas lacking good cash crops. Almost all

plant parts of this species are used in one or the other form. The juice of fresh leaves and young fruits of this plant are given in dysentery. Tender fruits are used for treatment of constipation. All parts of this tree, particularly fruit rind, stem bark and root bark contain tannin contents, which is utilized for manufacturing of dye, used for production of Morocco leather (Howes, 1953).

Genetic transformation is the latest method available for crop improvement. Hence, using the transformation technology, agronomically important characters can be introduced in short time (Jones, 1991). In this regard, *Agrobacterium* mediated genetic transformation hold the potential for inserting foreign genes in a directed manner (Lindroth *et al.*, 1993). The genetic transformation of plants have made possible the transfer of chimeric genes into genomes of a number of woody species (Khurana and Khurana, 1999) such as *Pinus taeda* and *P.echinata* (Huang and Tauer,1994), *Populus tremula* (Tzfira *et al..*,1997), *Robinia pseudocacia* (Igasaki *et al.*,2000), *Picea* species (Klimazewska *et al.*,2001) and *Hevea brasiliensis* (Montoro *et al.*,2003). Out of different reporter genes, β-glucuronidase (GUS) and *npt-II* genes have been transferred successfully in a number of woody species including *Robinia pseudoacacia* (Kanwar *et al.*, 2003) and *Morus alba* (Agarwal *et al.*, 2004) from our own laboratory.

Agrobacterium mediated genetic transformation work has also been carried out on wild pomegranate in our laboratory. Experiments were done on the regeneration and genetic transformation of wild pomegranate (*Punica granatum* L.)

through *gus* marker and *npt-II* gene. The work has also been initiated on *cry1Ab* gene to produce insect resistant wild pomegranate. The regeneration of the transgenic tissue/shoots/plantlets were at different stages of growth and development. It is well established that although confirmation of putative transgenic material may be done biochemically/ histochemically, but confirmation through PCR is recent and reliable method. Confirmation of the transformation through PCR has been done in a large number of woody species such as *Castanea sativa* Mill. (Corredoira *et al.*, 2004), *Chamaecyparis obtusa* Sieb. Et Zucc. (Taniguchi *et al.*, 2004), *Eucalyptus tereticornis* (Prakash and Gurumurthi, 2005) *Robinia pseudocacia* (Kanwar *et al.*,2003) and *Morus alba* L. (Agarwal *et al.*, 2004). Therefore, the present proposal aims at DNA marker studies to fulfill the following objectives :

Objectives

The work is already going on the genetic transformation of wild pomegranate (*Punica granatum* L.) through *Agrobacterium tumefaciens* carrying *gus* marker and *cry1Ab* gene. The present investigation aims at the confirmation of putative plantlets for the *gus* and putative transgenic shoots carrying *cry1Ab* gene with the following objectives :

1. Confirmation of *npt-II* and *gus* marker
2. Confirmation of *cry1Ab* gene

REVIEW OF LITERATURE

Chapter-2

REVIEW OF LITERATURE

Agrobacterium system is extensively used for the production of most of the transgenic plants (Gasser and Fraley, 1989; Day and Lichtenstein, 1992; Linsey, 1992; Jain, 1993). The use of this approach for gene transfer has resulted in the crop improvement involving tissue culture. An efficient method for introducing cloned genes into plant cells and plant was given by Fraley et al. (1983), Herrera Estrella *et al.*, (1983) and Zambryski *et al.*, (1983). The preferred method for genetic engineering of higher plants may be *Agrobacterium* – mediated transformation because it results in a simpler integration pattern and limited re-arrangement in introduced DNA (Birch, 1997).

The recognition of the ability of the soil bacterium *Agrobacterium tumefaciens* to transfer a portion of its DNA to plants was perhaps the most important milestone in plant biotechnology (Klee *et al.*, 1987). The utility of this bacterium as a gene transfer system was first recognized by Chilton *et al.* (1977). Since the production of first transgenic plant of tobacco (Horsch *et al.*, 1985), the use of genetic engineering methods has increased by leaps and bounds. A number of binary vectors are commercially available which contain transcription promoters and screenable reporter genes such as those encoding β-glucuronidase, *gus* (Jefferson *et al.*, 1987). The *E. coli* β-glucuronidase gene was developed as a reporter gene

system for transformation of plants to overcome the difficulties faced in using the other reporter genes.

During the last 10 years, stable genetic transformation has been developed for a number of forest trees and used to transfer agronomically interesting genes conferring traits such as virus, insect or herbicide resistance (Jouanin *et al.*, 1993, Shin *et al.*, 1994 and Ellis, 1995). Bt technology has been used to develop transgenic insect resistance crops such as maize, cotton and potatoes that are now grown on a commercial scale (Dunwell, 2000; James, 2000). Insect resistance has also been engineered into a few tree species, such as eucalyptus (Harcourt et al., 2000) and poplars (Hu *et al.*, 2001).

The production of transgenic plants by *Agrobacterium* requires not only the availability of disarmed vectors, but also an efficient regeneration protocol. Hence, the present review has been sub-divided in three sections :

2.1 *In vitro* regeneration

2.2 Genetic transformation and confirmation of *gus* and *npt-II*

2.3 Genetic transformation and confirmation of Bt gene

2.1 *In vitro* regeneration

Kanchan and Mehra (1986) found MS supplemented with 4 ppm NAA, 2 ppm kinetin and 15 per cent coconut water, best for callus induction. Different explants used were hypocotyls, cotyledons, stem, shoot tips, leaves and embryos of *Punica granatum* L. cv. Kandhari anar. A high frequency of direct regeneration of

roots, shoots and whole plants without callus formation occurred with cotyledon, leaf and stem explant.

Moriguchi *et al.*, (1987) cultured anthers of *Punica granatum* L. (Issaizakuro pomegranate) on nutrient media containing BA and NAA. Callus from anther wall was observed after 30 days of culture. Subsequently adventitious shoots were formed by transferring callus to a medium containing BA and NAA.

Omura *et al.*, (1987a) reported adventitious shoot and plantlet formation from cultured pomegranate (*Punica granatum* L. var. nana) explants. Leaf was cultured on MS medium supplemented with BA and NAA and produced adventitious shoots after 2 months of culturing. Shoot elongation occurred in half strength MS medium supplemented with BA and rooted on ½ MS with NAA.

Omura *et al.*, (1987b) have obtained plantlet through adventitious bud from stem, carpel and root explants in dwarf pomegranate (*Punica granatum* L. var. nana). Regenerated plants differentiated flowers within 3 months after acclimatization.

Mahishni *et al.* (1991) investigated the effect of different media and growth regulators on *in vitro* propagation of pomegranate (*Punica granatum* L. cv. H.S. 4, UAS). Shoot tip was used as initial explant and plant regeneration was observed on MS, Llyod and McCrown Woody plant media supplemented with BAP and NAA.

Yang *et al.* (1991) reported plant regeneration using MS medium supplemented with IBA, when dormant bud of *Punica granatum* L. (cv. Ruanzi) was used as initiatory explant. Rooting was reported in half strength of MS medium.

Zhang and Stolz (1991) found MS medium, supplemented with NAA and BA, best for multiple shoot formation when terminal shoot was used as explant in case of *Punica granatum* L. var. nana.

Yang and Ludders (1993) reported *in vitro* regeneration of plantlets through callus from leaf and stem explants of dwarf pomegranate cv. Nana. Leaf segment and stem explants were initially cultured on modified MS basal medium supplemented with various growth regulators. Adventitious shoot elongation was stimulated most effectively when the initial calli were transferred from the shoot induction medium to MS basal medium supplemented with BA and IBA. Elongated shoots rooted easily on half strength MS medium.

Drazeta (1997) developed a protocol for plant regeneration of pomegranate (cvs. Slatki, Barski, Serbetas, Kanjski, Zubi and Davidis) using apical and axillary buds as explants. Explants were cultured on MS medium supplemented with BA and NAA for best results.

Faugat *et al.* (1997) reported callus induction using MS medium supplemented with 4.00 mg/l NAA, 2.00 mg/l kinetin and 15 per cent coconut water, using different explants of pomegranate. Subculturing of callus on MS medium supplemented with 2.00 mg/l BA resulted into various shoot primordia followed by

good shoot proliferation. Rooting was best on MS medium supplemented with 4.00 mg/l IBA, 2 mg/l kinetin and 15 per cent coconut water.

Kantharajah *et al.* (1998) investigated the effect of media and plant growth regulators on *in vitro* propagation of pomegranate cv. Wonderful. Leaf was used as explant for callus induction. Callus initiation was best on MS medium containing BAP (10.00 mg/l). Callus regenerated into shoots after 8 weeks of incubation. The best rooting was observed at 0.1 mg/l NAA.

Naik *et al.* (2000) developed a complete protocol for *in vitro* regeneration of pomegranate (*Punica granatum* L. cv. Ganesh) using cotyledonary nodes derived from axenic seedlings. Shoot development was induced on MS medium supplemented with 2.3 – 23.0 μM BA or kinetin. Both types and concentration of cytokinin significantly influenced shoot proliferation. Roots were developed on ½ MS supplemented with NAA. Plantlets were successfully acclimatized and established in soil.

Sharon and Sinha (2000) reported plant regeneraton from cotyledonary nodes of *Punica granatum* L. cultivar Ganesh and Kabul on B5 medium. For Ganesh best plant growth regulators concentration were 0.05 m/l NAA, 1.0 mg/l kinetin and 1.0 mg/l BA, whereas for Kabul it was 0.5 mg/l NAA and 0.5 mg/l BA. Multiple shoots were induced within 10 days. Elongation of shoots in both cultivars was done in same medium, i.e. B5 supplemented with 0.05 mg/l NAA and 0.5 mg/l BA with 1, 5, 10 and 15 per cent coconut milk. Coconut milk at 10 per cent concentration gave

best results in shoot elongation. Rooting of two month old shoots in B5 medium containing 3 mg/l IAA gave best results.

Murkute *et al.* (2002) gave method for callus induction and differentiation for leaf and cotyledonary explants of *Punica granatum* L. (cv. Ganesh). Profuse callus induction, proliferation and shoot differentiation was obtained in MS medium supplemented with 1 mg/l BAP and 0.5 mg/l NAA, whereas good rooting was obtained in ½ MS containing 1.0 mg/l IBA. Cotyledon explants respond extremely well as compared to leaf segments.

Naik and Chand (2003) presented a protocol for direct adventitious shoot organogenesis and complete plant regeneration from seedling – derived explants of pomegranate (*Punica granatum* L.). MS medium enriched with 8.95 mol/l benzyladenine (BA), 5.45 mol/l NAA and 10 % coconut water induced adventitious shoot bud proliferation in axenic seedling derived cotyledons as well as hypocotyls segments. Regenerated shoots were rooted in half-strength MS medium (1/2 MS) containing 0.54 mol/l NA. The well rooted plantlets were acclimatized and eventually established in soil.

A study on the *in vitro* culture of pomegranate showed that explants from twigs cultured on MS basal medium supplemented with 2 mg/l BA and 1 mg/l GA_3 generated callus 30 days after inoculation and that adventitious buds sprouted on the callus in succession. Leaf tissues inoculated on to MS medium supplemented with 1

mg/l NAA and 0.5 mg/l BA produced callus and sprouted 15 days after subculture. Half strength MS medium supplemented with 0.5 mg/l NAA, 1 mg/l activated charcoal and 20 g/l sucrose was optimum for the induction of adventitious roots, with the rooting rate reaching 95.8 per cent and the mean number of roots reaching 5.6 after 10 days of subculture (Zhu *et al.*, 2003).

Shelja *et al.*, (2005) compared *in vitro* regeneration pathways viz, *in vitro* germination of seeds, indirect induction of adventitious buds, direct induction of adventitious buds and proliferation of axillary buds and nodal segments for different media concentrations, combinations and response thereafter.

2.2 Genetic transformation and confirmation of *gus* and *npt-II*

The *E. coli* β-glucuronidase was developed as a reporter gene system for transformation of plants (Jefferson *et al.*, 1987). The β -glucuronidase gene has been cloned and sequenced and encoded as a stable enzyme that has desirable properties for the construction and analysis of gene fusions (Jefferson, 1985; Jefferson et al., 1986; Jefferson *et al.*, 1987). The plant expressing *gus* gene were very normal, healthy and fertile. The *gus* gene is very stable and tissue extracts continue to show high levels of *gus* activity after prolonged storage. The *npt-II* gene on the other hand confers resistance to the aminoglycoside antibiotics kanamycin and neomycin. The *npt-II* gene product, neomycin phosphotransferase, inactivates these antibiotics through their phosphorylation. Kanamycin resistance due to *npt-II* has been widely used as a selectable marker in many plant species. The selection of transformed cells is done on a medium containing kanamycin sulphate.

An efficient system for *A. tumefaciens* mediated-transformation of *E. camaldulensis* and production of transgenic plants. Histochemical assay revealed the expression of the *gus* gene in leaf, stem and root tissues of transgenic plants. Insertion of *gus* gene in the nuclear genome of transgenic plants was verified by genomic southern hybridization analysis (Ho *et al.*, 1988).

Cultures of nodular cell aggregates were established from callus derived from leaf pieces using woody plants. Transformation was verified by positive GUS staining of transformed tissues and the hybridization of genomic DNA from transformed shoots with a GUS Fragment. Southern hybridization showed that the insertion differed among transformed lines and occurred at multiple sites within one genome (Chen and Stomp, 1992).

Confalonieri *et al.* (1994) tested the clones of San Georgia and Jean Pourtel *in vivo* and *in vitro*. *Agrobacterium* mediated transformation of leaf discs and stem segments of the clones were analysed using *gus* gene transient expression. Polymerase chain reaction analysis and southern blot hybridization confirmed the integration of the *npt-II* gene into the genome.

Huang and Tauer (1994) demonstrated the genetic transformation in loblolly pine (*Pinus taeda*), short leaf pine (*P. echinata*) and loblolly x slash pine (*P. elliotti*) hybrid using an *Agrobacterium* mediated gene transfer system. Both PCR DNA amplification and southern hybridization confirmed that integrative transformation had occurred and that a specific foreign DNA fragment was present in the genome of transformed tissues.

Leaf discs from green house grown cuttings were used to develop transgenic plants containing *npt-II* gene and *gus* gene. Transformation was confirmed by amplification of *npt-II* and *gus* gene fragments from genomic DNA of regenerated transgenic plantlets using PCR and integration of these genes into the nuclear genome was confirmed by genomic southern hybridization analysis. Histochemical assay revealed GUS expression (Tsai *et al.*, 1994).

A transformation procedure was developed by hybrid local *L. kaempferi x L. decidua* embryogenic tissue using an *Agrobacterium tumefaciens* strain. Stable integration of the transgene was confirmed by PCR and southern hybridization on transformed tissue and acclimatized plants (Levee *et al.*, 1997).

Tzfira *et al.*, (1997) described *Agrobacterium* – mediated transformation procedures for an *in vitro* cultured aspen (*Populus tremula*) clones. Disarmed *Agrobacterium tumefaciens* strain EHA10**5** harbouring the binary plasmids pKIWI10S was used for the transformation of stem explants. The binary plasmids harboured genes encoding neomycin phosphotransferase II (*npt-II*) and beta-glucuronidase [*uidA*] (*gus*). Stem explants were highly susceptible to the bacteria, as indicated by high rates of transient *gus* expression. Over 90 % of regenerating roots were transformed and 65 % of adventitious shoots exhibited stable *gus* expression following analysis of transformed clones confirmed their transgenic nature.

Seabra and Pais (1997) performed the stable incorporation of the *npt-II* gene into *Castanea sativa* by *Agrobacterium tumefaciens* – mediated transformation. Transformation assays were performed by infecting wounded hypocotyls with *A. tumefaciens* strain LBA4404, harbouring a plasmid containing washer genes *uidA* and *npt-II*. PCR analysis confirmed *npt-II* gene integration into the plant genome and a *gus* histochemical assay revealed the expression of *uidA* in regenerated shoots.

Puite and Schaart (1999) studied the *Agrobacterium* – mediated transformation of the leaf segments of the apple cultivars. They were co-cultivated with a supervirulent *Agrobacterium* strain, containing the *npt-II* and *gus* genes. The transgenes were further confirmed by Southern blot analysis.

Seabra and Pais (1999) initiated genetic transformation studies using marker genes, namely the gene *uidA* of beta glucuronidase gene with a plant intron (PIV2), restricting its expression to plant cells, and the *npt-II* gene of neomycin phosphotransferase, conferring resistance to kanamycin and other antibiotics. The strain of *Agrobacterium tumefaciens* used was LBA4404, which harboured the plasmid p35SGUSINT with the referred genes. The integration of the genes in the plant genome was confirmed at molecular level by the use of technique such as PCR and Southern blotting.

Gartland *et al.*, (2000) studied the transformation system using *Agrobacterium tumefaciens* C58 pMP90 p35S *gus*/INTRON allowing the transfer of

foreign genes. PCR analysis indicated both *npt-II* and *uidA* genes inserted into the plant genome. The Southern hybridization further confirmed the presence of the *uidA* gene in regenerated plants.

Higgins *et al,*. (2000) reported the application of a PCR-based method in conjugation with automated sequencing for the reliable detection and verification of transgenes in crude extracts of leaf and callus tissue from different plant species. This protocol was used to confirm the different plant species. This protocol was used to confirm the presence and sequence of genes encoding selectable markers and reporters, etc.

Genetically transformed *Robinia pseudoacacia* (black locust) plants were regenerated after co-cultivation of stem and leaf segments with *Agrobacterium tumefaciens* strain GV3101 (pMP90) harbouring a binary vector that included genes for beta-glucuronidase (*gus*) and hygromycin phosphotransferase. Successful transformation was confirmed by the ability of stem and leaf segments to produce calli in the presence of hygromycin, by histochemical assay of *gus* activity in plant tissues, and by Southern blotting analysis (Igasaki *et al*., 2000).

Venkatachalam *et al.* (2000) studied an efficient transformation protocol for groundnut (*Arochis hypogea* L.) plants. Precultured cotyledons were co-cultured with *Agrobacterium tumefaciens* strain LBA4404 harbouring the binary vector pBI121 containing the *uidA* (*gus*) and *npt-II* genes. The transformation was confirmed by PCR analysis and by Southern hybridization with *npt-II* gene probe.

Ying *et al.*, (2000) studied a method for obtaining transgenic papaya (*Carica papaya*) plants by *Agrobacterium* – mediated transformation of somatic embryos with *Agrobacterium tumefaciens* strains LBA4404 harbouring Ti binary vectors pBI121 carrying *uidA* and *npt-II* genes. The presence of *npt-II* and *uidA* genes was further confirmed by PCR and Southern hybridization.

Transgenic plants of three *Picea* species, i.e. *P. glauca, P. moriana* and *P. abies* were produced having *npt-II* selectable state of the embryogenic tissue was initially confirmed by histochemical GUS assay followed by Southern hybridization (Klimazewska *et al.*, 2001).

Embryogenic masses were genetically transformed using *Agrobacterium tumefaciens*. The pBI121 vector containing beta-glucuronidase and the neomycin phosphotransferase II genes was introduced into this tissue. After co-cultivation with *Agrobacterium*, the embryogenic tissues were transferred to a selection media. *GUS* gene was detected by PCR analysis in genomic DNA isolated from transformed embryogenic tissues. These results indicate stable transformation of *Pinus radiata* somatic embryogenic tissues using *Agrobacterium tumefaciens* - mediated transformation (Cerda *et al.*, 2002).

Hong (2002) developed the *Agrobacterium* – mediated genetic transformation of *Acacia mangium* using rejuvenated shoots as the explant. The stem segments of rejuvenated shoots were co-cultured with *Agrobacterium*

tumefaciens strain LBA4404 harbouring binary vector pBI121. The southern hybridization confirmed the incorporation of the *npt-II* gene into the host genome.

Genetically transformed *Populus alba* plants were regenerated from calli which were derived from stem segments after co-cultivation with *Agrobacterium tumefaciens* strain GV3101 (pMP90) that harboured a binary vector into which genes for resistance to the herbicide bralophos [bilanafos] (bar) and for beta glucuronidase had been incorporated. The analysis by genomic PCR confirmed the success of transformation (Igasaki *et al.*, 2002).

Suzuki and Nakano (2002) developed the *Agrobacterium* mediated production of transgenic plants of *Muscari armerinacum* (grape hyacinth). The leaf derived embryogenic cultures were co-cultivated with *Agrobacterium tumefaciens* carrying the neomycin phosphotransferase II (*npt-II*), hygromycin phosphotransferase (*hpt*) and intron containing β -glucuronidase (*gus*-intron) genes in the T-DNA regions. The transgenic plants were verified by *gus* histochemical assay and polymerase chain reaction further confirmed by Southern blot analysis.

Aronen *et al.* (2003) demonstrated the production of a transgenic scots pine (*Pinus sylvestris* L.) seedling through the application of transformed pollen in controlled crossings. PCR amplification revealed the presence of both the *npt-II* and *gus* genes in one seedling. Results were confirmed by Southern blot analysis.

Bhatnagar and Khurana (2003) developed an efficient and reproducible protocol for the production of transgenic plants for *Morus indica* cv. K2 by *Agrobacterium tumefaciens*-mediated transformation. The hypocotyls, cotyledons, leaf and leaf callus explants precultured for 5 days on regeneration medium were co-cultivated with the bacterial suspension. The transformed explants were selected on 50 – 75 mg/l kanamycin for 1 month. Regenerants showed *gus* and *npt-II* activity by PCR chain reaction analysis after 6 months. Transgene integration into the nuclear genome of 1 year old regenerates was confirmed in 10 of the 18 transformants tested by Southern analysis.

Jayashree *et al.* (2003) reported genetic transformation and regeneration of rubber tree (*Hevea brasiliensis*). Immature anther derived calli were used to develop transgenic plants. These calli were co-cultivated with *A. tumefaciens* harbouring a plasmid vector containing the *H. brasiliensis*i superoxide dimutase gene (Hb SOD) under the control of CaMV-35S promoter. The β -glucuronidase gene was used for selection of transformed calli. Transformed calli surviving on a medium 300 mg l^{-1} kanamycin showed a strong *gus* positive reaction. Histochemical *gus* assay and Southern hybridization revealed transformation.

Montoro *et al.*, (2003) established a procedure for *Agrobacterium tumefaciens* mediated genetic transformation of *Hevea brasiliensis* embryogenic friable calli. The confirmation of *gus* and *npt-II* genes in the plant genome was confirmed by DNA amplification and by Southern hybridization.

Transgenic plants of the important citrus rootstocks *Swingle citrumelo* were obtained using *Agrobacterium* mediated transformation of seedling epicotyl tissue. Stable integration of the transgene sequence was confirmed by histochemical assay of GUS, PCR and Southern hybridization (Molinari *et al.*, 2004).

Tanguchi *et al.* (2004) developed a genetic transformation procedure for *Chamaecyparis obtuse* after co-cultivation of embryogenic tissues with disarmed *A. tumefaciens* strain C58/pMP90, which harbours the SgFp (Synthetic gram fluorescent protein) visual reported and *npt-II* (neomycin phosphotransferase-II) selectable marker genes. The integration of the genes into the genome of regenerated plantlets was confirmed by Southern blot analysis.

2.3 Genetic transformation and confirmation of Bt gene

Bacillus thuringiensis (Bt) formulations has been used for more than 50 years as biological insecticides to control agricultural pests (Ahmed *et al.*, 2002). The Bt insecticidal genes encode delta-endotoxin crystal proteins, which are active against lepidopteran insects. These genes are widely accepted as important components of integrated pest management programs (Endwistle *et al.*, 1993; Sticklen, 1991). A transgenic approach through the expression of Bt insect control protein genes in transgenic plants, offers a more targeted approach to insect control as the insecticidal proteins are exclusively ingested by feeding insects and are specific to the target pest. Further, inducible promoters could provide a mechanism to produce the toxin only when the plant is under insect attack (Lynette *et al.*, 2005).

Agarwal *et al.* (2004) studied *Agrobacterium tumefaciens* mediated genetic transformation in *Morus alba* L. Expression and presence of transgene (*gus* and *npt-II* genes) was confirmed by histochemical test and polymerase chain reaction.

Chang *et al.* (2004) established the acceptor system of transgene *Populus xiaohei, P. xiaohei* plants transformed with fusion gene which is composed of the *C. terminal* of Bt gene and the spider insecticidal were gained through *Agrobacterium tumefaciens* transformation. The transformed plants with insect resistance were marked as A1, A2, 13 and *gus* activity and PCR detection were conducted. Results showed that the fusion gene has been transformed into the plants.

Corredoira *et al.* (2004) described an innovative and efficient genetic transformation protocol for European chestnut in which embryogenic cultures were used as the target material. Transformation was confirmed by a histochemical β - glucuronidase (*gus*) assay, PCR and Southern blot analysis.

Pinus radiata cotyledon explants were cocultivated with *Agrobacterium tumefaciens* strain AGL1 containing a plasmid coding for the neomycin phosphotransferase II (*npt-II*) gene and the beta-glucuronidase (*gus*) gene (*uidA*). Transformed shoots were selected using either geneticin or kanamycin. Of the 105 putative transformants that were recovered from selection media, 70 % were positive for integration of the *npt-II* gene when analysed by PCR (Grant *et al.*, 2004).

Douches *et al.* (1998) augmented natural resistance in potato by transforming it with codon-modified *cryV*-Bt gene using *Agrobacteirum* – mediated techniques. 'Lemhi Russet' potato and two clones with different host plant resistance mechanisms were transformed with *cryB*-Bt gene. Gene integration of regenerated plants was confirmed by polymerase chain reactions and Southern analysis.

Lin *et al.*, (1999) obtained *Brassica napus* by introducing Bt toxic protein gene, using cotyledonary petioles from 4-5 days old plantlets of cv. Xiangyou 13. Explants were cultured on MS medium containing 4-5 mg/l BAP. Transgenic plantlets were obtained by transformation with the Bt toxin protein gene. Southern blotting showed that the Bt gene had been integrated into the plant's genome.

Genetically transformed poplar (*Populus deltoides x Populus simonii*) plants were regenerated after co-cultivation of leaf disc with *Agrobacterium tumefaciens* strain LBA4404 that harboured a binary vector pFWZ10 which induced *Bacillus thuriengenesis* delta-endotoxin gene (Bt gene) and *npt-II* gene. Transformations were confirmed by PCR and PCR-blotting (Rao *et al.*, 2000).

Xiang *et al.*, (2000) developed an effective plant regeneration procedure and a gene transfer system via *Agrobacterium tumefaciens* in *Brassica compestris* spp. *parachinensis*. Hypocotyls from 5 days old seedlings, with 2 days preculture were infected with *Agrobacterium* strain MOG301 harbouring a binary vector containing a synthetic *Bacillus thuringiensis* (Bt) *cry1Ab* or *cry1Ac* gene. PCR, Southern

blotting and western blotting were done to confirm the integration and expression of *cry1Ab* or *cry1Ac* gene.

Chakrabarty *et al.*, (2002) reported a protocol for *Agrobacterium* – mediated transformation of cauliflower. A number of factors that influence the genetic transformation were evaluated to optimize transformation of hypocotyls explants of variety Pusa Snowball K-1. Explant age, precultured period, bacterial strain and density were found to be critical determinants of transformation efficiency. Using the optimized protocol, the synthetic *cry1Ab* gene was mobilized in cauliflower. Molecular analysis of transgenic established the integration and expression of the transgene.

Kim *et al.*, (2002) introduced a synthetic *Bacillus thuringiensis* (Bt) *cry1Ac* gene, driven by two different kinds of promoter CaMV 35S and rbcS, into hot pepper line by *Agrobacterium* – mediated transformation. Polymerase chain reaction (PCR) analysis was performed to confirm *cry1Ac* gene integration into pepper genome.

Bhattacharya *et al.*, (2003) established an effective *Agrobacterium*-mediated transformation method for three varieties of vegetable *Brassica*. A synthetic *cry1Ab* gene coding for an insecticidal crystal protein of *Bacillus thuriingiensis* (Bt) was transferred to cabbage (cv. Golden acre), cauliflower (cv. Pusa Snowball K-1) and broccoli (cv. Pusa KTS-1) by cocultivating hypocotyls explants with two different strains (GV2260 and LBA4404) of *Agrobacterium tumefaciens*. Transformation

parameters were optimized by *Agrobacterium* strains harbouring *gus* intron gene in a binary vector. Transformed plantlets resistant to kanamycin were regenerated. Southern and northern hybridization experiments demonstrated gene expression and m-RNA expression. PCR analysis with *cry1Ab* gene specific primers showed inheritance of transformed gene in T_1 programmes.

A synthetic *Bacillus thuringiensis* (Bt) *cry1Ac* gene under the control of the 35S CaMV promoter was introduced into cauliflower (*Brassica oleracea* var. *botrytis*) by *Agrobacterium tumefaciens* mediated transformation with hygromycin selection. All the hygromycin-resistant transformants also carried the Bt gene, as shown by PCR with primers specific to the *cry1C* gene (Cao *et al.*, 2003).

A synthetic fusion gene of *Bacillus thuringiensis* encoding a translational fusion product of *cry1B* and *cry1Ab* delta-endotoxins was transferred to a tropical cabbage breeding line by *A. tumefaciens* mediated transformation. Selection of transformants was carried out on media containing kanamycin. Polymerase chain reaction (PCR) analysis revealed that twelve of the putative transformants contained the transgene (Paul *et al.*, 2003).

Santha *et al.*, (2003) reported transformation of pearl millet (immature inflorescences) using Bt gene. LS medium with 2,4-D and kinetin was used for induction of embryogenic calli. A synthetic Bt gene construct, *cry1Ac* which has protective role against lepidopteran insects, was used as the target gene. Molecular analysis by PCR, RT-PCR and Southern blotting confirmed the presence of the gene.

Tang and Tian (2003) introduced DNA containing synthetic *Bacillus thuriengenesis* (Bt) *cry1Ac* gene and neomycin phosphotransferase II (*npt-II*) gene into loblolly pine (*Pinus taeda* L.) tissues. The transformed tissues were proliferated and selected in media with kanamycin. The integration and expression of the internodal genes in the transgenic loblolly pine plants was confirmed by polymerase chain reaction (PCR) analysis and by Southern hybridization.

The acceptor system of transgene *Populus xiaohei* was established and *P. xiaohei* plants transformed with fusion gene which is composed of C-terminal of Bt gene and the spider insecticidial were gained through *Agrobacterium tumefaciens* transformation. The transformed plants with insect resistance were marked and *gus* activity of PCR detection were conducted. Results showed that the fusion gene has been transformed into the plant (Chang *et al.*, 2004).

A protocol was optimized for the transformation of hypocotyls explants of broccoli based on transient *gus* (beta-glucuronidase) expression. A synthetic *cry1A(b)* gene coding for an insecticidal crystal protein (ICP) of *Baccillus thuringiensis* (Bt) was transferred to the broccoli cultivar cv. Pusa broccoli KTS-1 by co-cultivating hypocotyls explants with *Agrobacterium tumefaciens*. Transformed plants resistant to kanamycin were regenerated. Molecular analysis demonstrated gene integration and expression(Vishwakarma *et al.*, 2004).

Jiang *et al.*, (2005) introduced a synthetic Bt (*Bacillus thuringiensis*) *cry1Ac* gene into the genome of cut flower chrysanthemum [*Chrysanthemum morifolium* (Syn. *Dendranthema morifolium*)] cv. Japanese Yellow *in vitro* using the explants of the internodal segments. The transformation was mediated by *Agrobacterium tumefaciens*. The results of PCR and Southern blot analysis showed that the gene was integrated as one copy into the genome.

Salehi *et al.*, (2005) reported *Agrobacterium* mediated transformation of 'Arizona Common' common bermudagrass with *Bacillus thuringiensis* Berliner *cry1Ac* gene encoding an endotoxin active against black cutworm. The integration of the gene into the genome was confirmed by PCR.

MATERIALS AND METHODS

Chapter-3

MATERIALS AND METHODS

The present investigations entitled "Confirmation of transgenes in putative transgenic tissue of wild pomegranate (*Punica granatum* L.)" was carried out in department of Biotechnology, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan and facilities were also availed at Central Potato Research Institute (CPRI), Shimla.

The details of the methodology followed to carry out the investigation have been described under the following headings :

3.1 Plant material

3.2 Cleaning of glassware

3.3 Media preparation

3.4 Aseptic manipulation and culture conditions

3.5 Maintenance of in vitro raised putative GUS transgenic plantlets

3.6 Multiplication and maintenance of control shoots

3.7 Multiplication and maintenance of putative Bt transgenic shoots

3.8 Confirmation of transformation

3.1 Plant material

Putative transgenic material carrying *gus* marker along with *npt-II* and Bt gene (*cry1Ab*) and non-transformed material was obtained from the laboratory of department of Biotechnology, College of Horticulture, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.).

3.2 Cleaning of glassware

All the new glassware were cleaned in a solution of teepol in water with the help of the test tube brush. The glassware was then soaked in chromic acid solution for overnight and rinsed thoroughly in tap water and then with double distilled water and dried in hot dry oven at 150°C for one hour.

The used glassware containing spent up media and contaminated cultures were first autoclaved to kill the contaminating micro-organisms. The warm medium was allowed to cool to a semi molten form and disposed off to empty the culture vessels. The culture vessels were then washed with water and teepol and rinsed with double distilled water followed by dry heat sterilization in oven at 150°C for one hour.

by filter sterilization added to molten medium (45 ± 1°C) under laminar air cabinet. The medium was poured in the pre-sterilized flasks and allowed to solidify.

3.4 Aseptic manipulation and culture conditions

All the operations were carried out aseptically in a laminar flow chamber fitted with ultra violet (UV) light (Klenzaids model 1594) and with two filters i.e. fine filter (HEPA filter) and coarse filter. Before starting with aseptic manipulations, the laminar air flow chamber was thoroughly wiped with rectified spirit and then culture vessels containing medium, culture equipments, spirit, spirit lamp, etc. were kept inside the chamber and switched on UV light for 15 – 20 minutes.

After switching off the UV light started the air flow. Hands were frequently wiped out with rectified spirit during aseptic manipulations. All metallic tools like scalpel handles, forceps and needles were autoclaved before use, inserted in a glass beads sterilizer and flame sterilized during the time of use. The rims of culture vessels were quickly flame sterilized before and after inoculations. In all the experiments, the culture were incubated in culture room at 25 ± 2°C under 16 hours photoperiod.

3.3 Media preparation

i) MS medium (Murashige and Skoog's medium)

Separate stock solutions of inorganic nutrients, vitamins and growth regulators were prepared, kept in the refrigerator and used within one month. The chemicals used were of analytical grade and obtained from reliable firms.

During medium preparation each stock (after bringing them to room temperature) was added one by one in required quantity. After addition of sucrose (30.0 g l^{-1}), vitamins and growth regulators, the pH of media was adjusted to 5.7. Thereafter agar-agar (8.00 g l^{-1}) was added and homogenized by heating the media.

The media was finally dispensed into desired culture vessels (culture tubes/flasks). After dispensing the media, culture vessels were plugged with cotton plugs wrapped in muslin cloth and autoclaved at a pressure of 15 lbs per $inch^2$ at 121°C for 15-20 minutes for proper sterilization of culture media. The media were stored in dark at 25 ± 2°C and used after 5-7 days of preparation.

ii) Selective medium

The selective medium was prepared with concentration of 30 mg/l kanamycin sulphate and 150 mg/l cefotaxime, after filter sterilization to the molten medium. The antibiotics were dissolved in minimum volume of sterilized water and

3.5 Maintenance of in vitro raised putative GUS transgenic plantlets

The information regarding the *Agrobacterium* strain and plasmid that was used for genetic transformation is as follows:

Disarmed *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983) contained a reporter/marker β-glucuronidase (GUS gene) in binary vector pBI121 (Jefferson *et al.*, 1987) along with a kanamycin resistance gene, npt II (neomycin phosphotransferase II). The coding sequence of GUS was connected to 35S promoter of cauliflower mosaic virus (CaMV 35S) and terminator from nopaline synthase gene (NOS). The coding sequence of npt II was connected to nopaline synthase gene promoter and terminator sequence (Fig 1).

Already regenerated putative GUS transgenic plantlets of wild pomegranate (*Punica granatum* L.) were maintained in the culture room of the laboratory of Department of Biotechnology, Dr Y S Parmar University of Horticulture and Forestry, Nauni- Solan H. P.

3.6 Multiplication and maintenance of control shoots

Explants with small shoot buds were transferred on shoot multiplication medium consisting of solid MS medium containing 2.0 mgl^{-1} BAP, 1.0 mgl^{-1} GA_3

and 1.0 mgl^{-1} NAA. After six weeks small shoots of size 1.0- 1.5 cm were separated from the explant and inoculated individually on the medium of same composition to observe the effect of subculturing on shoot multiplication. With each subculture passage the observations were recorded for (i). average number of shoots per explant and (ii) average shoot length (cm). Each treatment consisted of three replicates (culture flasks) and the experiment was repeated thrice.

3.7 Multiplication and maintenance of putative Bt transgenic shoots

Agrobacterium strain and plasmid used:-

Disarmed *Agrobacterium tumefaciens* strain containing binary vector pBin Btl with *cry 1Ab* (insect resistance gene) and npt II (neomycin phosphotransferase II), for selection in both bacteria and plant (Fujimoto *et al.*, 1993) was used for co-cultivation experiment to transfer *cry 1 Ab* gene in wild pomegranate cell (Fig 2a,b&c).

The already co cultivated cotyledonary explants with small shoot buds were transferred to selective multiplication medium containing 2.0 mgl^{-1} BAP, 1.0 mgl^{-1} GA_3, 1.0 mgl^{-1} NAA, 30 mgl^{-1} kanamycin and 150 mgl^{-1} cefotaxime. After six weeks small shoot of size 1.0- 1.5 cm were separated from the explant and inoculated individually on the medium of same composition to observe the

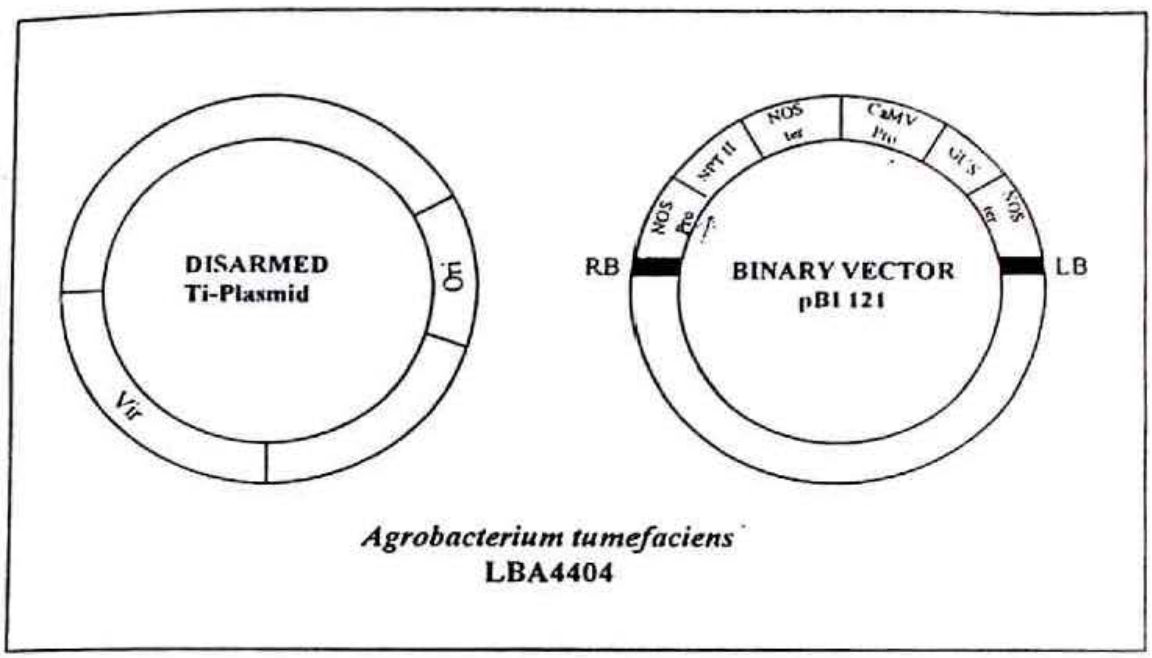


Fig. 1a. Outline diagram of disarmed *Agrobacterium tumefaciens* strain LBA4404

Fig. 1b. Schematic representation of pBI121 containing

i) **Transcriptional fusion of NOS promoter with coding region of NPT II and NOS terminator,**

ii) **Transcriptional fusion of CaMV35S promoter with coding region of GUS and NOS terminator**

LB -	Left border of T-DNA
RB -	Right border of T-DNA
CaMV 35S Pro -	Cauliflower mosaic virus 35S promoter
NOS pro -	Nopaline synthase promoter
NOS ter -	Nopaline synthase terminator
GUS	β -glucuronidase
NPT II -	Neomycin phosphotransferase II

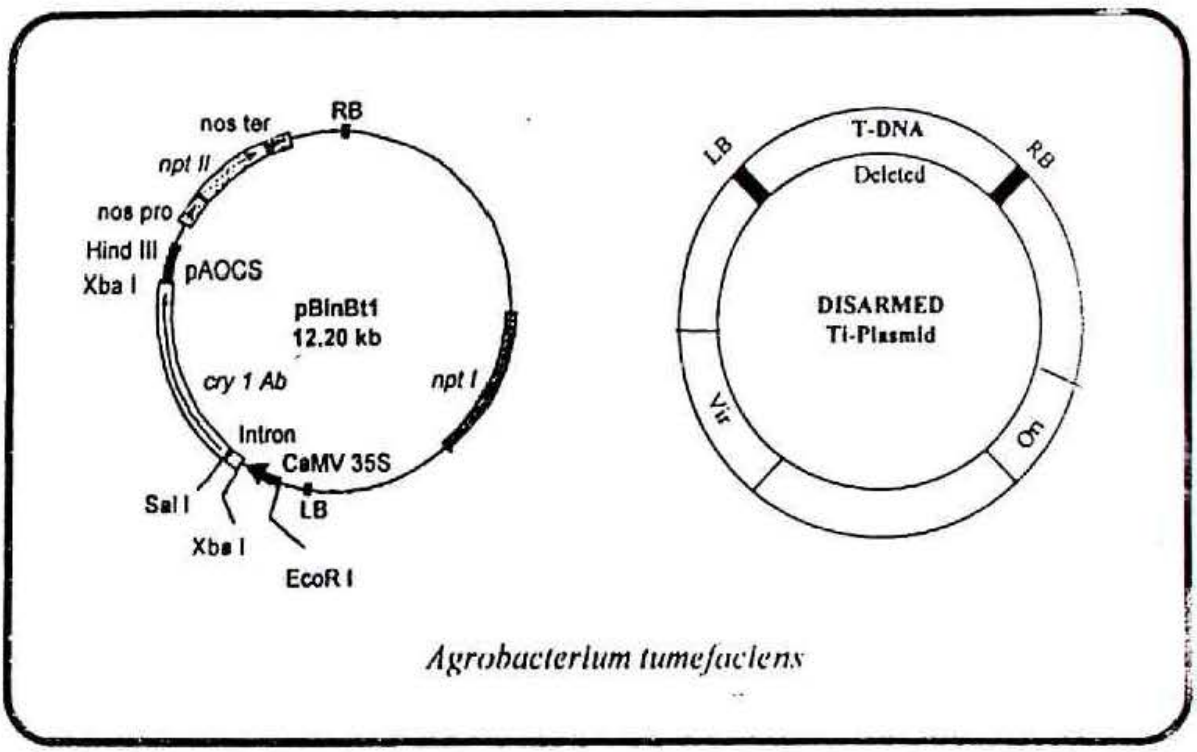


Fig. 2a. The outline diagram of disarmed Ti-plasmid and binary vector pBin Bt1 containing *cry1Ab* insect resistance gene and *npt-II* kanamycin resistance gene in *Agrobacterium tumefaciens* strain.

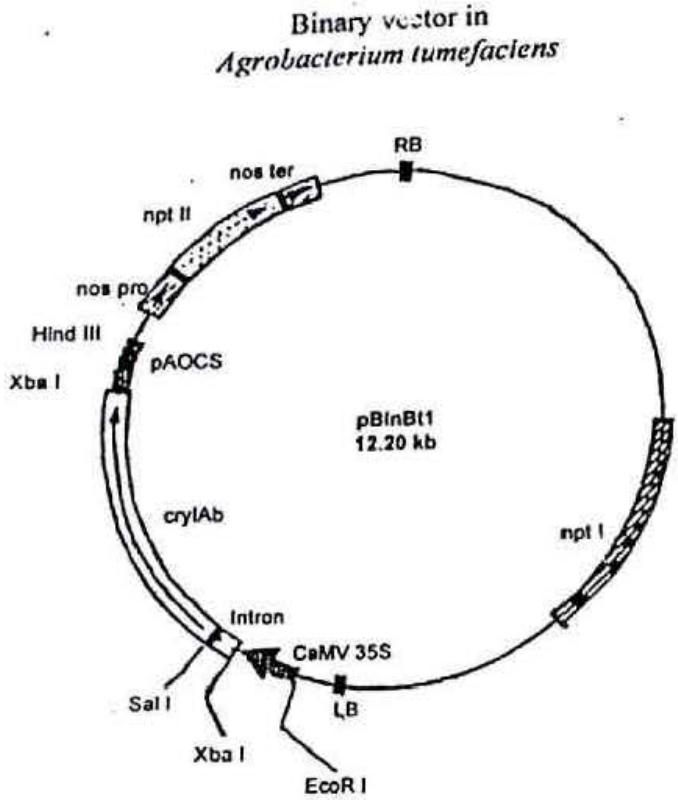


Fig. 2b. The outline diagram of binary vector pBin Bt1 containing *cryIAb* insect resistance gene in binary vector along with kanamycin resistance gene (*npt-II*) for selection in both bacteria and plant.

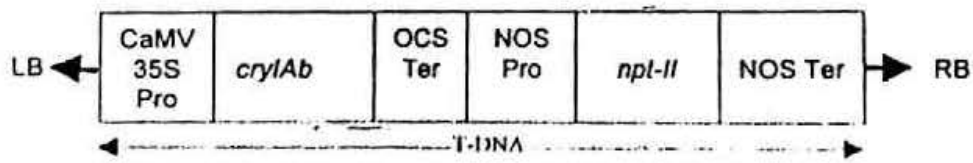


Fig. 2c. Schematic diagram of plasmid (binary vector) constructs: T-DNA region of pBin Bt1 containing transcriptional fusion of CaMV 35S promoter with the coding region of *cryIAb* and NOS promoter with the coding region of *npt-II*.

LB - Left border of T-DNA
RB - Right border of T-DNA
35S - CaMV 35S promoter
NOS ter - Nopaline synthase terminator
NOS pro - Nopaline synthase promoter
npt-II - Neomycin phospho transferase-II
cryIAb - Insect resistance gene
OCS Ter - Octopine synthase terminator

effect of subculturing on shoot multiplication. The green shoots growing on the selective medium were subcultured regularly after six weeks. The average shoot length and number of shoots per explant were recorded with each subculture.

3.8 Confirmation of transformation

For the confirmation of *npt-II* and *gus* marker and *cry1Ab* gene, the putative tissues were analysed by polymerase chain reaction.

i) DNA isolation and purification

Total genomic DNA was isolated from randomly selected, putative transformants and control shoots. The CTAB method (Doyle and Doyle, 1987) with some modifications was used.

DNA extraction buffer

a) 100 mM Tris-Cl (pH 8.0)

b) 20 mM EDTA (pH 8.0)

c) 1.4 M NaCl

d) 2.0 % (w/v) CTAB

e) 0.2 % (v/v) β-Marcaptoethanol

Plant DNA isolation and purification

- Ground plant material in liquid nitrogen to fine powder in a pre chilled pestle and mortar
- Transferred the powder to a 1.5 ml microtube containing 1.5 ml of pre-warmed (65°C) DNA extraction buffer. Mixed by gentle inversion.
- Incubated at 65°C for one hour.
- Added 700 µl chloroform : isoamylalcohol (24:1, v/v) and mix by inversion to emulsify.
- Pipetted out gently the aqueous phase without disturbing the interphase to another tube. Added 2/3rd volume of isopropanol and mixed by gentle inversion.
- Spun down the DNA pellet or take out the DNA using a bent Pasteur pipette.
- Washed in 70% ethanol and spun at 10000 rpm for 5-10 minutes at 4°C.
- Dried the pellet and dissolved in 50-60 µl of sterile water or TE buffer (pH 8.0) depending upon the yield of DNA.
- Added 5 µl of RNase (10 mg/ml) to the final concentration of 10 µg/ml, mixed gently and incubated for 1 hour at 37°C.
- Added equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v) and mixed thoroughly to emulsify.
- Spun at 12,000 rpm for 10 minutes and carefully took out the upper aqueous phase using a 200 µl pipette without disturbing the interphase.

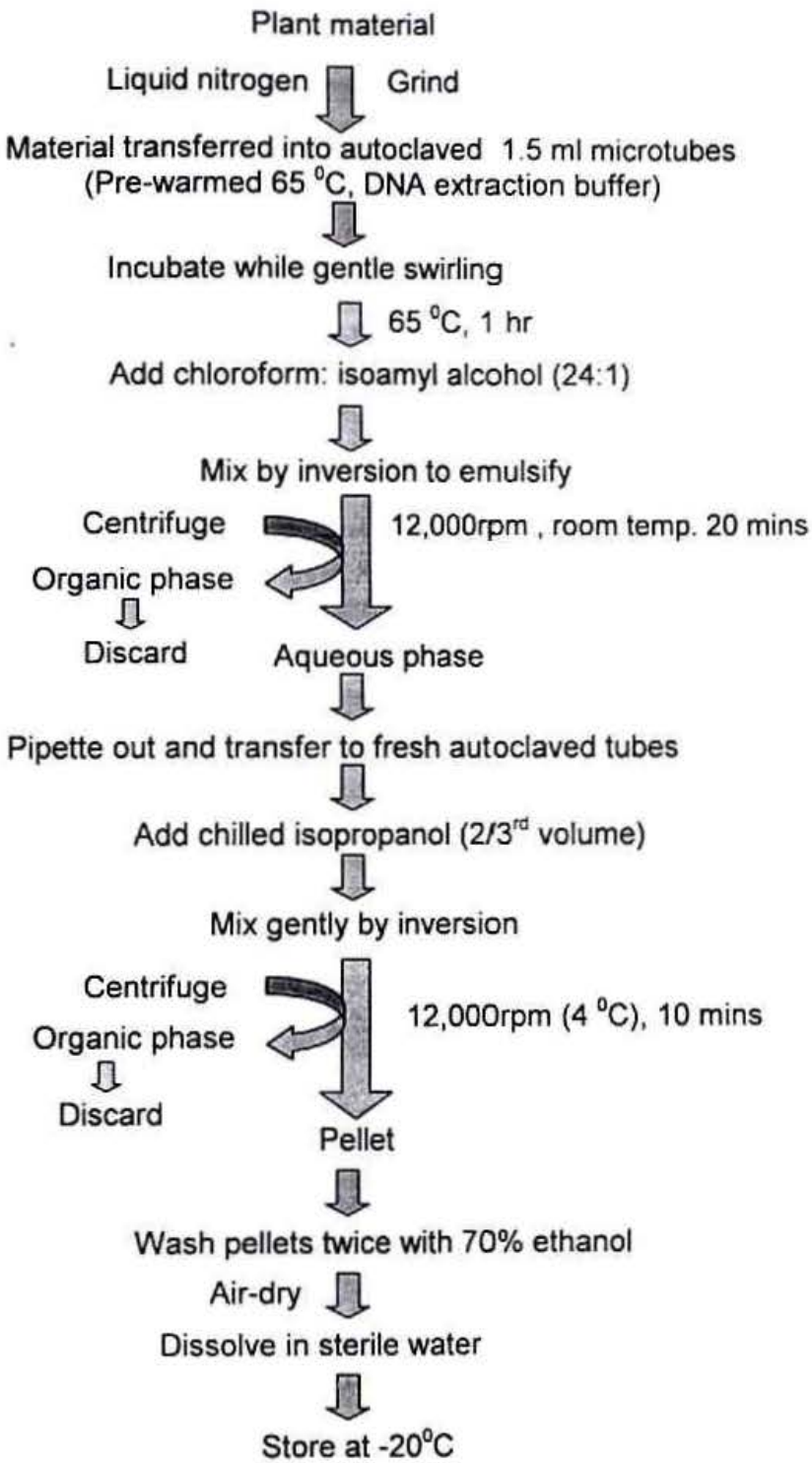


Fig 3. Flow chart for isolation of total DNA from Plant Material

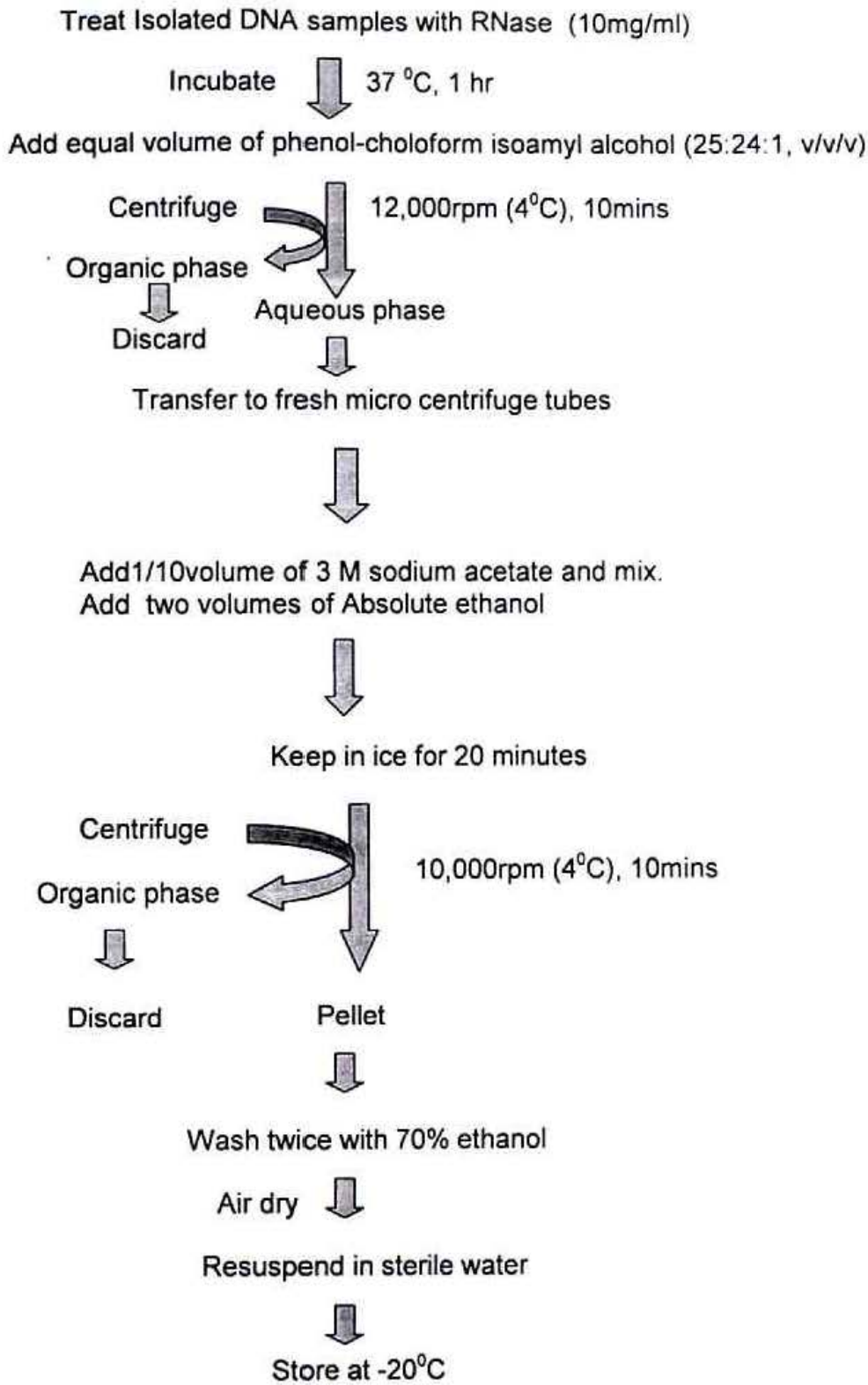


Fig 4. Flowchart for Purification of DNA

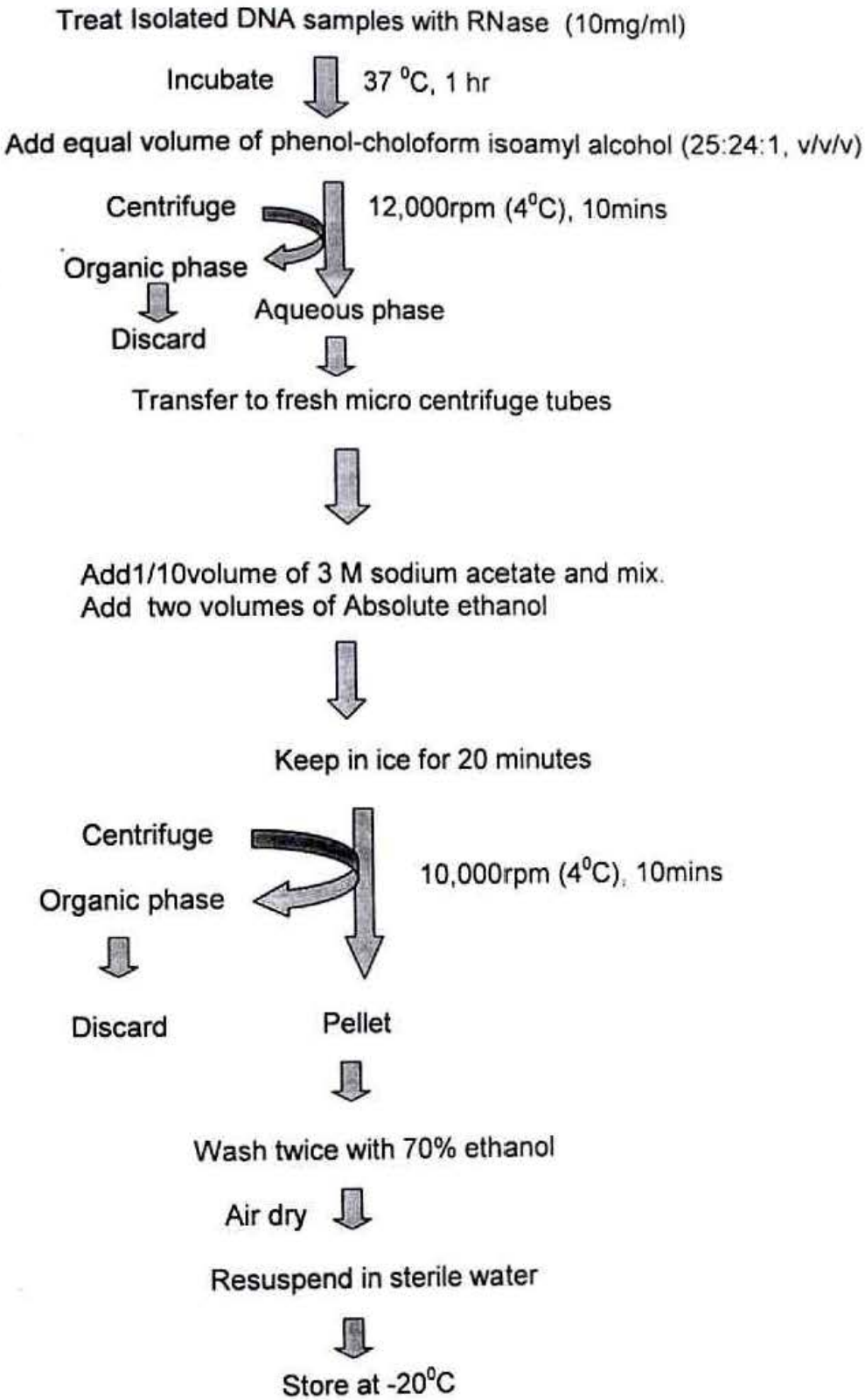


Fig 4. Flowchart for Purification of DNA

- Added 1/10 volume of 3 M sodium acetate, pH 5.2 and mixed thoroughly. Added two volumes of absolute ethanol (95 %). Mixd by gently inversion and kept in ice for 20 minutes.
- Spun at 10,000 rpm for 10 minutes to pellet the DNA.
- Washed the DNA pellet with 70 % ethanol.
- Spun for 2-3 minutes.
- Dried the DNA pellet and dissolved in sterile water or TE buffer.

ii) Electrophoresis of purified DNA

Reagents

1. Agarose 1 %
2. TBE buffer (25 X)
3. Ethidium bromide – 3 µl
4. Loading dye (0.25 % bromo phenol blue)

Protocol

Run the DNA samples which were isolated, for about I hour. The electrophoresis was done on 0.8 percent agarose gel. The TBE buffer (25 X) 120 ml, was used as the buffer tank solution. For DNA staining, 3 µl ethidium bromide was added in the gelling solution. The loading mixture for each well contained 8 µl DNA + 4 µl loading dye. The DNA samples were electrophoresed at 100 V for an hour and observed under UV transilluminator.

ii) Qualitative and quantitative estimation of DNA

Estimation of DNA was carried out by a spectrophotometer (BIO RAD – SMART SPEC™ 3000).

a) Qualitative estimation

The approximate purity of double stranded DNA was estimated by determination of the ratio of absorbance at 260 nm and 280 nm (A_{260} / A_{280}). This ratio is 1.8 for pure double stranded DNA. A_{260} / A_{280} ratio greater than 1.8 suggest RNA contamination, whereas one less than 1.8 suggests contamination with protein or phenol.

b) Quantitative estimation

For quantitating the amount of DNA, readings should be taken at wavelength of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 50 µg/ml for double stranded DNA. The concentration of the DNA samples was calculated spectrophotometrically in which 95 µl of autoclaved water and 5 µl of the DNA samples was taken to see the OD (optical density).

iv) Polymerase chain reaction

Amplification of the target genes were done by polymerase chain reaction in a thermal cycle.

a) DNA amplification for the *npt-II* gene confirmation

The PCR was run for the *npt-II* gene confirmation using two specific designed primer (one forward, one reverse primer – Table 3.1).

Reagents

1. Autoclaved distilled water - 16.75 µl
2. Buffer – 2.50 µl
3. $MgCl_2$ stock (25 mM) – 0.50 µl
4. dNTP's – 1.00 µl
5. F primer (forward primer) – 1.00 µl
6. R primer (reverse primer) – 1.00 µl
7. Taq polymerase – 0.25 µl
8. DNA – 2.00 µl

Protocol

Prepared a PCR master mix i.e. 25 µl consisting of the above reagents as follows :

The reaction mixture was calculated for 10 reactions on the basis of reaction volume of 25 µl .

ADW	-	167.50 µl
Buffer	-	25.00 µl
$MgCl_2$ stock	-	5.00 µl
dNTPs	-	10.00 µl
F primer	-	10.00 µl
R primer	-	10.00 µl
Taq polymerase	-	2.50 µl

Mix gently and dispense 23 µl of each of reaction mixture to autoclaved PCR tubes. 2 µl DNA was added separately to each PCR tube to make final reaction mixture 25 µl. The following thermocycle programme was used for the amplification of DNA (the npt-II gene).

Stage	Temperature	Time	Cycle
1. Initial denaturation	94°C	5 min	1
2. a) Denaturation	94°C	30 sec	
b) Annealing	55°C	30 sec	40
c) Extension	72°C	1 min	
3. Final extension Hold at Temp 4°C	72°C	10 min	1

Temperature profile

PCR was carried out with a total of 40 cycles. Each cycle consisted of 30 sec denaturation at 94°C, 30 sec annealing at 55°C and 1 min extension at 72°C and final extension at 72°C for 10 minutes. Prepared 1.6 % agarose gel of 250 ml volume. 4 µl 6x loading dye was added to each sample and loaded into the gel. A 2 µl of 100 bp ladder sequence was also loaded in the 1st and last well as standard and the gel was electropohoresed for one hour at 120 V. The image of the gel was taken through FlouS™ Multiimager. The images were stored in the computer software for further studies.

b) **DNA amplification for the *cry1Ab* gene confirmation**

The polymerase chain reaction was run for the *cry1Ab* gene confirmation using two specific primers (one forward, one reverse primer – Table 3.2).

Reagents

1. ADW – 17.25 µl
2. Buffer – 2.50 µl
3. $MgCl_2$ stock (25 mM) – 0.50 µl
4. dNTPs – 1.00 µl
5. F primer – 0.75 µl
6. R primer – 0.75 µl
7. Taq polymerase – 0.25 µl
8. DNA – 2.00 µl

Protocol

Prepared a PCR master mix i.e. 25 µl consisting of the above reagents as follows :

The reaction mixture was calculated for 10 reactions on the basis of reaction volume of 25 µl .

ADW	-	172.50 µl
Buffer	-	25.00 µl
$MgCl_2$ stock	-	5.00 µl
dNTPs	-	10.00 µl
F primer	-	7.5.00 µl
R primer	-	7.5.00 µl
Taq polymerase	-	2.50 µl

Mix gently and dispense 23 µl of each of reaction mixture to autoclaved PCR tubes. 2 µl DNA was added separately to each PCR tube to make final reaction mixture 25 µl . The thermocycle programme similar to that of DNA amplification of npt II gene was used. Prepared 1.6 % agarose gel of 250 ml volume. 4 µl 6x loading dye was added to each sample and loaded into the gel. A 2 µl of 100 bp ladder sequence was also loaded in the 1st and last well as standard and the gel was electropohoresed for one hour at 120 V. The image of the gel was taken.

Table 3.1 Specific (designed) primer used in the present study for the amplification of *npt-II* gene in the transgenic *Punica granatum* L. genome

S.No.	Primer	Sequence (S' – 3')
1.	Forward primer (*npt-II* F)	ATC GGG AGG GGC GAT ACC GTA
2.	Reverse primer (*npt-II* R)	GAG GGT ATT CGG CTA TGA CTG

Table 3.2 Specific (designed) primer used in the present study for the amplification of *cry1Ab* gene in the transgenic *Punica granatum* L. genome

S.No.	Primer	Sequence (S' – 3')
1.	Forward primer	TCT CCA ACT ACG ACA GCA GGA CCT
2.	Reverse primer	GGT GAA TCC ACG AGA ACA TGG GAG

EXPERIMENTAL RESULTS

Chapter-4

EXPERIMENTAL RESULTS

The results obtained from the present investigation have been discussed under the following headings:-

4.1 Molecular analysis of putative GUS transgenic plantlets

4.1.1 DNA isolation and purification

4.1.2 Quantitative estimation of DNA

4.1.3 Qualitative estimation of DNA

4.1.4 Confirmation of transformation by PCR

4.2 Molecular analysis of putative Bt transgenic shoots

4.2.1 Multiplication and maintenance of control shoots

4.2.2 Multiplication and maintenance of putative Bt transgenic shoots

4.2.3 Comparison of *in vitro* shoot proliferation in control and putative Bt transgenics.

4.2.4 DNA isolation and purification

4.2.5 Quantitative estimation of DNA

4.2.6 Qualitative estimation of DNA

4.2.7 Confirmation of transgene

a) npt-II gene amplification

b) Amplification of *cry 1 Ab* gene

The results of the present investigation were carried out in two parts. The first part deals with the molecular analysis of putative GUS transgenic plantlets, while the second part is on *in vitro* proliferation of control and Bt putative transgenic microshoots followed by molecular analysis.

4.1 MOLECULAR ANALYSIS OF PUTATIVE GUS TRANSGENIC PLANTLETS

In the present experiment five putative GUS plantlets and one control plantlet were randomly selected which were already raised *in vitro* in the laboratory of Department of Biotechnology, College of Horticulture, Dr Y.S. Parmar University of Horticulture & Forestry, Nauni- Solan (HP). The selected putative GUS plantlets were numbered G_1, G_2, G_3, G_4, and G_5. The non transformed control plantlet was numbered G_0. These selected plantlets were used to carry out further experiments.

4.1.1 DNA isolation and purification

Total genomic DNA was isolated from the shoots of the control plantlet G_0 as well as putative GUS plantlets (G_1, G_2, G_3, G_4, and G_5). About 1 g of each sample was ground separately in liquid nitrogen in order to extract the DNA. Thus the DNA pellet obtained at the end was dissolved in 200µl of autoclaved distilled water in 1.5 ml micro tubes separately and were labeled as G_0 and G_1, G_2, G_3, G_4, G_5, before storing at –20°C for carrying out further experiments.

4.1.2 Quantitative estimation of DNA

Before carrying out further experimentation it is important to check the quantity of the DNA isolated and purified from each sample. Therefore, the present experiment was conducted to check the quantity of DNA in the control (G_0) and GUS plantlets (G_1, G_2, G_3, G_4 and G_5). The concentration of DNA samples was calculated spectrophotometrically (separately for each sample) in which 95 µl of autoclaved water and 5 µl of the DNA sample was taken. For quantitating the amount of isolated DNA, readings were taken at wavelength of 260 nm and 280 nm. The reading at 260 nm allowed calculation of the concentration of the DNA samples. .

The results are presented in Table 4.1 which shows the concentration of DNA in µg/ml in each sample selected for molecular analysis. It was observed that there existed some variation in the DNA concentration of putative GUS plantlets and control plantlets.

Table- 4.1: Quantitative estimation of DNA of control and putative GUS plantlets

Plantlet type	Plantlet number	DNA concentration (µg /ml)
Untransformed	G_0	850
Transformed	G_1	750
	G_2	700
	G_3	700
	G_4	750
	G_5	750

4.1.3 Qualitative estimation of DNA

The experiment was carried out to check the purity of the DNA to be used for molecular analysis. The presence of impurities like protein, polysaccharides, phenols and RNA interfere with molecular analysis, during PCR studies. Thus, it is

important that the DNA should be free of all interfering agents in order to prevent PCR artifacts. The approximate purity of double stranded DNA was estimated by determination of the ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}). This ratio is 1.8 for pure double stranded DNA. Quality check of DNA was done by electrophoresis by loading the samples on 0.8% agarose gel. Current of 100 V was passed through it for 1 hr and after 1 hr the gel was viewed under the transiluminator and photographed.

The electrophoretic pattern of DNA is presented in Plate 1. It was observed that the genomic DNA of putative GUS transgenic was almost pure as the ratio of absorbance (A_{260}/A_{280}) was almost 1.8 in all the 5 samples ranging from G_1 to G_5. Whereas, RNA contamination was observed in case of control sample G_0, as the ratio of absorbance was above 2 in it. This could be seen in the DNA electrophoresis results where a little smear was obeserved during the electrophoresis in case of DNA of sample G_0 (Control) as shown in Plate 1. However, no smear was seen in case of DNA of putative GUS transgenics when electrophoresis was carried out on 0.8% agarose gel at 100V (Plate-1). Therefore, an additional step of purification was given to purify the DNA of sample G_0 (Control).

4.1.4 Confirmation of transformation by PCR

PCR was carried out to study the presence and the integration of the target gene in the putative transgenic plantlets of wild pomegranate. The isolated and purified DNA of samples G_1, G_2, G_3, G_4, G_5 and G_0 were used for PCR amplification. Target gene specific primers were used to amplify a 700 bp fragment of npt II gene by PCR. For this 2 primers were used, forward and reverse (npt-II F and npt II R). The npt II F had the sequence $^{5'}$ATC GGG AGG GGC GAT ACC GTA$^{3'}$ and the npt II R had the sequence $^{5'}$GAG GAT ATT CGG CTA TGA CTG$^{3'}$.

The products of PCR were visualized after electrophoresis in 1.6 per cent agarose gel and are shown in Plate 2. Lane 1 represent DNA size marker of 100 bp, lane 2- containing plasmid pBI121 was taken as positive control, lane 3 containing sterile water represents the negative control, lane 4 contain the DNA of none transformed control G_0 and lanes 5 –9 contain the DNA of transformed samples G_1, G_2, G_3, G_4 and G_5.

It may be observed in Plate 2 that lane 2 containing the plasmid pBI121 showed the band of the amplified npt II gene, while lane 3 containing sterile water taken as negative control did not show any such band. It may be seen that DNA of

the non-transformed control G_0 in lane 4 also did not contain any presence of the amplified band. It may be noted that lane 5-8 containing the DNA of the putative GUS transgenics showed the amplification of band of the integrated gene which is at par with the band of the positive control, thus showing the confirmation of the transgene (npt II). However, there was no amplification of the target gene in sample G_5 (lane 9).

Table 4.2 shows the results of gene amplification by PCR. Out of the 5 putative plantlets (G_1, G_2, G_3, G_4, and G_5) only 4 plantlets - G_1, G_2, G_3 and G_4 showed amplified gene bands, thereby indicating the presence and integration of npt II gene in these 4 transgenic plantlets. No amplified gene band was seen in case of G_5. The control plantlet (G_0) did not show any band for amplified gene.

Table 4.2: PCR characterization of the putative GUS transgenics by amplification of approximately 700 bp fragment from the npt II gene.

Plantlet type	Plantlet number	Molecular analysis of npt II by PCR
Untransformed	G_0	-
Transformed	G_1	+
	G_2	+
	G_3	+
	G_4	+
	G_5	-

amplication : +
no amplication : -

4.2 MOLECULAR ANALYSIS OF PUTATIVE Bt TRANSGENIC SHOOTS

Work is already in progress on the *in vitro* proliferation of Bt shoots to raise transgenic insect resistant plantlets in the laboratory of department of biotechnology, college of horticulture. Dr.Y.S.Parmar university of Horticulture and Forestry, Nauni, Solan, HP. Therefore, the already transformed micro shoots carrying Cry1*Ab* through *Agrobacterium tumefaciens* strain (Fig 2) from the present laboratory were used for the second part of the investigation. Therefore, in this experimentation the multiplication of the putative Bt transgenic shoot as well as the control shoots were carried out to get microshoots for DNA studies to see the integration of transgene in the transformed shoots.

4.2.1 Multiplication and maintenance of control shoots

The control shoots of size 1.0-1.5 cm were separated from clumps growing on cotyledon explants and cultured individually on solid MS medium containing 2.0 mgl^{-1} BAP, 1.0mgl^{-1} GA_3 and 1.0 mgl^{-1} NAA (Kanwar *et al.*, 2004). The cultures were incubated at 25±2^{0}C under 16 hours photoperiod for six weeks. The shoots were subcultured after an interval of six weeks on the same medium three times.

Observation were recorded after six weeks of incubation for number of shoots/shoot clump and length of shoots to see the effect of subculturing of shoot multiplication. Results are presented in (Table 4.3 and Plate 3). The results show that the number of shoots/shoot clump increased with each subculturing. The same trends was observed for the shoot length, which increased with increase in the n umber of subculturings.

4.2.2 Multiplication and maintenance of putative Bt transgenic shoots

The *in vitro* putative transgenic microshoots were separated from shoot clumps and cultured individually on selective medium which consisted of solid MS medium containing 2.0 mgl^{-1} BAP, 1.0 mgl^{-1} GA_3, 1.0 mgl^{-1} NAA and supplemented with kanamycin 30 mgl^{-1} and cefotaxime 150 mgl^{-1}. The cultures were incubated at 25±2^{0}C under 16 hours photoperiod for six weeks. The shoots were subcultured after an interval of six weeks on the same selective medium three times.

The results are presented in Table 4.3 and Plate 4. Results show that the number of shoots/shoot clump increased with each subculturing. However, escape shoots were removed at each subculturing. Although the length of the putative transgenic shoots increased with subsequent subculturings but the increase in length was less as compared to control.

4.2.3 Comparison of *in vitro* shoot proliferation in control and putative Bt transgenics.

The length of shoots and number of shoots/shoot clump of control and putative Bt transgenics were compared at an interval of 6 weeks. The experiment was repeated thrice and the results summarize the data of three independent experiments.

It may be seen in Table 4.3 that the *in vitro* proliferated shoots increased in number as well as in length with increase in number of subculturing in both control and on selective medium. In case of control maximum (6.88) shoots per shoot clump with maximum shoot length of 4.06 cm was observed after third subculture. Same trend was observed in case of shoots subcultured on selective medium with maximum (4.96) shoots per shoot clump and maximum average shoot length of 1.75 cm. It may be noted that the number of shoots as well as length of shoot at each subculturing was higher in control as compared to transgenic shoots.

Table 4.3 : Comparison of the *in vitro* shoot proliferation in control and putative Bt transgenic shoots (after an interval of 6 weeks)

Subculture passage	No. of shoots/shoot clump		Length of shoots (cm)	
	Control	Transformed	Control	Transformed
S_1	3.15	2.59	2.79	1.06
S_2	5.43	3.20	3.97	1.47
S_3	7.88	4.96	4.06	1.75
S.E.M.	0.15	0.04	0.06	0.04
$CD_{0.05}$	**0.37**	**0.10**	**0.16**	**0.09**

4.2.4 DNA isolation and purification

The genomic DNA was isolated from randomly selected putative Bt shoots and control shoots. Four putative Bt shoot clumps were randomly selected and labeled as B_1, B_2, B_3 and B_4. The non transgenic control shoots was labeled as B_0. About 1g of putative transgenic shoots from the selected samples were ground separately in liquid nitrogen and DNA was extracted. The DNA pellets thus obtained from each sample was dissolved separately in 200 µl autoclaved distilled water and stored at –20°C.

4.2.5 Quantitative estimation of DNA

Quantity of DNA was estimated by a spectrophotometer (BIO RAD-SMART SPEC™ 3000). The concentration of the DNA samples was calculated spectrophotometrically in which 95 µl of autoclaved water and 5 µl of the DNA samples was taken. For quantitating the amount of DNA, readings were taken at wave length of 260 nm, and 280 nm. The reading at 260 nm allowed calculation of the concentration of DNA in the sample.

The results are presented in Table 4.4 which shows the concentration of DNA in µg/ml in each sample selected for molecular analysis. It may be seen that some variation existed in DNA concentration among control and putative Bt transgenic shoots.

Table 4.4 : Quantitative estimation of DNA of control and putative Bt shoots

Shoot type	Shoot number	DNA concentration (µg/ml)
Untransformed	B_0	1100
Transformed	B_1	900
	B_2	950
	B_3	950
	B_4	900

4.2.6 Qualitative estimation of DNA

It was important to purify the DNA of all contaminants as the presence of impurities like, protein, polysaccharides, phenols and RNA interfere with molecular analysis. The ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}) was found out spectrophotometrically which helpd to determine the approximate purity of double standard DNA, which is 1.8 for pure double stranded DNA. Quality check of DNA was done by electrophoresis by loading the samples on 0.8% agarose gel. Current of 100 V was passed through it for 1 hr and after 1 hr the gel was viewed under the transiluminator and photographed.

Results are shown in Plate 5. It was observed that all genomic DNA of putative Bt transgenic shoots had phenol/protein contamination as the ratio of absorbance was between 1.6-1.7. RNA contamination was observed in case of control shoots as the ratio of absorbance was above 2.0. This could also be seen in the DNA electrophoresis results where a little smear was observed as shown in results presented in plate 5. Therefore, an additional step of purification was given to purify the DNA samples, which on further electrophoresis did not show any smear.

4.2.7 Confirmation of transgene

Target gene amplification was done by polymerase chain reaction. The

vector contains npt II and *cry 1 Ab* genes, therefore the confirmation was studied using npt II specific primers as well as *cry 1 Ab* specific primers.

a) npt-II gene amplification

Total genomic DNA isolated from putative Bt transgenic shoots growing on the selective medium was analyzed by PCR, using specific primers to verify the presence of npt-II gene in the transgenic shoots. A DNA fragment of approximate 700 bp, corresponding to expected span between the two primers (npt- II F and npt- II R as shown in Table-3.1) was amplified in the shoots where gene had integrated. The product of PCR were visualized after electrophoresis on 1.6 % agarose gel as shown in plate-6. Lane 1 represents the DNA size marker of 100bp, lane 2 containing plasmid pBI 121 was taken as positive control, lane 3 containing sterile water was taken as negative control, lane 4 contained the DNA of the non-transformed control B_0, lane 5-8 contain the DNA of transformed samples of B_1, B_2, B_3 and B_4.

Banding pattern in Plate 6 shows that lane 2 containing the plasmid pBI 121 showed the band of the amplified npt II gene, while lane 3 containing sterile water taken as negative control did not show any band. It was observed that the DNA of the non- transformed control B_0 in lane 4 did not show the specific band corresponding to npt II, although a band appeared which was not specific to npt II

amplified band. It is interesting to note that lane 5 and 6 containing the DNA of the putative Bt transgenics showed the amplification of band of the integrated gene which was at par with the band of the positive control, thus confirming the integration of the transgene (npt II). However, there was no amplification of the target gene in sample B_3 and B_4 as shown in Plate 6.

Table 4.5 shows the results of npt-II gene amplification in the putative Bt transgenic shoots. It was observed that amplified gene bands were seen only in B_1 and B_2 shoots. However, no amplification occurred in B_3, B_4 and control shoots.

Table 4. 5: PCR characterization of the putative Bt transgenic shoots by amplification of approximately 700 bp fragment from npt-II gene

Shoot type	Shoot number	Molecular analysis of npt-II by PCR
Untransformed	B_0	-
Transformed	B_1	+
	B_2	+
	B_3	-
	B_4	-

amplification : +
no amplification :-

b) Amplification of Cry 1 *Ab* gene

Polymerase chain reaction (PCR) was carried out to study the presence and integration of the target gene (*cry1Ab*) in the putative transgenic shoots of wild pomegranate and to confirm the transfer of *cry1Ab* gene from *Agrobacterium tumefaciens* into the genome of wild pomegranate shoots. Total genomic DNA was isolated from 4 putative kanamycin resistant *in vitro* grown transgenic shoots along with 1 control shoot. 2µl of DNA was used for PCR amplification from each sample. Gene specific primers were used to amplify a 700 bp fragment of *cry1Ab* gene by PCR. Forward primer had the sequence $^{5'}$TCT CCA ACT ACG ACA GCA GGA CUT$^{3'}$ and the reverse primes had the sequence $^{5'}$GGT GAA TCC ACG AGA ACA TGG GAG^{3}. PCR products were visualized after electrophoresis in 1.6 per cent agarose gel as shown in Plate 7.

Lane 8 represents the DNA size marker of 1 kb, lane 7 containing the DNA from a known *cry 1 Ab* transgenic was taken as positive control, lane 6 containing sterile water was taken as negative control, lane 5 contains the non-transformed samples B_0 and lane 1-4 contain the transformed samples B_1, B_2, B_3 and B_4.

The results presented in Plate 7 depicts that *cry 1 Ab* gene fragment of approximately 700 bp was amplified where gene integration had taken place. Lane 7 representing the positive control shows the presence of *cry 1 Ab* band of 0.7 kb.

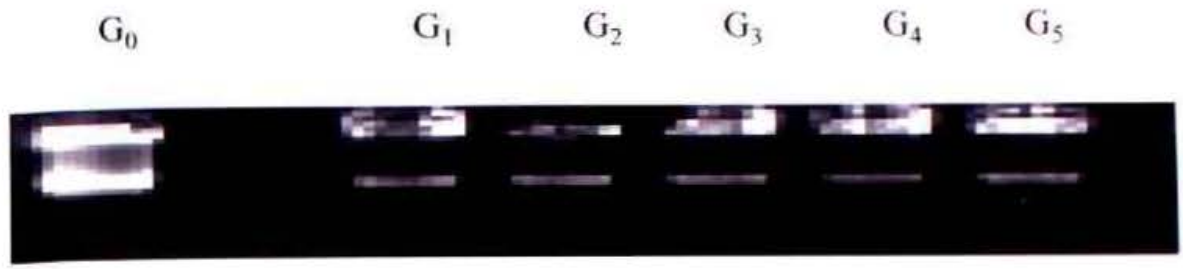
Lane 5 and 6 are without any band as these lanes contain the DNA of non transformed control B_0 and negative control. It is seen that lane 3 and 4 contain the amplified DNA of 0.7 kb which is at par with DNA band of the positive control. Although lane 1 and 2 contain the DNA of putative Bt transgenic shoots, but failed to show the amplification of the respective DNA fragment of the target gene.

The result of PCR amplification is shown in Table 4.6. It was observed that *cry1Ab* gene was amplified in only B_1 and B_2 shoots. Whereas, no bands appeared in B_3 and B_4 and control shoots.

Table 4.6: PCR characterization of the putative Bt transgenic shoots by amplification of approximately 700 bp fragment from *Cry1Ab* gene.

Shoot type	Shoot number	Molecular analysis of *Cry1Ab* by PCR
Untransformed	B_0	-
Transformed	B_1	+
	B_2	+
	B_3	-
	B_4	-

amplification : +
no amplification : -

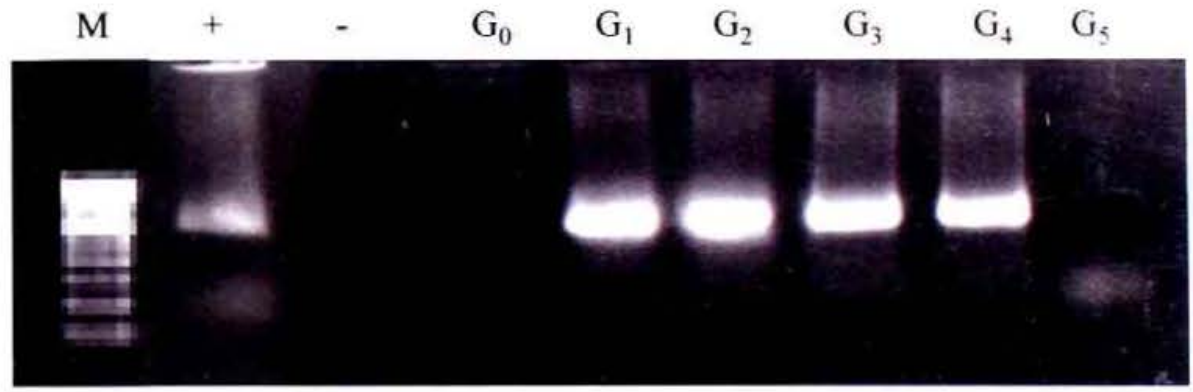


Quality assessment of DNA obtained from control and putative GUS plantlets during gel electrophoresis ón 0.8% agarose gel

Lane 1 – DNA of control plantlet

Lane 3-7 – DNA of putative GUS transgenics

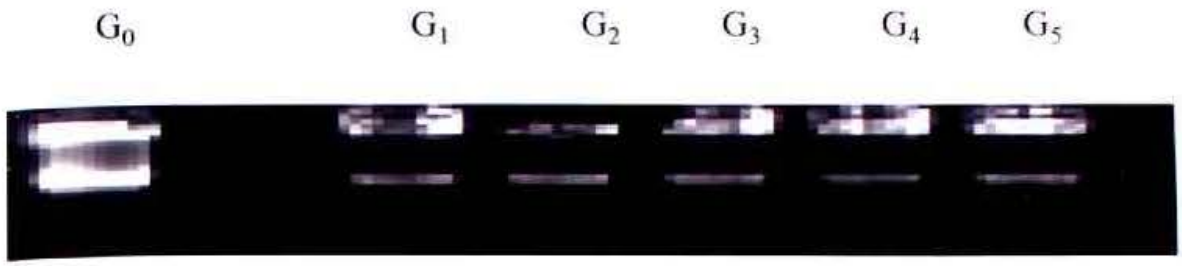
Plate - 1



PCR characterization of the putative GUS transgenics by amplification of approximately 700 bp fragment from npt-II gene

Lane 1 - DNA size marker(100bp)
Lane 2 – Positive control
Lane 3 – Negative control
Lane 4 – Non transformed control(G_0)
Lane 5-9 – Putative GUS transgenics

Plate- 2

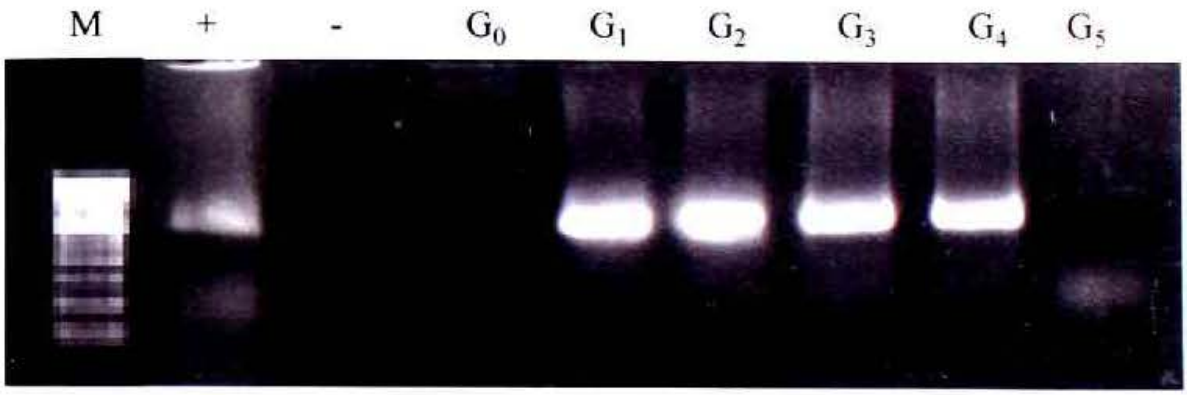


Quality assessment of DNA obtained from control and putative GUS plantlets during gel electrophoresis ón 0.8% agarose gel

Lane 1 – DNA of control plantlet

Lane 3-7 – DNA of putative GUS transgenics

Plate - 1



PCR characterization of the putative GUS transgenics by amplification of approximately 700 bp fragment from npt-II gene

Lane 1 - DNA size marker(100bp)
Lane 2 – Positive control
Lane 3 – Negative control
Lane 4 – Non transformed control(G_0)
Lane 5-9 – Putative GUS transgenics

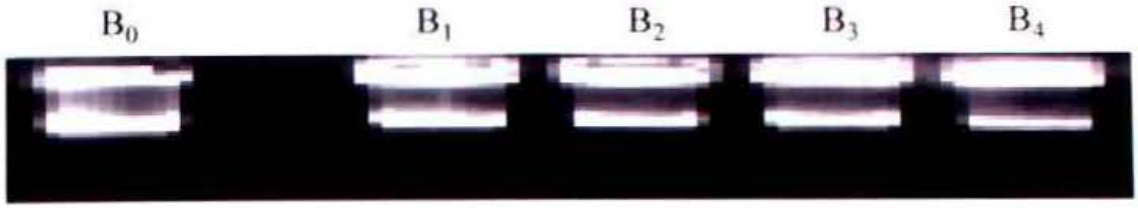
Plate- 2

Multiplication of control shoots on MS medium supplemented with 2.00 mgl^{-1} BAP +1.00 mgl^{-1} GA_3+ 1.00 mg l^{-1}NAA

Plate - 3

Multiplication of putative Bt transgenic shoots on selective medium consisting of MS medium supplemented with 2.00 mgl^{-1} BAP +1.00 mgl^{-1} GA_3+ 1.00 mg l^{-1}NAA + 30 mg l^{-1} Kanamycin

Plate - 4

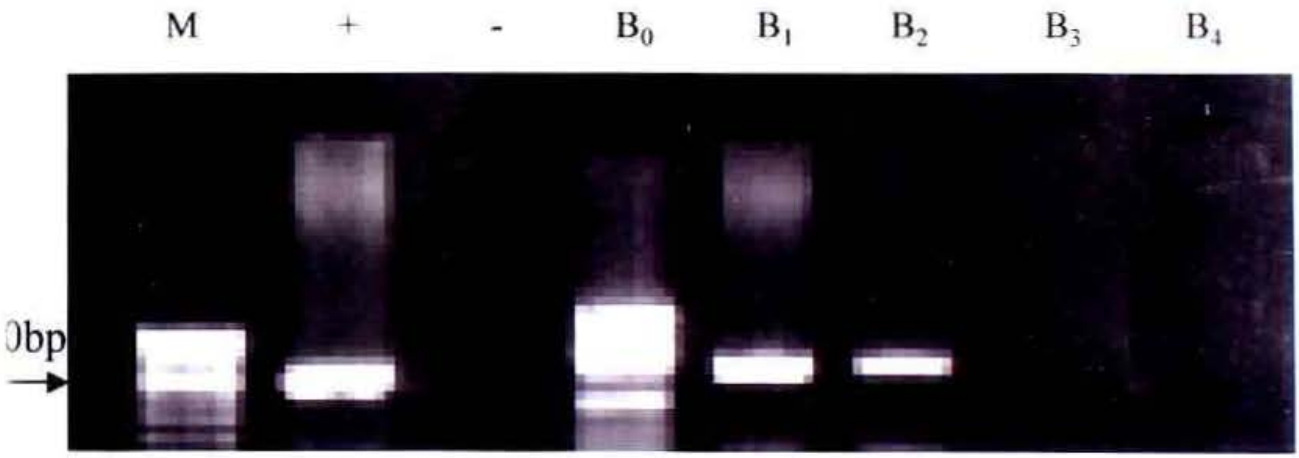


Quality assessment of DNA obtained from control and putative Bt shoots during gel electrophoresis on 0.8% agarose gel

Lane 1 – DNA of control shoot

Lane 3-7 – DNA of putative Bt transgenic shoots

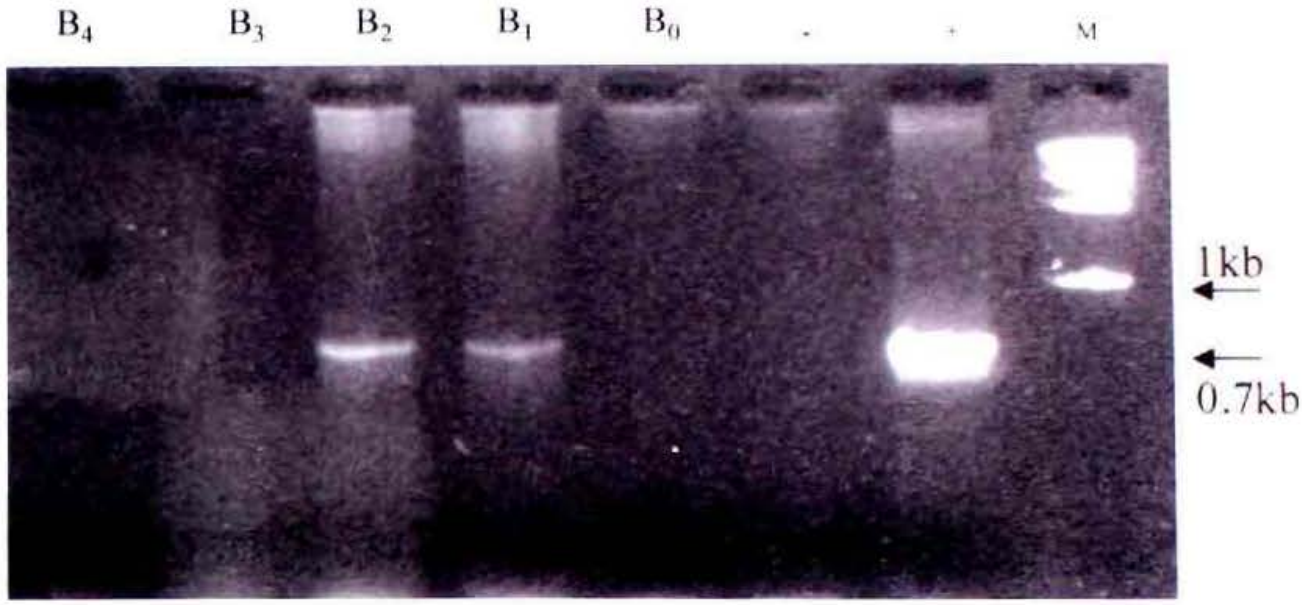
Plate -5



PCR characterization of the putative Bt transgenic shoots by amplification of approximately 700 bp fragment from npt-II gene

Lane 1 - DNA size marker(100bp)
Lane 2 – Positive control
Lane 3 – Negative control
Lane 4 – Non transformed control(B_0)
Lane 5-8 – Transformed shoots

Plate - 6



PCR characterization of the putative Bt transgenic shoots by amplification of approximately 700 bp fragment from Cry1Ab gene

Lane 1-4 – putative Bt transgenic shoots

Lane 5 –Non transformed control(B_0)

Lane 6 – Negative control

Lane 7 - Positive control

Lane 8 – DNA size marker(1kb)

Plate - 7

DISCUSSION

Chapter-5

DISCUSSION

Wild pomegranate (*Punica granatum* L.) is a multipurpose cash crop tree species having economic and ecological significance. It plays an important role in pushing up the economy of the farmers of the degraded hill slopes lacking other cash crops. The fruit is a good source of sugars and vitamin C. The seeds with aril are sun dried and commercially marketed as 'anardana' and are widely used as a condiment (Parmar and Kushal, 1982). Almost all plant parts of this species are used in one or the other form. However, wild pomegranate is attacked by pomegranate butterfly (*Virachola isocraty* Fb.) causing heavy damage and is a limiting factor in its cultivation (Parmar and Kushal, 1982). It is known that wild pomegranate is infested by several species of insects, out of which the pomegranate fruit borer, anar butterfly-*Deudorix isocrates* Fabr. (Lycaenidae:Lepidoptera) previously named as *Virachola isocrates* F. is considered to be the most important one based on the extent of damage caused in pomegranate crop (Bhutani, 1979). In mid hills of H.P. wild pomegranate is commonly attacked by *Deudorix epyarbas* Moore. which lays eggs on fruit, bores inside and then feed on the seeds (Rawat *et al.*, 1989) One of the ways to overcome this is genetic transformation for insect resistance.

The natural gene transferring ability of *Agrobacterium*, a consequence of specific protein DNA interaction can be exploited to a major extent to genetically modify the plants. Thus, it is now technically possible to transfer genes across all taxonomic boundaries in plants from other plants, animals, microbes and even use or introduction of totally artificial genes. This offers a considerable potential for genetic improvement of crop plants for insect pest resistance and more specifically for improved quality characteristic. But before carrying out genetic transformation to transfer agronomically important genes such as those of insect resistance, it is essential to standardize the protocol for genetic transformation which is done in the present laboratory by using reporter genes such as GUS and selectable marker gene npt II.

During the course of the investigation efforts were made to confirm the integration of target gene in putative GUS plantlets as well as in the putative Bt transgenic shoots of *Punica granatum* L. The result of the investigations are discussed under the following headings:

5.1 Molecular analysis of already *in vitro* raised putative GUS transgenic plantlets.

5.2. *In vitro* proliferation of control and putative Bt transgenic shoots followed by its molecular analysis.

5.1 Molecular analysis of already *in vitro* raised putative GUS transgenic plantlets

The putative transformed tissues already subjected to histochemical GUS assay (Kashyap, 2004) were taken to carry out polymerase chain reaction for the confirmation of transformation. The GUS gene acts as a scorable marker and in combination with constitutively expressed CaMV 35 S promoter this system achieves a high expression in nearly all types of tissues (Hull and Devic, 1995). The gus gene or β- glucoronidases catalysis the hydrolysis of a wide variety of β-glucoronides. The X-gluc is histochemically detected glucuronide used for tissue specific localization of Gus activity (Jefferson *et al.*, 1987). On the other hand npt II gene confers resistance to all aminoglycoside antibiotics kanamycin and neomycin. Kanamycin resistance due to npt II has been widely used as a selectable marker in many plant species. The selection of transformed cells is done on a medium containing Kanamycin sulphate.

In the present study control and randomly selected GUS plantlets (histochemically assayed earlier) were taken to carry out molecular analysis.

DNA isolation, quantitative and qualitative estimation

Genomic DNA was isolated from the shoots of *in vitro* raised five putative GUS plantlets and one control plantlet of wild pomegranate (*Punica granatum* L.)

using CTAB method (Doyle & Doyle, 1987) with some modification. The quantity and quality of DNA was assessed spectrophotometrically and by electrophoresis. There was some variation in the DNA concentration of putative GUS plantlets and control plantlets. The DNA concentration ranged between 850 µg/ml to 700 µg/ml. which may be due to the contaminants retained during DNA extraction. Therefore an additional step of purification was given to purify the DNA in the present studies, otherwise the impurities led to interface in molecular analysis.

As yet there is no such information available in the available literature that mentioned anything regarding quantity and quality of DNA extracted from *in vitro* or *in vivo* raised plantlets of wild pomegranate (*Punica granatum* L.).

For qualitative assessment of DNA, the five putative GUS plantlets showed good quality DNA as was indicated by the ratio of absorbance as well as the results of electrophoresis. DNA of control plantlet showed little smearing during electrophoresis which may be due to presence of impurities. Thus an additional step of purification was given after which no smearing was seen when electrophoresis was carried out again.

Confirmation of transformation by PCR

Polymerase chain reaction (PCR) is a technique to selectively amplify *in vitro* a specific segment of a total genomic DNA a billion fold (Saiki *et al.*, 1985; Mullis *et al.*, 1986). The most essential requirement is the availability of a pair of

short (typically 20-25 nucleotides) oligonucleotides called primers having sequence complementary to either end of the target DNA segments (called template DNA) to be synthesized in a large amount.

The transgenic nature of the histochemically assayed GUS plantlets was further supported by polymerase chain reaction with primers specific to npt II.

To avoid amplification of unspecific fragments the reaction was carried out at higher annealing temperature (55^0C). Therefore no recombinant products were formed due to annealing of incomplete elongation products during PCR, a phenomenon reported by Meyerhans *et al* (1990) and Marton *et al.*, (1991).

The results of gene amplification of PCR showed that out of the five putative plantlets (G_1, G_2, G_3, G_4 and G_5) only 4 plantlets (G_1, G_2, G_3, and G_4) showed amplified gene bands, thereby indicating the presence and integration of npt II gene in the 4 transgenic plantlets. No bands were detected in the non transformed control (G_0) and plantlet G_5 .Similar work was done by Agarwal *et al* (2004) in *Morus alba* L.; Charity *et al* (2005) in *Pinus radiata*; Matsuda *et al* (2005) in *Pyrus commumi* L.; Carredoira *et al* (2004) in *Castenea sativa*, Levee *et al* (1996) in hybrid larch (*Larix Kaempferi* X *L.decidua*) and Prakash and Gurumurthi (2005) in *Eucalyptus tereticornis*.

Thus the confirmation by PCR of putative GUS transgenic plantlets in wild pomegranate *(Punica granatum* L.) by using npt II specific primers is probably the first report .

5.2. *In vitro* proliferation of control and putative Bt transgenic shoots.

As mentioned earlier the work is already going on the genetic transformation of wild pomegranate through *Agrobacterium tumefaciens* to produce insect resistant plants. Therefore, the already transformed shoots were cultured on the selective multiplications medium and control shoots on simple multiplication medium in the present study. It was observed that the non- transformed shoots showed higher growth as compared to the transformed shoots after each subculture. Hammerschlag *et al* (1997) stated in *Malus domestica* the percentage of shoot regeneration from leaf explant was 67 percent from control and 11 percent on selective media from transformants. The same results have een obtained in case of *in vitro* proliferation of transformed and non transformed shoots of *Punica granatum* L. (Kashyap, 2004); in *Morus alba* L. (Agarwal, 2002) and *Robinia pseudocacia* (Kanwar *et al*.,2003).

5.2.2 Molecular analysis of putative Bt transgenic shoots

DNA was isolated from shoots of transformed shoots as well as the non transformed shoots of wild pomegranate (*Punica granatum* L.) using CTAB method (Doyle & Doyle, 1987) with some modification. Quantity of the DNA showed some variations. The DNA concentration of control shoots was higher as compared to all

transformed shoots. The DNA concentration ranged between 1100 µg/ml to 900 µg/ml. For qualitative assessment of DNA, the DNA from both transformed and non transformed shoots showed presence of phenol/protein contamination. This was indicated by the ratio of absorbance as well as the result of electrophoresis. Thus an additional step of purification was given to both, the DNA of transformed as well as non transformed shoots. Although no such reports are available but same type of observation were noted while quantification of DNA of putative GUS transgenics in the previous section.

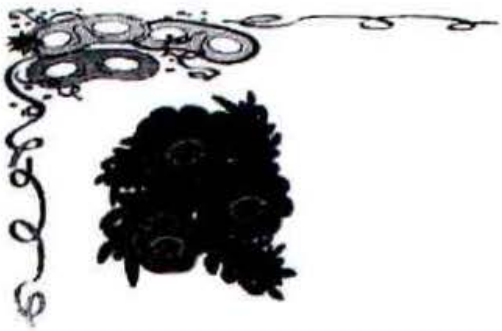
Confirmation of transformation by PCR

To confirm the integration of the target gene in the genome of the transformed shoots, PCR studies were carried out. The vector used for confirmation contained npt II and *cry 1Ab* genes, therefore the confirmation was studied using npt II specific primers as well as *cry 1 Ab* specific primers. Similar PCR analysis was carried out by Lynette *et al* (2005) by using primers specific for sequences within the npt II and *cry 1Ac* gene.

The results of the npt II gene amplification showed that two out of the four putative shoot clumps had the target genes integrated in it. No amplification was observed in control shoots. Verma and Chand (2005) also reported similar type of results in case of *Cajanus cajan* L. where two out of seven surviving Bt gene transformed shoots showed amplification of 700 bp fragment for the npt II gene.

To further confirm the integration of the target gene, amplification using *cry1Ab* specific primers was carried out which also showed band amplification in only two out of the four putative shoot clumps. Chakarbarty *et al* (2002) also analysed plants for the presence and expression of transgene (*cry 1Ab*) in cauliflower using PCR. Kaushik and Kumar (2003) confirmed presence of *cry 1Ab* gene in brinjal by PCR. Salehi *et al* (2005) confirmed cry 1Ab gene integration in common bermudagrass by PCR and Lynette *et al* (2004) also analysed plants for the presence and expression of *cry 1 Ab* gene in *Pinus radiata* using PCR.

Investigation revealed that the transformation frequency in case of genetic transformation using gus and npt II gene was fairly good. Whereas in case of *cry 1 Ab* gene, transformation frequency was low. Low transformation frequency has also been reported in various other plants such as various brassica species (Barfied and Pusa, 1991; Pental *et al*, 1993 and Dixit *et al*, 1998). Zhang *et al* (2000) reported transformation frequency of 1.6-2.7 percent in brassica. Thus the protocol developed for introducing insect resistance gene into wild pomegranate (*Punica granatum* L.) may be used in future experiments only after some modification so as to increase the transformation frequency.



SUMMARY AND CONCLUSION

Chapter-6

SUMMARY AND CONCLUSION

During the present investigation efforts were made to confirm the integration of the transgenes (npt II and gus; *cry 1Ab*) in to the tissues of putative transgenics of wild pomegranate (*Punica granatum* L.). This was necessary to see whether the protocol developed for *Agrobacterium* mediated transformation of wild pomegranate was efficient or needed any modifications.

From the observations so recorded, the important findings have been summarized below:

1. There existed some variation in the DNA concentration of putative GUS plantlets and controls plantlets.
2. Quality of the DNA of putative GUS plantlets was good as compared to DNA of control plantlets which needed another step of purification.
3. Temperature profile for PCR amplification was standardized.
4. Optimum concentration of different components of a 25µl reaction volume of the PCR master mix was standardized for amplification of the genomic DNA.
5. Successful amplification of npt II gene was confirmed by PCR amplification using primers specific to npt II gene. 700 bp npt II fragment lying between

the two primers was amplified in four out of five putative transgenic plantlets, non transformed plantlet did not show any reaction.

6. Putative Bt transgenic shoots, induced directly on the explants (cotyledons) were multiplied by repeated subculture on selective medium to increase the number of transformed shoots.
7. Non - transformed control shoots induced directly on the cotyledonary explants were multiplied by repeated subculture to increase the number of control shoots.
8. Comparison of the *in vitro* shoot proliferation in control and putative Bt transgenic shoots were done. It was observed that in both cases the number of shoots/ explant and the length of shoots increased but the number of shoots/ explant and the length of shoots was less in case of the transformed shoots as compared to the non transformed shoots.
9. The quality of both transformed and non transformed was not very good hence needed an additional step of purification.
10. Amplified gene bands specific to 700 bp npt II fragment was observed in case of only two out of the four transformed shoot clumps. Non transformed shoot did not show any band amplification specific to npt II gene.
11. Integration of Bt gene was further confirmed by using *cry 1 Ab* specific primers where similar results to that of the npt II gene amplification were observed.

The present investigation was carried on "Confirmation of transgenes in putative transgenic tissue of wild pomegranate (*Punica granatum* L.)". This was conducted in two parts. Firstly molecular analysis of already *in vitro* raised putative GUS plantlets through *agrobacterium tumefaciens* strain LBA4404. Second part deals with the *in vitro* proliferation of already co cultivated Bt transgenic shoots and control shoots followed by molecular analysis using npt II and *cry 1Ab* primers.

The results presented in the thesis shows that the amplification of transgene (npt II) was confirmed in four out of the five putative GUS plantlets. The *in vitro* raised untransformed and transformed Bt shoots were also subjected to quantitative and qualitative estimation of DNA before subjecting to PCR confirmation by npt II as well as *cry 1Ab* primers. It was observed that both npt II and *cry 1Ab* were confirmed by respective primers in two out of the four transformed Bt shoots.

The development of the protocol for raising genetically transformed plants of GUS seems to be promising as detected by the confirmation of transgene (npt II). However, the protocol developed for insect resistant shoots needs further modification to increase the transformation frequency.

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ABSTRACT

THESIS ABSTRACT

Title of Thesis :	:	"Confirmation of transgenes in putative transgenic tissue of wild pomegranate (*Punica granatum* L.)"
Name of the student	:	Ritika Chauhan
Admission number	:	H-2003-13-M
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Minor field(s)	:	Tree Improvement Biochemistry
Degree awarded	:	M. Sc (Biotechnology)
Year of award of degree	:	2005
No. of pages in the thesis	:	77+VI
No. of words in abstract	:	221

ABSTRACT

The integration of the transgene through *Agrobacterium tumefaciens*-mediated gene transfer in the already raised *in vitro* putative GUS plantlets of wild pomegranate (*Punia granatum* L.) was confirmed by PCR studies. Some variation existed in DNA concentration of putative GUS and control plantlets, which was subjected to another step of purification. Successful integration of npt II gene in four out of the five putative GUS trasngenics was confirmed by PCR. Putative Bt transgenic shoots already co cultivated through *Agrobacterium* carrying *cry 1Ab* gene as well as control shoots were multiplied, by repeated subculturing on selective multiplication and non selective multiplication medium respectively, and compared. It was observed that number of shoots per explant and length of shoots was less in case of the transformed shoots. The quality of DNA was not very good in case of both transformed and untransformed shoots and thus subjected to another step of purification. The integration of the transgene in the putative Bt transgenic shoots was confirmed by PCR amplification using npt II and *cry 1 Ab* specific primers. Amplified gene bands specific to 700 bp npt II fragment was observed in only two out of four transformed shoots. Similar results were obtained on gene amplification using *cry 1 Ab* specific primers thus confirming the integration of Bt gene in only two out of four transgenic shoots.

Major advisor

Ritika

Signature of the student

Countersigned

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APPENDICES

APPENDIX-I

Solutions for DNA extraction and gel electrophoresis

DNA extraction

Chloroform : Isoamyl alcohol (24 :1, v/v)	Mix 96 ml chloroform and 4 ml isoamyl alcohol and keep at room temperature in a closed container
10 % (w/v) CTAB	Add 10 g of CTAB to approximately 70 ml of water. Dissolve the detergent by warming the solution to 65°C. Adjust the volume to 100 ml.
0.5 M EDTA (pH 8.0)	Add 18.61 g of ethylenediaminetetraacetate. 2 H_2O to 80 ml of H_2O. Add approximately 2 g of NaOH pellets to adjust the pH to 8.0 (The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approximately 8.0 by the addition of NaOH). Adjust the volume to 100 ml and sterilize by autoclaving.
5 M NaCl	Dissolve 29.22 g NaCl in 80 ml water. Adjust the volume to 100 ml and sterilize by autoclaving.
Phenol : Chloroform – Isoamyl alcohol (25:24:1)	Mix 750 µl buffered phenol (pH approx. 7.8), 720 µl chloroform and 30 µl isoamyl alcohol. Mix by vortexing and keep at 4°C in coloured container (or this could be prepared just before use).
1 M Tris	Dissolve 12.11 g Tris base in approximately 60 ml water. Adjust the pH to 8.0 by adding HCl. Adjust the volume to 100 ml and sterilize by autoclaving.
3 M Sodium acetate	Dissolve 40.8 g sodium acetate. 2 H_2O in 60 ml water. Adjust the pH to 5.2 with glacial acetic acid. Adjust the volume to 100 ml with water and sterilize by autoclaving.

Gel electrophoresis

Ethidium bromide - Add 100 mg ethidium bromide (10 mg/ml) to 10 ml of sterile water. Stir on a magnetic stirrer until the dye is completely dissolved. Wrap the container in aluminium foil or transfer the solution to a dark bottle and store at room temperature. **Caution:** Ethidium bromide is a powerful mutagen; avoid contact with skin and inhalation.

6 X Loading dye - 0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in water. Keep at 4^0C.

TBE buffer (25X) - Dissolve 135 g Tris base in 300 ml water. Add 65.87 g boric acid and 50 ml of 0.5 M EDTA (pH 8.0). Adjust the volume to 500 ml and sterilize by autoclaving.

Appendix-II

Composition of modified Murashige and Skoog's (1962) basal medium (MS Medium)

Constituents	Amount (mgl^{-1})
Inorganic constituents	
Major constituents	
NH_4NO_3	1650.00
KNO_3	1900.00
KH_2PO_4	170.00
$CACL_2.2H_2O$	440.00
$MgSO_4$	370.00
Minor constituents	
H_3BO_3	6.20
KI	4.25
$Na_2MoO_4.2H_2O$	0.25
$CoCI_2.6\ H_2O$	0.025
$CuSO_45.H_2O$	0.025
$ZnSO_4.4\ H_2O$	8.60
$MnSO_4$	22.30
$FeSO_4.7H_2O$	27.80
Na_2EDTA	37.30
Organic constituents	
Nicotinamide	0.5
Thiamine HCl	0.1
Pyrodoxine HCl	0.5
Glycine	2.0
Meso Inositol	100.0

Appendix-III

Analysis of variance (ANOVA)

No of shoots/shoot clump (control)

Source	DF	SS	MS	F
Treatment	2	33.57	16.78	491.32
Error	6	0.02	3.41	
Total	8	33.79		

No of shoots/shoot clump (transformed)

Source	DF	SS	MS	F
Treatment	2	9.06	4.53	1933.67
Error	6	0.001	0.002	
Total	8	9.08		

Length of shoot (control)

Source	DF	SS	MS	F
Treatment	2	3.01	1.50	244.33
Error	6	0.03	0.006	
Total	8	3.05		

Length of shoot (transformed)

Source	DF	SS	MS	F
Treatment	2	0.72	0.36	164.23
Error	6	0.01	0.02	
Total	8	0.73		

All Tables are significant at 5% level of significance.

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